# Directed Biosynthesis of Peptaibol Antibiotics in Two *Trichoderma* Strains

## I. Fermentation and Isolation

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(Received for publication August 15, 1997)

Peptaibols are linear α-aminoisobutyric acid-containing peptide antibiotics originating from soil fungi mainly of the genus *Trichoderma* and biosynthesized in complex mixtures of closely related analogues by a polyenzymatic pathway. Addition of amino acids such as α-aminoisobutyric acid (Aib), glutamic acid or arginine, to the fermentation medium of two *Trichoderma* strains, *T. harzianum* and *T. longibrachiatum*, has been shown to result in the simplification of the natural peptaibol mixtures, leading in each case to the almost exclusive biosynthesis of a single peptide. Surprisingly, the obtained peptides are Aib-enriched, whether the added amino acid is Aib, Glu or Arg. By adding Aib to the fermentation medium of *T. harzianum*, two new Aib-rich peptaibols were isolated. Moreover, adding glutamic acid to the culture medium of *T. longibrachiatum*, which produces both neutral and acidic 20-residue peptaibols with either glutamine or glutamic acid at position 18, increases the production of the acidic peptides. However, arginine which is a positively charged amino acid generally absent from peptaibol sequences, is not incorporated in trichorzins when added to the fermentation medium of *T. harzianum*.

*Trichoderma* are widespread soil fungi characterized by the production of peptides which display antifungal and antibacterial activities. Belonging to the peptaibol class, these peptides interact with phospholipid bilayers, increasing their permeability properties[1,2] and forming voltage-dependent ion channels.[2~4] With a high proportion of α,α-dialkylated amino acids, such as α-aminoisobutyric acid (Aib, U) and isovaline (Iva, J), peptaibols are linear peptides which exhibit an *N*-terminal acylated residue and a *C*-terminal amino alcohol. According to their chain length and chemical characteristics, they are classified into three subclasses: the long-sequence peptaibols including the well-known alamethicin[5] with 18~20 residues,[6~9] the short-sequence peptaibols with 11~16 residues[9~12] and the lipopeptaibols with 7 or 11 residues and an *N*-terminal lipid chain.[13,14]

As frequently observed, peptide antibiotics originating from microbial organisms, bacteria and filamentous fungi, arise from a nonribosomal biosynthetic pathway implying large protein templates termed peptide synthetases.[15~17] The alamethicin synthetase extracted from *T. viride* has a molecular weight around 480 kDa; the *N*-acetyl and the *C*-terminal amino alcohol linkage steps have been studied in detail.[18] Such biosyntheses proceed by formation of a linear peptidyl intermediate, the termini of which may be either enzymatically modified or transformed into cyclic structures, as observed for the potent immunosuppressant, cyclosporin A.[19] A general observation for such biosynthesis mechanism is that the peptides are produced in mixtures of closely related sequence analogues, termed microheterogeneous mixtures and particularly exemplified by peptaibols.[11,20]

Previous studies on cyclosporins biosynthesis have shown that the course of their formation is strongly influenced by an exogenous supply of amino acids to the fermentation medium, resulting either in enhanced yields of some of the natural components at the expense of others, or in the incorporation of foreign amino acids, depending on the added amino acid.[21,22] Similarly, the biosynthesis of the Aib-containing efrapeptins by *Tolypocladium geodes* is affected by the amino acid composition of the culture medium.[23] However, such experiments have never been tried with peptaibols. We

thus analyzed the influence of amino acid supplies on the biosynthesis course of *Trichoderma* strains, in an attempt to incorporate exogenous amino acid and to synthesize new analogues, which could exhibit modified membrane properties.

For the present study, we selected two *Trichoderma* strains which had been previously studied for their peptide content and considered the influence of an Aib-supply on the biosynthesis of the 20-residue longibrachins by *Trichoderma longibrachiatum*[24] (Fig. 1-A) and of the 18-residue trichorzins PA (Fig. 1-B) and the 14-residue harzianins PC by *Trichoderma harzianum*.[25] We here describe the modifications of the microheterogeneous peptide mixtures composition arising from Aib-directed biosynthesis in these two *Trichoderma* strains. We also report on the effect of supplementing the standard growth medium of *T. longibrachiatum* and *T. harzianum* with charged amino acids such as glutamic acid and arginine, glutamic acid being a natural component of peptaibols, while the positively charged arginine is usually absent in these peptides.

