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Studies on the Fluorescent Substances in *Phoma Wasabiae*

By

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One of the authors has studied on the enzyme activities of *Phoma Wasabiae*[1,2] and on the metabolic products from the culture of *Phoma Wasabiae*[3]. It was recognized that *Phoma Wasabiae* has the activities of polyphenol oxidase. When exposed to ultraviolet light of wavelength 365 mμ, *Phoma Wasabiae* fluoresces a white blue color. In this report, these fluorescent substances were separated, and the presence of $\Delta^{5,7}$-sterols was recognized. In addition, a red pigment which *Phoma Wasabiae* produced by metabolization was also separated from culture medium of potato, which seemed a new trihydroxy-1, 4-naphthoquinone. As this work is in progress, it will be described later.

Experiments and Results

The culture medium was prepared as follow. In 1 l. of distilled water, 200 g. of thinly sliced potato was boiled for 30 minutes, and the mixture was filtered with two sheets of gauze. To the filtrate, 20 g. of glucose was added, the pH was adjusted to 7.0 with 20% potassium hydroxide solution, and 400 ml. of filtrate was poured into a 1-l. Erlenmeyer flask, then autoclaved at 120°C for 15 minutes.

Phoma Wasabiae was inoculated on the culture medium and incubated at 25°C for three weeks in the static state. After three weeks, blakish mold grew on the surface of the liquid culture medium. The mold was filtered, and then air-dried at room temperature. The dried materials were pulverized and extracted with methanol at 40–45°C. The methanol solution was dark brown under visible light and fluoresced a white blue under ultraviolet light. It was concentrated to give a brown viscous material (S-1).

Separation procedure 1. S-1 was extracted with chloroform and the solution was then extracted with 1% sodium carbonate. The sodium carbonate layer was adjusted to pH 4.0 with sulfuric

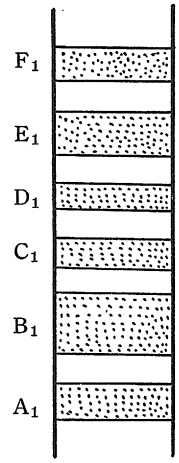

Fig. 1. Chromatogram of separation procedure 1
V : Under visible light
UV : Under ultraviolet light

acid and extracted with ether. The ether layer was concentrated and the residue was dissolved in ethyl acetate, and the solution was passed through a 2 cm.×10cm. column of alumina, the chromatogram being shown in Fig. 1. Fraction A_1 and B_1 could be eluted, but other fractions C_1, D_1, E_1 and F_1 could not be eluted. Concentration of fraction A_1 gave an oily liquid. Concentration of fraction B_1 gave a crystalline solid, and this was further purified by thin layer chromatography. Crystals thus obtained were repeatedly recrystallized from methanol and there was obtained 48 mg. of a substance melting at 139-140°C (V-I). Its crystal form was hexagonal thin plates as shown in Fig. 2. It reacted with antimony trichloride, conc. sulfuric acid and conc. sulfuric acid-acetic anhydride which are the color reagents of steroids.[4] The ultraviolet spectrum of it showed the maximum absorptions at 272, 282 and 294 mμ with characteristics of $\Delta^{5,7}$-

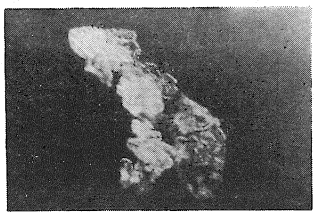

Fig. 2 Photograph of free sterol V-I.

