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IDENTIFICATION AND DIRECTED BIOSYNTHESIS OF EFRAPEPTINS IN THE FUNGUS *Tolypocladium geodes* GAMS (DEUTEROMYCOTINA: HYPHOMYCETES)[1]

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Abstract—HPLC analysis of crude dichloromethane extracts of shaken liquid cultures of the hyphomycetous fungus *Tolypocladium geodes* Gams revealed the presence of efrapeptins. These peptides, which have mitochondrial ATPase inhibitory activity as well as antifungal and insecticidal properties, are previously known only from the congeneric species, *T. niveum* Rostrup. The identity of efrapeptins was confirmed by fast atom bombardment mass spectrometry and by amino acid analysis. HPLC analyses of efrapeptins extracted from single isolates of both *T. geodes* and *T. niveum* indicated that both species produced the same efrapeptins but the profile of relative abundance of the compounds produced was diagnostic for the isolates examined. Efrapeptin F was the major component of the mixture from *T. geodes* with the order of abundance of the six efrapeptins detected being F > G > D ~ E > H > C. Efrapeptin D was the major component from *T. niveum* with the order of abundance of the six efrapeptins detected being D > E > F > C ~ G > H. Efrapeptin F differs from efrapeptin D by a single amino acid residue, efrapeptin F having an alanine where efrapeptin D has a glycine. Addition of alanine to the culture medium increased the relative abundance of efrapeptin F in the profile of both species. Conversely, addition of glycine increased the relative abundance of efrapeptin D in the profile of both species.

Key Words—*Tolypocladium geodes*, *T. niveum*, Deuteromycotina, Hyphomycetes, fungal toxins, efrapeptins, mitochondrial ATPase inhibition, antibiotic, directed biosynthesis.

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[1] Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.
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INTRODUCTION

The hyphomycetous fungal genus *Tolypocladium* contains a number of soil-inhabiting species, some of which are pathogenic to insects (Bisset, 1983). One species in the genus, *T. niveum* (Rostrup) is especially notable for its secondary metabolites. It produces the cyclosporins, a group of cyclic peptides with well-known immunosuppressive and antifungal activity (Dreyfuss et al., 1976, and references cited), and the efrapeptins (Figure 1), a comparatively lesser known group of antibiotics that have been shown to be potent inhibitors of ATPases from mitochondria, chloroplasts, and bacteria (Gupta et al., 1991a,b, and references cited).

Insecticidal activity has been reported for crude extracts of several species of *Tolypocladium*, including *T. geodes*, and has been attributed to an unidentified material referred to as "tolypin" (Matha et al., 1988). However, at present, the only chemically characterized compounds from *Tolypocladium* species with documented insecticidal activity are the cyclosporins (Weiser and Matha, 1988) and the efrapeptins (Krasnoff et al., 1991), and these two classes of compounds are known so far only from *T. niveum*.

As part of an ongoing investigation of the role that efrapeptins play in the biology of *Tolypocladium* species, we have been screening extracts of isolates of various species for insecticidal activity as a probe for the presence of efrapeptins. We recently detected insecticidal and feeding deterrent activity against adult tephritid fruit flies of several species (*Anastrepha suspensa*, *Ceratitis capitata*, and *Rhagoletis pomonella*) with crude dichloromethane extracts of culture broth from an isolate of *T. geodes* (Krasnoff and Gupta, unpublished data). In

C	Ac-PIP-AIB-PIP-AIB-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-GLY-LEU-AIB-X	(FW=1606)
D	Ac-PIP-AIB-PIP-AIB-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-GLY-LEU-IVA-X	(FW=1620)
E	Ac-PIP-AIB-PIP-IVA-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-GLY-LEU-IVA-X	(FW=1634)
F	Ac-PIP-AIB-PIP-AIB-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-ALA-LEU-IVA-X	(FW=1634)
G	Ac-PIP-AIB-PIP-IVA-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-ALA-LEU-IVA-X	(FW=1648)

