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PARACELSIN E, A NEW PEPTAIBOL FROM *TRICHODERMA SATURNISPORUM*

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ABSTRACT.—The structure of paracelsin E, a new peptaibol from *Trichoderma saturnisporum*, has been determined primarily by fabms. The well-known paracelsins A, B, C, and D were also found in a culture of this organism.

Fungi belonging to the genus *Trichoderma* (Hyphomycetes) have been studied extensively as potential sources of biocontrol agents (1), enzymes (2,3), and several bioactive secondary metabolites (4). In recent years, considerable effort has been directed toward the structural elucidation and investigation of the biological activity of a family of linear peptides, named peptaibols, produced by several *Trichoderma* species. Features of the peptaibols are a high content (up to 50%) of two uncommon amino acids, namely, α-aminoisobutyric acid (Aib) and isovaline (Iva); an acetylated *N*-terminal amino acid; a *C*-terminal amino alcohol, and a mol wt between *m/z* 1600 and 2000. Peptaibols exhibit varied biological activites, including antibiotic activity (5), hemolysis (6), and the uncoupling of oxidative phosphorylation (7), most of which are related to their membrane-modifying properties (8).

Among peptaibols, the paracelsins are characterized by the presence of phenylalaninol as the *C*-terminal amino alcohol, and by the specific amino acid content and sequence. The paracelsins were first isolated from cultures of *T. reseei* Simmons and found to exhibit membrane-modifing properties and antibiotic activity, particularly against Gram-positive bacteria (9). The structures of paracelsins A, B, C, and D were determined by fabms in combination with selective *in situ* hydrolysis (10). Recently, two further compounds related to paracelsins were isolated from cultures of a strain of *T. saturnisporum* Hammill and designated as saturnisporins SA II and SA IV (11). Paracelsins B and D (saturnisporins SA I and SA III) were also found in the same culture.

During the screening of isolates from various *Trichoderma* species, culture extracts from a strain of *T. saturnisporum* CBS 330.70 showed antibiotic and mycotoxic activity. Subsequent bioassay-guided purification of the culture filtrate resulted in the isolation of a new compound belonging to the paracelsin family. In this note we report the isolation of paracelsin E, the structure of which was elucidated primarily by fabms.

Paracelsins consist mainly of hydrophobic amino acids, together with one residue of proline and three of glutamine (Table 1). Although the isolation and purification of this new peptaibol was hampered by the absence of polar amino acid residues, it was possible to optimize the hplc purification procedures (11) and obtain reproducible chromatograms which showed several well-resolved peaks in the paracelsins region (Figure 1). Each peak was subsequently purified using the same hplc conditions and material suitable for fabms analysis resulted. Fabms

TABLE 1. Amino Acid Sequences of the Known Paracelsins.

Paracelsin A	AcAib-Ala-Aib-Ala-Aib-Ala-Gln-Ala-Val-Aib-Gly-Aib-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol
Paracelsin B (SA I)	AcAib-Ala-Aib-Ala-Aib-Ala-Gln-Ala-Leu-Aib-Gly-Aib-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol
Paracelsin C	AcAib-Ala-Aib-Ala-Aib-Aib-Gln-Aib-Val-Aib-Gly-Aib-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol
Paracelsin D (SA III)	AcAib-Ala-Aib-Ala-Aib-Ala-Gln-Ala-Leu-Aib-Gly-Aib-Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol
Saturnosporin SA II	AcAib-Ala-Aib-Ala-Aib-Ala-Gln-Ala-Leu-Aib-Gly-Aib-Aib-Pro-Val-Aib-Iva-Gln-Gln-Pheol
Saturnosporin SA IV	AcAib-Ala-Aib-Ala-Aib-Aib-Gln-Aib-Leu-Aib-Gly-Aib-Aib-Pro-Val-Aib-Iva-Gln-Gln-Pheol
Paracelsin E	AcAib-Ala-Aib-Ala-Aib-Ala-Gln-Ala-Leu-Aib-Gly-Aib-**Ala**-Pro-Val-Aib-Aib-Gln-Gln-Pheol

