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Secondary Metabolites from *Escovopsis weberi* and Their Role in Attacking the Garden Fungus of Leaf-Cutting Ants

Basanta Dhodary,[a] Michele Schilg,[b] Rainer Wirth,[b] and Dieter Spiteller*[a]

Abstract: The specialized, fungal pathogen *Escovopsis weberi* threatens the mutualistic symbiosis between leaf-cutting ants and their garden fungus (*Leucoagaricus gongylophorus*). Because *E. weberi* can overwhelm *L. gongylophorus* without direct contact, it was suspected to secrete toxins. Using NMR and mass spectrometry, we identified several secondary metabolites produced by *E. weberi*. *E. weberi* produces five shearinine-type indole triterpenoids including two novel derivatives, shearinine L and shearinine M, as well as the polyketides, emodin and cycloarthropsone. Cycloarthropsone and emodin strongly inhibited the growth of the garden fungus *L. gongylophorous* at 0.8 and 0.7 μmol, respectively. Emodin was also active against *Streptomyces* microbial symbionts (0.3 μmol) of leaf-cutting ants. Shearinine L instead did not affect the growth of *L. gongylophorus* in agar diffusion assays. However, in dual choice behavioral assays *Acromyrmex octospinosus* ants clearly avoided substrate treated with shearinine L for the garden fungus after a 2 d learning period, indicating that the ants quickly learn to avoid shearinine L.

Introduction

Attine ants (subfamily Myrmicinae, tribe Attini) evolved the practice to cultivate the fungus *Leucoagaricus gongylophorus* (Agaricales: Leucocoprineae). Among attine ants, the genera *Acromyrmex* and *Atta* are unique because they provide their fungal symbiont with fresh plant material as growth substrate. In turn the fungus serves the ants as primary food source.[1]

Microbial pathogens from the surrounding soil of the nest and microorganisms brought into the nest because of the foraging behavior[2] of the leaf-cutting ants potentially threaten the garden fungus as well as the leaf-cutting ants' survival.[3] Therefore, leaf-cutting ants control the growth of adverse microbes in their fungus gardens by chemical treatment[4] and weeding behavior.[5] In addition antibiotic-producing *Actinomyces* symbionts support leaf-cutting ants in the defense against noxious invaders.[6] Even so, a broad variety of detrimental bacteria, filamentous fungi and yeasts occur in the nest of leaf-cutting ants.[2,7] In particular, the specialized fungal parasite *Escovopsis* (Ascomycota, Pezizomycotina: anamorphic Hypocreales) attacks the fungus garden of leaf-cutting ants.[7b] *Escovopsis* fungi have never been isolated outside of the fungus-growing ants' ecosystem and have been established to have coevolved in association with fungus-growing ants and their cultivated fungi.[6a] In comparison to their closest known relatives, *Escovopsis* adapted to the mycoparasitic lifestyle, for example, by loss of genes coding for plant-material-degrading enzymes.[8] Moreover, different *Escovopsis* strains can destroy the fungus gardens of *Atta* and *Acromyrmex* leaf-cutting ants.[9]

Reynolds and Currie established in 2004 that *Escovopsis weberi* does not compete for the plant material brought into the nest by the leaf-cutting ants to feed *L. gongylophorus* but it directly consumes the mutualistic fungus.[10] *E. weberi* degrades the hyphae of *L. gongylophorus* before direct physical interaction, suggesting that the pathogen secretes toxins and/or enzymes that can break down host mycelium before contact occurs.[10] The recently sequenced *E. weberi* genome encodes for a variety of secondary metabolite biosynthesis gene clusters.[8] Some of these gene clusters including polyketide synthase gene clusters were significantly up-regulated when growing with *L. gongylophorus*.[8] Moreover, crude extracts of several *Escovopsis* species were reported to inhibit the growth of *L. gongylophorus*.[11] In a co-cultivation study of *E. weberi* with microbial symbionts of leaf-cutting ants Férnandez-Marín et al. discovered that *E. weberi* produces several shearinines.[12]

However, until now, no other secondary metabolites from *E. weberi* have been identified and it has not been established which compounds from *E. weberi* contribute to overpower the mutualistic fungus of leaf-cutting ants.[11,12] Here, we present the isolation and identification of secondary metabolites from *E. weberi* and evaluate their biological function, in particular their impact on *L. gongylophorus* and *A. octospinosus* leaf-cutting ants.

[a] B. Dhodary, Prof. Dr. D. Spiteller
Chemical Ecology/Biological Chemistry, University of Konstanz
Universitätsstraße 10, 78457 Konstanz (Germany)
E-mail: dieter.spiteller@uni-konstanz.de

[b] M. Schilg, Dr. R. Wirth
Plant Ecology and Systematics, Technical University Kaiserslautern
Erwin-Schrödingerstraße 13, 67653 Kaiserslautern (Germany)

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Results

Identification of secondary metabolites from *E. weberi*

Methanol extracts of *E. weberi* grown on SFM agar plates at 28 °C for 14 d were analyzed by high performance liquid chromatography (HPLC) coupled to a diode array detector and an electrospray mass spectrometer (LC-DAD-ESI-MS). The base peak chromatogram and the UV chromatogram (254 nm) (Figures 1 A and S1 in the Supporting Information) revealed a series of intense peaks. The compounds causing the most prominent peaks **1**–**7** were purified by silica gel column chromatography (CC), Sephadex LH-20 CC and reversed phase HPLC coupled with a fraction collector in order to elucidate their structures by mass spectrometry and NMR. The purified secondary metabolites comprised five shearinines including two novel shearinines (**1** and **2**), cycloarthropsone (**6**), and emodin (**7**) (Figure 1 B). *E. weberi* also produces these secondary metabolites when it grows together with *L. leucoagaricus* on PDA medium mimicking the attack of the garden fungus by *E. weberi* (Figure S31 B).

