# Induced Production of Depsipeptides by Co-Culturing *Fusarium tricinctum* and *Fusarium begoniae*

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**Abstract**

A co-culture of *Fusarium tricinctum* and *F. 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.

**Keywords** Co-culture; *Fusarium tricinctum*; *Fusarium begoniae; Fusarium equiseti*; Depsipeptide; Rotamer

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 *Fusarim* species (*F. tricinctum*, *F. begoniae* and *F. 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 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).

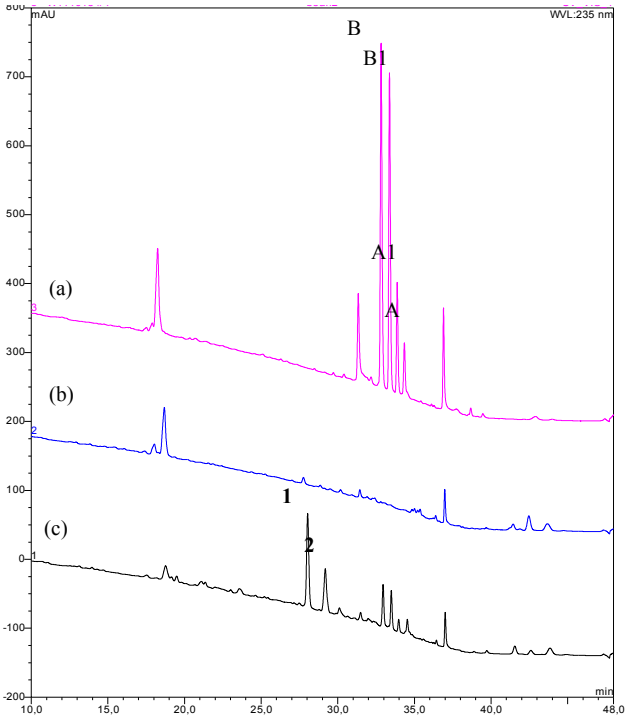


**Figure 1.** HPLC profiles of the EtOAc extracts of *F. tricinctum* (a), *F. begoniae* (b), and co-culture of *F. tricinctum* and *F. begoniae* (c) detected at UV 235 nm. B, B1, A1, and A refer to enniatins B, B1, A1, and A, respectively.

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 ($\delta_H$ 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 [$\delta_C$ 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 [$\delta_H$ 2.97 s, $\delta_C$ 31.0 q (6-Me); and $\delta_H$ 3.03 s, $\delta_C$ 32.0 q (17-Me)]. Moreover, the $^{13}C$ NMR spectrum showed the presence of four carbonyl groups ($\delta_C$ 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 showed two- or three-bond correlations to the adjacent carbonyl(s), which established the mutual linkage of the substructures. The correlations from *N*-methyl groups to the β-methine and carbonyls not only confirmed the peptide bond linkage, but also completed the connectivity of the whole structure. Thus, the major "isomer" of compound **1** was established as a linear depsipeptide

(Fig. 4). This result inspired us to investigate the structures of the other "isomers". Considering that the peptide bond has limited rotational freedom, rotamers around this bond may be observed. Since two peptide bonds are present in **1**, it would be not surprising if four rotamers (not isomers) were observed. Indeed, such a phenomenon was reported in a recent paper,[18] in which the authors successfully distinguished the rotamers and diastereoisomers by 1D-seletive chemical-exchange NMR experiments (eg. 1D NOE), based on the different NMR behavior between chemical exchange and normal NOE enhancement. By using the same method, a selective excitation of the resonance at 5.13 ppm (CH-13) in **1** was performed, which creates a peak at 5.13 ppm as well as three new peaks (at 5.33, 5.26, and 5.19 ppm) of the *same* phase (Fig. 2b) due to rotameric chemical exchange, since normal NOE enhancements appear in the *opposite* phase from the irradiated peak. Thus, these four peaks were attributed to the α-protons (CH-13) of the four rotamers. Alternatively, 2D ROESY (or NOESY) experiments can serve the same purpose, since the signals derived from NOE or from chemical exchange will appear in different phases. Hence, the ROESY spectrum of **1** was measured, which also clearly indicated the chemically exchangeable signals, for example, the cross-peaks between any of the four aforementioned α-H signals ($\delta_H$ 5.33, 5.26, 5.19, and 5.13) were observed (Fig. 2c). In addition, the normal NOE correlations were used to confirm the amide linkage (Fig. 3b).

