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ISOLATION OF NOVEL BEAUVERICIN ANALOGUES FROM THE FUNGUS *BEAUVERIA BASSIANA*

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ABSTRACT.—Beauvericin [**1**] and two novel cyclodepsipeptide analogues, beauvericin A [**2**] and beauvericin B [**3**], containing 2-hydroxy-3-methylpentanoic acid as one of the constituent residues, were isolated as insect toxic principles from a mycelial extract of the fungus *Beauveria bassiana*.

Cyclodepsipeptide toxin beauvericin [**1**] is known to be produced by several fungi, including *Beauveria bassiana* (Hyphomycetes) (1–8). During a screening program aimed at identifying extracts with biological activity of agrochemical interest, an isolate of *B. bassiana* showed toxic activity against mosquito larvae. Bioassay-guided separation resulted in the isolation of **1** as the most abundant bioactive component. However, all the activity could not be accounted for by **1** alone and further analysis resulted in the isolation of two novel analogues of **1**. One of these compounds was fully characterized, mostly by the analysis of its nmr data, as the cyclodepsipeptide **2** (beauvericin A).

A CH_2Cl_2 extract from the mycelial culture of *B. bassiana* showed activity when assayed against mosquito larvae at 100 ppm. Further separation resulted in an active fraction that showed the presence of three compounds by hplc analysis. Lc-ms analysis of this fraction showed that the major component had a mol wt of 783 daltons. This compound was purified and identified as **1** based on the data described below. The two minor components showed mol wts of 797 and 811 daltons, respectively. These three compounds exhibited similar uv absorption patterns indicating them to be structurally related.

Acid hydrolysis of **1** followed by tlc analysis showed the presence of one ninhydrin-positive spot that was identical to *N*-methyl-phenylalanine (Phe) obtained from the hydrolysis of an authentic sample of beauvericin [**1**]. The spectral data of **1** were in complete agreement with the reported data for this compound (1,2). Final confirmation of the identity of **1** as beauvericin came from a direct comparison (tlc, hplc) with an authentic sample. Compound **1** is a cyclic trimeric ester of *N*-methylphenylalanyl-α-hydroxyisovaleryl amide.

Acid hydrolysis of **2** followed by tlc analysis showed the presence of a single ninhydrin-positive spot that was chromatographically identical with *N*-methyl-phenylalanine. Compound **2** is a depsipeptide, as indicated by the presence of ir absorptions at 1742 (ester carbonyl) and 1659 (amide carbonyl) cm^{-1}. The mol wt difference between **1** and **2**, and the higher lipophilicity of the latter indicated the presence of an extra methylene group in **2**. The hrfabms of **1** showed a protonated molecular ion at *m/z* 798.4311, which indicated an elemental composition of $C_{46}H_{60}N_3O_9$. One of the major fragment ions, indicating a loss of a $C_6H_5CH_2$ unit, was observed at *m/z* 706.3712, which corresponded to an elemental composition of $C_{39}H_{52}N_3O_9$. The presence of only one amino acid in **2** suggested that this extra methylene could only be a part of one of the α-hydroxyisovaleric acid (Hiv) units in **1**. The two alternative, biosynthetically rational structures would include either a unit of 2-hydroxy-3-methylpentanoic acid (de-

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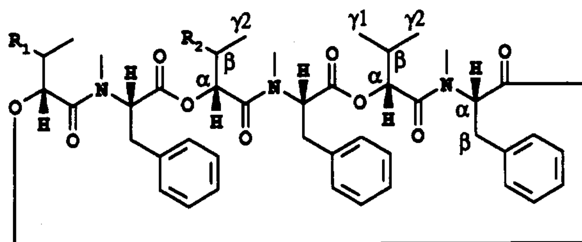

1 $R_1=R_2=CH_3$
2 $R_1=CH_2(\gamma1)CH_3(\delta)$, $R_2=CH_3$
3 $R_1=R_2=CH_2CH_3$

rived from the isoleucine biosynthetic intermediate α-keto-β-methylvaleric acid) or a unit of 2-hydroxy-4-methylpentanoic acid (derived from the leucine biosynthetic intermediate α-keto-isocaproic acid) (9). Compound **2** has 2-hydroxy-3-methylpentanoic acid (Hmp) as one of the components that was confirmed by analysis of its nmr data.

