Antiinsectan Natural Products from Fungal Sclerotia

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It is generally accepted that many plants and animals produce and/or sequester metabolites that serve as chemical defenses. By contrast, relatively little is known about chemical defense systems that have evolved among fungi. The fungi are well-known as prolific producers of secondary metabolites,[1] but most of these compounds have been discovered through random screening programs, or through studies of mycotoxins that contaminate food supplies of humans and livestock. Fungi commonly thrive in competitive environments, and it is often hypothesized that their secondary metabolic capabilities have been influenced by selection pressures exerted by other organisms. Indeed, fungal metabolites have been implicated in diseases of plants and insects, animal poisonings or intoxications, biocontrol of other fungi, and interspecies antagonism.[1] Reports of compounds imparting resistance to fungivory are rare, with most of the known examples coming from basidiomycetes (e.g., mushrooms). Many documented phenomena suggesting the presence of fungal "defensive" substances have never been followed up by chemical investigations.

Compelling evidence for the existence of fungal chemical defense systems has been documented and reviewed.[2] Mycelia or fruiting structures of various fungi are known to be avoided by fungivorous insects, presumably due to the presence of fungal metabolites.[2–7] It has also been proposed that the capability of fungi to produce mycotoxins evolved, and has been retained, partly because these toxins render fungal substrates (e.g., fruits, seeds, etc.) unpalatable to herbivores.[2,8,9] A related phenomenon is the production by grass endophytes of fungal ergot alkaloids that reduce consumption of the host grasses by herbivores.[10,11] Evidence for an ongoing coevolutionary process is provided by the fact that some fungivores have evolved detoxification mechanisms, allowing them to consume toxin-producing fungi and their substrates.[2,5,12,13]

In view of the extraordinary diversity of fungal species,[14] the proven track record of fungi as sources of valuable natural products,[1] and the relatively unexplored nature of most aspects of fungal chemical ecology, we initiated a research program designed to apply principles of chemical ecology to the search for novel bioactive fungal metabolites. Our initial efforts in this area focused on important fungal survival structures (sclerotia) that are exposed to potential predators (fungivorous insects) under natural conditions. Our results indicate that fungal sclerotia often contain unique antiinsectan metabolites that can help to protect them from predation.[15–39] This work has been characterized by a particularly high incidence of previously undescribed natural products, even in cases where the producing fungi have long histories of prior chemical study. This Account summarizes the results of these investigations.

On the basis of literature evidence that defensive metabolites of plants are often concentrated in reproductively important plant parts,[40] we began our studies of fungal chemical defense by targeting physiological structures that are particularly important to fungal survival and propagation. One such structure is the

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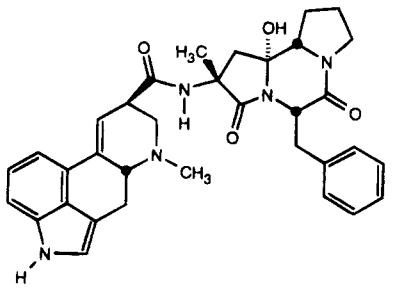
fungal sclerotium. Sclerotia are reproductive bodies produced by certain fungi as a mechanism for survival and propagation of the species.[41] They typically form in fungus-infected plant tissues and are separately dispersed onto the soil surface or remain attached to decaying plant parts. These important bodies survive harsh conditions that other fungal parts cannot withstand, and they typically lie dormant in soil for extended periods of time. Under favorable conditions, sclerotia eventually germinate to produce mycelia, spores, or fruiting bodies that lead to new fungal growth, thereby serving as a vital source of primary inoculum for the producing species. Generally, sclerotia survive under more severe conditions and for longer periods than any other kinds of fungal bodies, sometimes remaining viable in soil for periods of several years.[42] Sclerotia are by far the largest fungal propagules, ranging in size from 30 μm to 20 mm or more depending on species. Thus, sclerotia represent a substantial metabolic investment, and the production of these bodies is a key element of the survival strategy of the fungi which form them. During their dormant phase, sclerotia are exposed to large numbers of insects, many of which consume fungi as a part of their diet.[36] By analogy to plant seeds, some of which are known to contain toxins effective against seed-eating predators, sclerotia may contain metabolites which discourage their consumption by insects.

One conspicuous precedent provides a particularly useful introduction. The ergot alkaloids (e.g., **1**) comprise a class of medicinally useful compounds that were originally isolated from the ergots (sclerotia) of *Claviceps purpurea*.[43,44] It has been proposed that the evolutionary development of the ergot alkaloids may have been partly guided by selection pressures exerted by herbivores.[2,8] It is significant that sclerotia are not generally produced in liquid shake fermentation cultures used widely for metabolite screening. If defensive metabolites are concentrated in sclerotia, they may not be formed in liquid fermentations. Indeed, neither the ergot alkaloids nor *Claviceps* sclerotia were originally detected in submerged fermentation cultures, and many of the ergot alkaloids would not have been detected through traditional liquid culture screening of *C. purpurea*.



