assistance in the production and early work on isolation of the compounds.

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Structure of Triornicin, a New Siderophore†

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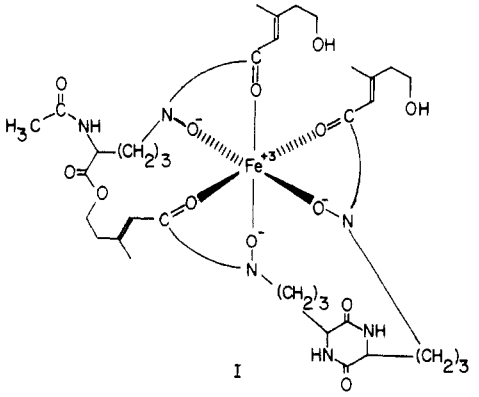
ABSTRACT: Spectroscopic analysis of the tumor inhibitory factor triornicin produced by *Epicoccum purpurascens* indicated that it was of similar structure to the known siderophore desferricoprogen, which is also produced by the fungus. The ^1H and ^{13}C NMR spectra indicated the replacement of an (*E*)-5-hydroxy-3-methyl-2-pentenoyl moiety of the desferricoprogen structure with an acetyl function. Cleavage of triornicin with basic methanol produced two fragments. The first was identified as a natural siderophore, dimerumic acid, which was also produced by basic cleavage of desferricoprogen. The second compound was identified as N^{α},N^{δ}-diacetyl-N^{δ}-hydroxyornithine. The structure of these fragments serves to define the structure of triornicin as a new siderophore.

A large number of iron-binding compounds which promote the growth of *Arthrobacter flavescens* JG-9 in low-iron media are produced by the soil fungus *Epicoccum purpurascens* Schlecht. Among the first seven compounds isolated, coprogen and ferricrocin were identified, but the others appear to represent new compounds (Frederick et al., 1980).

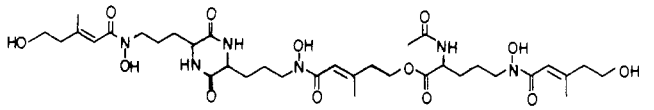
In general, the iron-transport agents produced by fungi are characteristically aliphatic acylhydroxamic acids with a high affinity for iron. Ferricrocin, the iron complex of a cyclic peptide [*cyclo*-(tri-N^{δ}-acetyl-N^{δ}-hydroxyornithinylglycylserylglycyl)] (Keller-Schierlein & Deër, 1963; Neilands, 1973), and coprogen (I)[1] (Keller-Schierlein & Diekmann, 1970) are



I

such examples and are produced by *E. purpurascens* (Frederick et al., 1980). Other previously characterized siderophores and related compounds have been reviewed (Nielands, 1973).

In this investigation, the structure of one of the new compounds, triornicin, which has slight antitumor activity, was determined. Spectral analyses, particularly ^1H NMR studies, indicated that triornicin was related structurally to desferricoprogen (II), and the structural characterization of triornicin



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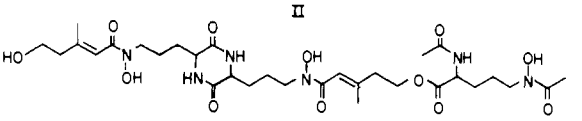

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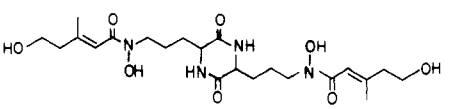

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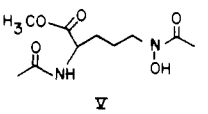

V

(III) was accomplished by the spectral analysis and chemical degradation which is subsequently described.

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[1] Although the structure shown in the Λ isomer, the absolute configuration of the ligand about the Fe^{3+} has not been established.

0006-2960/81/0420-2436$01.25/0

Table I: Chemical Shifts and Structural Assignments of ^1H NMR

structural assignments	chemical shifts of compound II	III	IV	V
$CHCH_2CH_2CH_2N(OH)$	1.51–1.90 (br,[a] 12)	1.51–1.98 (br, 8)	1.60–1.85 (br, 8)	1.66–1.81 (br, 4)
$C{=}CCH_3$	1.95 (br s, 9)	1.95 (br s, 6)	1.95 (br s, 6)	
CH_3CONH	2.03 (s, 3)	2.03 (s, 3)		2.03 (s, 3)
$CH_3CON(OH)$		2.14 (s, 3)		2.14 (s, 3)
$C{=}CCH_2$	2.41 (m, 6, J = 6.5 Hz)	2.28–2.68 (m, 4, J = 6.5 Hz)	2.41 (t, 4, J = 6.5 Hz)	
CH_2OH, $CH_2N(OH)$	3.61–3.91 (m, 10)	3.61–3.94 (m, 8)	3.59–3.89 (m, 8, J = 6.5 Hz)	3.66 (t, 2, J = 6.5 Hz)
$NHCHCO$ (ring)	4.21 (br s, 2)	4.21 (br s, 2)	4.21 (br s, 2)	
$NHCHCO$, CO; OCH_2	4.39 (br s, 3)	4.38 (br s, 3)		4.38 (br, 1)
$C{=}CH$	5.95, 6.21 (br, 3)	5.96, 6.20 (br, 2)	5.95–6.20 (br, 2)	
CO_2CH_3				3.73 (s, 3)

[a] Broad.

