METABOLITE PRODUCTION BY ENTOMOPATHOGENIC FUNGI

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ABSTRACT – The entomopathogenic fungi produce a wide assortment of metabolites, some of which are important to the host-specialization of this group of fungi. Very few entomopathogenic fungi have been examined in detail for their metabolites and in even fewer cases is there information on the role of these metabolites in the disease process. Nevertheless, it is clear that certain proteases and lipases are crucial to the invasion of insect cuticle by the fungus. These fungi have the unusual ability to hydrolyze branched alkanes. Also, they normally produce very active, broad-spectrum proteases. These are needed for penetration of the hydrophobic epicuticle and the highly proteinaceous cuticle of insects. Chitinases also are involved, but usually have only secondary importance as penetration enzymes. The best known of the penetration enzymes is Pr1 – a chymoelastase protease, the major enzymic product of *Metarhizium anisopliae* infection structures (appressoria). Growth in the hemocoel involves various hydrolytic enzymes; and, in addition, may lead to the formation of smaller compounds with detrimental effects on the host. These can be simple organic poisons, e.g. oxalic acid; but more often they are larger compounds, and many are depsipeptides. Metabolites of entomopathogenic fungi are currently being investigated as potential chemical insecticides and as pharmacological agents.

Index terms: Cuticle-Degrading Enzyme, Proteases, toxins, cuticular, differentation.

PRODUÇÃO DE METABÓLITOS POR FUNGOS ENTOMOPATOGÊNICOS

RESUMO – Os fungos entomopatogênicos produzem diferentes metabólitos, alguns dos quais são importantes para a especialização deste grupo de fungos. Poucos fungos entomopatogênicos foram examinados em detalhe com relação aos seus metabólitos, e existe ainda menos informação sobre o papel desempenhado por estes metabólitos no processo da doença. No entanto, está claro que algumas proteases são fundamentais para penetração do fungo pela cutícula do inseto. Estes fungos têm a capacidade rara de hidrolizar os derivados dos alcanos. Também produzem normalmente proteases muito ativas e de amplo espectro. Estas são necessárias para a penetração da epicutícula hidrofóbica e da cutícula de alto conteúdo protéico. As quitinases também estão envolvidas, mas normalmente têm uma importância secundária como enzimas de penetração. A mais conhecida enzima de penetração é a Pr1 – uma protease quimoelastase que é o maior produto enzimático das estruturas de infecção (apressórios) de *Metarhizium anisopliae*. O crescimento no hemocele envolve várias enzimas hidrolíticas, e pode levar à formação de compostos menores com efeitos prejudiciais para o hospedeiro. Estes podem ser simples venenos orgânicos, como por exemplo, o ácido oxílico; mas freqüentemente são compostos maiores e muitos são depsipeptídeos. Os metabólitos dos fungos estão atualmente sendo estudados como potenciais inseticidas químicos, e como agentes farmacológicos.

Termos para indexação: enzimas que degradam cutícula, proteases, toxinas, diferenciação celular.

INTRODUCTION

Fungi, as a group, are well known for their

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production of chemically diverse compounds; some of which are biologically very potent, causing severe adverse reactions in other organisms. Examples include antibiotics used to cure bacterial diseases, ergot alkaloids for vasoconstriction, and numerous mycotoxins produced in moldy grain which cause human, as well as domestic animal, mortality. The entomopathogenic fungi number some 700 species in approximately 100 genera (Roberts et al. 1991), and only a small fraction of these have been examined for metabolites directly toxic to their hosts (insects) or that aid the fungi in establishing infections in insects.. Nevertheless, a considerable number of such compounds have been reported (Roberts 1981).

The best known are probably the low-molecular-weight depsipeptides such as destruxins and bassianolide. Five such secondary metabolites of entomopathogenic fungi will be discussed in detail below. The entomopathogenic fungi produce enzymes for converting insect tissue into nutrients for their growth. Tanned insect cuticle is poorly utilizable by most fungi; but the entomopathogenic fungi, which invade the insect host through its cuticle, have evolved powerful cuticle-degrading enzymes. One of these, a chymoelastase produced by *Metarhizium anisopliae* and named Pr1, is much better than any of the others from this group of fungi. At the time of infection structure (appressorium) production, 80% of *M. anisopliae* protein synthesis is committed to Pr1 synthesis. This, and other observations, suggest that Pr1 is a key virulence factor for this entomopathogenic fungus. Accordingly, this enzyme will be treated in detail below as an example of enzymic involvement in pathogenesis.

TOXIC SECONDARY METABOLITES FROM ENTOMOPATHOGENIC FUNGI

Several entomopathogenic fungi are known to produce biologically active secondary metabolites possessing interesting biological properties. Secondary metabolites of fungi are usually small to medium size molecules (mw usually <2000). These are derived from primary metabolites incorporating, more often than not, unusual substructures which are often put together by unusual biosynthetic pathways occasionally involving multifunctional, multienzyme molecules capable of performing complex assemblies. The production of secondary metabolites is an inherent genetic property of the oragnism, but the overall production of a particular secondary metabolite can be significantly altered by optimizing the growth conditions such as nutrients, temperature, pH, etc. Aberrant biosynthesis of unnatural products from unnatural precursors can also be induced under certain circumstances.

In most cases, the significance to the fungus of production of its secondary metabolites is not well understood. It seems plausible, in the case of entomopathogenic fungi, which usually produce toxic/antibiotic secondar metabolites, that the toxins secreted into the medium by the fungi may act as antimicrobial or anti-immune agents, thus eliminating the competition for the nutrients or facilitating the establishment of the pathogen. This is the case for destruxins and leucinostatins. In certain instances where the metabolites are not secreted and stay inside the cell, they may have entirely different functions such as facilitating the movement of ions acorss lipophilic membranes by acting as ionophores (e.g. beauvericin, bassianolide), or by forming transcellular ion channels (e.g. peptaibols such as alamethicin). Following is a brief discussion of the chemical compositon, its elucidation, and the biological activity of some of the secondary metabolites produced by the entomogenous fungi.

Efrapeptins

Efrapeptins are a complex mixture of peptide antibiotics produced by the fungus *Tolypocladium niveum (syn. Tolypocladium*

inflatum, Beauveria nivea), a soil hyphomycete (Jackson et al. 1979).

