Iron Chelating Capability of Physcion, a Yellow Pigment from *Aspergillus ruber*

George W. Engstrom,* Donald J. McDorman, and Michael J. Maroney

A yellow pigment isolated from ether extracts of *Aspergillus ruber* (*Eurotium ruber*) was independently purified and characterized by spectral analysis. Its molecular weight was 284, empirical formula $C_{16}H_{12}O_5$, chemical name 1,8-dihydroxy-3-methoxy-6-methylanthraquinone, and it is identical with physcion (parietin). We report here the preparation of an iron complex that consists of the yellow fungal pigment (physicon) and ferric iron combined in the ratio of 2:1, respectively. The iron complex was dark red brown, soluble in chloroform, and insoluble in water. Elemental analyses and spectral and solubility changes provided evidence that a complex had been formed. The complex decomposes upon treatment with acidified (HCl) methanol. It also changes color and solubility upon being dried with heat (50 °C). To our knowledge, this is the first report about the iron chelation capability of physcion. It is one of the first pigments produced by this fungus and may have a function in iron transport or metabolism of the fungal cell.

INTRODUCTION

The need for iron by microorganisms induces them to secrete chelating agents with high affinity for iron into the surrounding environment. These chelating agents facilitate iron transport into the microbial cell (Neilands, 1974). A species with stronger iron chelating compounds would thus be able to secure the iron it needs from the environment for its growth requirements at the expense of other systems. This has been demonstrated in mammalian systems where competition between the microbial chelate and iron binding proteins (transferrin and lactoferrin) for iron determines the virulence of the bacteria (Sussman, 1974). It has also been demonstrated with blue-green algae in that they are able to dominate other algae because they secrete a stronger iron-selective chelator into their environment (Murphy et al., 1976). Low molecular weight compounds that are known to be involved in microbial iron transport contain hydroxamate or phenolate groups which then form coordination complexes with the ferric ion. Most of the natural hydroxamates have been isolated from fungi and actinomycetes, but a few have also been found in bacteria and green plants (Neilands, 1967). Mycobacteria produce another type of iron chelating compound called mycobactins which have both hydroxamate and phenolate groups in the same molecule. These are the only iron chelators which have good solubility in lipid solvents (Snow, 1970). McCullough and Merkel (1976, 1979) from this laboratory have reported characterization of iron chelating compounds from *Mycobacterium avium* and *Corynebacterium kutscheri*. This interest in iron chelators has led us to look at fungal pigments as a potential source of new iron chelating compounds.

The *Aspergillus glaucus* group is especially rich in pigments (Raper and Fennell, 1965). *Aspergillus ruber* (König, Spieckerman & Bremer) Thom et Church is included in the *A. glaucus* group. In 1934, Gould and Raistrick described yellow, orange, and red crystalline pigments produced by various species of *Aspergillus* in the *A. glaucus* group; these were named flavoglaucin, auroglaucin, and rubroglaucin. Cruickshank et al. (1938) showed that rubroglaucin was not homogeneous, and Ashley et al. (1939) showed that the compound was actually composed of physcion ($C_{16}H_{15}O_5$) and erythroglaucin ($C_{16}H_{12}O_6$) which are both anthraquinone derivatives. Four other papers have been published in the last 5 years which provide new spectral data for the chemical characterization of physcion (Suemitsu et al., 1975; Höfle, 1977; Podojil et al., 1979; Bachmann et al., 1979). In this paper we report the iron chelating capability of the fungal pigment physcion and our method for its isolation and purification.

The National Animal Disease Center, Federal Research, Science and Education Administration, U. S. Department of Agriculture, Ames, Iowa 50010 (G.W.E. and D.J.M.), and the Department of Chemistry, University of Washington, Seattle, Washington 98195 (M.J.M.).

EXPERIMENTAL SECTION

Materials. Sources of chemicals and supplies were as follows: malt extract and Noble agar from Difco Labs, Detroit, MI; potassium bromide from Harshaw Chemical Co., Cleveland, OH; silica gel for dry column chromatography from ICN Nutritional Biochemicals, Cleveland, OH; silica gel H from Brinkmann Instruments, Des Plaines, IL; Bio-Beads S-X8 from Bio-Rad Laboratories, Richmond, CA.

Culture Methods. Fungal samples isolated from moldy bread were transferred to malt–salt agar plates that had been prepared as follows: equal volumes of solution A (12 g of malt extract and 12 g of agar dissolved and suspended in 300 mL of distilled water) and solution B (36 g of sodium chloride dissolved in 300 mL of distilled water) were mixed together after autoclaving at 15 psi for 30 min. Pure cultures were isolated and identified as *A. ruber*. A predominantly cleistothecial isolate was selected and maintained on malt-salt agar plates with transfer every 30 days. After the culture had grown on the agar plates for 30 days, areas of the agar covered with cleistothecia and encrusted hyphae (as opposed to asexual sporulating areas) were harvested and added to culture flasks containing 200 mL of sterile 2% malt-salt medium. The culture flasks were incubated at room temperature (25 °C) in the dark for 30 days since these conditions favor increased growth and pigment production (Mislivec et al., 1975).

