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Effect of the cyclopeptolide 90-215 on the production of destruxins and helvolic acid by *Metarhizium anisopliae*

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The effect of the cyclopeptolide 90-215 on the production of destruxins and helvolic acid in various *Metarhizium anisopliae* strains was investigated. Addition of 10.0 mg L^{-1} of the cyclopeptolide to the production media increased the production of destruxins by 1.3-fold to 12.5-fold, whereas the production of helvolic acid was decreased by 1.6- to 11.0-fold. Fifty liters were fermented in Erlenmeyer flasks with the strain 86-23766 grown in medium supplemented with cyclopeptolide 90-215. The procedure for isolation and purification of destruxins was simplified due to the higher yield of destruxins. Good quantities of destruxins were obtained from this fermentation. The results of our studies show that the addition of small quantities of a suitable compound can drastically alter the production and relative ratios of secondary metabolites. This can have a wide range of potential applications in the area of metabolite production.

Keywords: cyclopeptolide; destruxin; helvolic acid; *Metarhizium anisopliae*; cyclodepsipeptide

Introduction

Filamentous fungi are a rich source of metabolites such as antibiotics, enzymes, vitamins, organic acids and alkaloids, which have a wide range of applications in the pharmaceutical industry [5]. Many parameters during cultivation (eg temperature, aeration and medium composition) can influence the production rate significantly.

We have found that the addition of certain compounds in small amounts to the medium may significantly influence the production rate of secondary metabolites. We report here how the cyclic peptolide 90-215 influences the titers and ratios of destruxins and helvolic acid in various *Metarhizium anisopliae* strains.

Destruxins are cyclodepsipeptides produced by the entomo-pathogenic fungus *Metarhizium anisopliae*. These compounds are composed of five amino acids and an α-hydroxy acid forming a cyclic hexadepsipeptide. To date 27 destruxins have been reported from different fungi, however most of them are produced by *M. anisopliae* [3]. Due to their wide range of activities such as immunosuppression, activation of calcium channels in insect muscles, insecticidal activity, etc, these compounds have been the target of biological and chemical studies and have led to the recent discovery of new destruxins: E-diol [1], destruxins A_3, F [9], destruxins A_4, A_5 [4] and desmethyldestruxin B_2 [10].

Helvolic acid is a tetracyclic triterpenoid antibiotic first isolated from the fungus *Aspergillus fumigatus* [8]. Later it was also discovered in the fermentation broth of *Metarhizium anisopliae* and from other species of fungi [7].

Cyclopeptolide 90-215 is a natural pipecolic acid-containing cyclopeptolide, wherein the peptidic backbone is composed of nine α-amino acid residues joined together by peptide bonds. It was isolated from a fungus belonging to the genus *Septoria*. This peptolide and its semisynthetic derivatives exhibit interesting pharmacological, antifungal, chemotherapeutic drug resistance-reversing properties and some extend immunosuppressant and anti-inflammatory actions [2].

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Materials and methods

Materials

The cyclic peptolide 90-215, the isolation of which has been reported elsewhere [2], as well as destruxins A and E and helvolic acid were obtained from Novartis Pharma AG, Basel, Switzerland.

Microorganism

The *Metarhizium anisopliae* strains and their sources are listed in Table 1. Frozen vegetative preparations were maintained in 10% glycerol stored at –80°C for use as working stocks.

Media and culture conditions

Seed medium SM1 is composed of bacto agar (1.0 g L^{-1}), malt extract (20.0 g L^{-1}) and yeast extract (4.0 g L^{-1}). Production medium PM1 is composed of bacto agar (1.0 g L^{-1}), peptone (20.0 g L^{-1}), yeast extract (1.0 g L^{-1}), mannitol (20.0 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 g L^{-1}), K_2HPO_4 (0.1 g L^{-1}) and $CaCl_2 \cdot 6H_2O$ (0.2 g L^{-1}). Production medium PM2 is composed of bacto agar (1.0 g L^{-1}), peptone (10.0 g L^{-1}), malt extract (5.0 g L^{-1}), yeast extract (1.0 g L^{-1}), maltose (20.0 g L^{-1}), $MgSO_4 \cdot 7H_2O$ (0.1 g L^{-1}), K_2HPO_4 (0.2 g L^{-1}), KH_2PO_4 (0.1 g L^{-1}) and $CaCl_2 \cdot 6H_2O$ (0.05 g L^{-1}). All media were prepared with deionized water and autoclaved at 121°C for 20 min. All chemicals used were of analytical grade.