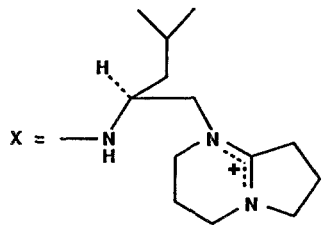

FIG. 1. Structure of efrapeptins C, D, E, F, and G (compounds C–G, respectively). Amino acid residues are abbreviated as follows: alanine (ALA), glycine (GLY), leucine (LEU), α-amino-isobutyric acid (AIB), β-alanine (β-ALA), isovaline (IVA), and pipecolic acid (PIP). Ac = acetyl, FW = formula weight.

this paper we present evidence that *T. geodes* produces efrapeptins and that the relative abundance of the efrapeptins produced by the strain studied is distinct from that produced by an isolate of *T. niveum*. We also present evidence that the amino-acid composition of the culture medium affects the profile of efrapeptins produced by both species.

METHODS AND MATERIALS

Growth of Fungi. Fungal inoculum was obtained from cultures maintained under liquid nitrogen in the USDA-ARS entomopathogenic fungi collection (ARSEF). To compare production of efrapeptins by *T. geodes* (ARSEF #2684) and *T. niveum* (ARSEF #616) under identical conditions, conidia were harvested in sterile 0.1% Tween-80 from 7- to 9-day-old cultures grown on slants of Sabouraud dextrose agar + 1% yeast extract (SDAY) at 22 ± 1°C. Concentrations of initial conidial suspensions were determined using an improved Neubauer hemacytometer. Suspensions of ca. 1×10^6 conidia/ml were made by dilution of initial suspensions in sterile 0.1% Tween-80. These were used to inoculate 100-ml batches of Czapek-Dox (Difco) liquid medium + 0.5% bactopeptone (Difco) in 250 Erlenmeyer flasks with 1×10^6 conidia. Liquid cultures were grown at 22 ± 1°C on a rotary shaker (160 rpm) for 11 days. Six replicates were produced for each species.

To investigate the effect of glycine and alanine on efrapeptin production, the fungi were grown in the above medium as well as media supplemented with either 1.0% L-alanine or 1.0% glycine (Sigma). Agar plugs from 13-to 14-day-old cultures grown on SDAY at 22 ± 1°C were used as inoculum for this experiment. Liquid cultures were grown at 22 ± 1°C on a rotary shaker (160 rpm) for 11 days. Each of the three treatments (control medium, alanine-supplemented medium, and glycine-supplemented medium) was duplicated.

Production of Extracts. Fungal mycelium, harvested after 11 days of growth, was separated from the broth by filtration through eight layers of cheese cloth. The broth was then centrifuged at 860 *g* for 10 mins. The pellet, containing conidia and mycelial fragments, was discarded and the supernatant was extracted three times with 100-ml portions of dichloromethane. The organic layer was concentrated *in vacuo*, then taken up in 60 ml of dichloromethane, washed twice with 10-ml portions of deionized water, dried over anhydrous sodium sulfate, filtered, and concentrated *in vacuo* once again. The extract was then transferred through a cotton filter to a vial in 3 × 2-ml portions of dichloromethane and concentrated to dryness with gentle heating under a stream of dry nitrogen. Extracts were stored at −20°C. Prior to HPLC analysis, samples were dissolved in methanol and filtered through a 0.45-μm membrane filter cartridge.

