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Identification of Two Pigments and a Hydroxystilbene Antibiotic from *Photorhabdus luminescens*

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Two yellow pigments were isolated for the first time from the entomopathogenic bacterium *Photorhabdus luminescens* in liquid culture and were identified as the anthraquinone derivatives 3,8-dimethoxy-1-hydroxy-9,10-anthraquinone (minor) and 1,3-dimethoxy-8-hydroxy-9,10-anthraquinone (major). A known antibiotic, 3,5-dihydroxy-4-isopropylstilbene, was also detected and for the first time showed strong fungicidal activity against several fungi of medical and agricultural importance.

Nematodes of the genus *Heterorhabditis* carry a luminous bacterial symbiont, *Photorhabdus luminescens*, in their gut (18). These parasitic nematodes penetrate insects and expel their bacterial symbionts into the insects' hemocoels (16, 17). Once released in an insect, the bacteria multiply rapidly, the insect dies within 24 to 48 h, and the nematodes feed on the multiplying bacteria. The insect cadaver becomes deep red but does not putrefy, apparently because of an antibiotic(s) produced by the bacteria (1, 9, 14, 15, 19). Recently, it was reported that antibiotics from *P. luminescens* and from *Xenorhabdus* spp., bacterial symbionts from *Steinernema* spp., have strong activities against fungal pathogens of plants (7, 13). These observations led to an investigation into the chemical natures of these substances, and we report here the identification of two pigments and one antibiotic from *P. luminescens.*

MATERIALS AND METHODS

Microorganisms. *P. luminescens* C9 and its nematode symbiont used in this study, *Heterorhabditis megidis* 90, were collected from soil in British Columbia, Canada, and maintained in culture in this laboratory (7). Last-instar larvae of the greater wax moth, *Galleria mellonella*, were infected with infective juvenile nematodes carrying *P. luminescens* at a rate of 25 infective juveniles per larva (7). After 24 to 48 h, the dead insect larvae were surface disinfected by being dipped into 95% ethyl alcohol and ignited. The cadavers were aseptically dissected, and hemolymph was streaked onto an NBTA medium (nutrient agar supplemented with 0.025 g of bromothymol blue and 0.04 g of 2,3,5-triphenyltetrazolium chloride per liter) and incubated in the dark at room temperature. Inocula of the primary form of the bacterium (7) were prepared by adding one loopful of the culture to 50 ml of tryptic soy broth in 100-ml Erlenmeyer flasks. Cultures were shaken at 120 rpm on a gyrorotary shaker for 24 h at 25°C. Bacterial fermentation was initiated by adding 100 ml of this bacterial culture to 900 ml of tryptic soy broth in a 2,000-ml flask, which was then incubated in the dark at 25°C on a gyrorotary shaker. After 96 h, the culture was centrifuged (12,000 × *g*, 20 min, 4°C) to remove the bacterial cells.

Isolation of chemical components. The cell-free culture broth (5 liters) was extracted with the same volume of ethyl acetate three times. The extracts were combined, dried over anhydrous sodium sulfate, and filtered through glass wool. The level of bioactivity was tested at this and each subsequent step in the isolation process, with *Bacillus subtilis* and *Botrytis cinerea* being used to detect antibacterial and antifungal activities, respectively (10). The filtrate was evaporated with a flash evaporator at 30°C. The concentrated, crude material (approximately 2 g) was then separated by flash silica gel chromatography (column dimensions, 4 by 30 cm) initially with 30% and then with 60% ether in hexane and finally with 100% ether as the eluting solvents. The most active fraction was washed out with 60% ether in hexane and was then concentrated to give active compound I, while a yellow fraction was eluted with 100% ether from the column. This yellow fraction (a mixture of pigments II and III) was not separable by high-performance liquid chromatography (HPLC) on a C_{18} preparative column (Spherisorb 10 [octadecyl silane 1]; column dimensions, 250 by 10 mm; particle size, 10 μm; Phenomenex, Torrance, Calif.), monitored at 254 nm, with either acetonitrile-water or methanol-water as the mobile phase.

