Aflavinines and Other Antiinsectan Metabolites from the Ascstromata of *Eupenicillium crustaceum* and Related Species

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This report describes the distribution of antiinsectan metabolites present in sclerotoid ascstromata produced by representative strains of *Eupenicillium crustaceum* and fungal taxa that are considered to be closely related. The hexane and chloroform extracts of *E. crustaceum* NRRL 3332 displayed significant antiinsectan activity in assays against the corn earworm, *Helicoverpa zea*. The major metabolite accounting for this antiinsectan activity was a known aflavinine analog, 10,23-dihydro-24,25-dehydroaflavinine, occurring at approximately 2.8 mg/g of dry ascstromata. In dietary assays at ca. 3,000 ppm, a 79% reduction in weight gain and a 42% reduction in feeding rate were observed in *H. zea* and *Carpophilus hemipterus* larvae, respectively. A new aflavinine analog, 10,23,24,25-tetrahydro-24-hydroxyaflavinine, was also identified. These aflavinine compounds are the first to be reported from a fungal genus other than *Aspergillus*. New macrophorin-type metabolites accounted for the antiinsectan activity of ascstromata produced by *E. crustaceum* NRRL 22307, which produced no aflavinines, while *Eupenicillium molle* NRRL 13062 produced both aflavinines and macrophorins. Sclerotia produced by *Penicillium gladioli* NRRL 938, NRRL 939, and QM 2743, a fungus reported to be conspecific with the anamorph of *E. crustaceum*, produced neither aflavinines nor macrophorins. *Eupenicillium reticulispurum* NRRL 3446 produced the aflavinine analog 10,23-dihydro-24,25-dehydroaflavinine and an unrelated compound called pyripyropene A, a potent inhibitor of acyl-coenzyme A-cholesterol acyltransferase. *Eupenicillium abidjanum* NRRL 5809, reported to be conspecific with *E. reticulispurum*, produced neither of these compounds. The *Eupenicillium* species that produced aflavinines are also known for their ability to grow rapidly with reduced water activity.

The genus *Eupenicillium* is distinguished by its production of globose to subglobose pseudoparenchymatous or sclerotoid ascstromata and a *Penicillium* anamorph (18, 28). Immature ascstromata of *Eupenicillium ochrosalmoneum* Scott and Stolk were shown to function like sclerotia in the fungal life cycle in maize fields, eventually ripening to produce ascospores (15, 40). Because sclerotia are important to the survival of some fungi, we theorized that sclerotia should be chemically defended against fungivorous arthropods (36, 39). Indeed, sclerotia produced by *Aspergillus* spp. have proven to be a rich source of novel antiinsectan compounds with activity against the corn earworm (*Helicoverpa zea*) and/or the dried fruit beetle (*Carpophilus hemipterus*) (38). Like the aspergilli, molds possessing the *Penicillium* anamorph state have historically yielded a wealth of biologically active fungal metabolites. In the present study, our objective was to determine if *Eupenicillium* ascstromata might also contain antiinsectan metabolites.

An initial survey of *Eupenicillium* species revealed that the crude hexane extract of ascstromata produced by *Eupenicillium crustaceum* Ludwig showed significant antiinsectan activity against *H. zea* and *C. hemipterus*. While several other *Eupenicillium* spp. produced ascstromata affording antiinsectan extracts, initial chemical investigation of the *E. crustaceum* extract showed that an aflavinine derivative is the most abundant active component. This was particularly intriguing because the only previously known sources of aflavinines were the sclerotia of certain *Aspergillus* spp. (13, 36, 39). On the basis of these preliminary results, representative strains of *E. crustaceum*, the type species of the genus *Eupenicillium* (28),

and species considered to be synonymous were selected for chemical evaluation. This report describes the distribution of a variety of antiinsectan metabolites produced by ascstromata from *E. crustaceum* and by ascstromata or sclerotia from *Eupenicillium* and *Penicillium* species that are considered to be closely related taxonomically.