The ^{1}H-nmr spectrum of **2** in $CDCl_3$ showed similarities to that of **1** in terms of the observed chemical shifts. Because of the dissymmetry introduced in the molecule by the presence of a new α-hydroxy acid residue, the nmr data showed an additional set of signals. The four methyl doublets from the two Hiv units resonated at 0.43, 0.45 (both shielded), 0.76, and 0.77 ppm. The methyl doublet from the Hmp residue resonated at 0.81 ppm. The expected triplet from the terminal methyl, however, appeared as a multiplet because of the overlap with the γ1-methylene resonance. A possible explanation for the unusual high-field absorption for these methylene protons (0.7 ppm, multiplet) is due to the aromatic shielding from the phenyl ring of the adjacent Phe residue. It is noteworthy that the Hiv methyls in **1**, which is a symmetrical molecule, resonated at 0.42 (shielded) and 0.79 ppm.

The other salient features of the ^{1}H-nmr spectrum were the presence of *N*-methyl resonances at 2.95 and 2.99 ($2\times CH_3$) ppm. The benzylic methylene protons resonated as multiplets at 2.99 and 3.35 ppm. The two α-protons from the Hiv units resonated as doublets at 4.93 and 4.96 ppm, and the doublet from the α-proton of the Hmp residue was at 5.04 ppm. The presence of this doublet was a direct evidence of the presence of the Hmp residue as opposed to the alternative 2-hydroxy-4-methylpentanoic acid where the α-proton would be expected to be a triplet. All the multiplicities were confirmed by a HOM 2D *J*-resolved experiment.

The ^{13}C-nmr data for **2** showed the presence of essentially two sets of signals. Signals at 11.31 (δ) and 14.34 (γ2) ppm were ascribed to the two methyls of the Hmp residue. The methyls from the two Hiv units resonated at 17.54 and 18.29 ppm ($2\times CH_3$ each). The signal at 24.47 ppm was assigned to the γ1-methylene of the Hmp unit. The signal from the β-methine carbon of the Hmp residue occurred at 35.81 ppm, and the β-methine carbon of the Hiv units resonated at 29.7 ($2\times CH$) ppm. The α-methine carbons of the Hiv residues were at 57.52 ($2\times CH$) ppm, and that of the Hmp residue was at 74.22 ppm. Except for the carbonyls, the signals from the Phe residues were also split. *N*-Methyl carbons resonated at 32.43 ($2\times CH_3$) and 32.37 ppm. The α-carbons were at 57.52 ($2\times CH$) and 57.35 ppm, and the β-methylene carbons showed chemical shifts of 34.68 ($2\times CH_2$) and 34.83 ppm. All the multiplicities were confirmed by DEPT analysis.

A COSY experiment revealed the presence of three isolated spin-systems in the molecule. The α-protons of Phe resi-

dues (multiplet at 5.43 ppm) showed a connectivity with the β-protons at 3.35 and 2.99 ppm which, in turn, were mutually coupled. The α-protons from the Hiv units (doublets at 4.93 and 4.96 ppm) were coupled to the multiplet at 2.04 ppm (β-C*H*), which were then coupled to methyl doublets at 0.43, 0.45, 0.76, and 0.77 ppm. The Hmp α-proton doublet at 5.04 ppm showed direct connectivity to the methine multiplet at 1.78 ppm, which, in turn, was coupled to the multiplet centered at 0.7 ppm (γ1-CH_2) and the doublet at 0.81 ppm (γ2-CH_3). All the direct carbon-proton connectivities were observed in a HETCOR experiment. Of particular significance were the connectivities between the carbons at 11.31 (δ-CH_3 of Hmp residue) and 24.47 ppm (γ1-CH_2 of Hmp residue) with the proton multiplet centered at 0.68–0.7 ppm, thereby confirming the above assignments.

Support for the assigned structure also came from the comparison of the reported ^{13}C-nmr chemical shifts for leucine (10) and isoleucine (11) side-chains in small peptides with the assigned chemical shifts for **2**. The ^{13}C-nmr data as shown in Figure 1 further substantiated the proposed structure of **2**.

