

Isolation of Cyclodepsipeptides from Plant Pathogenic Fungi

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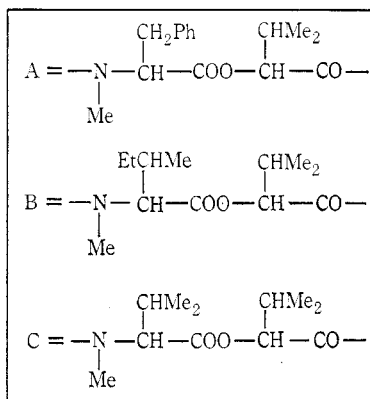
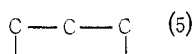
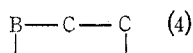
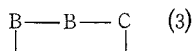
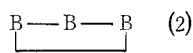
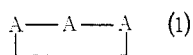
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Abstract

The cyclodepsipeptide beauvericin and a mixture of the cyclodepsipeptides, enniatin A₁, enniatin B₁, enniatin B and enniatin A, have been isolated from plant pathogenic fungi. Previously, beauvericin has only been isolated from the unrelated entomopathogenic fungi.

Introduction

Cyclodepsipeptides have been isolated from various bacteria, actinomycetes and fungi and are often produced by the cultures as a family of chemically related compounds, e.g., sporidesmolides, enniatins and triostins.¹ All cyclodepsipeptides are considered as species specific secondary metabolites and their functions are not yet clearly understood.² Beauvericin (1), an insecticidal cyclodepsipeptide, is reported to be produced by a few species of *Beauveria*^{3,4} and *Paecilomyces fumoso-roseus*.⁵



¹ Hook, D. J., in 'Handbook of Microbiology' (Eds A. I. Laskin and H. A. Lechevalier) Vol. 3, Microbial Products, p. 295 (Chemical Rubber Co.: Columbus, Ohio, 1973).

² Turner, W. B., 'Fungal Metabolites' p. 18 (Academic Press: London 1971).

³ Hamill, R. L., Higgins, C. E., Boaz, H. E., and Gorman, M., *Tetrahedron Lett.*, 1969, 4255.

⁴ Suzuki, A., Kanaoka, M., Isogai, A., Murakoshi, S., Ichinoe, M., and Tamura, S., *Tetrahedron Lett.*, 1977, 2167.

⁵ Bernardini, M., Carilli, A., Pacioni, G., and Santurbano, B., *Phytochemistry*, 1975, 14, 1865.

All these fungi are entomopathogenic and are classified as deuteromycetes. On the other hand, the enniatins (2)–(5) have previously been isolated from various *Fusarium* species.⁶

As part of our general search for cyclodepsipeptides from microorganisms we now report the occurrence of beauvericin in *Polyporus sulphureus* and of the enniatins in *Fusarium roseum acuminatum*. The isolation of beauvericin from *P. sulphureus* is particularly novel in that this plant pathogenic fungus is grouped in the class of basidiomycetes. If the process of secondary metabolism is species specific, this occurrence of beauvericin in a vastly different class of fungi indicates it may not be a secondary metabolite.

Results and Discussion

Isolation of Beauvericin

Lipids extracted from the mycelium of *P. sulphureus* were fractionated into neutral and polar lipids by chromatography on alumina and beauvericin was isolated from the neutral lipid fraction by p.l.c. The identity of this compound was established by acid hydrolysis which gave *N*-methylphenylalanine and α -hydroxyisovaleric acid, and by comparison of its spectral properties with those previously reported.^{3,7} In particular, beauvericin displays a very characteristic p.m.r. spectrum with an ABX spin system (δ_A 3.36, δ_B 3.00, δ_X 5.63, J_{AB} 15.0, J_{AX} 5.0, J_{BX} 12.0 Hz) for the aliphatic protons in the phenylalanine moiety and two doublets for three protons at unusually high chemical shift (δ 0.78 and 0.35) for the methyl group protons in the isovaleric acid unit. The parent peak in the mass spectrum (M^+ 783) confirms that the compound is a cyclic trimer (1) involving alternating *N*-methylphenylalanine and α -hydroxyisovaleric acid units.

Isolation of Enniatins

Extensive fractionation of the neutral lipids from *F. roseum acuminatum* yielded an enniatin fraction which was crystallized from aqueous alcohol. Acid hydrolysis of this crystalline preparation yielded α -hydroxyisovaleric acid, *N*-methylvaline and *N*-methylisoleucine. The mass spectrum obtained at 70 eV resembled closely the spectrum published for natural enniatin B⁸ in that all of the major peaks at M^+ 639, 624, 610, 596, 584, 528, 510 and 496 were present in intensities similar to those published, but in addition peaks at M^+ 681, 667 and 653 were also present. When the mass spectrum was run at 12 eV only peaks at M^+ 681, 667, 653 and 639 were present and their relative intensities were 7 : 40 : 100 : 95. Following the symbolism of Wipf *et al.*,⁹ it was thus concluded that the enniatin sample isolated contained enniatin A (2) (3%), enniatin A₁ (3) (17%), enniatin B₁ (4) (41%) and enniatin B (5) (39%).

Experimental

P. sulphureus (41 B), brown rot, was obtained from the Wood Technology Division of the Forestry Commission of New South Wales. *F. roseum acuminatum* (Gp 1, F-4029) was obtained

⁶ Audhya, T. K., and Russell, D. W., *J. Gen. Microbiol.*, 1974, **82**, 181.

⁷ Ovchinnikov, Yu. A., Ivanov, V. T., and Mikhaleva, I. I., *Tetrahedron Lett.*, 1971, 159.