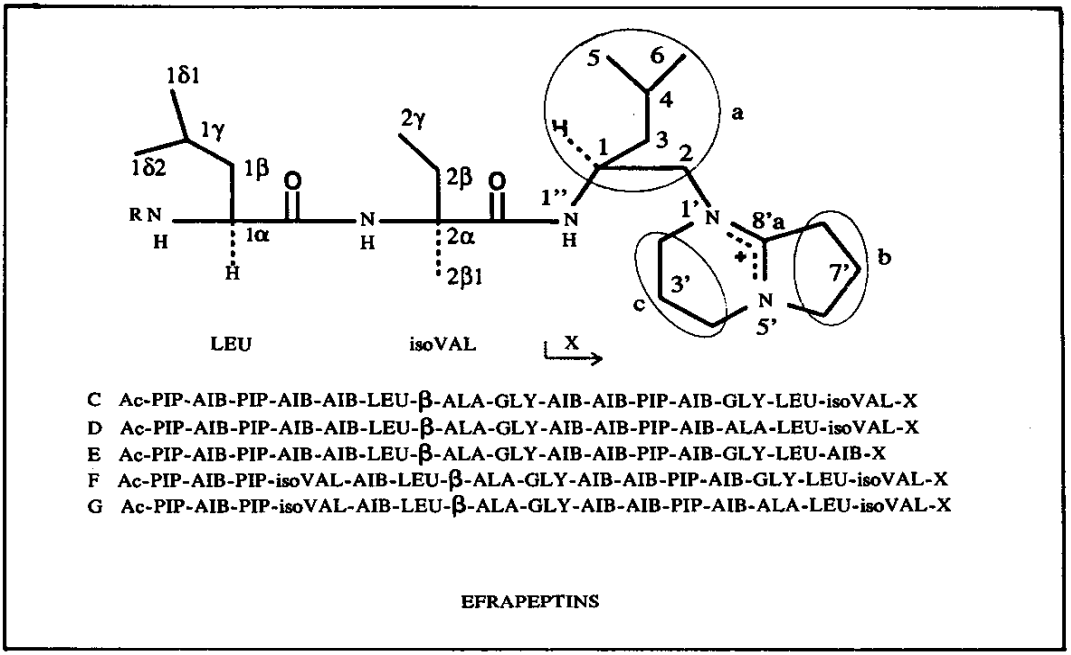
The structure of efrapeptins – Lipid soluble extract from the culture broth of an isolate of *Tolypocladium niveum* displayed toxic activity against the insect, Colorado potato beetle (*Leptinotarsa decemlineata,* Coleoptera) (Krasnoff et al. 1991). Subsequent bioassay-guided fractionation resulted in concentration of the activity in a peptidal fraction which was identified as a mixture of efrapeptins (Gupta et al. 1991). This mixture could be resolved by high performance liquid chromatography (HPLC) into five peaks (C-G) (Bullough et al. 1982). For separation of individual components, a methodology was developed that involved use of flash chromatography on silica gel followed by Sephadex LH-20 chromatography which furnished a clean mixture of peptides. By the use of preparative thin layer chromatography (TLC) on silica gel, two major peptides (D and F) could be enriched into different fractions. Final purification of individual components was achieved by semiperparative HPLC; reverse phase (RP) C8 column (solvent MeCN:12.5 mM $(NH_4)_2SO_4$(75:25, detection UV, 225 nm).

The linear peptides are rich in α-aminoisobutyric acid (Aib) and are composed of 15 amino acid residues with and acetylated N-terminus (Bullough et al. 1982). The C-terminal is amidated with a novel bicyclic amine which has been identified as N-peptido-1-isobutyl-2[1-pyrrolo-(1,2-α) pyrimidinium-2, 3, 4, 6, 7, 8-hexahydro] ethylamine (Gupta et al. 1991). Efrapeptins are different from the well-known group of fungal metabolites, peptaibols, which are α-Aib rich peptides with an acetylated N-terminus and an amino alcohol at the C-terminus (Rinehart et al. 1981). Structural elucidation of efrapeptins was accomplished by a combination of fast atom bombardment mass spectrometry (FABMS), gas chromatography-mass spectrometry (GC-MS) and high resolution nmr analyses. The final determination of the structure of the C-terminal blocking group came from a single crystal x-ray analysis of a hydrolytic C-terminal dipeptide fragment.

Amino acid analysis of the peptide mixture showed the presence of Aib, alanine (Ala), β-alanine (β-Ala), glycine (Gly), pipecolic acid (Pip), leucine (Leu) and isovaline (Iva). Presence of N-acetyl Pip was confirmed by the GC-MS analysis of the partial hydrolyzate [trimethyl silyl (TMS), protonated molecular ion $(M+H^+)$ at 244, chemical ionization (CI)]. Absolute configuration of all the chiral amino acids in efrapeptins is S(L). This was confirmed for Ala and Leu by GC analysis of their N-trifluoroacetyl (TFA), O-Me esters on a chiral column. Absolute configuration of Pip was confirmed by the direct isolation of the amino acid and the measurement of the optical rotation. Absolute configuration of Iva was confirmed by the x-ray analysis of a C-terminal dipeptide crystal and by its relative relationship to a Leu residue.

FABMS analysis of the peptides C-G showed molecular ion (M^+) at mass/charge (m/z) 1606, 1620, 1634, 1634, and 1648, respectively. Because of the presence of a quaternary nitrogen **(Fig. 1),** these peptides give unusually intense molecular ion peaks (M^+) instead of protonated molecular ion $(M+H^+)$ under FABMS conditions. High resolution FABMS of efrapeptin F gave an M^+ at 1634.0758 which corresponded to an elemental composition of $C_{82}H_{141}N_{16}O_{16}$ (calculated value, 1634.0773). The FAB spectra of the peptides showed fragment ions corresponding to C-terminal alkyl ('Z), amino (Y') and acyl (X), and N-terminal acylium ions (B). Notably, the most intense peaks in the clusters of C-terminal acyl ions (X) are 1 mass unit lower than the corresponding fragment mass probably because of a proton loss during fragmentation. Complete amino acid sequence of the peptides could be derived from analyses of the FAB mass spectra.

These sequence assignments were further substantiated by GC-MS [EI (electron imact) and CI] analysis of the partial hydrolyzates of



C Ac-PIP-AIB-PIP-AIB-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-GLY-LEU-isoVAL-X
D Ac-PIP-AIB-PIP-AIB-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-ALA-LEU-isoVAL-X
E Ac-PIP-AIB-PIP-AIB-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-GLY-LEU-AIB-X
F Ac-PIP-AIB-PIP-isoVAL-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-GLY-LEU-isoVAL-X
G Ac-PIP-AIB-PIP-isoVAL-AIB-LEU-β-ALA-GLY-AIB-AIB-PIP-AIB-ALA-LEU-isoVAL-X

EFRAPEPTINS

FIG 1. Structure of the efrapeptins: amino acid and the C-terminal blocking group.

the peptide mixture. The O-Me, N-TFA-derivative of the partial hydrolyzate (con. HCl, 37°, 12h) from the peptide mixture showed the fragments Aib-Aib, Aib-Ala, Aib-Gly, Pip-Aib and Pip-Aib-Ala. Similarly, GC-MS analysis of the TMS derivative of the partial hydrolyzate (6NHCl, 110°, 25m) showed the presence of the following fragments: Pip-Aib, Aib-Aib, Aib-Ala, Aib-Pip, Pip-Aib-Ala, Aib-Aib-Leu and Gly-Aib-Aib. The identification of the fragment TFA-Pip-Aib-Ala-OMe also confirmed the position of Ala in efrapeptin F.

Efrapeptins can neither be acetylated (pyridine-acetic anhydride) nor do they react with diazomethane, which suggests the absence of any free amino, hydroxyl or carboxyl functions. The peptides are also resistant to base treatment (1M NaOH or MeOH-K_2CO_3), Edman degradation and dansylation. Nuclear magnetic resonance (nmr) and ultraviolet spectrometry (UV) data for the peptides indicated the absence of any olefinic bonds and aromatic rings.

The partially purified toxin was hydrolyzed (6N HCl, 110°, 12h) and chromatographed on cellulose followed by HPLC (column RPC4, CH_3CN:H_2O:TFA 0.1% gradient). Major peak was pooled to afford a peptide fragment whose high resolution FABMS showed M^+ at m/z 436.3647 suggesting an elemental composition of $C_{24}H_{46}N_5O_2$ (calculated value, 436.3652). Amino acid analysis confirmed the presence of Leu and Iva. The molecular formula of the peptide requires five degrees of unsaturation with an ionized species. ^1H nmr of the peptide in deuterated methanol (CD_3OD), COSYPS (correlation spectroscopy phase sensitive) and COSY-45, established the presence of, in addition to Leu and Iva moieties, three spin systems: (a) $(CH_3)_2CHCH_2CH(CH_2)$-; (b) $-CH_2CH_2CH_2$-;

and (c) $-CH_2CH_2CH_2-$ as depicted in Figure 1. These results were further substantiated by the proton nmr and COSYPS in pyridine d_5 and by a long range proton-proton connectivity (RELAYH) 2D nmr experiment in deuterated methanol which showed long-range connectives supporting earlier assignments. ^{13}C nmr of the peptide in CD_3OD showed the presence of 24 carbons [distortionless enchancement by polarization transfer (DEPT), 6 methyls, 10 methylenes and 4 methines].

