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Cercosporin. A Pigment of *Cercosporina Kikuchii Matsumoto et Tomoyasu.* I. Cultivation of Fungus, Isolation and Purification of Pigment

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A pigment, cercosporin, has been isolated in good yield from the cultured mycelia of *Cercosporina Kikuchii M. et T.*, a fungus known as a pathogen of "soy bean purple speck disease." Its molecular formula is $C_{30}H_{28}O_{10}$, and the presence of two methoxy, several acidic hydroxyl groups and the quinoid system has been established. Conditions for the culture of the fungus and the isolation, purification and general properties of the pigment are described.

In 1924 Kikuchi[1] showed that the "soy bean purple speck disease" which had been considered to be an hereditary disease, was caused by a fungus belonging to the *Cercospora* species. In 1925 Matsumoto and Tomoyasu[2,3] confirmed Kikuchi's observation and the fungus was subsequently named *Cercosporina Kikuchii Matsumoto et Tomoyasu.* Although pathological and agronomical studies of this fungus have been reported, no attention has been paid to the pigments produced.

The authors have been able to isolate a deep red pigment from the dried mycelia of *Cercosporina Kikuchii M. et T.* cultured on a malt extract–peptone–glucose medium.

From the fact that this pigment dissolves in aqueous alkali with a green color, and in a reduced state it shows a bright yellow color with intense green fluorescence, it can be assumed that the pigment has a chromophoric system which differs remarkably from those of pigments which have so far been isolated from other fungi and lichens. Therefore "cercosporin" is proposed as the name of this pigment. A pigment isolated from infected soy beans was found to be identical with cercosporin. The present paper deals with the cultivation of *Cercosporina Kikuchii M. et T.* and the isolation, purification and general properties of cercosporin.

Considerable difficulty was encountered in the analysis and the assignment of a molecular formula to this substance since traces of the solvent of recrystallization were held tenaciously. The solvent was removed for the most part by drying the crystals at high temperature under high vacuum for a long time. After repeated purification of the crystals, the analysis indicated that cercosporin has a molecular formula of $C_{30}H_{28}O_{10}$; a molecular weight of 550–560 was obtained by Rast's method (with camphor as the solvent) and 520–550 by the Barger–Akiya method[4] (with azobenzene as the standard and pyridine as the solvent). The Signer–Clark vapor pressure method[5] gave an inconsistent value owing to the limited solubility of cercosporin in chloroform and other volatile solvents, and the retention of solvent by the crystals as mentioned above.

(1) R. Kikuchi, "The Scientific Researches of the Alumni Association of the Utsunomiya Agricultural College (Japan)," Vol. I, 1924, p. 7–25.

(2) R. Tomoyasu, "The Scientific Researches of the Alumni Association of the Morioka Agricultural College (Japan)," Vol. II, 1925, p. 53–73.

(3) J. Matsumoto and R. Tomoyasu, *Ann. phytopathological Soc. Japan*, **1**, No. 6 (1925).

(4) H. Akiya, *J. Pharm. Soc. Japan.*, **57**, 967 (1937).

(5) E. P. Clark, *Ind. Eng. Chem., Anal. Ed.*, **13**, 820 (1941).

Cercosporin contains two methoxy groups (Zeisel 11.6%) and the C-methyl determination by the Kuhn–Roth method suggests the presence of several C-methyl groups (4.7%). Reduction of cercosporin with zinc in glacial acetic acid or hydrogenation in the presence of Adams' catalyst gave a bright yellow solution with an intense green fluorescence; reduction with sodium hyposulfite or zinc dust in alkali gave an orange solution with a yellow fluorescence. The reduced solution was reversibly oxidized by air to give a solution of the original color; this suggests the presence of a quinoid system.

Cercosporin is optically active and shows a rotation of $[\alpha]^{20}_{7000}$ +470° which changes on heating in a high boiling solvent, such as toluene, or fusing the crystals at the melting point to a value of −152°. A levorotatory isomer, named isocercosporin, is separated from the solution by column chromatography on calcium hydrogen phosphate. It shows a rotation of $[\alpha]^{20}_{7000}$ −826° and reisomerizes on heating to give an equilibrium mixture ($[\alpha]^{20}_{7000}$ −152°) of approximately equal amounts of cercosporin and isocercosporin. Isomerization at low temperatures (below 50°) occurs to some extent; however, the higher the temperature, the more rapid is the isomerization. It is necessary, therefore, to avoid temperatures above 50° during the treatment of cercosporin. Both isomers are more stable in the crystalline state than in solutions. Thus, when crystalline cercosporin was heated at 130° for 30 hours under reduced pressure, no change was observed. Isocercosporin which is less stable was completely isomerized by heating at 130° for 6 hours; however, it remained unchanged at 100° for 30 hours.