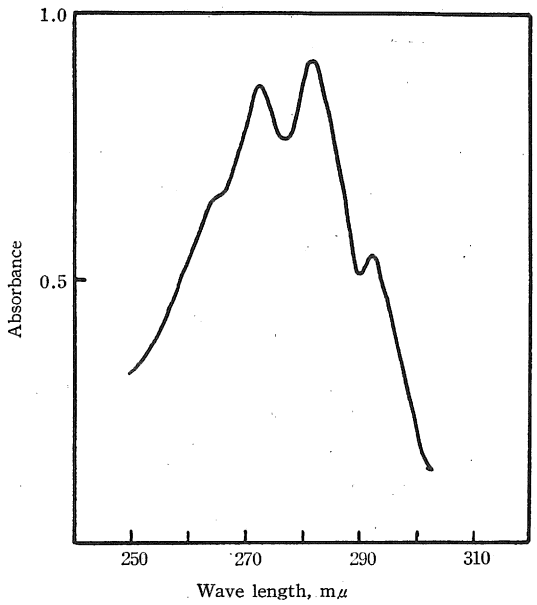

Fig. 3. Ultraviolet absorption spectrum of V-I. in Ethanol

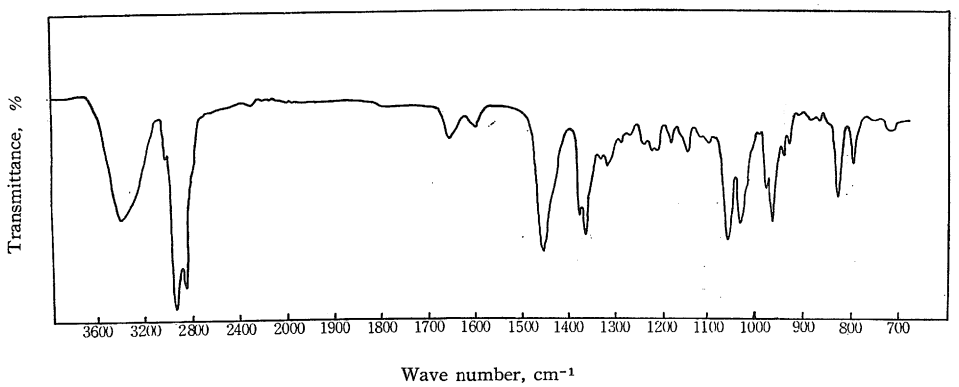

Fig. 4. Infrared spectrum of V-I.

sterols (Fig. 3). The infrared spectrum was shown in Fig. 4.

Separation procedure 2. S-1 (67 g, 34 % of dried mold) was extracted with ether, to give *a ether-soluble portion* (*X*) and *a ether-insoluble portion* (*Y*).

The ether-soluble portion (*X*) was concentrated and dissolved in ethyl acetate, and the solution chromatographed over basic alumina in a similar manner as above. The adsorption zones (A_2, B_2 and C_2 in Fig. 5) were then eluted with ethyl acetate. Fractions A_2 and C_2 gave small amounts of oily liquid. A crystalline solid V (418 mg., m.p. 140-143°C, $[\alpha]_D^{18}$ -112.5°, 0.21 % of dried mold) from fraction B_2 showed a positive Rosenheim reaction[5] and developed a blue green color very rapidly by Liebermann-Burchard reaction[6]. It was repeatedly recrystallized from acetone but the melting point was not constant, so that it was further purified by thin layer chromatography (Benzene : Methanol (90 : 10) was used as the developing solvent). When a substance thus obtained in crystals was recrystallized five times from acetone, it had m.p. 136-137°C and $[\alpha]_D^{18}$ -112.8° (V-II).

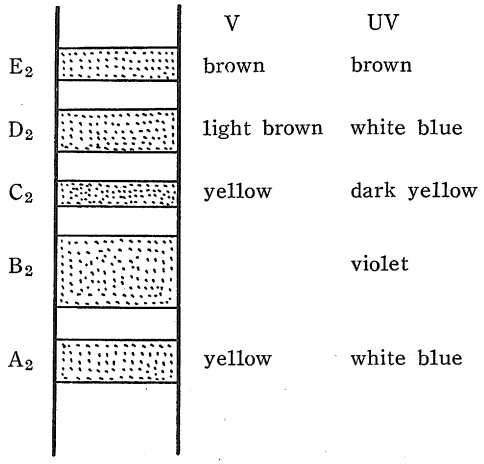

Fig. 5. Chromatogram of separation procedure 2.
V : Under visible light
UV : Under ultraviolet light

Its molecular weight (mass spectrum) was 396, from which the molecular formula was estimated to be $C_{27}H_{40}O_2$ (Found : C, 81.56 ; H, 11.06 ; $C_{27}H_{40}O_2$ requires C, 81.82 ; H, 10.10, O, 8.08%). Its ultraviolet absorption spectrum was shown in Fig. 6. It has the characteristic absorption maxima of 5 : 6-and 7 : 8-conjugated sterols ; λ_{max} 272(log ε : 3.85), 282(3.89), 294 (3.65) mμ. Its infrared spectrum was shown in Fig. 7.

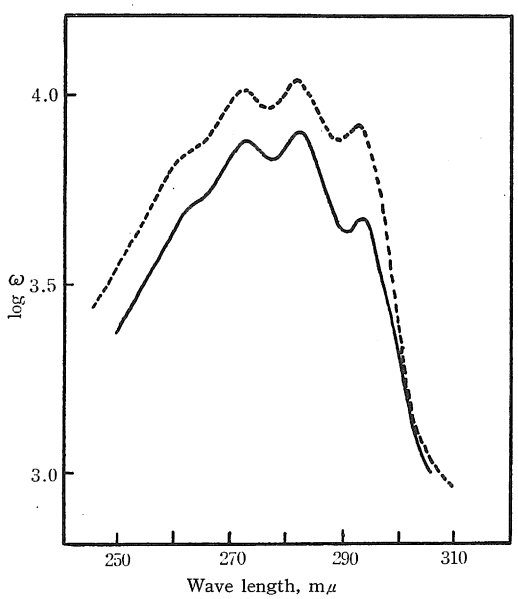

Fig. 6. Ultraviolet absorption spectra of V-II (——) and V-II-A (·····) in Ethanol.

