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Biosynthesis of depsipeptide mycotoxins in *Fusarium*

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Abstract

The cyclic hexadepsipeptide enniatin is known as a phytopathogenic compound from Fusaria causing necrosis and wilt. The molecule consists of three alternating residues each of a branched chain amino acid and D-hydroxyisovaleric acid (D-Hiv). Enniatins are synthesized by a 347 kDa multienzyme (enniatin synthetase) via a thiol template mechanism. The corresponding gene *esyn1* has an open reading frame of 9393 nucleotides and harbours two modules, one responsible for D-hydroxy acid activation and one for L-amino acid activation with an integrated *N*-methyltransferase domain. Such methyltransferases build an homologous group among *N*-methyl peptide synthetases. Enniatins are synthesized by step-wise condensation of dipeptidol building blocks in an iterative manner resembling fatty acid synthesis. A key enzyme in enniatin biosynthesis is the NADPH-dependent D-2-hydroxyisovalerate dehydrogenase, that supplies enniatin synthetase with D-Hiv. Enniatins contribute to the wilt toxic character of Fusaria. Virulence was significantly reduced in *F. avenaceum* after disruption of the *esyn1* gene.

Introduction

Fungi constitute a diverse and damaging group of plant pathogens and therefore have a major economic importance in agriculture (Knogge, 1996). Fungi of the genus *Fusarium* are widespread phytopathogens and produce a number of highly toxic compounds e.g. enniatin, trichothecene and the homologous T-toxin. However, relatively little is known about the possible involvement of these toxins in plant infection. Most of the mycotoxins produced by *Fusarium* are not host-specific and affect a wide range of plants, causing symptoms such as necrotic lesions, rots and wilts (Drysdale, 1982). The toxicity of enniatins presumably derives from their ability to increase the permeability of membranes to ions and from their action as uncouplers of oxidative phosphorylation (Shemyakin et al., 1969).

The biosynthesis of enniatins is accomplished nonribosomally by the multifunctional enzyme enniatin synthetase (Zocher and Keller, 1997; Glinski et al., 2001). The enzyme is a *N*-methylcyclopeptide synthetase and represents a hybrid system of a peptide synthetase and an integrated *N*-methyltransferase (Haese et al., 1993). Peptide synthetases are organized into coordinated groups of active sites termed modules, each encompassing a length of approximately 600 amino acids corresponding to a molecular mass equivalent of ~70 kDa. Each module is responsible for catalysis of one cycle of polypeptide chain elongation and associated functional group modifications (Zocher and Keller, 1997; Glinski et al., 2001). The modules show considerable sequence conservation to each other and the number of these units equals the number of the amino acids serving as substrates of the corresponding enzymes.

In this paper, we describe the biosynthesis of the enniatin and its role in plant pathogenicity.

Mechanism of enniatin biosynthesis

Enniatins belong to the class of *N*-methylated cyclodepsipeptides, found in various strains of the genus *Fusarium* (Plattner et al., 1948). They consist

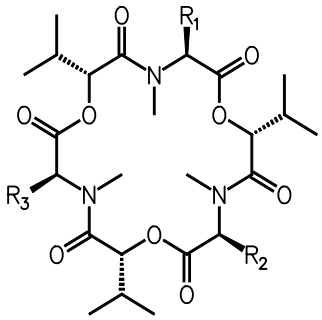

Figure 1. Structures of enniatins and beauvericin: Enniatin A: $R_1 = R_2 = R_3 =$ sec-butyl; Enniatin A1: $R_1 =$ isopropyl, $R_2 = R_3 =$ sec-butyl; Enniatin B: $R_1 = R_2 = R_3 =$ isopropyl; Enniatin B1: $R_1 = R_2 =$ isopropyl, $R_3 =$ sec-butyl; Beauvericin: $R_1 = R_2 = R_3 =$ benzyl.

of alternating residues of D-2-hydroxyisovaleric acid (D-Hiv) and a *N*-methylated branched chain L-amino acid, such as valine, leucine and isoleucine, linked by peptide and ester bonds (Figure 1). In the case of beauvericin, produced by entomopathogens, the branched chain L-amino acid is substituted by phenylalanine (Figure 1) (Hamill et al., 1969).