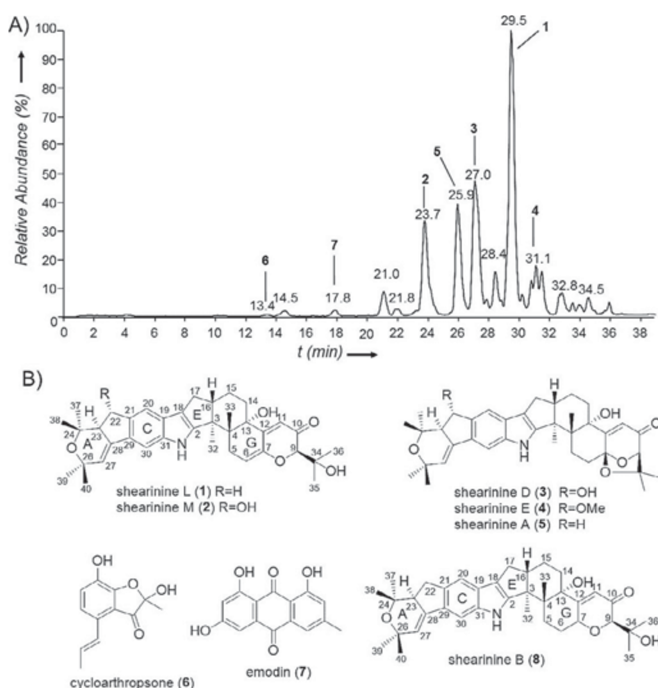

Figure 1. A) LC-negative-ESI-MS base peak chromatogram of a methanol extract of *E. weberi.* Labelled peaks were identified as shearinine L (**1**), shearinine M (**2**), shearinine D (**3**), shearinine E (**4**), shearinine A (**5**), cycloarthropsone (**6**), and emodin (**7**). B) Structures of the identified secondary metabolites from *E. weberi* and shearinine B (**8**) for comparison.

Shearinines

Several shearinines have been recently identified as secondary metabolites from *E. weberi*.[12] However, we observed additional prominent peaks **1** and **2** in the LC-DAD-ESI-MS profile that turned out to be novel shearinine derivatives.

Shearinines were detectable in SFM agar plates inoculated with *E. weberi* after 3 d at 28 °C. The amount of shearinines increased significantly with incubation time, reaching a maximum concentration of approximately 8 $\mu g\,mL^{-1}$ SFM medium of **1** after day 9. Shearinine L (**1**) was obtained as a white amorphous powder. Its molecular formula was established to be $C_{37}H_{45}NO_5$ by HR-ESI-MS corresponding to 16 double bond equivalents (DBE).

The ^1H NMR spectrum (Figure 2, Figure S3) showed the presence of signals for eight aliphatic methyl singlets which are characteristic for a shearinine-type indole triterpenoid as well as two indicative aromatic proton singlets at $\delta_H = 7.16$ (H-20) and 7.35 (H-30). The ^{13}C NMR spectrum (Figure S4) revealed 37 carbon resonances corresponding to eight methyl groups, five methylene groups, and eight methine groups. Furthermore, among the 16 quaternary carbons, the signal at $\delta_C = 197.6$ (C-10) indicated the presence of an unsaturated keto group. The ^1H and ^{13}C NMR data together with HMBC correlation analysis,

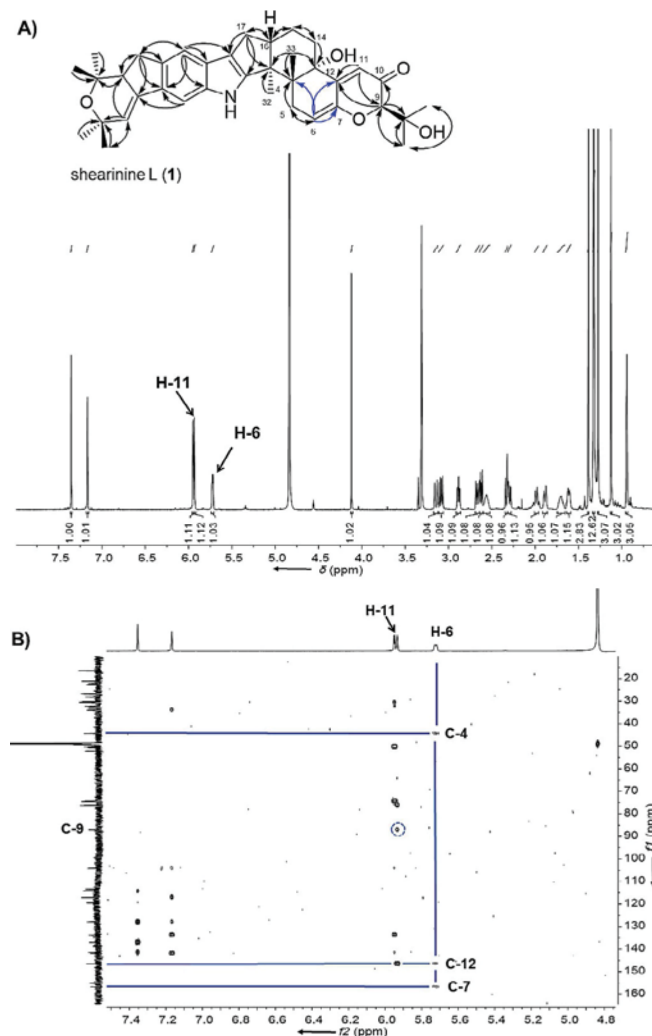

Figure 2. A) ^1H NMR of shearinine L (**1**), B) key ^1H–^1H COSY and ^1H–^{13}C HMBC correlations of shearinine L (**1**).