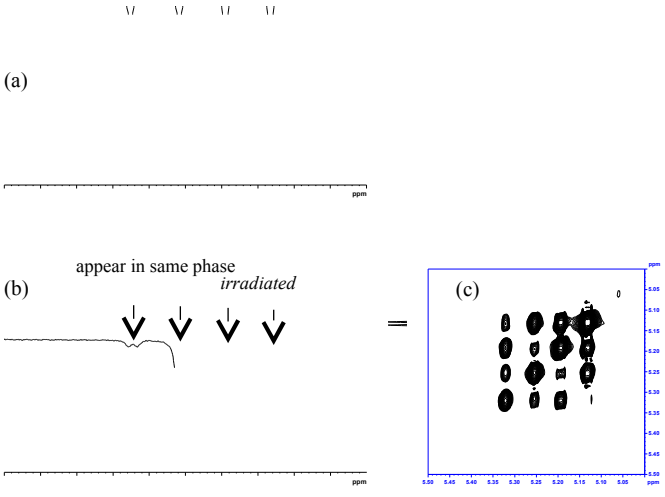

**Figure 2.** NMR spectrum of **1** (5.0-5.5 ppm region only) (a) $^{1}$H NMR spectrum, (b) 1D NOE difference spectrum (irradiated at 5.13 ppm), and (c) ROESY spectrum

**Table 1** $^{1}$H (600MHz) and $^{13}$C (150MHz) NMR data of compounds **1**, and **2** (DMSO-$d_6$) [a]

| | **1** | | **2** | |
|---|---|---|---|---|
| Position | $\delta_C$, type [b] | $\delta_H$, mult. (*J*) | $\delta_C$, type [b] | $\delta_H$, mult. (*J*) |
| 1 | 173.8, s | | 173.6, s | |
| 2 | 72.7, d | 4.11 d (5.2) | 72.7, d | 4.11 d (5.0) |
| 3 | 30.4, d | 1.90 m | 30.4, d | 1.90 m |
| 4 | 19.3, q | 0.90 [e] | 19.3, q | 0.90 [e] |
| 5 | 16.3, q | 0.79 [e] | 16.3, q | 0.79 [e] |
| 6-Me | 31.0, q | 2.97 s | 30.9, q | 2.97 s |
| 7 | 169.6, s | | 169.5, s | |
| 8 | 61.4, d | 4.82 d (10.3) | 59.9, d | 4.91 d (10.3) |
| 9 | 26.7, d [c] | 2.18 m | 32.4, d | 2.01 m |
| 10 | 19.7, q | 0.99 [e] | 24.4, t | 1.33 m, 1.00m |
| 10-Me | | | 10.2, q | 0.82 [e] |
| 11 | 18.82, q [d] | 0.79 [e] | 15.5, q | 0.93 [e] |
| 12 | 168.7, s | | 168.4, s | |
| 13 | 75.2, d | 5.13 d (4.7) [f] | 75.2, d | 5.13 d (4.9) [h] |
| 14 | 28.7, d | 2.09 m | 28.7, d | 2.10 m |
| 15 | 18.75, q [d] | 0.97 [e] | 18.7, q [g] | 0.96 [e] |
| 16 | 16.7, q | 0.87 [e] | 16.7, q | 0.88 [e] |
| 17-Me | 32.0, q | 3.03 s | 31.9, q | 3.03 s |
| 18 | 171.6, s | | 171.4, s | |
| 19 | 62.6, d | 4.37 d (10.3) | 62.7, d | 4.37 d (10.1) |
| 20 | 26.8, d [c] | 2.14 m | 26.8, d | 2.15 m |
| 21 | 20.0, q | 0.95 [e] | 20.0, q | 0.95 [e] |
| 22 | 18.83, q [d] | 0.77 [e] | 18.8, q [g] | 0.77 [e] |

[a] Assignments were based on DEPT-135, COSY, HSQC, HMBC, and ROESY experiments (only for the major rotamer).

[b] Multiplicities were indicated by DEPT-135, and HSQC experiments.

[c-d, g] Assignments may be interchanged within the same column.