Experimental

Culture media.—*Cercosporina Kikuchii M. et T.* was grown in the following liquid media: (a) Czapeck–Dox, (b) Pfeffer, (c) Raulin–Thom, (d) potato extract–glucose and (e) malt extract. (Extract 50 g. of malt with 1 liter of boiling water for 30 minutes; add 3 g. of peptone and 30 g. of glucose to the filtrate.) The sterilized medium was inoculated with fungus from medium e-agar slope and incubated at 25° for 2 weeks. Poor results were obtained with media a and b, but the fungus grew quite well on c, d and e. As e gave the highest yield of crude cercosporin (79 mg./g. dry fungus), it was adopted as the culture medium. The mycelia were harvested, washed with water and dried at room temperature.

Isolation and Purification of Cercosporin.—Dried mycelia (190 g.) obtained from 13 l. of culture medium were crushed and extracted with ether in a Soxhlet apparatus. After few hours deep red crystals of cercosporin began to separate in the receiving flask. The separated crystals were collected daily since cercosporin gradually isomerizes

to isocercosporin when it is refluxed for a long time even at a low temperature. The extraction was continued until the color of the pigment had disappeared from the extract. The total yield of crystals was about 34 g.

This crude cercosporin (800 mg.) was dissolved in chloroform and chromatographed on 250 g. of dehydrated calcium hydrogen phosphate (tube 37 × 500 mm.). (Superheating should be avoided in the dehydration of the calcium hydrogen phosphate.) The pigment adsorbed on the top of the column was developed with chloroform containing 0.5% of methanol. A small amount of a yellow substance and isocercosporin formed during extraction were discarded, and the developing solvent was changed to chloroform containing 1.5% of methanol. A dark red band of cercosporin was eluted, leaving a small amount of brown impurity at the top of the column. The eluted solution was concentrated *in vacuo* below 50° and kept overnight in an ice-box. Pure cercosporin separated as prisms (yield 720 mg.) which were recrystallized from chloroform and benzene.

Isolation of Pigment from Infected Soy Beans.—The epidermis (450 mg.) obtained from 30 grains of infected soy beans, was soaked in 8 ml. of glacial acetic acid at room temperature for 3 days; the pigment dissolved completely in the acetic acid. Ether (50 ml.) was then added to the solution which was washed several times with water to remove the acetic acid. The ether layer was dried and evaporated. When subjected to paper chromatography, the resulting pigment showed the same R_f value (0.61) as the cercosporin obtained from cultured mycelia.

General Properties of Cercosporin.—The samples for elementary analysis and measurement of molecular weight were recrystallized from benzene–chloroform, or alcohol, and dried at 130° (0.001 mm.) for 32 hours. Cercosporin contains no nitrogen, chlorine, sulfur or phosphorus and melts at 241° without sublimation or decomposition. $[\alpha]^{20}_{7000}$ +470° (*c* 0.5, chloroform); λ_{max}^{MeOH} 223, 260, 271, 275 and 470 mμ, ($E^{1\%}_{1 cm.}$ 940, 638, 652, 650 and 498); λ_{max} (in 1 *N* NaOH) 253, 299, 478, 610, 620 and 645 mμ, ($E^{1\%}_{1 cm.}$ 535, 802, 562, 263, 257 and 375). Major bands in the infrared absorption spectrum (KBr window): 3400m, 2940m, 1619vs, 1585vs, 1554vs, 1455m, 1428m, 1395w, 1348w, 1315m, 1268s, 1223m, 1170s, 1145m, 1113m, 1075m, 1055m, 1017m, 978w, 938w, 921w, and 860m cm.$^{-1}$; bands in the 1700–1550 cm.$^{-1}$ region (in chloroform): 1614vs, 1583s and 1551 cm.$^{-1}$ (in tetrahydrofuran): 1615, 1585 and 1651 cm.$^{-1}$.

Anal. Calcd. for $C_{28}H_{22}O_8(OCH_3)_2$ (548.52): C, 65.69; H, 5.15; OCH_3, 11.32. Found: C, 65.50; H, 4.83; OCH_3, 11.61 (from chloroform–benzene). C, 65.67; H, 4.85; OCH_3, 11.94 (from alcohol); C–CH_3 (by Kuhn–Roth), 4.70.