To prepare an inoculum, 2 ml of the frozen vegetative

Table 1 *M. anisopliae* strains used in this work

Sandoz No.	Provided by	Sample No.	Country
86-23757	Dr JO Pereira[a]	A4	Brazil
86-23758	″	DF	″
86-23759	″	DF19	″
86-23760	″	M	″
86-23761	″	M5	″
86-23762	″	RJ	″
86-23763	″	257	″
86-23764	″	299	″
86-23765	Dr S Keller[b]	370	Switzerland
86-23766	″	390	″
86-23854	″	389	″
86-23855	″	398	″
86-23856	″	376	″
86-23857	″	394	″
86-23858	″	427	″
86-23859	″	423	″
86-23860	″	430	″
86-23861	″	414	″
86-23862	″	425	″
86-23863	″	403	″
86-23864	″	404	″
86-23865	″	424	″
86-23866	″	422	″
86-23867	ATCC[c]	22099	USA

[a]Dr Jose Odair Pereira, Agricultural University, Piracicaba, Sao Paulo, Brazil.
[b]Dr Siegfried Keller, Eidgenössische Versuchsanstalt Reckenholz, Zürich, Switzerland.
[c]ATCC: American Type Culture Collection, Rockville, MD, USA.

stock was thawed at room temperature and transferred to a 500-ml Erlenmeyer flask containing 200 ml of medium SM1. The flasks were incubated at 24°C for 3 days on a rotary shaker at 200 rpm. Four milliliters of the seed culture were transferred to 500-ml Erlenmeyer flasks containing 200 ml of production media PM1 or PM2 which were incubated at 24°C for 9 days at 200 rpm.

The peptolide 90-215 was dissolved in methanol at a concentration of 1.0 mg ml^{-1}, and added to the production media prior to inoculation. Routinely, media were supplemented with 10 mg of 90-215 per liter medium. Media containing peptolide 90-215 are indicated with *.

Extraction and HPLC analysis of supernatants

The mycelium was removed by centrifugation of 20 ml of fermentation broth at $50 \times g$ for 10 min. The supernatant medium was decanted and mixed with an equal volume of ethyl acetate and shaken for 3 min. The ethyl acetate phase was removed by centrifugation at $50 \times g$ for 10 min and evaporated to dryness. The residue was weighed and redissolved in 1.0 ml of methanol.

HPLC analysis was performed using a Merck-Hitachi Model L-6200A pump equipped with a Hitachi L-4500 UV/Vis diode array detector (190–800 nm). A LiChroCART column (Merck, 125×4.0 mm i.d.) packed with LiChrospher 100 RP-18 (5 μm) was used at a flow rate of 1.5 ml min^{-1}. The mobile phase was a linear gradient of 0.1% H_3PO_4/MeCN 80 : 20 to 0 : 100 in 20 min with monitoring at 210 nm. Ten to twenty microliters of the samples were injected *via* the injector of the Hitachi Model AS-2000A HPLC. The production of destruxins and helvolic acid in the fermentation was calculated from the peak areas obtained by integration of the elution profiles. Destruxins A and E, helvolic acid and cyclopeptide 90-215 were used as standards.

Preparative isolation of destruxins and helvolic acid from fermentation broth

The isolation and purification of destruxins and helvolic acid process are summarized in Figure 1.

Chromatographic methods

Thin layer chromatography (TLC) was performed on precoated silica gel TLC plates (Kieselgel 60 F_{254}; thickness, 0.25 mm). The compounds on TLC plates were detected by UV light at 254 nm and subsequently by iodine vapors. The eluent used was MTBe-MeOH 9 : 1. Medium pressure liquid chromatography (MPLC) was performed using Labomatic and Büchi systems (Labomatic VS-200 gradient controller, Labocord 200 UV detector, Büchi 681 pump and 684 fraction collector). The column (6×50 cm) was packed with silica gel (Merck, 63–200 μm) and the eluent used was a gradient of MTBe-MeOH 100 : 0 to 50 : 50 in 5 h. Reverse phase C18 (RP-18) column chromatography was performed in a glass column packed with LiChroprep RP-18 (Merck, 40–63 μm). The column was eluted stepwise with mixtures of MeOH-H_2O 55 : 45, MeOH-H_2O 60 : 40 and 100% MeOH. The compounds eluted were detected using a Labomatic UV detector (Labocord 200).