## Results and Discussion

### Aib-directed Biosynthesis of Peptaibols

As Aib is the peculiar amino acid responsible for the special characteristics and properties of peptaibols, the Aib-directed biosynthesis of the selected peptaibols was first examined. Fermentation of *T. longibrachiatum* (M-853431) on a synthetic medium led to a crude peptaibol mixture, isolated from the culture broth extract by exclusion chromatography over Sephadex LH20. When submitted to silica gel chromatography, the crude peptide fraction provided two peptide groups, designated as longibrachins LGA (neutral) and LGB (acidic) (Table 1). The LGA and LGB HPLC profiles obtained on a $C_{18}$ reversed-phase column showed four (LGA I~IV) and five (LGB I~V) components, respectively (Fig. 2-A, 2-C). The sequences of the major longibrachins (LGA I~IV and LGB II, III) have been determined previously (Fig. 1-A).[24]

When *T. longibrachiatum* was grown on a 0.8% w/v Aib-supplied medium and the peptide mixture separated according to the usual procedure, two peptide groups were also obtained (Table 1) and termed longibrachins $LGA_U$ and $LGB_U$. Regarding their HPLC profile, they appeared much simpler, each characterized by one main peptide, $LGA_U$ 1 (Fig. 2-B) and $LGB_U$ 2 (Fig. 2-D). Both longibrachins were isolated by repetitive semi-preparative HPLC and submitted to a co-chromatography HPLC study in order to assign $LGA_U$ 1 and $LGB_U$ 2, as compared to the LGA and LGB natural mixtures obtained on the normal culture medium (Table 2). The HPLC results indicated $LGA_U$ 1 to be LGA I and $LGB_U$ 2 to be LGB II, in agreement with the NMR and MS data.

Fig. 1. Amino acid sequences.

A: Longibrachins LGA and LGB from *T. longibrachiatum*.

| | 1 | 2 | 3 | 4 | 5 | **6** | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | **17** | **18** | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **LGA I** | Ac | Aib | Ala | Aib | Ala | Aib | **Ala** | Gln | Aib | Val | Aib | Gly | Leu | Aib | Pro | Val | Aib | **AibGln** | Gln | Pheol |
| **LGA II** | Ac | Aib | Ala | Aib | Ala | Aib | **Ala** | Gln | Aib | Val | Aib | Gly | Leu | Aib | Pro | Val | Aib | **IvaGln** | Gln | Pheol |
| **LGA III** | Ac | Aib | Ala | Aib | Ala | Aib | **Aib** | Gln | Aib | Val | Aib | Gly | Leu | Aib | Pro | Val | Aib | **AibGln** | Gln | Pheol |
| **LGA IV** | Ac | Aib | Ala | Aib | Ala | Aib | **Aib** | Gln | Aib | Val | Aib | Gly | Leu | Aib | Pro | Val | Aib | **IvaGln** | Gln | Pheol |
| **LGB II** | Ac | Aib | Ala | Aib | Ala | Aib | **Ala** | Gln | Aib | Val | Aib | Gly | Leu | Aib | Pro | Val | Aib | **AibGlu** | Gln | Pheol |
| **LGB III** | Ac | Aib | Ala | Aib | Ala | Aib | **Ala** | Gln | Aib | Val | Aib | Gly | Leu | Aib | Pro | Val | Aib | **IvaGlu** | Gln | Pheol |

B: Trichorzins PA and $PA_U$ 4 and harzianin $PC_U$ 4 from *T. harzianum*.

