Journal of Chemical Ecology, Vol. 25, No. 4, 1999

ANTIFUNGAL DIKETOPIPERAZINES FROM SYMBIOTIC FUNGUS OF FUNGUS-GROWING ANT *Cyphomyrmex minutus*

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(Received May 5, 1998; accepted December 8, 1998)

Abstract—The attine fungus *Tyridiomyces formicarum*, the symbiont of the fungus-growing ant *Cyphomyrmex minutus*, produces several antifungal diketopiperazines. This represents the first identification of antifungal compounds from an attine symbiont and contradicts previous suggestions that attine fungi do not produce metabolites with antifungal activity. *T. formicarum* probably produces antifungal compounds in defense (1) against other fungi that invade the gardens and escape the weeding activity of the ants, or (2) against ant-pathogenic fungi that could harm the host ants. Fungi cultivated by fungus-growing ants may represent a rich source of additional bioactive metabolites.

Key Words—Antifungal, diketopiperazine, *Cyphomyrmex minutus*, fungus-growing ant, symbiosis, *Tyridiomyces formicarum*

INTRODUCTION

Microorganisms in mutualistic associations with larger organisms are a rich source of secondary metabolites. The importance of these associations for the discovery of novel compounds is illustrated by two recent examples, the isolation of the anticancer drug taxol from an endophytic fungus of the Pacific yew (Stierle et al., 1993) and the isolation of the antiinflammatory depsipeptide salin-

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0098-0331/99/0400-0935$16.00/0

amide from an actinomycete inhabiting the surface of the jellyfish *Cassiopeia xamachana* (Trischman et al., 1994). Symbiotic organisms are particularly likely to produce biologically active compounds because of the following reasons; (1) The raison-d'etre for symbiosis frequently rests on the chemical benefits that symbionts derive from each other (Douglas, 1994). (2) Symbiotic organisms are strong ecological interactors and are thus subject to strong selection and rapid evolution (Futuyma and Slatkin 1983; Thompson, 1994). (3) Coevolution between mutualistic partners leads to the evolution of metabolites with specific modulatory effects (Futuyma and Slatkin, 1983). As part of a program to explore these "biorational" guidelines in the search for novel compounds (Eisner, 1990; Beattie, 1992), we screened the symbiont of the fungus-growing ant *Cyphomyrmex minutus* (Attini: Formicidae) for bioactive metabolites.

Fungus-growing (attine) ants depend on cultivated fungus for food in a mutualistic dependency that originated about 50 million years ago (Weber, 1972; Schultz and Meier, 1995). The attine fungus chosen in this study, *Tyridiomyces formicarum* (Wheeler, 1907), is cultured only by ants in the *Cyphomyrmex "rimosus"* group (Snelling and Longino, 1992; Schultz and Meier, 1995) and is unique among the attine fungi by growing as a yeast form (unicellular) and not as the mycelial form (multicellular) typical for all other attine fungi. Because of its unique growth form, *T. formicarum* had been thought to be distinct taxonomically from all other attine fungi (Wheeler, 1907; Weber, 1972, 1982). However, *T. formicarum* reverts to a hyphal growth form in artificial culture and then shows morphological similarity to other attine fungi (Hervey et al., 1977), suggesting that *T. formicarum* is merely a unicellular growth variant of the typical mycelial attine symbionts. Indeed, a recent phylogenetic analysis groups *T. formicarum* together with other attine fungi and places them near free-living lepiotaceous fungi in the genera *Leucoagaricus* and *Leucocoprinus* (Chapela et al., 1994; Mueller et al., 1998).

Several attine fungi had previously been screened for antibiotic activity (Martin et al., 1969; Weber, 1972; Hervey et al., 1977), and Hervey and Nair (1979) reported the isolation of the antibacterial lactol lepiochlorin from the symbiont of the ant *Cyphomyrmex costatus*. Attempts to demonstrate antifungal activity of extracts from attine fungi were negative (Martin et al., 1969; Weber, 1972), however, and contradicted sporadic observations on pure symbiont cultures that revealed growth inhibition against unspecified contaminant fungi (Weber, 1955). The absence of antifungal activity is surprising from an ecological perspective, because the symbiotic fungi are expected to coevolve with pathogenic and parasitic fungi that invade the nutrient-rich gardens, evade the weeding activity of the ants, and thus compete with the symbionts for resources. Based on this biorational analysis (Eisner, 1990), we began screening attine fungi for antifungal metabolites despite the previously reported failure (Martin et al., 1969; Weber, 1972).

