Cyclodepsipeptides from *Beauveria bassiana* Bals. Part 1. Beauverolides H and I

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Beauverolide H, a secondary metabolite of a strain of the entomopathogenic fungus *Beauveria bassiana* is shown to be the cyclotetradepsipeptide *cyclo*-[(*R*)-β-hydroxynonanoyl-L-phenylalanyl-L-alanyl-D-leucyl]. An impurity, beauverolide I, is the homologue derived from β-hydroxyundecanoic acid.

In 1969, Hamill and his co-workers isolated the biologically active metabolite beauvericin from the entomopathogenic fungus *Beauveria bassiana* Bals., and identified it as the regular cyclohexadepsipeptide (1).[1] Because a fungal species can produce more than one cyclodepsipeptide (peptide-lactone),[2,3] we have examined two strains of *B. bassiana*, designated UICP22 and UICP32 respectively, for other novel related compounds which might be biologically active. In a preliminary communication[4] we reported isolating mixtures which appeared to be closely related cyclotetradepsipeptides, which we termed beauverolides. We now present evidence for the structure (2) of beauverolide H,[4] isolated from UICP32.

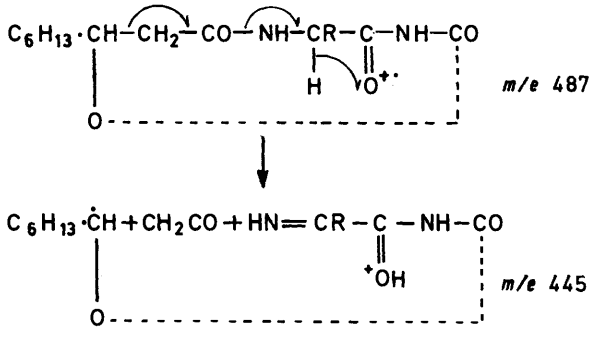
The fungus was cultured under conditions similar to those previously described,[1] and the ethyl acetate-

[1] R. L. Hamill, C. E. Higgins, H. E. Boag, and M. Gorman, *Tetrahedron Letters*, 1969, 4255.
[2] D. W. Russell, *Quart. Rev.*, 1966, **20**, 559.
[3] A. Taylor, *Adv. Appl. Microbiol.*, 1970, **12**, 189.
[4] J. F. Elsworth and J. F. Grove, *S. African J. Sci.*, 1974, **70**, 379.

soluble extract of the dried mycelium was chromatographed on basic alumina. After lipids had been eluted with benzene, benzene–ethyl acetate (3 : 1) eluted ergosterol, and the same solvent system (1 : 9) then eluted the fraction from which beauverolide H was obtained as a gelatinous precipitate, crystallising as needles, $C_{27}H_{41}N_3O_5$, $[\alpha]_D^{21}$ −11°. The high resolution mass spectrum established the molecular formula and also revealed the presence of a minor impurity, $C_{29}H_{45}N_3O_5$,[4] now designated beauverolide I.

Strong i.r. absorptions at 1 715 (lactone CO) and 1 675 cm^{-1} (amide CO) indicated the presence of a peptide-lactone structure in beauverolide H, and absorptions at 3 305 and 1 538 cm^{-1} were consistent with the presence of unsubstituted amide nitrogen.[2] An unsubstituted phenyl group was evident from bands at 750 and 700 cm^{-1}, and specific absorption in the u.v. spectrum at 250—267 nm. The ^1H n.m.r. spectrum confirmed the absence of substituents on the phenyl ring and on the amide nitrogen atoms.

Vigorous acidic hydrolysis of beauverolide H gave an equimolar mixture of L-alanine, D-leucine, and L-phenylalanine, the absolute configurations of the amino-acids having been determined spectropolarimetrically after their separation on an amino-acid analyser. Thus beauverolide H contains the three amino-acid units (4)—(6). The balance of the molecular formula, *viz.* $C_9H_{16}O_2$, is assigned to the 3-hydroxynonanoic acid residue (7) on the following grounds. From the acidic hydrolysis, an ether-soluble, laevorotatory, acidic, wax-like solid was isolated. The structure was deduced indirectly from the ^1H n.m.r. and mass spectra of beauverolide H. The integrated ^1H n.m.r. spectrum revealed four methyl signals as an unresolved multiplet near δ 0.9. Three of these can be assigned to the alanyl and leucyl residues, (4) and (5). The fourth must be terminal in the hydroxy-acid residue (7), thus implying an unbranched chain. This is confirmed by the relatively strong peak *s* at *m/e* 139 in the high resolution mass spectrum (Table 1), which on the basis of its composition is readily assigned to the ion (14). The n.m.r. spectrum contained a two-proton multiplet at δ 2.0 assigned to CH_2·CO of a β-hydroxy-acid residue. Furthermore, the relatively weak peak *c* at *m/e* 445 in the mass spectrum can best be rationalised in terms of the elimination of keten from the molecular ion (Scheme).



