segments are constantly rotated during the course of the experiment and they are, theoretically, bathed equally with the solution. Identical curvature was observed with deacetylcytochalasin H (Cole et al., 1981). However, in that case, the 10^{-3} M treated coleoptiles, which were inhibited 100%, were also markedly curved.

When the relative inhibitory activities of cytochalasins previously studied are compared, metabolite III was unlike any already tested (Cutler et al., 1980). Metabolite IV resembled the activity of deacetylcytochalasin H at all concentrations except 10^{-6} M (Cole et al., 1981).

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Spectral Identification, X-ray Structure Determination, and Iron-Chelating Capability of Erythroglaucin, a Red Pigment from *Aspergillus ruber*

George W. Engstrom,* Ronald E. Stenkamp, Donald J. McDorman, and Lyle H. Jensen

A red pigment from *Aspergillus ruber* was purified by thin-layer and column chromatography with three different solvent systems and crystallized from acetone. The pigment had a molecular weight of 300 and an empirical formula of $C_{16}H_{12}O_6$. X-ray crystallographic and spectral analysis showed it to be 1,3,5-trihydroxy-3-methoxy-6-methylanthraquinone, identical with the compound previously named erythroglaucin. The molecule crystallizes in space group *Pnma*, a = 23.862 (5) Å, b = 6.547 (1) Å, c = 8.149 (2) Å, and Z = 4. X-ray data include final atomic parameters, thermal ellipsoids, bond lengths, bond angles, and molecular numbering scheme. With ferric iron, the pigment formed a dark blue complex that was insoluble in chloroform, methanol, ether, water, and dimethyl sulfoxide. The IR spectrum of the complex was different in several areas from that of erythroglaucin, and elemental analysis showed it contained 15.70% iron.

A large number of bacterial and fungal pigments including ferrichrome from *Ustilago sphaerogena* (Neilands, 1952), skizokinen from *Bacillus megaterium* (Byers et al., 1967), and ferrineoaspergillin from *Aspergillus ochraceus* Wilh (Maebayashi et al., 1978) are known to form complexes with iron atoms. Our interest in microbial mineral metabolism led us to investigate the structure and iron-chelating capability of pigments from other sources. We selected the fungus *Aspergillus ruber* (König, Spieckerman, and Bremer) Thom et Church because it was one of the *Aspergillus glaucus* group especially rich in pigments (Raper and Fennell, 1965). Gould and Raistrick (1934) reported that various species of the *A. glaucus* group produced yellow, orange, and red pigments called flavoglaucin, auroglaucin, and rubroglaucin, respectively. Later, Ashley et al. (1939) demonstrated that rubroglaucin was a mixture of two pigments: physcion ($C_{16}H_{12}O_5$) and erythroglaucin ($C_{16}H_{12}O_6$).

Recently, we reported on the iron-chelating ability of physcion, a yellow anthraquinone derivative from ether extracts of *A. ruber* (Engstrom et al., 1980). This was the first report of complexes formed between iron and 1,8-dihydroxyanthraquinones. We have now isolated a dark red crystalline pigment from an ether extract of *A. ruber* grown in a liquid medium that contained adequate iron. This report presents data from the identification and X-ray structure determination of the red pigment which demonstrated that it was identical with erythroglaucin (Suemitsu et al., 1977; Bachmann et al., 1979; Podojil et al., 1979). The report also includes data that show the ability of erythroglaucin to form a complex with ferric iron.

The National Animal Disease Center, Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, Ames, Iowa 50010 (G.W.E. and D.J.M), and the Department of Biological Structure, School of Medicine, University of Washington, Seattle, Washington 98195 (R.E.S. and L.H.J.).