Production of component I. The bacterial cultures were prepared by adding 1 ml of inoculum of the bacterial isolate to 30 ml of tryptic soy broth in a 100-ml flask and culturing at 25°C (with shaking at 120 rpm). Samples were taken at 1, 2, 3, 4, and 5 days, with three replicated flasks for each sample. The culture samples were neutralized with 6 M HCl and centrifuged (11,000 × *g*, 20 min, 4°C). Then, 10 ml of the supernatants was extracted twice with 10 ml of ethyl acetate each time, and the extracts were combined and then dried by being passed through an anhydrous sodium sulfate column (column dimensions, 40 by 4 mm). The ethyl acetate extracts were evaporated to dryness and dissolved in 0.5 ml of methanol. The 20-μl sample of methanol solution was injected into the HPLC column for the determination of component I. The HPLC analysis was run with a C_{18} reverse analytical column (column dimensions, 250 by 6 mm; Lichrosorb 10 RP-18) at a rate of 1.5 ml/min with the following mobile phase gradient: 0 to 5 min, 10% CH_3CN in H_2O; 5 to 25 min, gradual increase to 90% CH_3CN in H_2O; 25 to 27 min, 90% CH_3CN in H_2O. Pure chemical I (retention time on HPLC, 19.8 min) was used as the external standard for determining chemical production, by comparing the peak areas.

Analysis of metabolites. Nuclear magnetic resonance (NMR) spectra of component I in $CDCl_3$ were recorded on a Bruker WM400 spectrometer, with residual $CHCl_3$ as the internal standard. Proton, carbon, and two-dimensional NMR spectra of components II and III in $CDCl_3$ were recorded on a Bruker AMX600 spectrometer. Low-resolution mass spectra (MS) were obtained on a Hewlett-Packard 5985B gas chromatography-MS system, operating at 70 eV with a direct probe. Chemical ionization MS were obtained with isobutane on the same instrument for low-resolution MS. Infrared spectra were recorded on a Perkin-Elmer S99B spectrometer. HPLC analysis was performed on a Waters 510 chromatograph with a Waters 484 UV detector.

Determination of MICs. Active compound I was dissolved in dimethyl sulfoxide, filter sterilized with a 0.2-μm-pore-size filter, and diluted into 2 ml of potato dextrose broth in a 5-ml vial, resulting in a final dimethyl sulfoxide concentration of <0.4% (vol/vol). The active compound was serially diluted twofold to produce culture media containing dilutions from 200 μg of active ingredient per ml in order to determine the MIC (10). Each dilution was replicated three times with both liquid media and agar plates.

The two yeast test candidates (*Candida tropicales* and *Cryptococcus neofor-*

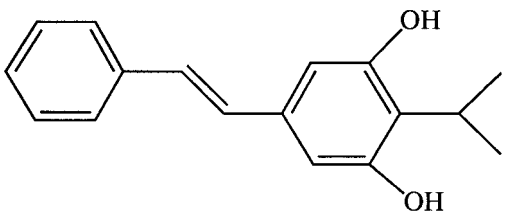

FIG. 1. Structure of 3,5-dihydroxy-4-isopropylstilbene (component I).