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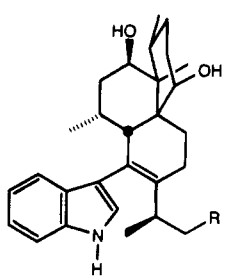
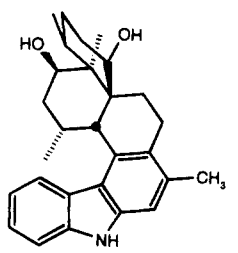


2 R = H

3 R = OH



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On the basis of these considerations, we set out to determine whether sclerotia produced by other fungi may have evolved chemical defenses effective against insect predation. In addition to the potential implications of such research in fungal ecology, new natural products with antiinsectan activity could be of practical value. The demand for new insecticides is compounded by the fact that problematic insect pests are developing resistance to many commonly used commercial pesticides.[45] Moreover, the use of some effective pesticides has been curtailed due to concerns about undesirable environmental impact.

Initial direction for this investigation was provided by the observation that sclerotia of the common fungus *Aspergillus flavus* are avoided by the dried-fruit beetle *Carpophilus hemipterus*, a fungivorous insect that often inhabits the same ecosystems as *Aspergillus* spp. *Aspergillus* spp. have been widely studied because of their ubiquitous nature and their tendency to produce mycotoxins, especially aflatoxins.[1] *C. hemipterus* adults and larvae will consume other parts of *A. flavus*, even when aflatoxins are present.[15] On the basis of these considerations, a dietary assay for feeding reduction in *C. hemipterus* was developed.[15] Sclerotial extracts are incorporated into a standard pinto bean test diet at levels approximating their concentration in sclerotia. Pure compounds are tested at specific concentrations, typically 100 ppm. The assay is evaluated in terms of percent feeding reduction compared to controls. The maximum rating for the assay is conser-

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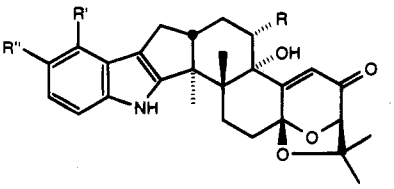
vatively estimated at 75%, since some exploratory feeding on the diet plug is nearly always observed.

Chemical investigations of cultures produced by solid-substrate fermentation on corn revealed that the sclerotia of *A. flavus* (NRRL culture No. 6541) contain a series of unique indole diterpenoids (aflavinines, e.g., **2**–**3**), most of which were previously undescribed at that time.[15,16,21,23] Indeed, the only prior reports of aflavinines had been from whole (probably sclerotium-containing) solid substrate fermentation extracts of *A. flavus*,[46,47] and one of these compounds was later shown to be found only in the sclerotia.[48] Our investigation also afforded a unique new aflavinine analog in which one of the side-chain vinylic methyl groups had become linked to the indole 2-position, leading to formation of a carbazole. This compound, aflavazole (**4**), contains a previously undescribed ring system.[21]

The most abundant sclerotial metabolite (**3**) completely prevented feeding by *C. hemipterus* in controlled studies at dietary levels far below its typical sclerotial concentration.[15,16,23] The other analogs were active as well, though to a lesser degree. Sclerotia produced by inoculation of corn in field plots also contained effective levels of the compounds.[16,23] All of these aflavinine analogs are confined to the sclerotia and were not found in the other tissues or in liquid shake cultures of *A. flavus*.[16,23] Examination of sclerotia from 11 other strains of *A. flavus* and its close relative *Aspergillus parasiticus* collected from various geographical locations indicated that the same compounds are present in each case.[23] In fact, the presence of high levels of these compounds in sclerotia can serve as a chemotaxonomic fingerprint for *A. flavus* and *A. parasiticus*, since none of the many other sclerotium-producing aspergilli we have examined produce this set of metabolites. The ergot alkaloids have been proposed to play a role in the ecology of *Claviceps* partly because of their high sclerotial concentrations (up to 1% dry weight). Interestingly, the aflavinines are present in *A. flavus* sclerotia at similar levels.

As might be expected, aflavinines are not the only antiinsectan metabolites present in *A. flavus* sclerotia. Aside from known compounds that were isolated [paspalinine (**5**), paspaline, kotanin, and aflatrem], two other new antiinsectan compounds representing different structural classes were also encountered.[24] The unsymmetrical bicoumarin polyketide aflavarin (**6**) was not as universally present as were the aflavinines, occurring in only about half of the *A. flavus* isolates examined. Compound **6** was nearly as effective as **3** against the fungivorous test insects, causing a 66% reduction in feeding rate among *C. hemipterus* adults in dietary assays at 100 ppm. Like the aflavinines, its sclerotial concentration in several *A. flavus* strains was much higher than 100 ppm, suggesting that it would be an effective contributor to chemical defense against *C. hemipterus* in those strains. A new representative of the paspalinine class, β-aflatrem (**7**), was also isolated from some strains of *A. flavus*. Paspalinine (**5**) is a tremorgenic mycotoxin, originally isolated from *Claviceps paspali*, whose structure and activity have inspired efforts at total synthesis.[49] As will be seen below, this general structure type occurs frequently among sclerotial metabolites and is often associated with considerable antiinsectan activity.

