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THE STRUCTURE OF THE FUNGAL SIDEROPHORE, ISOTRIORNICIN

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A siderophore isolated from *Epicoccus purpurascens* has been found to be an isomer of triornicin (I) which differs from I only in interchange of the positions of acetyl and (E)-5-hydroxy-3-methyl-2-pentenoyl hydroxamate groups. The structure of the new compound, isotriornicin (II), was determined by spectroscopic techniques and by cleavage with methanol-ammonia to a new dihydroxamic acid (III) and N-acetyl-*trans*-fusarinine methyl ester (IV).

The soil fungus, *Epicoccum purpurascens* (syn. *E. nigrum*), is a prolific source (1) of siderophores (2,3) which are produced by the organism in response to low iron concentrations. Siderophores strongly bind iron and transport it across cell membranes where it is released for intracellular utilization. Of the siderophores produced by the fungus, coprogen and ferricrocin were previously isolated from other organisms (4,5), but a large number apparently were previously unknown. The structure of one, triornicin (I), which possessed slight antitumor activity in mice injected with Ehrlich ascites tumor cells, was determined (6).

A second previously unknown siderophore produced by the fungus has now been shown to be an isomer of triornicin and has been given the name isotriornicin.

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ABBREVIATIONS: PMR, Proton nuclear magnetic resonance; ^{13}C-NMR, ^{13}C-nuclear magnetic resonance.

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METHODS AND MATERIALS

The fungal culture conditions and isolation methods have been described (1). In summary, they were based on culture of the fungus in an iron deficient medium to maximize siderophore production. The ligands were then chelated with Fe^{+3}, extracted from the medium with benzyl alcohol, and separated on a series of carboxylic ion exchange columns. The resulting siderophores were de-salted on a gel filtration column and the chelated iron was removed with a large excess of 8-hydroxyquinoline. As noted for coprogen (4) and triornicin (6), neither the iron-free ligand nor the chelate could be crystallized.

Structural analysis was conducted in the same manner and with the same instrumentation as that described for triornicin (6). The ester group of isotriornicin was cleaved with a methanol solution saturated with ammonia gas, and reaction products were separated by ion-exchange chromatography (6).

RESULTS AND DISCUSSION

The physical and chemical properties of isotriornicin (previously described (1) as chromatographic fraction A-5) are very similar to those of triornicin (6). The ultraviolet-visible spectrum of the iron-chelated compound in water has λ_{max} at 195, 250 (shoulder), and 430nm (log ε4.53, 3.86, and 3.25). The absorption at 430nm was not shifted to a longer wavelength when the solution was acidified to pH 3.0 as noted for other trihydroxamic acids (7). The proton magnetic resonance (PMR) and ^{13}C magnetic resonance (^{13}C NMR) spectra are nearly identical (within the resolution of these instruments) to those reported for triornicin.

Reductive hydrolysis with hydriodic acid (8) produced a single ninhydrin-positive spot that co-migrated with ornithine by thin layer chromatography on silica gel with three solvent systems.[1] Quantitative ninhydrin analysis with an ornithine standard gave a calculated equivalent weight of 700±31 for the parent compound versus a calculated molecular weight for II of 698. Similar hydrolysis with 6N hydrochloric acid produced a single ninhydrin-positive spot that co-migrated with authentic

[1] 1-Butanol, acetic acid, water (60:15:25); 1-butanol, 2-butanone, water (40:40:20); and 1-butanol, acetone, water, triethylamine (40:40:20:8) with Rf's of 0.22, 0.03, and 0.20 (ornithine) and 0.26, 0.16, and 0.29 for N^{δ}-hydroxyornithine.