The presence of 4 unprotonated carbons was also confirmed by the quaternary only sequence which showed the resonances of four carbons at 176.83, 169,94, 166.11 and 61.76 ppm. Considering the two downfield signals as the amide carbonyls of Leu (169.94 ppm) and Iva (176.83 ppm), the molecules must have a C=N moiety to account for the third carbon signal at 166.11 ppm. This also suggested the presence of a bicyclic system in the molecule which must be the unknown C-terminal blocking group because the whole fragment is a linear peptide as suggested by the acetylation reaction and the FABMS fragmentation pattern. Carbon-proton connectivities were determined by a heteronuclear correlation (HETCOR) experiment.

A literature search revealed that the reported carbon chemical shifts of the pyrroline moiety of a cyclic amidine closely matched with the chemical shifts as observed for the spin system b of the peptide fragment. This suggested the presence of 1-pyrroline ring system in the blocking group which accounted for the C=N moiety as well as the COSY fragment b. The presence of such a moiety was further substantiated by long range carbon-proton connectitivy (COLOC) experiments. On the basis of several COLOC experiments, coupled with the rest of the spectral data, the structure could be constructed into an N-peptido-isobutyl-[1-pyrrolo-(1,2-α) pyrimidinium 2,3,4,6,7,8-hexahydro] ethylamine system as shown (Fig. 1).

The only remaining question was the position of the isobutyl side chain which was unambiguously confirmed by a COLOC experiment where a $^3J_{ch}$ connectivity was observed between the methylene resonances at 3.39 and 3.58 ppm ($2\text{-}CH_2$) and the sp2 carbon of the pyrrolo-pyrimidine moiety at 166.11 ppm. This clearly established the position of the isobutyl side chain as being next to the amide nitrogen. On the basis of the combined evidence, we could assign the final structure of the fragment. Further support for the structure came from the observation that the efrapeptins show a prominent peak at m/z 138 in their FAB mass spectra. This peak can be generated by a homolytic cleavage of the C1-C2 bond only if the isobutyl chain is on the carbon next to the amide nitrogen.

Unambiguous determination of the structure of the blocking group came from a single crystal x-ray analysis of the trifluoroacetyl derivative of the dipeptide fragment. This analysis revealed that one peptide-fragment molecule, one molecule of water, and one trifluoroacetate comprise the asymmetric unit. The occurrence of sharp single resonances, chemical shift differences between the diastereotiopic geminal protons, and vicinal coupling constants that differ from the mean values, indicate the existence of a single predominant conformation in solution and that probably, there is minimal nitrogen flipping and ring inversion of the heterocyclic ring.

On the basis of biosynthetic considerations and structural analogy, is seems plausible that part of the blocking group with an isobutyl side chain is derived from S-Leu (amide formation with Iva followed by modification and reduction of the carboxyl) where the chiral center of Leu remains undisturbed during the bioconversion. This is the first reported occurrence of a peptide linked to hexahydropyrrolo-(1,2-α) pyrimidine (also known as 1,5-diazabicyclo[4.3.0] non-5-ene, DBN) which is particularly interesting because of the unique biological activity associated with these peptides. The blocking

group is formally an alkylation product of DBN with S-leucinol.

Biological activity of efrapeptins – Efrapeptins C-G showed insect toxicity against Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera) (foliar spray assay, LC^{50} at 18.9 and 8.4 ppm respectively for efrapeptins D and F) (Krasnoff et al. 1991). All the peptides showed mitochondrial ATPase inhibitory activity when tested against preparations from entomopathogenic fungi (eg. *Metarhizium anisopliae* and *Tolypocladium niveum*) and insects (eg. flight muscles from *Musca domestica*). An interesting unanswered question is how does the fungus protect itself from the toxin as, unlike some other ATPases, *in vitro* assays show that the mitochondrial ATPase of *T. niveum* is inhibited by efrapeptins. Also, the acetylated derivative of a peptide fragment Ac-Aib-Gly-Leu-Iva-X showed a clear dose-response when bioassayed for ATPase inhibitory activity, although the specific activity was lower than the parent peptides. This observation suggests a possible role for the blocking group in the biological activity of efrapeptins. These peptides are strong inhibitors of mitochondrial oxidative phosphorylation and ATPase activity (Kohlbrenner & Cross 1979).

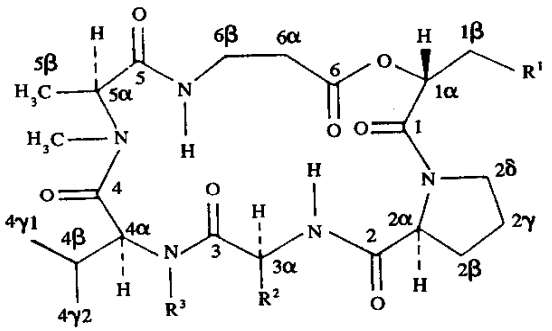
Efrapeptins also inhibit photophosphorylation in chloroplasts (Lucero et al. 1976). The peptides are probably catlytic-site competitive inhibitors which bind do the soluble F^1 part of the mitochondrial ATPase (Lardy et al. 1975). It has been proposed that efrapeptins bind at the catalytic site and block accessibility of an essential arginine residue on the enzyme at the adenine nucleotide binding site (Kohlbrenner & Cross 1978). Pi-ATP exchange catalyzed by modified F^1 can be inhibited by the F^1-specific efrapeptins (Bossard & Schuster 1981). In some cases, it has also been shown that ATPases and certain mutant cell lines are resistant to efrapeptins (Clark & Shay 1982; Wise et al. 1984; Dean et al. 1986).

Destruxins

Destruxins, the insecticidal cyclodepsipeptides, were originally isolated from the entomogenous fungus, *Metarhizium anisopliae* (Kodaira 1961, 1962; Roberts 1966, 1969). Recently, destruxins were isolated as the active principle from the chlorosis-causing plant pathogenic fungus, *Alternaria brassicae* (Ayer & Pena-Rodrigues 1987; Bains & Tewari 1987). Roseotoxin B, the *trans*-3-methyl proline analog of destruxin A, is known to be produced by *Trichothecium roseum* (Engstrom et al. 1975; Engstrom 1978; Springer et al. 1984).

The structure of destruxins – For the isolation of destruxins, *Metarhizium anisopliae* was grown in sterilized Cpzapek-Dox medium enriched with 0.5% bactopeptone (Gupta et al. 1989a). The culture was allowed to grow at ambient temperature for ten days on a rotary shaker (150 rpm). The culture was filtered and the clear filtrate was treated with dil. HCl to adjust the PH to 5.5. Aqueous solution was then extracted with methylene chloride and the organic layer washed with 10% aqueous sodium bicarbonate. Solvent was removed from the organic layer to afford the neutral destruxin-containing extract. The extract was flash chromatographed on silica gel and destruxins were separated by HPLC using a reverse phase C18 column and a gradient combination of acetonitrile-water as the eluting solvent.

