NEW INSECTICIDAL CYCLODEPSIPEPTIDES FROM THE FUNGUS
ISARIA FELINA

I. PRODUCTION, ISOLATION AND INSECTICIDAL PROPERTIES
OF ISARIINS B, C AND D

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(Received for publication May 12, 1981)

Three new cyclodepsipeptides related to the previously described isariin were isolated
from a strain of Isaria felina. They were named isariins B, C and D. Isariin D and, to a
lesser extent, isariin C, exhibited insecticidal activity against Galleria mellonella larvae, whereas
isariin B and isariin itself proved inactive.

Cyclodepsipeptides are a group of heteromeric peptides which, according to the definition given
by Russe1, “comprises a number of cyclic compounds, in which the ring is composed entirely of
residues of amino- and hydroxy-acids joined by amide and ester bonds”. Such compounds have
been isolated from true bacteria, actinomycetes and fungi. Some fungal cyclodepsipeptides exhibit
toxic properties to insects or other invertebrates, especially beauvericin from Beauveria bassiana2)
, bassianolide from the same fungus and from Verticillium lecanii3), beauvellide from B. tenella4) and
destruxins from Metarrhizium anisopliae5,6).

Among fungi producing cyclodepsipeptides are the Isaria, whose main characteristic is the pos-
session of synnemata (a synnema is a group of hyphae, joined together, generally upright and produc-
ing spores). A common situation in fungal taxonomy, the name Isaria is fairly ambiguous. Most
of the so-called fifty “species” which had been described by mycologists are only conidial stages of
various fungi; in such cases, the name “Isaria” has no taxonomic meaning, but only a morphological
one. However, the latest review of these fungi (by De Hoog7 in 1972) showed that two Isaria could
not find a place in other genera and therefore are likely authentic species: Isaria felina (DC per Fr.)
Fr. and I. orthopterorum Petch. Since they seem to have no perfect stage, they are regarded as Fungi
imperfecti (order Moniliales and family Stilbaceae).

I. felina, under the synonymic denomination of I. cretacea van Beyma, was the subject of physio-
logical and chemical investigations by Taber and Vinin from 1957 to 1963. They isolated from
this fungus a new cyclodepsipeptide, isariin, to which they assigned the structure of Fig. 18), i.e.
a pentapeptide cyclized through a molecule of β-

hydroxydodecanoic acid.

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A study by Wolstenholme and Vining9) of the amino acids sequence by mass spectrometry brought in 1966 additional evidences of this structure, which was definitely confirmed when synthesis of isariin was achieved in 1972 by Okada et al.10) and in 1974 by Hardy et al.11)

On the basis of variations they had observed in the ratios of the amino acids in isariin hydrolyzates, Vining and Taber had anticipated the presence of other isariin-like cyclodepsipeptides, but they failed in their attempt to isolate them by paper chromatography.

On the other hand, another Isaria belonging to a “new” species (but which was neither described nor named, and proved later to be a Beauveria tenella4) gave in 1966 to Briggs et al.12) a mixture of three “isarolides” which were not separated; from a complex mass spectrometry study of that mixture, the authors concluded that isarolides were cyclodepsipeptides with a β-hydroxyundecanoic acid unit, and a tripeptide containing isoleucine, phenylalanine and valine (isarolides A and B), or only the first two amino acids (isarolide C).

Concerning the biological properties of the cyclodepsipeptides of Isaria, Taber and Vining13) showed in 1963 that “antibiotic factors” active against Staphylococcus aureus and Candida albicans are synthetized by a variant of I. cretacea which does not produce synnemata (“strain B”); two of these factors gave on hydrolysis several amino acids and unidentified acidic products, which suggested to the authors that they were presumably cyclodepsipeptides related to isariin. Neither the latter nor the “isarolides” seem to have been subjected to biological investigations, especially regarding a possible insecticidal activity.

In consideration of these biological as well as chemical imprecisions, we decided to undertake a new study of the cyclodepsipeptides of Isaria felina, a strain of which we had isolated several years ago.

