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Analytical Studies on Isoxazoles. IV.[1] Fluorometric Determination of Isouron[2]

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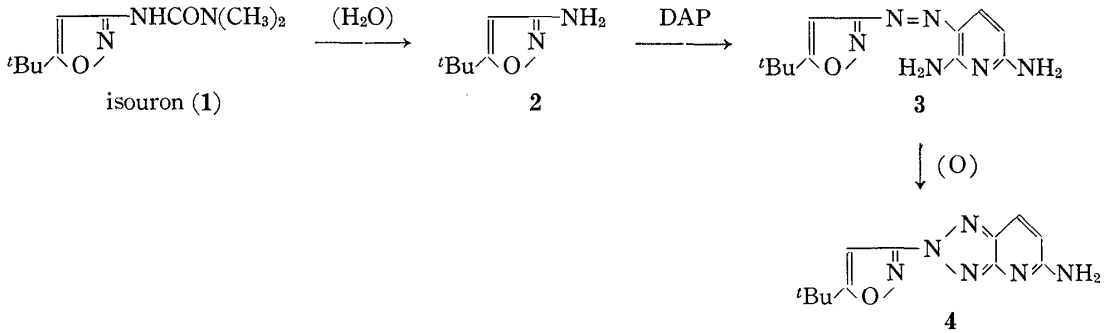
A fluorometric method for the determination of isouron (**1**) was established by modifying Dombrowski's method. Isouron was hydrolyzed to 3-amino-5-*tert*-butylisoxazole (**2**) followed by diazo coupling with 2,6-diaminopyridine (DAP), and the resulting azo derivative (**3**) was oxidized to a fluorescent triazole (**4**). A simple nitrosation method was developed in order to overcome the interference by the strongly fluorescent reagent DAP. The oxidation yield of **3** to **4** was improved by employing the neutral condition.

A linear relationship between the fluorescence intensity and isouron concentration was obtained in the range of 0.1—0.4 μg/ml. Isouron could be determined with a coefficient of variation of 2—3% (ten determinations), overall recovery of 85—90%, and quantitation limit of 40 ng/ml.

Keywords——isouron; fluorometric determination; 3-amino-5-*tert*-butylisoxazole; 2,6-diaminopyridine; diazo coupling; triazolopyridine; nitrosation of 2,6-diaminopyridine

In the previous paper, a colorimetric method for the assay of isouron (**1**), a potential herbicide, was established.[1] However, because the sensitivity of the method was not sufficient to permit estimation of the residual **1** in soil, we attempted to establish a sensitive fluorometric method. The fluorometric assay method is in principle based on Dombrowski's method[3] for primary aromatic amines as shown in the following scheme. It has been pointed out that the remaining reagent, 2,6-diaminopyridine (DAP), interferes with the assay by Dombrowski's method because of its intense fluorescence. In fact, the assay of **1** is impossible in the presence of DAP, although the final product **4** is an intense fluorophor. Dombrowski *et al.*[3] attempted to separate excess DAP by repeated extractions. The effect of DAP, however, could not be removed completely even by such a troublesome procedure. In the present paper, a simple method involving nitrosation was used to eliminate the interference by DAP, and suitable conditions for the oxidation from the azo derivative (**3**) to **4** were also examined.



isouron (**1**) **2** **3** **4**

Chart 1

Experimental

Apparatus——A Hitachi MPF-2A recording spectrofluorometer was used for fluorescence measurement. A Hitachi digital spectrophotometer, model 624, was used for absorbance measurement.

Reagent——Commercially available DAP (Nakarai Chemical Ltd.) was purified by recrystallization

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from MeOH–ether, mp 122°C. Commercially available ethyl acetate (Spectrosol of Dojindo Laboratories) was used. The other chemicals were of reagent grade. 2,6-Diamino-3-nitrosopyridine (**5**) was prepared by the known method,[4] mp 267°C (dec.).