The biosynthesis of enniatins proceeds non-ribosomally via the thiol template mechanism catalysed by the multifunctional enzyme enniatin synthetase in an iteratant process (Figure 2) (Zocher and Keller, 1997; Glinski et al., 2001). The substrates are activated as acyladenylates by the corresponding modules. Subsequently, they are covalently attached to enzyme-bound 4′-phosphopantetheine residues as thioesters. The *N*-methylation step occurs prior to peptide bond formation (Billich and Zocher, 1987). In this process, S-adenosyl-L-methionine (AdoMet) acts as the methyl group donor. Enniatin synthetase is a two-module enzyme (D-Hiv and L-amino acid activation module) (Haese et al., 1993; 1994). By interaction of the two modules a dipeptidol unit is formed, which is transferred to a thiol-waiting position (Figure 2A). This thiol group acts as the acceptor of the growing peptide chain (condensation of dipeptidol units) and picks up the intermediates of enniatin synthesis. The enniatin molecule is synthesized by three successive condensations of enzyme-bound dipeptidols. The process of enniatin formation is terminated by the cyclization reaction of the linear hexadepsipeptide (Figure 2B). Enniatin synthetases from *F. scirpi*, *F. lateritium* and *F. sambucinum* differ in their amino acid specificity, which results in the formation of various enniatins (Figure 1) (Pieper et al., 1992). This may be caused by mutations in the amino acid recognition sites of the various enniatin synthetases. The amino acid sequences of the amino acid activation domains of Esyns from *F. scirpi* and activating L-valine, and *F. sambucinum,* preferably activating L-isoleucine, are nearly identical with the exception of three regions showing significant differences (Doller et al., 1996).

Molecular structure and function of enniatin synthetase

The enniatin synthetase corresponding gene, *esyn1,* from *F. scirpi* encoding an ORF of 9393 bp (3131 amino acids) has been isolated and sequenced (Haese et al., 1993). The multifunctional enzyme is a single polypeptide of 347 kDa. Sequence analysis of the *esyn1* gene, biochemical characterization and expression studies indicated that the enzyme consists of two modules (**EA** and **EB**), which are each approximately 420 amino acid residues of size (Figure 3) (Haese et al., 1993; 1994; Pieper et al., 1992).

The N-terminal module **EA** is responsible for the activation and binding of D-Hiv. The second module **EB**, C-terminal to **EA**, activates and preferably binds L-valine. The two modules **EA** and **EB** contain a conserved 4′-phosphopantetheine binding site at the C-terminus with a highly conserved serine residue. Module **EB** has an additional 4′-phosphopantetheine group, the so-called waiting position (Figure 2). Between both modules a putative condensation (**C**) domain can be found such as in the N-terminal and C-terminal part of enniatin synthetase (Figure 3) (Glinski et al., 2001).

Module **EB** contains a *N*-methyltransferase domain **M** of 434 amino acids length (Figure 3). Domain **M** is highly conserved among *N*-methyl peptide synthetases from prokaryotic and eukaryotic origin and shows only local sequence similarities to structural elements of other AdoMet-dependent methyltransferases (motif I, II/Y, IV and V in Figure 3) (Burmester et al., 1995; Billich and Zocher, 1987; Hacker et al., 2000).

In Figure 4, an unrooted phylogenetic tree of *N*-methyltransferase domains of various peptide synthetases is shown (Glinski et al, 2001; Hacker et al., 2000). Sequence comparison revealed that the seven *N*-methyltransferase domains of the multifunctional enzyme cyclosporin synthetase, the *N*-methyltransferase domain from PF1022A synthetase (*Mycelia sterilia*) (Futamara et al., 2001)

