half of the bilayer may be impeded by the increased rigidity of the hydrocarbon chains.

The results obtained with adapted *M. capricolum* cells treated with DMS (Table III) support the conclusion we made previously with ionophore- and chloramphenicol-treated cells, i.e., that in the adapted cells part of the free cholesterol is incorporated and translocated in a growth-dependent process (Clejan et al., 1978). The mechanism of inhibition by DMS differs from the actions of ionophores and chloramphenicol. The cross-linking reagent DMS probably inhibits uptake and translocation of free cholesterol (and cell growth in these cholesterol-requiring organisms) by modulating the packing of membrane components at or within the membrane surfaces. Alternatively, we cannot exclude the possibility that a surface protein is involved in the uptake and movement of cholesterol in the *M. capricolum* membrane.

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Production and Isolation of Siderophores from the Soil Fungus *Epicoccum purpurascens*†

Clay B. Frederick,‡ Paul J. Szaniszlo, Paul E. Vickrey,§ Michael D. Bentley,⊥ and William Shive*

ABSTRACT: A large number of iron transport agents, siderophores, which stimulated the growth of *Arthrobacter flavescens* JG-9, were isolated during a study of the antitumor activity associated with the metabolic products of the fungus *Epicoccum purpurascens*. The production of the siderophores was significantly enhanced in a variety of media by culture of the fungus in the near absence of ferric iron. A novel method of purification involving a carboxylic ion-exchange resin separated the siderophores into four subgroups. The first subgroup, which contained the majority of the activity, was subsequently resolved in a similar manner with the carboxylic resin into seven individual siderophores. Of these, two were characterized as ferricrocin and coprogen whereas the others appeared to represent new compounds. One of the latter was given the name triornicin and exhibited slight antitumor activity in mice injected with Ehrlich ascites tumor cells.

During preliminary studies of the biologically active compounds produced by a common soil fungus, both antitumor activity and siderophore activity were discovered. The purpose of this report is to describe the production, isolation, and nature of the siderophores detected among the metabolic products of the fungus identified as *Epicoccum purpurascens* Schlecht.

Siderophores are high-affinity, microbial ferric transport molecules secreted into the environment by bacteria and fungi for the purpose of sequestering iron to facilitate their growth. The criteria for preliminary assignment of a natural product to the siderophore category are (a) the repression of its formation by iron, (b) the large differential in its avidity for ferric vs. ferrous iron, the former being greatly favored, and (c) the inability of mutants or natural strains incapable of synthesizing either the particular ligand or its "permease" to take up exogenous iron (Neilands, 1973). Molecules satisfying these criteria vary in structure, but generally can be classed as secondary hydroxamic acids, or catechols. Generally, bacteria produce ligands based on catechol coordination whereas fungi

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produce hydroxamic acids exclusively.

In the production of siderophores by the strain of *Epicoccum purpurascens* isolated from soil, the fungus was routinely cultured in a medium nearly devoid of iron because all preliminary production experiments suggested that high iron concentrations significantly repressed siderophore production. An unusually effective method of chromatographic separation of the active principles on a carboxylic ion resin was developed which provided four different groups of active principles. One of the groups was subjected to higher resolution chromatography on the same resin and separated into seven different siderophores. Two of these compounds were found to be identical with known fungal siderophores, ferricrocin (Zähner et al., 1963; Neilands, 1973) and coprogen (Pidacks et al., 1953). A new siderophore, which has been given the name triornicin, was isolated and found to cause a marginal, but reproducible, extension of the mean life span of mice injected with Ehrlich ascites tumor cells.

Materials and Methods

Organism and Cultural Conditions. Cultures of *E. purpurascens* [originally isolated by Clark (1970) The University of Texas, Austin, TX, personal communication] were maintained on a V-8 agar slanted medium (V-8A) consisting of juice filtrate in distilled water (1:5 v/v) and (in grams per liter) maltose (5), yeast extract (2), mannitol (1), $CaCO_3$ (0.5), and agar (20). The pH of the V-8A was adjusted to 6.5 prior to autoclaving.