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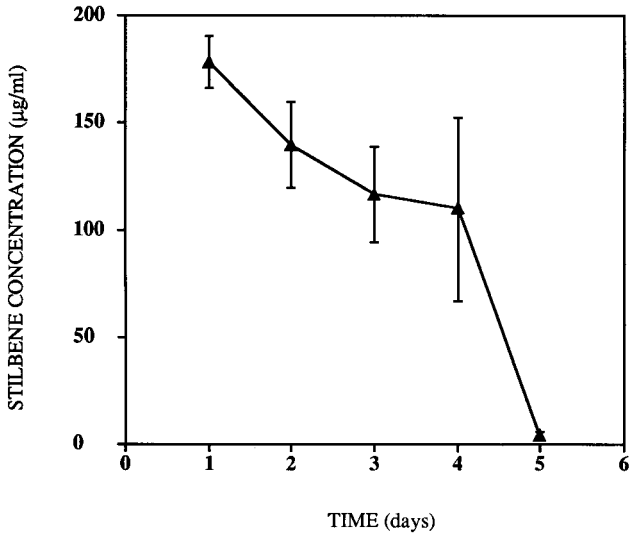
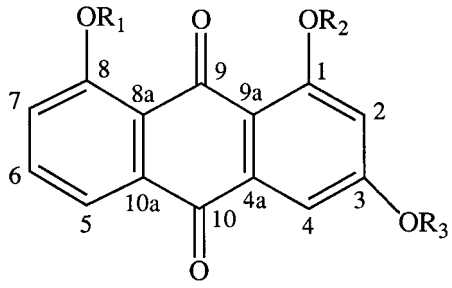


FIG. 2. Hydroxystilbene concentrations in the tryptic soy broth culture of *P. luminescens* over 5 days at 25°C.

mans) were cultured on potato dextrose agar plates for 24 h at 25°C, the plates were flooded with 0.8% saline, the yeasts were scraped off, and the saline suspension was made up to inocula containing 2.5×10^7 to 2.8×10^7 cells per ml. *Aspergillus* spp. and *Botrytis cinerea* were grown on potato dextrose agar for 7 days at 25°C before the conidia were harvested by flooding the plates with sterile, distilled water and diluting the cultures to make final inocula of 2.5×10^6 to 3.0×10^6 conidia per ml. The various inocula were then added separately to the respective culture media with the test chemicals. Replicates were incubated at 35°C except for those on *Botrytis cinerea*, which were inoculated at 24°C, and the MICs were visually determined after 24 h (48 h for *Botrytis cinerea*). The MIC is defined as the lowest concentration of active compound which prevents growth of the test organism at the above-mentioned conditions.



II: R_1=CH_3, R_2=H, R_3=CH_3

III: R_1=H, R_2=CH_3, R_3=CH_3

IV: R_1=H, R_2=CH_3, R_3=H

V: R_1=H, R_2=H, R_3=CH_3

VI: R_1=H, R_2=H, R_3=H

FIG. 3. Structures of pigments from *P. luminescens*.

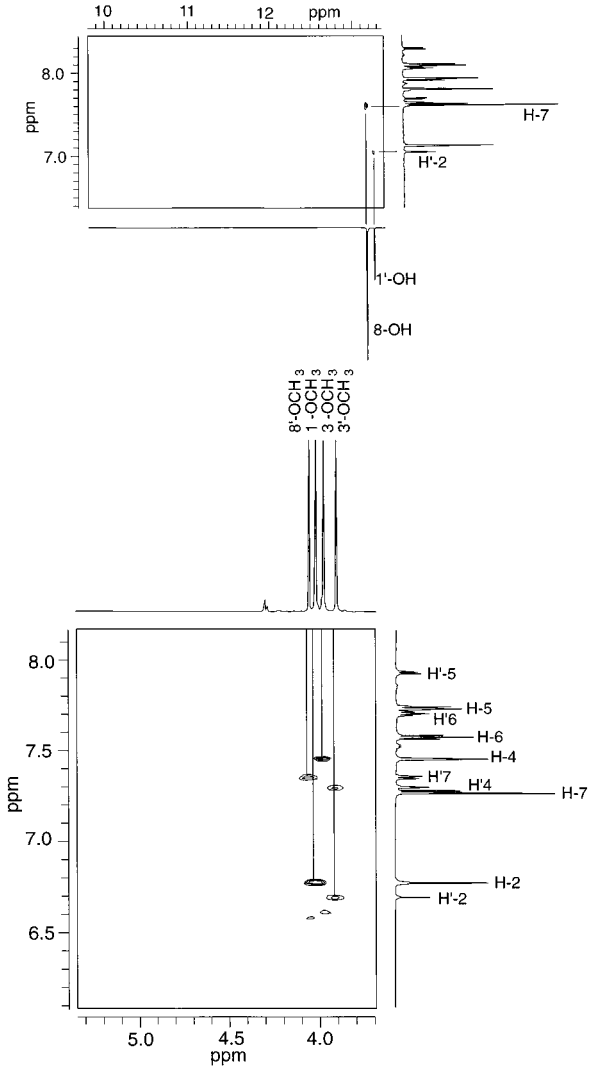


FIG. 4. Nuclear Overhauser-exchange spectroscopy spectra of a mixture of components II and III. The positions in the minor compound are indicated by prime marks.