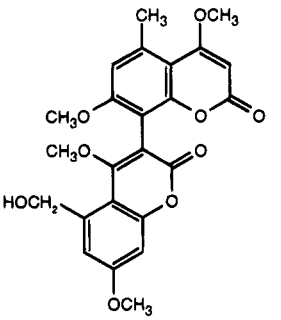


5 R = R' = H ; R'' = $C(CH_3)_2CH{=}CH_2$

7 R = R' = R'' = H

9 R = OH ; R' = R'' = H

10 R = OCOCH(i-pr)$N(CH_3)_2$; R' = R'' = H



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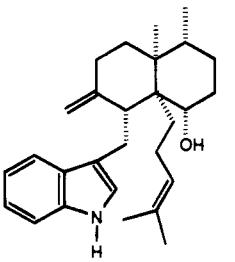

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These encouraging results inspired studies of other sclerotium-producing species of *Aspergillus* in search of additional naturally-occurring sclerotial compounds that cause feeding deterrence or insecticidal effects. Our research focused on a systematic investigation of fungi classified within major taxonomic subgroups of *Aspergillus* (*A. flavus* group, *A. niger* group, and *A. ochraceus* group).[50] Fungal strains to be examined were obtained from the NRRL culture collection at the USDA National Center for Agricultural Utilization Research. During the course of this work, the economically important crop pest *Helicoverpa zea* was added as a second assay organism. Although it is unlikely to be ecologically relevant to the sclerotia of *Aspergillus* spp., the discovery of agents with potent activity against *H. zea* could be of practical significance, since *Helicoverpa* spp. (formerly known as *Heliothis* spp.) have been cited as the most important insect pests in the United States in terms of economic loss.[45,51] A dietary assay similar to that described for *C. hemipterus* was employed, but in this case, *H. zea* larvae were evaluated for mortality and reduction in weight gain among survivors relative to controls, and effectiveness was compared to insecticide standards. Neither the aflavinines nor aflavarin showed significant activity in assays against *H. zea*. However, a variety of new compounds with activity against *H. zea* were discovered through further investigations of sclerotial metabolites, and many of these compounds are described in the remainder of this Account.

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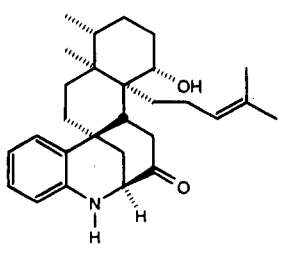
Sclerotia of *Aspergillus nomius* (NRRL 13137), a member of the *A. flavus* taxonomic group, yielded nominine (**8**),[17] 14-hydroxypaspalinine (**9**),[30] 14-[(*N,N*-dimethylvalyl)oxy]paspalinine (**10**),[30] and the particularly novel compound aspernomine (**11**),[25] all of which exhibit activity against *H. zea*. Despite its obvious structural and biogenetic similarity to the aflavinines, nominine (**8**) had little effect on *C. hemipterus*. However, it did show potent activity against *H. zea*, causing 40% mortality and 96% reduction in weight gain among survivors at 100 ppm. These values are comparable to those obtained in the same assay using the plant-derived insecticide azadirachtin and are within an order of magnitude of the potency of commercial pyrethroid insecticides (e.g., permethrin) in the assay. Unfortunately, although nominine does show some topical activity, it is not competitive with commercial insecticides when administered in this manner. Interestingly, the new paspalinine derivatives **9** and **10** also caused *ca.* 90% reduction in weight gain by *H. zea*, while paspalinine itself (**5**), a known tremorgen, caused essentially no effect at the same dietary concentration.[30] Compounds **9** and **10** are the only paspalinine analogs known that contain substitution at C-14, and *N,N*-dimethylvaline had been reported only once previously as a constituent of a natural product. Aspernomine (**11**), which possesses a novel ring system, was less potent against *H. zea*, although it did show significant cytotoxicity.[25] All of these compounds were found to be concentrated in the sclerotia of *A. nomius*.[25,30]



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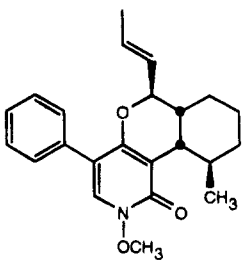

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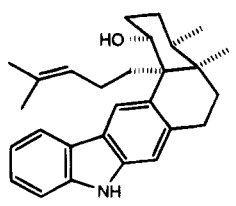

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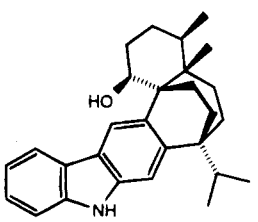

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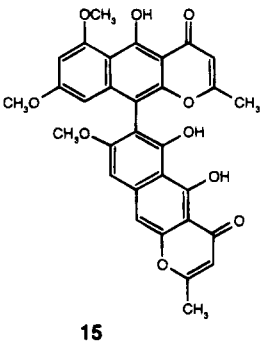
Studies of *A. leporis*, an additional *A. flavus* group member, afforded two aflavinine analogs, together with leporin A (**12**), an unrelated, new *N*-methoxypyridone of mixed biosynthetic origin.[22] Leporin A showed only moderate dietary activity at 100 ppm against *H. zea*, causing 36% reduction in weight gain relative to controls after 1 week.