suggested that the structure of **1** is closely related to that of shearinine B (**8**).[13] Compound **1** and shearinine B (**8**)[13] differ only in the ring G (Figure 2). Compound **1** bears a double bound between C-6 and C-7 that causes the appearance of one characteristic olefinic proton at $\delta_H = 5.72$ (H-6). The HMBC correlations between H-6 and C-4 ($\delta_C = 44.3$), C-7 ($\delta_C = 156.8$) and C-12 ($\delta_C = 146.5$) (Figure 2B, Figure S7) further confirmed the position of the double bond. Thus, **1** was determined as Δ6,7-shearinine B (**8**) and called shearinine L. The relative configuration of shearinine L (**1**) was clarified by NOESY spectrum analysis (Figure S8). The presence of NOE correlations between H_3-32 to H-17α and also between H-16 to H_3-33 indicated a *trans*-3,16-ring fusion.

NOE correlations between H_3-33 and H-14β as well as between H-11 and H-14α suggested the α-orientation of the hydroxy group at C-13 (Figure S9). Based on the study by Xu et al.[14] and biosynthetic considerations we suggest the absolute configuration of shearinine L (**1**) to be identical to that of shearinine D (**3**) which is also produced by *E. weberi*.

The HR-ESI-MS of compound **2** confirmed its molecular formula to be $C_{37}H_{45}NO_6$ (16 DBE). Thus, **2** contains one oxygen atom (16 amu) more compared to shearinine L (**1**). The ^{1}H and ^{13}C NMR signals of **2** (Figure S11 and S12) were similar with those of shearinine L (**1**) except for the presence of a single broad doublet signal at $\delta_H = 4.90$ (1H, $J = 5.7$ Hz, H-22) instead of the two signals $\delta_H = 3.09$ (1H, $J = 15.3$, 9.2 Hz, H-22), 2.60–2.64 (1H, m, H-22) in shearinine L (**1**) (Figure S3 and S11). This suggested that compared to shearinine L (**1**), compound **2** contains a hydroxylated methine moiety at carbon C-22. The position of the hydroxy group at C-22 was further confirmed by HMBC correlations between H-22 and C-23 ($\delta_C = 60.3$), C-24 ($\delta_C = 75.6$), C-20 ($\delta_C = 114.2$), C-21 ($\delta_C = 132.0$), C-28 ($\delta_C = 137.3$) and C-29 ($\delta_C = 139.2$) (Figure S15). Thus, the structure of **2** was determined as 22-hydroxy-shearinine L. We named the new compound shearinine M. The NOESY spectrum exhibited correlations between H-22 and H_3-38 and between H-23 and H_3-37 (Figure S16), which implies that H-22 and H-23 are on opposite faces of ring B. The ^{1}H and ^{13}C NMR data at C-22 and C-23 of **2** were also in good agreement with those of shearinine D,[15] indicating the relative H-22β and H-23α configurations. The relative configuration of ring fusions in shearinine M (**2**) is identical to shearinine L (**1**) based on the NOESY correlations: H_3-32/H-17α, H-16/H_3-33, H_3-33/H-14β and H-11 and H-14α (Figure S17B). Because shearinine M (**2**) is closely related to shearinine L (**1**) and D (**3**) it is expected to have the same absolute configuration as shearinine D (**3**).[14]

Compounds **3**–**5** were identified as shearinine-type indole triterpenoids, namely shearinine D (**3**), shearinine E (**4**), and shearinine A (**5**) (SI), which have been characterized before from *Penicillium* species or its teleomorph *Eupenicillium* species.[13–15]

Cycloarthropsone (6)

3.2 mg of compound **6** (ca. 6 μg mL^{-1} SFM medium) were isolated. **6** was an aromatic compound with the molecular formula $C_{12}H_{12}O_4$ and seven DBE. Its ^{1}H NMR spectrum exhibited two *ortho*-coupled aromatic protons, signals corresponding to an (*E*)-propenyl chain and a methyl singlet (Figure S23). The NMR data and MS/MS fragmentation (Figure S25) indicating loss of H_2O and CO suggested **6** to be a benzofuranone derivative. Moreover, GC-MS analysis after derivatization with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) (Figure S26) exhibited a molecular ion ($M^{+\bullet}$) at 364, which pointed to the presence of two hydroxy groups. Thus, compound **6** was identified as cycloarthropsone that had been identified previously from the fungus *Arthropsis truncata*. The reported NMR data matched perfectly with ours.[16]

Emodin (7)

Compound **7** was obtained as orange needles (ca. 6 μg mL^{-1} SFM medium). For the aromatic compound the molecular formula $C_{15}H_{10}O_5$ and 11 DBE were deduced. The UV spectrum of **7** in methanol (Figure S30) revealed four absorption maxima at 216, 256, 308 and 461 nm that are characteristic for anthraquinones. The ^{1}H NMR spectrum exhibited four *meta*-coupled aromatic proton signals and a methyl singlet signal (Figure S28). Further analysis of the ^{13}C NMR data (Figure S29) and comparison of the retention time with an authentic reference confirmed the compound to be emodin (**7**).

Comparison of secondary metabolite profiles from *E. weberi* with *Escovopsis aspergilloides*

E. aspergilloides that has also been identified from nests of attine ants[17] is closely related to *E. weberi*. This parasitic fungus morphologically differs from *E. weberi* by its globose shaped, rather than clavate shaped phialide-vesicles.[18] However, the metabolite profiles of methanol extracts of both *Escovopsis* strains turned out to be very similar (Figure S31A). Both strains produced shearinines (**1**–**5**), cycloarthropsone (**6**), and emodin (**7**). The amount of the compounds in both *Escovopsis* strains may slightly vary.

