Department of Biochemical Technology[1], Institute of Biotechnology and Food Industry and Department of NMR and Mass Spectroscopy[2], Institute of Analytical Chemistry, Faculty of Chemical and Food Technology, Slovak University of Technology; Department of Cell and Molecular Biology of Drugs[3], Faculty of Pharmacy, Comenius University, Bratislava; Zentiva a.s.[4], Hlohovec, Slovak Republic

# Enniatins produced by *Fusarium dimerum*, an endophytic fungal strain

S. Firáková[1], M. Šturdíková[1], T. Liptaj[2], N. Prónayová[2], L. Bezáková[3], B. Proksa[4]

*Received December 1, 2007, accepted March 14, 2008*

*Silvia Firáková, Department of Biochemical Technology, Institute of Biotechnology and Food Industry, Faculty of Chemical and Food Technology, Slovak University of Technology, Radlinského 9, 812 37 Bratislava, Slovak Republic*
*silvia.firakova@gmail.com*

*Pharmazie 63: 539–541 (2008)* *doi: 10.1691/ph.2008.7831*

Cyclohexadepsipetides enniatin B, B1 and G were isolated from the cultivation broth of *Fusarium dimerum* Penzig, an endophyte of *Magnolia x soulangeana*. Their production was about 350 mg $l^{-1}$ after 96 h of submerged cultivation in Sabouraud maltose medium. Isolated enniatins inhibited growth of selected microorganisms and activity of 12-lipoxygenase with $IC_{50} = 0.73$ mg $l^{-1}$.

## 1. Introduction

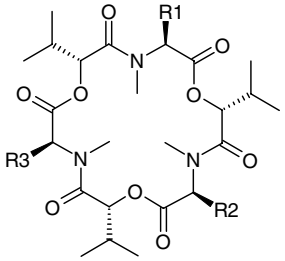
*Fusarium dimerum* Penzig is known as producer of siderophores (Dave and Dube 2000) and this strain posseses an ability to modify the side chain of steroids, e.g. progesterone (Adham Nehad 2002). However, *F. dimerum* was identified also as a potential pathogen occurring in immuno compromised patients (Biglev et al. 2004). Cyclohexadepsipetide enniatins are a group of microbial secondary metabolites with proven antibiotic effect and their activity as inhibitors of drug efflux pump was also studied. Biosynthesis and pharmacological activity of these cyclohexadepsipeptides has recently been reviewed by Firáková et al. (2007). So far no information was found about production of enniatins by *F. dimerum* inhibiting the activity of 12-lipoxygenase which is described in this paper for the first time.