Isouron (**1**) and 3-amino-5-*tert*-butylisoxazole (**2**) were prepared as described in the previous paper.[1]

3-[(5-*tert*-Butylisoxazol-3-yl)azo]pyridine-2,6-diamine (**3**)——An aqueous solution of 5 ml of $NaNO_2$ (1 g) was added dropwise to a solution of **2** (1 g) in 30 ml of hydrochloric acid (*ca.* 35%) under ice cooling. After addition of H_2O (30 ml), ammonium sulfamate (8 g) was added and the mixture was stirred for 5 min. After addition of DAP (1 g), the mixture was further stirred for 20 min. The reaction mixture was extracted with benzene (50 ml), and the benzene phase was discarded. Next, 10 N NaOH was added to the aqueous phase until the color changed to green. The solution was extracted with two 100 ml portions of AcOEt. The combined AcOEt extracts were washed with two 20 ml portions of pH 5 acetate buffer, dried over Na_2SO_4, and evaporated to dryness under reduced pressure to give a crystalline residue. Recrystallization from MeOH gave 0.38 g (20%) of **3** as orange prisms, mp 247—250°C. IR cm^{-1}: 3416, 3146, 2962, 1657, 1600 (KBr). UV and visible absorption spectra λ_{max}^{EtOH} nm (log ε): 282 (4.14), 427.5 (4.47). *Anal.* Calcd for $C_{12}H_{16}N_6O$: C, 55.37; H, 6.20; N, 32.29. Found: C, 55.46; H, 6.24; N, 32.16.

2-(5-*tert*-Butylisoxazol-3-yl)-2*H*-1,2,3-triazolo[4,5-*b*]pyridine-5-amine (**4**)——A solution of **3** (40 mg) in pyridine (3 ml) was treated with 4% aqueous $CuSO_4$ (1 ml). The solution was heated for 1 h on a boiling water bath. After cooling to room temperature, the solution was evaporated to dryness under reduced pressure. The residue was extracted with two 50 ml portions of ether. The ether extract was washed with H_2O (10 ml) and dried over Na_2SO_4. The ether was distilled off and the residue was recrystallized from $CHCl_3$–*n*-hexane to give 10 mg (25%) of colorless needles, mp 203—204°C. IR cm^{-1}: 3384, 2965, 1627, 1597 (KBr). UV $\lambda_{max}^{H_2O}$ nm (log ε): 257 (3.70), 339 (4.33). *Anal.* Calcd for $C_{12}H_{14}N_6O$: C, 55.80; H, 5.46; N, 32.54. Found: C, 56.07; H, 5.26; N, 32.54.

Assay Procedure——Prepare a sample solution by dissolving **1** in dimethylformamide followed by dilution with H_2O (10% aqueous dimethylformamide). Pipet 2.0 ml of the sample solution (0.2 μg/ml) into a hydrolysis tube.[5] Seal the tube and heat it at 145—155°C for 1 h. After cooling to room temperature, open the tube and pipet 1.0 ml of the solution into a 40 ml centrifuge tube, in which hydrochloric acid (*ca.* 35%; 1 ml) was previously placed. Cool the tube in an ice-cold water bath and allow to stand for 5 min. Add 0.5 ml of 0.2% $NaNO_2$ solution and allow to stand for 3 min. Add 1.0 ml of 0.5% ammonium sulfamate solution and allow to stand for 5 min. Then, add 0.5 ml of 0.01% DAP solution and allow to stand for 10 min. Take the tube out of the ice-cold water bath and add 1.5 g of $NaHCO_3$. Add 1.0 ml of 20% NH_4Cl solution and then 1.0 ml of 10% $CuSO_4$ solution. Heat the mixture on a boiling water bath for 10 min. After cooling to room temperature, add 5.0 ml of AcOEt to the reaction mixture, shake for 5 min with a shaker (an Iwaki KM shaker, type V-S) and centrifuge (a Sakuma centrifuge, model 90-4) for 5 min. Pipet 3.0 ml of the AcOEt phase into a 12 ml centrifuge tube. Add 2.0 ml of pH 3 acetate buffer (1 M) and then 1.0 ml of 0.5% $NaNO_2$ solution. Shake for 10 min and centrifuge for 5 min. Discard the aqueous phase and add 1.0 ml of 1 N NaOH. Shake for 5 min and centrifuge for 5 min. Measure the fluorescence intensity of the AcOEt solution at 403 nm with excitation at 351 nm and that of the reagent blank prepared in the same manner using 10% aqueous dimethylformamide instead of the sample solution.