MATERIALS AND METHODS

Fungal strains. All cultures were obtained from the Agricultural Research Service Culture Collection, Peoria, Ill. *E. crustaceum* NRRL 3332 (anamorph state = *Penicillium kewense* Smith) is the type culture of *P. kewense*, described as a new ascosporic species of the genus *Penicillium* (27). Although no type specimen or type culture of *E. crustaceum* exists, this species is typified by Brefeld's (4) illustrations and description of *Penicillium glaucum*. Stolk and Scott (30) identified *E. crustaceum* with the type culture of *P. kewense* NRRL 3332. This fungus was isolated in 1959 by G. Smith as a contaminant of a mineral oil culture at the London School of Hygiene and Tropical Medicine. The Agricultural Research Service Culture Collection received this strain in 1968 as CBS 344.61 from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. *E. crustaceum* NRRL 3556 (= CBS 581.67) was isolated by S. H. Iqbal from a soil sample collected in Lahore, Pakistan. *E. crustaceum* NRRL 22307 (= CBS 457.72) was isolated by J. Mouchacca from desert soil collected in Egypt. *E. crustaceum* NRRL 22755 (= CBS 653.82; NRRL 2094) was isolated at the Northern Regional Research Laboratory in 1944 from a soil sample sent by B. B. Mundkur, Imperial Agricultural Research Institute, New Delhi, India, and was considered by Raper and Thom (22) to be intermediate between *Penicillium egyptiacum* and *Penicillium brefeldianum* Dodge. Stolk and Samson (28) examined the strain and placed it in the species *E. crustaceum*. *E. egyptiacum* NRRL 2090 (ex type culture) was isolated by Y. S. Sabet from soil collected in Cairo, Egypt. *E. molle* NRRL 13062 (ex type culture; = CBS 456.72) was isolated by S. A. Lodhi from soil collected in Pakistan. Pitt (20) distinguished *E. crustaceum*, *E. egyptiacum* (van Beyma) Stolk and Scott, and *Eupenicillium molle* Malloch and Cain by the ornamentation and size of the ascospores. However, Stolk and Samson (28) regarded *E. egyptiacum* and *E. molle* as small-spored strains of *E. crustaceum*, the latter representing a variable species.

The anamorphic state of *P. kewense* was considered by Pitt (20) to be conspecific with sclerotium-forming strains of *Penicillium gladioli* McCulloch and Thom. At the same time, while *P. gladioli* appears to be similar to the anamorphs of *E.*

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crustaceum, no ascospores have ever been observed in *P. gladioli* isolates (23). Stolk and Samson (28) regarded *P. gladioli* as a separate species from *E. crustaceum*. They noted that strains of *E. crustaceum* all had smooth stipes, while those of *P. gladioli* were coarsely roughened. *P. gladioli* NRRL 939 (ex type culture) was isolated in 1926 by L. McCulloch from a *Gladiolus* corm imported from the Netherlands. *P. gladioli* NRRL 938 was isolated in 1931 by F. T. Brooks from a *Gladiolus* corm in Cambridge, England, and was identified by P. W. Brian. *P. gladioli* NRRL A-1828 (= QM 2743) was isolated by G. F. Weber in 1944 from book lice that were collected from a tent (Florida QM Storage Deterioration Testing, Camp Indian Bay, Florida) and was identified by K. B. Raper as "near *P. gladioli*." *E. crustaceum* NRRL 22306 (= CBS 215.71 = CSIR 1026) was isolated from soil collected at Jan Smuts Airport, Johannesburg, South Africa, by J. P. van der Walt, and was identified by Scott (25). Stolk and Samson (28) listed CBS 215.71 with *Penicillium raciborskii* Zaleski, because it is sclerotial and has strongly vesiculate metulae with apices up to 8 μ m wide. The Agricultural Research Service Culture Collection received this strain in 1993 as CBS 215.71. *Penicillium raistrickii* NRRL 2039 (type culture) (= *P. raciborskii*) was isolated from cotton yarn by G. Smith and is considered a synonym of *P. raciborskii* by Stolk and Samson (28).

Eupenicillium reticulisporeum NRRL 3447 (type culture; = CBS 122.68 = NHL 6105) was isolated by S.-I. Udagawa in 1967 from a soil sample collected in Moto-machi, Oshima Island, Japan. *E. reticulisporeum* NRRL 3446 (= CBS 121.68 = NHL 6102) was isolated by S.-I. Udagawa in 1966 from a soil sample collected in Jukkoku, Kajikazawa-machi, Japan. *Eupenicillium abidjanum* NRRL 5809 (type culture; = CBS 246.67) was isolated by J. C. Renard in 1966 from savannah soil collected in Abidjan, Ivory Coast.

Production of ascostromata and sclerotia. *Eupenicillium* ascostromata and *Penicillium* sclerotia were produced by solid substrate fermentation on autoclaved, whole corn kernels by the methods reported earlier for *Aspergillus* sclerotia (39), except that cultures were incubated at 25°C for 28 to 35 days. Ascstromata and sclerotia were stored at 4°C until extracted. The formation of ascstromata by *E. ochrosalmonum* on autoclaved corn kernels was first demonstrated by Wicklow et al. (41).

