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A New Fluorometric Analysis of Dulcin using Sodium Nitrite. II.[1] Isolation and Structural Investigation of the Fluorescent Compound[2]

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From the reaction of dulcin and sodium nitrite, a fluorescent compound (colorless crystals, mp 183—184°) was obtained. The structure of this fluorescent compound was presumed to be 1,3-bis(4-ethoxyphenyl)-5-tetrazolone by nuclear magnetic resonance, infrared, mass spectroscopy and by several reactions.

The present authors have reported a fluorometric determination of dulcin using sodium nitrite.[1] This paper dealed with the isolation and structural investigation of the fluorescent compound obtained by this reaction.

To obtain the fluorescent compound preparatively, dulcin was treated under the same conditions as in the case of the fluorometric measurement. Namely, a solution of dulcin in water was treated with sodium nitrite in the presence of hydrochloric acid at room temperature, the reaction mixture was made alkaline with sodium hydroxide, and extracted with chloroform. The chloroform extract was chromatographed on a silica gel column, and, from the fluorescent fraction, the colorless crystals were obtained (mp 183—184°).

This compound was thin-layer chromatographically homogeneous with three developing solvents and agreed entirely with the main fluorescent substance contained in the reaction mixture by means of overlapping application (Fig. 1).

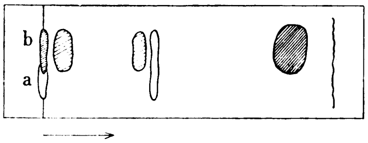

Fig. 1. Thin-Layer Chromatograms of the Fluorescent Compound and the Chloroform Extract of the Reaction Mixture with Overlapping Application

a : fluorescent compound isolated
b : chloroform extract of the reaction mixture
(a and b were spotted to overlap each other)
thin-layer plate: Kiesel gel G (Merck), 250 μ activated at 110°
developing solvent: benzene-ethyl acetate (35:15)
⬭: fluorescent spot by radiation at 365 mμ

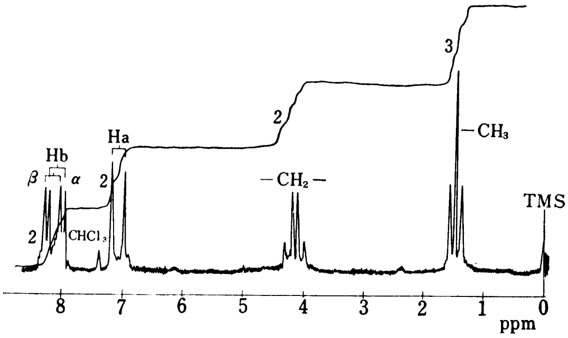

Fig. 2. NMR Spectrum of the Fluorescent Compound in $CDCl_3$

Since its fluorescence spectrum (excitation maximum at 354 mμ, emission maximum at 446 mμ) also agreed with that of the reaction mixture obtained by the determination

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[1] Part I: S. Uchiyama, T. Kondo, and I. Kawashiro, *Yakugaku Zasshi*, **89**, 828 (1969).

[2] This work was presented at the 90 th Annual Meeting of Pharmaceutical Society of Japan, Sapporo, July 1970. A portion of this work was reported as preliminary communication. Z. Tamura, Y. Iitaka, H. Tanabe, and S. Uchiyama, *Chem. Pharm. Bull.* (Tokyo), **18**, 2359 (1970).

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procedure, this fluorescent compound should be the essential fluorescent product in the determination.

The infrared (IR) spectrum of this fluorescent compound ($C_{17}H_{18}O_3N_4$) showed an absorption band, due to carbonyl group (C=O), at 1680 cm^{-1}, but any band, due to hydroxy (OH) or amines (NH, NH_2), was not observed. The ultraviolet (UV) spectrum showed three absorption maxima at 287 mμ (log ε: 4.01), 296 mμ (3.99) and 348 mμ (4.25). The 60 Mc nuclear magnetic resonance (NMR) spectrum was shown in Fig. 2.

The proton signals due to the methyl and methylene of an ethoxyl group appear at 1.46 and 4.13 ppm, respectively, and the signals in the region of 7.0—8.0 ppm were assigned to the benzene ring protons. In this spectrum an A_2B_2 pattern is observed, and the coupling constants for Ha-Hbα and for Ha-Hbβ are both 9.15 cps. These NMR data strongly supported the existence of a 4-ethoxyphenyl moiety. Consequently, the fluorescent compound must have two 4-ethoxyphenyl groups from the facts that its molecular formula is $C_{17}H_{18}O_3N_4$ and that, in the NMR spectrum, there is no signal except those due to the ethoxyl group and the benzene ring.