Physical methods

Mass spectra (MS) were obtained on a VG TS 250 mass spectrometer operating in the FAB/positive ion mode with xenon atoms (8 KeV) and using a matrix of glycerol. ^{1}H-nuclear magnetic resonance (^{1}H-NMR) spectra were taken on Bruker AM 360 and AMX 500 MHz spectrometers, using $CDCl_3$ with tetramethylsilane as an internal standard. Ultraviolet (UV) spectra were recorded on a Perkin Elmer Lambda 19 UV spectrophotometer. Infrared (IR) spectra were recorded on a Bruker IFS 66 FTIR instrument (KBr pellets).

Results

Among the 24 strains of *M. anisopliae* initially grown in media PM1 and PM2 and analyzed by HPLC, strains 83-23765, 83-23766, 83-23855, 83-23861, 86-23863, 86-23867, 86-23760 and 86-23762 are good producers of destruxin A and E and helvolic acid. However strain 83-23766 in medium PM1 appeared to be the best destruxin A and E producer and was selected for further studies.

In several preliminary experiments with strain 86-23766, we observed that the addition of small amounts of cyclopeptolide 90-215 to the production medium PM1 at the time of inoculation had a pronounced effect on the production of secondary metabolites. As compared to the control medium PM1, medium PM1* increased the production of destruxins and suppressed production of helvolic acid. Ten milligrams per liter of cyclopeptolide 90-215 and 9 days incubation were optimal (results not shown).

Similarly, as evaluated by analytical HPLC, the seven other *M. anisopliae* strains 83-23765, 83-23855, 83-23861, 86-23863, 86-23867, 86-23760 and 86-23762 grown in

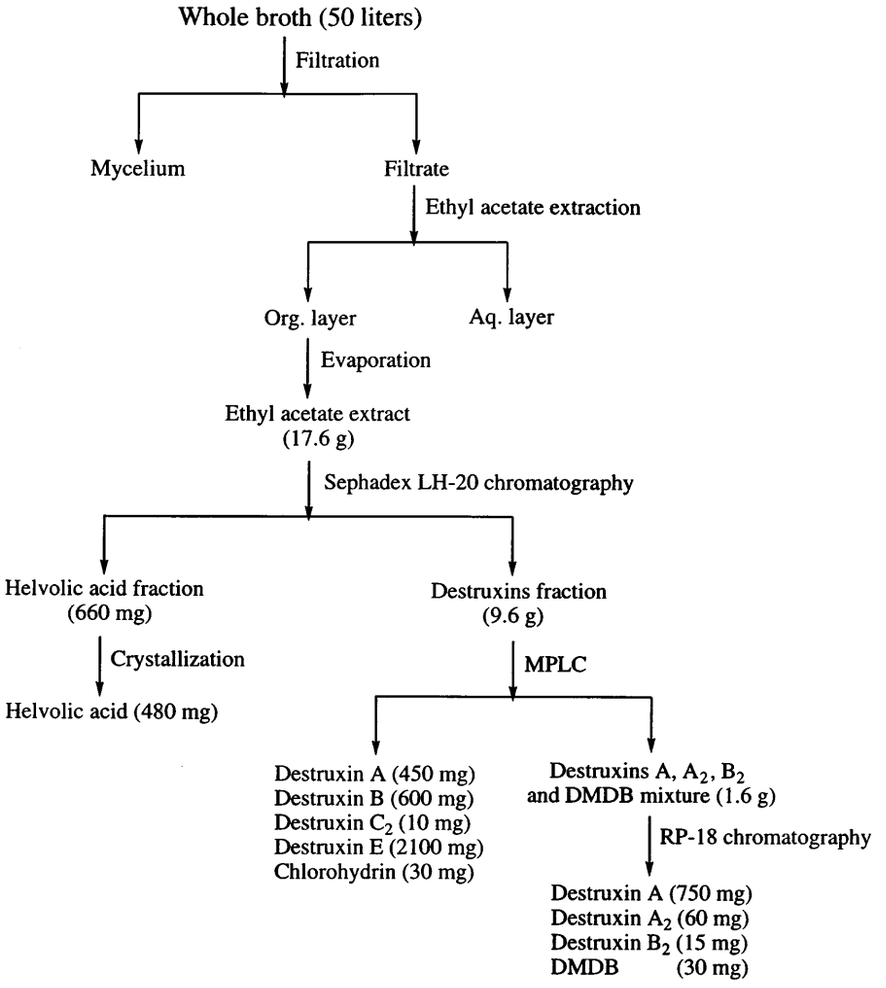

Figure 1 Procedure for isolation and purification of destruxins and helvolic acid.

media PM1, PM1*, PM2 and PM2* reacted in the same way: the addition of cyclopeptolide 90-215 promoted production of destruxins and suppressed production of helvolic acid (Tables 2 and 3). For all strains tested, media PM1* and PM2* significantly enhanced the production of destruxins A, B and E over the original production media PM1 and PM2, and caused a reduction of total helvolic acid production. Likewise, the production of other minor compounds, isolated later and identified as destruxins (A_2, B_2, DMDB, C_2 and chlorohydrin), was increased.