Pigment Extraction. Diethyl ether (200 mL) was added to each culture flask containing fungal mat and liquid medium and was allowed to stand with occasional mixing for 24 h. The ether layer from each flask was filtered through Whatman No. 42 filter paper, all were combined, and solvent was removed by flash evaporation.

Chromatography Methods. Thin-layer chromatography (TLC) plates (20 × 20 cm) were prepared with silica gel H at a thickness of 0.3 mm with an adjustable Desaga applicator distributed by Brinkmann Instruments, Inc., Des Plaines, Il, and dried at 110 °C for 1 h or overnight. An equal volume mixture of hexane and ethyl acetate was used as the TLC solvent system. TLC plates were de-

0021-8561/80/1428-1139$01.00/0 © 1980 American Chemical Society

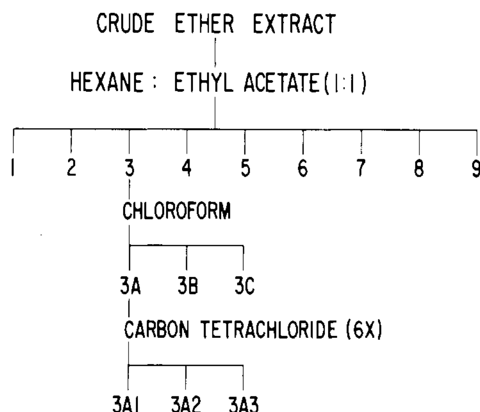

Figure 1. Flow chart for purification of physcion, a yellow pigment from *Aspergillus ruber*.

veloped by the ascending method at room temperature in unlined unequilibrated glass tanks in a fume hood. Resolved bands were scraped from the TLC plates and eluted with chloroform, and the solvent was removed by flash evaporation. Long columns (85 × 0.9 cm) were set up with Woelm silica gel for dry column chromatography (activity III). The concentrated ether extract was dissolved in a minimum volume of ether. One milliliter of this extract was transferred to a 25-mL flask containing 1.5 g of Woelm silica gel and mixed by flash evaporation. This procedure resulted in adsorption of the sample onto the silica gel which was then placed on top of the column. This column was eluted with a 50/50 mixture of hexane–ethyl acetate which resolved the sample into ten fractions (Figure 1). The yellow pigment, physcion, was found in fractions 2 and 3. It was purified by TLC on silica gel H with chloroform as the developing solvent. The sample was resolved into three bands; the fastest moving band, designated 3A (R_f 0.61), contained physcion and erythroglaucin. Physcion was purified by TLC on silica gel H with carbon tetrachloride (multiple development). This fraction was resolved into three bands: a red orange band, designated 3A1 (R_f 0.17), erythroglaucin), the yellow band, designated 3A2 (physcion) (R_f 0.09), and a band at the origin. Chromatographic purity of this preparation was checked by TLC with silica gel H and three different solvents, (1) carbon tetrachloride, (2) carbon disulfide, and (3) trichloroethylene.

The chloroform-soluble physcion–iron complex was purified by gel filtration chromatography on Bio-Beads S-X8 in chloroform, and the purity was checked by TLC on silica gel H with methanol or chloroform as the developing solvent.

Melting Point. Melting point determinations were made in sealed capillary tubes heated to give a temperature increase of 1 °C/min.

Elemental Analyses. Carbon, hydrogen, oxygen, and iron analyses were determined by the Galbraith Labs, Knoxville, TN.

Spectral Analyses. Ultraviolet and visible absorption spectra were obtained with a Beckman DB spectrophotometer. Infrared spectra were obtained with a Beckman IR-9 spectrophotometer, and the samples were prepared in KBr pellets. The molecular weight, elemental composition, and fragmentation patterns were determined by electron ionization with a Model MS- double-focusing, high-resolution mass spectrometer of Associated Industries at Shrader Analytical and Consulting Labs, Detroit, MI. The proton and natural abundance carbon-13 nuclear magnetic resonance (NMR) spectra were determined with a Varian T60A spectrometer and a Bruker HX-90 NMR spectrometer interfaced with a Nicolet Model 1080 computer. Both proton and carbon-13 NMR spectra were run in deuteriochloroform with an internal standard of tetramethylsilane (Me_4Si).

