Tetrahedron Letters 54 (2013) 2492–2496

Contents lists available at SciVerse ScienceDirect

Tetrahedron Letters

journal homepage: www.elsevier.com/locate/tetlet

Induced production of depsipeptides by co-culturing *Fusarium tricinctum* and *Fusarium begoniae*

Jian-ping Wang [a,b], Wenhan Lin [c], Victor Wray [d], Daowan Lai [a,*], Peter Proksch [a,*]

[a] Institute of Pharmaceutical Biology and Biotechnology, Heinrich-Heine University, Düsseldorf, Universitätsstrasse 1, Geb. 26.23, 40225 Düsseldorf, Germany
[b] Key Laboratory of Natural Medicinal Chemistry and Resources Evaluation of Hubei Province, College of Pharmacy, Huazhong University of Science and Technology, Wuhan, PR China
[c] State Key Laboratory of Natural and Biomimetic Drugs, Peking University, Health Science Center, 100191 Beijing, PR China
[d] Helmholtz Centre for Infection Research, Inhoffenstraße 7, D-38124 Braunschweig, Germany

ARTICLE INFO

Article history:
Received 20 December 2012
Revised 27 February 2013
Accepted 4 March 2013
Available online 13 March 2013

Keywords:
Co-culture
Fusarium tricinctum
Fusarium begoniae
Fusarium equiseti
Depsipeptide
Rotamer

ABSTRACT

A co-culture of *Fusarium tricinctum* and *Fusarium begoniae* induced the production of two new linear depsipeptides, subenniatins A and B (**1**–**2**), which were not detected when either of the two fungi was cultured alone. The structures of the new compounds were unambiguously determined by analysis of 1D, 2D NMR, and mass spectra, as well as by chemical transformation. Complex NMR spectra were observed for compounds **1** and **2**, which were attributed to the presence of rotamers as revealed by 1D NOE and ROESY measurements. Structurally, compounds **1** and **2** are biogenetic building blocks of the cytotoxic enniatins B, B1, A1, and A, which are the major metabolites of *F. tricinctum* when this fungus is cultured alone. Compounds **1** and **2** were found to be inactive in cytotoxic and antibacterial assays.

In recent years, secondary metabolites from fungi have attracted considerable interest, since many of them possess unique structures and pronounced biological activities.[1–4] However, finding new and promising microbial natural products is becoming increasingly difficult as the rate of rediscovery is getting gradually higher. Several strategies have been reported to trigger biosynthetic pathways which remain silent under standard laboratory culture conditions. Such manipulations may result in the formation of 'cryptic natural products'.[5] One of these strategies involves co-cultivation of two or more microbes which may result in the production of bioactive secondary metabolites upon elicitation in a competitive environment. Examples include the formation of pestalone that shows potent antibiotic activity and is produced by the marine fungus *Pestalotia* sp. in response to challenge by a marine bacterium,[6] and cytotoxic diterpenoids, libertellenones A–D, produced when co-culturing a marine-derived fungus *Libertella* sp. with a marine α-proteobacterium,[7] as well as the recent discovery of *N*-formyl alkaloids from mixed fermentation of *Aspergillus fumigatus* with *Streptomyces peucetius*.[8] Finally, the induced production of cyclic depsipeptides, emericellamides A and B, by the marine-derived fungus *Emericella* sp. in co-culture with the marine actinomycete (*Salinispora arenicola*) was reported.[9] Induced accumulation of natural products by co-cultivation of two or more fungi has in contrast only rarely been reported, with the only two examples being marinamide obtained by co-culturing of two mangrove derived fungi,[10] and enhanced production of several metabolites of *Penicillium pinophilum* by mixed fermentation with *Trichoderma harzianum*.[11]

Fusarium spp. are known to produce several strongly bioactive mycotoxins, such as trichothecenes, fusaproliferin, beauvericin, moniliformin, and enniatins.[12] The latter are cyclic hexadepsipeptides consisting of alternating residues of D-2-hydroxyisovaleric acid and branched-chain *N*-methyl-L-amino acids linked by peptide and ester bonds. These compounds are known for their ionophoric, phytotoxic, and anthelmintic effects, as well as for their antibiotic activity and potent cytotoxic activity against cancer cell lines.[13] With the aim of isolation and identification of new metabolites, mixed fermentations were performed in this study between three different *Fusarium* species (*Fusarium tricinctum*, *Fusarium begoniae* and *Fusarium equiseti*). *F. tricinctum* is a well-known source of enniatins,[14] and *F. begoniae* was only reported to produce fusaproliferin,[15] while *F. equiseti* mainly produces trichothecenes and fusarochromanone.[16]

