NEW INSECTICIDAL CYCLODEPSIPEPTIDES FROM THE FUNGUS *ISARIA FELINA*

II. STRUCTURE ELUCIDATION OF ISARIINS B, C AND D

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(Received for publication May 12, 1981)

Isariins B, C and D, isolated from a strain of *Isaria felina*, were shown to be cyclodepsipeptides constituted by a pentapeptide cyclized through a β-hydroxyacid. The nature of the latter and the sequence of the peptide were determined for each compound. Relations between insecticidal activity and structure of the depsipeptides were pointed out.

We reported in the precedent paper[1] the isolation of four compounds (I ~ IV) from synnemata of the imperfect fungus *Isaria felina* (=*I. cretacea*), two of which exhibited insecticidal properties against *Galleria mellonella* larvae. We now bring evidences of their cyclodepsipeptidic structure, and of identity of compound I with the previously described isariin.

Results

UV spectra of the four compounds showed end absorption. Their IR spectra were very similar. Several intense bands between 2850 and 2970 cm^{-1} were in favour of an aliphatic component. A broad band between 3030 and 3300 cm^{-1} (NH) and two bands at 1640 and 1540 cm^{-1} (CO of a secondary amide group) were characteristic of peptidic bonds. An absorption at 1740 cm^{-1} could be assigned to the CO of an ester group. All these data, which were similar to those obtained by VINING and TABER with isariin[2], were consistent with a cyclodepsipeptidic structure.

Molecular mass and empirical formula of each compound were determined by high resolution mass spectrometry and were in agreement with elementary analysis. These results, and its melting point, showed that I was presumably identical with isariin, and that the other three compounds were closely related to it. This was confirmed by the study of the peptide and hydroxyacid of all four compounds.

Vigorous acid hydrolysis yielded the same four amino acids: glycine, alanine, valine and leucine. The latter was shown to be of the D-configuration, as in isariin, whereas alanine and valine were of the L-. Quantitative analysis of the hydrolysates showed that the amino acids constituted a pentapeptide with the same composition in I and II on the one hand, III and IV on the other hand.

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The ether-extractable fraction of each acidic hydrolyzate yielded a compound whose IR spectrum was consistent with an aliphatic acid possessing a secondary alcohol group, which, taking isariin as a model, would have the structure $HOOC–CH_2–CHOH–(CH_2)_n–CH_3$, n beeing easily deduced from the molecular mass and the pentapeptide composition. This was confirmed, for all four depsipeptides, by comparing (by gas chromatography after silylation) the compound extracted from the hydrolyzate with the synthetic assumed β-hydroxyacids.

Mass spectra of the depsipeptides (electron impact, 70 eV) gave informations on their molecular ion and showed a fragmentation pattern which was consistent with the expulsion of amino acids residues (*m/z* 86, $Me_2CH–CH_2–CH=NH_2^+$, leucine; *m/z* 72, $Me_2CH–CH=NH_2^+$, valine; *m/z* 44, $Me–CH=NH_2^+$, alanine; *m/z* 30, $CH_2=NH_2^+$, glycine). Relative intensity of the other peaks was too weak for interpretation, which prevented us making hypothesis on the peptidic sequences.

On the contrary, the spectrum of the methyl ester (chemical ionization, CH_4) obtained after opening the ring of each depsipeptide by alkaline hydrolysis and methylation of the free acid, showed the ions $(M+1)^+$ and successive expulsions of amino acids residues from the methylated end of the molecule up to the aliphatic hydroxyacid, this resulting from successive fissions of the peptide bonds; for compounds I and II, a dehydration occurs before expulsion of amino acids (Fig. 1). The deduced sequences

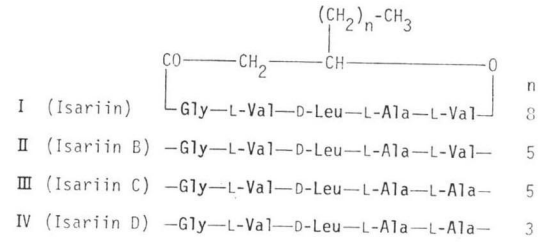
Fig. 1. Fragmentation of the methyl esters obtained from the four isariins.

							$[(M-H_2O)+1]^+$	$[M+1]^+$
I	R_1	181 Gly	238 L-Val	337 D-Leu	450 L-Ala	521 L-Val-OMe	652	670
II	R_2	139 Gly	196 L-Val	295 D-Leu	408 L-Ala	479 L-Val-OMe	610	628
III	R_3	157 Gly	214 L-Val	313 D-Leu	426 L-Ala	497 L-Ala-OMe		600
IV	R_4	129 Gly	186 L-Val	285 D-Leu	398 L-Ala	469 L-Ala-OMe		572

were confirmed through metastable transitions which were determined by linked scan (B/E) with a double focusing mass spectrometer.

