REVIEW

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Biosynthesis and biological activity of enniatins

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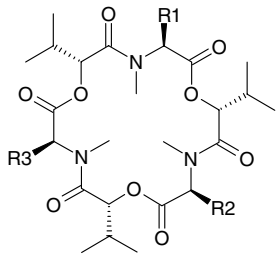
Enniatins are N-methylated cyclohexadepsipeptides (CHDPs), composed of three units each of N-methylated branched-chain L-amino acid and D-2-hydroxy acid arranged in an alternate fashion. These low-molecular secondary metabolites are produced by *Fusarium* species, typical mycotoxin producing fungi. Enniatins are known for their ionophoric, phytotoxic and anthelmintic effect, antibiotic activity and recently their potent cytotoxic activity against cancer cell lines was shown. They act also as inhibitors of drug efflux pumps. Biosynthesis, biological activity, and the structural characteristics of these microbial metabolites were summarized in this review.

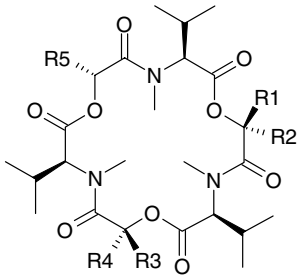
1. Introduction

Fusarium is a typical mycotoxin (beauvericin, moniliformin, enniatins, trichothecens, zearalenone) producing fungus of the northern temperate regions. *Fusarium* sp., pathogens of maize and small grains, cause stem and ear rot with enormous crop yield reduction. In samples of Finnish grain (wheat, barley, oat, rye) enniatin B and B1 were detected in all of them, enniatin A and A1 were found in 74% and 95% of the samples, respectively. *F. avenaceum* was the most abundant strain in the aforementioned collection (Jestoi et al. 2004a). Another study by Jestoi et al. (2004b) demonstrated that in selected grain-based products occuring on the market in Finland and Italy during spring 2002 enniatins and other detected toxins did not pose any obvious health risk. However, enniatins with different substitutions on CHDPs ring are known for their antibiotic activity (Kamyar et al. 2004, 2006), recently were shown as potent cytotoxic compounds and inhibitors of drug efflux pumps (Hiraga et al. 2005; Yamamoto et al. 2005; Dornetshuber et al. 2006).

Enniatins were named and for the first time isolated from *F. orthoceras* App. var. *enniatinum* by Gäumann et al.



Enniatin	R1	R2	R3
A	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$
A1	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)_2$
A2	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$	$CH_2CH(CH_3)_2$
B	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)_2$
B1	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH(CH_3)CH_2CH_3$
B4	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$	$CH(CH_3)_2$
C	$CH_2CH(CH_3)_2$	$CH_2CH(CH_3)_2$	$CH_2CH(CH_3)_2$
D	$CH(CH_3)_2$	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$
E	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$	$CH(CH_3)CH_2CH_3$
	$CH(CH_3)_2$	$CH(CH_3)CH_2CH_3$	$CH_2CH(CH_3)_2$
F	$CH_2CH(CH_3)_2$	$CH(CH_3)CH_2CH_3$	$CH(CH_3)CH_2CH_3$
G	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$	$CH_2CH(CH_3)_2$



Enniatin	R1	R2	R3	R4	R5
H	H	$CH(CH_3)_2$	H	$CH(CH_3)_2$	$CH(CH_3)CH_2CH_3$
I	H	$CH(CH_3)_2$	$CH(CH_3)CH_2CH_3$	H	$CH(CH_3)CH_2CH_3$
L	$CH(CH_3)_2$	H	$CH(CH_3)_2$	H	$C(CH_3)OHCH_2CH_3$
M1	$CH(CH_3)_2$	H	$CH(CH_3)CH_2CH_3$	H	$C(CH_3)OHCH_2CH_3$
M2	$CH(CH_3)CH_2CH_3$	H	$CH(CH_3)_2$	H	$C(CH_3)OHCH_2CH_3$
N	$CH(CH_3)CH_2CH_3$	H	$CH(CH_3)CH_2CH_3$	H	$C(CH_3)OHCH_2CH_3$