The extracts obtained from mixed fermentation of the different fungi on white bean medium were analyzed by HPLC, and compared to those from fermentation of only one fungus on the same medium. The results showed that extracts of the three fungi are

* Corresponding authors. Tel.: +49 211 81 14187 (D.L.); tel.: +49 211 81 14163; fax: +49 211 81 11923 (P.P.).
E-mail addresses: laidaowan123@gmail.com (D. Lai), proksch@uni-duesseldorf.de (P. Proksch).

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http://dx.doi.org/10.1016/j.tetlet.2013.03.005

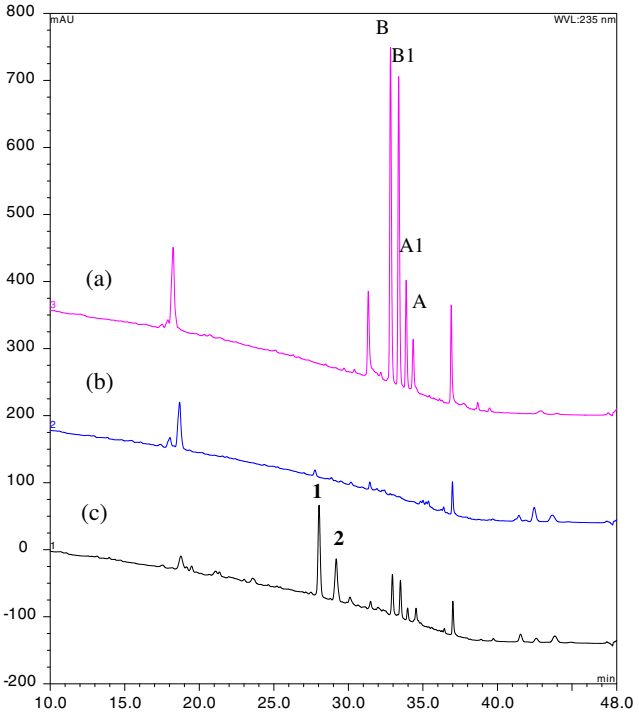

Figure 1. HPLC profiles of the EtOAc extracts of *F. tricinctum* (a), *F. begoniae* (b), and co-culture of *F. tricinctum* and *F. begoniae* (c) detected at UV 235 nm. B, B1, A1, and A refer to enniatins B, B1, A1, and A, respectively.

vastly different as indicated by their HPLC chromatograms (Fig. S1, see Supplementary data). No significant differences in the HPLC traces were found when *F. equiseti* was co-cultured with either *F. tricinctum* or *F. begoniae* (Fig. S1). Interestingly, two new peaks (rt 27.8, 29.0 min) were observed in the HPLC profile of an extract resulting from co-culturing *F. tricinctum* and *F. begoniae*, which were not detected when either of the two fungi was cultured alone (Fig. 1).