Sclerotia from members of the *A. niger* taxonomic group contained some compounds arising from biosynthetic pathways similar to those of the *A. flavus* group, as well as some with quite different origins. Three more new aflavinine analogs, as well as two new carbazole alkaloids with previously undescribed ring systems (tubingensins A and B, **13** and **14**), were isolated from the sclerotia of *Aspergillus tubingensis* (NRRL 4700).[18-20] The same compounds were also detected in sclerotia produced by strains of a more common relative, *A. niger*.[23] These metabolites are not as effective against the test insects, even though **13** and **14** are more cytotoxic than the aflavinines. *A. tubingensis* and *A. niger* sclerotia also contain considerable amounts of a series of (mostly known) bis-naphthopyrones of the aurasperone/fonsecinone class (e.g., **15**),[1] which are responsible for most of the effects of the sclerotial extracts on the fungivorous beetle *C. hemipterus*.[23,36]



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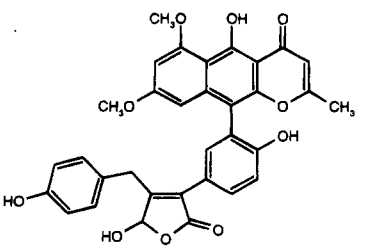

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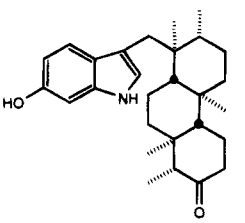

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Recent studies of an additional member of the *A. niger* group, *Aspergillus carbonarius* (NRRL 369), again afforded aurasperone and fonsecinone derivatives, but also provided members of a new class of aromatic compounds, which we named carbonarins A–G (e.g., carbonarin A; **16**).[32] The carbonarins presented an interesting problem in structure determination because of poor solubility, and because several of them showed doubled NMR signals due to atropisomerism in combination with the presence of a chiral center. The carbonarins consist of two dissimilar subunits that appear to arise from different biosynthetic pathways, and they represent a new family of compounds with no close literature analogs. The carbonarins, together with the bis-naphthopyrones, account for the potent activity of *A. carbonarius* extracts and fractions against *H. zea* and *C. hemipterus*. In this case, the activity of the extracts appears to be due to high concentrations of these compounds, because the individual metabolites are not especially potent. For example, compound **16** caused a 31% feeding reduction by *C. hemipterus* adults at the 100 ppm dietary level. It did not affect *H. zea* when tested at the same concentration, although it did cause 26% mortality and 52% reduction in leaf-damage in a cotton leaf disk diet assay against the fall armyworm *Spodoptera frugiperda* (another important agricultural pest) at 50 μg/75 mg disk. In contrast to the results described above for *A. flavus*, *A. parasiticus*, and *A. nomius*, the aurasperones, fonsecinones, and carbonarins could be detected in

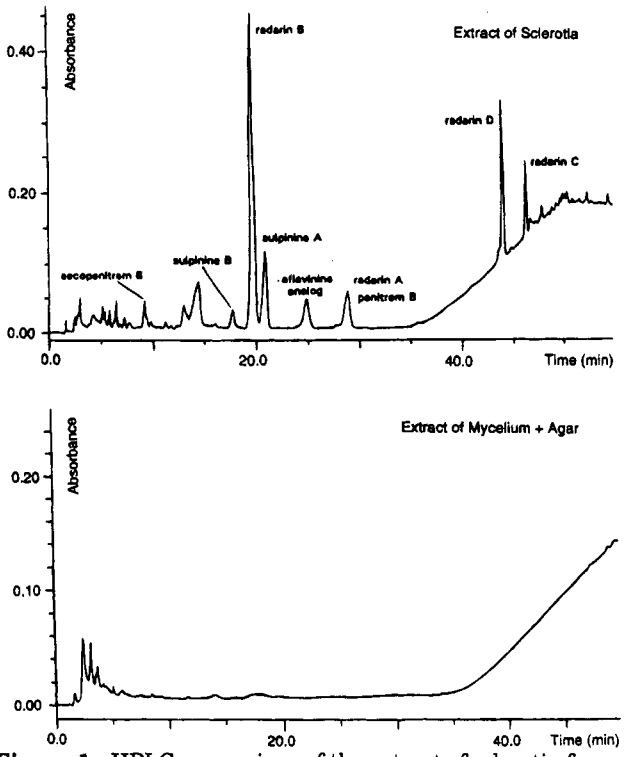
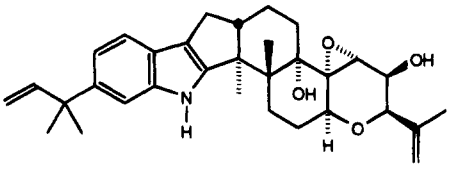


Figure 1. HPLC comparison of the extract of sclerotia from a Petri plate culture of *A. sulphureus* (NRRL 4077) with the extract of all other material (mycelium + agar) from the same plate. Chromatograms were obtained by injecting 50 μg of each extract, representing 0.3% of the total sclerotial extract and 1.3% of the total mycelium + agar extract. (Conditions: C_{18} column; 80:20 MeOH–H_2O for 30 min, followed by linear gradient to 100% MeOH over 10 min.)