Insect feeding response to ascostromatal and sclerotial metabolites. To locate secondary metabolites that might function as insect antifeedants or toxins, portions of the crude extracts, column fractions, or purified metabolites obtained from ascstromata or sclerotia were incorporated into a standard agar-based pinto bean diet used in rearing *C. hemipterus* and *H. zea* (7, 39). Testing of crude extracts was performed by incorporation of the extract into the diet at levels approximating the concentrations found in the ascstromata or sclerotia.

Isolation and characterization of fungal metabolites. High-performance liquid chromatography (HPLC) separations employed a binary gradient system made up of two Beckman 110A pumps, a Beckman model 166 variable-wavelength UV detector, a Rheodyne model 7125 injector, and column sizes and types as specified below. Details of nuclear magnetic resonance (NMR), mass spectrometry (MS), and other instrumentation used in this work have been reported elsewhere (3).

Intact, dry ascstromata of *E. crustaceum* NRRL 3332 (480 g) were extracted at room temperature with hexane (4,800 ml four times) and then CHCl_3 (600 ml three times). After removal of solvents under vacuum, the hexane extract weighed 4.4 g and the CHCl_3 extract weighed 4.5 g. A sample of the hexane extract (1.2 g) was fractionated by silica gel column chromatography (3.5 by 50 cm) with elution with a step gradient from 30% CHCl_3 in hexane to 100% CHCl_3 , followed by a gradient from 0.5 to 10% MeOH in CHCl_3 . The fractions that eluted with 2 to 2.5% MeOH in CHCl_3 were combined and concentrated to give 62 mg of residue, which was then triturated with MeOH. A portion of the MeOH-soluble material (21 of 26 mg obtained) was purified by semipreparative reversed-phase HPLC (Beckman Ultrasphere 5- μ m-particle-size C_{18} column; 1 by 25 cm; 91% MeOH- H_2O at 2.5 ml/min) to give compound 1 (20 mg, retention time [R_t], 17.8 min). Compound 1 was identified by comparison with an authentic sample isolated from *Aspergillus tubingensis* NRRL 4700 (on the basis of HPLC coinjection, melting point, $[\alpha]_D$, UV, MS, ^1H NMR, and ^{13}C NMR data) (34). The fractions that eluted with 5 to 6% MeOH in CHCl_3 were combined to give 295 mg of brown oil upon removal of solvent. A portion of this sample (176 mg) was fractionated through a column of Sephadex LH-20 (20 to 100 μ m particle size, 2.5 cm inside diameter by 40 cm) with elution with 30% MeOH in CH_2Cl_2 and collection of 25-ml fractions. Fraction 6 (22.8 mg) was subjected to semipreparative HPLC (Shandon Hyperprep 100 HS BDS 8- μ m-particle-size C_{18} column, 1 by 25 cm; 75% CH_3CN - H_2O at 2.5 ml/min), to afford the known metabolite fumitremorgen A (compound 3, Fig. 1 [2.1 mg; R_t , 25 min], which was identified on the basis of ^1H NMR and MS data (5)).

Three portions of the crude CHCl_3 extract from *E. crustaceum* (350, 500, and 379 mg) were fractionated separately through a column of Sephadex LH-20 (20 to 100 μ m pore diameter, 2.5 cm inside diameter by 47 cm) with 50% CH_2Cl_2 -MeOH followed by MeOH as eluents. Similar fractions eluting with CH_2Cl_2 -MeOH were combined (64 mg) on the basis of their thin-layer chromatography behavior. Further separation of the combined fractions was carried out on a Harrison Chromatotron employing a silica gel 60 PF-254-precoated plate (stepwise gradient from 5 to 100% CH_2Cl_2 in MeOH at a flow rate of 2.5 ml/min). Similar fractions eluting at 5% CH_2Cl_2 were combined and purified by semipreparative reversed-phase HPLC (Beckman Ultrasphere 5- μ m-particle-size C_{18} column; 1 by 25 cm; 85% MeOH- H_2O at 2.5 ml/min) to afford compound 2