METHODS AND MATERIALS

Colonies of the fungus-growing ants *Cyphomyrmex minutus* and *C. rimosus* were collected in March 1994 at the Archbold Biological Station, Lake Placid, Florida. Fungal isolations followed the methods described in Mueller et al. (1996). Individual nests (fungus garden and ants) were kept in containers for at least one week. This allowed ants to eliminate contaminants from gardens and thus minimized contamination during the fungal isolation. Single bromatia (clumps of yeast cells) were taken from gardens with a sterilized needle and transferred to acidified (pH 4.5–5.0) potato dextrose agar plates (PDA; Difco, Detroit, Michigan). Plates were monitored daily to eliminate fast-growing contaminants. Dense, fine mycelium typical for attine fungi covered uncontaminated inocula within one or two days. Isolates were taken from growth fronts after three weeks and transferred to PDA slants.

Preliminary agar-plug assays of fungal isolates from the ants *C. rimosus* and *C. minutus* revealed strong antifungal activity against the yeasts *Saccharomyces cerevisiae* and three strains of the human pathogen *Candida albicans* (ATTC strains C109, 406, and Wisconsin). Because fungi from *C. rimosus* and *C. minutus* are closely related (Chapela et al., 1994) and may in some cases represent recent descendants from the same fungal clone (Mueller et al., 1998), only one fungus from *C. minutus* (UGM940317-01) was chosen for further analysis. The fungus was grown at room temperature in potato dextrose broth supplemented with 0.5% tryptone (Difco). Liquid cultures were shaken at 200 rpm. Seven 1-liter flasks (600 ml broth/flask) were inoculated each with a small inoculum of the yeast symbiont and shaken for 14 days. The cultures were then filtered through Celite, and the filtrate was extracted twice with equal volumes of ethyl-acetate.

The crude extract (450 mg) was subjected to Sephadex LH-20 column chromatography and eluted with methylene chloride and methanol (1 : 1). Fractions were combined based on their TLC profile and tested for antifungal activities. The antifungal fraction (317 mg) was further fractionated on a reverse-phase flash column (C_{18}) and eluted with increasing amounts of MeOH in H_2O (0, 30, 50, 75, 100% MeOH). Reverse-phase (C_{18}) HPLC (19% MeCN in H_2O) of the antifungal fraction (153 mg) afforded three antifungal metabolites: compound A (6 mg), compound B (20 mg), and compound C (4 mg).

RESULTS

The high-resolution FAB mass spectrum of the compound A ($[M+H]^+$ 211.145) indicated the formula $C_{11}H_{18}N_2O_2$. DEPT, ^{13}C, and ^1H NMR data indicated the presence of two amide carbonyls, three methines, four methylenes, and

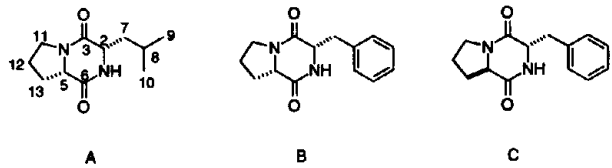

FIG. 1. Structures of the three antifungal compounds isolated from *Tyridiomyces formicarum*, the fungal symbiont of the fungus-growing ant *Cyphomyrmex minutus*.

two methyl groups. COSY data revealed $CHCH_2CH(CH_3)_2$ and $CHCH_2CH_2CH_2$ subunits. Long-range carbon–proton correlations were provided by HMBC data. Long-range carbon–proton correlations were provided by HMBC data. Long-range C-H correlations of a methylene (1.51/1.94/39.5 ppm, $C7H_2$) to two methyl doublets (0.95/22.3 and 0.96/23.4 ppm, $C9H_3$ and $C10H_3$) and a carbonyl carbon (169.1 ppm, C3) indicated a valine unit. The presence of a proline was confirmed by the unsaturation number and HMBC spectrum, which showed cross peaks of a methylene (2.02/2.30/29.2 ppm, $C7H_2$) to a carbonyl carbon (172.9 ppm, C6), and a methylene (3.50/46.6 ppm, $C11H_2$) to a carbonyl carbon (169.1 ppm, C3). Based on the unsaturation number and nitrogen count, the remaining atoms must be arranged to form a diketopiperazine ring leading to the structure shown in Figure 1 for compound A. Compound A presumably is derived from valine and proline.