RESULTS AND DISCUSSION

Isolation. The bioactive component and the mixture of yellow pigments were obtained by flash silica gel chromatography. Further separation of the pigments into single, pure compounds was unachievable with the column and conditions used. Therefore, the mixture was analyzed as follows.

Identification. Spectral analyses (^{1}H NMR, Fourier transform IR, and MS analyses were identical to those described in references 15 and 19) showed that bioactive component I had the structure shown in Fig. 1, which has been identified previously as that of an antibacterial agent from *P. luminescens* Hb (15) and HK (19). However, we showed for the first time that this component has strong antifungal activity. Further, it was observed that component I was produced rapidly by the bacteria during the first day of culture; then its concentration in the culture declined until it was undetectable on the fifth day under the fermentation conditions described above (Fig. 2). This hydroxystilbene has been detected for all strains of *P. luminescens* studied by both us and other scientists (15, 19) but was not reported by Sztaricskai et al. (20). However, the long

TABLE 1. ^{1}H NMR data for pigments II and III from *P. luminescens* in $CDCl_3$

Pigment and position	Chemical shift (ppm)
II	
1′-OH	13.22 (s)
2′-H	6.70 (d, J = 2.5 Hz)
3′-OCH_3	3.92 (s)
4′-H	7.30 (d, J = 2.5 Hz)
5′-H	7.94 (d, J = 7.7 Hz)
6′-H	7.71 (dd, J = 8.4, 7.7 Hz)
7′-H	7.35 (d, J = 8.4 Hz)
8′-OCH_3	4.07 (s)
III	
1-OCH_3	4.03 (s)
2-H	6.78 (d, J = 2.4 Hz)
3-OCH_3	3.99 (s)
4-H	7.46 (d, J = 2.4 Hz)
5-H	7.75 (d, J = 7.4 Hz)
6-H	7.58 (dd, J = 8.2, 7.4 Hz)
7-H	7.28 (d, J = 8.2 Hz)
8-OH	13.14 (s)

culture period (20 instead of 3 to 5 days) used in the experiment of Sztaricskai et al. would provide an explanation as to why they did not detect the hydroxystilbene(s).

The ^{1}H NMR spectrum of the yellow fraction shows that it

TABLE 2. ^{13}C NMR data for pigments II and III from *P. luminescens* in $CDCl_3$

Pigment and position	Chemical shift (ppm)
II	
1′	165.7 (165.5, 161.0)
2′	107.6
3′	165.5 (165.7, 161.0)
4′	106.7
5′	120.5
6′	135.6
7′	118.6
8′	161.0 (165.7, 165.5)
9′	187.7
10′	182.9
8′a	134.4 (135.9)
9′a	135.9 (134.4)
4′a	121.0 (111.9)
10′a	111.9 (121.0)
3′-OCH_3	56.2
8′-OCH_3	56.9
III	
1	165.7 (163.3, 162.7)
2	105.0
3	162.7 (165.7, 163.3)
4	104.3
5	119.1
6	135.6
7	125.2
8	163.3 (165.7, 162.7)
9	188.0
10	182.9
8a	132.9 (137.9)
9a	137.9 (132.9)
4a	117.1 (115.1)
10a	115.5 (117.1)
3-OCH_3	56.3
1-OCH_3	56.9