Destruxins are cyclic depsipeptides consisting of five amino acids and a D-α-hydroxy acid (Suzuki et al. 1970; Suzuki & Tamara 1972; Pais et al. 1981) **(Fig. 2).** Two of the amino acids are N-methylated amino acids: N-methyl-L-alanine (replaced by L-alanine in protodestruxin) and N-methyl-L-valine (replaced by L-valine in desmethyldestruxin B and protodestruxin). The other three aminoacids are β-alanine, L-leucine (eg. in destruxin A and A1, but replaced by L-valine in destruxin A2), and L-proline (eg. in destruxin A and A2, but



DESTRUXIN	R1	R2	R3
A (1)	$-CH=CH_2$ (1γ, 1δ)	$-CH(CH_3)CH_2CH_3$ (3β, 3γ2, 3γ1, 3δ)	CH_3
A2 (2)	$-CH=CH_2$	$-CH(CH_3)_2$ (3β, 3γ1, 3γ2)	CH_3
B (3)	$-CH(CH_3)_2$ (1γ, 1δ1, 1δ2)	$-CH(CH_3)CH_2CH_3$	CH_3
B2 (4)	$-CH(CH_3)_2$	$-CH(CH_3)_2$	CH_3
E (5)	$-CH(O)CH_2$ (1γ, 1δ)	$-CH(CH_3)CH_2CH_3$	CH_3
E2 (6)	$-CH(O)CH_2$	$-CH(CH_3)_2$	CH_3
C (7)	$-CH(CH_3)CH_2OH$ (1γ, 1δ2, 1δ1)	$-CH(CH_3)CH_2CH_3$	CH_3
C2 (8)	$-CH(CH_3)CH_2OH$	$-CH(CH_3)_2$	CH_3
DMDB (9)	$-CH(CH_3)_2$	$-CH(CH_3)CH_2CH_3$	H
CHL (10)	$-CH(OH)CH_2Cl$ (1γ, 1δ)	$-CH(CH_3)CH_2CH_3$	CH_3

FIG. 2. Structure of destruxins isolated from *Metarhizium anisopliae*.

replaced by L-pipecolic acid in destruxin A1, and replaced by trans-3-methyl L-proline in roseotoxin B).

D-α-hydroxy acid is the variable structural residue of destruxins, for example, it is D-2-hydroxy-4-pentenoic acid in destruxin A, D-2-hydroxy-4-methylpentenoic acid in destruxin B, and D-2-hydroxy-4,5-epoxypententoic acid in destruxin E. So far 18 different structurally-related, naturally-occurring destruxins have been characterized (Gupta et al. 1989b). Destruxins were identified by their physical and spectral data, and chemical conversions. Lactone ring of the destruxins can be opened by mild base treatment to afford the linear product which then can be subjected to mass spectral analysis to get the sequence data. Complete proton and carbon nmr assignments for destruxins A and B were done using different 1D and 2D nmr techniques (Gupta et al. 1989b).

Destruxin A is cyclo (2(R) - hydroxy - 4 - petenoyl - L - prolyl - L - isoleucyl - N - methyl - L - valyl - N - methyl - L - alanyl - β - alanyl). Four α-amino acids have the L(S)

configuration while the α-hydroxy-4-pentenoic acid has the D (R) configuration. The ester linkage and four of the five peptide bonds are trans, while the N-methyl alanyl-N-methyl valyl peptide bond is cis. It has been observed that N-methylated cyclic peptides tend to possess cis amides, and destruxin B was speculated to have one cis peptide bond on the basis of nmr studies. Though the peptide backbone of destruxin A is essentially asymmetric, the overall geometry of the molecule appears to be roughly rectangular. This is commonly observed in the case of cyclic hexapeptides characterized by 4--> 1 cis chain reversal at one end and a β-turn at the other. Isoleucine and β-alanine are the linking units, and β-alanine assumes the gauche conformation about the C(6α)-C(6β) bond. Isoleucine and proline side chains protrude towards the end of the molecule which is more hydrophobic, while N-methyl alanine and N-methyl valine, at the other end, are linked by a cis peptide bond.

This arrangement results in a conformation where the amide nitrogens of isoleucine and β-alanine project towards the interior of the molecule and allow the formation of two transannular 4-->1 type hydrogen bonds. The formation of two intramolecular hydrogen bonds between the amide protons and the carbonyl oxygens of isoleucine and β-alanine results in the formation of two ten membered rings inside the covalent 19-membered macrocyclic lactone. This leads to a stabilized conformation with two ends of the molecule constrained to β-turns. It was suggested for roseotoxin B that the peptide conformation actually leads to the formation of cross ring bridges which probably are less significant for the rigidity of the backbone. Usually, the peptide backbone in cyclo- and depsipeptides is a conformationally flexible structure capable of having nonplanar geometries.

Occurrence of well-resolved, sharp signals in the ^1H nmr spectrum of destruxin A suggests the presence of a stable conformation in solution. However, the completely N-demethylated analog of destruxin B, the protodestruxin, gives a complex pattern of resonances. This suggests the presence of multiple conformers in solution and that N-methylation results in the formation of stable conformation, probably by limiting the number of free amides available for intramolecular hydrogen bonding. The monodemethylated analog of destruxin B, desmethyldestruxin B, gives a well resolved[1] nmr spectrum suggesting little or no role for N-methylation of valine in the conformational stability. Yet, unlike the other common destruxins, desmetryldestruxin B cannot be readily crystallized.

Solution conformation of destruxin A in deuterated chloroform was studied by ^1H 2D NOE (NOESY, nuclear Overhauser spectroscopy) and compared with its crystal conformation (Gupta et al. 1989c). The results suggest that the peptide backbone conformation of destruxin. A in solution resembles its crystal conformation. However, a difference seems to occur in the orientation of the N-methyl of alanine which, in solution, is apparently tilted more towards the inside of the ring, aided by an extra twist of the peptide backbone bringing it close to the β-alanine β-proton on the one side and to the γ1 methylene of the isoleucine on the other. In the crystal conformation, the N-methyl points away from the center of the macrocycle. The formation of the two intramolecular hydrogen bonds is probably responsible for the conformational stability of the molecule in solution. Total synthesis of destruxin B and protodestruxin have been reported (Kuyama & Tamura 1965; Lee et al. 1975; Izumiya 1982).

Biological activity of destruxins – Destruxins are insecticidal compounds with a wide range of activity. Is has been reported that destruxin B is the chlorosis-causing principle of the plant pathogenic fungus, *Alternaria brassicae* (Ayer & Pena-Rodriguez 1987; Bains & Tewari 1987), which was also found to produce desmethyldestruxin B and homodestruxin B. Roseotoxin B, the

trans-3-methylproline analog of destruxin A, was isolated as the toxic metabolite of *Trichothecium roseum* (Engstrom et al. 1975; Engstrom 1978; Springer et al. 1984). Apart from well-documented insecticidal activity, destruxins have also been shown to possess immunodepressant activity in insect model systems (Vey et al. 1985; Huxham et al. 1989; Cerenius et al. 1990).

This finding is important in view of the fact that cyclosporins, a group of cyclic peptides derived from fungi, are clinically important immunodepressant drugs. Destruxin E was found to exhibit cytostatic and cytotoxic effects on mouse leukemia cells (Morel et al. 1983; Odier et al. 1987). Destruxins can also activate calcium channels in insect muscles (Samuels et al. 1988c) but, unlike beauvericin, a cyclodepsipetide produced by the entomopathogenic fungus *Beauveria bassiana*, destruxins do not seem to posses ionophoric properties (Abalis 1981; Samuels et al. 1988c). A relationship between the production of destruxins and the pathogenicity of the fungus has been suggested, and the role of the destruxins may be to facilitate establishment of the pathogen in the host (Fargues et al. 1985; Samuels et al 1988a). These insect toxins apparently can be the cause of insect mortality after fungal infection (Suzuki et al. 1971; Samuels et al. 1988b).