Cultures were grown for inoculum in 50 mL of either a malt extract, peptone, corn steep, mineral salts medium (MPC), or Mycological broth (MB; Difco). The MPC (1000 mL) contained 5 mL of corn steep liquor (a gift from the Eli Lilly and Co.), 20 g of malt extract (Difco), 10 g of Bacto peptone (Difco), 0.25 g of $MgSO_4 \cdot 7H_2O$, 1 g of K_2HPO_4, 0.5 g of KH_2PO_4, 0.1 g of $CaCl_2 \cdot 2H_2O$, and 6.25 mL of a trace metal solution which consisted of (in grams per liter) $CuSO_4 \cdot 5H_2O$ (1.60), $MnCl_2 \cdot 4H_2O$ (1.30), and $ZnSO_4 \cdot 7H_2O$ (0.24). The pH of the medium was adjusted to 6.5 prior to autoclaving. Inoculum cultures were established by transferring the surface growth from one V-8A slant to 50 mL of inoculum broth. After a 2-day incubation at 25 °C on a New Brunswick G50 gyrotory shaker operating at 250 rpm, the mycelium was fragmented and uniformly dispersed in a sterile Waring blender microcup. A protion of the mycelial suspension (10% v/v) was then used to establish a second inoculum culture which was incubated as before. The resulting mycelium was harvested by centrifugation (7710*g* for 15 min), washed 2 times with low-iron production medium, fragmented and dispersed in a sterile Waring blender microcup, and finally used to inoculate the low-iron production media.

A variety of production media were employed during attempts to maximize siderophore yields. The media were rendered iron deficient by deletion of all iron from the media formulations and also by removal of Fe^{3+} contamination by either treatment of most media components with Chelex 100 chelating resin (Bio-Rad No. 1422852, 400 spherical dry mesh designation) or by extraction with 8-hydroxyquinoline and chloroform. Typically, the low-iron medium (LIM) used for large-scale production of siderophores consisted of (in grams per liter) NH_4NO_3 (1.5), K_2HPO_4 (1.0), KH_2PO_4 (0.5), KCl (0.5) thiamin chloride hydrochloride (0.002), proline (1), l-ornithine hydrochloride (1), $MgSO_4$ (0.25), and glucose (25). Trace metals for this medium were added as 3.3 mL of a solution consisting of (in grams per liter) $CuSO_4 \cdot 5H_2O$ (1.02), $MnCl_2 \cdot 4H_2O$ (1.3), and $ZnSO_4 \cdot 7H_2O$ (0.24). The first seven components of this medium were dissolved in 2 L of H_2O and extracted after addition of 8-hydroxyquinoline (5 g) with 100-mL batches of chloroform to remove residual iron. After extraction with chloroform until no ligand was detected in the chloroform, medium components were added, the solution was diluted to the appropriate volume, and the pH was adjusted to 6.5. Distilled deionized water was used in this and all other media, and all media were dispensed into acid-cleaned glassware. Most other media employed for production were similar in composition. Sucrose was substituted in some for glucose, and yeast extract often replaced the thiamin–amino acid mixture.

Isolation of Siderophores. At the end of the incubation period, the mycelia were removed by centrifugation, the broth was decanted and pooled, 2 g/L $FeCl_3 \cdot 6H_2O$ was added, and the solution was saturated with $(NH_4)_2SO_4$. The broth was extracted with benzyl alochol according to the method of Neilands (1952) until 90–95% of the visible absorption at 425 nm had been removed. The dark rust-brown organic layer was mixed with 5 volumes of ether and extracted with 0.25 volume of water. The rust-brown aqueous layer was washed 3 times with ether and reduced to dryness in vacuo to yield a dark brown viscous liquid.

The broth extract was dissolved in acetone–acetic acid solvent consisting of acetone–water–acetic acid (50:50:0.0015 v/v). This solution was chromatographed on a 5 × 25 cm column of carboxylic ion-exchange resin (200–400 mesh Bio-Rex 70, acid form) with the same solvent as eluant (column I). Fractions were collected, pooled based on their optical density at 425 nm and biological activity, and reduced to dryness in vacuo.