EXPERIMENTAL SECTION

Materials. Sources of chemicals and supplies were as follows: potassium bromide from Harshaw Chemical Co. (Cleveland, OH); malt extract and Noble agar from Difco

0021-8561/82/1430-0304$01.25/0

Table I. Atomic Parameters for Nonhydrogen Atoms

	values of positional parameters × 10^4			values of thermal parameters[a] × 10^3					
atom	x	y	z	U_{11}	U_{22}	U_{33}	U_{12}	U_{13}	U_{23}
C(1)	3323 (1)	7500	896 (4)	35 (2)	40 (2)	30 (2)	0	2 (1)	0
C(2)	3862 (1)	7500	1547 (4)	25 (2)	46 (4)	39 (2)	0	3 (1)	0
C(3)	3949 (1)	7500	3211 (4)	26 (2)	45 (2)	41 (2)	0	−2 (1)	0
C(4)	3505 (1)	7500	4300 (4)	34 (2)	45 (2)	28 (2)	0	−8 (1)	0
C(5)	1476 (1)	7500	5359 (4)	39 (2)	37 (2)	26 (2)	0	0 (1)	0
C(6)	918 (1)	7500	4789 (2)	30 (2)	44 (2)	34 (2)	0	4 (2)	0
C(7)	821 (1)	7500	3127 (4)	27 (2)	59 (2)	39 (2)	0	−2 (1)	0
C(8)	1261 (1)	7500	1982 (4)	33 (2)	45 (2)	29 (2)	0	−2 (1)	0
C(9)	2285 (1)	7500	1365 (4)	35 (2)	32 (2)	25 (2)	0	−2 (1)	0
C(10)	2498 (1)	7500	4869 (4)	33 (2)	33 (2)	27 (2)	0	−2 (1)	0
C(11)	1920 (1)	7500	4247 (4)	30 (2)	38 (2)	28 (2)	0	−2 (1)	0
C(12)	1821 (1)	7500	2529 (4)	26 (2)	41 (2)	30 (2)	0	−2 (1)	0
C(13)	2856 (1)	7500	1975 (3)	30 (2)	32 (2)	31 (2)	0	0 (1)	0
C(14)	2967 (1)	7500	3682 (3)	34 (2)	36 (2)	27 (2)	0	−1 (1)	0
C(15)	4621 (2)	7500	5408 (4)	32 (2)	85 (3)	43 (2)	0	−10 (2)	0
C(16)	445 (2)	7500	6010 (4)	37 (2)	67 (2)	39 (2)	0	9 (2)	0
O-C(1)	3279 (1)	7500	−735 (3)	36 (1)	62 (2)	25 (1)	0	2 (1)	0
O-C(3)	4504 (1)	7500	3682 (3)	24 (1)	85 (2)	40 (1)	0	−6 (1)	0
O-C(5)	1550 (1)	7500	6982 (3)	50 (2)	71 (1)	36 (1)	0	2 (1)	0
O-C(8)	1112 (1)	7500	395 (3)	29 (1)	82 (4)	27 (1)	0	−3 (1)	0
O-C(9)	2193 (1)	7500	−144 (2)	35 (1)	62 (2)	22 (1)	0	0 (1)	0
O-C(10)	2595 (1)	7500	6351 (3)	38 (1)	74 (2)	23 (1)	0	−3 (1)	0

[a] Thermal parameters defined by the expression $\exp[-2\pi^2(U_{11}h^2a^{*2} + U_{22}k^2b^{*2} + U_{33}l^2c^{*2} + 2U_{12}hka^*b^* + 2U_{13}hla^*c^* + 2U_{23}klb^*c^*)]$.

Laboratories (Detroit, MI); silica gel for dry column chromatography (Woelm-Activity III) from ICN Nutritional Biochemicals (Cleveland, OH); silica gel H from Brinkmann Instruments (Des Plaines, IL); deuteriochloroform from Aldrich Chemical Co. (Milwaukee, WI).

Methods. Culture methods, pigment extraction, and purification procedures were the same as previously reported (Engstrom et al., 1980).