(4.6 mg; R_t , 31.8 min) as a white solid with the following characteristics: melting point, 115 to 118°C; $[\alpha]_D$, -22° (c = 0.1 g/dl; CHCl_3); electron impact mass spectrometry (70 eV) 423 (M^+ ; relative intensity, 2.8%), 405 (9.9), 387 (1.6), 330 (1.5), 234 (1.0), 210 (4.9), 196 (20), 184 (12), 182 (15), 168 (13), 156 (21), 143 (12), 130 (100), 117 (17). ^1H NMR (multiplicity; J in hertz; assignment): 8.22 (br s; H-1), 7.32 (d; 2.4; H-2), 7.15 (ddd; 8.0, 7.1, 0.9; H-6), 7.21 (ddd; 8.1, 7.1, 1.0; H-7), 7.37 (br d; 8.1; H-8), 3.80 (dd; 13, 5.2; H-10), 2.10 (ddd; 5.2, 5.2, 2.2; H-11), 1.57 (m; H-12), 1.57 (m; H_a -13), 1.16 (m; H_b -13), 1.54 (m; H_a -14), 0.92 (m; H_b -14), 1.15 (m; H-16), 1.69 (dddd; 13, 13, 13, 3.6; H_a -17), 1.27 (m; H_b -17), 1.82 (m; H_a -18), 2.05 (dd; 4.4, 2.9; H_b -18), 4.75 (br s; H-19), 2.00 (m; H_a -21), 1.82 (m; H_b -21), 1.47 (ddd; 13, 13, 13, 5.1; H_a -22), 1.90 (m; H_b -22), 2.60 (ddd; 13, 13, 6.4; H-23), 1.09 (s; H-25), 1.13 (s; H-26), 1.36 (d; 7.1; H-27), 0.72 (d; 6.8; H-28), 0.95 (s; H-29). ^{13}C NMR: 123.2 (C-2), 117.4 (C-3), 127.1 (C-4), 118.0 (C-5), 119.8 (C-6), 122.5 (C-7), 111.5 (C-8), 136.0 (C-9), 33.1 (C-10), 40.8 (C-11), 29.3 (C-12), 28.1 (C-13), 28.2 (C-14), 39.2 (C-15), 31.1 (C-16), 25.2 (C-17), 29.5 (C-18), 69.1 (C-19), 43.7 (C-20), 24.1 (C-21), 23.7 (C-22), 45.3 (C-23), 75.5 (C-24), 24.0 (C-25), 29.3 (C-26), 21.8 (C-27), 15.7 (C-28), 18.3 (C-29). Heteronuclear multiple bond correlations (H no. $\rightarrow$ C no.): H-2 $\rightarrow$ C-3, 4, 9; H-5 $\rightarrow$ C-3, 7, 9; H-6 $\rightarrow$ C-4, 8; H-7 $\rightarrow$ C-5, 9; H-8 $\rightarrow$ C-4, 6; H-10 $\rightarrow$ C-2, 3, 4, 11, 12, 23, 24; H-11 $\rightarrow$ C-10, 12, 20, 21, 23, 27; H-12 $\rightarrow$ C-13; H_a -13 $\rightarrow$ C-14, 15, 27; H_b -13 $\rightarrow$ C-12; H_a -14 $\rightarrow$ C-13, 15, 16; H_b -14 $\rightarrow$ C-12, 15; H_a -18 $\rightarrow$ C-20; H_b -18 $\rightarrow$ C-17; H-19 $\rightarrow$ C-15, 17; H_a -21 $\rightarrow$ C-11, 22; H_b -21 $\rightarrow$ C-20, 22; H_a -22 $\rightarrow$ C-21, 23; H_b -22 $\rightarrow$ C-10, 20, 21; H-23 $\rightarrow$ C-3, 10, 22, 24, 25, 26; H-25 $\rightarrow$ C-23, 24, 26; H-26 $\rightarrow$ C-23, 24, 25; H-27 $\rightarrow$ C-11, 12, 13; H-28 $\rightarrow$ C-15, 16, 17; H-29 $\rightarrow$ C-14, 15, 16, 20.

Intact, dry ascstromata of *E. crustaceum* NRRL 22307 (15 g) were extracted with CH_2Cl_2 to provide 989 mg of crude extract after evaporation of the solvent. A portion of the extract (138 mg) was processed directly by preparative reversed-phase HPLC (Rainin 8- μ m-particle-size C_{18} column; 2.1 by 25 cm; 93% CH_3CN - H_2O at 10 ml/min) to afford 33.1 mg of macrophorin analog 4 (Fig. 1; R_t , 23.5 min). Complete characterization of compound 4 and minor analogs also obtained from this procedure will be reported elsewhere.