Isolation and Characterization of Efrapeptins. A sample of the crude dichloromethane extract of culture broth of the *T. geodes* isolate was analyzed on a reverse-phase C8 analytical column (0.4 × 15 cm, particle size 5 μm) eluted with MeCN–12.5 mM $(NH_4)_2SO_4$ (63:37) at a flow rate of 1.2 ml/min. Detection was by monitoring UV absorption at 225 nm (0.2 aufs). Material for fast atom bombardment mass spectrometry (FABMS) and amino acid analysis was obtained from repetitive semipreparative HPLC on a reverse-phase C8 column (0.9 × 50 cm) eluted with MeCN–12.5 mM $(NH_4)_2SO_4$ (75:25) at a flow rate of 5.5 ml/min. Detection was by monitoring UV absorption at 225 nm (0.5 aufs). Amino acid analysis was performed on an analyzer in which amino acid composition of the hydrolyzate was determined as the phenylthiohydantoin derivatives by comparing HPLC retention times with those of standards. Efrapeptin profiles were evaluated by HPLC analyses using an integrating chart recorder (Hewlett-Packard 3390 A) for quantitative comparison of peak areas. Semiquantitative comparisons among samples of relative amounts of efrapeptins produced per unit of crude extract were made by comparing total efrapeptin peak area and correcting for amount of crude extract injected (product of injection volume and concentration of extract solution). A standard curve was generated to verify that the response of the UV detector to efrapeptins was linear with respect to the amount of extract injected onto the HPLC.

RESULTS

Isolation and Characterization of Efrapeptins. HPLC analysis of crude dichloromethane extracts of *T. geodes* indicated the presence of a mixture of efrapeptins (Figure 2). Peaks from the *T. geodes* extracts cochromatographed with efrapeptins C, D, E, F, and G from a partially purified mixture from *T. niveum* (ARSEF #616) (Gupta et al., 1991b). A peak eluting after efrapeptin G possibly corresponds to efrapeptin H as shown by Jackson et al. (1979). Peak H made up ca. 6% of the total area of the efrapeptin fraction from *T. geodes*. The amino acid sequence of this peak remains to be determined.

Identification of efrapeptins from *T. geodes* extracts was confirmed by data from FABMS analysis and amino acid analysis of an HPLC-purified mixture of efrapeptins C, D, E, F, G, and H. FAB mass spectra showed molecular ions (M^+) at *m/z* 1606, 1620, 1634, 1648, and 1662. The quaternary nitrogen in the C-terminal blocking group of the efrapeptins (Figure 1) is responsible for producing unusually intense molecular ion peaks (M^+) instead of the usual $M+H^+$ under FABMS conditions (Gupta et al., 1991b). This spectrum is consistent with the presence of efrapeptins C (1606), D (1620), E (1634), F (1634), G (1648), and a sixth compound of molecular weight 1662, probably efrapeptin H.