Table 2 Effect of cyclopeptolide 90-215 on the production of destruxins and helvolic acid by *M. anisopliae* strains in medium PM1

Strains	Destruxin A (mg L^{-1})			Destruxin B (mg L^{-1})			Destruxin E (mg L^{-1})			Other destruxins[a] (mg L^{-1})			Helvolic acid (mg L^{-1})		
	PM1	PM1*	Δ[b]	PM1	PM1*	Δ[b]	PM1	PM1*	Δ[b]	PM1	PM1*	Δ[b]	PM1	PM1*	Δ[b]
86-23766	15.1	60.8	4.0	14.1	36.7	2.6	13.4	56.5	4.2	9.4	24.6	2.6	49.8	23.1	–2.1
86-23765	9.5	18.3	1.9	4.0	15.0	3.7	10.0	16.0	1.6	3.8	19.8	5.2	11.5	2.7	–4.2
86-23855	9.7	12.8	1.3	10.5	14.0	1.3	6.4	8.5	1.3	7.1	10.1	1.4	13.0	5.4	–2.4
86-23861	14.6	19.2	1.3	10.7	17.6	1.6	11.1	14.7	1.3	10.0	24.2	2.4	14.6	2.2	–6.6
86-23863	13.2	17.2	1.3	9.3	16.0	1.7	13.4	17.6	1.3	6.5	19.1	2.9	13.7	6.2	–2.2
86-23867	13.5	17.6	1.3	12.0	17.1	1.4	13.6	17.7	1.3	10.5	17.1	1.6	15.3	5.0	–3.0
86-23760	13.4	20.1	1.5	10.5	19.2	1.8	13.5	17.8	1.3	7.2	19.0	2.6	7.6	0.8	–9.5
86-23762	2.3	4.2	1.8	1.1	3.3	3.0	2.0	5.2	2.6	NC[c]	NC[c]	NC[c]	7.0	1.2	–5.8

[a]Destruxins A_2, B_2, DMDB and Chlorohydrin.
[b]Increment (fold).
[c]Not calculated.

Table 3 Effect of cyclopeptolide 90-215 on the production of destruxins and helvolic acid by *M. anisopliae* strains in medium PM2

Strains	Destruxin A (mg L^{-1})			Destruxin B (mg L^{-1})			Destruxin E (mg L^{-1})			Other destruxins[a] (mg L^{-1})			Helvolic acid (mg L^{-1})		
	PM2	PM2*	Δ[b]	PM2	PM2*	Δ[b]	PM2	PM2*	Δ[b]	PM2	PM2*	Δ[b]	PM2	PM2*	Δ[b]
86-23766	1.0	5.5	5.5	1.1	3.0	2.7	0.8	10.0	12.5	NC[c]	NC[c]	NC[c]	18.0	11.2	–1.6
86-23765	11.3	20.4	1.8	6.5	16.4	2.5	9.0	16.5	1.8	4.5	23.4	5.2	7.0	0.8	–8.7
86-23855	4.2	6.4	1.5	2.8	4.0	1.4	2.5	3.8	1.5	3.3	5.0	1.5	16.0	4.2	–3.8
86-23861	13.6	20.5	1.5	8.8	18.0	2.0	12.5	18.8	1.5	10.5	36.0	3.4	17.8	2.0	–8.9
86-23863	14.3	19.0	1.3	12.5	19.5	1.5	12.5	16.6	1.3	17.5	30.5	1.7	14.7	4.2	–3.5
86-23867	12.8	21.8	1.7	9.3	20.6	2.2	13.4	19.2	1.4	13.5	37.8	2.8	16.8	4.4	–3.8
86-23760	13.6	17.7	1.3	5.1	16.5	3.2	13.3	17.5	1.3	10.4	29.6	2.8	6.6	0.6	–11.0
86-23762	8.5	13.1	1.5	4.0	14.8	3.7	7.6	14.2	1.8	6.0	9.1	1.5	7.2	1.8	–4.0

[a]Destruxins A_2, B_2, DMDB and Chlorohydrin.
[b]Increment (fold).
[c]Not calculated.