## 2. Investigations, results and discussion

### *2.1. Isolation and identification of enniantins*

*Fusarium dimerum* was isolated as an endophytic microorganism from the plant tissue of *Magnolia x soulangeana* Soul.-Bod. (Magnoliaceae), an ornamental tree growing in Bratislava. The most suitable medium for production of fungal secondary metabolites was Sabouraud maltose (SM) broth as was deduced from preliminary studies. The broth after cultivation of *F. dimerum* was extracted with a series of solvents with increasing polarity. The most promising results were obtained with hexane, ethyl acetate and *n*-butanol. Cultivation medium was further processed with hexane due to positive results in TLC, HPLC analysis and dry mass of extract. The residue (0.8 g) received after evaporation of hexane from the extract of the cultivation broth was dissolved in diisopropylether, the solution was treated with activated charcoal, the filtrate was concentrated and after addition of hexane (1 : 1 v/v) left to crystallize affording white crystals (0.6 g). TLC in methylethylketone (S1), tetrahydrofurane (S2), toluene (S3) and toluene : methanol 95 : 5 v/v (S4) on Kieselgel 60 F254 and aluminiumoxid 150 F254 revealed only one spot after visualization with iodine vapor, however, three major peaks were observed in HPLC. Further purification of this mixture by column chromatography on silica gel eluted with diisopropylether with increasing concentration of methanol was not successful, therefore we tried to analyse this mixture. Three peeks, eluted in 22.43 min, 22.66 min and 22.72 min were recorded by GC and compounds represented by these peaks showed similar fragmentation in MS, with minute differences in *m/z* of fragments and ion abundance as well. Comparison of mass spectra with those in library and published data (Shemyakin et al. 1966; Kiryushkin et al. 1968; Wulfson et al. 1965) confirmed the identity of the appropriate compounds with 3,6-diisopropyl-N-methyl-2,5-dioxomorpholine, 3-(2-methylpropyl)-6-isopropyl-N-methyl-2,5-dioxomorpholine and 3-(1-methylpropyl)-6-isopropyl-N-methyl-2,5-dioxomorpholine. These substituted 2,5-dioxomorpholines are not genuine microbial metabolites, but represent products of pyrolysis of more complex compounds – depsipeptides (Kiryushkin et al. 1968; Wulfson et al. 1965). LC/MS/MS was used for confirmation of this hypothesis. This technique revealed the presence of three major compounds with M-6+H at *m/z* 633.5, 647.5 and 661.5, respectively. Such spectra are typical for enniatins previously isolated from *Fusarium* sp. (Plattner and Nager 1948; Bergendorff et al. 1994; Nilanonta et al. 2003) and compounds with the summary formula $C_{33}H_{57}N_3O_9$, $C_{34}H_{59}N_3O_9$ and $C_{35}H_{61}N_3O_9$ were identical with enniatin B, B1 and G, respectively. The UV spectrum with only one weak peak at 207 nm ($A^{1\%}_{1\,cm} = 1.156$) as well as the IR spectrum with bands at 2976 (C–H), 1740 (ester C=O), 1675 (amide C=O), 1480 (C–H of a methyl groups), 1262 (C–O–C) are in accord with the spectra published for other homologous enniatins (Nilanonta et al. 2003). The final confirmation of enniatin structure af-

forded LC/NMR of the second dominant peak ascribed to enniatin B1 which is composed of three α-hydroxyisovaleric, two *N*-methylvaline and one *N*-methylisoleucine units. Appropriate signals of these three units were assigned according to $^{13}C$, $^{1}H$, HSQC, HMBC and COSY experiments with shifts of i) α-hydroxyisovaleric acid unit ((δ/ppm): 5.100, 1 H d (7.43), 5.096 1 H d (7.86), 5.080 1 H d (7.71) H-2; 2.221 3 H (m), 3 × H-3; 0.898 m 18 H (m) 6 × $CH_3$); ii) *N*-methylvaline: ((δ/ppm) 4.659 2 H d, (9.6) 2 × H-2; 2.221 2 H m, 2 × H-3; 3.171 3 H (s), 3.170 3 H (s) 2 × N−$CH_3$; 0.966 12 H (m) 4 × $CH_3$); iii) *N*-methylisoleucine: ((δ/ppm) 4.800 1 H, d, (9.0) H-2; 3.156 3 H, s, N−$CH_3$, 1.420 + 1.024 2 H (m), H-3; 0.973 3 H d, C-2−$CH_3$, 0.834 3 H, t, $CH_3$).




| Enniatin | R1 | R2 | R3 |
|---|---|---|---|
| B | $CH(CH_3)_2$ | $CH(CH_3)_2$ | $CH(CH_3)_2$ |
| B1 | $CH(CH_3)_2$ | $CH(CH_3)_2$ | $CH(CH_3)CH_2CH_3$ |
| G | $CH(CH_3)_2$ | $CH_2CH(CH_3)_2$ | $CH_2CH(CH_3)_2$ |