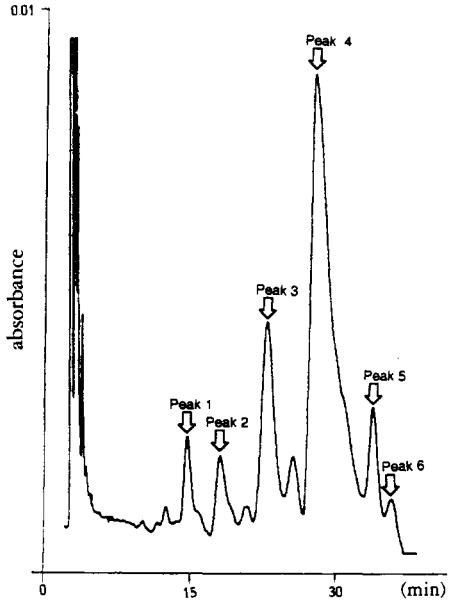

FIGURE 1. Hplc chromatogram of the natural mixture of paracelsins purified as described in the Experimental using a Bio-sil C_{18}, 4.6×250-mm (Bio-Rad) column and uv detection at 205 nm. Elution was performed isocratically at a flow rate of 1 ml/min with MeOH-H_2O (86:14) for 28 min, then the MeOH concentration was increased up to 90% in 9 min. Peaks 1 and 2, mixture of unidentified paracelsins; peak 3, paracelsin E; peak 4, paracelsins A and B; peak 5, paracelsin C; peak 6, paracelsin D.

data showed that the main peak (R_t 29 min) contained a mixture of paracelsins A and B; while paracelsins C and D were found in peaks 5 and 6. On the other hand, the spectra of the first two peaks (R_t 15 and 18 min) showed that a mixture of peptaibols with undescribed nominal monoisotopic relative molecular mass was present. The peak that eluted with R_t 23 min (i.e., peak 3) was highly enriched in a new metabolite, paracelsin E.

This peptide showed a fragmentation pattern very similar to that of paracelsin B, but had a nominal monoisotopic relative molecular mass of *m/z* 1908, the same as that of paracelsin A (Table 1).

The first structural difference between paracelsins A and E was revealed by the presence in the spectrum of an ion peak at *m/z* 1064. This ion peak is due to substitution of the expected Aib in position 13 with an Ala (and a loss of 71 mass units instead of 85 mass units). Hence the ion peak at *m/z* 1050 was not detected. The remaining 12 residue-*N* terminal sequence is identical to that of paracelsin B while the last eight residues are common for the three paracelsins A, B, and E. Paracelsins E and B differ from paracelsin A in the presence of a Leu instead of Val at position 9 (Table 1).

The fabms for all previously known paracelsins show a diagnostic peak at *m/z* 774. This was also the case for paracelsin E and is due to the Pro residue at position 14, which provides a characteristic fragmentation point in this class of peptaibols. This evidence confirmed that paracelsin E has the same *C*-terminal sequence as that of the previously described paracelsins (Pro-Val-Aib-Aib-Gln-Gln-Pheol; *m/z* 774).

In summary, the unequivocal molecular mass assignment of the new paracelsin, together with the fragmentation pattern of its fabms spectrum, similar to those of known analogues, provided strong evidence for the following sequence: AcAib-Ala-Aib-Ala-Aib-Ala-Gln-Aib-Leu-Aib-Gly-Aib-Ala-Pro-Val-Aib-Aib-Gln-Gln-Pheol.

In addition to paracelsin E, at least three other analogues of the paracelsins

were present in culture extracts of *T. saturnisporum* CBS 330.70, but their sequences remain uncertain as they were always recovered as mixtures. This observed microheterogeneity of the peptaibols due to single and multiple exchange of amino acids in the peptide sequence is a characteristic feature of these compounds and arises from their nonribosomal biosynthesis (13).

Thus far, studies of biological activity have not shown significant differences between the various paracelsins. However, an in-depth study of structure-activity relationships will be carried out when sufficient pure materials are available.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—All solvents were of spectral grade. Fabms were recorded on a VG Analytical ZAB-SE double-focusing mass spectrometer fitted with a VG cesium gun operating at 25 keV (2 μA). Samples were dissolved in 5% HOAc and loaded onto a glycerol-coated probe tip; thioglycerol was added to the matrix just before the introduction of the probe into the source. Amplification of the electric signal was decreased during the magnet scan according to the intensity of the mass signals observed on the oscilloscope; mass spectra were recorded on uv-sensitive paper and counted manually. Tlc analyses were performed using precoated Si gel $60F_{254}$ plates and the spots were visualized by exposure to uv light and/or to I_2 vapor. Hplc analyses were obtained using a Dionex pump and LDC 3100 uv detector.