Table: Enniatins, producers and physico-chemical properties

Compound	Microbial producer	Molecular formula/weight	M.p. (°C)	References
ENNIATIN A	*Fusarium sambucinum*	$C_{36}H_{63}N_3O_9$	122–124	Pieper et al. (1992)
	F. sp. HA 43–88	681.91		Bergendorff et al. (1994)
	F. oxysporum			Ivanova et al. (2006)
	F. avenaceum			Jayasinghe et al. (2006)
ENNIATIN A1	*F.* sp. HA 43–88	$C_{35}H_{61}N_3O_9$	66–67	Bergendorff et al. (1994)
	F. oxysporum	667.88		Logrieco et al. (2002)
	F. avenaceum			Ivanova et al. (2006)
	F. tricinctum			Jayasinghe et al. (2006)
	F. culmorum			
	F. poae			
ENNIATIN A2	*F. avenaceum*	$C_{36}H_{63}N_3O_9$	123–125	Blais et al. (1992)
		681.91		
ENNIATIN B	*F.* sp.	$C_{33}H_{57}N_3O_9$	173–175	Plattner and Nager (1948)
	F. sp. Y-53	639.83		Audhya and Russell (1974)
	F. sp. F31			
	F. lateritium var.stiboides			Pieper et al. (1992)
	F. avenaceum			Altomare et al. (1995)
	F. sambucinum			Trenin et al. (2000)
	F. scirpi			Jiang et al. (2002)
	F. torulosum			Lin et al. (2002)
	F. tricinctum			Logrieco et al. (2002)
	F. culmorum			Nilanonta et al. (2003)
	F. poae			Pohanka et al. (2004)
	Verticillium hemipterigenum			Vongvilai et al. (2004)
	Halosarpheia sp. 732			Hiraga et al. (2005)
	Unidentified fungus, MOBCOF-1			Yamamoto et al. (2005)
	Unidentified fungus, BCC2629			Ivanova et al. (2006)
ENNIATIN B1	*F.* sp. HA 43–88	$C_{34}H_{59}N_3O_9$	178.5	Bergendorff et al. (1994)
	F. sp. Y-53	653.85		Hiraga et al. (2005)
	F. sp. F31			Yamamoto et al. (2005)
	F. oxysporum			Pohanka et al. (2004)
	F. avenaceum			Ivanova et al. (2006)
				Jayasinghe et al. (2006)
ENNIATIN B2	*F. avenaceum*	–	–	Ivanova et al. (2006)
ENNIATIN B3	*F. avenaceum*	–	–	Ivanova et al. (2006)
ENNIATIN B4	*F.* sp. F31	$C_{34}H_{59}N_3O_9$	140–143	Vongvilai et al. (2004)
	V. hemipterigenum	653.85		Pohanka et al. (2004)
	Halosarpheia sp. 732			Nilanonta et al. (2003)
	Unidentified fungus, BCC2629			Lin et al. (2002)
	Unidentified fungus, MOBCOF-1			Jiang et al. (2002)
ENNIATIN C	*F.* sp.	$C_{36}H_{63}N_3O_9$	160–161	Plattner and Nager (1948)
	V. hemipterigenum	681.91		Nilanonta et al. (2003)

Table Cont.