| | 1 | 2 | 3 | **4** | **5** | 6 | **7** | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | **18** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **PA II** | Ac | Aib | Ser | Ala | **AibIva** | Gln | **Aib** | Val | Aib | Gly | Leu | Aib | Pro | Leu | Aib | Aib | Gln | **Trpol** |
| **PA IV** | Ac | Aib | Ser | Ala | **AibIva** | Gln | **Iva** | Val | Aib | Gly | Leu | Aib | Pro | Leu | Aib | Aib | Gln | **Trpol** |
| **PA V** | Ac | Aib | Ser | Ala | **IvaIva** | Gln | **Aib** | Val | Aib | Gly | Leu | Aib | Pro | Leu | Aib | Aib | Gln | **Trpol** |
| **PA VI** | Ac | Aib | Ser | Ala | **AibIva** | Gln | **Aib** | Val | Aib | Gly | Leu | Aib | Pro | Leu | Aib | Aib | Gln | **Pheol** |
| **PA VII** | Ac | Aib | Ser | Ala | **IvaIva** | Gln | **Aib** | Val | Aib | Gly | Leu | Aib | Pro | Leu | Aib | Aib | Gln | **Trpol** |
| **PA VIII** | Ac | Aib | Ser | Ala | **AibIva** | Gln | **Iva** | Val | Aib | Gly | Leu | Aib | Pro | Leu | Aib | Aib | Gln | **Pheol** |
| **PA IX** | Ac | Aib | Ser | Ala | **IvaIva** | Gln | **Aib** | Val | Aib | Gly | Leu | Aib | Pro | Leu | Aib | Aib | Gln | **Pheol** |
| **PAU 4** | Ac | Aib | Ser | Ala | **AibAib** | Gln | **Aib** | Val | Aib | Gly | Leu | Aib | Pro | Leu | Aib | Aib | Gln | **Trpol** |
| **PCU 4** | Ac | Aib | Asn | Leu | Aib | Pro | Ser | Ile | Aib | Pro | Aib | Leu | Aib | Pro | **Valol** | | | |

Bold-faced letters indicate those amino acids which differ in the sequences; Aib, U: α-aminoisobutyric acid, Iva, J: isovaline, Pheol, Fol: phenylalaninol, Trpol, Wol: tryptophanol, Valol, Vol: valinol.

Table 1. Weights of peptaibols (mg) for typical *T. longibrachiatum* (M-853431) 20 liter cultures performed on normal culture medium (NM) and on the same culture medium supplemented either with Aib (NM+U)[a] or with Glu (NM+E)[a].

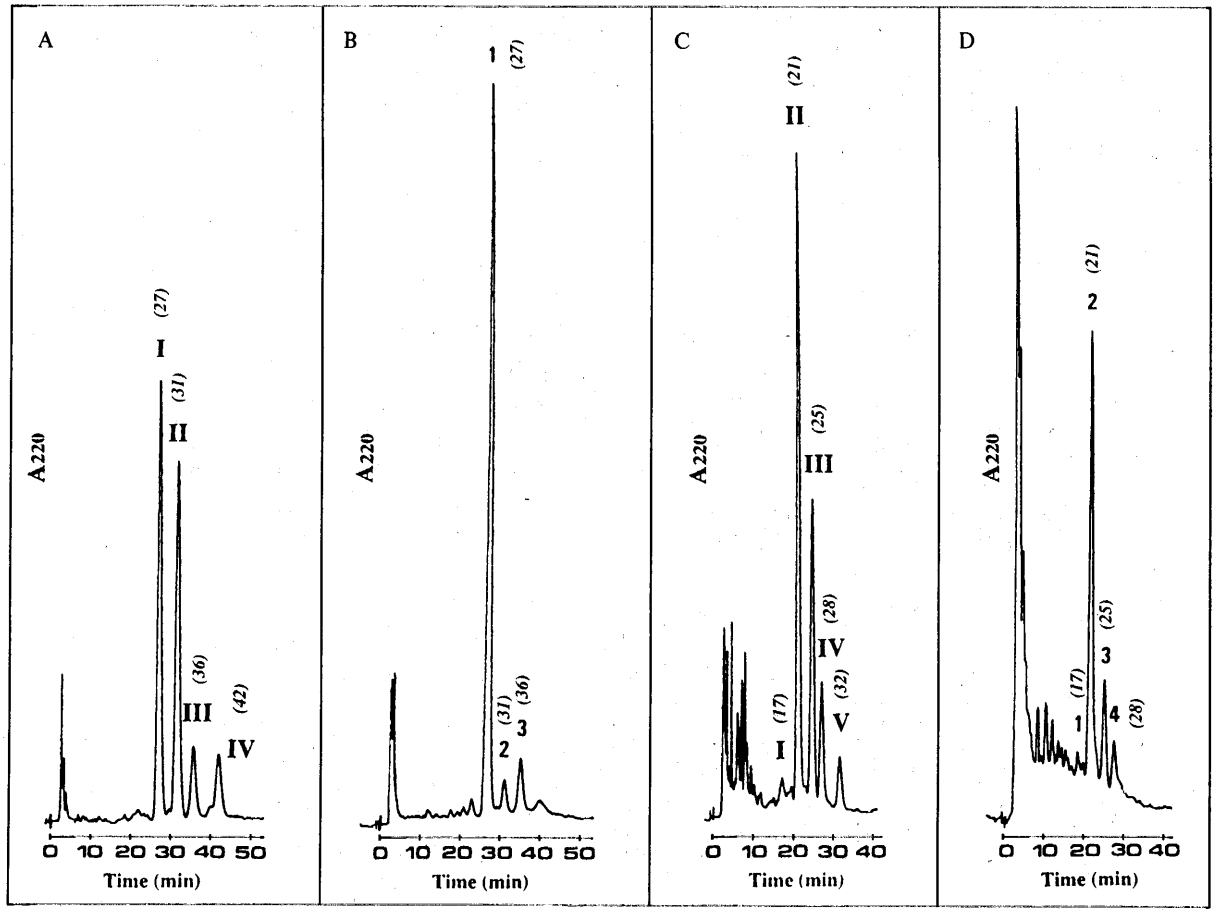
| | NM (mg) | NM+U (mg) | NM+E (mg) |
|---|---|---|---|
| Crude extract | 2120 | 1500 | 1610 |
| Crude peptide mixture[b] | 140 | 95 | 51 |
| LGA | 40 | 13 | 9 |
| LGB | 58 | 50 | 22 |

