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NOTES

Identification of an Anthraquinone Pigment and a Hydroxystilbene Antibiotic from *Xenorhabdus luminescens*

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The entomopathogenic bacterium *Xenorhabdus luminescens* produces a red pigment and an antibiotic in insect carcasses in which it grows and in axenic cultures. The pigment was purified and identified as the anthraquinone derivative 1,6-dihydroxy-4-methoxy-9,10-anthraquinone, which exhibits a pH-sensitive color change, i.e., it is yellow below pH 9 and red above pH 9. The antibiotic was also purified and identified as the hydroxystilbene derivative 3,5-dihydroxy-4-isopropylstilbene.

Nematodes of the genus *Heterorhabditis* carry a luminous bacterial symbiont, *Xenorhabdus luminescens*, in their gut. These parasitic nematodes reproduce in insects after infecting them and expelling their bacterial symbionts into the hemocoels of the insects (13, 14). Once they are released, the bacteria multiply rapidly, and the insect dies within 24 h. The insect carcass is initially luminous because of the bacteria (15). As luminescence decreases, the carcass becomes deep red (11) and does not putrefy, apparently because of antibiotic(s) produced by the bacteria (1, 12).

Bacteria isolated from infected insects are luminescent (15) and produce both an antibiotic (1) and a red pigment (8, 18). We report here the identification of both the pigment and the antibiotic purified from the culture supernatant of *X. luminescens* HK.

Cultures of strain HK were provided by Wayne Lanier (Biosis, Palo Alto, Calif.) and maintained on L agar (10). To purify the pigment and the antibiotic, cultures were grown to stationary phase of 10 liters of LB medium (10) with 5 ml of antifoam B (Baker no. B531-5) in a Braun Biostat V fermentor at 25°C with vigorous aeration. After 48 to 72 h, the cells were removed from the medium by centrifugation at 5,900 × *g* for 10 min. The supernatant was divided into four 2.5-liter fractions, and then each fraction was extracted with 3 500-ml volumes of ethyl acetate. The combined ethyl acetate fractions were dried with anhydrous sodium sulfate to eliminate aqueous emulsions and then filtered through Celite (Johns-Manville Products Corp.). The ethyl acetate phase was concentrated on a rotary evaporator at 30 to 40°C.

Approximately 0.6 to 0.8 g of the ethyl acetate extract residue was loaded onto a chromatography column (28 mm by 60 cm) slurry packed with ca. 67 g of silica gel. Thirty to forty 250-ml volumes of eluent were collected in the following order: 10% CH_2Cl_2–hexane, 20% CH_2Cl_2–hexane, 50% CH_2Cl_2–hexane, 70% CH_2Cl_2–hexane, CH_2Cl_2, and, last, ethyl acetate or diethyl ether.

A pigment was isolated from the ethyl acetate eluent and purified by recrystallization from 95% ethanol and by flash chromatography with 80% ethyl acetate–hexane. Thin-layer chromatography revealed one major peak after purification (R = 0.51, 80% ethyl acetate–hexane, silica gel).

The structure that best fits the spectral data for the pigment is shown in Fig. 1a; this is based on the following analyses. Mass spectra of the pigment, measured with a Finnegan 3000 spectrometer and a Mass Spectrometer Services Ltd. MS-9 spectrometer, showed the following molecular ion and fragment ions (20 eV): 270 (M, $C_{15}H_{10}O_5$, 86.0%), 252 (M − H_2O, $C_{15}H_8O_4$, 38.2%), 241 (M − CHO, $C_{14}H_9O_4$, 38.2%), 224 (M − [H_2O + CO]), $C_{14}H_8O_3$, 100%), 212 (M − [CHO + CHO], $C_{13}H_8O_3$, 18.6%), and 184 (M − [CHO + CHO + CO], $C_{12}H_8O_2$, 47.7%). Loss of CHO and CO from phenols and anthraquinones is a typical fragmentation route (5). The broken lines in Fig. 1 indicate a common fragmentation route (5), and this can be used to distinguish between the two isomers shown in Fig. 1a and b. The fragment ions associated with the isomer in Fig. 1a are 120 (HOC_6H_3CO, 12%) and 150 [$HO(CH_3O)C_6H_3CO$, 1.9%]. In contrast, only one of the corresponding fragment ions for the isomer shown in Fig. 1b is observed in low abundance, i.e., 136 [$(HO)_2C_5H_2CO$, 2.24%] but not 134 ($CH_3OC_6H_3CO$, 0.0%).