Table II: Chemical Shifts and Structural Assignments of ^{13}C NMR

structural assignments	chemical shifts of compound II	III	IV	V
$CH{=}CCH_3$	18.6	18.6	18.7	
$N(OH)COCH_3$		20.1		20.1
$NHCOCH_3$	22.3	22.3		22.3
γ-C(orn[a])	22.6, 23.4	22.3, 23.1	22.7	23.1
β-C(orn)	28.3, 31.4	28.3, 31.3	31.5	28.2
$C{=}CCH_2$	37.8, 42.7	39.1, 42.9	42.5	
δ-C(orn)	48.2, 52.1	48.0, 51.8	50.4	48.0
α-C(orn)	53.4	53.4		53.4
α-C(ring)(orn)	55.0	55.0	55.0	
CH_2OH	60.1	60.1	60.2	
CH_2OCO	64.1	64.2		
$C{=}CHCO$	118.0	117.7	117.8	
$C{=}CHCO$	152.5	151.0	152.2	
unassigned carbonyl	168.0, 169.8 170.0, 170.8 174.5, 175.0	168.0, 170.0 170.8, 174.6 175.1	170.9	174.8, 175.3

[a] Hydroxyornithine moiety.

Materials and Methods

The siderophores were isolated from 14-day cultures of *E. purpurascens* as previously described (Frederick et al., 1980). Both coprogen and triornicin were recalcitrant to crystallization as noted previously for coprogen (Keller-Schierlein & Diekmann, 1970).

Analytical Methods. Proton magnetic resonance (^1H NMR) spectra were recorded at 100 MHz on a Varian HA-100 spectrometer. Samples were dissolved in D_2O, and the peak chemical shifts are reported in δ units with tetramethylsilane as an external reference in a coaxial capillary. Chemical shifts are corrected for differences in sample bulk susceptibility. ^{13}C Magnetic resonance (^{13}C NMR) spectra were obtained with a Bruker WH-90 spectrometer. Samples were dissolved in D_2O, and chemical shifts were recorded in δ units relative to an internal reference signal from dioxane at δ 67.4. Chemical shift assignments were facilitated by using the swept off resonance decoupling (SORD) technique of Shoulders & Speer (1978). The ultraviolet–visible (UV–vis) absorption spectra were recorded on a Beckman Acta III spectrometer with the samples dissolved in water. Low-resolution electron impact mass spectra were obtained on a CEC-491 spectrometer, and high-resolution spectra were provided by a CEC 21-110 spectrometer. Optical rotation was determined with a Perkin-Elmer 141 polarimeter with the samples dissolved in methanol.

Methanol–Ammonia Cleavage of Esters. The iron-free siderophore (20–100 mg) was dissolved in 3 mL of methanol in a Teflon-lined screw-cap test tube. The sample solution was saturated with ammonia gas, capped, and allowed to remain at room temperature for 9 h. The reaction mixture was reduced to dryness in vacuo. The residue was dissolved in methanol–water (1:9 v/v) and chromatographed on a Bio-Rex 70 carboxylic ion-exchange column (Bio-Rad, 200–400 mesh, 1.5 × 24 cm, acid form) in the same solvent. The column effluent was monitored at 254 nm with an Isco UA-2 column monitor. Fractions that produced a rust-brown or violet color when aliquots were mixed with an aqueous 1% $FeCl_3$ solution were pooled and reduced to dryness in vacuo.

Results and Discussion

The ^1H NMR spectrum of the known natural product desferricoprogen (II) is summarized in Table I along with that of triornicin (III) from *E. purpurascens*. The spectra are very similar; however, there is a new peak at δ 2.14 which corresponds to the methyl group of an acetylhydroxamic acid (Llinás et al., 1970). Correspondingly, the integration of the spectrum indicates that only two (*E*)-5-hydroxy-3-methyl-2-pentenoyl (*trans*-dehydromevalonyl) moieties are present. These results suggest that one of the terminal *trans*-dehydromevalonic units of desferricoprogen has been replaced by an acetyl function.

The ^{13}C NMR spectra of desferricoprogen and triornicin are listed in Table II. Again, the spectra are very similar with the exception of a new peak for triornicin at δ 20.1 that corresponds to the methyl carbon of an acetylhydroxamic acid. Since these spectra were collected under identical conditions, the area of the peak for the methine carbons of the diketopiperazine ring (δ 55.0) may be used as an internal reference to compare the peak areas. Those peaks assigned to the *trans*-dehydromevalonyl moieties of triornicin are reduced in intensity by one-third, corresponding to a loss of one unit relative to desferricoprogen.

The UV–vis spectrum of the iron chelate of triornicin has λ_{max} 195, 250, and 430 nm (log ϵ 4.61, 4.11, and 3.27). These values correspond to those reported for coprogen (Keller-Schierlein & Diekmann, 1970). On acidification to pH 3.0, the absorption at 430 nm was not shifted as previously noted for other trihydroxamic acids (Moore & Emery, 1976).