SCHEME A mode for the elimination of keten

The laevorotatory character of the hydroxy-acid in chloroform solution points to a 3*R*-configuration.[5] This assignment agrees with the observation that long chain β-hydroxy-acids isolated from fungal depsipeptides possess the same chirality.[2,6]

The foregoing results all point to a cyclotetradepsipeptide structure for beauverolide H. Similar structures have been proposed for isarolides A—C, a mixture isolated from a new *Isaria* sp., on the basis of acidic hydrolysis and low resolution mass spectrometry.[7] Mass spectrometry is recognised as a powerful aid in elucidating the sequence of hydroxy- and amino-acid residues in cyclodepsipeptides;[2,3] consequently we resorted to high resolution mass spectrometry to establish the structure of beauverolide H. Because of the many possible sites for ionisation and cleavage under electron impact,

[5] K. Serck-Hansenn, *Chem. and Ind.*, 1958, 1554.
[6] L. C. Vining and W. A. Taber, *Canad. J. Chem.*, 1962, **40**, 1579; W. A. Wostenholme and L. C. Vining, *Tetrahedron Letters*, 1966, 2785.
[7] L. H. Briggs, B. J. Fergus, and J. S. Shannon, *Tetrahedron*, Supp. 8, I, 1968, 269.

extensive fragmentation occurred. The significant fragment ions, together with their molecular formulae, are shown in Table 1.

The molecular ion (peak *b*) is capable of undergoing protonation, the intensity ratio $(MH)^+ : M^+$ being *ca.* 1 : 2 in this and subsequent studies.[8] Fragmentation of the molecular ion occurred in a number of ways, consistent with the thirteen-membered ring [2,3] eliminating, initially, the small species CONH (peak *d*) and CO_2 (peak *e*).[9] Briggs *et al.* suggested that the $M - 28$ peaks for the peaks *p* and *q*, and further in the piperazinedione fragment ion (13) (peak *n*), a product previously observed in the fragmentation of cyclodepsipeptides under electron impact.[9] (iii) Peaks *g* (*m/e* 416) and *h* (*m/e* 401) can be rationalised in terms of structures (8) and (9), respectively, originating from the loss of alanyl or of (alanyl + NH_2) residues from beauverolide H. (iv) The two ions assigned to peaks *t* and *u* confirm the phenylalanyl residue, and peaks *v* and *w* confirm the leucyl and alanyl residues, respectively.

TABLE 1

Structures of significant fragment ions in the high resolution mass spectrum of beauverolide H

Peak	Rel. int. (%)	Accurate mass	Molecular formula C	H	N	O	Calc. mass − obs. mass ($\times 10^3$)	Structure
a	7.2	488.308 2	27	42	3	5	−4.2	$(MH)^+$
b	19.5	487.305 4	27	41	3	5	0.8	M^+
c	1.3	445.295 4	25	39	3	4	1.4	$(M - CH_2CO)^{+\cdot}$
d	5.5	444.296 3	26	40	2	4	2.5	$(M - CONH)^{+\cdot}$
e	11.1	443.314 6	26	41	3	3	−0.1	$(M - CO_2)^{+\cdot}$
f	4.1	431.243 6	23	33	3	5	1.5	$(M - C_4H_8)^{+\cdot}$
g	5.4	416.267 8	24	36	2	4	0.3	$C_4H_9\cdot CH(\dot{N}H)\cdot CO_2\cdot CH(C_6H_{13})\cdot CH_2CONH\cdot CH(CO^+)\cdot CH_2\cdot C_6H_5$ (8)
h	5.7	401.256 5	24	35	1	4	−0.1	$C_3H_7\cdot CH{=}CH\cdot CO_2\cdot CH(C_6H_{13})\cdot CH_2\cdot CONH\cdot CH(CO^+)\cdot CH_2\cdot C_6H_5$ (9)
j	28.8	358.225 5	21	30	2	3	0.0	$C_6H_{13}\dot{C}H\cdot CH_2\cdot CONH\cdot CH(CH_2\cdot C_6H_5)\cdot CONH\cdot CH(CO^+)\cdot CH_3$ (10)
k	36.3	286.179 0	18	24	1	2	−1.7	$C_6H_{13}\cdot CH{=}CH\cdot CONH\cdot CH(CO^+)\cdot CH_2\cdot C_6H_5$, (11)
l	22.8	258.186 3	17	24	1	1	0.5	$C_6H_{13}\cdot CH{=}CH\cdot \overset{+}{C}NH{=}CH\cdot CH_2\cdot C_6H_5$, (12)
m	4.4	254.188 2	15	26	0	3	0.0	$C_4H_9\cdot \dot{C}H\cdot CO_2\cdot CH(C_6H_{13})\cdot CH_2CO^+ \xrightarrow{-H^\cdot}$ *m/e* 253 (1.3%)
n	4.7	218.106 7	12	14	2	2	1.2	$CH_3\cdot CH\cdot CONH\cdot CH(CH_2\cdot C_6H_5)\cdot CONH^{+\cdot}$, (13)
p	1.6	203.091 1	12	13	1	2	−3.5	$C_6H_5\cdot CH_2\dot{C}H\cdot CONH\cdot CH(CO^+)\cdot CH_3 \xrightarrow{-H^\cdot}$ *m/e* 202 (11.9%)
q	31.5	175.100 1	11	13	1	1	0.4	$C_6H_5\cdot CH_2\dot{C}H\cdot \overset{+}{C}NH{=}CH\cdot CH_3 \xrightarrow{-H^\cdot}$ *m/e* 174 (11.4%)
r	4.4	167.143 9	11	19	0	1	0.3	$C_8H_{17}\cdot CH{=}CHCO^+$
s	38.6	139.112 7	9	15	0	1	0.5	$C_6H_{13}\cdot CH{=}CHCO^+$ (14)
t	15.4	131.050 1	9	7	0	1	0.4	$C_6H_5\cdot CH{=}CHCO^+$
u	99.9	120.081 6	8	10	1	0	0.3	$C_6H_5\cdot CH_2\cdot CH{=}NH_2^+$
v	79.5	86.093 9	5	12	1	0	−3.0	$C_4H_9\cdot CH{=}NH_2^+$
w	100.0	44.050 0	2	6	1	0	0.0	$MeCH{=}NH_2^+$