The nuclear magnetic resonance (NMR) spectrum [$(CD_3)_2$ CO], measured with a Chemenergetics 200-MHz spectrometer, is consistent with the assignment to the pigment of the resonance structure (Fig. 2) for the isomer in Fig. 1a: 4.00 (s, 3H, OCH_3), 6.93 (d [J = 2.6H_3], 1H, H_2), 7.36 (d [J = 2.6H_3], 1H, H_4), 7.28 (3 lines [J = 4.9 Hz], 1 H, H_6), 7.67 (2 lines [J = 0.9 Hz], 1H), 7.70 (ca. s, 1H), 7.70 to 7.67 (J = 4.9 Hz). The 7.67 and 7.70 absorptions are attributed to H_7 and H_5, where the doublets due to splitting by H_6 are nearly superimposable. The absorption at 7.28 for H_6 is a doublet of doublets in which the central lines coincidentally overlap to give three lines. Although the absorption for H_7 may appear to be too far downfield, the resonance structure in Fig. 2b would give carbonyl deshielding at H_7.

Fourier transform infrared (FTIR) spectra, measured with a Perkin-Elmer FT1750 spectrometer, also provide support for the structure shown in Fig. 1a, with the following absorptions (KBr, per centimeter): 3333 (broad β-OH), 3080, 3030 (aromatic C—H stretch), 2957, 2849 (aliphatic C—H

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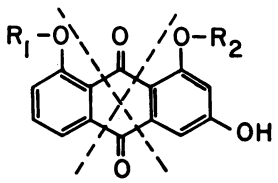
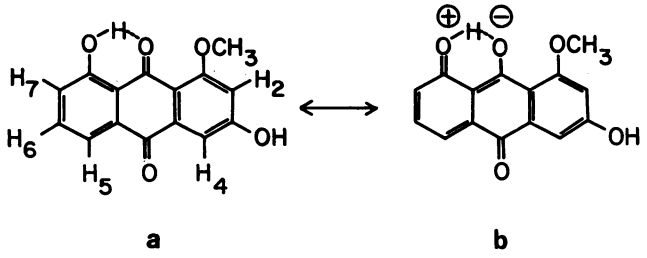


a) R_1 = H, R_2 = CH_3
b) R_1 = CH_3, R_2 = H

FIG. 1. Structure assignments for anthraquinone pigment.

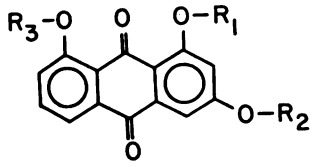
stretch), 2730 (weak, β-OH), 1673 (moderate, C=O), 1631 (strong, C=O), 1600 (strong, aromatic skeletal vibration), 1353, 1279, and 1230 (strong, C—O stretch). The low-frequency carbonyl absorption at 1631 cm^{-1} is characteristic of an alpha-hydroxy anthraquinone, as is a weak O—H absorption near 2700 cm^{-1} (4). The strong broad absorption at 3333 cm^{-1} also requires a beta-hydroxy group.

The UV-visible spectrum of the pigment offers further evidence for the structure shown in Fig. 1a, where λ_{max} (nanometers) (ε, methanol) is as follows: 218(6.25 × 10^3), 241(2.89 × 10^3), 268(sh), 279(3.99 × 10^3), and 419(1.66 × 10^3). When a drop of saturated aqueous KOH solution is added to this yellow methanolic solution, the color changes to red: 247(4.45 × 10^3), 301(3.72 × 10^3), and 480(1.72 × 10^3). The color change upon the addition of base is typical of phenols. A survey of reported UV-visible spectra of hydroxy and methoxy anthraquinones (2, 7, 16) indicates that our pigment must have only one alpha-hydroxy group and that there is no 1,2-dihydroxy array characteristic of alizarin (4).



a b

FIG. 2. Structure assignments for resonance structures (a and b) of the anthraquinone pigment shown in Fig. 1a.



a) R_1 = R_2 = R_3 = CH_3
b) R_1 = R_2 = R_3 = H
c) R_1 = R_3 = H, R_2 = CH_3
d) R_1 = R_2 = CH_3, R_3 = H
e) R_1 = R_2 = H, R_3 = CH_3

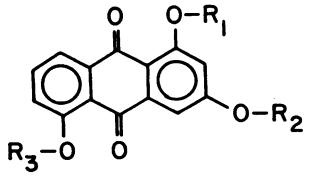
FIG. 3. Possible structures of the anthraquinone pigment.