Iron Chelation. Compound 3A2 (physcion) (28.4 mg, 0.100 mmol) was dissolved in 10–20% chloroform in methanol (300 mL). Upon addition of potassium hydroxide (16.8 mg, 0.300 mmol) the color of the solution changed from yellow to red. When ferric chloride hexahydrate (27.0 mg, 0.100 mmol) was added, the solution changed to a reddish brown color and became turbid. The volume of the solution was reduced to 25 mL by flash evaporation and a dark red brown material precipitated. The solvent was carefully decanted, and the precipitate was washed several times by alternately suspending it in methanol, centrifuging, and decanting the solvent. This dark red-brown precipitate, still wet with methanol, was soluble in chloroform, giving an intense dark red solution. After the precipitate was dried at 50 °C under partial vacuum, the material turned to a purple color and was insoluble in chloroform and methanol. Elemental analyses were obtained on this purple material.

RESULTS

Chemical Identification of Physcion. The material contained in fraction 3A2 (Figure 1) formed long yellow-orange needlelike crystals from acetone. These crystals had a sharp melting point at 204–205 °C which was in good agreement with that reported by Shibata and Natori (1953) for an authentic sample of physcion (parietin). It was soluble in chloroform, diethyl ether, ethyl acetate, 1,4-dioxane, tetrahydrofuran, and acetone and had limited solubility in methanol, ethanol, carbon tetrachloride, acetonitrile, and hexane. It was insoluble in water. Anal. Calcd: C, 67.60; H, 4.26; O, 28.14. Found: C, 67.43; H, 4.51; O, 28.30.

The UV spectrum had absorption maxima at 223, 248, 253, 264, and 285 nm (methanol); IR (KBr pellet) 3300–3600, 2930, 2855, 2360, 1680, 1625, 1565, 1485, 1370, 1320, 1300, 1275, 1225, 1160, 1105, 1035, 980, 930, 900, 870, 855, 775, 740, 670, 635 615, and 555 cm^{-1}; MS, molecular ion peak at m/e 284; fragmentation ions [(relative intensity, composition, fragments lost from molecular ion) m/e 284 (100, $C_{16}H_{12}O_5$, M^+), 256 (5, $C_{15}H_{12}O_4$, M − CO), 255 12, $C_{15}H_{11}O_4$, M − CO − H), 254 (6, $C_{15}H_{10}O_4$, M − CO − 2H), 241 (8, $C_{14}H_9O_4$, M − CO − CH_3), 227 (6, $C_{14}H_{11}O_3$, M − 2CO − H), 226 (6, $C_{14}H_{10}O_3$, M − 2CO − 2H), 213 (5, $C_{13}H_9O_3$, M − 2CO − CH_3), 185 (5, $C_{12}H_9O_2$, M − 3CO − CH_3) 139 (5, $C_{11}H_7$), 128 (6, $C_{10}H_8$); ^{13}C NMR C-1 δ 165.5, C-2 108.2, C-3 166.8, C-4 107.1, C-5 121.3, C-6 148.5, C-7 124.5, C-8 162.0, C-9 191.0, C-10 173.3, C-11 110.6, C-12 135.6, C-13 133.6, C-14 113.9, O–Me 56.1, *C*–Me 22.1, ^1H NMR (60 MHz, $CDCl_3$): δ 2.44 (s, 3 H, aromatic Me), 3.94 (s, 3 H, aromatic OMe), 6.69 (d, J = 1.8 Hz, 1 H, aromatic), 7.07 (br s, 1 H, aromatic), 7.35 (d, J = 1.8 Hz, 1 H, aromatic), 7.62 (br s, 1 H, aromatic), 12.12 (s, OH, aromatic, chelated), 12.32 (s, OH, aromatic, chelated). The spectral data were in good agreement with those published by Bachmann et al. (1979) and Höfle (1977).

Chemical Properties of the Physcion–Iron Complex. Elemental analyses of the complex gave the following results: Calcd for a complex consisting of 2 molecules of physcion and 1 of ferric iron: C, 61.53; H, 3.88; Fe, 8.96. Found: C, 58.04; H, 3.93%; Fe, 8.16%. During the chelation reaction, the visible spectrum changes from an absorption maxima at 504 nm for the deprotonated form of physcion (red color) to 462 nm for the complex; IR (KBr pellet) 3300–3600, 2925, 2855, 2365, 1738, 1670, 1600, 1570, 1495, 1470, 1435, 1390, 1367, 1319, 1275, 1227,

1173, 1055, 1000, 880, 755, 680, and 550 cm^{-1}. The complex decomposes upon treatment with acidified (HCl) methanol to give the original reactants, physcion and ferric chloride.