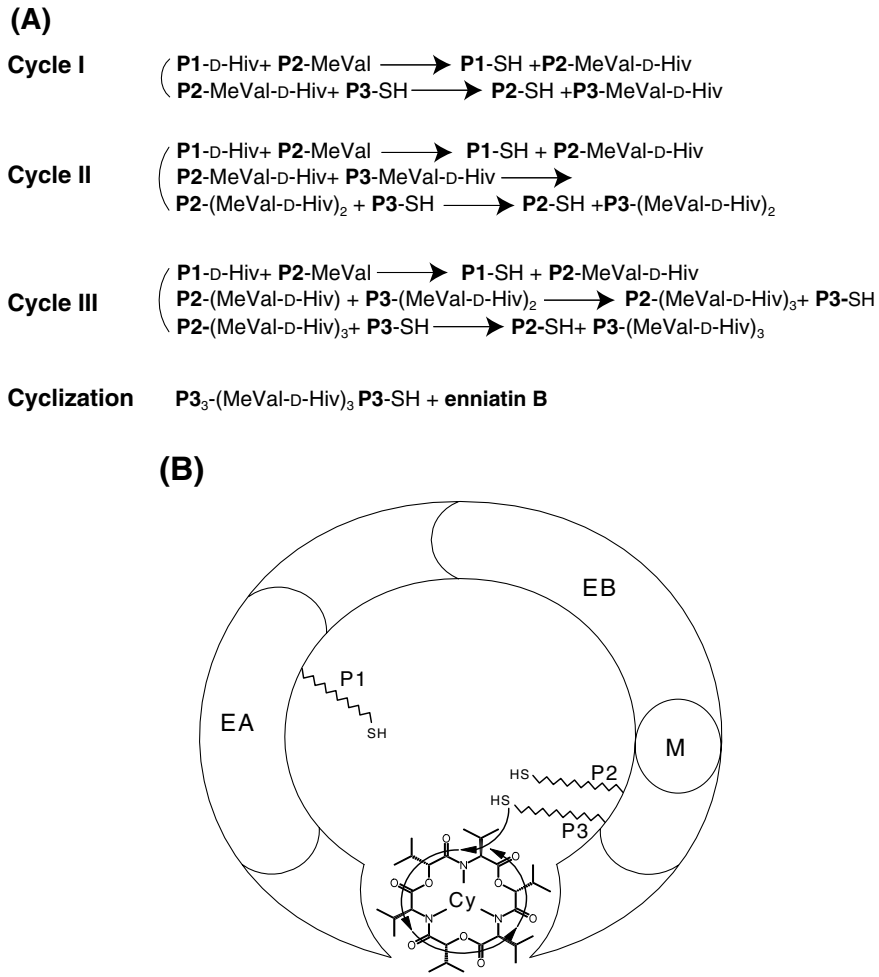

Figure 2. Mechanism of enniatin B biosynthesis. (A) Scheme of partial reactions leading to enniatin B. P1, P2, P3 = 4′-phosphopantetheine. (B) Model of arrangement of catalytic sites of enniatin synthetase. Cy: cyclization cavity; EA: D-Hiv-activation module; EB: L-valine-activation module; M: *N*-methyltransferase domain.

and the *N*-methyltransferase domains of Esyns from *F. scirpi*, *F. sambucinum* and *F. pallidoroseum* build one group (Burmester et al., 1995). The domains of the different *Fusaria* share 65% identity. The second group (prokaryotic origin) includes the *N*-methyltransferase domains from various *Streptomyces* and *Microcystis spp*. The overall sequence similarity between the two major groups is in the range of 18–25%.

Mutational analysis of the *N*-methyltransferase domain of enniatin synthetase

Figure 3 shows the sequential order of conserved motifs within the *N*-methyltransferase domain of enniatin synthetase. Deletion of the first 21 N-terminal amino acid residues of the *N*-methyltransferase domain of enniatin synthetase had no influence on AdoMet-binding in a photolabelling test with ^{14}C-AdoMet (Hacker et al., 2000). Further deletions of additional 26 amino acids resulted in a total loss of binding activity. Truncation of a short portion (38 amino acids) from the C-terminus and also deletions of internal sequences containing the conserved motifs led to complete loss of AdoMet-binding activity.