A sample of air-dried ascstromata of *E. reticulisporeum* NRRL 3446 (170 g) was extracted with hexane (1.8 liters three times) and then CHCl_3 (1.8 liters three times). After removal of solvent, a portion of the crude CHCl_3 extract (531 mg of 1.58 g) was fractionated by column chromatography on Sephadex LH-20 (2.5 by 40 cm) with elution with 40% MeOH in CH_2Cl_2 and collection of 8-ml fractions. Fractions 22 to 25 were combined to give 31 mg of material, which was further purified by preparative HPLC (Rainin 8- μ m-particle-size C_{18} column; 2.1 by 25 cm; 58% CH_3CN - H_2O at 10 ml/min) to afford 14.2 mg of pyripyropene A (compound 5, Fig. 1 [R_t , 42 min]). Compound 5 was identified by comparison of ^1H NMR, ^{13}C NMR, and MS data to values in the literature (16).

Quantitative analysis of the major aflavinine analog 1 in ascstromata of *E. crustaceum* NRRL 3332. A series of 10 standard MeOH solutions of the major component 1 were prepared, covering the range from 0.5 to 5 mg/ml. HPLC analysis of each sample was carried out with a Beckman 5- μ m-particle-size Ultrasphere C_{18} column (4.6 mm by 25 cm) with elution with 85% MeOH- H_2O at 1.0 ml/min and UV detection at 215 nm. Ten-microliter aliquots of each sample were injected, and a calibration curve was generated by plotting concentration versus peak area. Duplicate solutions of the hexane extract were made in MeOH to a concentration of 10 mg/ml. HPLC analyses were performed for each sample in duplicate under the same conditions used for calibration. The approximate concentrations of compound 1 in the extract samples were calculated from the corresponding peak area with the calibration curve.

Presence of metabolites in the mycelium and culture medium. To determine if ascstromatal metabolites are also products of the fungal mycelium, petri dishes containing an agar medium that supports abundant sclerotium formation in *Aspergillus* spp. (19) were inoculated with conidial suspensions of *E. crustaceum* NRRL 3332. These dishes were incubated in the dark at 25°C for 21 days. After incubation, all ascstromata were removed with a microspatula with the aid of a dissecting microscope. The remainder of the culture, consisting of both agar medium and fungal mycelium, was freeze-dried and then ground with a porcelain mortar and pestle prior to solvent extraction. Extracts of the ascstromata and the agar/mycelium material were separately analyzed by ^1H NMR and HPLC for the presence of the major metabolite 1 (Fig. 2).

Liquid shaken cultures of *E. crustaceum* NRRL 3332 were grown in a medium nutritionally identical to that used to produce the metabolites on petri dishes as described above. Cultures (three cultures of 50 ml of broth in 250-ml Erlenmeyer flasks) were aerated by agitation on a New Brunswick model G-53 orbital shaker at 200 rpm. The culture broths and mycelia were collected after 30 days and analyzed separately by ^1H NMR and HPLC for aflavinine analog 1.

RESULTS AND DISCUSSION

The hexane and CHCl_3 extracts of the ascstromata produced by *E. crustaceum* NRRL 3332 displayed significant activity in assays against the corn earworm, *H. zea*. Chromatography of the extracts on silica gel, followed by reversed-phase HPLC, afforded compounds 1 and 2 (Fig. 1). The ^1H NMR spectrum of compound 1 showed signals characteristic of a 3-substituted indole, while ^{13}C NMR data showed 28 carbon

FIG. 1. Structures of 10,23-dihydro-24,25-dehydroaflavinine (compound 1), 10,23,24,25-tetrahydro-24-hydroxyaflavinine (compound 2), fumitremorgen A (compound 3), macrophorin analog (compound 4), and pyripyropene A (compound 5).

signals. These data were suggestive of indole diterpenoid metabolites encountered previously as antiinsectan constituents of *Aspergillus sclerotia* (12, 32–34, 39). Indeed, upon comparison of NMR and MS data with those of an authentic standard

(34), compound 1 was identified as 10,23-dihydro-24,25-dehydroaflavinine. This compound has been previously reported only from the sclerotia of *A. tubingensis* NRRL 4700 (34).

Compound 2 exhibited very similar spectral data. The elec-

FIG. 2. (A) HPLC elution profile of chloroform extract of ascostromata harvested from agar cultures of *E. crustaceum* NRRL 3332 (10 µg injected). (B) HPLC elution profile of chloroform extract of mycelium and agar medium of the same agar culture (same conditions, 1,000 µg injected).

tron impact mass spectrum contained a molecular ion at m/z 423, along with prominent ions at m/z 405 ($M-H_2O$) and 130 (characteristic of an alkyl-substituted indole). The electron impact MS results, together with the ^{13}C NMR and distortionless enhancement by polarization transfer (DEPT) spectra, indicated the molecular formula $C_{28}H_{41}NO_2$. Compound 2 was identified as 10,23,24,25-tetrahydro-24-hydroxyaflavinine, primarily on the basis of analysis of two-dimensional NMR data (correlation spectroscopy, heteronuclear multiple bond correlation (2), and heteronuclear multiple quantum correlation [26]) and comparison with the data for compound 1. This new metabolite and known compound 1 are the first aflavinine derivatives to be reported from a fungal genus other than *Aspergillus*.