Compound **1**[17] was isolated as a colorless oil. The ESI-MS spectra of **1** displayed ion peaks at *m*/*z* 445 $[M+H]^+$, and 443 $[M-H]^-$, indicating a molecular weight of 444. The molecular formula of **1** was established as $C_{22}H_{40}N_2O_7$ by HR-ESIMS, as it showed a pseudomolecular ion peak at *m*/*z* 467.2734 $[M+Na]^+$ (calcd for $C_{22}H_{40}N_2O_7Na$, 467.2728). The ^1H NMR spectrum showed several methyls in the upfield region, and α-protons of amino acids in the region of 4.0–5.5 ppm, indicating a peptide nature of **1**. However, the spectra suggested that compound **1** was a mixture of four 'isomers' (Fig. 2a), although the HPLC and LC/MS profiles of **1** showed only one symmetric peak. Several attempts to further purify this compound were made. After several unfruitful trials, we realized that these 'isomers' could not be separated. Nevertheless, with the aid of 2D NMR spectra, including ^1H–^1H COSY, HSQC, and HMBC, the major 'isomer' was unambiguously determined. By analysis of the ^1H–^1H COSY spectrum, four spin systems (from C-2 to C-5, C-8 to C-11, C-13 to C-16, and C-19 to C-22) were established, which start from the four α-protons (δ_H 4.11, 4.82, 5.13, 4.37, each d) via the β-methine, and extend to the terminal methyl groups (Fig. 3a). The chemical shifts of the α-C were indicative of the presence of two oxygenated methine groups [δ_C 72.7 (C-2), 75.2 (C-13)] (Table 1). In addition, the structure of **1** also contained two *N*-methyl groups as suggested by their NMR data [δ_H 2.97 s, δ_C 31.0 q (6-Me); and δ_H 3.03 s, δ_C 32.0 q (17-Me)]. Moreover, the ^{13}C NMR spectrum showed the presence of four carbonyl groups (δ_C 173.8 s, 169.6 s, 168.7 s, 171.6 s). These functionalities satisfy all degrees of unsaturation required by the molecular formula, thus compound **1** must have a linear structure. The connectivity of the respective moieties was accomplished by analysis of the HMBC correlations (Fig. 3a). The α-protons in the four spin systems

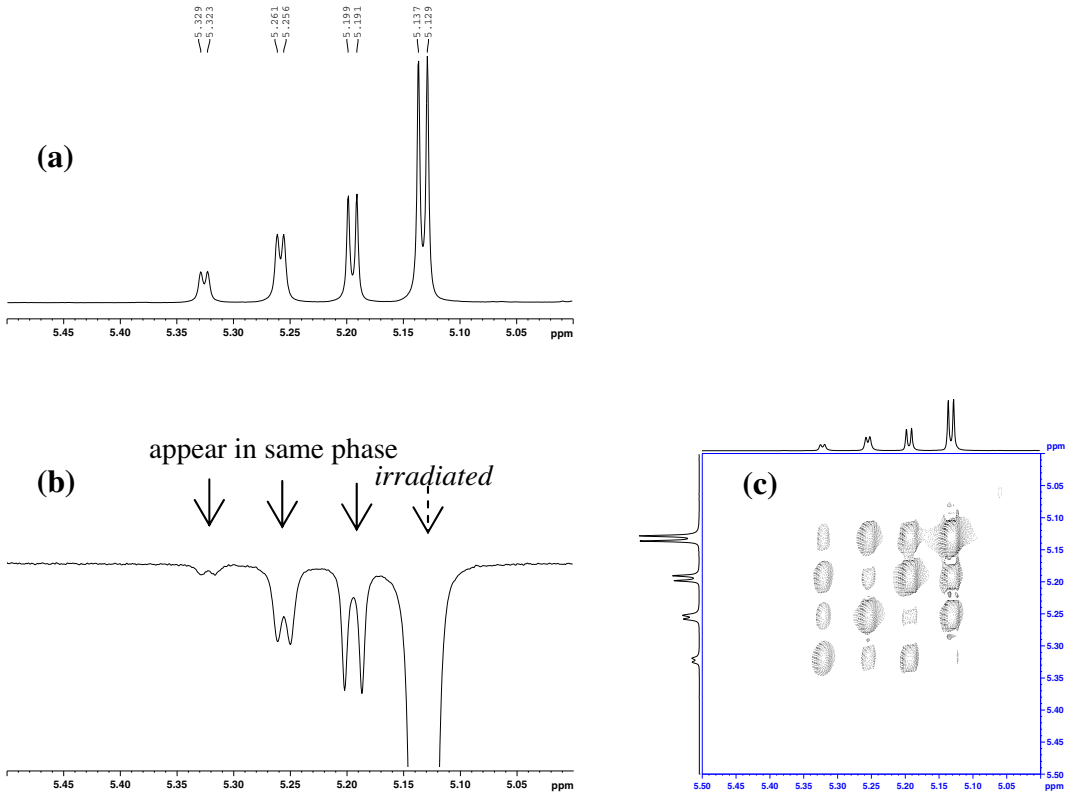

Figure 2. NMR spectrum of **1** (5.0–5.5 ppm region only) (a) ^1H NMR spectrum, (b) 1D NOE difference spectrum (irradiated at 5.13 ppm), and (c) ROESY spectrum.