Conclusions from all these data are the structures (shown in Fig. 2) of the four cyclodepsipeptides of *Isaria felina*. Identity of I with VINING and TABER's isariin is confirmed; we therefore propose to name the other three compounds: isariins B, C and D.

Fig. 2. Structure of the four isariins from *Isaria felina*.



Discussion

Isariins B, C and D contain a β-hydroxyacid, just as isariin itself. When the latter was discovered, most of the known cyclodepsipeptides were of the α-hydroxyacid type, except a few of bacterial origin (esperin, serratamolide)[2]. Since that time, new cyclodepsipeptides of the β-hydroxyacid type have been isolated, especially from fungi: beauvellide from *Beauveria tenella*[3], beauverolides H and I from *B. bassiana*[4], not to mention BRIGGS *et al.*'s "isarolides"[1], in which the structure of the acid was deduced

rather from biosynthetic analogies than from analytical evidences. The three new isariins are thus enlarging the family of the β-hydroxyacid type cyclodepsipeptides.

It seems very likely that isariins B, C and D are the compounds which had been anticipated by VINING and TABER[2]. These authors had noticed that "analysis of the components in various preparations of isariin did not yield whole numbers of amino acids. In particular, the content of alanine was frequently higher, and of valine lower, than found in samples which had been repeatedly recrystallized from aqueous ethanol". Presence of alanine instead of valine at COOH terminal position in isariins C and D throws light on that observation.

VINING and TABER had also admitted that isariin "was not the major constituent under all conditions of culture". Our results are quite consistent with that opinion, since in our strain isariin accounts for only 11.5% of the whole, far behind isariins B (59%) and C (23.1%) and preceding only isariin D (6.3%), as shown by HPLC[1].

Insecticidal properties of isariins C and D, compared with inactivity of the other two members of the group, afford an interesting problem of relations between chemical structure and biological activity. Obviously, the nature of the β-hydroxyacid is not of prime importance, since the same acid is found in isariin B (inactive) and isariin C (active). On the contrary, presence of alanine at the COOH end of the pentapeptide seems preponderant. And when this condition is realized (isariins C and D), insecticidal activity appears to vary contrary to the length of the carbon chain of the hydroxyacid.

Taking those results into account for a synthesis program of isariin-like compounds would presumably be advantageous.

Experimental

The four depsipeptides were extracted and purified as described in the precedent paper[1].

Physical Characteristics

The four compounds are nearly insoluble in water and easily soluble in methanol, ethanol or chloroform.

I (isariin): m.p.=251°C; $[\alpha]_D^{20}$ −31°; II (isariin B): 268°C, −45°; III (isariin C): 240°C, −30°; IV (isariin D): 235°C, −19°. Optical rotations were determined on a Jobin-Yvon digital polarimeter.

IR spectra were recorded on a Perkin-Elmer 157G spectrometer. (KBr, ν cm^{-1}); 3300, 3060 (w), 2950 (shoulder), 2920, 2855, 1735, 1650 (broad), 1532 (broad), 1470, 1450 (shoulder), 1380 (broad), 1190.

Mass spectra were obtained on a AEI MS 9 spectrometer at 70 eV, using direct insertion probe. Found: I: *m/z* 637.8674, $C_{33}H_{59}N_5O_7$; II: 595.7862, $C_{30}H_{53}N_5O_7$; III: 567.7320, $C_{28}H_{49}N_5O_7$; IV: 539.6778, $C_{26}H_{45}N_5O_7$.

Elementary Analysis

I:	Calcd. for $C_{33}H_{59}N_5O_7$:	C, 62.11; H, 9.32; N, 10.98%
	Found:	C, 62.3; H, 8.81; N, 11.41%
II:	Calcd. for $C_{30}H_{53}N_5O_7$:	C, 60.5; H, 8.90; N, 11.76%
	Found:	C, 60.93; H, 8.75; N, 11.53%
III:	Calcd. for $C_{28}H_{49}N_5O_7$:	C, 59.25; H, 8.64; N, 12.34%
	Found:	C, 59.12; H, 8.82; N, 12.11%
IV:	Calcd. for $C_{26}H_{45}N_5O_7$:	C, 57.88; H, 8.34; N, 12.98%
	Found:	C, 57.71; H, 8.53; N, 13.11%

Acid Hydrolysis

Each depsipeptide (20 mg) was hydrolysed for 72 hours at 110°C in a sealed tube with 6 N HCl (1 ml). The reaction mixture was diluted with water to 4 ml and extracted with ether (2×5 ml).