Calibration Curve——A calibration curve was made according to the standard procedure. A linear relationship between the concentration of **1** and the fluorescence intensity was obtained in the range of 0.08—0.4 μg/ml.

Measurement of the Recovery of 3 after Oxidation——Oxidation of **3** was carried out under various conditions as described below.

(a) Oxidation in Alkaline Solution: Compound **3** (dissolved in 10% dimethylformamide–H_2O (1 ml)) was added to a mixture of hydrochloric acid (*ca.* 35%; 1 ml), H_2O (2 ml) and aqueous ammonia (*ca.* 25%; 1—3 ml). After addition of 10% ammoniacal $CuSO_4$ (1 ml),[3] the whole was heated on a boiling water bath.

(b) Oxidation in Neutral Aqueous Solution: To a mixture of hydrochloric acid (*ca.* 35%; 1 ml) and H_2O (3 ml) was added 1.5 g of $NaHCO_3$. Then, **3** was added. Ammonium chloride (0.05—1.25 g) and 10% aqueous $CuSO_4$ (1 ml) were added and the whole was heated on a boiling water bath.

(c) Oxidation using Various Amounts of $CuSO_4$: After preparing the same medium as described in (b), **3** was added, followed by $CuSO_4$ in the concentration range of 2.5—30% (1 ml) and NH_4Cl (0.2 g). The mixture was heated on a boiling water bath for 10 min.

(d) Oxidation for Various Reaction Times: Oxidation with 20% NH_4Cl (1 ml) and 10% $CuSO_4$ (1 ml) was carried out for 5—60 min.

The fluorescent compound produced by the procedures (a)—(d) was extracted with AcOEt. The extract was treated according to the standard assay procedure, and the recovery of **3** was measured fluorometrically. A calibration curve was made by using a standard solution of **4** (0.02—0.08 μg/ml) prepared in AcOEt.

Measurement of UV Spectra——The spectrum of DAP was measured in 0.001 N HCl (DAP; 2.22×10^{-5} M). An aqueous solution of DAP was treated with 1 ml of 0.01 N HCl and 1 ml of 0.1% $NaNO_2$, and then the solution was diluted immediately to 10 ml with H_2O (DAP; 2.22×10^{-5} M). The spectra were measured at the time intervals shown in Fig. 5.

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The pKa values of **5** were measured by the method described in the previous paper.[6] The values were −0.59, 4.25 and 13.11.

Results and Discussion

Diazo Coupling of 3-Amino-5-*tert*-butylisoxazole (2)

Compound **2** was diazotized under the same conditions as described in the previous paper.[1] The diazonium salt was coupled with DAP. The effect of DAP concentration on the coupling reaction was examined. Since the product **3** has absorption maximum at 410 nm, the absorbances of the diazo coupling solutions obtained by the use of various amounts of DAP were measured. When DAP was present in a two-fold molar excess over 2, a constant absorbance was obtained as shown in Fig. 1. The yield of **3** was 96%.