Antimicrobial activity of secondary metabolites from *E. weberi*

In order to evaluate the function of the identified secondary metabolites, cycloarthropsone (**6**), emodin (**7**), and shearinine L (**1**) were tested in agar diffusion assays against selected organisms present in the leaf-cutting ants' nests. In particular, the potential to overwhelm *L. gongylophorus* was addressed.

Cycloarthropsone (**6**) and emodin (**7**) inhibited the growth of *L. gongylophorus*, causing 14 and 10 mm inhibition zones, at 0.82 and 0.66 μmol, respectively (Figure 3, Figure S35–36). However, shearinine L (**1**) was not active at 0.31 μmol (Figure S37). 0.30 μmol emodin (**7**) inhibited the *Streptomyces* symbionts of leaf-cutting ants *Streptomyces* sp. 28_1 and *Streptomyces* sp. 26_1 causing inhibition zones of 10 mm and 7 mm, respectively (Figure S32–S33). Moreover, none of the compounds tested showed activity against *Pseudonocardia* sp. Ao19, *Fusarium equiseti*, and *Phialophora fastigiata*.[19]

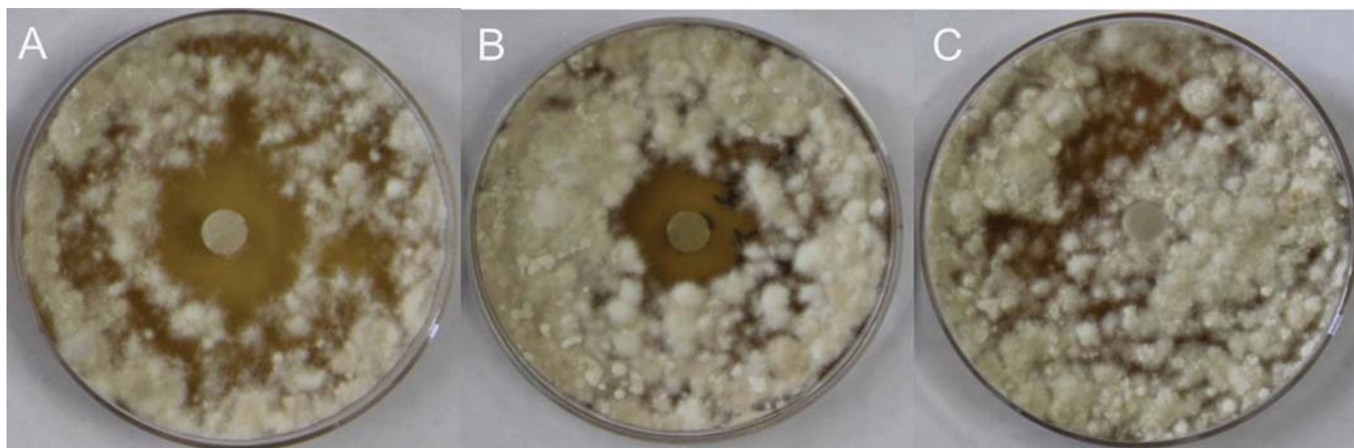

Figure 3. Agar diffusion assays against *L. gongylophorous*, on PDA agar plates (5.5 cm diameter) after 18 d of growth: A) 0.82 μmol cycloarthropsone (**6**) on paper disk; B) 0.66 μmol emodin (**7**) on paper disk, and C) control.

Response of *A. octospinosus* ants to shearinine L (2)

A. octospinosus ants from subcolonies were offered oat flakes as a substrate for their garden fungus. These flakes were either impregnated with shearinine L (**1**) (10 μg/flake) or control flakes in a dual-choice assay.[20] The percentage of flakes removed by the ants from the treated and the untreated pile of oat flakes, respectively, was determined (removal rate). The oat flakes were transported by *A. octospinosus* workers into the subcolonies and processed into small pieces that were incorporated across the garden to cultivate *L. gongylophorus*. Oat flake choice by *A. octospinosus* workers was strongly influenced by the shearinine L (**1**) treatment [Figure 4, GLMM (general linear mixed models)$_{\text{choice assay}}$, effect of treatment: $F_{1,18}=19.59$; $p<0.001$]. Notably, preference for the oat flakes reversed during the course of the assay from treated to untreated oat flakes (GLMM$_{\text{choice assay}}$, effects of the interaction between treatment and day: $F_{4,18}=20.93$; $p<0.001$), although day per se had no significant effect due to low replication (GLMM$_{\text{choice assay}}$; effect of day: $F_{4,18}=0.00$; $p>0.05$). On day one, the ants significantly preferred treated over control flakes (ca. 70 % versus 30 %). Reversely, from day 3 onwards, shearinine L treated oat flakes were strongly rejected (ca. 75 % on days 3, 4, and 5) (Figure 4). This switch in preference occurred on day 2, with no significant difference in removal rates (42 % vs. 58 %). All subcolonies (i.e., replicates) responded similarly to the treatment (GLMM$_{\text{choice assay}}$, effect of subcolony: $F_{2,18}=0.00$; $p>0.05$).