observed in their studies on the isarolides originated from the elimination of CO.[7] However, in this and subsequent studies, the initial elimination of CO was not observed; indeed when isoleucine is present C_2H_4 can be eliminated.[8]

Beauverolide H is assigned the cyclic structure (2) on the following grounds. (i) Peak *j* is consistent with the elimination of both CO_2 and $C_5H_{11}N$, leaving fragment (10). Leucine therefore forms the ester bond with the hydroxy-acid, a proposition which is substantiated by peak *m*. (ii) Peak *k* at *m/e* 286 is consistent with the loss of the alanyl residue from fragment (10), thereby establishing the amide bond between the hydroxy-acid and phenylalanyl residues, as represented by the ion (11), which by subsequent loss of CO would give the ion (12) (peak *l*). In the absence of peaks corresponding to the fragment ions $C_6H_{13}\dot{C}HCH_2CONH\cdot CHRCO^+$ (R = Me or Bu), the sequence must be N–Phe–Ala–Leu–O. The presence of the amide bond between the phenylalanyl and alanyl residues is confirmed in the fragment ions

[8] J. F. Elsworth and J. F. Grove, unpublished results.

[9] N. S. Wulfson, V. A. Puchkov, B. V. Rozinov, A. M. Zyakoon, M. M. Shemyakin, Yu. A. Ovchinnikov, A. A. Kiryushkin, and V. T. Ivanov, *Tetrahedron Letters*, 1965, 2793; N. S. Wulfson, V. A. Puchkov, B. V. Rozinov, Yu. V. Denisov, V. N. Bochkarev, M. M. Shemyakin, Yu. A. Ovchinnikov, A. A. Kiryushkin, E. I. Vinogradova, and M. Yu. Feigina, *ibid.*, p. 2805.

A synthesis of beauverolide H is in progress.

In addition to the molecular ion at *m/e* 515.335 9 (beauverolide I), fragment ions of composition $(X + C_2H_4)$ corresponding to the major fragments *e*, *j*, *k*, *l*, and *s* (composition X) of beauverolide H were also present in the high resolution mass spectrum of crude beauverolide H. On this evidence, beauverolide I is considered to be the homologue (3) derived from β-hydroxyundecanoic acid.

EXPERIMENTAL

I.r. spectra were determined for mulls in Nujol and u.v. spectra for methanolic solutions. N.m.r. spectra were obtained at 100 MHz with tetramethylsilane as internal standard. Molecular weights and molecular formulae were recorded with a Varian CH5D (double-focusing) mass spectrometer coupled to a Varian 620L computer. Amino-acids were identified quantitatively with a Locarte amino-acid analyser. Amino-acids were separated with a Beckman 120c amino-acid analyser fitted with a preparative column. Aluminium plates coated with Merck silica gel F_{254} were used in t.l.c.; for preparative layer chromatography up to 50 mg of material was applied to glass plates (20 × 20 cm) coated with 1 mm of silica gel Merck $HF_{254+366}$, which were developed in chloroform–methanol (95 : 5). Optical rotations were measured with a Perkin-Elmer 141 spectropolarimeter (1 dm cell).