Results

Our strain of Isaria felina produces an abundance of synnemata on various solid media, especially on carrot agar. In agitated liquid media (in conical flasks on a rotary incubator, or in a fermentor), growth is rapid (particularly at 30°C), and leads to the formation of spore-producing mycelial pellets mixed with very numerous free conidia; the whole biomass can be collected by centrifugation.

Preliminary tests confirmed the already-known absence of depsipeptides in the supernatant. In other respects, their yield from a submerged-grown biomass proved not to be higher than that afforded by synnemata; sometimes it was even lower. For the purpose of obtaining small quantities of pure depsipeptides, we therefore decided in favour of solid media. However, a liquid medium derived from “Waksman’s glucose broth”14) proved to be an amelioration compared with other liquid media, and should allow a production by fermentation in case of need.

An ethanolic extract of fresh synnemata contains, besides usual substances like mannitol and ergosterol, four major compounds detectable by TLC and HPLC: I, II, III and IV in the order of decreasing Rfs on TLC plates. They were obtained as partially purified substances by PLC, and their purification was completed by semipreparative HPLC, yielding four crystallized compounds. Further structural studies5) showed that I was identical with Vining and Taber’s isarin, and that II, III and IV were novel isarin-like cyclodepsipeptides, which therefore were named isariins B, C and D.

Insecticidal properties of these four depsipeptides were investigated using as the test-insect the greater wax moth Galleria mellonella, as in similar investigations5-19). Tests were carried out on larvae just before pupation. Ethanol and Tween 80 helped to dissolve the depsipeptides in water, in
which they are nearly insoluble.

Two techniques were used; intrahemocoelic injection and topical application. In the former, doses (10 and 20 µg per larva) were chosen on the analogy of the works on beauvericin from *Beauveria bassiana*<sup>18</sup> and on destruxin from *Metarrhizium anisopliae*<sup>19</sup>. For the latter technique, the dose of 400 µg per insect was similar to that at which the toxin of *M. anisopliae* is active on mosquito larvae<sup>20</sup>.

Injection of 10 µg per larva induced no effect. At doses of 20 µg and whichever depsipeptide was tested, the following symptoms were observed; the larvae became flaccid within five minutes, then paralysis reached the prolegs and finally the whole insect. But these recovered sufficiently within 24 hours to move slightly<sup>17</sup>; thus, *Galleria* larvae seem to be able to detoxify isariins, as it was pointed out by ROBERTS<sup>16</sup> with destruxins.

In the case of topical application, the results which were observed appear in Table 1.

### Table 1. Insecticidal activity of isariins on *Galleria mellonella* larvae.

<table>
<thead>
<tr>
<th>Depsipeptides</th>
<th>% of mortality within</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>24 hrs</td>
</tr>
<tr>
<td>I (isariin)</td>
<td>0</td>
</tr>
<tr>
<td>II (isariin B)</td>
<td>0</td>
</tr>
<tr>
<td>III (isariin C)</td>
<td>20</td>
</tr>
<tr>
<td>IV (isariin D)</td>
<td>45</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
</tbody>
</table>

### Experimental

**Strain**

Our strain of *Isaria felina (=*I. cretacea) was collected in the course of an atmospheric survey at PAU (France) in 1964. It was preserved by transfers on malt agar or carrot agar, and exhibited no morphological changes since it was isolated.

**Culture for Depsipeptides Production**

Carrots (200 g) were ground in a Waring blendor, 20 g of agar were added and the volume was made up to 1 liter with distilled water. Immediately after sterilization (by autoclaving at 120°C for 15 minutes), the medium was poured into sterile glass vessels 20 cm in diameter so that the layer was approximately 2 cm-thick. After cooling, the vessels were inoculated by plating a suspension of spores, covered with a lid and incubated at 25°C. After 2 days, the lids were half-lifted to improve aeration, and incubation was carried on for 2 weeks, leading to a thick mycelial layer covered with synnemata about 2 cm-high.