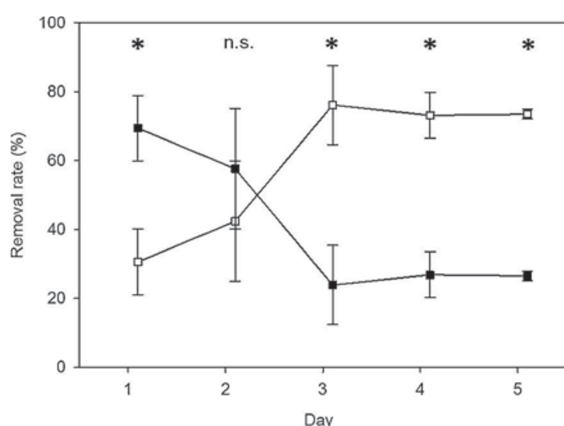

Figure 4. Dual choice assays with *A. octospinosus* leaf-cutting ants. The removal rate (percentage of oat flakes removed by the ants) of shearinine L treated oat flakes versus untreated flakes is shown. Black squares (shearinine L treated oat flakes) and white squares (untreated oat flakes) represent the mean values, error bars indicate the standard deviation based on 3 replicates. * = statistically significant, n.s. = not significant.

Leaf-cutting ant performance in shearinine L (**1**) treated versus control subcolonies was investigated for 10 d, starting on day 2 of the choice assay, by determining the daily amount of waste deposition (fresh weight) and worker mortality (number of dead ants). Subcolonies treated with shearinine L (**1**) revealed similar worker mortality and waste deposition as the control subcolonies, and both measures of colony performance remained constant throughout the 10 d experiment (GLMM$_{\text{waste deposition}}$, effect of treatment: $F_{1,38}=0.000036$; $p>0.05$; effect of day: $F_{9,38}=1.37$; $p>0.05$; effects of day in combination with treatment: $F_{9,38}=0.62$; $p>0.05$; effect of subcolony: $F_{2,38}=1.99$; $p>0.05$; GLMM$_{\text{worker mortality}}$, effect of treatment: $F_{1,38}=0.34$; $p>0.05$; effect of day: $F_{9,38}=8.42$; $p>0.05$; effects of day in combination with treatment: $F_{9,38}=0.62$; $p>0.05$; effect of subcolony: $F_{2,38}=8.42$; $p<0.001$).

Discussion

How the major pathogen of leaf-cutting ants, *E. weberi*, overwhelms *L. gongylophorus* and thus threatens survival of the ants has not been addressed in detail so far. Using metabolite profiling by LC-DAD-MS of *E. weberi* extracts, we identified seven of the major compounds produced by both *E. weberi* and *E. aspergilloides* as shearinines (**1**–**5**), cycloarthropsone (**6**), and emodin (**7**) (Figure 1, S1, and S31 A). The major metabolites **1**–**3**, **6**, and **7** were also detected when *L. gongylophorus* growing on PDA medium was challenged with *E. weberi* (Figure S31 B). Since we did not observe the formation of other secondary metabolites in these co-cultivation experiments—that might have been produced in response to the presence of *L. gongylophorus*, we assumed that the identified com-

pounds comprise those used by *E. weberi* to overpower the leaf-cutting ants' nest.

The finding of shearinines is in line with Boya et al., who recently observed shearinines D, F, and J in a co-cultivation experiment with mutualistic *Streptomyces* symbionts of *Acromyrmex echinatior* leaf-cutting ants against *E. weberi* TZ49.[12] Also, genome mining using antismash[21] revealed that the *E. weberi* genome[8] comprises genes involved in indole terpenoid biosynthesis fitting to the formation of shearinines.

However, our *Escovopsis* strains mainly released two novel shearinines L (**1**) and M (**2**) alongside the known shearinines D (**3**), E (**4**) and A (**5**) with maximal production after 9–12 d of growth on SFM agar plates (Figure 1). Shearinines have been originally identified from *Eupenicillium shearii.*[13] Shearinines exhibit anticancer activities;[15] they block large-conductance calcium-activated potassium channels,[14] and are known to act against insects.[13] Furthermore, shearinines have been found to suppress *Candida albicans* biofilm formation and potentiate the antifungal activity of the polyene macrolide antifungal amphotericin B.[22] However, in agar diffusion assays against *L. gonglyophorus* 0.3 μmol shearinine L (**1**) did not stop the growth of *L. gongylophorus.*

Due to the reported activity of shearinines against insects,[13] we suspected that they may rather act against the leaf-cutting ants than the garden fungus and thus indirectly weaken the fitness of the garden fungus. In order to address how shearinines may affect the leaf-cutting ants' colony we investigated how *A. octospinosus* subcolonies responded to the major component shearinine L (**1**). Shearinine L (**1**) impregnated oat flakes and control oat flakes were offered to *A. octospinosus.* Initially, *A. octospinosus* accepted both treated and untreated flakes even preferring the shearinine L (**1**) treated flakes. Consequently, the ants did not immediately recognize shearinine L (**1**) as detrimental. However, after a short learning period the ants clearly rejected shearinine L treated oat flakes as substrate for *L. gongylophorus* from day 3 onwards (Figure 4). Thus, *A. octospinosus* ants quickly learned to avoid shearinine L (**1**) treated oat flakes. However, when we evaluated the state of the subcolonies (daily waste production) as well as the death rate of *A. octospinosus* workers no clear signs of a direct toxicity of shearinine L (**1**) could be observed indicating that shearinine L (**1**) does not quickly kill *A. octospinosus* workers. Still after 2 d *A. octospinosus* ants strongly rejected the substrate treated with shearinine L (**1**).