Amino Acids

The aqueous phase from each hydrolyzate was subjected to descending paper chromatography (Whatman No. 1, *n*-butanol - acetic acid - water, 12: 3: 5) and gave four spots which were identified with alanine, glycine, leucine and valine.

Quantitative analysis of the hydrolyzates was performed with a Technicon TSM autoanalyser. Found: Ala: 1.06 (I), 0.93 (II), 1.91 (III), 1.88 (IV); Gly: 1.06, 1.03, 1.02, 1.01; Leu: 1.00, 1.00, 1.00, 1.00; Val: 1.90, 1.87, 1.03, 1.12.

To determine the configuration of the amino acids, 3 ml of the concentrated acidic hydrolyzate of each depsipeptide were streaked on Whatman 3MM paper and chromatographed with the same mixture as above. Bands were detected by ninhydrin test on their ends and eluted by distilled water. Eluates were filtered and evaporated to dryness in tared vessels. After weighing, the residues were taken up with 6 N HCl (6 ml), and their optical rotations were determined with a Jobin-Yvon digital polarimeter. Found: $[\alpha]_D^{22}$ +11.0°, L-alanine (*c* 2.5); −15.1°, D-leucine (*c* 5); +25.3°, L-valine (*c* 2.7).

Identification of the β-Hydroxyacids

The ether extract of the acidic hydrolyzate of each depsipeptide, obtained as above, was evaporated to dryness and the resulting residue (5 mg) was silylated by trimethyl-silylimidazole in pyridine (Pierce Tri-Sil-Z, ref. 49231, 1 ml); similar procedure was applied to reference β-hydroxyacids which were synthetized for that purpose (see below). A gas chromatography study of the silylated compounds was carried out with a 5730 A Hewlett-Packard gas chromatograph (column: 15% methyl silicone SE 30 on Chromosorb W, 4 mm×200 cm; column temperature: 180°C; carrier gas: N_2, flow rate 75 ml/minute). Retention time of the silylated derivatives of the natural and synthetic homologous β-hydroxyacids were identical. Found: 11.4 minutes (acid from depsipeptide I and β-hydroxydodecanoic acid), 3.8 minutes (acid from depsipeptide II or III and β-hydroxynonanoic acid) and 2 minutes (acid from depsipeptide IV and β-hydroxyheptanoic acid).

Synthesis of Reference β-Hydroxyacids

A solution of *tert*-butyl bromoacetate (0.03 mole) and of the aldehyde CH_3–$(CH_2)_n$–CHO (0.02 mole) in anhydrous tetrahydrofuran (50 ml), was added drop by drop to a suspension of zinc powder in 50 ml of the same solvent. The mixture was heated at 75°C for 1 hour, then the THF was removed by distillation. One hundred ml of benzene were added and the mixture was refluxed for 2 hours. After concentration to 5 ml, the mixture was poured into 100 ml of ice-cold 2 M HCl, and the aqueous layer was extracted by ethyl acetate (3×30 ml). Combined organic extracts were washed with 1 M $NaHCO_3$ (4×10 ml), dried over anhydrous Na_2SO_4 and evaporated under vacuum. β-Hydroxyheptanoic acid (n=3) was obtained as an oil; β-hydroxynonanoic (n=5) and β-hydroxydodecanoic (n=8) acids were crystallized from petroleum ether. m.p.=57~59°C for the former (Ref.: 60~61°C[5]), 67~68°C for the latter (Ref.: 68~69°C[5]).

Alkaline Hydrolysis of the Depsipeptides, Methylation and Mass Spectrometry

To a solution of each depsipeptide (10 mg) in 3 ml of methanol, 1 ml of 0.1 N KOH was added and the mixture was allowed to stand at 40°C for 48 hours. After acidification with 0.1 N HCl, the solution was cooled to 0°C and the precipitate was separated, washed with water and dried. The residue was taken up in acetone and methylated by diazomethane.

Mass spectrometry of the methyl esters was carried out with a VG Micromass 70–70 F double focusing mass spectrometer.

Acknowledgements

The authors are indebted to Prof. NEUZIL and Dr. HOMBRADOS for autoanalyser determination of amino-acids; they are grateful to Mr. SORIANO and Mr. FONDEVILLE for technical assistance.

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