DISCUSSION

Physcion (1,8-dihydroxy-3-methoxy-6-methylanthraquinone) has been isolated from a variety of organisms besides fungi including *Xanthoria* and *Caloplaca* and different types of plants (Ashley et al., 1939). Physcion is one of the first pigments synthesized by *A. ruber*, which gives the young culture its yellow color; physcion may play a part in iron metabolism. Results of our iron chelation experiments including elemental analysis of the reaction product and spectral and color differences between reactants and products provide evidence that physcion (3A2) does form a complex with ferric iron in methanol–chloroform mixtures. It is important to note that the iron complex is reactive in some way, as evidenced by the fact that drying with heat changes the complex. There is a change in color (red brown to purple) and in solubility in chloroform. This is the basis upon which we propose that physcion (3A2) may have a function in iron transport and or metabolism in the fungal cells of *A. ruber*. Siderophores are microbial high affinity iron carriers or chelators. Although this fungal pigment may not meet all the criteria needed to qualify as a siderophore (National Research Council, 1979), it could be one of the many other molecular species which transport iron.

Recently Akers et al. (1980) reported that α, β, γ-thujaplicin and certain other planar cyclic hydroxy ketones can function as iron-transport compounds for strains of *Salmonella typhimurium* which were unable to synthesize their own normal iron transport agent (enterobactin). They suggested that these compounds occupy a chemical "middle ground" between the hydroxamate and catechol classes of siderophores. Our results suggest that anthraquinones with two hydroxyl groups on ring positions adjacent to the same quinone carbonyl (i.e., 1,8-dihydroxy) may be another example of a planar cyclic α-hydroxy keto compound with iron-transport function or capability. Since physcion and its iron complex are soluble in chloroform (lipid solvent), they could exist and function in the cell membrane in a manner similar to that described by Raymond and Carrano (1979) for one of their iron-transport mechanisms.

It is fairly likely that the complex is not a monomer but rather a dimer or polymer, especially in view of the nature of the ligand (hydroxyl and carbonyl oxygens) and the fact that there are water molecules available from the ferric chloride hexahydrate.

Two papers have been published recently reporting that physcion given by intraperitoneal injection produces some cytotoxic effects in mice, 1-day-old cockerals (Bachmann et al., 1979), and HeLa cells (Podojil et al., 1979). Physcion was not toxic in the mouse or day-old cockeral when given orally. Bachmann et al. (1979) felt that the mutagenic action of many anthraquinones was of greater concern, but concluded that the small amount that might be ingested from contamination does not represent a risk to human health.

ACKNOWLEDGMENT

We are grateful to Dr. W. G. McCullough and Dr. Norman J. Rose for helpful discussions.

LITERATURE CITED

Akers, H. A.; Abrego, V. A.; Garland, E. *J. Bacteriol.* **1980**, *141*, 164.

Ashley, J. N.; Raistrick, H.; Richards, T. *J. Biochem. (Tokyo)* **1939**, *33*, 1291.

Bachmann, M.; Lüthy, J.; Schlatter, C. *J. Agric. Food Chem.* **1979**, *27*, 1342.

Cruickshank, J. H.; Raistrick, H.; Robinson, R. *J. Chem. Soc.* **1938**, 2056.

Gould, B. S.; Raistrick, H. *Biochem. J.* **1934**, *28*, 1640.

Höfle, G. *Tetrahedron* **1977**, *33*, 1963.

McCullough, W. G.; Merkal, R. S. *J. Bacteriol.* **1976**, *128*, 15.

McCullough, W. G.; Merkal, R. S. *J. Bacteriol.* **1979**, *137*, 243.

Mislivec, P. B.; Dieter, C. T.; Bruce, V. R. *Mycologia* **1975**, *67*, 1187.

Murphy, T. P.; Lean, D. R. S.; Nalewajko, C. *Science* **1976**, *192*, 900.

National Research Council, Division of Medical Sciences. "Iron"; University Park Press: Baltimore, Md., 1979.

Neilands, J. B. *Science* **1967**, *156*, 1443.

Neilands, J. B. In "Microbial Iron Metabolism"; Academic Press: New York, 1974.

Podojil, M.; Sedmera, P.; Vokoun, J.; Betina, V.; Barathova, H.; Durackova, Z.; Horakova, K.; Nemec, P. *Folia Microbiol.* **1979**, *23*, 438.

Raper, K. B.; Fennell, D. I. "The Genus Asperillus"; Williams and Wilkins: Baltimore, MD, 1965.

Raymond, K. N.; Carrano, C. J. *Acc. Chem. Res.* **1979**, *12*, 183.

Shibata, S.; Natori, S. *Pharm. Bull.* **1953**, *1*, 160.

Snow, G. A. *Bacteriol. Rev.* **1970**, *34*, 100.

Suemitsu, K.; Iwai, J.; Kawaguchi, K. *Agric. Biol. Chem.* **1975**, *39*, 2249.

Sussman, M. N. "Iron in Biochemistry and Medicine"; A. Jacobs and M. Worwood, Eds; Academic Press: New York, 1974.

Received for review February 13, 1980. Accepted June 17, 1980. M.J.M. acknowledges support from the University of Washington Graduate School Research Fund.