Compound	Microbial producer	Molecular formula/weight	M.p. (°C)	References
ENNIATIN D	*F.* sp. Y-53 *F.* sp. FO-1305	$C_{34}H_{59}N_3O_9$ 653.85	–	Tomoda et al. (1992) Hiraga et al. (2005) Yamamoto et al. (2005)
ENNIATIN E	*F.* sp. FO-1305	$C_{35}H_{61}N_3O_9$ 667.88	–	Tomoda et al. (1992)
ENNIATIN F	*F.* sp. FO-1305	$C_{36}H_{64}N_3O_9$ 682.92	–	Tomoda et al. (1992)
ENNIATIN G	*V. hemipterigenum* *Halosarpheia* sp. 732	$C_{35}H_{61}N_3O_9$ 667.88	143–145	Nilanonta et al. (2003) Lin et al. (2002)
ENNIATIN H	Unidentified fungus, BCC2629 *V. hemipterigenum*	$C_{34}H_{59}N_3O_9$ 653.85	105–106	Vongvilai et al. (2004) Nilanonta et al. (2003)
ENNIATIN I	Unidentified fungus, BCC2629 *V. hemipterigenum*	$C_{35}H_{61}N_3O_9$ 667.88	–	Vongvilai et al. (2004) Nilanonta et al. (2003)
ENNIATIN J1–J3	*F.* sp. strain 31	–	–	Pohanka et al. (2004)
ENNIATIN K 3	*F.* sp. strain 31	–	–	Pohanka et al. (2004)
ENNIATIN L	Unidentified fungus, BCC2629	$C_{34}H_{59}N_3O_{10}$ 669.85	132–133	Vongvilai et al. (2004)
ENNIATIN M1	Unidentified fungus, BCC2629	$C_{35}H_{61}N_3O_{10}$ 683.88	–	Vongvilai et al. (2004)
ENNIATIN M2	Unidentified fungus, BCC2629	$C_{35}H_{61}N_3O_{10}$ 683.88	–	Vongvilai et al. (2004)
ENNIATIN N	Unidentified fungus, BCC2629	$C_{36}H_{63}N_3O_{10}$ 697.91	98–99	Vongvilai et al. (2004)
ENNIATIN O1–O3	*V. hemipterigenum*	$C_{35}H_{61}N_3O_9$ 667.88	–	Supothina et al. (2004)
MK 1688	Unidentified fungus, BCC2629 *V. hemipterigenum*	$C_{36}H_{63}N_3O_9$ 681.91	–	Vongvilai et al. (2004) Nilanonta et al. (2003)

(1947). Enniatins are secondary metabolites produced predominantly by various *Fusaria*. Enniatins producing strains were isolated from different sources, e.g. marine algae *Codium fragile* (Jiang et al. 2002), walnut tree leaves (Bergendorff et al. 1994), foliage of balsam fir *Abies balsamea* (Strongman et al. 1988). These metabolites are produced also by strains of some species of *Verticillium* (Nilanonta et al. 2003) and *Halosarpheia* (Lin et al. 2002). Microbial producers of enniatins are summarized in the Table.

2. Biosynthesis of cyclohexadepsipeptides, enniatins

The primary precursors for enniatin biosynthesis are L-valine, L-leucine or L-isoleucine, D-2-hydroxy-3-methylbutanoic, D-2,3-dihydroxy-3-methylpentanoic and D-2-hydroxy-3-methylpentanoic acids, ATP, and *S*-adenosylmethionine. Biosynthesis of enniatins is catalysed by an enzyme enniatin synthetase (Esyn), which was purified from *Fusarium scirpi, F. oxysporum, F. lateritium* and *F. sambucinum* (Zocher et al. 1982; Pieper et al. 1992). Enniatin synthetases isolated from various microbial sources showed differences in their amino acids specificities. The enzyme from *F. sambucinum,* producer of enniatin A, exhibits high affinity to L-leucine and L-isoleucine. On the contrary, Esyn from *F. lateritium* and *F. scirpi,* which preferably produce enniatin B accepts L-valine as substrate (Pieper et al. 1992; Krause et al. 2001). Biochemical analysis of the Esyn-encoding gene *esyn1* from *F. scirpi* revealed that the enzyme consists of one polypeptide chain with a molecular mass of 347 kDa comprising 3131 amino acids (Zocher et al. 1982; Billich and Zocher 1988). Esyn is a multifunctional enzyme which belongs to the class of nonribosomal peptide synthetases. These multifunctional enzymes (enniatin, cyclosporin synthetase) represent hybrid enzyme systems of peptide synthetases and *S*-adenosyl-L-methionine-dependent N-methyltransferases (Hacker et al. 2000). Like cyclosporin synthetase, Esyn exists in solution as a monomer and product assembly is achieved in an intramolecular manner. Enniatin biosynthesis proceeds by a stepwise condensation of three dipeptidol building blocks and it follows so-called thiol template mechanism (Pieper et al. 1995; Glinski et al. 2002).

Many enzymes of secondary metabolism are presented during growth of microbial producers and are induced at the end of the trophophase (penicillin acyltransferase). Enniatin synthetase behaves like a constitutive enzyme of primary metabolism and it does not seem to be regulated by a specific mechanism either on a transcriptional or a translational level (Billich and Zocher 1988). Another key enzyme in depsipeptide synthesis is a highly specific D-hydroxyisovalerate dehydrogenase purified from *F. sambucinum* (Lee et al. 1992).