Preparative isolation of destruxins and helvolic acid from strain 86-23766

In order to obtain sufficient quantities of destruxins A and E for further studies, 50.0 L were fermented in Erlenmeyer flasks with strain 86-23766 in medium PM1*, which is superior to PM2*. Nine-day-old strain 86-23766 grown in PM1* was suction filtered. The filtrate was extracted twice with 40 L of ethyl acetate. The organic layer was dried over anhydrous Na_2SO_4 and evaporated to dryness to give 17.6 g of crude extract. This extract was subjected to a Sephadex LH-20 column (9×80 cm) using MeOH as the mobile phase. Sixty-five 80-ml fractions were collected and aliquots of each fraction were analyzed by TLC. Fractions 19–25 which contained a mixture of destruxins were pooled and evaporated to give 9.6 g of a yellow powder while fractions 26–27 which mainly contained helvolic acid, were pooled and evaporated to yield 660 mg of a pale yellow powder. The fraction composed of destruxins was purified by medium pressure liquid chromatography with a gradient of MTBe-MeOH 100 : 0 to 50 : 50 at a flow rate of 10 ml min^{-1} in 5 h. Sixty 50-ml fractions were collected. Elution was monitored by TLC and fractions containing the same components were pooled and evaporated to yield the following destruxins: 600 mg of B (fractions 9–11), 450 mg of A (fractions 17–20), 30 mg of chlorohydrin (fractions 24–25), 2100 mg of E (fractions 32–36) and 10 mg of C_2 (fraction 40). Fractions 14–16 and 21–22 which contained destruxins A, A_2, B_2 and desmethyldestruxin B (DMDB) mixture (1.6 g), were pooled and further purified by column chromatography on RP-18 (5×60 cm). The column was first eluted with MeOH : H_2O 55 : 45 (2.5 L) and then with MeOH : H_2O 60 : 40 (1.5 L) and 100% MeOH (1.0 L). In this way, fractions eluted with the first mobile phase yielded 60 mg of destruxins A_2 and 750 mg of A while fractions eluted with the second mobile phase yielded 15 mg of destruxins B_2 and 30 mg of DMDB, respectively.

The fraction containing helvolic acid gave 480 mg of this compound, which was crystallized from methanol.

Identification of destruxins and helvolic acid

Destruxins A and E and helvolic acid were first analyzed by FAB-MS and identified by analytical HPLC comparison with authentic samples and by spectroscopic data (^{1}H-NMR UV and IR). The structures of destruxins B, B_2, DMDB, A_2, C_2 and chlorohydrin were assigned by a comparison of their ^{1}H-NMR, FAB-MS, UV and IR data with those reported in the literature [3,6].

Discussion

The effect of cyclopeptolide 90-215 on the production of destruxins and helvolic acid by various *M. anisopliae* strains (Tables 2 and 3) is surprising. When this cyclopeptolide was added to the production media PM1 and PM2 at a concentration of 10.0 mg L^{-1}, the production of destruxins was increased by 1.3- to 12.5-fold, whereas the production of helvolic acid was inhibited by 1.6- to 11.0-fold. Although this report is based on the results obtained from the analysis of the culture broth supernatants, similar effects were observed in the mycelium (results not shown). We have also found that higher concentrations of cyclopeptolide 90-215 (12.0 to 15.0 mg L^{-1}) neither yield further improvement of destruxin titers nor affect growth of the cultures. Cyclopeptolide 90-215 promotes an increase of destruxins from the third day of the fermentation onwards and the maximum level of production occurs around the ninth day.

When the cyclopeptolide was added on the fourth day of the fermentation, the production of destruxins was only modestly increased over that observed without it and it did not inhibit the production of helvolic acid (results not shown). This means that for optimal stimulation of destruxin production and helvolic acid suppression by *M. anisopliae* strains, cyclopeptolide 90-215 must be added at the beginning of the fermentation.

This process has been successfully reproduced with strain 86-23766 in medium PM1 where cyclopeptolide 90-215 shows a remarkable enhancing effect on destruxins production. The amounts of destruxins A and E isolated from 50.0 L fermentation in Erlenmeyer flasks with medium PM1 supplemented with 90-215 (PM1*) are in agreement with the analytical results. The procedure for isolation and purification of destruxins was simplified due to the higher yield of destruxins and the lower yield of helvolic acid compared to the original medium PM1. In fact, the isolation and purification of destruxins from

fermentation with medium PM1 (results not shown) required much more effort and the use of additional chromatographic techniques.

The results of these studies show that the addition of small quantities of a suitable compound can drastically alter the production and relative ratios of secondary metabolites. Potentially, this can have a wide range of applications in the area of metabolite production.

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