Structure of enniatins produced by *F. dimerum*

### 2.2. Production of enniatins by F. dimerum

Production of enniatins, growth kinetics of the studied endophytic strain in SM broth and also utilization of carbon source are shown in the Fig. The highest production of enniatins was observed after 96 h of cultivation. At this time the source of the carbon in the medium was completely depleted, the growth of microorganism ceased (dry weight of biomass was 17 g $l^{-1}$) and concentration of enniatins was 350 mg $l^{-1}$. Production of enniatins by *Fusarium* sp. reached 1300–1724 mg $l^{-1}$, but after 21–34 days of cultivation on the solid agar medium (Audhya and Russell 1974). *Verticillium hemipterigenum* in experiments with various amino acids precursors added to medium

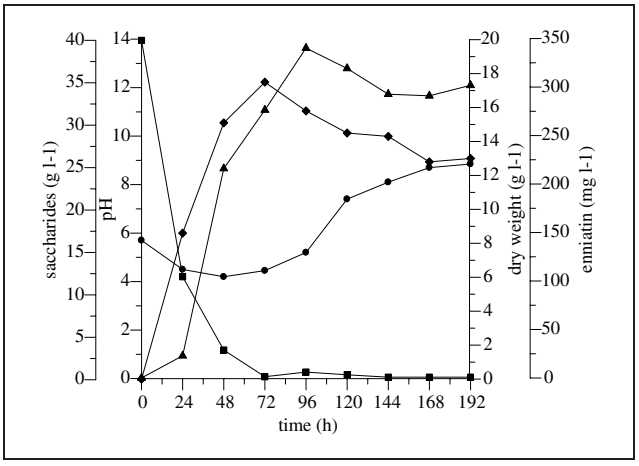



Fig.: Enniatin production, fermentation parameters of *F. dimerum* grown in Sabouraud maltose medium
◆ – dry weight of biomass (g $l^{-1}$), ■ – utilization of saccharides (g $l^{-1}$), ● – pH, ▲ – enniatins (mg $l^{-1}$)

synthesized enniatins in the range 20–120 mg $l^{-1}$ after 4 week cultivation (Nilanonta et al. 2003; Supothina et al. 2004).

### 2.3. Biological activity of enniatins

Increased activity of 12-lipoxygenase (12-LOX) is tightly connected with imflammation, alergy and immunity disorders (Deschamps et al. 2006). Dried hexane extract of culture media of *F. dimerum* and purified enniatins were tested as potential inhibitors of 12-LOX, type D isolated from the lung cytosolic fraction of diabetic rats (Bezáková et al. 2005). Enniatins suppressed activity of this enzyme in the concentration range of 0.625 mg $l^{-1}$–1.25 mg $l^{-1}$; $IC_{50}$ of purified enniatins was 0.73–0.82 mg $l^{-1}$. Enniatins were effective against *Bacillus subtilis* CCM 1999, *Candida albicans* CCY 29391, *Trychosporom cutaneum* CCY 30510 and *Cryptococcus neoformans* CCY 1716, but did not inhibit the growth of *Escherichia coli* CCM 3988 in agar diffusion test.

### 2.4. Conclusion

Isolated endophytic strain *F. dimerum* produced enniatins B, B1 and G so far not identified as metabolites of this species of microorganism. Isolated compounds lowered activity of 12-LOX and proved antibiotic activity. Level of enniatins production by *F. dimerum* is promising for further optimization of cultivation conditions.