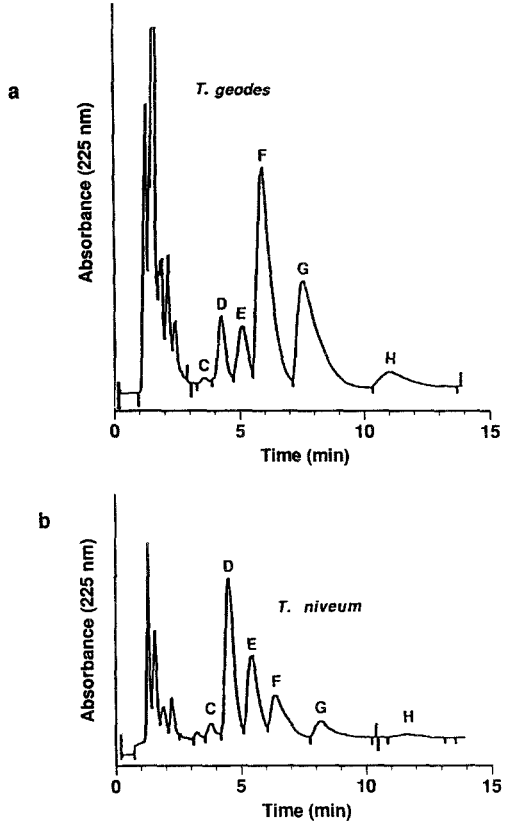

FIG. 2. HPLC chromatograms of crude extracts from *Tolypocladium geodes* (5 μl of 5 μg/μl solution in methanol) and *T. niveum*. (4 μl of 5 μg/μl solution in methanol). Samples were injected on a reverse-phase C8 analytical column (0.4 × 15 cm, particle size, 5 μm; solvent MeCN–12.5 mM $(NH_4)_2SO_4$ (63:37); flow 1.2 ml/min; detection UV 225 nm, 0.2 aufs. Fungi were grown on Czapek-Dox liquid medium + 0.5% bactopeptone.

Amino acid analysis of the HPLC-purified mixture confirmed the presence of α-aminoisobutyric acid (aib), leucine (leu), isovaline (iva), glycine (gly), β-alanine (β-ala), and alanine (ala) in a molar ratio of 672.2 (aib):257.3 (leu):203.2 (iva):185.1 (gly):148.8 (β-ala):123.8 (ala). This corresponds well with the molar ratio calculated for a mixture of efrapeptins C, D, E, F, and G, by taking into account their amino acid content (Figure 1), their relative abundance in *T. geodes* crude extracts as determined by HPLC analyses (see below), and standardizing to the molar estimate for leucine from the analysis: 717.0 (aib):257.3 (leu):183.6 (iva):153.2 (gly):128.7 (β-ala):104.1 (ala).

Comparison of Efrapeptin Profiles. Efrapeptin F was the major component of the efrapeptin profile of *T. geodes* with the order of abundance of the six detectable efrapeptins being F > G > D ~ E > H > C (Table 1, Figure 2a). Efrapeptins F and G accounted for >75% of the efrapeptin fraction of *T. geodes*. The major component in the efrapeptin profile of *T. niveum* was efrapeptin D with the order of abundance of the six efrapeptins being D > E > F > C ~ G > H (Table 1, Figure 2b). Efrapeptins D, E, and F accounted for >85% of the efrapeptin fraction of *T. niveum*. Efrapeptin G, a major component in the *T. geodes* profile, was a minor component in the *T. niveum* profile. Conversely, efrapeptin E, a major component in the *T. niveum* profile, was a minor component in the *T. geodes* profile.

Although *T. geodes* produced almost twice as much extract per unit of broth, 18.7 ± 1.2 mg/100 ml (±standard error for $N = 6$) compared with *T. niveum*, 8.9 ± 0.2 mg/100 ml (±standard error, $N = 6$), it produced only 0.63 times as much of the efrapeptins per unit mass of extract as *T. niveum*. Thus *T. geodes* produced 1.3 times more efrapeptins per unit volume of broth than *T. niveum*.

Effect of Supplemental Alanine and Glycine on Efrapeptin Profiles. Efrapeptins F and D differ only by an amino acid substitution at position 13 (Figure 1), D having a glycine and F having an alanine. With the standard medium (Czapek-Dox + 0.5% bactopeptone), *T. geodes* produced a ratio of efrapeptin F to D of 5.3:1 (ratios presented are based on the average of two replicates; Figure 3a). Cultured with 1% alanine this ratio increased to 11.3:1 (Figure

TABLE 1. RELATIVE ABUNDANCE OF EFRAPEPTINS C, D, E, F, G, H IN DICHLOROMETHANE EXTRACTS OF CULTURE BROTH FROM *T. geodes* AND *T. niveum* EXPRESSED AS AVERAGE PERCENTAGE OF TOTAL EFRAPEPTIN FRACTION BASED ON HPLC PEAK AREA $n = 6$ SAMPLES; (RANGE OF PERCENTAGES SHOWN IN PARENTHESES)[a]

Efrapeptin	Species	
	T. geodes	*T. niveum*
C	0.6(0.4–0.7)	6.0(4.8–7.7)
D	8.3(6.6–9.5)	39.9(37.1–41.8)
E	9.1(8.4–10.0)	24.5(22.3–26.1)
F	44.5(42.9–46.1)	21.9(20.3–23.1)
G	31.7(30.3–33.6)	6.8(4.4–9.4)
H	6.0(5.1–7.4)	1.0(0.0–0.4)

[a] Means for all six compounds were significantly different between species at $\alpha = .01$ (T-tests with α-levels adjusted by Bonferoni's inequality).