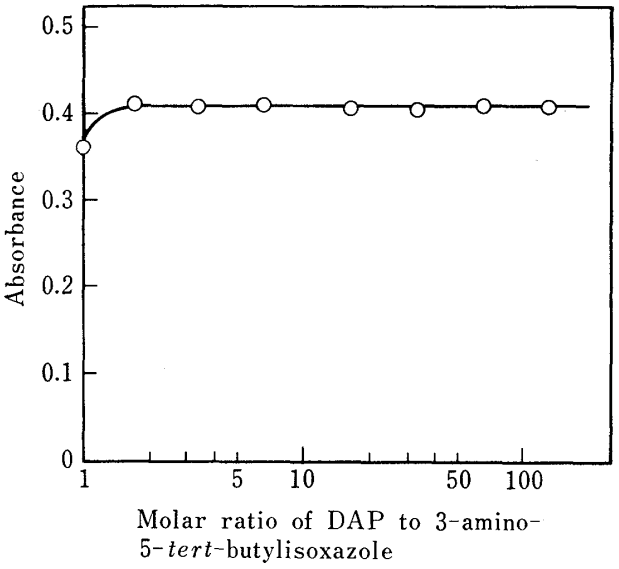

Fig. 1. Effect of DAP Concentration on the Diazo Coupling

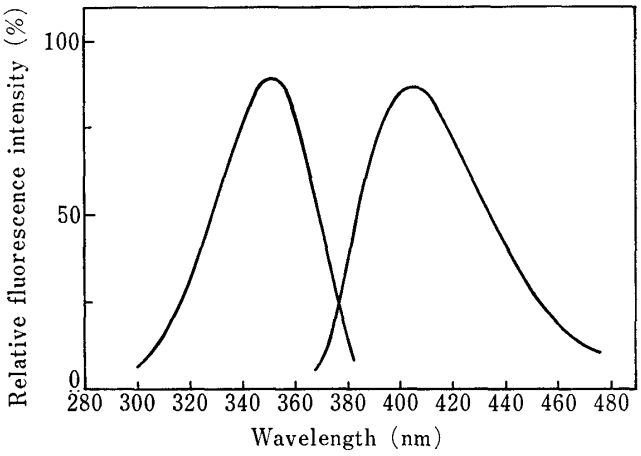

Fig. 2. Excitation and Fluorescence Spectra of the Triazole in AcOEt

Oxidation of 3-[(5-*tert*-Butylisoxazole-3-yl)azo]pyridine-2,6-diamine (3)

Oxidation of **3** was carried out in acidic, ammoniacal alkaline and neutral aqueous solutions using $CuSO_4$. The fluorescent compound was produced under neutral or alkaline solution.

The excitation and fluorescence spectra in the AcOEt extract are shown in Fig. 2. The spectra coincided with those of compound **4** which was prepared separately.

No fluorescence was observed when the oxidation was carried out in 2N HCl. In ammoniacal alkaline solution, **3** was converted to **4** in a yield of less than 76%. The yield became lower with increase of ammonia concentration. The fluorescence values obtained when **3** was oxidized for 5 min and 10 min in neutral aqueous solutions containing various amounts of NH_4Cl are shown in Fig. 3. The oxidation was greatly affected by the amount of NH_4Cl added, especially at 5 min reaction time. A constant fluorescence intensity was obtained by the use of 0.10—0.30 g of NH_4Cl. The yields were found to be 102—105%.

Effect of Amount of $CuSO_4$ on the Oxidation

Figure 4 shows the effect of amount of $CuSO_4$ on the oxidation. A constant fluorescence intensity was observed by the use of 0.075—0.3 g of $CuSO_4$. The yields were in the range of 100 to 102.5%.

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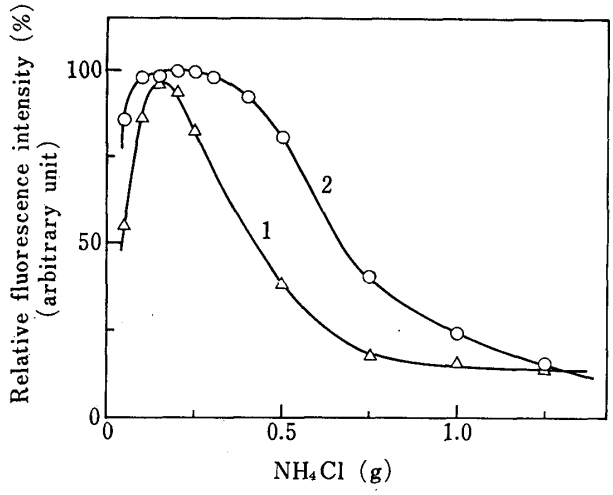

Fig. 3. Effect of Amount of NH_4Cl on the Oxidation

Curve 1: reacted for 5 min.
Curve 2: reacted for 10 min.
Fluorescences observed for both reaction solutions are plotted in the same intensity units.