[e] overlapped signals

[f] The signals for all the four rotamers are as follows: $\delta_H$ 5.33 d (*J*=3.6 Hz, 0.08 H), 5.26 d (*J*=3.4 Hz, 0.19 H), 5.19 d (*J*=4.6 Hz, 0.23 H), 5.13 d (*J*=4.7 Hz, 0.50 H).

[h] The signals for all the four rotamers are as follows: $\delta_H$ 5.32 d (*J*=2.8 Hz, 0.08 H), 5.25 d (*J*=2.8 Hz, 0.18 H), 5.20 d (*J*=4.6 Hz, 0.24 H), 5.13 d (*J*=4.9 Hz, 0.50 H).

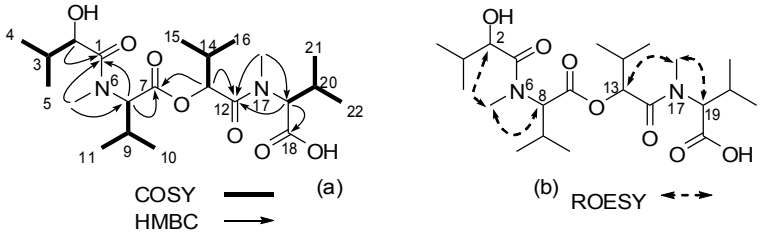


**Figure 3.** Selected $^1$H-$^1$H COSY, HMBC (H→C), and ROESY correlations of **1**

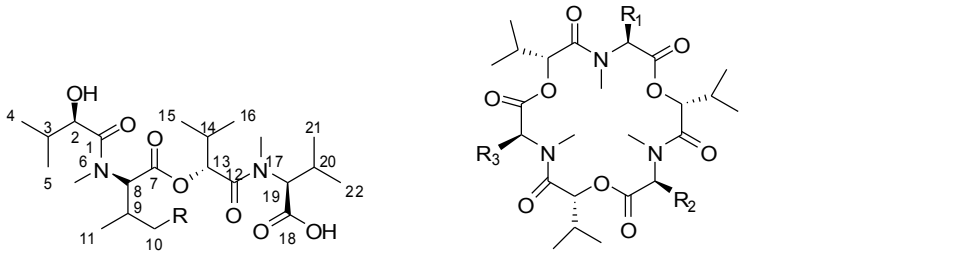


**Figure 4.** Structures of **1** and **2**, and enniatins A, A1, B, and B1

Compound **2**[19] was also isolated as a colorless oil. The molecular formula of **2** was deduced as $C_{23}H_{42}N_2O_7$ by HRESIMS, indicating one further $CH_2$ group compared to **1**. The NMR data of **2** (Table 1) are closely related to those of **1**, implying that both compounds share a similar skeleton. The DEPT-135 spectrum of **2** showed an additional methylene group ($\delta_C$ 24.4), which was not present in **1**. This methylene group was deduced to be located at C-10 by inspection of the $^1$H-$^1$H COSY correlations from $H_2$-10 to H-9, and 10-Me, and due to the HMBC correlations from $H_3$-11 to C-8, C-9, and C-10. Therefore, the *N*-methylvaline residue (N6-C10) in **1**

was replaced by *N*-methylisoleucine in **2** (Fig. 4). The four rotamers of **2** were revealed by 1D NOE difference experiments and ROESY experiments as well (Fig. 5).

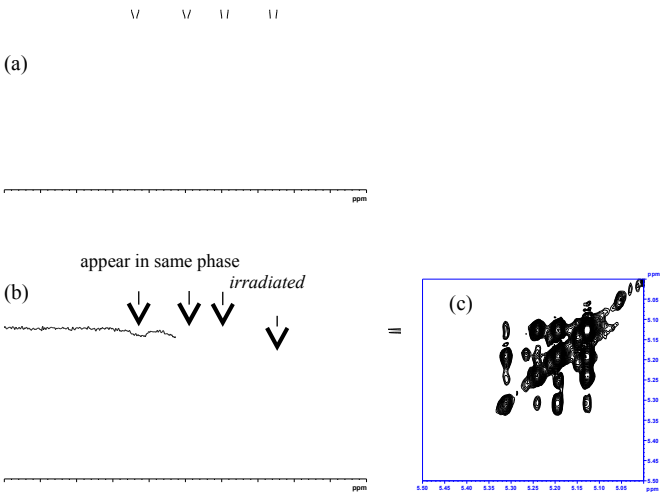


**Figure 5.** NMR spectrum of **2** (5.0-5.5 ppm region only) (a) $^{1}$H NMR spectrum, (b) 1D NOE difference spectrum (irradiated at 5.13 ppm), and (c) ROESY spectrum