Production of enniatins in submerged cultivation was in the range of $0.8–1.2\ g \cdot l^{-1}$ (Minasian et al. 1978). Enniatins were prepared also by total synthesis which involves formation of depsipeptide hexamers from three dimeric fragments by a $(2 + 4)$-fragment condensation, e.g. using N-terminal protected dipeptides and O-terminal protected tetrapeptide fragment. Several other methods are known for enniatin synthesis (Jeschke et al. 2003, 2006). Chemical synthesis is able to prepare novel enniatin derivatives with biological activity different from that of the parent compound. Another strategy for preparation of new derivatives is *in vitro* cell-free synthesis exploits multienzyme Esyn and/or *in vivo* precursor feeding of enniatin producing *Fusarium* strains (Madry et al. 1984; Peeters et al. 1984; Krause et al. 2001).

3. Biological activities of enniatins

Antiobiotic activity is characteristic for enniatins. Nowadays, toxins in low non-toxic concentrations are tested for application in human and veterinary medicine. In many studies enniatin showed perspectives for further development.

3.1. Ionophoric activity

The most studied property of CHDPs and structurally related compounds is their ionophoric effect. Change in cell membrane permeability and disruption of ionic gradient is involved in antibiotic effect and local therapeutic properties of several polypeptide antibiotics and also enniatins. Enniatin modulated ionophoric activity affecting action potential parameters and cell homeostasis. CHDPs are lipophilic ionophores which are easily incorporated into the cell membrane forming cation selective pores and transmembrane spanning. Cation selectivity in the order $K^+ > Ca^{2+} > Na^+ > Mg^{2+} > Li^+$ correlates well with the ionic radius of the respective cations. Enniatins, like gramicidin, form passive channel. Single channel properties are different for the enniatin A1, B and B1. The largest conductivity showed enniatin B, followed by enniatin A1 and B1 (Kamyar et al. 2004, 2006). In solution, enniatins form complexes with a cation in ratio 1 : 1, 2 : 1 or 3 : 2. In the 1 : 1 complex, the ion is docked in the molecular cavity, in the other two complex conformations cation is sandwiched between two molecules of enniatin (Kamyar et al. 2004). Enniatins transport cations through a mobile carrier mechanism selective for K^+ vs. Na^+ involves two antibiotic mols. The transport efficiency of the various enniatins appears to be related to their hydrophobicity, in agreement with sandwich transport model (Levy et al. 1995). In the study by Doebler (2000) it was shown that alteration in action potential in neuroblastoma X glioma hybrid treated with enniatin appear not to be related to K^+ transport activity. Ennitins also play a role in the intracellular calcium concentration (Kouri et al. 2003).

3.2. Anthelmintic, antimicrobial and phytotoxic effect

Parasitic nematodes cause significant problems worldwide to the life of many animals and humans. 18-Membered CHDPs are of interest with regard to their activity against the gastrointestinal nematodes like *Haemonchus contortus* Rudolphini in sheep. Enniatins with strong *in vivo* anthelmintic activity exist in solution as conformers with restricted flexibility. It is assumed that the inflexibile region of the major conformer might mimic the active conformation of the CHDPs (Jeschke et al. 2005, 2006). Newly synthetized anologues of enniatin A showed enhanced anthelmintic activity. The most potent derivative contained D-lactic acid and was active against *Haemonchus contortus* in sheep at an intravenous dose rate of $0.5\ mg \cdot kg^{-1}$ (Jeschke et al. 2003). Enniatin A $5\ \mu g \cdot ml^{-1}$ was effective against parasites *Nippostrongylus brasiliensis* and *Trichinella spiralis* in vitro (Pleiss et al. 1996). Enniatins producing *Fusarium* sp. act as entomopathogenic fungi or biological control fungi. Toxicity of fungal metabolites including enniatins was evaluated on lepidopteran *Spodoptera frugiperda* (SF-9) cell line by Trypan blue dye exclusion and MTT-colorimetric assay. No statistical difference was found between results obtained by MTT test and Trypan blue dye exclusion. In the MTT assay, the cytotoxicity of enniatin was IC_{50} 6.6 μM (Fornelli et al. 2004).