A similar delayed rejection and avoidance learning—most likely induced by *L. gongylophorus*—has been studied previously with antifungal (cycloheximide) treated plant substrate[23] as well as defense-induced plant material.[20]

Currently, it remains elusive how *A. octospinosus* workers learn to avoid shearinine L (**1**) a major compound produced by *E. weberi.* It is conceivable that the avoidance is induced due to a signal by *L. gongylophorus* indicating a detrimental substrate or a cue that links to the pathogen *E. weberi.* Alternatively, the ants recognize themselves, for example, during the processing of the oat flakes into small pieces for their garden fungus, that shearinine L (**1**) is noxious or associated with the detrimental *E. weberi.*

In contrast to shearinine L (**1**), both cycloarthropsone (**6**) and emodin (**7**) strongly inhibited the growth of *L. gongylophorus* in agar diffusion assays (Figure 3, Figure S35, Figure S36) suggesting that *Escovopsis* fungi can make use of these polyketides to overpower *L. gongylophorus.* Cycloarthropsone (**6**) has been identified from *Arthropsis truncata.*[16] However, its biological activity has not been evaluated so far. In contrast, emodin (**7**), that is produced by plants and microorganisms, is well known for its wide range of pharmacological activities, such as antibacterial (against *Bacillus subtilis*),[24] antifungal (against *Alternaria*, *Fusarium*, *Fomesannosus* etc.),[25] antiviral,[26] anticancer,[27] anti-inflammatory,[28] and anti-ulcerogenic properties.[29] Thus, emodin (**7**) may help *Escovopsis* to outcompete other fungi that are known to occur in the leaf-cutting ants' nests.[30] However, our test strain for alternative pathogens, *Fusarium equiseti*, was not clearly inhibited in our agar diffusion assays (0.3 μmol, Figure S34). But emodin (**7**) (0.3 μmol) strongly inhibited some Actinomyces symbionts of leaf-cutting ants (Figure S32 and S33) indicating its use to fight against the protectors of the fungus garden.

Conclusions

The production of emodin (**7**) and cycloarthropsone (**6**) helps *E. weberi* to overpower its prey *L. gongylophorus* as well as to fight against the defenders, in particular the mutualistic *Actinomyces* symbionts.

However, *A. octospinosus* leaf-cutting ants—probably communicated by *L. gongylophorus*—can quickly learn to recognize shearinine L (**1**) from *E. weberi* and thus may initiate defense reactions against the pathogen demonstrating the arms race between the organisms.

With a multitude of organisms—the ants, their garden fungus, the mutualistic microbial symbionts, and pathogens, in particular *E. weberi*,—comprising the leaf-cutting ants' microcosmos, a detailed picture of the complex interactions can only be obtained if the involved chemicals, signaling compounds, toxins and antimicrobials, of the different partners are identified and their ecological function is addressed. In addition to straightforward in vitro bioassays, (behavioral) studies under close to natural conditions can help to reveal a more complete picture of the interactions and the diverse functions of the involved metabolites—as observed in the case of shearinine L (**1**).

Future experiments are needed to further reveal the biological function(s) of secondary metabolites from *Escovopsis* and thus shed more light on the fascinating interactions in the leaf-cutting ant microcosmos.

Experimental Section

General

Chemicals were purchased from Sigma Aldrich. IR detection was conducted on an ALPHA FT-IR spectrometer (Bruker, Germany). CD spectra were recorded with a Jasco J-815 CD-spectrometer and the optical rotation was determined with a Jasco P2000 polarimeter.

NMR spectra were recorded with a Bruker AVANCE AV-600 NMR spectrometer or a Bruker AV-600 NMR spectrometer fitted with a TCI cryoprobe (Bruker, Karlsruhe, Germany). Chemical shifts δ (ppm) were referenced to the solvent signals (^{1}H NMR CD_3OD: 3.31, ^{13}C NMR CD_3OD: 49.00, ^{1}H NMR [D$_6$]acetone: 2.05, ^{13}C NMR [D$_6$]acetone: 29.84 ppm). HR-ESI-MS were acquired using a Bruker Impact II (Bruker, Daltonics, Germany). UV/Vis spectra were recorded using an Implen nanophotometer. For column chromatography silica gel (40–63 mesh, Macherey–Nagel, Germany) and Sephadex LH-20 (GE Healthcare, Uppsala, Sweden) were used. Spots were detected under UV light and visualized by spraying with 5% H_2SO_4 in EtOH (v/v), followed by heating the plates. Silica GF254 (Merck KGaA, Germany) was used for analytical thin-layer chromatography. High performance liquid chromatography (HPLC) was performed on an Agilent 1100 liquid chromatography system fitted with a Phenomenex polar-RP column (250×2 mm, 4 μm or 250×4.6 mm, 4 μm) hyphenated to a Gilson FC204 fraction collector. LC-MS measurements were conducted with an Agilent 1100 HPLC connected to a Finnigan MAT LCQ. For GC-MS analysis, a Thermofisher Trace GC Ultra hyphenated with an ISQ was used.

Fungal and microbial cultures

E. weberi CBS 110660 and *E. aspergilloides* CBS 423.93 were obtained from the Westerdijk Fungal Biodiversity Institute (Utrecht, the Netherlands). *F. equiseti* FSU 5459 was from the Jena Microbial Resource Collection (JMRC, Jena, Germany). *P. fastigiata* DSM 2692 originated from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The *Streptomyces* and *Pseudonocardia* strains used for bioassays were isolates from *A. volcanus*, *A. octospinosus*, and *A. echinatior* leaf-cutting ants.[6e,h] *L. gongylophorus* was isolated from an *A. octospinosus* nest maintained at University of Kaiserslautern by Dr. Rainer Wirth and verified by 18S rDNA sequencing (Supporting Information). *L. gongylophorus* was grown on potato dextrose agar medium (PDA) purchased from Sigma Aldrich at 30 °C. All other strains were cultivated on soy flour medium (SFM) agar plates (20 g soy flour, 20 g mannitol, 15 g agar, 1 L ddH_2O) at 28 °C.