Beauvericin

Beauvericin has been isolated from the following sources: the entomopathogenic deuteromycetous fungi *Beauveria bassiana* (Hamill et al. 1969), *Paecilomyces fumosoroseus* (Bernardini et al. 1975), *Fusarium semitectum*, and *Fusarium moniliforme* var. *subglutinans*, and the plant pathogenic basidiomycetous fungus *Polyporus sulphureus* (Deol et al. 1978).

The structure of beauvericin – Isolation of beauvericin from a culture of *F. semitectum* was accomplished in the following manner: a culture of *F. semitectum* was filtered through several layers of cheesecloth to separate the mycelium and the broth. The mycelium was soaked in ethanol overnight, blended, and filtered to afford a clear filtrate which was evaporated *in vacuo*. The residue was suspended in water and extracted with methylene chloride. The organic layer was evaporated *in vacuo* to afford nonpolar material from the mycelium. The mycelial extract was flash chromatographed on silica gel followed by reverse phase HPLC under the following conditions: column, RPC_{18}, 0.9x50 cm; solvent, MeCN:H_2O (30:70) to MeCN in 30m, linear gradient; flow, 3.3 ml/m; detection, UV, 225 nm. Beauvericin was eluted as one major peak (retention time 18 min).

Structure elucidation of beauvericin was accomplished by a combination of degradative and spectral methods (Hamill et al. 1969). The hydrolysis of beauvericin furnished L-N-methylphenylalanine and D-γ-hydroxy isovaleric acid as determined by the comparison of their optical rotary dispersion (ord) curves with known standards. Depsipeptide nature of the toxin was inferred from the presence of two carbonyl bands in the infrared (ir) spectrumat 1740 (ester) and 1670 (amide) cm^{-1}. Simplicity of the ^1H nmr spectrum of beauvericin was indicative of the symmetry and cyclic nature of the depsipeptide.

The characteristic features of the spectrum are the presence of two C-methyl doublets at 0.42 and 0.79 ppm, N-methyl singlet at 2.98 ppm, and the multiplet from the aromatic protons at 7.23 ppm. The α-protons of N-methyl phenylalanine and α-hydroxyisovaleric acid show characteristic double doublet (5.42 ppm) and doublet (4.93) respectively. The ^{13}C nmr of beauvericin shows 13 signals. The characteristic resonances are the α-carbons of N-methyl phenylalanine and α-hydroxyisovaleric acid which resonate at 57.46 and 75.4 ppm respectively. Electron impact MS of beauvericin gave a molecular ion peak at 783 (M^+). Based on the combined evidence as

above, beauvericin was assigned the structure as shown in Figure 3, which is a cyclic lactone trimer of amide of N-methyl L-phenylalanine and D-α-hydroxyisovaleric acid. The monomer-derived diol (Fig. 3) can be isolated by lithium aluminum hydride reduction of beauvericin.

Biological activity of beauvericin – Beauvericin probably acts as an ionophore capable of making complexes with divalent cations (Prince et al. 174; Dorschner & Lardy 1968). It is cytotoxic (Vey et al. 1973) and has been reported to possess some insecticidal properties against mosquito larvae and blowflies (Grove & Pople 1980). Beauvericin was originally isolated as a toxin against brine shrimp (Hamill et al. 1969). There are no data available on the mammalian toxicity of beauvericin.

Another structurally-related group of antibiotic cyclodepsipeptides, enniatins A-C, have been reported from *Fusarium* sp. (Ovchnnikov et al. 1971). In enniatins A-C, the aromatic amino acid N-methyl phenylalanine of beauvericin has been replaced by N-methyl derivatives of the aliphatic amino acids isoleucine, valine, and leucine respectively **(Fig. 3)**. Despite the structural similarity, beauvericin and enniatins have never been found to co-occur in nature, probably because of the difference in the multienzymes responsible for their biosynthesis (Zocher et al. 1982; Peeters et al. 1983). Beauvericin has toxic activity against the insect, Colorado potato beetle, with LC_{50} of 633 ppm (95% fiducial limits, 530-748 ppm) and LC_{90} of 1196 ppm (95% fiducial limits, 954-1863 ppm).

Bassianolide

Bassianolide is a toxic metabolite of *Beauveria bassiana* and *Verticillium lecanii* (Suzuki et al. 1977). The toxin was originally isolated from strains of *B. bassiana* and *V. lecanii* which were entomogenous on the cadavers of *Bombyx mori* pupae (Murakoshi et al. 1978).

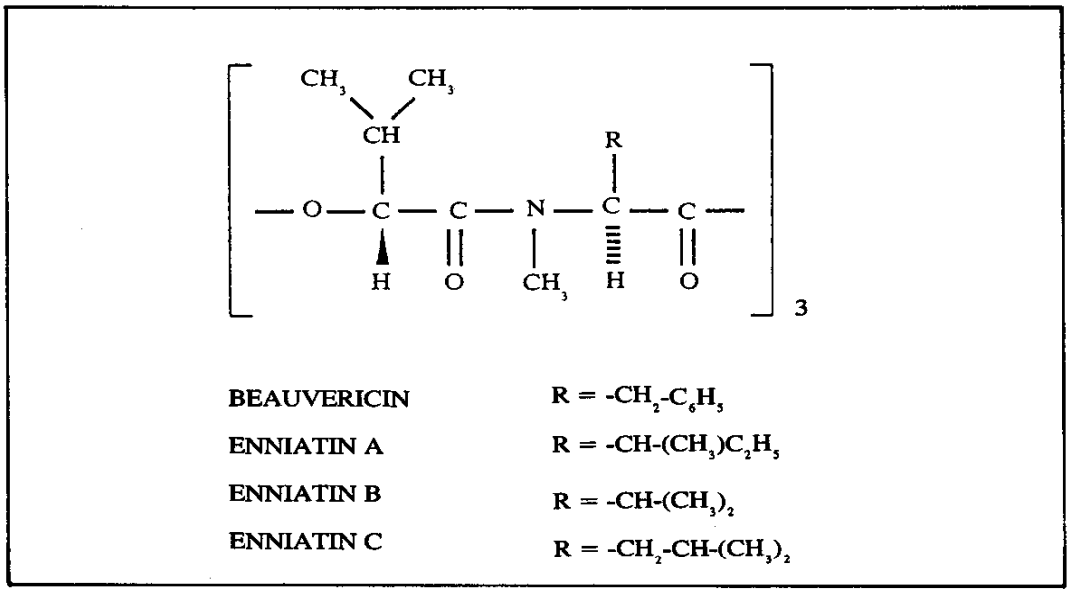


FIG. 3. Structure of beauvericin and enniatins.

The structure of bassianolide – Bassianolide was isolated from the mycelium obtained from the culture broth of *B. bassiana* and *V. lecanii* (Kanaoka et al. 1977). The isolation procedure involves the extraction of the mycelium with methanol. The ethyl acetate soluble fraction from the extract was washed with dil. HCl and saturated aqueous sodium bicarbonate to furnish neutrals which were subjected to silica gel chromatography followed by chromatography on neutral alumina. Crude toxin so obstained was subjected to preparative thin layer chromatography on silica gel and Sephadex LH-20 chromatography to afford pure bassianolide. Bassianolide can be resolved by HPLC s follows: column, RPC18; solvent, CH_3CN:H_2O (30:70) to CH_3CN (linear gradient in 25 min), detection, UV 220 nm.