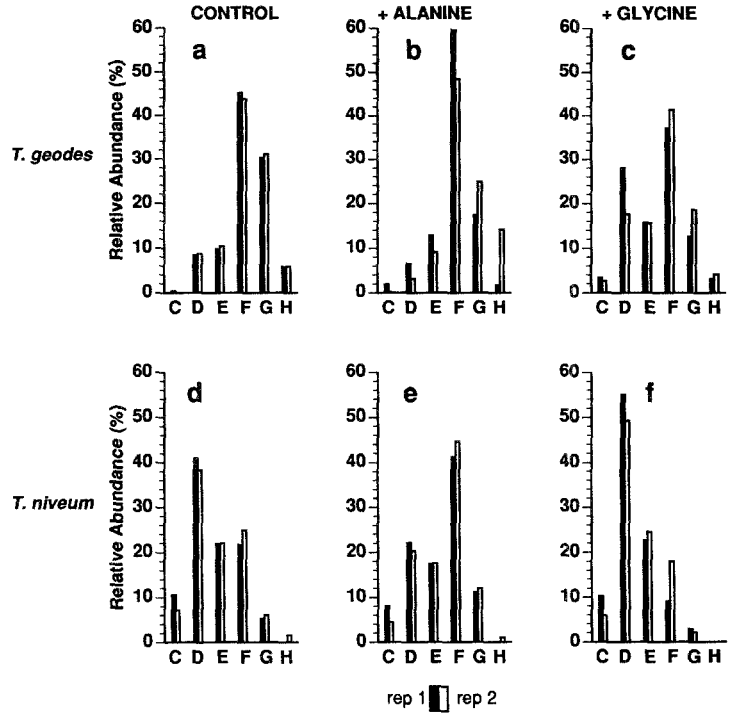

FIG. 3. Effect of supplementing standard growth medium (Czapek-Dox + 0.5% bactopeptone) with 1% alanine or 1% glycine on the efrapeptin profiles of *Tolypocladium geodes* and *T. niveum*. Relative abundance of efrapeptins C, D, E, F, G, and H (labeled on the *x* axes) in dichloromethane extracts of culture broth expressed as percentage of efrapeptin fraction based on HPLC peak area. Shown are results from two replicates.

3b). Cultured with 1% glycine, this ratio decreased to 1.7:1 (Figure 3c). With the standard medium, *T. niveum* produced a D to F ratio of 1.7:1 (Figure 3d). Grown with 1% alanine, this ratio decreased to 0.5:1 (Figure 3e). Grown with 1% glycine this ratio increased to 3.9:1 (Figure 3f). Thus in both species supplemental glycine had the effect of increasing the production of D relative to F while supplemental alanine had the effect of increasing the production of F relative to D.

Some analogous effects, although not as dramatic and consistent, were noted with the secondary components of the two species, efrapeptins E and G, which also differ from each other only by the replacement of glycine with alanine at position 13 (Figure 1). With additional alanine, the ratio of G to E in *T. geodes* extracts actually decreased from 3.1:1 to 1.9:1 counter to the expected pattern (Figure 3a,b). With additional glycine, however, the ratio decreased

predictably from 3.1:1 to 1:1 (Figure 3c). In *T. niveum* extracts the E to G ratio, which was 3.9:1 in the controls (Figure 3d), decreased predictably to 1.5:1 with additional alanine (Figure 3e) and increased predictably to 9.2:1 with additional glycine (Figure 3f).