Elemental and Spectral Analyses. Carbon, hydrogen, and iron analyses were determined by the Galbraith Laboratories (Knoxville, TN). Untraviolet- and visible-range absorption spectra were obtained with a Beckman DK-2A spectrophotometer. Infrared spectra were obtained with a Beckman IR-9 spectrophotometer, and the samples were prepared in KBr pellets. Proton 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. The molecular weight, elemental composition, and fragmentation patterns were determined by electron ionization with an Associated Industries Model MS-30 double-focusing, high-resolution, mass spectrometer at Shrader Analytical and Consulting Laboratories (Detroit, MI).

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

X-ray Structure Determination. Three-dimensional single crystal diffraction data were collected from a crystal of the red pigment of approximate dimensions: 0.16 × 0.21 × 0.50 mm by using a computer-controlled, four-circle diffractometer (λ = 1.54179 Å; ω − 20 scans; scan rate 2 °C/min; 20-s backgrounds on either side of the reflection; 10 standard reflections). Photographs showed orthorhombic symmetry, and the systematic absences ($0kl$; $k + l = 2n$; $hk0$; $h = 2n$) indicated the space group to be $Pnma$ or $Pn2_1a$. A least-squares fit to several centered reflections yielded cell constants of a = 23.862 (5) Å, b = 6.547 (1) Å, and c = 8.149 (2) Å with four molecules in the unit cell; the calculated density, 1.565 g/cm^3, was in the range normally found for crystals of organic compounds. Intensity measurements of the 0,18,0 reflection at various degrees of rotation about its diffraction vector yielded no significant systematic absorption correction. A small radiation decay correction (up to 3%) was obtained from multiple measurements of 10 standard reflections collected at regular time intervals.

The structure was solved in space group $Pnma$ by application of the direct methods program GENTAN (Hall, 1979) and refined by use of the X-RAY system of programs (Stewart, 1976). The hypercentric distribution of normalized structure factors indicated the centrosymmetric space group, but refinement was carried out in both space groups to confirm the assignment. In the initial refinement in $Pnma$, atom type assignments were made on the basis of bond lengths and thermal parameters. The anisotropic thermal parameters for 22 nonhydrogen atoms and isotropic thermal parameter for the hydrogen atoms (unit weights) was reduced to 0.114 for the 1010 reflections in the data set ($R = \sum||F_o| - |F_c||/\sum|F_o|$). At this point, a model suitable for refinement in $Pn2_1a$ was generated by arbitrarily shifting several of the atoms away from the molecular plane. Full matrix least-squares reduced R for the model in the noncentrosymmetric space group to 0.069. Similar refinement in $Pnma$ reduced R to 0.067.

In the early stages of the refinement, five low-order reflections (2,0,0; 0,1,1; 0,2,0; 2,2,0; 0,4,0) were discovered to have overflowed the counter while collecting data. Due to the stacked planar molecules in the structure, these reflections were so much more intense than most of the remaining data that their measurement at low X-ray flux could not be accurately scaled to the other reflections. Furthermore, when R had reached about 0.07, F_c for three moderately intense reflections (2,1,1; 2,3,1; 2,4,0) was found to be considerably less than F_o. Since they could suffer from multiple reflection related to the very intense reflections already noted, they were omitted from the last stage of refinement. Weights of $1/\sigma F$ were applied in the final three cycles, leading to an R of 0.057 for the 1007 reflections remaining in the data set. The final "goodness of fit" was 4.65. Because of the smaller number of parameters in the centrosymmetric space group and the slightly lower R value, we believe the structure is adequately described in space group $Pnma$.

Tables I and II list the final atomic parameters and Figure 1 shows the molecular numbering scheme, thermal ellipsoids, bond lengths, and bond angles. Structure factor

Table II. Atomic Parameters for Hydrogen Atoms

atom	positional parameters × 10^3 x	y	z	thermal parameters[a] × 10^2, U
H-C(2)	418 (1)	750	76 (4)	43 (10)
H-C(4)	355 (1)	750	536 (4)	35 (9)
H-C(7)	41 (1)	750	268 (3)	26 (8)
H-C(15)	497 (2)	750	558 (5)	86 (14)
H-C(15)	443 (1)	611 (4)	594 (3)	67 (8)
H-C(16)	5 (3)	750	568 (6)	127 (18)
H-C(16)	47 (1)	638 (5)	681 (3)	95 (10)
H-O-C(1)	293 (2)	750	−106 (5)	79 (16)
H-O-C(8)	136 (2)	750	−29 (5)	55 (11)

[a] Thermal parameters defined by the expression $\exp(-8\pi^2 U^2 \sin^2 \theta/\lambda^2)$.