other fungal parts of both *A. tubingensis* and *A. carbonarius*, albeit in somewhat lower concentrations than are found in the sclerotia.[23,32]

Representatives of the *A. ochraceus* taxonomic group have also afforded interesting results.[26–29,31,33] For example, sclerotia of *Aspergillus sulphureus* (NRRL 4077) contained a variety of additional new indole-derived antiinsectan compounds, including radarin A (**17**),[26] sulpinine A (**18**),[27] a ring-opened analog of penitrem B (**19**),[27] an oxidized penitrem derivative,[31] and several other related metabolites.[26–28] Compounds **17**–**19** cause reductions in weight gain in *H. zea* comparable to that caused by nominine (**8**), although they do not induce significant mortality. Biosynthetically-related compounds have been isolated from liquid cultures of other fungi.[1] However, as was the case with members of the *A. flavus* group, detailed analysis of *A. sulphureus* cultures indicated that the active compounds are concentrated in the sclerotia and are relatively scarce or absent in other fungal parts or liquid cultures.[28] Results from a representative experiment are shown in Figure 1. For this analysis, sclerotia were manually separated from Petri plate cultures of *A. sulphureus*, extracted, and analyzed by reversed-phase HPLC in comparison with the extracts of the remaining fungal material (mycelium) and agar from the Petri plates. The results clearly indicated that the antiinsectan compounds are again, selectively, if not exclusively, found in the sclerotia.

One of the metabolites isolated from *A. sulphureus* (sulpinine C; **20**) clearly arises from oxidative ring opening of the indole portion of sulpinine A (**18**).[27] This structural change results in a significant loss of activity against *H. zea* larvae. While **18** causes 96% reduction in weight gain in *H. zea* at 100 ppm, the



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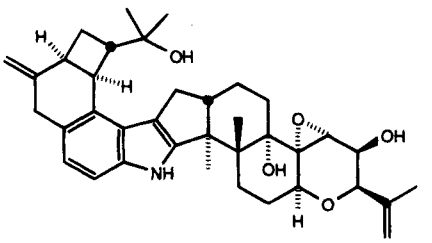

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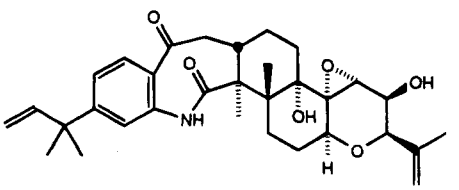

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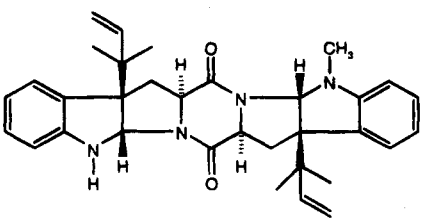

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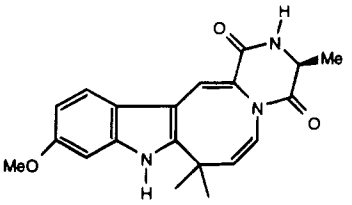
corresponding figure for **20** is only 26%. After analogous oxidation products of two related compounds were encountered in other organisms, it became clear that the indole ring in most of the paspalinine-type compounds is quite susceptible to autoxidation. For example, simply stirring a pure sample of paspalinine (**5**) in methanol in air for 3 days resulted in approximately 15% oxidation to the ring-opened analog.[38] Although **20** was found in freshly prepared sclerotial extracts, this does not preclude an autoxidative origin. This oxidation process may be an important degradation (and detoxification) pathway for the paspalinine class of compounds, and it probably occurs to some degree in all members of this important class.

Sclerotia produced by other members of the *A. ochraceus* group were found to contain several new metabolites not related to those described above. These compounds exhibited only moderate antiinsectan activity, although most of them were rather abundant constituents of the sclerotia.[29,37] Diketopiperazine-derived metabolites **21** and **22** and several bis-indolyl benzenoids (e.g., ochrindole A; **23**) were isolated as major constituents of the sclerotia of *A. ochraceus* (NRRL 3519). *Aspergillus melleus* (NRRL 5226) was also found to produce bis-indolyl benzenoids, as well as the known diterpenoid variecolin (**24**), which was previously reported as an angiotensin-II receptor binding inhibitor from Aspergillus *variecolor*.[52] *Aspergillus alliaceus* (NRRL 315) produces

paspalinine (**5**) and a series of bis-coumarin metabolites (e.g., isokotanin A; **25**).[33]



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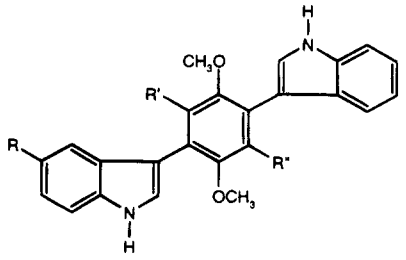
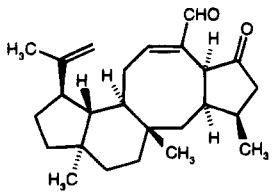