[a] The culture medium was supplemented with 8 g/liter of the concerned amino acid.
[b] The crude peptide mixtures contained both peptides and others metabolites.

Using the same multistep chromatography procedure as described above, fermentation of *T. harzianum* (M-902608) led to three main peptide groups of different polarity designated as PC, PD and trichorzins PA (Table 3). HPLC analysis of the PA mixture was performed on a $C_{18}$ reversed-phase column, exhibiting at least nine components (Fig. 3-A, Table 4), while PC and PD mixtures, which are not described here, were more complex. The sequence of the isolated trichorzins PA (Fig. 1-B) have been determined in a previous work.[25]

When the medium was Aib-enriched, the *T. harzianum* strain led only to two peptide groups, a minor group termed harzianins $PC_U$ and a major group termed trichorzins $PA_U$ (Table 3), revealing the simplification of the biosynthesized peptide mixture and the complete inhibition of the PD group production. HPLC analysis of the $PA_U$ mixture showed mainly one peptide $PA_U$ 4 and few minor components (Fig. 3-B). After purification by repetitive semi-preparative HPLC, the different tri-

Fig. 2. HPLC chromatograms of the longibrachins LGA (A), $LGA_U$ (B), LGB (C), $LGB_U$ (D), from *T. longibrachiatum*.




Kromasil $C_{18}$ (5 $\mu$), 4.6 × 250 mm, flow rate 1 ml/minute, absorption monitored at 220 nm. Solvent systems for A and B: MeOH-$H_2O$, 85:15; for C and D: MeOH-$H_2O$, 86:14.
Retention times of the peptides are given in brackets.

Table 2. HPLC retention times (minute) of the longibrachins LGA, $LGA_U$, $LGA_E$ and LGB, $LGB_U$, $LGB_E$ from *T. longibrachiatum* (M-853431).

| Peptides | LGA[a] | | | | $LGA_U/LGA_E$[a] | | | LGB[b] | | | | | $LGB_U/LGB_E$[b] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | I | II | III | IV | 1 | 2 | 3 | I | II | III | IV | V | 1 | 2 | 3 | 4 |
| Rt (minute) | 27 | 31 | 36 | 42 | 27 | 31 | 36 | 17 | 21 | 25 | 28 | 32 | 17 | 21 | 25 | 28 |

[a] Solvent system: MeOH - $H_2O$ (85 : 15).
[b] Solvent system: MeOH - $H_2O$, TFA 0.05% (86 : 14).