The first group of siderophores (A complex), which began eluting closely behind the void volume of the column, contained the largest amount of siderophores and was recovered by evaporation in vacuo. The combined A complex was dissolved in a minimum of a second acetone–acetic acid solvent containing acetone–water–acetic acid (25:75:0.0015 v/v) and was then chromatographed on a 2.5 × 50 cm Bio-Rex 70 column (200–400 mesh, acid form) with the same solvent (column II). This column resolved the A complex into several distinct bands. Fractions of each band were collected, pooled, and reduced to dryness in vacuo.

Each of the combined fractions from the second column was chromatographed on a 2.5 × 53 cm gel filtration column (Bio-Gel P-2, 200–400 mesh) in water to remove low molecular weight contaminants. The rust-brown siderophore fractions were reduced to dryness in vacuo to yield rust-brown glasslike solids.

For facilitation of structure determination of the siderophores, the chelated iron was removed with 8-hydroxyquinoline acording to the method of Keller-Schierlein (1963). Each iron-free siderophore was dissolved in methanol–water (1:10 v/v) and chromatographed on a 1.5 × 25 cm Bio-Rex 70 column (200–400 mesh, acid form) with the same solvent. The eluant was monitored at 254 nm. The residual chelated siderophore eluted more slowly than the iron-free ligand.

Siderophore Bioassay. This assay is a modification of an assay devised by Burnham & Neilands (1961) and is based on the growth stimulation of the siderophore auxotroph *Arthrobacter flavescens* JG-9. The bacterium was incubated in a low-iron and siderophore-free medium to deplete intracellular iron. The growth-arrested cells were dispersed in a soft nutrient agar medium (20 mL) dispensed in petri dishes. Serial dilutions of the siderophore sample were either prepared in 70% ethanol and 10-μL aliquots tranferred to sterile 0.25-in. filter paper disks or transferred as broth aliquots directly to

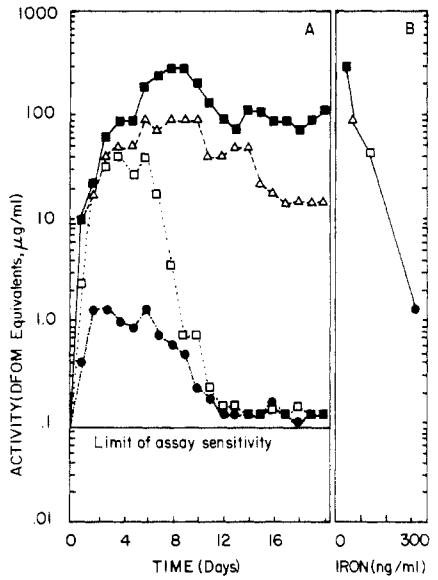

FIGURE 1: Production of *Arthrobacter flavescens* JG-9 stimulating compounds by *Epicoccum purpurascens*: effects of iron concentrations in a LIM–glucose–yeast extract medium initially deferrated with Chelex 100. (A) Concentrations in μg/mL DFOM equiv in relation to four Fe^{3+} concentrations, and (B) maximum concentrations in relation to total iron. Symbols: ng/mL added Fe^{3+} as $FeCl_3 \cdot 6H_2O$ and total iron (in parentheses) as determined by atomic absorption spectrophotometry: (■) 0 (36); (▲) 50 (68); (□) 100 (137); (●) 300 (314).

disks by using 0.05-mL microtiter loops. After the disks dried, they were transferred to assay plates and allowed to incubate for 24–36 h. The diameter of the dense growth zone in millimeters around each disk was recorded. Concurrently, standard dilutions were prepared of the commercially available siderophore desferrioxamine B methanesulfonate (DFOM; Ciba Corp., Desferal). A standard curve was plotted of the zone diameter vs. the log of the DFOM concentration (ng/mL). The concentration of the unknown siderophore solution is expressed in ng/mL DFOM equiv by extrapolation from the standard curve run concurrently with each experiment.