In compound **1**, it has been established that the absolute stereochemistry at the α-carbon of Phe is *S* and the α-carbon of Hiv is *R* (2). Conformationally, **1** is a stable, spherical molecule because of its cyclic nature and alternating residues with *R* and *S* absolute configuration (12,13). In accordance with the structural analogy between **1** and **2**, it is safe to assume that the absolute stereochemistry for the Phe and Hmp residues in **2** is also *S* and *R*, respectively. It has also been suggested that the biosynthesis of **1** involves a multienzyme complex known as beauvericin synthetase (14,15). The relatively nonspecific nature of multienzymes in their substrate requirement usually results in the production of secondary metabolites with analogous structures (16). The presence of **2** in *B. bassiana* supports this hypothesis, indicating that during the biosynthesis, one unit of Hiv was substituted with a Hmp residue. The presence of the Hmp residue results in the introduction of an extra chiral center at the β-carbon. Apparently, this residue is biosynthetically derived from the intermediates involved in the isoleucine biosynthesis, indicating the absolute stereochemistry at the β-carbon as *S*.

The other minor metabolite **3** (beauvericin B) could be isolated only in small amounts. Ir absorptions at 1739 (ester carbonyl) and 1650 (amide carbonyl) cm^{-1} indicated that it is a depsipeptide. The mol wt (*m/z* 811) suggested it to be a higher analogue of **2**. A comparison of the ^{1}H-nmr data of **3** with those of **2** indicated that **3** contained two units of Hmp. The salient features of the data were as follows. The *N*-methyls from the Phe residues resonated at 2.94 (2×CH_3) and 2.98 ppm. Interestingly, in **2**, the two-methyl singlet was at 2.99 ppm (from the Phe residues adjacent to Hiv) and one methyl singlet was at 2.95 ppm. Similarly the two α-proton doublets for the Hmp residues appeared at 4.99 and 5.01, respectively, and the single α-proton from the Hiv residue was observed at 4.9 ppm. The β-proton of the Hiv residue reso-

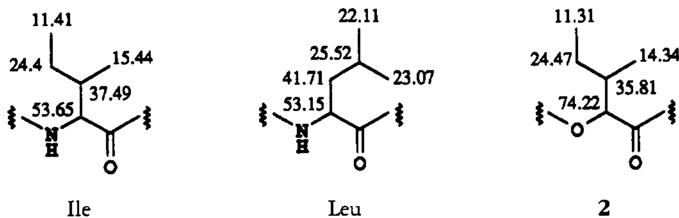

FIGURE 1. Selected ^{13}C-nmr chemical shifts for isoleucine, leucine, and **2** (Hmp residue).

nated at 2.01 ppm and the two β-protons of the Hmp residues had a chemical shift of 1.73 ppm. The only shielded methyl from the Hiv unit resonated at 0.4 ppm, and the γ1-methylenes and δ-methyls of the Hmp residues were observed as a multiplet centered at 0.65 ppm. Methyl doublets at 0.73, 0.75, and 0.76 ppm represented the rest of the methyl signals. Based on the above evidence, the compound was assigned structure **3** as shown. On the basis of the same reasoning as discussed for **2**, the absolute configuration for the Phe and the Hmp residues was assigned as *S* and *R*, respectively, and the chirality at the β-carbon of the Hmp residues was assigned as *S*.

Compound **1** and the structurally related enniatins (17) are cationophoric bioactive cyclodepsipeptides which have been isolated from different biological sources. In enniatins A–C, the Phe residue of **1** is replaced by *N*-methylisoleucine, valine, or leucine, respectively. However, in all the related compounds reported so far, the α-hydroxy acid unit has always been Hiv. The identification of **2** and **3** from *B. bassiana* represents the first report of natural products in this group of depsipeptides containing Hmp as one of the constituent residues.