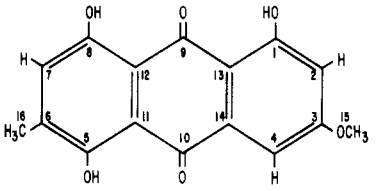

Figure 1. Chemical structure of erythroglaucin.

tables are available (see paragraph at end of paper regarding supplementary material).

Ferric Iron Chelation. One equivalent of purified red pigment was dissolved in 20% chloroform in methanol, and 3 equiv of potassium hydroxide (in methanol) was added to deprotonate the three hydroxyl groups. A solution of ferric chloride hexahydrate (in methanol) was added to give a molar ratio of red pigment to ferric chloride hexahydrate (300 to 270). The volume of the solvent was reduced by flash evaporation. When an insoluble product formed, it was transferred to special tubes for centrifugation and washed 3 times with fresh methanol prior to use for elemental analysis.

RESULTS

Identification of Erythroglaucin. Fraction 3A1 contained a pigment which formed flat red crystals from acetone. It had a sharp melting pont at 205–206 °C. The red pigment, 3A1, was soluble in chloroform, benzene, methylene chloride, tetrahydrofuran, and 1,4-dioxane. It was less soluble in ether, ethyl acetate, acetone, acetonitrile, and carbon tetrachloride, sparingly soluble in methanol, ethanol, hexane, and cyclohexane, and insoluble in water.

Results obtained from elemental analysis were as follows: found, 64.02% C and 4.09% H; calculated, 63.99% C and 4.03% H. Fragmentation ions from the mass spectrum, their molecular weight (m/e), relative intensity, empirical formula, and fragments lost from the molecular ion were as follows: 300 (100, $C_{16}H_{12}O_6$, M^+), 285 (1.3, $C_{15}H_9O_6$, M − CH_3), 271 (3.2, $C_{15}H_{11}O_5$, M − CO − H), 257 (6.7, $C_{14}H_9O_5$, M − CO − CH_3), 243 (1.1, $C_{14}H_{11}O_4$, M − 2CO − H), 229 (2.4, $C_{13}H_9O_4$, M − 2CO − CH_3), 201 (1.3, $C_{12}H_9O_3$, M − 3CO − CH_3).

The proton NMR spectrum of the red pigment (3A1) was run in deuteriochloroform with an internal standard of tetramethylsilane (Me_4Si) (Figure 2). It contained six relatively small peaks, three (6.701, 7.125, and 7.405 ppm) representing aromatic protons and three (12.268, 12.334, and 13.290 ppm) hydroxyl protons. The spectrum also contained two larger peaks, an aromatic *C*-methyl group at 2.359 ppm (three protons) and an aromatic *O*-methyl group at 3.945 ppm (three protons). The peak at 7.258 ppm was the deuteriochloroform solvent and the peak at 0 ppm was the reference Me_4Si.

An ethanol solution of the red pigment (erythroglaucin) produced a UV spectrum with absorption maxima at 232, 254, and 283 nm and in the visible spectrum at 4.65 (sh), 493, 510 (sh), and 523 nm. The infrared spectrum (KBr pellet) had absorption bands at 3600–3200, 2930, 2370, 1645, 1600, 1495, 1445, 1390, 1310, 1260, 1230, 1210, 1190, 1180, 1165, 1135, 1090, 1025, 990, 950, 920, 875, 855, 810, 755, 710, 640, 620, and 565 cm^{-1}. The chemical structure of the red pigment (erythroglaucin) is presented in Figure 1.