Tumor Inhibition Assay. Antitumor activity was based on the extension of survival time of 30-g Swiss Tex mice after 1×10^6 Ehrlich ascites tumor cells had been injected intraperitoneally (Greene & Greenberg, 1960). Intraperitoneal injections of the test substance (0.1 mL in aqueous solution) began 24 h later and continued daily for a total of 14 days. Test substances and the carrier control were coded, and the experiment was conducted under a double-blind protocol. The day of death was recorded for 45 days, and all mice surviving to the end of the experiment were considered statistically to have died on day 45. However, the number of survivors was recorded.

The siderophore fractions that were tested were deferrated with cold 1 N KOH by the method of Emery & Neilands (1960). The iron-free ligands were desalted by benzyl alcohol extraction (Neilands, 1952). The resulting aqueous phase was reduced to dryness in vacuo and resuspended in water for assay. Some degradation of the samples was observed by thin-layer chromatography of this extract.

Results

The production of exogenous siderophores by *Epicoccum purpurascens* is greatly diminished by increasing iron concentrations in various media. In general, variation in media components other than iron had only minor effects on the final exogenous siderophore concentration. Substitution of sucrose or yeast extract for amino acid mixtures had little effect on production as long as iron concentrations among the media remained about the same and the total amounts of carbon and nitrogen in the media were not drastically altered.

Table I: Weight and Relative Biological Activity of the Iron-Chelated Siderophore Fractions of *E. purpurascens* Broth

fraction	total solids[a] (mg)	biological act. (mg of DFOM)	sp act.
broth	2.8×10^5	1636.0	0.0058
benzyl alcohol extract	3100.0	776.0	0.25
column I			
A complex	1540.0	315.0	0.21
B complex	645.0	230.0	0.36
C complex	72.2	6.6	0.09
D complex	16.9	0.6	0.04
column II			
factor A1	13.5	1.0	0.08
factor A2	13.1	4.0	0.31
factor A3	7.5	2.3	0.31
factor A4	37.2	16.3	0.44
factor A5	59.0	52.5	0.89
factor A6	33.8	12.0	0.35
factor A7	278.9	150.8	0.54

[a] Based on 4.8 L of fungal broth.

Typical production experiments provided results such as presented in Figure 1. In this experiment the fungus was grown in LIM having sucrose (50 g) substituted for glucose and yeast extract (0.25 g) substituted for thiamin, proline, and ornithine. Siderophore production in this medium and most others tested was greatest when the medium was prepared without iron (Figure 1A). Atomic absorption spectrophotometry indicated that this medium contained about 36 ng of total iron/mL of medium (Figure 1B). Significant reductions in exogenous siderophore concentrations were noted in all media containing iron added as $FeCl_3$. Less than a 2-fold increase in total iron repressed siderophore synthesis about 3-fold whereas a 4-fold and 10-fold increase in total iron caused an 8- and 20-fold decrease, respectively. At all concentrations of iron, siderophore production increased very rapidly during the short period of rapid growth of the fungus (data not shown). At the two lowest concentrations of iron, exogenous siderophores remained high throughout the growth period. At these iron concentrations, growth of the fungus was severely limited. The intermediate iron concentrations produced moderately high concentrations of exogenous siderophores which disappeared rapidly as the time course of production continued. Production occurred similarly at the highest iron concentration, but at much reduced levels. Considerable growth was associated with the two highest iron concentrations.

The siderophores produced by *Epicoccum purpurascens* in the fermentation broth were concentrated approximately 40-fold by the benzyl alcohol extraction procedure as indicated in Table I to obtain 3.1 g of solids with a biological activity equivalent to 776 mg of DFOM, or a specific activity of 0.25, with a 47% yield.