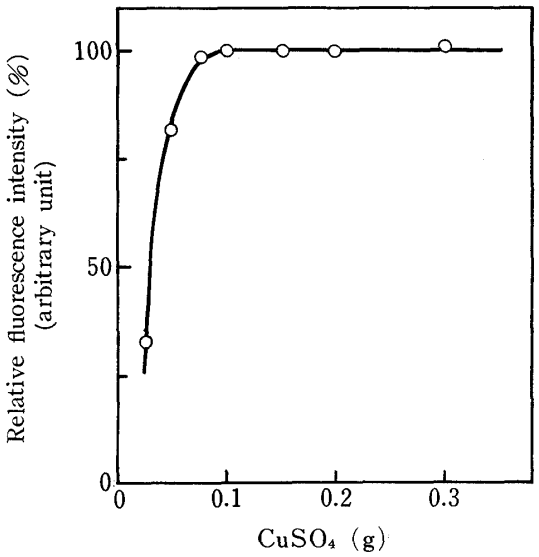

Fig. 4. Effect of Amount of $CuSO_4$ on the Oxidation

Effect of Time on the Oxidation

The oxidation was carried out for 5—60 min. A constant fluorescence intensity was observed at reaction times greater than 10 min.

Effect of pH on the Extraction

The effect of pH on the extraction of the oxidation product was examined. Standard solutions of **4** in the buffer (pH 2.0—8.0) were extracted with AcOEt. The recoveries measured at intervals of 1 pH unit in the range of 3.0 to 8.0 were 103—105%.

Effect of Excess DAP on the Fluorescence Development

The reagent DAP is strongly fluorescent. The maximum wavelengths of the excitation and fluorescence spectra in AcOEt are 309 and 342 nm, respectively. The intensity of DAP at 342 nm was about one-half of the value of **4** observed at the maximum wavelength of