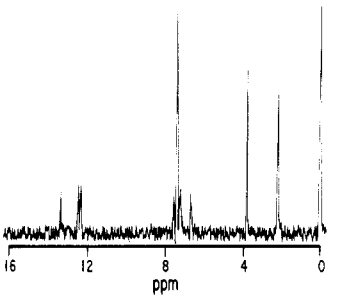

Figure 2. The 60-MHz proton NMR spectrum of the red pigment (erythroglaucin) in $CDCl_3$ at 25 °C. The chemical shifts (ppm) are measured from a tetramethylsilane (Me_4Si) internal standard.

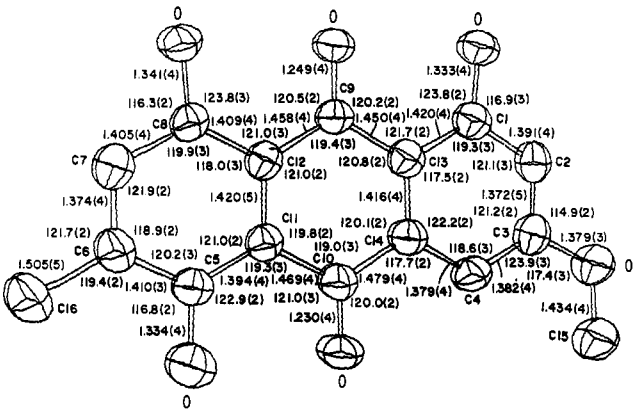

Figure 3. Atomic numbering scheme, bond length, bond angles, and thermal ellipsoids of red fungal pigment (erythroglaucin).

Erythroglaucin-Iron Complex. The addition of 1 equiv of ferric chloride hexahydrate to a solution containing 1 equiv of erythroglaucin and 3 equiv of potassium hydroxide resulted in the formation of a dark blue complex that was insoluble in methanol–chloroform, ether, water, and dimethyl sulfoxide. The IR spectrum had the following peaks: 3700–3100, 2370, 1635, 1560, 1505, 1400, 1320, 1245, 1165, 1105, 1040, 1000, 960, 925, 890, and 780 cm^{-1}. Elemental analysis values were 15.70% Fe, 53.75% C, and 3.17% H. It decomposed in acid (HCl) to give the original reactants ferric chloride and erythroglaucin.

DISCUSSION

The melting point of the crystalline red pigment (205–206 °C) was in good agreement with those reported by Ashley et al. (1939) and Chandrasenan et al. (1960) for erythroglaucin. Our spectral data on the red pigment was in good agreement with those published by Fujimoto et al. (1971), Suemitsu et al. (1977), Podojil et al. (1979), Bachmann et al. (1979), and Anke et al. (1980). Fujimoto et al. (1971) and Anke et al. (1980) reported NMR chemical shifts for the hydroxyl protons ($CDCl_3$) that were in good agreement with our values.

The crystallographic results presented in Tables I and II and in Figure 3 established the structure of the red pigment as 1,5,8-trihydroxy-3-methoxy-6-methylanthraquinone which was identical with erythroglaucin. In addition to locating and identifying the substituents on the

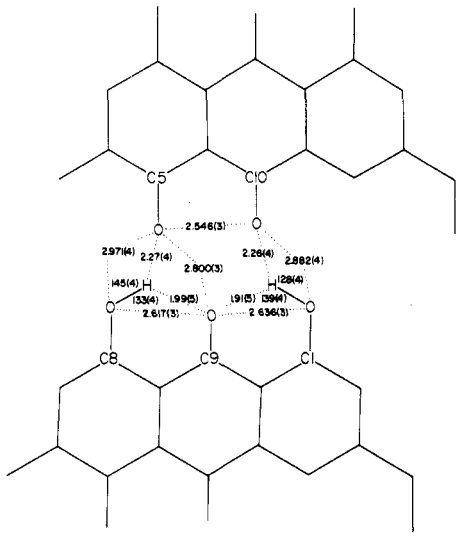

Figure 4. Schematic of the inter- and intramolecular hydrogen bonds.