The ascostromatal extracts from *E. crustaceum* NRRL 3332 were thoroughly examined for the presence of further aflavinines. None were detected, although the known compound fumitremorgen A (compound 3; Fig. 1) (5) was observed in trace amounts. Verruculogen, a close analog of compound 3 lacking the prenyl group on the oxygen alpha to the indole, has been reported as a metabolite of several *Eupenicillium* spp., including *E. crustaceum* CBS 344.61 (14), and is active against *H. zea* at 25 ppm (8). Interestingly, compound 3 showed no activity against *H. zea* at 50 ppm.

Because of some difficulty in cleanly separating (on a pre-

parative scale) the nonpolar compounds 1 and 2 from lipids found in the extracts, the isolated yields of these compounds were not representative of their true concentrations in *E. crustaceum* ascostromata. In order to determine the approximate concentration of the major component (compound 1) in the ascostromata, quantitative analysis was performed directly with the hexane extract by analytical-scale HPLC peak integration, since nearly all of compound 1 was found in this fraction. The results indicated that compound 1 is rather abundant, making up 30% of the crude hexane extract and occurring in the ascostromata at approximately 2,800 ppm (dry weight [2.8 mg/g of dry ascostromata]).

Assays of major components 1 and 2 in insect dietary tests against *C. hemipterus* and *H. zea* at 100 ppm showed little or no activity in either assay. However, when compound 1 was tested at concentrations closer to natural levels (ca. 3,000 ppm), a 79% reduction in weight gain was observed in *H. zea* larvae, and a 42% reduction in feeding rate was observed in *C. hemipterus* larvae. These data suggest that the naturally occurring concentration of compound 1 in *E. crustaceum* ascostromata may serve a defensive role against insect predation, by analogy to that proposed for the aflavinine derivatives isolated from sclerotia of *Aspergillus flavus* and related species (13, 38). While it is not clear at this stage whether these compounds show selectivity for insects, it is known that 20,25-dihydroxyaflavinine is nontoxic to 1-day-old chickens at 300 mg/kg of body weight (6) but deters feeding in *C. hemipterus* at 100 ppm (13, 39). Some aflavinine analogs (not including compound 1) are also known to display mild antibacterial activity (13, 34).

Ascostromata obtained from agar cultures of *E. crustaceum* NRRL 3332 were found to contain aflavinine 1 at levels comparable to those produced in solid substrate fermentation. Significantly, the HPLC trace of the extract obtained from the residual mycelium or agar from the same agar plates (Fig. 2) indicated that only a trace amount of compound 1 was detected ($\leq 0.1\%$ of the level detected in ascostromata on a per-milligram-of-extract basis). No other indole metabolites were observed. It is possible that this trace amount could be accounted for by leaching of material from the ascostromata as they rested on the agar surface during the culture process, although the results of a control experiment were inconclusive. Even so, these results indicate that aflavinine 1 is concentrated heavily, or perhaps exclusively, in the ascostromata of *E. crustaceum*. Again, this finding is analogous to results obtained for *A. flavus*, wherein the antiinsect aflavinine derivatives were found to be concentrated primarily or exclusively in the sclerotia (13, 38, 41).

The association of aflavinines with ascostromata suggested that the compounds might not be produced under liquid fermentation conditions, since ascostromata of *E. crustaceum* are not formed in liquid shaken cultures. Indeed, neither compound 1 nor any other indole metabolites were detected by HPLC or NMR analysis in the culture filtrate or the mycelium of *E. crustaceum* NRRL 3332 liquid cultures.

Ascostromatal extracts from three other *E. crustaceum* isolates (NRRL 3556, NRRL 22307, and NRRL 22755) and several other related species were analyzed by NMR and HPLC in order to determine whether compounds 1 and 2 (or other aflavinine analogs) could be detected (Table 1). Ascostromata of *E. crustaceum* NRRL 3556 and NRRL 22755 were found to contain levels of compound 1 similar to those present in NRRL 3332. However, neither compound 1 or 2 nor any other aflavinines were detected in ascostromata produced by the NRRL 22307 isolate. Instead, this isolate was found to produce a major antiinsect metabolite representative of a different

TABLE 1. Types of antiinsectan metabolites present in ascostromata of *E. crustaceum* and related species