Table 3. Weights of peptaibols (mg) obtained for typical *T. harzianum* (M-902608) 20 liter cultures performed on normal culture medium (NM) and on the same culture medium supplemented either with Aib (NM + U)[a] or with Arg (NM + R)[a].

| | NM (mg) | NM + U (mg) | NM + R (mg) |
|---|---|---|---|
| Crude extract | 1670 | 1460 | 1370 |
| Crude peptide mixture[b] | 920 | 233 | 255 |
| PC | 170 | 43 | — |
| PA | 347 | 115 | 76 |
| PD | 168 | — | — |

[a] The culture medium was supplemented with 8 g/liter of the concerned amino acid.
[b] The crude peptide mixtures contained both peptides and others metabolites.

chorzins $PA_U$ were co-injected with the natural mixture of known trichorzins PA obtained on the standard culture medium. That indicated $PA_U$ 4 and $PA_U$ 6 to exhibit the same retention time as PA I and PA III respectively (Table 4), as shown by a significant enhancement in their peak intensity. As both PA I and PA III peptides have been previously shown to be heterogeneous and each composed of at least two homologous peptides, from MS and NMR data,[25] the present data suggested $PA_U$ 4 and $PA_U$ 6 to be one of the two components making up the PA I and PA III peaks, respectively. In addition, new peptides were produced; three of them, $PA_U$ 1, $PA_U$ 2 and $PA_U$ 3, exhibited shorter retention time than those of the trichorzins PA obtained on the normal medium (Fig. 3-B, Table 4); less hydrophobic than trichorzins PA, they appeared original but were not isolated in sufficient amount to examine their structures. Moreover, HPLC analysis of the $PC_U$ mixture showed more than 12 peaks (Fig. 4); among the 12 peptide fractions isolated by further semi-preparative HPLC, only one corresponding to $PC_U$ 4 was homogeneous, as shown by further analyses (see the following paper).

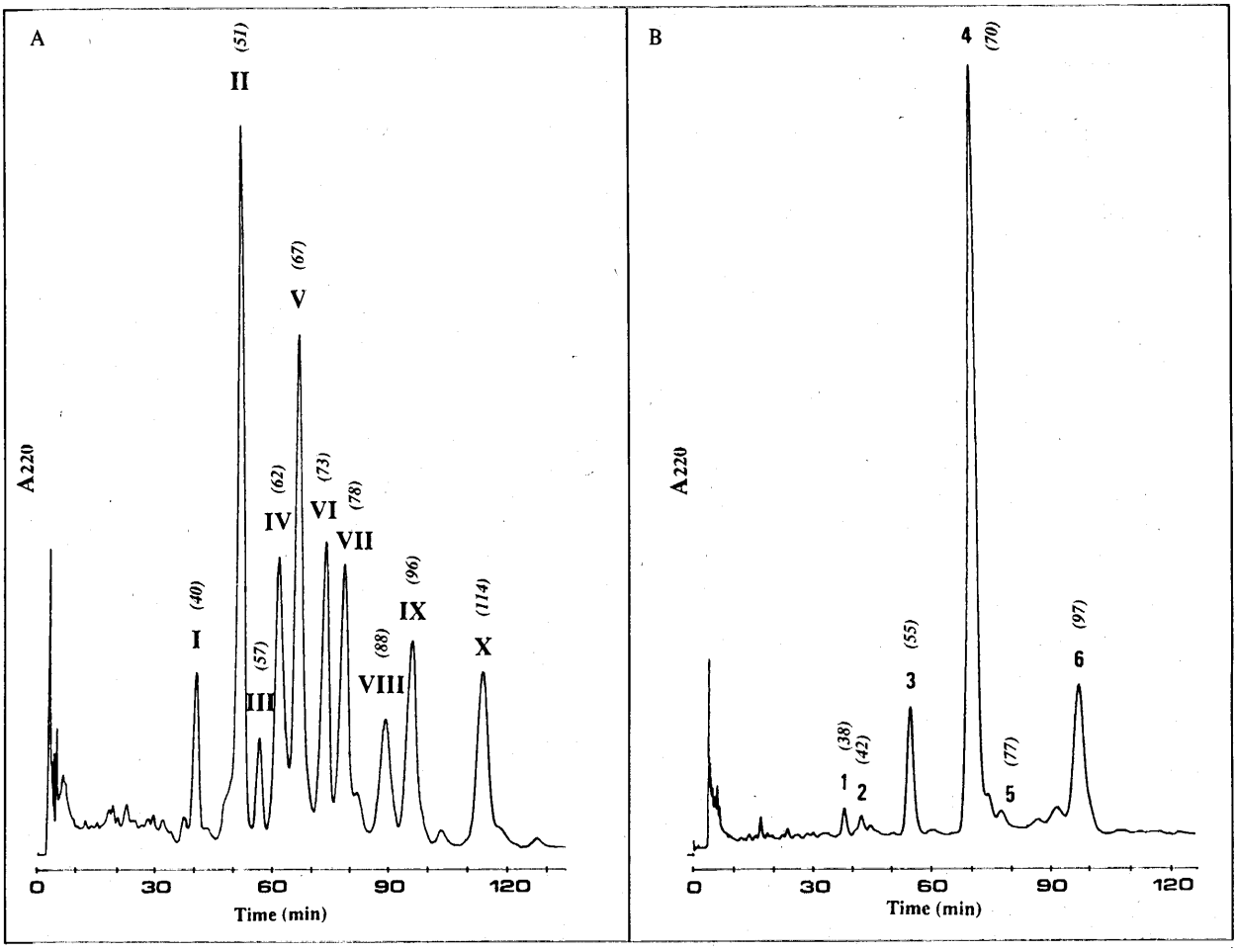
As exemplified by the two studied *Trichoderma* strains, these results give evidence that an exogenous Aib-supplied medium results in peptaibol biosynthesis modifications, leading to peptide mixture simplification. Indeed, the addition of Aib to the *T. harzianum* strain cultures led to the almost exclusive biosynthesis of a single peptide, in which all Iva residues have been replaced by Aib.