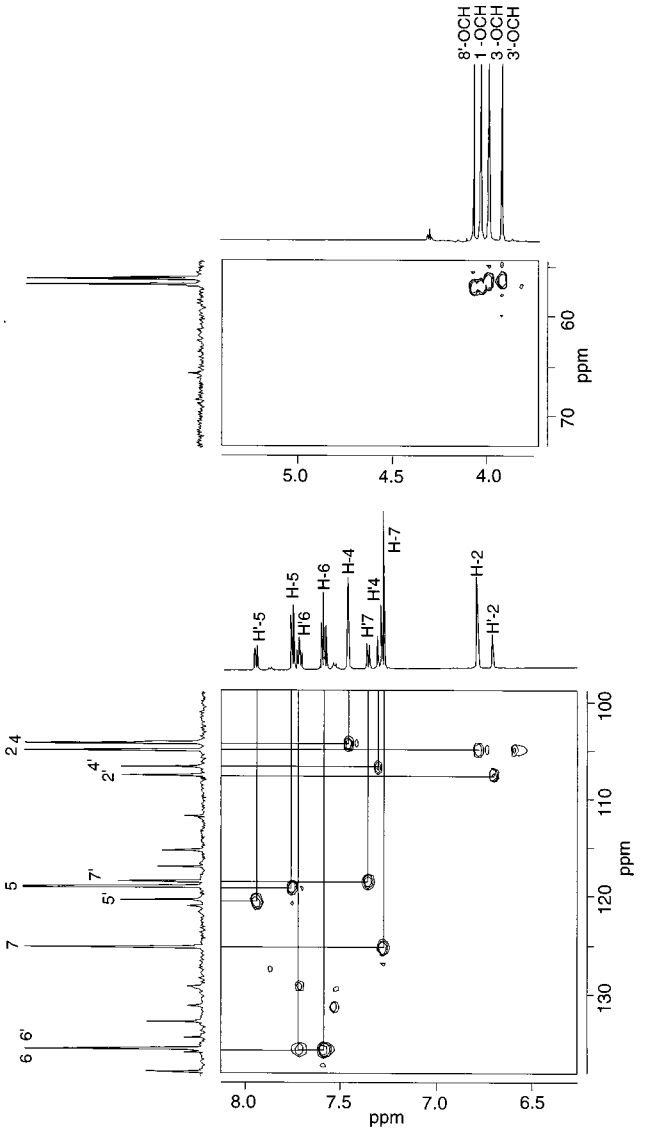


FIG. 5. Heteronuclear multiple-quantum coherence spectra of a mixture of components II and III. The positions in the minor compound are indicated by prime marks.

was a mixture of two compounds in a ratio of approximately 1:3. As its further chromatographic separation was unsuccessful, the analysis proceeded with a mixture of the pigments. The structures that best fit the spectral data for the pigments (Fig. 3) are based on the analysis summarized below. The MS of the pigment mixture (II and III) is described here for the first time and shows the following molecular ion and fragment ions: 285 (M^+ + 1, 21%), 284 (M, 100%), 267 ($M^+ - H_2O$ + 1, 23%), 266 ($M^+ - H_2O$, 46%), 255 (M^+ − CHO, 27%), 239 ($M^+ - [H_2O + CO]$ + 1, 33%), 238 ($M^+ - [H_2O + CO]$, 75%), 223 ($M^+ - [H_2O + CO + CH_3]$, 17%), and 128 (10%). These data differ from those for pigments IV, V, and VI (Fig. 3) from *P. luminescens*, which were identified previously (15, 19, 20), by the presence of an extra methyl group(s). Loss of CHO and CO from phenols and anthraquinones is a typical fragmentation route (6). The UV-visible spectrum of the pigment mixture offers further evidence for the structures shown in Fig. 3, for which the maximum wavelengths (solvent, methanol) are

TABLE 3. MIC of 3,5-dihydroxy-4-isopropylstilbene from *P. luminescens* against fungi of medical and agricultural importance

Fungus	MIC (μg/ml)
Aspergillus flavus	25
Aspergillus fumigatus	12
Botrytis cinerea	12
Candida tropicales	25
Cryptococcus neoformans	12