Site-directed mutagenesis of the conserved ^{2106}Tyr in motif II/Y into valine, alanine and serine significantly reduced the AdoMet-binding activity of the domain. Replacement of ^{2106}Tyr by phenylalanine restored AdoMet-binding activity to about 70%,

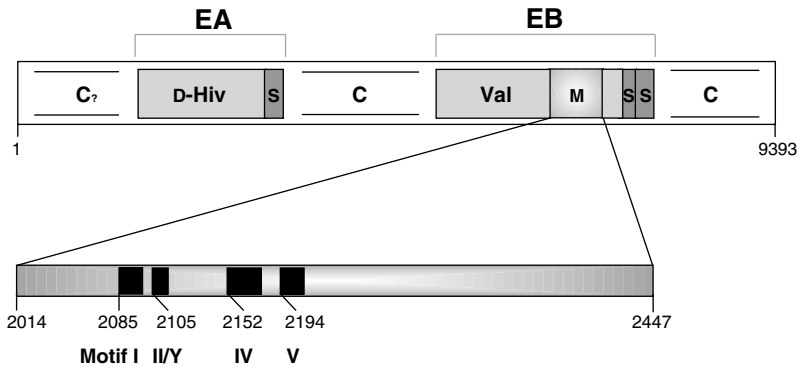

Figure 3. Physical map of enniatin synthetase (esyn1). EA represents the L-valine-activating module; EB represents the D-Hiv-activating module; S indicates the 4′-phosphopantetheine binding sites. M stands for *N*-methyltransferase domain. Putative condensation domains C are represented by an open white box. The numbers indicate the amino acid position in the sequence of enniatin synthetase. The black boxes indicate conserved motifs which can be found within various methyltransferases and *N*-methyltransferase domains of peptide synthetases.

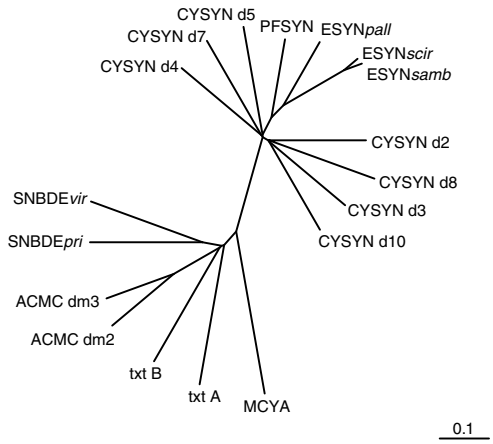

Figure 4. Unrooted phylogenetic tree of *N*-methyltransferase domains of peptide synthetases. The analysis was done by using a sequence alignment of *N*-methyltransferase domains of peptide synthetases (Clustal W). The tree was built using the neighbour-joining method of PHYLIP. The branch length marker of 0.1 gives the distance in which an amino acid substitution is found in every tenth position (10% difference), e.g. ESYN*scir*/ESYN*samb* with a distance of 0.05 have a sequence difference of 5%. The *N*-methyltransferase portions of the following sequences with their accession numbers were used: ESYN*scir*: Esyn from *Fusarium scirpi* Z18755, ESYN*samb*: Esyn from *F. sambucinum* Z48743, ESYNpall: Esyn from *F. pallidoroseum* AJ345016, CYSYN: Cyclosporin synthetase from *Tolypocladium niveum* Z28383, PFSYN: PF1022A synthetase from *Mycelia sterilia* (Futamura et al., 2001); MCYA: Microcystin synthetase A from *Microcystis spp.* AB019578, ACMC: Actinomycin synthetase II from *Streptomyces crysomallus*, SNBDE*pri*: Pristinamycin synthetase from *S. pristinaespiralis* Y11548 and SNBDE*vir*: Virginiamycin synthetase from *S. virginiae* Y11547. txt A: thaxtomin synthetase A, txt B: thaxtomin synthetase B from *S. acidiscabies* AF255732.

indicating that the tyrosine residue plays a crucial role in AdoMet-binding and that the aromatic residue of ^{2106}Tyr may be critical for AdoMet-binding in *N*-methyl peptide synthetases.