Structural elucidation of bassianolide involved the use of spectroscopic and degradative methods (Kanaoka et al. 1978). The ^1H nmr of bassianolide in benzene-d_6 at 70°C shows one N-methyl signal at 2.84 ppm. The signals observed and their assignments are as follows: 5.9 (1H, dd, 6, 10 Hz, 1α), 5.4 (1H, d, 8 Hz, 2α), 2.84 (3H, s, N-CH_3), 2.4 (1H, m, 1β), 1.82 (2H, m, 2β, 1.4 (1H, m, 2γ), and 0.8-1.1 (12H, m, C-CH_3) ppm. The ^{13}C nmr spectrum of bassianolide in benzene-d_6 at 70°C revealed 12 carbon signals at 171.2 (s, 2), 169.3 (s, 1), 75.1 (d, 1α), 54.9 (d, 2α), 37.3 (t, 2β), 30.7 (q, N-methyl), 30.4 (d, 1β), 25.5 (d, 2,γ), 23.4, 21.7, 18.7 and 18.0 (each q, C-methyl). Hydrolysis of bassianolide with 6N HCl at 115°C (16 hr) followed by ether extraction afforded α-hydroxy isovaleric acid which was identified by comparison with a standard (^1H nmr, GC of methyl ester). Only one amino acid was isolated from the water layer.

This was identified as N-methyl leucine (nmr, ir, optical rotation). These results suggested that the bassianolide is composed of these two constituents. Treatment of bassianolide with $LiBH_4$ afforded one product which was identified as D-α-hydroxyisovaleryl L-N-methylleucinol on the basis of spectral data (HRMS, nmr). That this product is D-α-hydroxyisovaleryl L-N-methylleucinol and not L-α-hydroxyisovaleryl L-N-methylleucinol, was confirmed by a direct comparison with synthetic standards where L-α-hydroxyisovaleryl L-N-methylleucinol was clearly distinguishable from the natural product-derived material by tlc and cmr. This result confirmed that the bassianolide is a cyclic polymer of D-α-hydroxyisovaleryl L-N-methylleucinol. That the toxin is a tetramer of this unit was confirmed by FD and EI mass spectra where bassianolide gives a molecular ion at m/z 908 which is in accordance with the molecular formula $C_{48}H_{84}N_4O_{12}$ confirming the structure of bassianolide as shown **(Fig. 4)**. A total synthesis of bassianolide has been reported (Kanaoka et al. 1977).

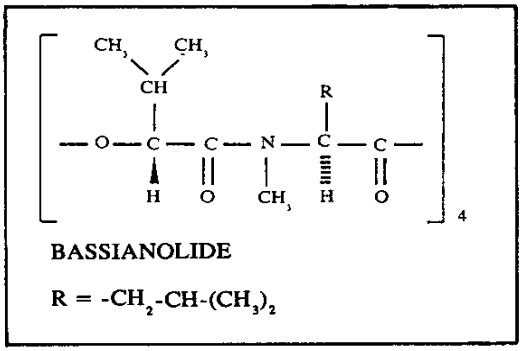


FIG. 4. Structure of bassianolide.

Bassianolide in solution exists as a mixture of two conformers (Kanaoka et al. 1978). One of these conformers apparently has a four-fold symmetry where the four N-methyls in the molecule resonate as a singlet in the ^1H nmr (eg. in CCl_4 or benzene d_6 at 70°C). In the other conformation, the molecule is spatially asymmetric and this is reflected in ^1H nmr where N-methyls resonate as 5 singlets (4 singlets from the assymmetric conformation and 1 from the symmetric conformation) (eg. in $CDCl_3$). The ratio of these two conformers,

as illustrated by the intensity of the N-methyl signals, is solvent and temperature dependent. For example, the symmetric conformation predominates in benzene d_6 at 70°C. Apparently, the rate of interconversion of the two conformations is slow on the nmr time scale under certain conditions, thus allowing the resolution by nmr. The two conformers could be separated on tlc (silica gel, -24°C, $CHCl_3$:acetone, 9:1).

Biological activity of bassianolide – Bassianolide, like certain other depsipeptides, probably acts as an ionophore. The molecular conformation of such depsiptides results in a structure where the molecule has a relatively hydrophilic interior (because of the amide carbonyls) and a hydrophobic exterior (because of the hydrophobic side chains). Inorganic cations can be ligated in the hydrophilic center of the molecule. Ions can then be transported across lipophilic membranes because of the overall hydrophobic nature of the complex. Bassianolide is toxic to insects (Kanaoka et al. 1978). Fifth instar larvae of *Bombyx mori* showed mortality when fed upon an artifical diet containing bassianolide at a dose of 13 ppm. Injection of bassianolide, suspended in water, to *B. mori* larvae caused atonic symptoms at a dose of 2 ug/larva. The toxin was lethal at a dose of >5 ug/larva.

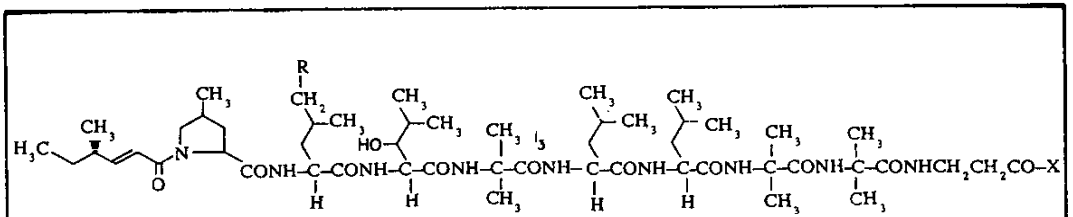
Leucinostatins

Leucinostatins have been isolated from the submerged cultures of *Paecilomyces lilacinus* (Arai et al. 1973), *Paecilomyces marquandi* (Rossi et al. 1983), and *Paecilomyces farinosus*.

The structure of leucinostatins – Leucinostatins A and B were originally isolated from an ethyl acetate extract of the culture broth of *P. lilacinus* by a combination of silica gel chromatography followed by alumina chromatography (Fukushima et al. 1983a,b). Minor leucinostatins have been separated by extensive flash chromatography on silica gel using chloroform with increasing proportions of MeOH and ammonium hydroxide as eluent (Casinovi et al. 1986). Major leucinostatins can be nicely resolved by HPLC on a C18 column with MeOH:isopropanol:H_2O:MeCN:triethylamine (4:3:2:1:0.01) as an eluent with UV detection at 230 nm (Stroh et al. 1986).

Six chemically distinct natural leucinostatins A-F have so far been identified **(Fig. 5)**. There appears to be some confusion in the nomenclature of leucinostatins as two identical compounds have been described in separate reports as leucinostatins H and K (Radics et al. 1987) and leucinostatins E and F (Stroh et al. 1986) respectively. Leucinostatins have also been referred to as paecilotoxins in the literature (Mikami et al. 1989). Leucinostatins are linear peptides which, along with α-aminoisobutyric acid, L-leucine, and β-alanine, have the following three unusual amino acids as constituent residues: L-threo-β-hydroxy leucine, 2-amino-6-hydroxy-4-methyl-8-oxodecanoic acid (AHMOD), and cis-4-methyl-L-proline (Mori et al. 1982). The N-terminal of the peptides is acylated with an α,β-unsaturated aliphatic acid (4S, 2E-4-methylhex-2-enoic acid) and the C-terminal is amidated with N^1, N^1-dimethylpropane-1,2-diamine (in leucinostatin A) and N^1-methylpropane-1,2-diamine (in leucinostatin B) (Mori et al. 1983; Casinovi et al. 1983).