The molecular formula of B was deduced as $C_{14}H_{16}O_2N_2$ from the high-resolution FAB mass spectrum ($[M+H]^+$ 245.129). DEPT, ^{13}C, and ^1H NMR data indicated the two amide carbonyls, a monosubstituted phenyl ring, two methines, and four methylenes. A methylene (3.15/38.3 ppm, $C7H_2$) shows long range C-H correlations to an amide carbonyl carbon (167.0 ppm, C3), two aromatic carbons (137.5 and 131.2 ppm), and a methine (4.44/57.8 ppm, C2), indicating the presence of a phenylalanine subunit. COSY and HMBC data also revealed a proline subunit. These results, along with the unsaturation number, allowed the structural assignment of compound B (Figure 1). The high-resolution FAB mass spectrum of compound C ($[M+H]^+$ 245.129) indicated the formula $C_{14}H_{16}O_2N_2$, identical to that of compound B. ^1H NMR, DEPT, ^{13}C, COSY, and HMQC data gave similar data and the same correlations. These data suggest that compound C is a stereoisomer—most likely an epimer—of compound B (Figure 1).

Acid hydrolysis of compounds A–C afforded the constituent amino acids. Preparation of the *N*-(pentafluoropropionyl)isopropyl ester derivatives, followed by chiral GC analysis on a Chirasil-Val column, showed that all of the amino acids from compounds A and B have the L absolute configuration. Compound C gave D-proline and L-phenylalanine, indicating that it is epimeric to compound C at the proline alpha carbon.

In a standard agar-diffusion assay against *Candida* 406 (see above), compound A caused a 9-mm zone of inhibition at 150 μg/disk, while compounds B and C gave 7-mm zones of inhibition at 300 μg/disk. In a positive control, Nystatin (100 units) induced a 19-mm zone of inhibition, indicating that compounds A–C are weakly antifungal.

DISCUSSION

The organic extracts of the attine fungus *Tyridiomyces formicarum* yielded three antifungal diketopiperazines (Figure 1) that are apparently derived from common amino acids (valine and proline). To our knowledge, this is the first identification of antifungal metabolites from an attine symbiont. The three isolated diketopiperazines showed only moderate antifungal activity in standard bioassays. The observed high antifungal activity of live cultures and extracts of *T. formicarum* suggests that the fungus employs a strategy of producing large amounts of a moderately potent mixture, rather than smaller amounts of more potent compounds. Indeed, the total amount of diketopiperazines produced by *T. formicarum* accounts for 9% of the weight of the crude extract, and compound B alone constituted almost 5% of the crude extract.

Diketopiperazines are widespread bioactive compounds of fungi that have been isolated from deuteromycetous, ascomycetous, and basidiomycetous fungi (Chen, 1960; Kodaira, 1961; Trigos et al., 1995; Arnone et al., 1996; Kozlovskii et al., 1997), and stereoisomeres of the compounds obtained from *T. formicarum* had been isolated previously from other fungi (Chen, 1960; Kodaira, 1961). The production of diketopiperazines by attine fungi therefore is not surprising and may indicate a general antifungal defense, rather than a specialized defense against particular kinds of fungal competitors. Fungus gardens present a rich resource base for parasitic and contaminant fungi, and the symbiotic fungi therefore are likely to compete with many types of alien fungi that escape the weeding activities of the ants. The high levels of antifungal diketopiperazines may therefore suppress or eliminate such fungal competitors in the garden. Alternatively, and not exclusive with the first hypothesis, antifungal compounds of the symbiont may serve a sanitary function and may protect the ant hosts against ant-pathogenic fungi, as has been hypothesized by Kermarrec et al. (1986) for leaf-cutting ants. Additional functions of the symbiont diketopiperazines cannot be excluded at this point. For example, fungal diketopiperazines have been shown to possess a variety of properties, including not only antifungal properties, as discussed above, but also antibacterial (Arnone et al., 1996) and antiviral properties (Tomassini et al., 1996). Diketopiperazines therefore may also serve additional functions in the symbiosis between ants and their cultivated fungi.

Acknowledgments—Field work was supported by the National Geographic Society; chemical analyses were funded by the NIH CA24487. We thank the Archbold Biological Station, Mark Deyrup, and Thomas Eisner for logistical support; and Jack Longino for verification of ant species identities.

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