On reduction with zinc and concentrated hydrochloric acid, the fluorescent compound gave a mixture of *p*-phenetylurea (dulcin) and *p*-phenetidine which was identified by thin-layer chromatography. This result suggested that it contained a group of $C_2H_5O{\cdot}C_6H_4\text{–}\overset{|}{N}\text{–}CO\text{–}\overset{|}{N}\text{–}$. As shown in Fig. 3, the mass (Mass) spectrum showed fragment peaks at *m/e* 149 and 121. From these spectral and chemical data, the structure of the fluorescent compound must be one of the following three, I, II, and III (Chart 1).

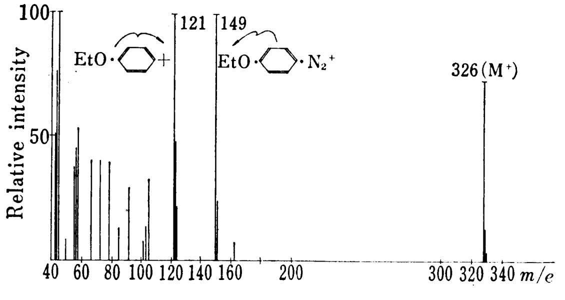

Fig. 3. Mass Spectrum of the Fluorescent Compound

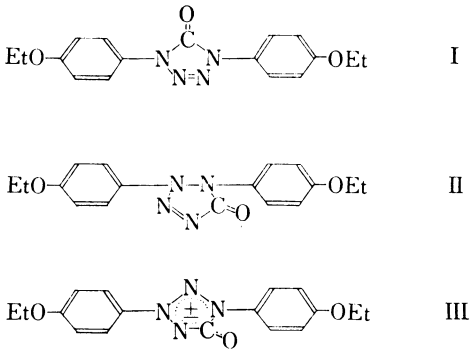

Chart 1

In order to select a true structure out of the above three, measurement conditions of the NMR were changed. In a deuterochloroform solution containing a small amount of trifluoroacetic acid, the signals at 7.03, 8.02 and 8.06 ppm shifted to 7.15, 8.05 and 8.18 ppm, (J= 9.15), respectively (Fig. 4). When nitromethane was used as a solvent, the doublet, due to Hb and Ha, changed to a quartet (J=9.15). Also, when the measurement temperature was changed from 25° to 100° in nitromethane, the signals in the region of 7.0—8.0 ppm remained unaltered. No steric hindrance was therefore observable in this system. Since the NMR data mentioned above indicate that there are two kinds of A_2B_2-pattern, the structure must be asymmetrical. Thus, the structure I was ruled out.

In addition to these facts, the reaction of 4-ethoxybenzene diazonium fluoroborate and potassium dinitromethane, which was carried out under the same conditions as Hönig's 5-oxytetrazoliumbetaine synthesis,[4] gave the same fluorescent compound as that obtained

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[4] S. Hünig and O. Boes, *Ann. Chem.*, **579**, 28 (1953).

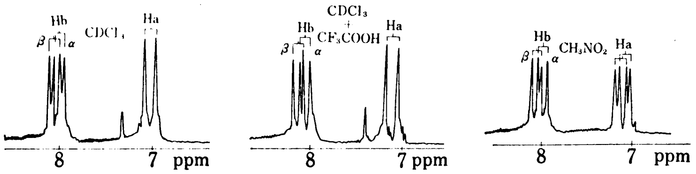

Fig. 4. Effect of Solvent on the Benzene Ring Protons

from the reaction of dulcin and sodium nitrite. The fact suggests that the fluorescent compound is made from two molar equivalent of the diazonium ion and one molar equivalent of dinitromethane, and that the structure III (1,3-bis(4-ethoxyphenyl)-5-tetrazolone) is more presumable than II.

Experimental[5]

Fluorescent Reaction of Dulcin and Isolation of the Fluorescent Compound——To a solution of dulcin (7g) in H_2O (3500 ml) was added conc. HCl (200 ml) and 10% $NaNO_2$ (1000 ml), over a period of 30 minutes at room temperature. The reaction mixture was made alkaline with 20% sodium hydroxide (1000 ml) at room temperature, and extracted twice with chloroform. The extract was washed with H_2O and dried over anhyd. Na_2SO_4, and concentrated under a reduced pressure.

The chloroform extract concentrated was chromatographed on a silica gel column (Mallinchrodt, 100 mesh, 100 g, eluting solvent: $CHCl_3$). Then, the fluorescent fraction was rechromatographed on a thin-layer prepared from silica gel (Kiesel gel, 500μ, activated at 110°, developing solvent; $CHCl_3$: EtOH=50: 3). From the fluorescent fraction, the crude red crystals were obtained and refined by chromatography using a column prepared from a mixture of Celite and charcoal (1: 1).