Enniatins exhibited significant antimicrobial and antifungal properties in several studies. Enniatin B, B1, B2, B4 and J1, J2, J3 and K3 were isolated from *Fusarium* F31 by bioassay-guided method using *Botrytis cinerea*. The minimum inhibitory concentration of pure enniatins was $75\ \mu g \cdot ml^{-1}$ (Pohanka et al. 2004), moderate antimicrobial activity was observed with, enniatin A, A1 and B1 against *Candida albicans*, *Cryptococcus neoformans* and *Mycobacterium intracellulare* (Jayasinghe et al. 2006). Enniatin B, B4, C, G, H, I isolated from *Verticillium hemipterigenum* BCC1449 collected from *Homoptera* adult leafhopper were active against *Mycobacterium tuberculosis* (H37Ra strain) and *Plasmodium falciparum* (K1 strain); growth of later mentioned microorganism was inhibited by enniatin B4 and enniatin H with IC_{50} $0.20\ \mu g \cdot ml^{-1}$ and $1.90\ \mu g \cdot ml^{-1}$, respectively. The mixture of enniatins O1, O2 and O3 showed biological activity similar to those of enniatin B, with IC_{50} $3.2\ \mu g \cdot ml^{-1}$. Antibacterial activity against *M. tuberculosis* (H37Ra strain) was not so significant IC_{50} $3.12–6.25\ \mu g \cdot ml^{-1}$ as that of isoniazide ($0.05\ \mu g \cdot ml^{-1}$). In this assay the most potent was enniatin B, B4 and the mixture of enniatins O1, O2, O3 (Nilanonta et al. 2003; Supothina et al. 2004). *Fusarium lateritium* Nees is a natural antagonist of the plant pathogen *Eutypa armeniacae*. Investigation of bioactive metabolites isolated from this strain revealed that strong antifungal activity against *E. armeniacae* was exhibited by enniatin B, B1 and A1 (Tsantrizos et al. 1993).

Enniatins are also phytotoxic compounds. Using enniatin-nonproducing mutants of *F. avenaceum* enniatins have been shown to play a role during infection process of plants and they contribute to the virulence of the fungal strain on potato tuber tissue (Herrmann et al. 1996a). A mixture of enniatins A, A1, B, B1 at 50 and 100 μg/slice caused necrotic damage of tested potato tuber tissue in a ratio 5 : 15 : 35 : 45, respectively; only the superficial tissue was affected at lower concentrations (Herrmann et al. 1996b). Enniatins at IC_{50} 10^{-4} M effect on seed germination of the parasitic weed *Striga hermonthica* and may have a potential for use as natural and safe herbicide (Zonno and Vurro 1999).

3.3. Cytotoxic properties

Beside aforementioned effects of enniatins cytostatic activities against human cancer cells are of a great interest. HCT116 (human colon carcinoma) cells with homozygously disrupted p53, p21, or bax genes were analyzed to investigate the impact of treatment with enniatins on apoptosis and cell cycle-regulating proteins activity. The studies by Dornetshuber et al. (2006, 2007) showed profound apoptosis-inducing effects at low micromolar concentrations (already after 24 h of treatment). Several cellular changes characteristic for programmed cell death such as cell shrinkage, chromatin condensation, DNA fragmentation, apoptotic body formation were observed. Correspondingly, the cleavage of poly(ADP-ribosyl)polymerase and the activation of multiple caspases accompanied a distinct loss of mitochondrial membrane potential. A potent cell cycle arrest in G0/G1 phase in cell cycle was also observed after treatment of HCT116 cells with enniatins. In contrast, short-term exposure to very low enniatin concentrations might have tumor-promoting functions based on its growth stimulation. It is assumed that the cytotoxic effects of enniatins are mediated by p53 mechanism. Cytotoxicity of enniatin A, A1, B, B1, B2 and B3 was tested in two cell lines of human origin – Hep G2 (hepatocellu-