## Charged Amino Acid-directed Biosynthesis of Peptaibols

In a second step, the feasibility of incorporating charged amino acids into peptaibol sequences has been investigated by examining the effects of adding glutamic acid and arginine to *T. longibrachiatum* and *T. harzianum* fermentations, respectively. As the negatively charged glutamic acid naturally occurs in the LGB group of longibrachins biosynthesized by *T. longibrachiatum*, the influence of a Glu-supplied medium on the course of the longibrachin biosynthesis was examined, expecting an increased or exclusive production of acidic longibrachins LGB. Indeed, a larger production of longibrachins $LGB_E$ to the detriment of the neutral $LGA_E$ (Table 1) was observed. The composition of each peptide group was also affected by the Glu-supply, as a single peptide was synthesized inside each longibrachin group. These two peptides, designated as $LGA_E$ 1 and $LGB_E$ 1 were assigned to LGA I and LGB II, respectively (Table 2) by HPLC co-chromatography confirmed by further NMR and MS data.

The positively charged arginine is normally absent from any peptaibol sequence and the possibility of incorporation of this amino acid in a peptaibol could affect considerably the membrane and antibiotic prop-

Fig. 3. HPLC chromatograms of the trichorzins PA (A) and $PA_U$ (B), from *T. harzianum*.




Kromasil $C_{18}$ (5 $\mu$), 4.6 × 250 mm, flow rate 1 ml/minute, absorption monitored at 220 nm. Solvent system for A: MeOH - $H_2O$, 84 : 16; for B: MeOH - $H_2O$, 83 : 17.
Retention times of the peptides are given in brackets.

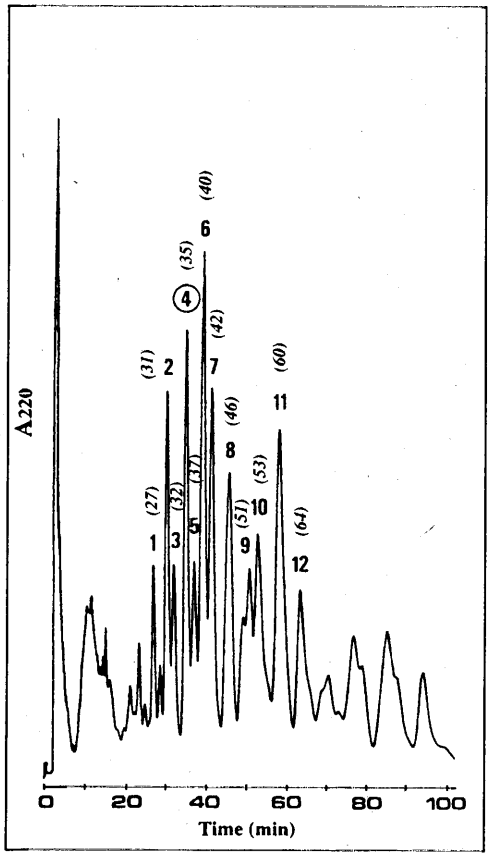
Table 4. HPLC retention times (minute) of the trichorzins PA, $PA_U$ and $PA_R$ from *T. harzianum* (M-902608).

