CELL-FREE SYNTHESIS OF THE DEPSIPEPTIDE BEAUVERICIN

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The enzymatic formation of the cyclodepsipeptide beauvericin was demonstrated in cell-free extracts from *Beauveria bassiana*. In analogy to the enniatin synthetase system formation of beauvericin is strictly dependent on the presence of the constituent amino and hydroxy acid, *S*-adenosylmethionine, and ATP/Mg^{2+}.

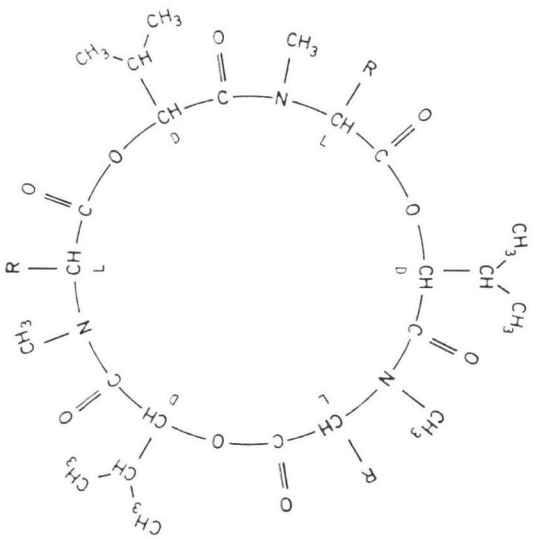
Synthesizing activity could be enriched about 12-fold by fractional ammonium sulfate precipitation. Besides the enniatin synthetase system this represents another example of the cell-free synthesis of a depsipeptide from eucaryotic origin.

Beauvericin is a cyclodepsipeptide with insecticidal properties produced by certain strains of the fungi *Beauveria bassiana* and *Paecilomyces fumosoroseus*[1,2,3]. It is composed of three residues each of *N*-methyl-L-phenylalanine (MePhe) and D-2-hydroxyisovaleric acid (HYIV), and thus may be regarded as a homologue of the ionophoric antibiotics enniatin A~C (Fig. 1).

The biosynthesis of the enniatins has already been elucidated[4]. The multienzyme, enniatin synthetase, catalyzes the formation of the depsipeptide in the presence of the substrate amino acids and D-2-hydroxyisovaleric acid, *S*-adenosylmethionine (SAM) and ATP, involving several activation, methylation and condensation reactions, similar to the non-ribosomal formation of peptide antibiotics like gramicidin S[5].

Fig. 1.

Beauvericin, R=$-CH_2C_6H_5$; Enniatin A, R=$-CH(CH_3)CH_2CH_3$; Enniatin B, R=$-CH(CH_3)_2$; Enniatin C, R=$-CH_2CH(CH_3)_2$.



In view of the close structural relationship between beauvericin and the enniatins we wondered whether the biosynthesis of beauvericin might also proceed in a manner similar to enniatin biosynthesis. A preliminary study providing ^{14}C-labelled precursors *in vivo* to *P. fumosoroseus* indicated such a similarity[6].

In this publication we describe the formation of beauvericin *in vitro* by cell-free extracts of *B. bassiana*.

Materials and Methods

Chemicals and Radioisotopes

Radioactive DL-HYIV and unlabelled D-HYIV were prepared from DL-[1-^{14}C]valine

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(specific activity: 60 Ci/mol) and D-valine, respectively, by reaction with HNO_2[7]. Specific activity of L-[*U*-^{14}C]phenylalanine was 513 Ci/mol. Radioactive compounds were purchased from Amersham. All other reagents were of highest purity commercially available.

Organism and Growth Conditions

The fungus identified as *B. bassiana* (Bals.) Vuill. was isolated from living larvae of the Australian sawfly *Lophyrotoma cyanea* (Pterigophorinae) feeding on *Eucalyptus melanophloia*. It was grown on agar slants (3% molasses, 1% cornsteep liquor, 2% agar) for 10~12 days. Spore suspensions (10^8 spores/ml) were prepared by filtration of a suspension of slant surface growth in sterile water. Submerged cultivation was carried out as described[6]. An inoculum of 10^7 spores per flask containing 100 ml cornsteep-molasses medium was used. [^{14}C]Phenylalanine incorporation into beauvericin *in vivo* was measured as described[6]. The cultures were harvested by suction filtration about 50 hours after inoculation (time of maximum *in vivo* incorporation). The mycelial cake was washed with 0.3 M KCl and stored at −80°C.