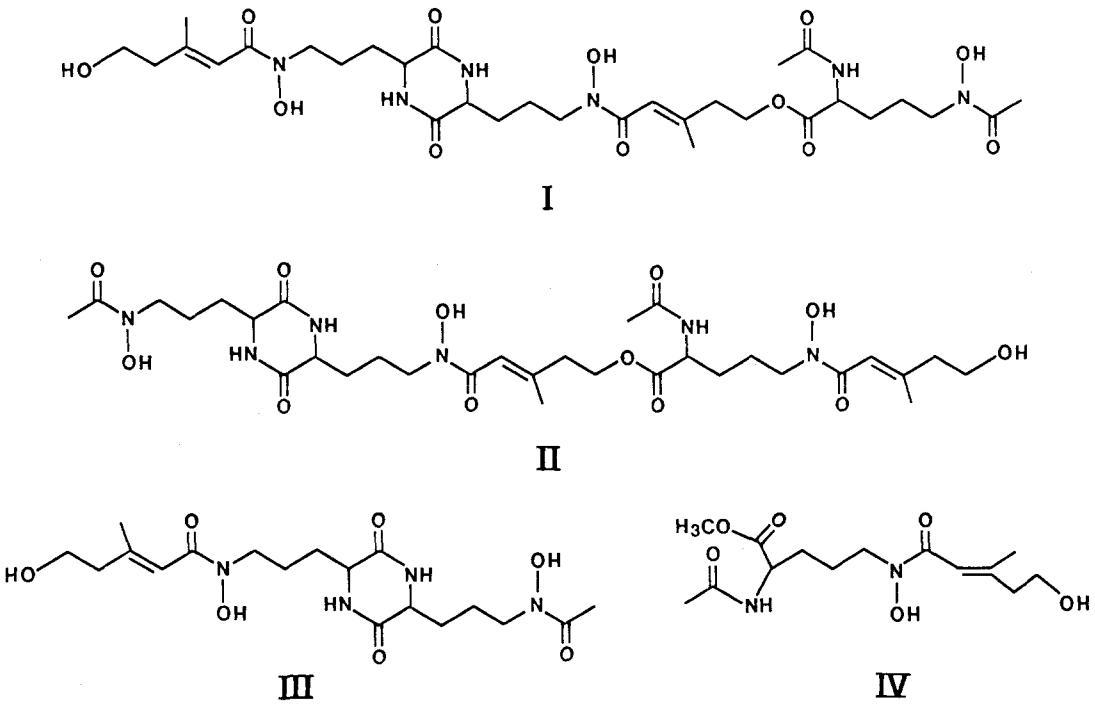

Figure 1

δ-N-hydroxyornithine derived from desferricoprogen. This product also exhibited a red color when sprayed with triphenyltetrazolium chloride in a reaction characteristic of a free hydroxylamine moiety (9).

These analytical results are essentially identical to those obtained with triornicin (I) and are compatible with structure II. An ammonia-methanol cleavage of the presumed ester function would give structures III and IV which would be amenable to further structural analysis. Structure III is a previously unknown dihydroxamic acid related to rhodotorulic acid and dimerumic acid (2). Structure IV is N-acetyl-*trans*-fusarinine methyl ester which may be derived from a similar basic methanolysis of desferricoprogen.

Structure of Fragment III. The methanol-ammonia cleavage and subsequent ion exchange chromatography of 193.6 mg of isotriornicin produced 75.1 mg (81% yield) of a white crystalline

solid with a decomposition point of 160-165°C. Some starting material was recovered unchanged. The PMR spectrum of III was assigned as follows: δ 1.55 -1.90 (br,8, -CH-C*H*$_2$-C*H*$_2$-CH$_2$-N-C-); δ 1.95 (br sh,3, -CH=C-C*H*$_3$); δ 2.15 (s,3, C*H*$_3$-C-N-); δ 2.41 (t,2, -CH=C-C*H*$_2$-); δ 3.59 -3.87 (m,4 -C*H*$_2$OH, -C*H*$_2$-N-C-); δ 4.22 (t,2, -NH-C*H*-C-); δ 5.95, 6.21 (br,1 -C=C-*H*). The ^{13}C NMR spectrum was assigned as: δ 18.5 (-CH=C-*C*H$_3$); δ 20.1 (*C*H$_3$-C-N-); δ 22.2 (-CH-CH$_2$*C*H$_2$-CH$_2$-N-C-); δ 31.2 (-CH-*C*H$_2$-CH$_2$-CH$_2$-N-C-); δ 41.9, 42.9 (-C=C-*C*H-); δ 48.2, 51.9 (-CH-CH$_2$-CH$_2$-*C*H$_2$-N-C-); δ 54.9 (-*C*H-CH$_2$-CH$_2$-CH$_2$-N-C-); δ 60.0 (-*C*H$_2$OH); δ 117.6 -118.5 (-C=*C*H-C-); δ 152.3 (-*C*=CH-C-); δ 170.8, 173.6, 174.7 (unassigned carbonyl). These spectral assignments are very similar to those of dimerumic acid (10), but the PMR integrations support the net loss of an (E)-5-hydroxy-3-methyl-2-pentenoic acid moiety and its replacement by an acetyl group. The appearance of a new peak relative to the dimerumic acid spectrum at δ 2.15 (PMR) and δ 20.1 (^{13}C NMR) confirms this substitution. The UV-Vis spectrum of the iron-free ligand provided λ_{max} 195 and 215nm (shoulder), log ε= 4.49 and 4.30. The optical rotation is $[\alpha]_D$ = -22.95° (c 0.780, methanol). This value is very close to that observed for the structurally similar dimerumic acid (-20.4°), suggesting an L-configuration for the ornithyl residues. The iron-free compound produced no reaction with ninhydrin or tetrazolium reagents, but gave an intense blue color with Folin-Ciocalteu reagent (11) and a rust-brown color with a ferric chloride solution as predicted for Structure III.