23 R = H; R' = OH; R" = $CH_2CH{=}C(CH_3)_2$

26 R = OH; R' = R" = OCH_3



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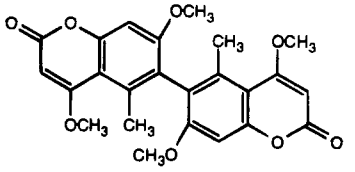

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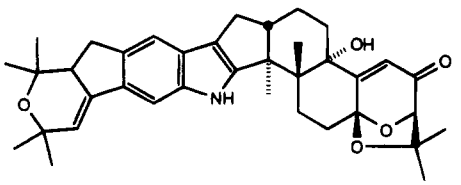
Knowledge of sclerotial metabolite profiles could have chemotaxonomic significance, as have secondary metabolite profiles generated in other ways.[53] However, unlike *A. flavus* and *A. nomius*, which afford virtually the same sclerotial chemistry regardless of the strain examined, different strains of *A. sulphureus*, *A. melleus*, and *A. ochraceus* appear to produce significantly different metabolite profiles. The type strain of *A. sulphureus* (NRRL 4077) consistently produces the sulpinines, radarins, and penitrem analogs. However, none of the other strains examined appear to form any of these compounds in significant quantities. Instead, we have found them to produce bis-indolyl benzenoids similar to those described above from *A. ochraceus* (e.g., **26**). In one case, we isolated nominine (**8**) as an *A. sulphureus* metabolite. In another instance, we encountered an aflavinine analog. We have now found nominine, aflavinines, and paspalinine-type metabolites in members of the *A. ochraceus*, *A. flavus*, and *A. niger* taxonomic groups. Thus, the biosynthetic pathways leading to these antiinsectan compound classes operate in representatives of each major taxonomic group of sclerotium-producing aspergilli. The presence this common set of biosynthetic pathways among these different taxa argues for the evolutionary significance of the compounds.

The success of these studies led to expansion of the scope of our efforts to encompass sclerotia from other fungal genera, as well as similar fungal bodies that are functionally analogous to sclerotia. Investigation of the sclerotial metabolites of *Penicillium* and *Eupenicillium* spp. was viewed as the next logical step in this research because of ecological similarities with *Aspergillus* and because of the well-known ability of *Penicillium* spp. to produce diverse bioactive chemical structures. In addition, certain species of the genus *Eupenicillium* (the sexual stage of *Penicillium*) produce physiological structures analogous to sclerotia, which are referred to as sclerotioid ascostromata. Ascostromata serve essentially the same function as sclerotia and would seem similarly likely to produce defensive metabolites. Sclerotia and ascostromata from representatives of *Penicillium* and *Eupenicillium* were produced by solid-substrate fermentation, and many of their extracts displayed antiinsectan activity. Chemical studies of *Eupenicillium* spp. have begun to afford interesting results.[35,36,38] The ascostromata of *Eupenicillium shearii* were found to contain a series of antiinsectan compounds that closely resemble metabolites isolated from sclerotium-producing *Aspergillus* spp., including the new compounds shearinines A–C (**27**–**29**) and isopentenylpaxilline (**30**).[38] The



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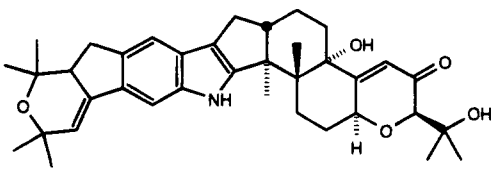

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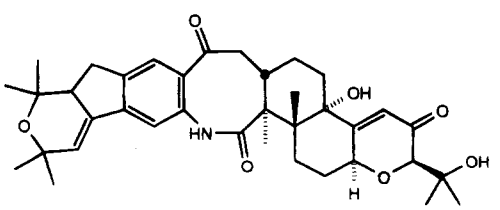

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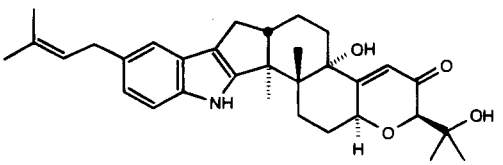

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shearinines are closely related to the janthitrems, a series of mycotoxins produced by *Penicillium jan-*

(52) Hensens, O. D.; Zink, D.; Williamson, J. M.; Lotti, V. J.; Chang, R. S. L.; Goetz, M. A. *J. Org. Chem.* **1991**, *56*, 3399.
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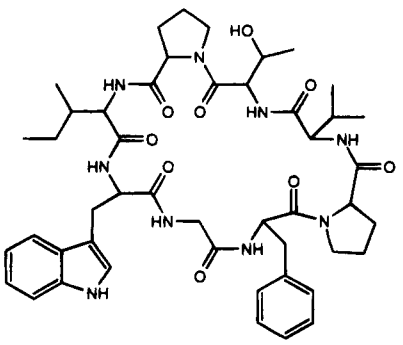
(54) deJesus, A. E.; Steyn, P. S.; vanHeerden, F. R.; Vleggaar, R. *J. Chem. Soc., Perkin Trans. 1* **1984**, 697.
(55) Wilkins, A. L.; Miles, C. O.; Ede, R. M.; Gallagher, R. T.; Munday, S. C. *J. Agric. Food Chem.* **1992**, *40*, 1307.