A. octospinosus leaf-cutting ants

The *A. octospinosus* ants originated from Paratebueno, Medina, Colombia (4°23′38.4′′N 73°16′07.5′′W). The ants were kept as laboratory culture at the Technical University Kaiserslautern at 25 °C under a 12 h:12 h light:dark regime. The ants were offered fresh blackberry leaves as substrate for *L. gongylophorus*.

Extraction and isolation of secondary metabolites from *E. weberi*

Secondary metabolites were isolated from *E. weberi* grown on SFM agar plates at 28 °C for 14 d. 20 SFM agar plates (9 cm) were cut in pieces and extracted with ethyl acetate (500 mL) for 2 h twice. After filtration, the combined solvent extracts were dried in vacuo. The crude extract (0.459 g) was subjected to silica gel column chromatography (column dimension 3×25 cm) eluting with *n*-hexane and ethyl acetate (7:1, 5:1, 3:1, 2:1, 1:1, 1:3, 1:6, 0:1 v/v, 200 mL each) to give six fractions (Fr1–Fr6). Fr2 (11.3 mg) was passed three times through a Sephadex LH-20 column with 90% MeOH to give pure compound **7** (3.1 mg). Likewise, Fr3 (16 mg) was subjected to Sephadex LH-20 column chromatography with 90% MeOH and further purified by HPLC using a Phenomenex polar RP column (250× 4.6 mm, 4 μm) at a flow rate of 0.8 mL min^{-1} eluting the compounds with a gradient of solvent A (H_2O 0.1% acetic acid) and solvent B (MeOH 0.1% acetic acid). HPLC conditions: 2 min 15% B, in 28 min to 100% B, 5 min 100% B. Compound **1** (1.90 mg) and compound **3** (1.45 mg) were obtained. A similar fractionation procedure was used for Fr4 (31.0 mg) to yield compounds **4** (1.3 mg), **5** (1.25 mg) and **6** (3.2 mg). Furthermore, Fr5 (6.5 mg) was directly purified by HPLC with a gradient of solvent A (H_2O 0.1% acetic acid) and solvent B (MeOH 0.1% acetic acid): HPLC conditions: (2 min 50% B, in 28 min to 100% B, 5 min 100% B) to yield compound **2**.

Spectroscopic data of shearinine L (1)

1.9 mg, white amorphous powder; IR (KBr):$\tilde{\nu}_{max}$ = 3441, 2971, 2617, 2343, 2051, 1668, 1462, 1365, 1253, 1149 cm^{-1}; UV (CH_3OH) λ_{max} = 255, 329 nm; ^{1}H NMR (600 MHz, CD_3OD) δ_H = 7.35 (1H, s, H-30), 7.16 (1H, s, H-20), 5.95 (1H, d, *J* = 2.96, H-27), 5.93 (1H, br s, H-11), 5.72 (1H, dt, *J* = 6.3, *J* = 2.2, H-6), 4.12 (1H, s, H-9), 3.14 (1H, d, *J* = 18.5, H-5), 3.09 (1H, dd, *J* = 15.3, *J* = 9.2, H-22), 2.86–2.91 (1H, m, H-23), 2.67 (1H, dd, *J* = 12.5, *J* = 6.2, H-17), 2.60–2.64 (1H, m, H-22), 2.52–2.59 (1H, m, H-16), 2.30–2.35 (1H, m, H-17), 2.27–2.31 (m, H-5), 1.96–2.02 (1H, m, H-15), 1.89 (1H, d, *J* = 13.6, H-14), 1.66–1.73 (1H, m, H-14), 1.61 (1H, d, *J* = 12.2, H-15), 1.38 (3H, s, H-40), 1.30–1.36 (12H, m, H-32, H-36, H-37, H-39), 1.27 (3H, s, H-35), 1.13 (3H, s, H38), 0.94 ppm (3H, br s, H-33); ^{13}C NMR (150 MHz, CD_3OD) δ_C = 197.6 (C-10), 156.8 (C-7), 155.1 (C-2), 146.5 (C-12), 141.9 (C-31), 141.5 (C-29), 137.0 (C-28), 133.7 (C-21), 128.1 (C-19), 119.4 (C-27), 117.1 (C-11), 117.0 (C-18), 114.3 (C-20), 113.3 (C-6), 104.1 (C-30), 87.1 (C-9), 76.3 (C-24), 76.1 (C-13), 74.6 (C-34), 74.4 (C-26), 52.1 (C-3), 50.7 (C-16), 50.3 (C-23), 44.3 (C-4), 34.0 (C-22), 33.4 (C-14), 32.4 (C-5), 32.2 (C-39), 30.6 (C-40) 30.2 (C37), 28.0 (C-17), 26.8 (C-36), 26.7 (C-35), 22.7 (C-15), 22.3 (C-38), 21.3 (C-33), 16.6 ppm (C-32); HR-ESI-MS: calcd for $C_{37}H_{44}NO_5$: 582.32250 $[M-H]^-$; found: 582.32053.

Spectroscopic data of shearinine M (2)