Figures 3 and 4 show a variety of possible structures of the two isomeric forms of the pigment obtained by substitution of the three R groups. Without α-hydroxy groups (Fig. 3a), the long-wavelength band is too low (λ_{max} = 395 nm) (2). With 1,8-dihydroxy substitution as shown in Fig. 3b (λ_{max} = 430 nm) (4) and Fig. 3c (λ_{max} = 428 nm) (2), the long-wavelength band is shifted too far to the blue to fit the pigment. Although the λ_{max} of the structure shown in Fig. 4a (415 nm) is similar to that of the pigment, the infrared (IR) spectrum of the compound shown in Fig. 4a is inconsistent with that of the pigment (2). This conclusion is based on the fact that there is only one carbonyl absorption associated with the isomer shown in Fig. 4a (1610 cm^{-1}) (2), whereas the pigment shows two bands at 1673 and 1631 cm^{-1}.

An excellent model compound for the pigment is shown in Fig. 3d, where λ_{max} (nanometers; ethanol) is 222, 242, 270(sh), 279, and 420. The pigment λ_{max} (nanometers; methanol) values match extremely closely, as follows: 218, 241, 268(sh), 279, and 419. The reported IR spectra of the structure shown in Fig. 3d (1666, 1625, and 1695 cm^{-1}) (2) are also in close agreement with those of the pigment (1673, 1631, and 1600 cm^{-1}). In contrast, an isomeric model compound (Fig. 4b) shows poor correspondence to the UV-visible spectrum of the pigment (λ_{max} of the structure in Fig. 4b [nanometers; ethanol]: 228, 248, 278, and 404) (2). In addition, the correspondence of the IR spectra of this structure (1665, 1645, and 1605 cm^{-1}) (2) with the pigment is less satisfactory.

In summary, both the UV-visible and IR spectra of the pigment indicate an α-hydroxy group, which is hydrogen-bonded to the anthraquinone carbonyl, and one β-hydroxy group. The NMR spectrum indicates one methoxy group, which is consistent with the mass spectrum where the molecular ion is 270 ($C_{15}H_{10}O_5$). This molecular formula corresponds to an anthraquinone which is substituted with one methoxy and two hydroxy groups. The NMR spectrum further indicates *meta* orientation between the two methoxy groups, as seen from the 6.93 and 7.36 doublets with a typical *meta J* value of 2.6 Hz (17). The choice between the isomer shown in Fig. 1a and that shown in Fig. 3e is based on the ArCO fragments observed in the mass spectrum. Placement of the OH group at the 8 position (cf. Fig. 1a) is based on an analysis of the coupling of the H_5, H_6, and H_7 protons. With the model compounds shown in Fig. 3d and 4b, the UV-visible and IR spectra indicate that the isomer shown in Fig. 1a is most consistent with the pigment, as opposed to the one shown in Fig. 4c.

In order to relate the pigment to a known compound, approximately 1 mg of the pigment was added to a melt of 0.1 g of aluminum chloride and 20 mg of sodium chloride at 185°C under a nitrogen atmosphere to effect demethylation.



a) R_1 = R_2 = R_3 = H
b) R_1 = R_2 = CH_3, R_3 = H
c) R_1 = CH_3, R_2 = R_3 = H

FIG. 4. Possible structures of the anthraquinone pigment.

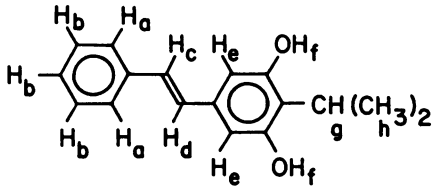


FIG. 5. Structure of the antibiotic compound produced by *X. luminescens* HK.