thinellum.[54,55] As might be expected from their structural resemblance to active compounds described above, these metabolites exhibit potent antiinsectan effects against *H. zea* in dietary, topical, and leaf-disk bioassays. For example, compound **28** causes 92% reduction in growth rate in the *H. zea* pinto bean diet assay (100 ppm) and 84% mortality in a leaf-disk diet assay against *S. frugiperda* (50 μg/75 mg leaf disk). Compound **27** causes an 80% reduction in *H. zea* larval growth rate in topical assays (2 μg/insect). Some activity was also observed against *C. hemipterus* larvae. Other compounds isolated from *E. shearii* include several new tryptophan-containing cyclic octapeptides (e.g., **31**).[34] These compounds show moderate activity in dietary assays against *H. zea* and *C. hemipterus*. A qualitative survey of eight different *E. shearii* isolates showed both the shearinines and the peptides to be present in each case. By analogy to the restriction to sclerotia of several of the *Aspergillus* metabolites described earlier, analytical experiments showed that the compounds are concentrated in the ascostromata.



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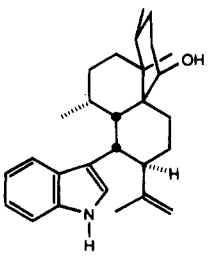

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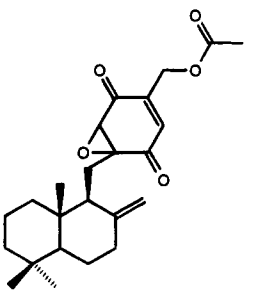
Studies of *Eupenicillium crustaceum* led to a particularly intriguing discovery. The ascostromata of several different isolates of *E. crustaceum* were found to contain approximately 0.3% by weight of a known aflavinine derivative that we had isolated earlier from *A. tubingensis* (**32**),[18] together with a previously unreported minor analog.[36] Prior to this discovery, the only known sources of aflavinines had been the sclerotia of *Aspergillus* spp. Moreover, by analogy to the concentration of aflavinines in *A. flavus* sclerotia, **32** is heavily concentrated in the ascostromata of *E. crustaceum* and is present at high levels that result in reduction in feeding by *C. hemipterus* (*ca.* 2800 ppm in NRRL 3332 ascostromata). Thus, it appears that sclerotium-producing *Aspergillus* spp., and at least some of the ascostroma-producing *Eupenicillium* spp., have evolved (or retained) very similar chemical defense systems. Although *Aspergillus* and *Eupenicillium* are not closely related taxonomically, they do occupy very similar ecological niches.

These initial findings suggested that the chemistry of *Eupenicillium* ascostromata might not differ significantly from that of *Aspergillus* sclerotia. However, more recently, we have discovered compounds representing biosynthetic classes quite different from those we have encountered in *Aspergillus* sclerotia. *Eupenicillium molle* (NRRL 13062) ascostromata contain, in addition to aflavinines, a series of major components (e.g., **33**) related to the known compound macrophorin A,[56] while ascostromata of *Eupenicillium reticulisporum* (NRRL 3446) contain the terpenoid-substituted pyridine pyripyropene A (**34**), which was reported recently by other workers from an isolate of *Aspergillus fumigatus* as a potent inhibitor of acyl-CoA–cholesterol acyltransferase.[57] Again, both compound types cause antiinsectan effects. For example, **33** was found to be present in *E. molle* ascostromata at levels comparable to those found for the aflavinines in *E. crustaceum*. It exhibited significant antiinsectan activity in dietary assays at the ascostromatal concentration (*ca.* 2300 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.



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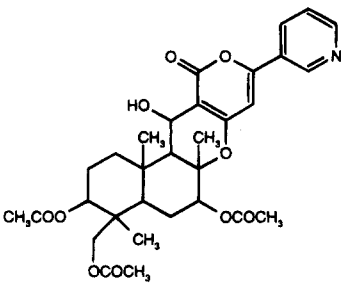

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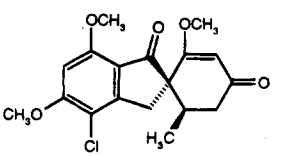

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The only antiinsectan compounds that we have isolated thus far from *Penicillium* sclerotia are griseofulvin (**35**; from *Penicillium raistrickii*) and a desmethyl analog, both of which are well-known compounds. Griseofulvin is very abundant in the sclerotia of *P. raistrickii*, shows activity in our assays (*ca.* 40% reduction in weight gain vs *H. zea* at 250 ppm),[58] and is quite different from the sclerotial metabolites of *Aspergillus*.