222, 242, 263, 279, and 420 nm, which are very similar to those found for pigment IV (19). The signals at 13.22 and 13.14 ppm (for the minor and major compounds, respectively) in the proton NMR suggest that the pigments each have a hydrogen-bonded proton, while the signals at 6.70 and 6.78 ppm (for the minor and major compounds, respectively) suggest that two electron-rich substitutes are present around 2-H in each pigment. On the basis of our analysis, structures II and III are suggested for the pigments. The final assignment of the major and minor pigments is based on different two-dimensional NMR analyses of the mixture.

The nuclear Overhauser-exchange spectroscopy spectrum (Fig. 4) of the mixture (II and III) clearly shows that there are nuclear Overhauser-exchange correlations between 1′-OH and 2′-H, 3′-OCH_3 and 2′-H, 3′-OCH_3 and 4′-H, and 8′-OCH_3 and 7′-H for the minor pigment (II), while there are nuclear Overhauser-exchange correlations between 1-OCH_3 and 2-H, 3-OCH_3 and 4-H, and 7-H and 8-OH for the major compound (III). Therefore, the minor compound is assigned as 3,8-dimethoxy-1-hydroxy-9,10-anthraquinone (II) and the major compound is assigned as 1,3-dimethoxy-8-hydroxy-9,10-anthraquinone (III). The full ^{1}H NMR assignments for the pigments (II and III) that are summarized in Table 1 are fully consistent with these structures.

The heteronuclear multiple-quantum coherence spectrum (Fig. 5) of the mixture (II and III) was obtained, and it shows the relationship between the carbon and the hydrogen attached to it. These results not only confirmed the identification of the two pigments but also allowed the assignment of ^{13}C NMR spectra for both pigments, which are summarized in Table 2.

Anthraquinones are known, but not common, metabolites of bacteria (12, 19, 20), and only 1,3,8-trihydroxy-9,10-anthraquinone and two of its monomethyl ether derivatives, 1,8-dihydroxy-3-methoxy-9,10-anthraquinone and 3,8-dihydroxy-1-methoxy-9,10-anthraquinone, have been recorded as being from *P. luminescens* (15, 19, 20). Our research shows the existence of two different pigments, 3,8-dimethoxy-1-hydroxy-9,10-anthraquinone (II) and 1,3-dimethoxy-8-hydroxy-9,10-anthraquinone (III), which are dimethyl ethers and have not previously been reported to come from bacteria, although compound III has been synthesized (4). The functions of the pigments are not known. As the preliminary screen bioassay in this isolation process showed that these pigments have antimicrobial activities, they probably function as antagonistic agents against other microorganisms in the insect cadaver. Closely related anthraquinones have been isolated from the leaves and stems of *Xyris semifuscata* (8) and strains of the fungus *Aspergillus glaucus* (2).

Antifungal test. The preliminary screen test showed strong antifungal activity for antibiotic I, and its MICs for several fungi of medical and agricultural importance were obtained by standard methods (Table 3). As this fungicidal substance can be synthesized relatively easily by chemical methods (3, 11), it has good potential for development as a commercial fungicide. Further tests on the pigments against different microorganisms were not carried out because they form a mixture that is very difficult to dissolve in test media. The present results strongly reinforce the hypothesis that antimicrobial substances produced by *P. luminescens* play an essential role in minimizing microbial putrefaction of the insects infected by the *Heterorhabditis-Photorhabdus* complex (5).

In conclusion, two anthraquinone pigments were isolated from *P. luminescens* C9 for the first time and by extensive two-dimensional NMR analyses were identified as 3,8-dimethoxy-1-hydroxy-9,10-anthraquinone (minor) and 1,3-dimethoxy-8-hydroxy-9,10-anthraquinone (major). As well, a known stilbene antibiotic, 3,5-dihydroxy-4-isopropylstilbene, was isolated and was shown for the first time to have strong fungicidal activity against several fungi of medical and agricultural importance.

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