0.98 mg, white amorphous powder; IR (KBr):$\tilde{\nu}_{max}$ = 3409, 3297, 2987, 2369, 2183, 1668, 1476, 1386, 1309, 1186 cm^{-1}; UV (CH_3OH) λ_{max} = 259, 328 nm; ^{1}H NMR (600 MHz, CD_3OD) δ_H = 7.37 (1H, s, H-30), 7.36 (1H, s, H-20), 5.99 (1H, d, *J* = 3.0, H-27), 5.94 (1H, br s, H-11), 5.72–5.75 (1H, m, H-6), 4.90 (1H, br d, *J* = 5.7, H-22), 4.12 (1H, s, H-9), 3.16 (1H, d, *J* = 18.4, H-5), 2.69 (1H, dd, *J* = 12.5, *J* = 6.2, H-17), 2.67 (1H, dd, *J* = 5.7, *J* = 2.8, H-23), 2.65–2.67 (1H, m, H-16), 2.36–2.41 (1H, m, H-17), 2.30–2.36 (1H, m, H-5), 1.99–2.06 (1H, m, H-15), 1.90–1.96 (1H, m, H-14), 1.76–1.83 (1H, m, H-14), 1.67 (1H, d, *J* = 12.3, H-15), 1.46 (3H, s, H-37), 1.37 (3H, s, H-40), 1.34 (3H, s, H32), 1.32–1.34 (6H, s, H-36, H-39), 1.27 (3H, s, H-35), 1.15 (3H, s, H38), 0.99 ppm (3H, s, H-33); ^{13}C NMR (150 MHz, CD_3OD) δ_C = 197.6 (C-10), 156.7 (C-7), 155.5 (C-2), 146.5 (C-12), 142.9 (C-31), 139.2 (C-29), 137.3 (C-28), 132.0 (C-21), 128.2 (C-19), 120.2 (C-27), 117.4 (C-18), 117.1 (C-11), 114.2 (C-20), 113.3 (C-6), 103.6 (C-30), 87.2 (C-9), 76.7 (C-22), 76.0 (C-13), 75.6 (C-24), 74.7 (C-34), 74.1 (C-26), 60.3 (C-23), 52.2 (C-3), 50.7 (C-16), 44.3 (C-4), 33.4 (C-14), 32.3 (C-5), 32.2 (C-39), 30.4 (C-40), 30.3 (C-37), 28.0 (C-17), 26.8 (C-36), 26.7 (C-35), 23.7 (C-38), 22.4 (C-15), 21.2 (C-33), 16.6 ppm (C-32); HR-ESI-MS: calcd for $C_{37}H_{44}NO_6$: 598.31741 $[M-H]^-$; found: 598.31763.

Agar diffusion assays

L. gongylophorus, F. equiseti, P. fastigiata, Streptomyces sp. 25_4, Streptomyces sp. 28_1, Streptomyces sp. 26_3 and *Pseudonocardia sp. Ao19 were* used as test organisms in the agar diffusion assays against the purified secondary metabolites or reference compounds. For the agar diffusion assays, 100 μL of mycelium suspensions of the test organisms were spread onto SFM or PDA agar

plates (5.5 cm diameter). Holes (6 mm diameter) were cut into the agar plates into which 50 μL of the test solutions or solvent controls (MeOH) were applied. For paper disk diffusion assays, sterilized filter paper disks (6 mm diameter) were impregnated with the test compounds (0.3, 0.66, and 0.82 μmol). The test organisms were cultivated at 28 °C except *L. gongylophorus*, which was grown at 30 °C. The inhibition zones were monitored daily from day 3 to 18. All assays were performed at least in triplicate and were compared to equally prepared solvent controls.

Artificial infection of *L. gongylophorus* with *E. weberi*

L. gongylophorus was cultivated for 15 d on PDA agar at 30 °C. Then *E. weberi* was added to the plates and both strains were cultivated for additional 7 d. Single plates (three replicates) were extracted with MeOH (20 mL), concentrated to 1 mL and subjected to LC-MS analysis (10 μL injection volume, see above).

Establishment of *A. octospinosus* subcolonies

Bioassays with *A. octospinosus* leaf-cutting ants were performed with 6 subcolonies (3 treatments, 3 controls). Both treatment and control subcolonies were cultivated equally apart from the shearinine L treatment. The subcolonies were established by isolating ca. 400 cm^3 of fresh fungus garden from three large laboratory mother colonies. The subcolonies were kept in experimental nests, consisting of 3 plastic boxes (feeding box, fungus garden box, and refuse chamber) connected by plastic tubes. The *A. octospinosus* subcolonies were kept at 25 °C under a 12 h:12 h light:dark regime. All subcolonies were established at least 5 d before the dual choice experiment and supplied with fresh blackberry leaves.

Coating of oat flakes with shearinine L (1)

Oat flakes (Blüten-zarte Flocken, Kölln, Germany) of approximately equal size were used for the experiment. The flakes were impregnated with 10 μL of shearinine L (**1**) (1 mg mL^{-1}) dissolved in acetone and air-dried. Control flakes were prepared analogously applying 10 μL acetone.

Dual-choice bioassays[20] with *A. octospinosus* leaf-cutting ants

For the dual-choice preference tests, 10 treated and 10 control flakes were simultaneously placed 3 cm apart in the foraging chamber of *A. octospinosus* subcolonies. Control subcolonies were only offered control flakes.

The subcolonies were monitored continuously and the number of flakes carried inside the nest was noted until 8 out of 10 control or shearinine L-treated flakes were picked up or 5 h had elapsed. All collected flakes were readily processed and incorporated into the fungus garden. The foraging preferences of each subcolony were monitored daily over 5 d. Starting from the second day of feeding preference tests, the number of dead ants (i.e., worker mortality) and the amount of waste deposited by the ants into the waste chamber were recorded daily for 10 d. The influence of shearinine L (**1**) on the choice behavior of ants, their mortality, and waste deposition was statistically analyzed using general linear mixed models ($GLMM_{choice\ assay}$, $GLMM_{worker\ mortality}$, and $GLMM_{waste\ deposition}$, respectively) with treatment, day and their interaction as fixed factors and subcolony as random factor. The F values represent the actual test results of the GLMM (equivalent to F values in an Anova); p values are calculated based on these F values. The subscript values are the degrees of freedom. Ant mortality was square-root transformed to achieve homogeneity of variance. Statistical tests were performed with Statistica software (Statistica 13, TIBCO Software Inc., Palo Alto, USA).

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Conflict of interest

The authors declare no conflict of interest.

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