Studies of sclerotia from other fungi have yielded other antiinsectan compounds that await publication, and further investigations are continuing in our laboratories. Despite the fact that the chemistry of *Aspergillus* had been heavily studied for many years, selective investigation of *Aspergillus* sclerotia has proven to be a fruitful approach to the discovery of new antiinsectan natural products. The structural relationship of many of these metabolites to compounds known to display physiological effects in mammals suggests that they may display additional bioactivities as well. In relatively limited testing, individual metabolites isolated in this project have been shown to exhibit antibacterial, antifungal, and antiviral activities, as well as cytotoxicity toward tumor cell lines.[16,23–26,37]

Many of the compounds isolated would not completely deter feeding by *C. hemipterus* at sclerotial

(56) Ayer, W. A.; Altena, I.; Browne, L. M. *Phytochemistry* **1990**, *29*, 1661.
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(58) Dowd, P. F. *Entomol. Exp. Appl.* **1993**, *69*, 5.

concentrations, but other active metabolites are generally present. Indeed, the natural levels of the complex cocktails of compounds found in the sclerotia of most of the aspergilli we have screened would undoubtedly cause considerable feeding reduction by *C. hemipterus*. Few of the compounds have been tested in combinations, but there is also precedent for synergistic effects against insects caused by fungal metabolite mixtures.[36,59] In addition, there are a great many other potential predators that would likely be affected by the presence of these metabolites. Only two to three insect species are used in our screens, and many compounds that show no activity against one cause considerable effects against another at the same dietary concentrations.

Conclusions

To date, our studies in this area have afforded over 70 new chemical structures, nearly all of which display some degree of activity against insects. Approximately 25 previously known compounds have also been isolated, many of which also cause antiinsectan effects. Interestingly, these bioassay-guided studies have not led us to isolate a single aflatoxin analog, although other known mycotoxins such as ochratoxin A, paspalinine, and penitrem B have been encountered. The success rate in finding new compounds has been exceptionally high, despite extensive prior studies of the chemistry of *Aspergillus* spp. The effectiveness of these studies clearly argues for continued selective study of other fungal structures that serve similar functions. In addition, these results strongly argue that assumptions about the identity of compounds causing ecological effects should not be based on previous knowledge of the chemistry of a fungal species. Given the vast body of prior work on *Aspergillus* metabolites, such assumptions might have seemed warranted in the case of *Aspergillus* sclerotia, but would clearly not have been valid on the basis of the results described above.

Although our results do not unequivocally prove that sclerotium-producing fungi have evolved chemical defenses that render a survival advantage, this work clearly demonstrates that observations in fungal ecology can be employed as leads to the discovery of novel natural products with predictable types of bioactivity. These findings help to validate the application of fundamental principles of chemical ecology to studies of fungi and provide additional tools with which to study fungal ecology and taxonomy. Carefully controlled ecological studies are needed to determine whether these compounds are truly significant in the life histories of the producing species. In any event, the discovery of new structural types with antiinsectan effects and possibly new modes of action is obviously of considerable interest. The potential for these compounds as natural agrochemicals appears to lie in their oral toxicity to insects. The most potent compounds isolated in this project to date display activity comparable to that of malathion or azadirachtin in dietary assays against *H. zea* larvae. None of the compounds are competitive with the most potent commercial contact insecticides (e.g., fourth-generation pyrethroids), but could be useful as lead compounds, as were the pyrethrins.

The high percentage of novel compounds isolated through these studies is due in part to the assay systems employed. The use of "ecologically relevant" assays employing fungivorous insects, in addition to agriculturally relevant test organisms, would understandably lead to some discoveries not likely to be made by those employing only the latter assays. However, other contributing factors include the nonrandom selection of organisms for study, the relatively unexplored nature of these fungal bodies, and the fact that "sclerotial" chemistry is often different from that observed in liquid culture. The presence of different metabolites in sclerotia or other nonmycelial physiological structures is consistent with commonly observed differences in metabolite profiles produced by many fungi on solid substrates, as opposed to liquid shake culture, and helps to rationalize such differences. These results argue strongly for employment of solid-substrate fermentation in screening for new fungal metabolites, and for consideration of habitat, ecological characteristics, and taxonomy when selecting fungi for screening.[60]

There is much current interest in enhancing chemical diversity for screening programs. Strategies employing ecological rationale of the type described here could be particularly valuable as a complement to ongoing random screening programs in the search for new bioactive fungal metabolites. As discussed in some recent reviews, there already appears to be a growing recognition of the potential merits of combining an increased level of rationale in organism selection with sophisticated, high-throughput bioassays.[60-62] There are many ecological niche groups among the fungi that remain virtually unexplored from a chemical standpoint.[60,62] If the results described above are any indication, chemical studies that target such areas are likely to be very fruitful.

Support for our work in this area from the National Science Foundation (CHE-9211252 and CHE-8905894), Biotechnology Research and Development Corporation (24-1-078), and the Herman Frasch Foundation (351-HF92) is appreciated. I would also like to acknowledge Drs. D. T. Wicklow and P. F. Dowd of the USDA National Center for Agricultural Utilization Research, Peoria, IL, whose collaboration with our group on this project has been both enjoyable and invaluable. Finally, I wish to acknowledge the graduate students and other research associates who conducted these studies: their names appear frequently among the references.

AR950013V

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