Upon chromatography on the carboxylic resin in acetone, water, and acetic acid, the siderophores were separated into four distinct bands consisting of groups of compounds. The first group, A, was collected in 7.5-mL fractions from fraction 30 to fraction 80, a second group, B, in fractions 140–230, and a third group, C, in fractions 250–330. A fourth group of factors, D, was then eluted with 0.1% trifluoroacetic acid. The overall yield accounted for 71% of the activity, as indicated by Table I, showing also total solids and specific activities. The retention volume of the A complex suggests that these siderophores are probably relatively neutral compounds as they begin eluting closely behind the column void volume. Side-

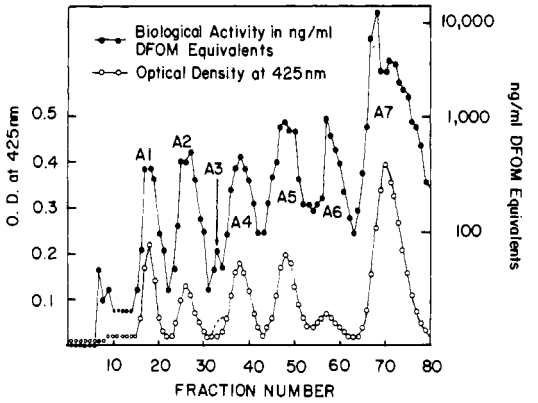

FIGURE 2: Separation of A complex group of siderophores from *Epicoccum purpurascens* on carboxylic acid ion-exchange resin in acetone–water–acetic acid (25:75:0.0015 v/v). Dashed line in optical density indicates variations in the amount of A3 obtained in different experiments.

rophores with three hydroxamate functions provide full octahedral coordination about a ferric ion under these conditions and typically exhibit a rust-brown color with a $\lambda_{max} \approx 425$ nm (Neilands, 1973; Diekmann, 1973). Both the A and the B complexes, which are the most abundant species, have this color. In contrast, mono- and dihydroxamates possess a purple color complexed with ferric ion (Neilands, 1973; Diekmann, 1973). The C and D complexes are distinguished by this color ($\lambda_{max} \approx 500$ nm).

The components of the A band, upon chromatography on the carboxylic resin in the more dilute acetone with a trace of acetic acid, were resolved into seven distinct bands as indicated in Figure 2. The degree of resolution of factor A3 from factor A4 varied in different experiments. Each of the A factors was further purified as described above and was compared by thin-layer chromatography in three solvent systems with a group of authentic siderophores. Factors A1 and A7 were chromatographically identical with ferricrocin and coprogen, respectively. The fractions also provided ^{1}H and ^{13}C nuclear magnetic resonance spectra identical with those of the authentic samples (data not shown). The remaining siderophores in the A complex appear to be newly discovered natural products based upon the evidence available.

The siderophores of the B complex are more basic than their A counterparts and are more difficult to separate. The siderophores of the C and D complexes appear to be mono- and dihydroxamic acids similar to the fusarinines and dimerumic acid.

A preliminary antitumor assay with a small number of mice indicated a 25% increase in mean survival time of those mice treated with the pooled A complex siderophores (after the removal of chelated iron with cold base) in comparison with the carrier-treated controls. No effect was observed with the other fractions. A subsequent assay of the individual A fractions with eight mice per group resulted in a 27% increase in the mean life span of those animals treated with factor A6 at 0.019 mg/mL vs. the carrier-treated control ($P > 0.995$ by t test). No other A fraction produced significant antitumor activity. This result was confirmed in a second assay of factor A6 with seven animals per group and a range of concentrations from 0.02 to 0.002 mg/mL to provide a modest increase in lifespan from 10% to 36% vs. the control group with $P > 0.975$ for the higher value.

Discussion

The highly pigmented fungus *E. purpurascens* is ecologically associated with the dead parts of plants and is frequently found in soils rich in humus. The strain used in this study was isolated from garden soil and taxonomically identified by one of us (P.J.S.) and confirmed by C. J. Alexopoulus and P. E. Powell (The University of Texas at Austin). Preliminary studies indicated that it was a source of a great variety of siderophores. Furthermore, the production of these factors was greatly enhanced by culture in a variety of low-iron media in accordance with previous studies (Neilands, 1973). In general, by use of the inoculum and medium conditions previously described, exogenous concentrations of siderophores supported the large amounts of growth associated with the high-iron cultures. Since the low-iron cultures did not have enough iron to ferrate all the siderophore molecules produced by the fungus and because fungi do not appear to transport deferrated siderophores into their mycelia, exogenous siderophore concentrations remain high in low-iron cultures.