The product was recrystallized from a mixture of $CHCl_3$ and EtOH (=2: 5) to give colorless leaflets, mp 183—184°. *Anal.* Calcd. for $C_{17}H_{18}O_3N_4$: C, 62.57; H, 5.52; N, 17.17. Found: C, 62.68; H, 5.42; N, 17.17. Millimass Spectrum *m/e*; *Anal.* Calcd. for $C_{17}H_{18}O_3N_4$, 326.137 (M^+). Found; 326.134 (M^+).

Thin-Layer Chromatography of the Fluorescent Compound——The thin-layer chromatography (TLC) of the crystals isolated was carried out in following three kinds of developing solvents to test its singleness. Developing solvent: H_2O-EtOH (=35-15), *Rf*=0.74; $CHCl_3$-EtOH (=50-3), *Rf*=0.71; benzene-ethyl acetate (=35-15), *Rf*=0.42. Plate: Merck, Kiesel gel G, 250 μ, activated at 110°. Also, in the overlapping method of TLC using above developing solvent, there was good agreement between the fluorescent band of the reaction mixture and that of the isolated crystals.

Reduction of the Fluorescent Compound with Zinc and Hydrochloric Acid——To a solution of the fluorescent compound (50 mg) in EtOH (10 ml) were added carefully zinc granules (5 g) and conc. HCl (10 ml). After standing for 10 min at a room temperature, the mixture was refluxed for 30 min and made alkaline with 20% NaOH, and extracted with benzene. The benzene extract was concentrated, and the solution was chromatographed on a silica gel thin-layer. The extraction of the band of dulcin, followed by recrystallization from benzene, afforded dulcin as colorless leaflets (5 mg), mp 173—174°. The identity of dulcin was carried out by means of a mixed fusion, TLC, and by IR spectroscopy. On the other hand, *p*-phenetidine isolated from the thin-layer plate was identified by TLC which was carried out under the following conditions. Plate: Merck, Kiesel gel G 250 μ, activated at 110°. Developing solvent; benzene-ethyl acetate (=1-1), *Rf*=0.40; $CHCl_3$-propionic acid (=4-1), *Rf*=0.06. Color reagent: *p*-dimethylamino benzene aldehyde.

Reaction of 4-Ethoxybenzene Diazonium Fluoroborate with Potassium Dinitromethane.[4]——To a solution of 4-ethoxybenzene diazonium fluoroborate (0.9 g) in 2N CH_3COOH (15 ml) was added potassium dinitromethane (0.35 g). After stirring for 20 min, the reaction mixture was stood overnight at room tem-

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5) Melting points were determined in aYanagimoto melting point apparatus and are not corrected; the UV spectrum was taken in $CHCl_3$ solution on a Hitachi EPU-2 Spectrophotometer; the flurorescence spectrum was taken in $CHCl_3$ solution on a Hitachi MPF-2A Fluorescence Spectrophotometer; the IR spectrum was measured on a Nihon Bunko DS-301 Spectrophotometer in KBr discs; the NMR spectra were run on a Japan Electron Optics Lab. JNM-C-60HL High Resolution NMR Instrument (60MC) and recorded in ppm values with TMS as the internal standard; the mass spectra were measured by the direct sample introduction techique on a Hitachi RMU-7 Double-focussing Mass Spectrometer and a Hitachi RMS-4 Mass Spectrometer (the heating temperature was at 200° and 300°, respectively. the ionizing current was maintained at 70eV and 80eV, respectively).

perature. The mixture was made alkaline with dil. NaOH, the alkaline solution was extracted with chloroform, and the extract was chromatographed on a silica gel thin-layer. The colorless crystals (5 mg), mp 183—184°, were obtained from the fluorescent band, and this compound was identical with the fluorescent compound obtained from ducin, by means of TLC and a mixed fusion, and by IR, fluorescence and UV spectroscopy.

Potassium Dinitromethane: This compound was synthesized from tribromoanilline by Losanitsh's method[6] as yellow crystals, mp 200—205° (decomp.), (Reported, 205° (decomp.)[7]).

4-Ethoxybenzene Diazonium Fluoroborate: This compound was synthesized from *p*-phenetidine by the method reported in "Organic Syntheses"[8] as colorless crystals, mp 89—90° (103° (decomp.)), (Reported, 103° (decomp.)[9]).

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6) S.M. Losanitsh, *Chem. Ber.*, **15**, 471 (1882).
7) P. Duden, *Chem. Ber.*, **26**, 3004 (1893).
8) G. Schiemann, "Organic Syntheses," Coll. Vol. II, ed. by A.H. Blatt, John Wiley and Sons, Inc., New York, N.Y., 1943, p. 299.
9) A. Roe and J.R. Grahan, *J. Am. Chem. Soc.*, **74**, 6297 (1952).