⁸ Kiryushkin, A. A., Rozynov, B. V., and Ovchinnikov, Yu. A., *Chem. Nat. Compd. (USSR)* (Engl. transl.), 1968, **4**, 157.

⁹ Wipf, H. K., Pioda, L. A. R., and Simon, W., *Helv. Chim. Acta*, 1968, **51**, 377.

from Dr L. W. Burgess, Department of Plant Pathology, University of Sydney, N.S.W. In our laboratory, the cultures were maintained on potato-sucrose agar slopes. Surface cultures in Roux bottles were prepared in lactose medium,¹⁰ and organisms were grown at 20°C for 20 days. All cultures were illuminated by six 40-W fluorescent tubes (cool, daylight) fitted 20 cm apart at a height of 1 m from the surface of the Roux bottles. Illumination was provided for 12 h followed by 12 h of complete darkness daily. Cultures were filtered and the lipids were extracted from the slightly wet mycelium by homogenizing three times in 2 vols of methanol. The extracts were pooled and methanol was evaporated over a rotary evaporator at 50°C. The residue was dissolved in chloroform (1 g/100 ml), and stirred for 1 h with cellulose powder (Whatman Chromedia, C.F. 11; 10 g/g lipid) and filtered. The chloroform was evaporated from the filtrate and the residue was redissolved in chloroform and passed through a column of activated charcoal powder supported over alumina (B.D.H., Brockmann activity II, 5 g/100 mg lipids). The neutral lipid fraction containing the cyclodepsipeptides was eluted through the column with 2 vols of chloroform. Neutral lipids were further fractionated by p.l.c. on 0.75 mm thick silica gel HR plates. Solvent systems used were either (A) acetone, chloroform, water [65/25/2 (v/v)] or (B) ethyl acetate, hexane, methanol, water [75/200/17/1 (v/v)]. Lipids from each band were analysed for their amino acid composition after hydrolysis with 7 M HCl in a sealed tube under nitrogen at 110°C for 24 h. The hydrolysates were extracted with ether. The ether extracts were pooled, dried briefly over anhydrous Na₂SO₄ and the ether was evaporated. The residue redissolved in ether was esterified with ethereal diazomethane at 0° and was analysed for fatty acid composition on g.l.c. (15% ethylene glycol succinate on Chromosorb W 60/80 mesh). The aqueous layer was taken to dryness on a rotary evaporator. The residual amino acids were identified and quantitatively estimated by amino acid analyser (Jeol, JLC-6AH) and by descending paper chromatography.¹¹

Fractionation of the neutral lipids of *P. sulphureus* on p.l.c. in solvent system (A) gave a fraction (R_F 0.6) as a white amorphous solid, m.p. 80°C (Found: C, 68.6; H, 7.3; N, 5.2. C₄₅H₅₇N₃O₉ requires C, 68.9; H, 7.3; N, 5.4%). ¹H n.m.r. δ (CDCl₃): 7.20, s, ArH; 4.80, d, >CHCHMe₂, 8.4 Hz; ABX spectrum δ_A 3.36, δ_B 3.00, δ_X 5.63, J_{AB} 15.0, J_{AX} 5.0, J_{BX} 12.0 Hz, >CHCH₂; 3.01, s, NCH₃; 1.90, m, CHCHMe₂; 0.78 and 0.35, d, CHMe₂, 8.0 Hz. M⁺ 783. $\nu_{\max}$ 1740, 1670 cm⁻¹. This spectrum was identical with that published for beauvericin (lit. m.p. 93–94°).³ Acid hydrolysis of the white amorphous solid afforded only *N*-methylphenylalanine and α -hydroxyisovaleric acid (identified by g.l.c. through comparison of its methyl ester with an authentic sample).

The culture medium (1000 ml) yielded dry mycelium (10.7 g) from which was obtained the lipid fraction (1.2 g) and finally beauvericin (0.036 g).

The ennatiins were separated similarly from the neutral lipids of *F. roseum acuminatum* by p.l.c. in solvent system (B). The band, R_F 0.3, was rechromatographed in solvent system (A) and afforded a sample (R_F 0.5) which was homogeneous by t.l.c. This sample was dissolved in ethanol (60 mg/ml) and then water (0.3 ml/ml) was added and the solution allowed to stand at 4° for 5 days. The colourless crystals obtained had m.p. 132°. M⁺ (12 eV): 681 (7%), 667 (40), 653 (100), 639 (95). M⁺ (70 eV) was similar to that previously published except that additional peaks appeared at M⁺ 681, 667 and 653. ¹H n.m.r. (CDCl₃) was complex since the sample was a mixture as evidenced by doublets at δ 5.12 (J 8.8 Hz), 4.80 (J 10.0 Hz) and 4.60 (J 10.0 Hz) (>CHO–, >CHN< and >CHN< in homologue respectively). The integral for the former doublet equalled the combined integrals for the latter two doublets. In addition, there appeared a singlet at δ 3.13 (NMe), a broad band centred at 2.25 (CHCH₂ and CHMe₂) and six lines between 1.10 and 0.94 (methyl adjacent to methine and methylene group protons). $\nu_{\max}$ 1735, 1670 cm⁻¹. The culture medium (1000 ml) yielded dry mycelium (10.0 g) from which was obtained the lipid fraction (2 g) and the ennatiin fraction (0.3 g).

Acknowledgment

We wish to express our sincere thanks to Associate Professor J. Done for his valuable help and criticism during these studies.

Manuscript received 16 January 1978

¹⁰ Audhya, T. K., and Russell, D. W., *Can. J. Microbiol.*, 1973, **19**, 1051.

¹¹ Audhya, T. K., and Russell, D. W., *J. Chromatogr.*, 1973, **84**, 361.