FUNGAL STRAIN AND CULTIVATION.—The fungal strain used in this work was *Trichoderma saturnisporum* CBS 330.70, originally isolated from forest soil in the state of Georgia. After reisolation from a single germinated conidium, the fungus was deposited in the collection of the Istituto Tossine e Micotossine da parassiti vegetali, CNR, Bari, Italy, under accession number ITEM 1325. For the production of paracelsins, the fungus was grown in still liquid culture on Adams-Hanson medium (AH: glucose 25 g, NH_4 tartrate 2 g, NH_4Cl 2 g, KH_2PO_4 2 g, $M_gSO_4.7H_2O$ 1 g, $FeSO_4.7H_2O$ 0.01 g, distilled H_2O 1 liter; adjusted to pH 6) (14). Roux bottles containing 100 ml of autoclaved AH were inoculated with a conidial suspension of the fungus. Cultures were incubated at 25° in the dark for 14 days and then filtered through filter paper (Whatman No. 1).

EXTRACTION AND ISOLATION.—The culture filtrates (1.8 liters) were lyophilized and then redissolved in distilled H_2O (200 ml) and partitioned with EtOAc (100 ml×4). The organic layers were combined, then dried over Na_2SO_4, and concentrated under reduced pressure. The oily residue (1.61 g) was chromatographed by gel filtration on Sephadex LH-20 eluted with MeOH and the column was monitored by tlc in comparison with paracelsin standard (Calbiochem, San Diego, CA) [$CHCl_3$-MeOH-H_2O-HOAc (65:25:4:3) solvent system A]. Fractions 10–16 were combined and solvent was removed *in vacuo* to give a residue of 51.5 mg with R_f 0.8 [solvent system A].

In addition, an aliquot of the aqueous phase (100 ml) was directly loaded onto a 600×10-mm column packed with Amberlite XAD-2. The column was eluted with H_2O and then with MeOH. Paracelsin-containing fractions were concentrated to yield 73.1 mg of material that exhibited a single spot at R_f 0.8 in solvent system A. This material was used for further hplc purification because it was purer than that obtained using LH-20 gel filtration. The purification process was monitored by testing the fractions on brine shrimp larvae and *B. megaterium*.

The 73.1-mg aliquot of the peptaibol mixture was purified further by prep. tlc developed with solvent system A. The Si gel was scraped off and the paracelsins dissolved in solvent system A; this procedure yielded 24.1 mg of residue which was subjected to reversed-phase hplc using a Biosil C_{18}, 4.6×250-mm column (Bio-Rad) and uv detection at 205 nm. Elution was performed isocratically with a flow rate of 1 ml/min using MeOH-H_2O (86:14) for 28 min, then the MeOH concentration was increased to 90% over 9 min.

Paracelsin E.—Fabms (25 keV) mol wt and relative intensities of the *N*-terminal fragment ions *m/z* $[M]^+$ 199 (50), 284 (100), 355 (75), 440 (80), 51 (40), 724 (15), 837 (10), 922 (25), 979 (7), 1064 (10), 1135 (25), 1908 $[M+H]^+$; mol wt and relative intensity of the *C*-terminal fragment ion *m/z* 774 (10).

BIOLOGICAL TESTING.—Bioactivity of the fractions obtained during the purification process was assayed on brine shrimp larvae (*Artemia salina* L.) (12). Assays were performed in cell culture plates (Corning) with 24 wells containing about 30–40 shrimp in 500 μl sea water and 1% MeOH solution of the purification fractions per well. The number of dead shrimp was recorded after incubation at 27° for 36 h and the percentage mortality calculated.

The antibiotic activity of the fractions was assayed with the Gram-positive bacterium *Bacillus megaterium* by the following procedure. Bacteria were incubated for 24 h at 26° in a test tube containing potato-dextrose-agar (PDA) and then suspended in 4 ml of sterile distilled H_2O. Aliquots

of 0.5 ml from this suspension were used to inoculate 3 ml of melted (45°) sterile soft H_2O/agar medium (0.7% agar) that were subsequently poured into Petri dishes containing 8 ml of PDA. The MeOH fractions were adsorbed on 6-mm concentration disks (Difco) and after solvent evaporation the disks were placed onto the substrate. Antibiotic activity was evaluated after 24 h by the bacterial growth inhibition halo.

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