The melt was cooled to 120°C, and 3 g of ice with 0.1 ml of concentrated HCl was added. The mixture was extracted with chloroform, and after the removal of this solvent, the residue was subjected to flash chromatography on silica gel (Baker, no. 40) with 40% ethyl acetate–60% hexane eluent. From this chromatography, an NMR spectrum of a very dilute carbon tetrachloride solution was obtained which was in qualitative agreement with the reported NMR spectrum of 1,3,8-trihydroxy-9,10-anthraquinone (2). Only the aryl protons were detected, because of the low concentration of the sample, and the areas of the aryl protons were approximately equal. Reported absorptions (2) were measured in dimethyl sulfoxide (DMSO)-d_6, and they are given in parentheses: 6.45 (6.55) H_2; 7.22 (7.09) H_4; 7.73 H_5, 7.47 H_6, and 7.25 H_7 (7.20 to 7.90). The CH_3O absorption at 4.00 in the pigment is no longer present.

Anthraquinones are known, but not common, metabolites of bacteria (9); however, to our knowledge, the pigment identified from *X. luminescens* has not previously been reported to come from bacteria. The function of the pigment is not known. Closely related anthraquinones have been isolated from the leaves and stems of *Xyris semifuscata* (7) and from the fungus *Trichoderma polysporum*, where it may act as an antagonistic agent of other fungi (6).

Antibiotic activity was present in the 70% CH_2Cl_2–hexane fraction from the open-column silica gel chromatography. The antibiotic was purified by preparative high-pressure liquid chromatography on a silica Resolve column (3.9 mm by 15 cm) with a solvent gradient of 60% CH_2Cl_2–hexane (3 m), 80% CH_2Cl_2–hexane (2 m), and 90% CH_2Cl_2–hexane (6 m) and with a flow rate of 1 ml/m (400 lb/in^2). The fraction with t_r = 2.9 m (area percent = 85%) was collected upon repeated injections of the sample in CH_2Cl_2 solution. Analysis of the pooled fractions on a C_{18}-Resolve column (3.9 mm by 15 cm) showed only one major component (t_r = 1.48 m, area percent = 96%) with a solvent gradient starting with 95% CH_3CN–5% H_2O and stepping the profile every 2 m with a 5% increase in water until 70% CH_3CN–30% H_2O was reached. The flow rate was 1.0 ml/min (2,000 lb/in^2).

Spectral analysis showed that the antibiotic had the structure shown in Fig. 5, which is identical to that reported for *Xenorhabdus* strain Hb (12). The structure is coded for the NMR assignments. The FTIR (evaporated on NaCl windows, per centimeter) is consistent with the structure described above, as follows: OH (3387, broad), aromatic C—H stretch (3083, 3060, and 3028), aliphatic C—H stretch (2960, 2929, and 2873), C=C bond stretch (1613), aromatic skeletal vibration (1582 and 1496), aliphatic C—H bond deformations (1450 and 1360), aromatic C—O stretch (1263). The NMR spectrum is also consistent with the structure ([parts per million], coupling, area, proton): 7.40 (d [J = 7.3 Hz], 2H, a), 7.25 (m, 3H, b), 6.87 (AB [J = 16 Hz], 2H, c + d), 6.35 (s, 2H, e), 4.68 (bs, 2H, f), 3.40 (m [J = 6 Hz], 1H, g), 1.36 (d [J = 6 Hz], 6H, k). The mass spectrum (70 eV) agrees with the structure shown in Fig. 3 as well: 254 (M, 47.6%), 255 (M + 1, 9.53%), 256 (M + 2, 1.09%), 239 (M − CH_3, 100%), 211 [M − $CH(CH_3)_2$, 1.8%], 179 (M − C_6H_5, 8.7%). The (M + 1)/(M + 2) abundance ratio (8.74) agrees closely with that calculated for $C_{17}H_{18}O_2$ (9.11) (3), which is the molecular formula of the antibiotic.

Two antibiotics have now been identified from different *X. luminescens* strains, and they fall into two distinct chemical groups, indole derivatives (12) and hydroxystilbene derivatives (12; this report). Since there may be subdivisions of the strains now designated as *X. luminescens* (8), it may be of interest to see if the antibiotics provide distinguishing taxonomic characteristics.

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