Beauvericin [**1**] has been reported to be toxic to mosquito larvae (18). A quantitative estimation of the toxicity on mosquito larvae was made by bioassaying a dosage series of **1** and **2** (19–21). Probit analysis (22) of the observed data gave LC_{50} values of 26 ppm (95% fiducial limits, 17–47 ppm) for **1** and 31 ppm (95% fiducial limits, 20–64 ppm) for **2**.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Nmr spectra were measured on a Varian Gemini-300 spectrometer (300 MHz for ^1H and 75 MHz for ^{13}C) and the chemical shifts are reported in ppm. Either TMS or residual solvent signal was used as internal standard. Ir spectra were obtained on a Perkin-Elmer 1600 series Ft-ir spectrophotometer. Uv absorptions were recorded on a Perkin Elmer diode array detector 235C on-line with a hplc system. Low-resolution mass spectra were obtained on a Finnigan MAT SSQ-710 quadrupole system and lc-ms data were obtained under thermospray or particle beam mode. High-resolution mass spectra were obtained under fab conditions. Flash chromatography was conducted on J.T. Baker Si gel (30–70 μm). Thin-layer chromatograms were run on Whatman LHPKF glass coated plates with fluorescent indicator. Analytical hplc was performed on a Phenomenex IBSIL C_{18} column (4.6×300 nm, particle size 5 μm), and preparative separations were done on a Macherey Nagel semiprep Nucleosil C_{18} column (10×300 mm, particle size 7 μm).

BIOASSAYS.—A mosquito larvae assay (*Aedes aegypti*) was performed as described in the literature (20,21). Test samples were dissolved in MeOH (100–200 μl) and added to 10 ml distilled H_2O in plastic cups. Ten fourth-instar larvae were then transferred to the cups. Controls with distilled water and methanol were similarly treated. The cups were covered and kept at ambient temperature. Mortality was assessed at 24 h. Only those results were used for calculations where all the controls survived. LC_{50} values were calculated using probit analysis.

FUNGAL MATERIAL.—*Beauveria bassiana* was isolated as a contaminant from a fungal culture obtained from the USDA-ARS collection of entomopathogenic fungi located at Ithaca, New York. The isolate has been redeposited with the collection and has been assigned the ARSEF No. 4122.

EXTRACTION AND ISOLATION.—The fungus was maintained on Sabouraud Dextrose agar slants at 5°. For liquid culturing, a preculture was prepared in 2×100 ml Erlenmeyer flasks (100 ml medium, Sabouraud Dextrose Broth, Difco) and was allowed to grow on a rotary shaker (120 rpm) at 22° for 3 days. This was then used to inoculate 5 liters of medium (50×250 ml Erlenmeyer flasks) which was allowed to grow on a rotary shaker (70 rpm) for 10 days. The culture was harvested by filtration through several layers of cheese cloth and the mycelial mat was soaked in MeOH overnight. After blending, the solvent was filtered and the clear filtrate was evaporated *in vacuo*. The residue was suspended in H_2O and extracted with CH_2Cl_2. The combined extract (364 mg) was loaded on a flash Si gel column in CH_2Cl_2 and eluted with increasing proportions of MeOH in CH_2Cl_2. Bioassay of the pooled fractions revealed that the fraction eluted with CH_2Cl_2-MeOH (98:2) (104 mg) contained all the activity. Analytical hplc of this fraction (CH_3CN-H_2O linear gradient, 70:30 to 90:10 in 30 min, 1 ml/min) revealed the presence of **1** (R_t 18.4 min), **2** (R_t 20.6 min), and **3** (R_t 22.9 min). Elution was monitored at 215 nm. The three compounds were then isolated (**1**, 7.7% yield

from the crude extract; **2**, 2.6%; **3**, 0.1%) by the hplc on a semi-prep. column with similar gradient elution (3.3 ml/min). Also, the lc-ms data on these peaks were obtained by running the sample on a C_{18} column (Phenomenex, Selectosil, 2×250 mm, 0.2 ml/min) with a similar gradient and post-column addition of 0.1% NH_4OAc, by using the thermospray ionization mode or alternatively using the particle-beam mode of sample introduction and positive-ion chemical ionization in the presence of CH_4.

Beauvericin [**1**].—Pb-cims *m/z* 784 $[M+H]^+$, 692, 592, 505, 441, 392, 331, 262, 244, 217, 134, 91; ^{13}C nmr ($CDCl_3$) δ 17.46 (γ1-CH_3, Hiv), 18.30 (γ2-CH_3, Hiv), 29.67 (β-*C*H, Hiv), 32.36 (N-CH_3), 34.73 (β-CH_2, Phe), 57.32 (α-*C*H, Phe), 75.44 (α-*C*H, Hiv), 126.73 (ar *C*H, Phe), 128.51 (ar *C*H, Phe), 128.86 (ar *C*H, Phe), 136.63 (ar *C*, Phe), 169.33 (*C*O), 169.89 (*C*O).