Acetylation of V : Free sterol V (100 mg.) was heated under reflux with pyridine (1 ml.) and acetic anhydride (1 ml.) on a water-bath for 2 hours. The mixture was cooled and poured into the ice water, and the precipitate was filtered and washed with 2% hydrochloric acid and water. The crude acetate m.p. 147-149°C) was recrystallized six times from ethanol, when it had m.p. 158-159°C and $[\alpha]_D^{18}$ -79.8° (V-II-A). It showed a

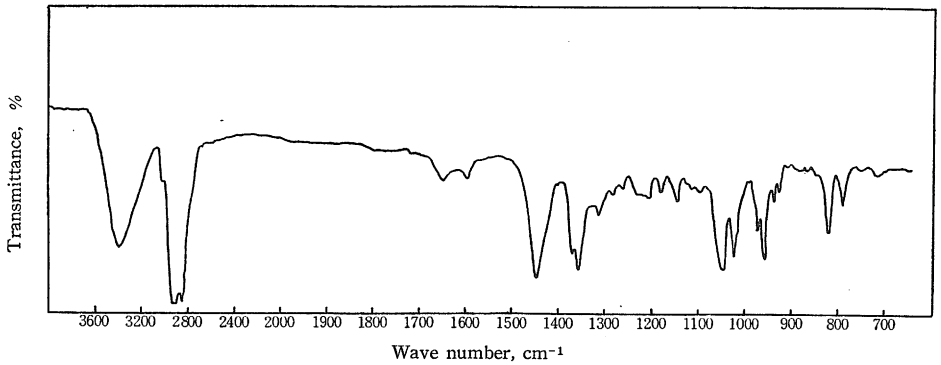

Fig. 7. Infrared spectrum of V-II.

positive Rosenheim reaction and a Liebermann-Burchard reaction. Its ultraviolet absorption spectrum was shown in Fig. 6. It absorbed the ultraviolet ray at 272 ($\log \varepsilon$: 4.00), 282 (4.02) and 294 mμ (3.80). Its infrared spectrum was shown in Fig. 8.

V-Benzoate : Free sterol V (37 mg) was dissolved in pyridine (0.37 ml.) and to it was added benzoyl chloride (0.025 ml), and the mixture was allowed to stand overnight and then was added water and cooled. The product was filtered and recrystallized three times from ethanol, twice from acetone, giving a benzoate m.p. 149-150°C, $[\alpha]_D^{18}$ -34.8°.

The ether-insoluble portion (Y) which was a brown viscous material was saponified, and the unsaponifiable matter was extracted with ether, and the extract was washed with 2N hydrochloric acid and water, and recrystallized three times from acetone, twice from acetone-ethanol (1 : 1), and finally from ethanol, to give a substance, m.p. 117-118°C, $[\alpha]_D^{18}$ -34.4° (**V-III**). It showed a positive Rosenheim reaction and a Liebermann-Burchard reaction. It absorbed the ultraviolet ray at 272, 282 and 294 mμ. It (10 mg.) was acetylated by refluxing with pyridine and acetic anhydride as above. The acetate was recrystallized four times from acetone, twice from acetone-ethanol (1 : 1), and finally from acetone to give an acetate, m.p. 151-152°C, $[\alpha]_D^{18}$ -74.4°.

Ultraviolet spectra were determined on ethanolic solution with a Hitach double-beam spectrophotometer Model 124 instrument, and infrared spectra were measured with a Japan spectroscopy DS-402 instrument in potassium bromide disk. Mass spectrum was mesured Hitachi RMU-7 instrument. Melting points were taken on the Yanagimoto melting point mesuring apparatus Model MP-S2. The colors under ultraviolet light were observed with wavelength 365-mμ light source. All optical rotations were measured with the samples dissolved in chloroform.