planar fused rings, the structure analysis indicated that the C(9) and C(10) carbonyl bonds had considerable double bond character. The C(10)–C(11), C(10)–C(14), C(9)–C(12), and C(9)–C(13) bond distances also indicated some double-bond character in the middle ring although this was not as aromatic as the other rings. The C(1)–O, C(5)–O, and C(8)–O identified these as hydroxy oxygens. A hydrogen atom bound to the C(5)–O appeared to be disordered and could not be located in residual difference electron density maps. All three hydroxyl groups were involved in hydrogen bonding as shown schematically in Figure 4; C(1)–O and C(8)–O in intramolecular hydrogen bonds to the C(9) carbonyl oxygen atom. The O–H···O angles for these were 139 (4)° and 133 (4)°. An intramolecular hydrogen bond was also possible between the C(5)–O and the C(10) carbonyl oxygen atoms, but it could not be confirmed since it would involve the unobserved hydrogen. Three intermolecular hydrogen bonds between C(8)–O and C(5)–O of a neighboring molecule [O···O distance of 2.971 (4) Å; O–H···O angle of 145 (4)°], between the C(9) carbonyl oxygen and the same C(5)O [O···O distance of 2.799 (3) Å], and between the C(10) carbonyl oxygen atom and C(1)–O [O···O distance of 2.881 (4) Å; O–H···O angle of 128 (4)°] were the major crystal packing interactions.

Physcion, a yellow pigment produced by *A. ruber*, has been shown to form a complex with ferric iron that was soluble in chloroform and therefore could function in the lipid environment of the cell wall (Engstrom et al., 1980). This observation, together with the fact that it was produced relatively early in the life of this culture, led us to propose that it may have a function in iron transport or metabolism of the fungal cells. The iron complex of erythroglaucin was insoluble in chloroform, methanol, ether, water, and dimethyl sulfoxide. It was also different in color (dark blue) from erythroglaucin (red-violet) or ferric chloride (yellow) at pH 8.5–9.0, and the elemental analyses indicated the presence of 15.70% iron. In addition, the IR spectrum of the complex was different from that of the red pigment. The important carbonyl peak at 1600 cm^{-1} (erythroglaucin) was shifted to 1560 cm^{-1}, and there were several other changes in peak location and strength. These changes provided evidence that a complex had been formed. Although erythroglaucin was not synthesized as early in the life of the fungal cell as physcion, it could be involved in some other aspect of iron metabolism such as iron storage or detoxification.

We interpreted the insolubility of the erythroglaucin–ferric iron complex as evidence that it was a polymer. The chemical structure of erythroglaucin has a hydroxy group(s) adjacent to both quinone carbonyls. This structure seems to offer more potential for forming a polymer than other anthraquinone derivatives with hydroxyl groups adjacent to one carbonyl (i.e., physcion). Polymer formation could also explain why the elemental analysis values of the complex did not correlate well with any simple stoichiometric ratio of erythroglaucin to ferric iron.

A recent paper by Akers et al. (1980) reported that selected planar cyclic α-hydroxy ketones such as α-, β-, and γ-thujaplicin, tropolone, 2-hydroxy-1,4-naphthoquinone, 3-hydroxy-2-methylpyran-4-one, and a few other closely related compounds were shown to have iron-chelator activity with certain strains of *Salmonella typhimurium*. These compounds which have a free hydroxyl adjacent to the keto group seemed to be different than the two major classes of siderophores—hydroxamates and catechols (Akers et al., 1980). The red pigment, erythroglaucin, with three hydroxyl groups adjacent to quinone carbonyls has a structure similar to the α-hydroxy ketone and, therefore, might belong to the same class of iron chelators.

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Supplementary Material Available: Material including all the structure factor information tabulated in five columns with table entries as l, $10|F_o|$, $10F_c$, $10\Delta F$, and 10σ (F_o). Reflections with $|F_o|$ less than $2\sigma(F_o)$ denoted with asterisks (9 pages). Ordering information is given on any current masthead page.

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