Beauvericin A [**2**].—Pb-cims *m/z* 798 $[M+H]^+$, 706, 606, 519, 441, 344, 331, 276, 262, 244, 230, 180, 134, 91; uv (CH_3CN-H_2O) λ max 205, 260 (sh) nm; ir (neat on NaCl plate) ν max 2964, 2931, 1742, 1659, 1484, 1456, 1415, 1371, 1326, 1263, 1196, 1109, 1059, 1017, 751, 699 cm^{-1}; ^1H nmr ($CDCl_3$) δ 0.43 (3H, d, *J*=6.4 Hz, γ1-CH_3, Hiv), 0.45 (3H, d, *J*=6.5 Hz, γ1-CH_3, Hiv), 0.68 (3H, m, δ-CH_3, Hmp), 0.70 (2H, m, γ1-CH_2, Hmp), 0.76 (3H, d, *J*=6.6 Hz, γ2-CH_3, Hiv), 0.77 (3H, d, *J*=6.8 Hz, γ2-CH_3, Hiv), 0.81 (3H, d, *J*=6.8 Hz, γ2-CH_3, Hmp), 1.78 (1H, m, β-C*H*, Hmp), 2.04 (2H, m, β-C*H*, Hiv), 2.95 (3H, s, N-CH_3), 2.99 (6H, s, N-CH_3 and 3H, m, β-C*H*(H), Phe), 3.35 (3H, β-CH(*H*), Phe), 4.93 (1H, d, *J*=6.2 Hz, α-C*H*, Hiv), 4.96 (1H, d, *J*=6.5 Hz, α-C*H*, Hiv), 5.04 (1H, d, *J*=7.7 Hz, α-C*H*, Hmp), 5.43 (3H, m, α-C*H*, Phe), 7.22 (15H, m, ar-C*H*, Phe); ^{13}C nmr ($CDCl_3$) δ 11.31 (δ-CH_3, Hmp), 14.34 (γ2-CH_3, Hmp), 17.54 (γ1-CH_3, Hiv), 18.29 (γ2-CH_3, Hiv), 24.47 (γ1-CH_2, Hmp), 29.70 (β-*C*H, Hiv), 32.37 (N-CH_3), 32.43 (N-CH_3), 34.68 (β-CH_2, Phe), 34.83 (β-CH_2, Phe), 35.81 (β-*C*H, Hmp), 57.35 (α-*C*H, Phe), 57.52 (α-*C*H, Phe), 74.22 (α-*C*H, Hmp), 75.4 (α-*C*H, Hiv), 126.76 (ar *C*H, Phe), 128.53 (ar *C*H, Phe), 128.92 (ar *C*H, Phe), 136.64 (ar *C*, Phe), 169.24 (*C*O), 169.94 (*C*O).

Beauvericin B [**3**].—Tspms *m/z* 830 $[M+NH_4]^+$; uv (CH_3CN-H_2O) λ max 205, 260 (sh) nm; ir (neat on NaCl plate) ν max 2927, 1739, 1650, 1454, 1415, 1381, 1277, 1210, 1117, 1014, 744, 699 cm^{-1}; ^1H nmr ($CDCl_3$) δ 0.40 (3H, d, *J*=6.2 Hz, γ1-CH_3, Hiv), 0.65 (6H, m, δ-CH_3, and 4H, m, γ1-CH_2, Hmp), 0.73 (3H, d, *J*=6.9 Hz, γ2-CH_3, Hiv), 0.75 (3H, d, *J*=7.2 Hz, γ2-CH_3, Hmp), 0.76 (3H, d, *J*=6.3 Hz, γ2-CH_3, Hmp), 1.73 (2H, m, β-C*H*, Hmp), 2.01 (1H, m, β-C*H*, Hiv), 2.94 (6H, s, N-CH_3), 2.98 (3H, s, N-CH_3, and 3H, m, β-C*H*(H), Phe), 3.33 (3H, m, β-CH(*H*), Phe), 4.90 (1H, d, *J*=8.5 Hz, α-C*H*, Hiv), 4.99 (1H, d, *J*=6.3 Hz, α-C*H*, Hmp), 5.01 (1H, d, *J*=6.6 Hz, α-C*H*, Hmp), 5.44 (3H, m, α-C*H*, Phe), 7.21 (15H, m, ar-C*H*, Phe).

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