Preparation of Cell-free Extracts

All operations were carried out at 4°C. Buffers: Buffer B, 50 mM potassium phosphate (pH 7.0), 4 mM dithiothreitol, 0.25 mM EDTA; Buffer A, 0.3 M KCl in Buffer B.

Crude Extract

Lyophilized mycelium (1~2 g) was homogenized with quartz sand in a mortar and extracted with 20 ml buffer A per gram dry weight. After 20 minutes of gentle stirring the homogenate was centrifuged for 15 minutes at 2,5000×*g*.

Poly(ethyleneimine) (PEI) precipitation[5]

A neutral solution of Polymin P (BASF, Ludwigshafen, FRG) (5%) was added to give a final concentration of 0.1%. After 20 minutes the extract was centrifuged as described above.

Ammonium Sulfate Precipitation

Saturated ammonium sulfate solution in buffer B was gradually added to the supernatant. Precipitates were pelleted by centrifugation (10 minutes 20,000×*g*). The precipitate between 25 and 35% saturation was dissolved in a minimal volume of buffer B and desalted by passage through a small Ultrogel AcA 44 column previously equilibrated with buffer B.

Assay of Beauvericin — Synthesizing Activity in Cell-free Extracts

The assay mixture contained ATP (4 mM), $Mg(OAc)_2$ (4 mM), HYIV (0.2 mM), SAM (0.2 mM) and 0.5 μCi L-[^{14}C]phenylalanine in a total volume of 120 μl. After incubation for 10 minutes at 25°C, 2 ml each of H_2O and EtOAc were added to the assay mixture.

EtOAc-extractable compounds were separated by TLC on silica gel (authentic beauvericin was added as a carrier) (Merck Kieselgel 60, EtOAc - MeOH - H_2O, 100: 5: 1).

After visualisation of the beauvericin bands by iodine vapor, they were scraped off the plates and the antibiotic was eluted twice with 2 ml EtOAc. Radioactivity of the beauvericin bands was measured in a Packard Tri-Carb scintillation counter. The activity of the beauvericin-synthesizing system is either directly expressed as cpm or calculated from these data as pmol beauvericin formed taking into account the specific radioactivity of the labelled compounds applied and the counting efficiency.

Characterization of the products by two dimensional separation of acid hydrolysates was carried out as described earlier[6]. Protein concentrations were measured by a modified Bradford procedure with bovine serum albumin (Serva, Heidelberg, FRG) as a standard[9].

Results and Discussion

Cell-free extracts prepared from lyophilized mycelium of *B. bassiana* as described in "Materials and Methods" were able to synthesize beauvericin *in vitro*. Several other beauvericin-producing strains of *B. bassiana* (other isolates) and *P. fumosoroseus*, were found to be unsuitable sources for active cell-free

Table 1. Partial purification of the beauvericin synthesizing activity.
3.4 g of lyophilized mycelium were the starting material.

Purification step	Volume (ml)	Protein total (mg)	Total units*	Specific activity (units/mg)	Recovery (%)
Crude extract	88.0	1,214.0	30.8	0.025	100
PEI-precipitation	85.0	892.5	26.5	0.029	86
$(NH_4)_2SO_4$-precipitation	5.5	77.5	24.9	0.321	80.8

* 1 unit is defined as 1 pmol beauvericin formed per second.

Fig. 2. *In vitro* formation of beauvericin.
Autoradiogram of a thin-layer chromatogram (see Materials and Methods). 100 μl of enzyme (ammonium sulfate step) were incubated for 10 minutes at 25°C in the presence of: A: [^{14}C]-HYIV, ATP/Mg^{2+}, Phe, SAM; B: [^{14}C]HYIV, Phe, SAM; C: [^{14}C]Phe, HYIV, SAM, ATP/Mg^{2+}; D: [^{14}C]Phe, HYIV, SAM.