## 3. Experimental

*HPLC:* 1100 LC (Agilent), column 125 × 4 mm Hypersil ODS 5 μm (Merck); acetonitrile-water (65 : 35 v/v), pH 2.4 with $H_3PO_4$; flow rate 0.7 ml $min^{-1}$; 210 nm.
*GC/MS:* 6890 GC Series (Agilent) with MS detector 5973N (Agilent); capillary HP-5 MS 30 m × 0.25 ×, 0.25 μm (Agilent); flow rate He 0.9 ml $min^{-1}$; injection 280 °C; oven temperature: 50 °C 2 min, then ramp to 300 °C 10 °C $min^{-1}$; EI energy 70 eV.
*LC/MS/MS:* 1100 Series LC/MSD Trap VL(G2445C) (Agilent); column 50 × 2.1 mm, 1.8 μm Eclipse XDB C18 (Agilent); mobile phase A: acetonitrile, B: 0.2% formic acid in water, gradient A/B: 0–36 min 10/90, 36–40 min 100/0; flow rate 0.3 ml $min^{-1}$.
*UV:* UV-1601 (Shimadzu); length path 1 cm, samples dissolved in methanol.
*IR:* FT-IR Nicolet Magna 750 spectrometer, KBr technique.
*LC-NMR:* INOVA-650 (Varian) with IFC TR/PFG 4(C/N) microflux NMR probes; frequecy 599,783 MHz. HPLC column 150 × 4.6 mm 5 μm, Microsorb C18 (Varian); mobile phase: acetonitrile, flow rate 0.7 ml $min^{-1}$, UV detector at 210 nm.
*Cultivation of F. dimerum*: Approximately $10^6$ spores inoculated into liquid Sabouraud maltose media (HiMedia), grown for two days on a rotary shaker (3.7 Hz; 180 rpm) at 28 °C in the dark. A portion of vegetative culture 10% (v/v) was inoculated into the Sabouraud maltose media, pH 5.6 ± 0.2 (before sterilization) incubation at 28 °C for 120 h on rotary shaker (3.7 Hz, 180 rpm) in the dark.
*LOX assay*: Spectrophotometric method according to Bezáková et al. (2005).

Acknowledgement: This work was supported by the Slovak Research and Development Agency under the contract No. APVV-20-014105 and by VEGA grant agency 1/2390/05. NMR measurements were enabled by the Slovak State Programme Project No. 2003SP200280203.

#### References

Adham NZ, El-Tayeb OM, Hashem AGM, El-Refai HA, Sallam LA (2002) Microbial side-chain degradation of progesterone I: Optimization of the transformation conditions. Pakistan J Sci Ind Res 45: 250–255.
Bezáková L, Obložinský M, Sýkorová M, Paulíková I, Košt'álová, D (2005) Antilipoxygenase activity and the trace elements content of Aloe vera in relation to the therapeutical effect. Česká Slov Farm 54: 43–46.
Bigley VH, Duarte RF, Gosling RD, Kibbler CC, Seaton S, Potter M (2004) *Fusarium dimerum* infection in a stem cell transplant recipient

treated successfully with voriconazole. Bone Marrow Transplant 34: 815–817.

Dave BP, Dube HC (2000) Chemical characterization of fungal siderophores. Indian J Exp Biol 38: 56–62.

Deschamps JD, Kenyon VA, Holman TR (2006) Baicalein is a potent *in vitro* inhibitor against both reticulocyte 15-human and platelet 12-human lipoxygenases. Bioorg Med Chem 14: 4295–4301.

Firáková S, Proksa B, Šturdíková M (2007) Biosynthesis and biological activity of enniatins. Pharmazie 62: 563–568.

Nilanonta C, Isaka M, Chanphen R, Thongorn N, Tanticharoen M, Thebtaranonth Y (2003) Unusual enniatins produced by the insect pathogenic fungus *Verticillium hemipterigenum*: isolation and studies on precursor-directed biosynthesis. Tetrahedron 59: 1015–1020.

Plattner PA, Nager U (1948) Welkstoffe und Antibiotika. 8. Mitteilung. Über die Konstitution von Enniatin B. Helv Chim Acta 31: 665–671.

Puchkov VA, Wulfson NS, Rozinov BV, Denisov YV, Shemyakin MM, Ovchinnikov YA, Ivanov VT (1965) Thermal effect in the mass spectrometry of organic compounds. Tetrahedron Lett 6: 543–553.

Wulfson NS, Puchkov VA, Rozinov BV, Zyakun AM, Shemyakin MM, Ovchinnikov YA, Kiryushkin AA, Ivanov VT (1965) Mass spectrometric study of cyclodepsipeptides. Fragmentation types of regular and irregular cyclohexadepsipeptides. Tetrahedron Lett 6: 2793–2804