**Extraction of the Depsipeptides**

Synnemata were harvested by scraping the cultures (approximately 60 g per batch, fresh weight), suspended into 200 ml of ethanol and homogenized in a Waring blendor. A 10-minute centrifugation at 4,000 rev./minute yielded a precipitate which was re-extracted twice as above. Combined alcoholic extracts (600 ml) were filtered, dried over Na₂SO₄, concentrated under reduced pressure, filtered again to remove mannitol which precipitates on concentration, then evaporated to dryness. This residue (300 mg) was taken up by 50 ml of CHCl₃ - CH₃OH (90: 10), which were filtered and concentrated to 10 ml.

**Rough Separation of the Depsipeptides by PLC**

200 x 200 mm plates coated with 0.5 mm-thick silica gel layers (Merck 7747) received 1 ml each of the previous solution as a 190 mm-long streak, and were developed by a system of CHCl₃ - CH₃OH (92: 8). Spraying an aqueous 0.1 N iodine solution delimited 4 closely spaced bands: I (Rf 0.7), II (0.6), III (0.5) and IV (0.3). Ergosterol migrated as a fluorescent band with the solvent front. After evaporation of iodine, each band was collected and eluted in a small column with CHCl₃ - CH₃OH (90: 10). Eluates were chromatographed twice again as previously described and the final homologous solutions were pooled.
Analytical HPLC

It was carried out with a Waters chromatograph on a 30 cm × 3.9 mm Porasil C18 column, eluted with CH₃CN - H₂O (63: 37) flow rate was 2 ml/minute, pressure 1,500 psi and detection was performed at 210 nm. Injection of a mixture of the eluates of I, II, III and IV gave four major peaks. Retention time and percentage of each compound; I: 5.92 minutes, 11.5%; II: 3.02 minutes, 59%; III: 2.3 minutes, 23.1%; IV: 1.9 minutes, 6.3%.

Purification of the Depsipeptides by Semi-preparative HPLC

The eluate of each band from PLC was concentrated and injected as 50 µl aliquots; only the central part of each peak was collected. Repeating this procedure afforded, after evaporation of the combined homologous eluates, the four depsipeptides as pure crystalline compounds: I, 20 mg; II, 33 mg; III, 10 mg and IV, 6 mg. Melting points and optical rotations will be given in the following paper.¹⁵)

Rearing of Galleria mellonella

The insects were reared in an incubator under controlled conditions of 28°C and 60% humidity. The larvae fed on old beeswax previously disinfected by exposure to formaldehyde vapors for 15 days. The developmental cycle was approximately 45 days (incubation: 7～8 days, larvae: 25～28 days, pupae: 8～9 days, adults: 3～4 days). Compounds were tested on larvae just before pupation. Average weight of larvae was 200 mg; those weighing less than 90 mg and those spinning cocoons were rejected.

Determination of Toxic Activity

Either 10 or 20 mg of the depsipeptide dissolved in 1 ml of ethanol were mixed with 2 drops of Tween 80 and 9 ml of distilled water.

For intrahemocoelic injection, each larva received 10 µl of one of the precedent solutions, i.e. 10 or 20 µg of depsipeptide per insect. Three replicates of 10 insects each were carried out. Controls were injected with the solvent alone. Injection needle was inserted through the planta of the left member of the third pair of prolegs and the insect was pushed up until the needle’s point was in the area of the first two abdominal segments.¹⁶)

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Immediately after injection, the larvae were put under observation.

For topical application, the larvae were placed in individual cages and were directly moistened with 0.2 ml of the 2 mg/ml solution, i.e. each insect received 400 µg of the depsipeptide. Control larvae received the solvent alone. Then the larvae were returned in the incubator and observed. Four replicates of 10 larvae each were carried out.

Acknowledgements

The authors are indebted to Prof. Neuuzil and Dr. Allard for HPLC analysis; they also thank Mr. Soriano and Mr. Fondeville for technical assistance.

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