Structures of leucinostatins were established essentially by partial hydrolysis and isolation of the smaller fragments which were then used for detailed chemical and spectral analysis. Amino acid sequences of the peptides were also established by FDMS and FABMS analysis (leucinostatin A, M^+ 1218, nominal mass, leucinostatin B, M^+ 1204). Leucinostatins C (M^+ 1118) (Casinovi et al. 1986) and D (M^+ 1104) (Rossi et al. 1987) are analogs of leucinostatins A and B respectively where AHMOD has been replaced by leucine. Leucinostatins E (M^+ 1134) and F (M^+ 1234)



LEUCINOSTATIN	R	X
A	$CH(OH)CH_2COCH_2CH_3$	$NHCH(CH_3)CH_2N(CH_3)_2$
B	$CH(OH)CH_2COCH_2CH_3$	$NHCH(CH_3)CH_2NHCH_3$
C	H	$NHCH(CH_3)CH_2N(CH_3)_2$
D	H	$NHCH(CH_3)CH_2NHCH_3$
E	H	$NHCH(CH_3)CH_2NO(CH_3)_2$
F	$CH(OH)CH_2COCH_2CH_3$	$NHCH(CH_3)CH_2NO(CH_3)_2$

FIG. 5. Structure of leucinostatins.

(Stroh et al. 1986) are N-oxides (at the N-1 of N-methylpropane 1,2-diamine) of leucinostatins C and A respectively. Conformational studies on leucinostatin A in solution using circular dichroism (cd) and infrared (ir) spectroscopy have been reported. It has been proposed, on the basis of ir data in $CDCl_3$, that the molecule has intramolecular hydrogen bondings while the cd data in lipophilic solvents suggests the existence of a helical structure (Vertuani et al. 1989).

Biological activity of leucinostatins – Leucinostatins have antimicrobial properties against gram positive bacteria and a wide range of fungi (Fukushima et al. 1983a, b). They have also been shown to have anticancer activity against Ehrlich solid carcinoma (Arai et al. 1973). Leucinostatins have intraperitoneal and oral toxicity in mice. Intraperitoneal LD_{50} for leucinostatin A and B hydrochlorides is 1.8 mg/kg body weight. The oral LD_{50} values for leucinostatin A and B hydrochlorides have been reported to be 5.4 and 6.3 mg/kg body weight, respectively. These antibiotics act as uncouplers of oxidative phosphorylation in mitochondria (Fukushima et al. 1983a,b) and there are several reports of use of leucinostatins as tools to probe mitochondrial activity. In our laboratory, leucinostatins were isolated as the active principle from an extract of *P. farinsosus* which showed insecticidal activity against Colorado potato beetle.

THE ROLE OF ENZYMES IN FUNGAL PATHOGENICITY

As mentioned perviously, in addition to low-molecular-weight biologically active metabolites, entomopathogenic fungi rely on enzymes to overcome their hosts. This is particularly true in the initial phases of infection, e.g. penetration of insect cuticle.

Germination and differentiation of fungal spores

The germination of fungal spores is the first and most crucial event in the propagation of the species. The transition from dormancy to active growth is accompanied by profound structural and biochemical changes. Early events in germination include the mobilization of stored carbon sources. However, the spores of many entomopathogenic fungi require additional exogenous nutrients from the host surface to germinate and develop (Smith & Grula 1981; Fargues 1981). Watersoluble nutrients, primarily amino acids, are present on cuticles from some soft bodied insects (Woods & Grula 1984), but not Coleoptera (Hunt et al. 1984). Inability to utilize the lipids, particularly hydrocarbons, which predominate on the surface of the cuticle (epicuticle) may consequently prevent some fungi from being pathogenic (Kerwin 1984; Latgé et al. 1987). Adaptation to utilize these substrates involves inducible extracellular enzymes, as demonstrated in *Metarhizium anisopliae* and *Nomuraea rileyi*, which perform both β-oxidation and ω-oxidation pathways to grow on a variety of hydrocarbons which are refractory to most other microbes (St. Leger et al. 1988a).

Mode of penetration into hosts

Most pathogenic fungi need to enter the insect body to obtain the nutrients for their growth and reproduction. Direct penetration of intact cuticle is the normal mode of entry by most fungi adapted for entomopathogenicity and often involves the precise differentiation of an organized series of structures on the host surface. Most fungi are prevented from being entomopathogenic because they lack one or more of the necessary morphological or biochemical cuticle-attacking mechanisms or they have not evolved ways to overcome disease resistance mechanisms in the cuticle.

The mode of penetration of fungal pathogens into their hosts has been a matter of controversy for many years. Penetration was considered to be accomplished by mechanical force if the cuticle was depressed inward at the point of penetration. Lack of such depression and the appearance of digestion was taken as evidence of enzymic penetration. However, evidence obtained from *M. anisopliae* has shown that these two mechanisms are not mutually exclusive. Ultrastructural studies with gold-labelled antibody prepared against a pathogen protease (Pr1) demonstrated that, while penetration of the epicuticle is primarily by enzymic degradation, penetration of the procuticle involves both enzymic degradation and the mechanical separation of the lamellae (Goettel et al. 1989a).

Many pathogens can produce a wide range of cuticle-degrading enzymes (CDE) corresponding to the diverse polymers in insect cuticle viz. protein, chitin and lipids (St. Leger et al. 1986a, Charnley & St.Leger 1991). The contact between extracellular CDE of pathogens and cuticle is often one of the first molecular interactions between host and pathogens, the outcome of which can modify the type or balance of the relationship.

This account will concentrate on the degradation of protein, the major structural component of cuticle, as this is probably of greatest significance in pathogenicity. The chymoelastase (Pr1) of *M. anisopliae* is reviewed in detail because it provides the best understood model and its role in pathogenesis has been established.

Regulation of cuticle-degrading enzyme (CDE) production

Potentially, pathogenic specialization may operate by way of regulatory controls which allow expression of genes under conditions in which similar genes in nonpathogens are not expressed. In *M. anisopliae*, cuticle degrading enzymes are inductive and/or subject to catabolite repression. Chitinase will only be required for a brief period during penetration of host cuticle and is subject to a high degree of regulatory control, being mediated by a

component of the cuticle (chitin degradation products) (St. Leger at. al. 1986b). By contrast, Pr1 with biological functions prior to and after infection, is produced rapidly in culture by carbon and nitrogen derepression alone (St. Leger et al. 1988b). Likewise Pr1 synthesis on infection structures (appressoria), produced on an artifical surface or during growth on host cuticle, is over-ridden by addition of readily utilized nutrients (St. Leger et al. 1989a,b). Studies utilizing [^{35}S] methionine incorporation and "Western" blot analysis demonstrated that Pr1 is the major protein produced when *M. anisopliae* produces appressoria against smooth polystyrene plastic or in situ during penetration of host (*Manduca sexta*) cuticle (St. Leger et al. 1989b). Synthesis during maturation of apperssoria and production of penetration pegs far exceeded synthesis of all other proteins. Such rapid protease synthesis is only possible in host tissues where the concentration of readily metabolizable compounds is low.

This is the case with most insect cuticles as the components are largely insoluble until released by cuticle-degrading enzymes(St. Leger et al. 1986c, 1987a,b). Repression could operate, however, if ever the release from the cuticle of degradation products exceeded fungal requirements. This was confirmed by addition to inocula of readily utilized nutrients, e.g. alanine, which resulted in extensive growth on the host cuticle but repressed penetration and synthesis of Pr1 (St. Leger et al. 1989b). Thus, with the *M. anisopliae* isolate ME1, the pathogenic process involving infection-related morphogenesis and enzyme production occurs only when lack of extracellular nutrients and depletion of endogenous reserves make it necessary for the pathogen to establish a nutritional relationship with the host.