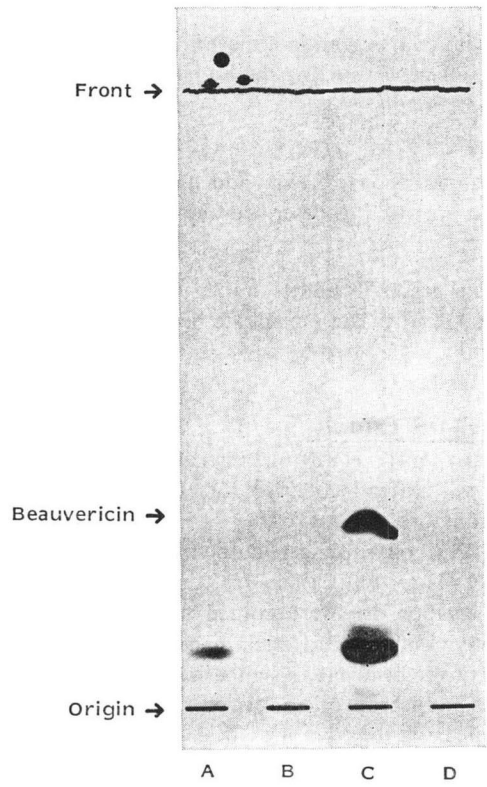
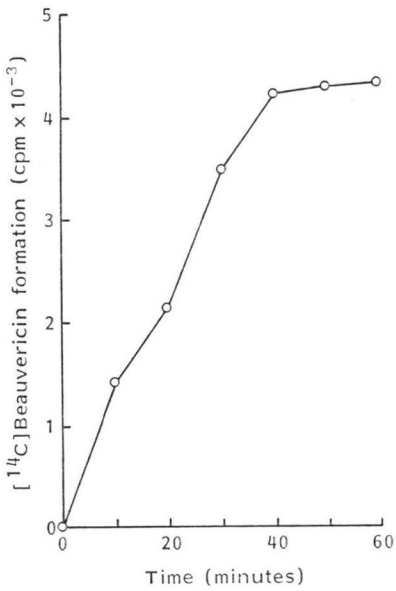


Table 2. Dependence of cell-free beauvericin synthesis on different substrates.
Assay conditions were as described in Materials and Methods with the exception of the indicated omission.

Substrates	[^{14}C]Phe (cpm)	[^{14}C]HYIV (cpm)
Complete	56,558	15,963
- ATP	725	113
- Mg^{2+}	11,992	5,951
- SAM	512	96
- HYIV	70	—
- Phe	—	75

Fig. 3. Time dependence of beauvericin formation (assay as described under Materials and Methods except that 130 μM phenylalanine was used).



extracts. Incubation of cell-free extracts with L-[^{14}C]phenylalanine or [^{14}C]HYIV in the presence of ATP, Mg^{2+}, SAM, and unlabelled HYIV or phenylalanine, respectively, yielded radioactive beauvericin and other unidentified EtOAc-extractable compounds (Fig. 2). Alternatively, [*methyl*-^{14}C]SAM could be used as the labeling substrate (not shown). *In vitro* beauvericin formation proceeded linearily for

at least 30 minutes (Fig. 3).

The beauvericin synthesizing activity could be enriched about twelve-fold by subsequent PEI and ammonium sulfate precipitations (Table 1). The experiments described below were carried out with such partially purified preparations. There was no significant loss of activity when preparations obtained after the ammonium sulfate step were stored at −80°C in buffer B containing 20% glycerol for several months.

In vitro product formation was absolutely dependent on ATP (see Fig. 3), HYIV, and L-phenylalanine (Table 2). Omission of Mg^{2+} resulted in a decrease of beauvericin formation by about 70% (Table 2). In an incubation mixture containing L-[^{14}C]phenylalanine, HYIV, magnesium acetate, and ATP but not SAM, a new radioactive product with a lower Rf value was formed instead of beauvericin (not shown), which was tentatively identified as unmethylated beauvericin. Acid hydrolysis of this compound yielded [^{14}C]phenylalanine exclusively; no [^{14}C]MePhe could be detected. This result is analogous to that obtained with purified enniatin synthetase, which catalyzed the formation of unmethylated enniatin in the absence of SAM[4].

As already shown by *in vivo* experiments with another fungus, *P. fumosoroseus*[6], free MePhe was not incorporated into the depsipeptide by active cell-free preparations from *B. bassiana*. Incubation of active preparations with [^{14}C]HYIV, ATP, Mg^{2+}, and MePhe did not yield any radioactive EtOAc-extractable compounds. Presumably, *N*-methylation is an obligatory intermediate step in beauvericin formation; only the unmethylated amino acid is accepted as a substrate for beauvericin synthesis. We conclude, that MePhe is not a free intermediate. Methylation of Phe residues is a later step in beauvericin synthesis occurring anywhere between amino acid binding to the hypothetical synthetase and release of the final product. In the case of enniatin biosynthesis, *N*-methylation of the amino acids only occurs after their activation as enzyme-bound thioesters[10].

In conclusion, the experiments reported demonstrate the cell-free formation of beauvericin. In addition to enniatin synthetase from *Fusarium oxysporum* the beauvericin system represents another example of depsipeptide synthesis *in vitro*. Further purification of the beauvericin-synthesizing activity is necessary to determine more closely whether the hypothetical "beauvericin synthetase" has additional features in common with enniatin synthetase.

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