B. bassiana *Culture*.—The strain employed had been isolated from a dead moth, *Nudaurelia cythera capenensis*, from the Saasveld Forest Research Station, George, Cape Province, South Africa, and was assigned the identification number UICP 32. The strain was maintained on agar slopes.

Cultivation and Harvesting.—The following nutrient medium (pH 6) was employed: sucrose (250 g), corn steep liquor (50 ml), malt extract (100 g), casein hydrolysate (100 g), dipotassium hydrogen phosphate (20 g), and water (10 l). A portion (100 ml) of the medium was measured into each of 100 250 ml conical flasks which were heated at 121 °C for 20 min. Each flask was innoculated with 1 ml of a spore suspension of the fungus under sterile conditions, and finally the whole batch was agitated (rotary shaker at 125 rev. min^{-1}) at 25 °C for 3—5 days. The mycelium was filtered off, dried in a vacuum oven at 25 °C for 7 days, and pulverised.

Extraction and Separation.—The dry powdered extract (216 g) was covered with methanol, filtered off after 1 day, and then subjected to continuous extraction with methanol for 3 days. The combined methanolic extracts on evaporation gave a dark viscous gum (97.7 g) which was extracted repeatedly with hot ethyl acetate. The combined extracts yielded a dark oil (28.2 g). This was adsorbed onto basic alumina (Merck; 40 g; activity 3) and applied to a dry column of the same alumina (810 g; 84 × 4 cm). The polarity of the eluants was increased in stepwise manner. U.v. (366 nm) light revealed a number of distinct bands, which facilitated separation into fractions.

Beauverolide H.—Ethyl acetate–benzene (90 : 10) eluted fraction 8 (418 mg). Hot methanolic solutions of this fraction deposited a gelatinous precipitate on cooling. Repeated recrystallisation from hot methanol gave *beauverolide H* as needles, m.p. 258—259° (Found: C, 66.3; H, 8.5; N, 8.5%; M^+, 487.305 4. $C_{27}H_{41}N_3O_5$ requires C, 66.5; H, 8.5; N, 8.6%; M, 487.306 2), $\nu_{max.}$ 3 305, 1 715, 1 675, 1 632, 1 538, 1 255, 750, and 700 cm^{-1}; $\lambda_{max.}$ 250 (log ε 1.68), 257 (1.67), 263 (1.66), and 267 nm (1.65), $[\alpha]_D^{21}$ −11° [*c* 0.36 in $CHCl_3$–MeOH, (1 : 1)], δ[$(CD_3)_2SO$] 0.9 (12 H, m, 4 CMe), 1.1—1.6 (13 H, m), 2.0 (2 H, m, CH_2CO), 2.9 (2 H, d, *J* 7 Hz, CH_2Ph), 3.9—4.4 (3 H, m, CO·C*H*·NH), 4.8 (1 H, m, CH·O), 7.2 (5 H, s, Ph), and 8.3 (2 H, m, NH).

Acidic Hydrolysis of Beauverolide H.—Beauverolide H (15.2 mg) was heated in a sealed tube with 6M-hydrochloric acid (6 ml) at 105 °C for 70 h; hydrolysis was then complete. The hydrolysate was extracted with diethyl ether; washing, drying (Na_2SO_4), and evaporation of the extract gave the waxy 3-hydroxynonanoic acid (2 mg), m.p. 50—55°, $[\alpha]_D^{23}$ −7.2° (*c* 0.25 in $CHCl_3$) (lit.,[5] m.p. 50—51°, $[\alpha]_D^{21}$ −19.6°). This acid gave a negative reaction with ninhydrin.

The aqueous acidic hydrolysate was evaporated to dryness and the amino-acids in the residue were separated by preparative chromatography in an amino-acid analyser. Alanine, leucine, and phenylalanine were recovered in the ratio 1 : 1 : 1. The amino-acid was recovered from each fraction by acidifying the eluate and passing it through a short column of Zeocarb 225. The amino-acid was recovered from the column by washing it with aqueous ammonia–ethanol (1 : 3) and evaporating the solution to dryness. The residue was dissolved in hydrochloric acid (2.0 ml) and the solution clarified by centrifugation for optical rotation measurements (Table 2).

TABLE 2

	[HCl]/ M	$[\alpha]_D$ (°)	Ref.	$\alpha_{obs.}$ (°)	Recovery (%)
L-Ala	1	+17	6	+0.020	85
D-Leu	6	−13	6	−0.005	19
L-Phe	6	−4.5	10	−0.005	43

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[10] 'Handbook for Biochemistry,' ed. A. H. Sober, Chemical Rubber Co., Ohio, 1970, p. C742.