DISCUSSION

We have documented the production of efrapeptins by *T. geodes* and have described a distinct difference between the efrapeptin profile of the isolate studied and that of an isolate of *T. niveum*. Two independent HPLC analyses of an efrapeptin fraction from another *T. niveum* isolate (Jackson et al., 1979; Krishna et al., 1990) show that efrapeptin D is the primary component with the order of abundance of efrapeptins essentially the same as reported here for *T. niveum*. This points to the possibility that the strain-specific differences we have documented here are representative of species-level characteristics. However, we recognize the need to evaluate a broader range of isolates of each species to support such a claim and are currently expanding our survey of isolates to provide a better overall picture of the intra- and interspecific patterns of variation in efrapeptin production in *T. geodes* and *T. niveum*.

The literature on antibiotics and mycotoxins is replete with reports of species-specific or strain-specific profiles based on the presence or absence of various compounds (e.g., Frisvad, 1986; Katz and Demain, 1977; Moss, 1982). However, documentation of strain- or species-specific differences of the type we have described here, i.e., where there is a diagnostic pattern of relative abundance in a microheterogeneous group of secondary metabolites, is relatively rare. Examples are the actinomycin profiles of *Streptomyces chrysomallus* and *S. antibioticus* (Katz, 1974) and the xanthomegnin/viomellein profiles of *Aspergillus ochraceus*, *Penicillium cyclopium*, and *P. viridicatum* (Stack and Mislivec, 1978).

There are precedents in the antibiotic literature for what has been called "directed" or "controlled" biosynthesis, i.e., the favoring of particular components of a microheterogeneous mixture by providing exogenous precursors (Katz, 1974). It is intriguing that this phenomenon has been previously reported to occur with cyclosporins, the other class of antibiotics known to be produced by *T. niveum* (Kobel and Traber, 1982).

As mentioned above, efrapeptin D, the primary product of *T. niveum*, differs from efrapeptin F, the primary product of *T. geodes*, by one amino acid substitution, efrapeptin D having a glycyl residue in position 13 where F possesses an alanyl residue. In analogous fashion, the second most abundant efrapeptins for each species, E in *T. niveum* and G in *T. geodes*, differ in precisely the same way, E having glycine in position 13 where G has an alanine. Although

nothing has been published regarding the biosynthesis of the efrapeptins, the difference observed here between the two species points to the existence of a mechanism whereby *T. niveum* favors the inclusion of glycine vs. alanine at position 13 and vice versa for *T. geodes*. This could involve differences in the levels of biosynthesis of the constituent amino acids, differences in the preference for one amino acid over another at a particular step in biosynthesis, or a combination of both scenarios. The only firm conclusion that can be made based on our findings regarding the biosynthesis of efrapeptins is that both fungi studied can incorporate exogenous amino acids into the peptide chains. This suggests the possibility that similar effects might be observed with α-aminoisobutyric acid and isovaline supplements in that several differences among the known efrapeptins are due to substitutions of these two amino acids at positions 4 and 15 (Figure 1). The effects of glycine and alanine supplements on the production of the major components in the efrapeptin profiles of the two species also have a practical implication; they point to the feasibility of directing biosynthesis toward either efrapeptin D or F, thus making purification more efficient in any large-scale effort to produce efrapeptins.

T. geodes is the second species in the genus *Tolypocladium* for which efrapeptin production has been documented. The presence of efrapeptins in *T. geodes* may account for the antifungal activity observed by Lundgren et al. (1978) in isolates of *T. geodes* and the insecticidal activity observed in extracts of *T. geodes* reported by Matha et al. (1988). Our results suggest the possibility that efrapeptins may occur elsewhere in the genus and their presence (and quantitative profiles) may be of taxonomic relevance. A more complete view of the pattern of efrapeptin distribution among species of *Tolypocladium* should offer insights into the evolution and function of these secondary metabolites.

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