| Peptides | PA[a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | I | II | III | IV | V | VI | VII | VIII | IX | X |
| Rt (minute) | 40 | 51 | 57 | 62 | 67 | 73 | 78 | 88 | 96 | 114 |

| Peptides | $PA_U$ | | | | | | $PA_R$[a] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 1 | 2 | 3 | 4 | 5 |
| Rt (minute) | 24[a]<br>38[b] | 26[a]<br>42[b] | 30[a]<br>55[b] | 40[a]<br>70[b] | 41[a]<br>77[b] | 57[a]<br>97[b] | 32 | 40 | 51 | 57 | 73 |

[a] Solvent system: MeOH - $H_2O$ (84 : 16).
[b] Solvent system: MeOH - $H_2O$ (83 : 17).

Fig. 4. HPLC chromatogram of the harzianins $PC_U$ from *T. harzianum*.




Kromasil $C_{18}$ (5 $\mu$), 4.6 × 250 mm, flow rate 1 ml/minute, absorption monitored at 220 nm. Solvent system: MeOH - $H_2O$, 80 : 20.

Retention times of the peptides are given in brackets.

erties of these peptides. The effect of adding arginine was studied on the *T. harzianum* strain. Upon the Arg-enrichment of the medium, *T. harzianum* only produced one peptide group, designated as trichorzins $PA_R$, showing that biosynthesis of the PC and PD groups was more strongly inhibited than with an Aib-supply (Table 3). The HPLC profile of trichorzins $PA_R$ exhibited mainly two peptides, $PA_R$ 2 and $PA_R$ 3, and three minor compounds, $PA_R$ 1, $PA_R$ 4 and $PA_R$ 5 (Table 4). A HPLC co-chromatography indicated $PA_R$ 2 and $PA_R$ 3 to be $PA_U$ 4 and PA II respectively, which was confirmed by further NMR and MS data. Similarly, the minor compounds, $PA_R$ 1, $PA_R$ 4 and $PA_R$ 5, were shown to be $PA_U$ 3, PA III and PA VI, respectively.

As observed with the Aib-supply, the adjunction of charged amino acids to the fermentation medium led to the simplification of the peptaibol mixtures. The Glu-supply favored the synthesis of acidic longibrachins, as expected from the presence of glutamic acid in their sequences. However and more surprisingly, it also affected the microheterogeneous mixture composition, leading to the biosynthesis of a single peptide inside each longibrachin class, this peptide being the same as that obtained with the Aib-supply. At last, the arginine incorporation by *T. harzianum* failed, as it only resulted in the synthesis of trichorzins PA already described and in the absence of Arg-containing peptides.

## Conclusion

The microheterogeneous mixtures of peptaibols produced by two *Trichoderma* strains, *T. harzianum* and *T. longibrachiatum* are composed of closely related sequence analogues differing from each other by amino acid substitutions, such as Aib/Iva, Glu/Gln or Trpol/Pheol at fixed positions. In the present study, we have given evidence that an exogenous amino acid supply results in simplification of the microheterogeneous peptide mixtures, as illustrated by both *Trichoderma* strains when Aib, Glu, or Arg is added to the fermentation medium. Furthermore, the amino acid supplies lead to enhanced yields of Aib-rich peptides, as unexpectedly observed for the Glu- and Arg-supplies. Our attempt to incorporate into the trichorzin sequences an amino acid normally absent from the sequences of peptaibols, such as the positively charged arginine, failed. However, a number of amino acids unknown in the sequences of cyclosporins have been previously incorporated by similar feeding experiments.[22] Further experiments are now in progress in our laboratory towards understanding the mechanisms by which the multienzymatic complexes responsible for the trichorzin and longibrachin production generate the corresponding peptide products.