Fungal strains	Presence of type of compound	
	Aflavinine (e.g., compound 1)	Macrophorin (e.g., compound 4)
<i>E. crustaceum</i>		
NRRL 3332	+	—
NRRL 3556	+	—
NRRL 22755	+	—
NRRL 22307	—	+
<i>E. egyptiacum</i>		
NRRL 2090 ^a	—	—
<i>E. molle</i>		
NRRL 13062	+	+
<i>E. abidjanum</i>		
NRRL 5809	—	—
<i>E. reticulispurum</i>		
NRRL 3446	+	—
NRRL 3447	+	—
<i>P. gladioli</i>		
NRRL 938	—	—
NRRL 939	—	—
QM 2743	—	—
<i>P. raciborskii</i>		
NRRL 2039	—	—
NRRL 22306	—	—

^a Extracts of freeze-dried agar cultures (21 days at 25°C).

structural class. This major component, 4, is closely related to the known compound macrophorin A, which had been previously reported from a *Macrophoma* sp. (24) and from *Penicillium brevi-compactum* Dierckx (1). Compound 4 was present in the ascostromata of NRRL 22307 at very high concentrations (15,000 ppm isolated yield), approximately five times the level of aflavinine 1 found in *E. crustaceum* NRRL 3332. This metabolite exhibited significant antiinsectan activity in dietary assays, even when tested at only 15% of the ascostromatal concentration (2,300 ppm), causing 63% reduction in weight gain among *H. zea* larvae, 40% reduction in feeding rate among *C. hemipterus* adults, and 69% reduction in feeding rate among *C. hemipterus* larvae.

Several other fungal isolates bearing some assigned taxonomic relationship to *E. crustaceum*, including several *Penicillium* spp., were also examined chemically (Table 1). *E. molle* NRRL 13062 was found to produce both compounds 1 and 4. Neither aflavinines nor macrophorins were detected in extracts of freeze-dried cultures of *E. egyptiacum* NRRL 2090. None of the *Penicillium* anamorphs most closely related to *E. crustaceum* (i.e., *P. gladioli* NRRL 938, NRRL 939, and QM 2743; *P. raciborskii* NRRL 2039 and NRRL 22306) were found to produce aflavinines or macrophorins. The micromorphology of the penicillius in *P. raciborskii* is much different from that of the anamorph state of *E. crustaceum*, and it is unclear whether the strain identified as *E. crustaceum* by Scott (25) is the same strain received by the Centraalbureau voor Schimmelcultures as CBS 215.71. As chemotaxonomic evidence, our results support the hypothesis of Stolk and Samson (28) that *E. molle* is closely related to *E. crustaceum* but that *P. gladioli* is not. Unfortunately, *E. egyptiacum* NRRL 2090 produces only

sparse ascostromata in culture, and this may explain our inability to detect aflavinines or macrophorins in this taxon.

As part of our larger survey of *Eupenicillium* species, *E. reticulispurum* Udagawa was also found to produce antiinsectan metabolites. Stolk and Samson (28) placed *E. reticulispurum* in synonymy with *E. abidjanum* Stolk. Therefore, we also examined the ascostromata produced by these taxa for antiinsectan metabolites.

Two *E. reticulispurum* isolates (NRRL 3446 and NRRL 3447) were found to produce aflavinine analog 1 as a minor component. One of these (NRRL 3446) also produced an additional, unrelated compound called pyripyropene A (compound 5), recently reported from an isolate of *A. fumigatus* as a potent inhibitor of acyl-coenzyme A-cholesterol acyltransferase (16). Pyripyropene A was responsible for much of the antiinsectan activity of the extracts, causing a 62% reduction in weight gain among *H. zea* larvae at a dietary level of 50 ppm. Neither pyripyropene A, aflavinines, nor macrophorins were detected in extracts of ascostromata produced by *E. abidjanum* NRRL 5809. Udagawa (35) distinguished *E. reticulispurum* from *E. abidjanum*, in part because of larger ascospore size. Our evaluation of ascostromatal chemistry supports the view that these are distinct taxa.

Despite the fact that antiinsectan aflavinine 1 is concentrated in *E. crustaceum* ascostromata, it is possible that the ascostromata of some *Eupenicillium* species may not contain unique metabolites. Frisvad et al. (1) found that the production of secondary metabolites in *Eupenicillium javanicum*, *Eupenicillium ehrlichii*, and other *Eupenicillium* species was not dependent on the production of ascostromata. No additional secondary metabolites were detected in media in which ascostromata were present compared with media in which no ascostromata were produced. Furthermore, the secondary metabolite profiles of ascostromatal isolates from these species were identical to those produced by strictly conidial isolates from non-ascostromatal colony sectors. However, it may be significant that *E. javanicum* and *E. ehrlichii* produce soft, pseudoparenchymatous ascostromata which ripen to form ascospores within 2 weeks, while most other *Eupenicillium* species produce very hard sclerotoid ascostromata (28).