D-2-Hydroxyisovalerate dehydrogenase

In some fungal species, namely the enniatin producers of the genus *Fusarium*, there is a pathway leading from the primary precursor L-valine to D-2-hydroxyisovaleric acid via 2-ketoisovalerate (Lee et al., 1992; Zocher et al., 2000). D-Hiv dehydrogenase catalyses the reversible reaction of 2-ketoisovalerate to D-Hiv, which is an intermediate in the biosynthetic pathway of enniatins in *Fusarium*. D-Hiv dehydrogenase consists of one polypeptide chain with a molecular mass of about 53 kDa. It is strictly dependent on NADPH and exhibits a high substrate specificity with respect to 2-ketoisovalerate. This may explain the fact that D-Hiv is the exclusive hydroxy acid component in enniatins isolated from *Fusaria* (Lee et al., 1992).

Phytotoxic properties of enniatin-producing *Fusaria*

In contrast to the regulation of other secondary metabolites, the expression of the enniatin synthetase during fermentative growth follows a constitutive manner (Billich et al., 1988). To elucidate the role of enniatins in plant virulence, 36 enniatin-producing *Fusarium*

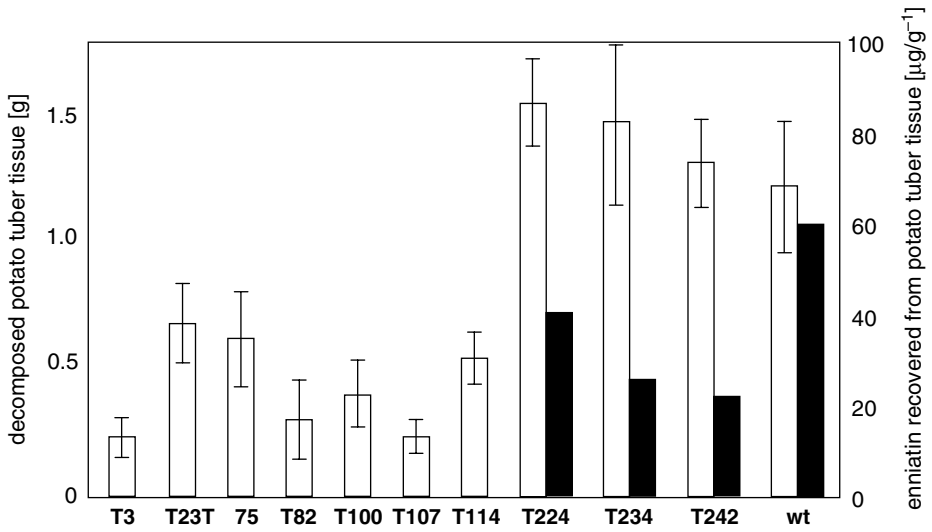

Figure 5. Virulence assay of *Fusarium avenaceum* on potato tuber tissue. Mean amounts of decomposed potato tuber tissue per slice (white bars with standard deviations), from 24 single values and means of enniatin recovered from the decomposed tissue (black bars). Values were obtained from four independent tests with six slices per test for all mutants and the wild-type (wt) strain.

strains were characterized regarding enniatin production and virulence on potato tuber tissue. Interestingly, seven enniatin-producing and 16 non-producing strains induced necrosis in the test, so that enniatin synthesis seemed not to be essential for the infection of potato tuber tissue (Herrmann et al., 1996a).

Fusarium avenaceum, a plant pathogenic strain and enniatin B producer, causes necrosis on knapweed (*Centaurea maculosa*) and also on potato tuber tissue. After disruption of the *esyn1* corresponding gene from *F. avenaceum* by homologous recombination, no enniatin production could be detected in the resulting transformants T3, T23, T75, T82, T100, T107 and T114 (Figure 5) (Herrmann et al., 1996b). The virulence of these enniatin non-producing transformants in the potato tuber tissue test was significantly reduced compared with the virulence of the group of enniatin-producing mutants (T224, T234 and T242) and the wild-type strain (Figure 5). Enniatins could be recovered from the tissue decomposed by the ectopic transformants T224, T234 and T242 and the wild-type. No enniatin was detected in tissues infected with the null mutants (Figure 5). In contrast to the findings with knapweed and potato, these results indicate a specific role of enniatin in plant pathogenesis. Further studies on other hosts, e.g. wheat, need to be carried out to obtain more detailed information on phytopathogenic mechanisms of enniatin-producing *Fusarium* strains.

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