## Materials and Methods

### Fermentation

The *T. longibrachiatum* (M-853431) and *T. harzianum* (M-902608) strains were obtained from the "Collection de souches fongiques du Muséum National d'Histoire Naturelle", Paris (France). Stocked as lyophilized samples, the preparations were transfered to agar slants that contained 2% w/v malt-agar at 27°C for 5 days. Fermentations were performed in Roux flasks (1 liter), each containing 170 ml of sterilized synthetic medium (glucose 0.5%; $KH_2PO_4$ 0.08%; $KNO_3$ 0.07%; $Ca(H_2PO_4)_2$ 0.02%; $MgSO_4 \cdot 7H_2O$ 0.05%; $MnSO_4 \cdot 5H_2O$

0.001%; $ZnSO_4 \cdot 7H_2O$ 0.001%; $CuSO_4 \cdot 5H_2O$ 0.0005%; $FeSO_4 \cdot 7H_2O$ 0.0001%). The exogenous supply of α-aminoisobutyric acid, glutamic acid or arginine, was 0.8%. The pH was controlled and set to 4.5. The stationary 20 liter cultures were incubated at 27°C, for 13 days in the case of *T. harzianum* and for 20 days in the case of *T. longibrachiatum*.

## Isolation

Separated from the mycelia by filtration, the filtrated broths were extracted three times with *n*-butanol. The crude extracts were evaporated to dryness. The residues were then submitted to gel chromatography over Sephadex LH20 with MeOH as eluent. Crude peptide mixtures, which contained peptides and others metabolites such as carbohydrates, were then chromatographed on silica gel (Kieselgel 60H, Merck, Darmstadt) with $CH_2Cl_2$-MeOH (9:1→3:7) as eluent. In the case of *T. longibrachiatum* cultivated on normal medium, the LGA group eluted first with $CH_2Cl_2$-MeOH (8:2), followed by LGB with $CH_2Cl_2$-MeOH (3:7); for the amino acid-supplemented media, two groups eluted in similar conditions, $LGA_U$ and $LGB_U$ for the Aib supply, $LGA_E$ and $LGB_E$ for the Glu supply (E and U designate the Glu- and Aib-supplies by the amino acid one-letter code). In the case of *T. harzianum* cultivated on normal medium, the PC group eluted first, followed by PA and PD, with $CH_2Cl_2$-MeOH (8:2); for the Aib-supplemented medium $PC_U$ and $PA_U$ groups eluted with $CH_2Cl_2$-MeOH (8:2), whereas the $PA_R$ group was only present with the Arg-supply, eluting with $CH_2Cl_2$-MeOH (8:2) (Tables 1 and 2).

## HPLC Separations

High-Performance Liquid Chromatography was carried out with a Waters liquid chromatograph (717 plus Autosampler, 600E Multisolvent Delivery System and 486 Tunable Absorbance Detector) on an analytical $C_{18}$ column (Kromasil $C_{18}$, 5 μ, 4.6 mm × 250 mm; AIT France).

Retention times of longibrachins (LGA, $LGA_U$, $LGA_E$ and LGB, $LGB_U$, $LGB_E$) and of trichorzins (PA, $PA_U$, $PA_R$) are given in Table 2 and Table 4, respectively.

*Harzianins $PC_U$*: eluent, MeOH-$H_2O$ (80:20); flow rate, 1 ml/minute. Rt (minute): $PC_U$ 1=27, $PC_U$ 2=31, $PC_U$ 3=32, $PC_U$ 4=35, $PC_U$ 5=37, $PC_U$ 6=40, $PC_U$ 7=42, $PC_U$ 8=46, $PC_U$ 9=51, $PC_U$ 10=53, $PC_U$ 11=60, $PC_U$ 12=64.

The different trichorzins $PA_U$ and $PA_R$ and harzianin $PC_U$ were further purified by repetitive HPLC on a semi-preparative $C_{18}$ column (Kromasil $C_{18}$, 5 μ, 7.8 mm × 300 mm; AIT France). Regarding the *T. harzianum* strain, three cultures were performed independently with an Aib-supply and the results were shown to be reproducible.

Acknowledgments

We thank Pr. M. F. ROQUEBERT (Laboratoire de Cryptogamie, Muséum National d'Histoire Naturelle, Paris, France) for providing the *Trichoderma* strains.

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