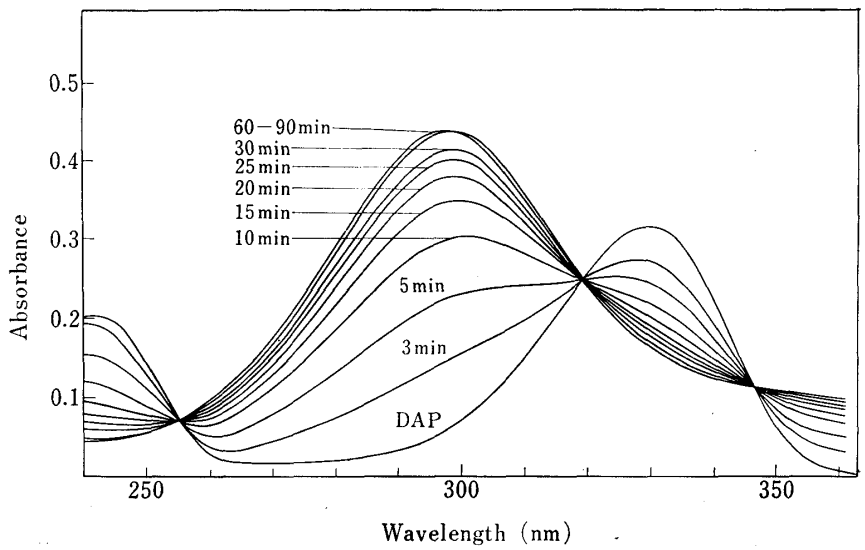

Fig. 5. UV Spectra of DAP and Its Nitroso Derivative

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the compound. To remove the effect of excess DAP, DAP was nitrosated with HNO_2. Figure 5 shows the spectral change of DAP on treatment with $NaNO_2$ in 0.001 N HCl. The spectrum of DAP changed with time and after more than 1 h coincided with that of 2,6-diamino-3-nitrosopyridine (**5**) in 0.001 N HCl. Nitrosation of DAP was immediately completed by the addition of 0.5% $NaNO_2$. No fluorescence was observed for the DAP solution treated with $NaNO_2$. In the assay procedure, since DAP was extracted together with **4** in AcOEt, the extract was treated by shaking with a mixture of pH 3 buffer and 0.5% $NaNO_2$. Although fluorescence of DAP was removed by this procedure, the resulting **5** also quenched the fluorescence development (the intensity was decreased to 43%). To avoid this quenching, **5** was extracted with 1 N NaOH. The dissociation constant of **5** was found to be 13.11. By the use of nitrosation followed by extraction with NaOH solution, the fluorescence of **4** was measurable without interference even in the presence of DAP at 40—400-fold excess.

Precision, Overall Recovery and Detection Limit of the Assay

Isouron samples were determined by the present method. The precision of the assay procedure was examined on replicate runs, and the data listed in Table I were obtained. The coefficient of variation was 2.22%. The overall recovery of **1** throughout all the processes in the assay method was evaluated using **4** as a standard. Sample solutions containing **1** at various concentrations (0.08—0.40 μg/ml) were assayed. As shown in Fig. 6, **1** was recovered as the fluorophor **4**, yield 85.4%. By repeated determinations of sample solutions at a constant concentration (0.242 μg/ml), the overall recovery was found to be 85.5±3.36% (n=9). The lower limit of the assay taken as the concentration giving a fluorescence signal amounting to twice the background noise was 40 ng of isouron per ml of the solution.

Table I. Determination of Isouron Samples

No.	Taken (μg/ml)	Found (μg/ml)	Content (%)
1	0.2608	0.2490	95.5
2	0.2608	0.2555	98.0
3	0.2608	0.2561	98.2
4	0.2608	0.2567	98.4
5	0.2608	0.2596	99.5
6	0.2608	0.2596	99.5
7	0.2608	0.2685	102.9
8	0.2608	0.2578	98.8
9	0.2608	0.2655	101.8
10	0.2608	0.2655	101.8
		$\bar{x}$	99.4
		c.v.	2.22

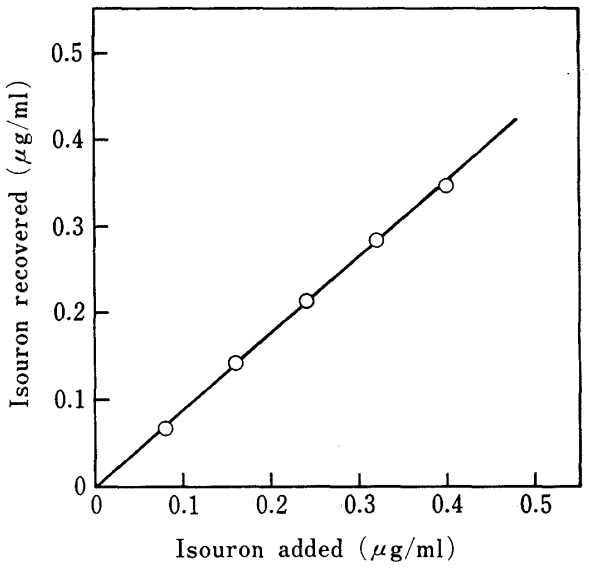

Fig. 6. Overall Recovery of Isouron throughout the Assay Procedure

Regression equation: $y=0.8535x+0.0057$, $s=0.0052$
The intercept was regarded as zero.

References and Notes

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