Reductive hydrolysis of 0.8 mg of triornicin with hydriodic acid provided a quantitative yield, based on structure III, of ornithine identified by thin-layer chromatography in those solvent systems and determined by quantitative ninhydrin analysis (Emery & Neilands, 1961). Hydrolysis with 6 N hydrochloric acid of samples of triornicin and desferricoprogen produced, in each case, a single ninhydrin-positive spot which migrated identically on three thin-layer chromatography systems.[2] This hydrolysis product exhibited a red color when sprayed with triphenyltetrazolium chloride in a reaction characteristic of a free hydroxylamine function (Snow, 1954). These results infer that the structure of the hydrolysis product of triornicin is N^{δ}-hydroxyornithine, as previously determined for coprogen (Keller-Schierlein & Diekmann, 1970).

A colorimetric titration of triornicin with a freshly made standard solution of ferric chloride provided an equivalent weight of 710 ± 75. Gel permeation chromatography on Bio-Gel P-4 (Bio-Rad, 1.5 × 80 cm column, 200–400 mesh) against a series of molecular weight standards indicated an exclusion molecular weight of approximately 660.

In summary, these results are congruent with a structure very similar to that of the previously characterized siderophore desferricoprogen, but with replacement of one of the terminal *trans*-dehydromevalonyl moieties with an acetyl group. For determination of which of the side chains had been modified, a sample of the triornicin was cleaved at the ester function with methanol–ammonia in a similar manner to that reported for coprogen (Keller-Schierlein & Diekmann, 1970). A sample of desferricoprogen was similarly hydrolyzed to provide fragments for spectral comparison.

Two products were isolated from the cleavage reaction of 21.0 mg of triornicin in methanol–ammonia followed by chromatography on a carboxylic ion-exchange column. The first was a white crystalline solid, mp 161–164 °C dec (12.3 mg; 85% yield). Its ^{1}H NMR and ^{13}C NMR are identical with those of dimerumic acid (IV), mp 162–164 °C dec, derived from the analogous cleavage of desferricoprogen (Tables I and II). Its chromatographic properties, UV–vis spectrum, optical rotation, and reaction with a variety of colorimetric reagents were identical with those previously reported for natural and synthetic dimerumic acid (Keller-Schierlein & Diekmann, 1970; Widmer & Keller-Schierlein, 1974) and with those observed for an authentic sample.

The second compound was isolated from the ion-exchange column as a clear, colorless oil (6.1 mg; 93% yield). The ^{1}H NMR (Table I) showed a characteristic peak at δ 2.09 for the methyl group of an acetylhydroxamic acid and none of the peaks characteristic of a *trans*-dehydromevalonyl unit. The ^{13}C NMR (Table II) confirmed the presence of the acetylhydroxamic function (δ 20.1) and the loss of the corresponding *trans*-dehydromevalonyl moiety occurring in the cleavage product of desferricoprogen. The optical rotation was determined to be $[\alpha]_D$ −10.5° (*c* 0.19, methanol). The UV–vis spectrum of the iron-free material provided λ_{max} 195 (log ϵ 3.29). The compound gave a positive reaction with Folin–Ciocalteu reagent (Subramanian et al., 1968) and a rust-brown color with aqueous ferric chloride, suggesting the presence of the hydroxamate functionality. The high-resolution mass spectrum provided a mass of the parent ion of 246.1218 dalton vs. a calculated mass for $C_{10}H_{18}N_2O_5$ of 246.1215 daltons. These results are compatible with structure V, N^{α},N^{δ}-diacetyl-N^{δ}-hydroxyornithine methyl ester, for this cleavage product.

The structure of the fragmentation products of triornicin determines its structure to be that of III. The absolute configuration about the α carbon of the N^{δ}-hydroxyornithyl residues may be inferred to be L by the correspondence in optical rotation of the parent compounds $[\alpha]_D$ −22.0° for coprogen and −22.1° for triornicin and by the identical rotation exhibited by the dimerumic acid cleaved from each molecule.

The identification of the desferricoprogen-like siderophore triornicin from *E. purpurascens* suggests that it is formed in a branch of the biosynthetic pathway to desferricoprogen. In fact, the dominant siderophore isolated from *E. purpurascens* is desferricoprogen (Frederick et al., 1980), and our preliminary investigations into several other siderophores produced by this organism suggest that several fall into this structural class. The key element in this structure is the core of three N^{δ}-hydroxyornithyl residues, two of which form a diketopiperazine ring. When the organism is grown in an iron-deficient medium, these siderophores are synthesized and excreted into the environment as the iron-free ligand. The name triornicin has been used to identify the iron-free molecule with the expectation that other analogous compounds will be discovered.

The slight but statistically significant antitumor effect for triornicin (Frederick, et al., 1980) was not observed for desferricoprogen. Thus, the N^{α},N^{δ}-diacetyl-N^{δ}-hydroxyornithine moiety is implicated as essential for the slight antitumor activity.

[2] 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 R_f's of 0.22, 0.03, and 0.20, respectively, on silica gel.

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