Structure of Fragment IV. The cleavage reaction and subsequent chromatography of isotriornicin produced 51.4 mg (77% yield) of N-acetyl-*trans*-fusarinine methyl ester (IV). The PMR and ^{13}C-NMR spectra were identical to the corresponding compound derived from methanol-ammonia cleavage of desferricoprogen (4).

Assignments for the PMR spectrum were: δ 1.45-1.7 (br,4, -CH-CH$_2$-CH$_2$-C-); δ 1.86 (s,3, CH$_3$-C(=O)-N-); δ 2.04 (s,3, -C=C-CH$_3$); δ 2.26 (t,2, -C=C-CH$_2$-); δ 3.54 (br,4, -CH$_2$-N(-OH)-C(=O)-, -CH$_2$OH); δ 3.64 (s,3, -CO$_2$CH$_3$); δ 4.21 (br s,1, -NH-CH-CO-); δ 4.55 (br s,1, -CH$_2$OH); δ 6.22 (s,1, -C=C-H); δ 8.24 (d,1, -CO-NH-CH-); δ 9.59 (br s,1, -CO-NOH-). Similarly the ^{13}C-NMR spectrum was assigned as: δ 18.6 (-C=C-CH$_3$); δ 22.4 (CH$_3$-CO-NH-); δ 23.4 (-CH-CH$_2$-CH$_2$-CH$_2$-NOH-); δ 28.3 (-CH-CH$_2$-CH$_2$-CH$_2$-NOH-); δ 42.4, 42.8 (-C=C-CH$_2$-); δ 48.0, 51.5 (-CH-CH$_2$-CH$_2$-CH$_2$-NOH-); δ 53.3 (-CH-CH$_2$-CH$_2$-CH$_2$-NOH-); δ 53.7 (-CO$_2$CH$_3$); δ 60.1 (-CH$_2$OH); δ 118.0 (C=CH-CO-); δ 149.0, 152.4 (-C=CH-CO-); δ 167.8, 170.2 (-CO-NOH-); δ 175.3 (-CO$_2$CH$_3$, CH$_3$CO-NH-). Electron impact mass spectrometry provided a fragmentation pattern consistent with the assigned structure, and high resolution peak matching of the parent ion indicated a mass of 316.1642 *vs* a calculated mass for $C_{14}H_{24}N_2O_6$ of 316.1634.

In summary, the spectral and chemical degradation data presented for isotriornicin, and its cleavage fragments III and IV, are consistent with structure II. The A-complex of siderophores produced by *E. purpurascens* is composed of seven biologically active siderophores as determined by our chromatographic techniques. The structure of four of the major components (ferricrocin, coprogen, triornicin, and isotriornicin) have been determined and these compounds comprise 87% of the total weight and 90% of the total biological activity in this fraction. In contrast to triornicin, which produced some prolongation of life in an antitumor assay (1), isotriornicin had no significant effect.

The biological significance of these compounds is still under investigation, but it may be noted that this is an ubiquitous fungal genus that is most often associated with vegetation (12). The strain used in this study was isolated from the

soil, and association of such a bountiful source of siderophores with plants may indicate that this genus is important in iron-complexation within the rhizoplane (13).

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