Discussion

The present investigation was undertaken to separate the fluorescent substances in *Phoma Wasabiae.* The incubated mold, which grew on the surface of the liquid culture medium of potato after three weeks, fluoresced a white blue under ultraviolet light. The fluorescent substances were formed in physiologically infected plants. For example, Shirley et al. separated scopoletin (6-methoxy-7-hydroxycoumarin) from potato infected with

root-leaf virus, and Uritani et al. separated scopoletin and umbelliferone (7-hydroxy-coumarin) from the black-rotted sweet potato.[7,8] We imagined, therefore, that these fluorescent substances were coumarins such as scopoletin or umbelliferone, and separated these substances with reference to the procedure of Uritani et al.[8] (separation procedure 1). But the obtained substance was not coumarin, but was a $\Delta^{5,7}$-sterol. The yield of free sterol was 48 mg. (0.04% of dried materials) in the separation procedure 1, and 418 mg. (0.21% of dried materials) in separation procedure 2. Identity of **V-I** and **V-II** was established by comparison of their infrared and ultraviolet spectra (Fig. 4 and 7). Their infrared spectra have the characteristic absorption bands at about 3418 (s.) and 1065 (s.) cm^{-1} (hydroxyl group), and at about 2955, 2869 (v. s.) and 1457 cm^{-1} (metylene and methyl group), and at about 1657 and 1603 (w.) cm^{-1} (double bonds between carbon and carbon atoms), and 1381 cm^{-1} (gem-dimetyl group), they also have strong absorption bands at 1457, 1367 and 966 cm^{-1}, as are shown in Fig. 4 and 7. From the result of the mass spectrum of **V-II**, its molecular weight was 396. The ultraviolet absorption spectrum showed absorption peaks at 272, 282 and 294 mμ, therefor, it has conjugated double bonds at 5 : 6 and 7 : 8 positions in ring-B.[9] The infrared spectrum of the acetate (**V-II-A**) has the characteristic absorption bands at 1735 cm^{-1} (carbonyl group), and 1240 cm^{-1} (-C-O- group), and it also has absorption bands at 3415 (w.) and 1035 (s.) cm^{-1} (Fig. 8). Therefor, **V-II-A** is estimated to be mono-acetate, and

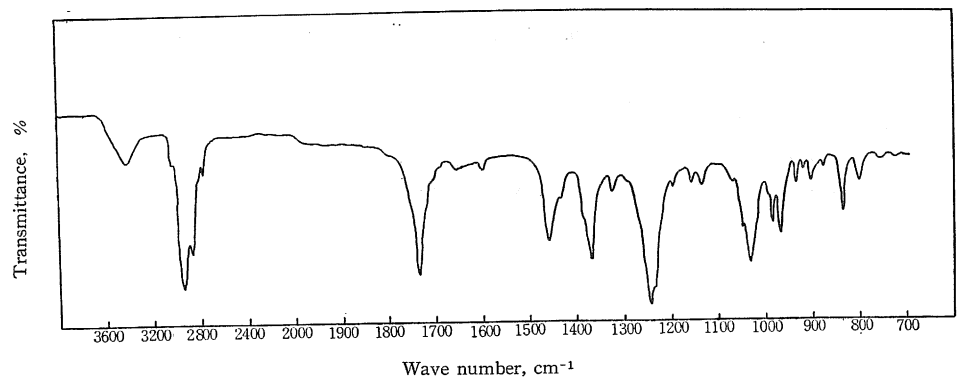

Fig. 8. Infraved spectrum of **V-II-A**.

from the disappearance of the absorption band at 1657 cm^{-1} is presumed that the acetylated hydroxyl group is a neighboring hydroxyl group of a double bond between carbon and carbon atoms. It seems that another hydroxyl group is difficult to be acetylated by the above method.

On the basis of these data, it seemed resonable to assume that **V-II** is a sterol with unsaturated divalent alcoholic hydroxyl groups. Since the estimated molecular formula is $C_{27}H_{40}O_2$, it seems that the side chain of 17-position has two double bonds. A sterol with such a structure is particularly interesting in a medical science.

Summary

The investigation was undertaken in order to separate the fluorescent substances in *Phoma Wasabiae*. The components of the fluorescent substances contained predominantly

$\Delta^{5,7}$-sterols. The $\Delta^{5,7}$-sterol (**V-II**) had m.p. 136-137°C, $[\alpha]_D^{18}$ -112.8°. From the ultraviolet, infrared and mass spectra, it is presumed that the C_{27}-sterol has two hydroxyl groups, and two double bonds at 5 : 6 and 7 : 8 in ring-B, and two double bonds in its side chain at 17-position. The mono-acetate of it had m.p. 158-159°C, $[\alpha]_D^{18}$ -79.8°. The other $\Delta^{5,7}$-sterol (**V-III**) which was separated from the unsaponifiable portion had m.p. 117-118°C, $[\alpha]_D^{18}$ -34.4°. About the molecular structure, we will describe in latter reports.

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