The solubilities of cercosporin in aqueous solutions are as follows: it is insoluble in sodium hydrogen carbonate, partially soluble in sodium carbonate, readily soluble in ammonia and very soluble in dilute alkali giving a clear green solution. It is readily soluble in pyridine, dioxane, chloroform, alcohol and acetone, soluble in ether and benzene, and insoluble in petroleum ether, its solution in concentrated sulfuric acid has a stable purple-blue color and shows no fluorescence; when water is added, a black-purple precipitate is formed. Cercosporin gives a red color with ferric chloride and a green color with magnesium acetate[6] in alcohol solution.

Paper chromatography was carried out on one-dimensional paper strips (2 × 15 cm.) which were developed (ascending flow) with the upper layer of a benzene–glacial acetic acid–water mixture (2:1:1). Cercosporin shows a spot with an R_f value of 0.61.

Isomerization of Cercosporin.—Cercosporin (200 mg.), dissolved in 40 ml. of toluene, was refluxed for 15 minutes. The reaction was followed by paper chromatography. Soon after the refluxing was begun, a new spot due to the formation of isocercosporin (R_f 0.74) appeared in addition to the spot due to cercosporin. The isomerization reached equilibrium within 15 minutes with equal amount of the two isomers present. On cooling purple-black crystals separated, each of which contained the same amount of cercosporin and isocercosporin. These "mixed crystals" were also obtained when cercosporin was fused at its melting point (241°). To separate isocercosporin from this mixture, it was dissolved in chloroform and chromatographed on calcium hydrogen phosphate with chloroform containing 0.5% of methanol as the developing solvent. The eluted isocercosporin was concentrated *in vacuo*. When small amount of petroleum ether was added, isocercosporin separated as dark red prisms which were recrystallized from chloroform and benzene containing a small amount of petroleum ether, and dried at 100° (0.001 mm.) for 32 hours. λ_{max}^{MeOH} 223, 260, 267, 329, 470 and 475 mμ, ($E^{1\%}_{1 cm.}$ 800, 670, 70, 544 and 560), λ_{max} (in 0.1 *N* NaOH) 256, 299, 472, 610, 635 and 645 mμ, ($E^{1\%}_{1 cm.}$ 472, 755, 475, 202, 245 and 316); Major bands in the infrared absorption spectrum (KBr window): 3400m, 2940m, 1619vs, 1584s, 1554s, 1462m, 1434m, 1397w, 1349w, 1319w, 1279vs, 1227m, 1174s, 1154m, 1119w, 1076m, 1054m, 1024s, 981w, 937w, 920m and 863m cm.$^{-1}$.

Anal. Calcd. for $C_{28}H_{22}O_8(OCH_3)_2$: C, 65.69; H, 5.15; OCH_3, 11.32. Found: C, 65.64; H, 5.25; OCH_3, 11.79.

Isocercosporin dissolves more readily in chloroform, benzene and alcohol than cercosporin; its solubility in carbonate and alkali solutions is about the same; it gives the same color reactions with ferric chloride and magnesium acetate.

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(6) Color reaction for hydroxyquinones; S. Shibata, *J. Pharm. Soc. Japan.*, **61**, 320 (1941).

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Cercosporin. A Pigment of *Cercosporina Kikuchii Matsumoto et Tomoyasu.* II. Physical and Chemical Properties of Cercosporin and its Derivatives

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Several acetyl and methyl derivatives of cercosporin were prepared; the results indicate that cercosporin contains two quinoid carbonyl, two phenolic hydroxyl and two alcoholic hydroxyl groups. The infrared, ultraviolet and the visible spectra of these derivatives suggest that cercosporin is a polyhydroxy derivative of a polycyclic quinone having an extended quinone system in which two phenolic hydroxyl groups are present in positions *peri* to the quinone carbonyls. By treatment with concentrated sulfuric acid, cercosporin is converted into noranhydrocercosporin, $C_{28}H_{20}O_8$, which contains no methoxy or alcoholic hydroxyl groups. The physical and chemical properties of noranhydrocercosporin and its derivatives are described.

In a previous paper[1] we reported the isolation of a new crystalline red pigment, cercosporin, from the cultured mycelia of *Cercosporina Kikuchii M. et T.*; $C_{30}H_{28}O_{10}$ was proposed as the molecular formula, and the presence of two methoxy and several

(1) S. Kuyama and T. Tamura, THIS JOURNAL, **79**, 5725 (1957).