From a biogenetic point of view, the configurations of the *N*-methylvaline residue in **1** and **2**, and *N*-methylisoleucine in **2** were deduced as *L*, while *D* configuration for the 2-hydroxyisovaleric acid moiety was assumed for both compounds. One evidence for this assumption rests on the fact that compounds **1** and **2** are detected along with enniatins A, A1, B and B1, which are present as minor metabolites in the same extract of *F. tricinctum* co-cultured with *F. begoniae* (Fig. 1). In structural terms, compounds **1** and **2** are obviously building block of the enniatins. This conclusion was confirmed by chemical transformation. A mild alkaline hydrolysis of enniatin B, followed by acid treatment, gave (3*S*,6*R*)-3,6-diisopropyl-4-methyl-2,5-morpholinedione (**1a**) as the only product. Similarly, enniatin A1 was processed in the same way to give (3*S*,6*R*)-3-*sec*-butyl-6-isopropyl-4-methyl-2,5-morpholinedione (**2a**) in addition to **1a**. Following the same protocol, compound **1** only produced **1a**, and compound **2** yielded **1a** and **2a**, as expected (Fig. 6).

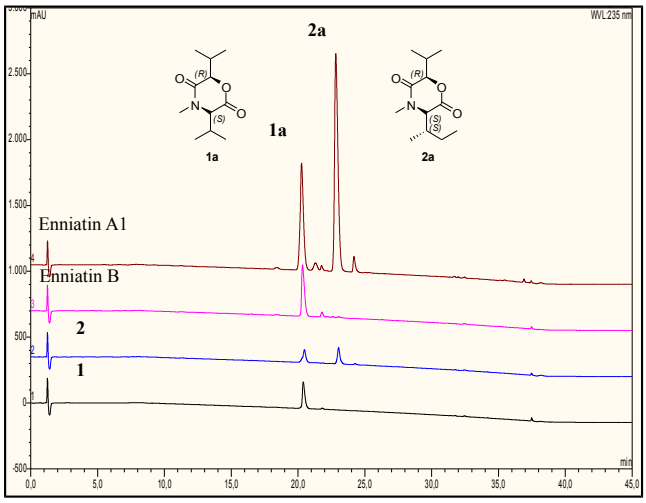


**Figure 6.** HPLC chromatograms of the alkaline hydrolysates of compounds **1**, **2**, enniatins B, and A1 (detected at UV 235 nm)

Compounds **1** and **2** were evaluated for their antibiotic activities against *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*, however, no inhibition was observed at a concentration of 64 μg/mL. It was reported that (mycotoxin) enniatins A1, B, and B1 induce apoptotic cell death in H4IIE hepatoma cells,[20] and A1, and B1 showed cytotoxicity effects in Caco-2 cells,[14] whereas all of the tested enniatins caused acute toxicity to brine shrimp.[21] Thus, compounds **1** and **2** were tested for their cytotoxicity against the mouse lymphoma cell line L5178Y. However, none of them were active ($IC_{50}$>10 $\mu$g/mL).

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**Supplementary data**

Experimental section, copies of $^{1}$H and $^{13}$C NMR, COSY, HSQC, HMBC, ROESY, 1D NOE, and MS spectra for **1** and **2**, as well as characterization data and copies of $^{1}$H and $^{13}$C NMR spectra for **1a** and **2a**.

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17. Subenniatin A (**1**): colorless oil; $[\alpha]^{20}_{D}$ -108.6 (*c* 0.2, MeOH); UV $\lambda_{max}$ (PDA) 201.9 nm; $^{1}H$ and $^{13}C$ NMR, see Table 1; ESIMS: *m/z* (positive) 445.2 $[M+H]^+$, 889.2 $[2M+H]^+$, (negative) 443.2 $[M–H]^–$; HRESIMS *m/z* 467.2734 $[M+Na]^+$ (cacl. for $C_{22}H_{40}N_2NaO_7$, 467.2728).
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