The reasons for such differences among various *Eupenicillium* spp. are not clear. Stolk and Samson (28) observed that most cultured strains of *Eupenicillium* soon lose their ability to develop ascospores, the ascostromata becoming smaller and more reduced after each transfer, and eventually the cultures may become completely conidial. It is not known whether there is a corresponding qualitative or quantitative decline in the chemical defense systems of these ascostromata upon repeated transfer. It is also possible that some differences in metabolite profiles between ascostromata and other fungal material may not have been observed because of a relatively small contribution of ascostromatal extract to overall colony extract or the presence of unknown metabolites for which standards were not available.

It is interesting to note that similar contrasts have arisen in studies of *Aspergillus* sclerotia. Wicklow and Cole (36) reported that 20,25-dihydroxyaflavinine was a product of *A. flavus* sclerotia, and not of the fungal mycelium or conidia. At the same time, they observed that the mycotoxins aflatoxin and cyclopiazonic acid were detected in the culture medium, mycelium, and sclerotia of *A. flavus*. Subsequent, detailed chemical investigations have shown that other *Aspergillus* spp. (*A. nomius* and *A. sulphureus*) also produce antiinsectan secondary metabolites that are confined predominantly to sclerotia (12, 17, 38), although some species (*A. tubingensis* and *A. niger*)

produce antiinsectan metabolites that are abundant in both the mycelium and sclerotia (31).

The high levels of aflavinine derivative 1 present in *E. crustaceum* ascostromata would clearly result in significantly reduced feeding by the fungivorous *C. hemipterus*. In our previous studies with various *Aspergillus* spp., we found that powdered sclerotia from which antiinsectan extracts were later obtained invariably showed antiinsectan effects when provided as a diet to the insects. Even so, the potency of compound 1 in the dietary assay is considerably lower than that of the major aflavinine derivative encountered in *A. flavus* sclerotia (39). It is possible that compound 1 may display more potent activity against detritivorous and/or fungivorous invertebrates that colonize the same substrates or soil habitat as *E. crustaceum*. Unfortunately, details of the fungal life cycle or ecology of *Eupenicillium* spp., apart from *E. ochrosalmoneum* (15, 40) are unknown. *Eupenicillium* species are typically isolated from soil, and knowledge is very limited with regard to the specific organic substrates these fungi colonize or the species of insects or arthropods that could threaten ascostromatal survival.

Other secondary metabolites have been reported as products of *Eupenicillium* spp., including agroclavine, brefeldin A, citreoviridin, citromycin, curvularin, gliotoxin, kojic acid, spinulosin, and verruculogen (14, 37), as well as fulvic acid, janthitrem, palitantin, paspaline, paspalinine, and penicillic acid (10). *Eupenicillium shearii* is reported to produce the tremorgenic paxilline (29). Some of these compounds may be products of the ascostromata, because use of the agar plug method of Filtenborg et al. (9) to determine secondary metabolite production of fungal isolates includes ascostromata in the extractable culture material. Few of these metabolites have reportedly been tested for activity against insects. Recent studies in our laboratories of extracts from ascostromata of *E. shearii* have afforded a series of new janthitrem-like compounds and several paxilline-type metabolites with antiinsectan activity (3).

The occurrence of antiinsectan metabolites, especially aflavinines, as major constituents of the ascostromata of *Eupenicillium* spp. provides an intriguing parallel to their occurrence (also as major metabolites) in the sclerotia of *Aspergillus* spp. Those *Eupenicillium* species that produced aflavinines are also known for their ability to grow rapidly with reduced water activity (20), not unlike *Aspergillus* spp. (21). It is not surprising that sclerotoid ascostromata, which have an important role in long-term fungal survival, should contain antiinsectan metabolites. The capacity to produce such compounds may have evolved (or may have been retained) in part because they serve as effective chemical defenses against insects, thereby rendering a survival advantage to these vital fungal bodies.

Although the results presented here do not prove that sclerotia and ascostromata generally produce chemical defenses, they do suggest that further studies of the chemistry of ascostromata, sclerotia, and other important physiological structures may be a valuable approach to the discovery of new bioactive fungal metabolites and may also be useful increasing understanding of fungal ecology and taxonomy.

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