Role of CDE in pathogenicity

There are numerous criteria which should be fulfilled before a CDE can be implicated in pathogenesis. These ideals have been achieved only in the case of *Metarhizium* Pr1.

Ability to produce CDE in vitro – Synthesis of CDE *in vitro* is no proof of involvement in disease. The demonstration that many entomopathogens besides *M. anisopliae* produce Pr1-type enzymes in culture on insect cuticle media (St. Leger et al. 1987b) is inconclusive, given that enzyme regulation may be complicated by host and pathogen metabolism and by environmental factors. Nevertheless, an indication of the potential range and characteristics of CDE can be obtained from cultures grown on insect cuticle, although it should also eventually be demonstrated that the properties of these enzymes do not differ from the forms detected *in vivo* (St. Leger et al. 1986a 1987a).

For workers investigating pathogens showing low growth or poor pontetial for CDE synthesis, it may be worthwhile to investigate the ability of intact or broken cells to reduce the viscosity of a model substrate (e.g. gelatin or glycolchitin) or hydrolyse a chromogenic substrate (e.g. blocked peptides such as succinyl-(alanyl)$_2$-prolyl-phenylalanine p-nitroanilide), or to grow on a cuticle component which implies production of the relevant polymerase.

Detection of CDE in infected cuticle – Obviously, enzymes should be detected at the site of penetration before or coincident whit symptoms. *Metarhizium* Pr1 is produced at high levels by the pathogen *in situ* during infection as shown by histoenzymological techniques (St. Leger et al. 1987a; St. Leger et al. 1989a; Goettel et al. 1989a). Esterase, lipase, trypsin-like protease (Pr2) and exo-chitinase have also been detected co-incident with cuticle infection by *Metarhizium* (St. Leger et al. 1987a).

Determination of enzyme activity *in vivo* usually requires the characterization of enzyme activity *in vitro* so that specific substrates or antibody probes can be prepared, a difficult task if the pathogen is hard to

cultivate. Here, the use of broader spectrum histoenzymological techniques, or antibodies and cDNA probes from other sources of the enzyme might be useful tools. It should be remembered that conventional histochemical localization by light microscopy will not allow designation of activity to a particular isozyme and that host enzymes can give interference.

Alteration of cuticle polymers – Purfied Pr1 readily degrades insect cuticles from a variety of insect sources (St. Leger at al. 1987c). The ability of pathogen chitinase to degrade host cuticle is enhanced after proteolytic degradation of encasing cuticle proteins (St. Leger et al. 1986 c). Alternatively, the presence of free-degradation products can give a qualitative reflection of prior activity of a CDE, e.g. amino acids (from Pr1) in *Metarhizium*-infected *Manduca sexta*. By contrast, chitin degradation products were not detected (St. Leger et al. 1987a).

Microscopic alterations in infected cuticles – Transmission electron microscopy can give valuable indications as to the participation of CDE and,in fact, much of the current discussion is based on EM studies and the physical appearence of the cuticle in contact with the invading pathogen. Obviously such lines of evidence are suggestive but require additional biochemical and immunocytochemical studies. Ultrastructural localization of specific CDE during infection has particular merit. Such an approach involving immuno-cytochemical techniques for Pr1 demonstrated substantial hydrolysis of cuticle protein during enzyme production by infection structures (Goettel et al. 1989a). Application of this approach for other CDE awaits production of appropriate antisera.

Correlation of enzyme production with pathogenicity – Isolates of *M. anisopliae* most pathogenic to *Nilaparvata lugens* are characterized by high endoprotease activity (Samuels, et al. 1990). Nevertheless, comparisons of natural isolates for pathogenicity and production of CDE usually only reveal the great variability within a species for numerous factors, many of which may influence, but may be unrelated to, CDE synthesis (Charnley 1984). Induction of point mutants with a common genetic background is a powerful, but underutilized, approach in which current knowledge of enzyme regulation would allow the choice of media to select mutants deficient, super-productive, or constitutive for Pr1, chitinase and other CDE. This has proved problematic. A considerable effort with chemical mutagens to obtain viable mutants lacking Pr1 failed, with evidence that mutations were either extremely enfeebled or lethal. The wild type allele of a Pr1 mutant locus could have been isolated by complementing (transforming) the mutant with a wild type genomic library, and the role of Pr1 in disease tested by transforming the Pr1 mutant with the wild type allele. Nevertheless, with the development of satisfactory transformation technology in *M. anisopliae* (Goettel et al. 1989b) and the cloning of several putative virulence genes it will be possible to use *in vitro* mutagenesis to improve our understanding of the molecular mechanisms that control pathogenicity.

Effects of CDE inhibition during cuticle invasion – Repression of enzyme synthesis (via catabolite repression affected by increasing nutrient levels on cuticle surfaces) blocked formation of infection structures by *Metarhizium* as well as Pr1 synthesis, so the effects are too non-specific to allow clear interpretation of the role of Pr1 (St. Leger et al. 1989a,b). However, simultaneous application of a specific Pr1 inhibitor (TEI; turkey egg white inhibitor) and conidia significantly delayed mortality of Manduca larvae compared with larvae inoculated with conidia alone, supporting the importance of Pr1 in penetration (St. Leger et al. 1988c). The inhibitor also reduced melanization of cuticle (a host reaction to infection) and invasion of the hemolymph as well as

maintaining the host's growth rate.TEI or antibodies raised against Pr1 delayed penetration of the cuticle but did not affect spore viability or prevent growth and formation of appressoria on the cuticle surface. This suggests that inhibition of Pr1 reduced infection by limiting fungal penetration of the insect cuticle. *In Vitro* studies using TEI showed the accumulation of protein degradation products from the cuticle, including ammonia, was dependent on active Pr1. This confirms the major role of Pr1 in solubilizing cuticle proteins and making them available for fungal nutrition.

An overview of enzyme involvement in pathogenesis

The great diversity of fungi and the many routes by which they have achieved their success as pathogens render it imprudent to consider *M. anisopliae* as an all encompassing model for pathogenicity of all entomopathogenic fungi. Nevertheless, given that the proteinaceous cuticle is a barrier to all entomopathogenic fungi which penetrate the cuticle directly, *prima facie* the involvement of proteases is likely to be ubiquitous.

CONCLUSIONS

Pathogenic fungi possess an intriguing array of mechanisms that permit them to acquire nutrients for growth and reproduction from the host environment. For the most part, the fungal components assist the pathogen with: (1) physical aspects of ingress, e.g. cuticle-degrading enzymes that actively destroy or modify the structural integrity of the host; (2) by inhibiting selective processes or enzymes of the host; and (3) by interfering with the regulatory systems of the host. Such cellular damage, with disease symptoms may be produced both by the pathogen's enzymes and by its low-molecular-weight metabolites (toxins). It is likely that in every disease numerous fungal mechanisms of attack are brought into play, each fulfilling particular functions necessary to elicit the full concert of events in pathogenesis. Because of the dynamics of both the pathogen and host affect the result of an encounter, no single trait or mechanism is likely to determine pathogenicity; and many components may be essential. Nevertheless, studies on the role of individual, well-characterized enzymes and toxins in the expression of virulence provides a powerful tool and useful starting point in the ultimate goal of defining insect pathogenesis at the molecular level. The pathogen and the host biochemistry may be altered as the pathogenic process progresses. Studies on the phenomena have gained impetus in recent years with the application of rigorous microbiological and enzymatic techniques, and meaningful information is available on the biological significance and function of some enzymes and toxins in insect-pathogen interactions.

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