lar carcinoma) and MRC-5 (fibroblast-like foetal lung cancer) using the BrdU (bromodeoxyuridine) incorporation assay and Alamar Blue assay for cell proliferation measurements. The lowest inhibitory concentration for MRC-5 cells treated with enniatins was 0.8 μM (enniatin A) and 3.6 μM (enniatin B) (Ivanova et al. 2006). Enniatin B, B4, C, G, H, I using colorimetric method revealed cytotoxic activity which was comparable with used chemotherapeutic in an assay with two cancer cell-lines KB (human epidermoid carcinoma cells), BC-1 (human brest carcinoma cells) and Vero cells (kidney fibroblast from African green monkey) (Nilanonta et al. 2003). Mixture of enniatins O1, O2, O3 showed cytotoxic activity against cancer cell lines KB, BC-1 and NCI-185, with respective IC_{50} 2.4 $\mu g \cdot ml^{-1}$, 1.4 $\mu g \cdot ml^{-1}$, 0.78 $\mu g \cdot ml^{-1}$ (Supothina et al. 2004).

3.4. Inhibition of drug efflux pumps

Active efflux of drugs out of cells by membrane transporter proteins (drug efflux pump) has been recognized as a major cause of bacterial resistance against antibiotics. Moreover multidrug resistance (MDR) proteins in cancer cells cause clinical problems in cancer therapy, because chemotherapeutics are transported out of the cells. Thus, reversal of MDR in cancer is a desperately needed clinical requirement and a scientific challenge. Recently, enniatins were proved to be potent inhibitors of ABC (ATP-binding cassette) transporters. The MDR phenotype can be mediated by adenosine triphosphate ABC transporter molecules, which lower the intracellular accumulation of structurally and functionally unrelated anticancer drugs (Stein and Walther 2006). Enniatin B, B1 and D at a non-toxic concentration of each molecule (5 $\mu g \cdot ml^{-1}$) were potent and specific inhibitors of Pdr5p, a functional homologue of mammalian P-glycoprotein, with mechanism similar to that of potent immunossuppresant FK506 (tacrolimus, fujimycin), however, enniatins were less toxic (Hiraga et al. 2005; Yamamoto et al. 2005). P-glycoprotein (PGP) is a 170 kDa membrane-bound protein which has been implicated as a primary cause of multidrug-resistance in tumors. An understanding of the physiological regulation of this transporter is key to designing strategies for the improvement of therapeutic efficacy of drugs that are their substrates for PGP activity (Sukhai and Piquette-Miller 2000).

3.5. Anti-HIV activity

Enniatins were assessed for *in vivo* anti-HIV activity in the hollow fiber assay with IC_{50} 0.78 μM for enniatin B (McKee et al. 1997).

3.6. Hypolipidemic effect

Acyl coenzyme A cholesterol acyltransferase (ACAT), EC 2.3.1.26, is an enzyme, located in the endoplasmic reticulum in cells playing an important role in the esterification of cholesterol and fatty acyl coenzyme A to facilitate both intracellular storage and intercellular transport. The inhibition of ACAT activity has been associated with decreased plasma cholesterol level by suppressing cholesterol absorption and by diminishing the assembly and secretion of apolipoprotein B-containing lipoproteins such as very low density lipoprotein. Moreover, ACAT inhibitors were shown to prevent formation of macrophage-derived foam cells in the arterial walls (Miyazaki et al. 2005). Enniatin B showed strong hypolipidemic activity. In the assay with cell line HepG2 it inhibited activity of ACAT, triglyceride biosynthesis and diminished free fatty acids pool in the cells (Trenin et al. 2000). Enniatin D, E and F inhibited ACAT activity in an enzyme assay using rat liver microsomes with IC_{50} 87, 57 and 40 μM, respectively (Tomoda et al. 1992).

4. Conclusion

Fungi are an abundand source of novel natural products. Many of these compounds are toxins but in low non-toxic concentrations they possess remarkable biological activities and they are often used as potent therapeutics in human or veterinary medicine. Enniatins are mycotoxins produced especially by *Fusarium* spp. In many studies enniantins were strong inhibitors of proliferation of human malaria parasite (*Plasmodium falciparum*), inhibited growth of *Mycobacterium tuberculosis*; a non-toxic concentration of enniatins suppressed effect of drug efflux pump, which cause multidrug resinstance also in cancer cells. Enniatins possess cytotoxic activity against several human tumor cells. *Fusarium* is a microorganism useful for the biotechnological production of natural enniatins which may play more significant role in the future.

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