The isolation of such a large number of siderophores from a single species was unprecedented. Previously, the most prolific sources of siderophores have been two fungal species which produce four structurally related fusarinines (Sayer & Emery, 1968; Moore & Emery, 1976). Concern was expressed that our unique isolation methods may have introduced structural artifacts into the natural products. The procedure was validated by the efficient isolation of ferricrocin and coprogen from *Neurospora crassa* as previously reported (Horowitz et al., 1976). Each of the compounds isolated from *E. purpurascens* and several authentic siderophores from other laboratories were stable to the chromatographic conditions of this study. Therefore, it appears that the individual siderophore species isolated from *E. purpurascens* represented the natural products present in the incubation medium.

Two of the siderophores, ferricrocin and coprogen, isolated from the broth of *E. purpurascens* have been characterized as natural products from several other fungal species (Diekmann, 1973). They are both trihydroxamic acids but differ significantly in structure and presumably in biosynthetic origin. The elution profile from column I clearly indicated that many other siderophores were produced by this organism, but the significance of this observation with regard to the biosynthetic pathway, metabolism, or survival of the fungus is not apparent.

The slight antitumor activity of factor A6 in the Ehrlich ascites assay appears to be the first tumor inhibitory effect observed for a siderophore; however, hadacidin (*N*-formyl-*N*-hydroxyglycine) is a hydroxamate with antitumor activity which does not appear to repress its synthesis in *Penicillium aurantio-violaceum* F 4070b (Nielands, 1973). The amount of inhibition noted (10–36%) by A6 was marginal, but repeatable with reasonably high statistical probability. Interestingly, only a single-colum fraction (A6) produced this activity, with all other factors having no effect. Structural studies reported separately on factor A6 suggested that it is related to coprogen, and this compound was designated triornicin.

Ong & Neilands (1979) have recently reported the presence of the common fungal siderophores ferrichrome and coprogen in commercial fungally ripened cheese. Tadenuma & Soto (1967) have previously identified several ferrichrome-type siderophores from sake and rice-koji that had been produced with *Aspergillus oryzae*. These observations imply that siderophores are reasonably common contaminants of the human diet. In this regard, *E. purpurascens* has been frequently associated with dead vegetation and grain products, and the siderophores produced by this species may contribute yet another element to the human diet.

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Structure of Triornicin, a New Siderophore†

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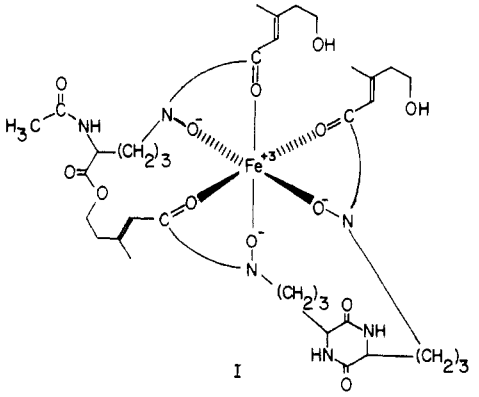
ABSTRACT: Spectroscopic analysis of the tumor inhibitory factor triornicin produced by *Epicoccum purpurascens* indicated that it was of similar structure to the known siderophore desferricoprogen, which is also produced by the fungus. The ^1H and ^{13}C NMR spectra indicated the replacement of an (*E*)-5-hydroxy-3-methyl-2-pentenoyl 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



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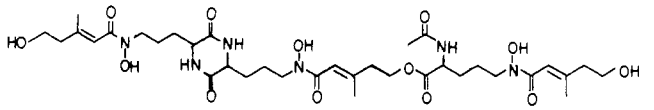
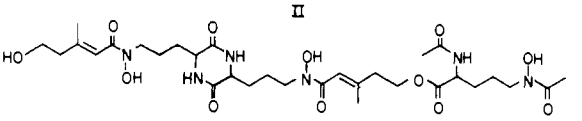
such examples and are produced by *E. purpurascens* (Frederick et al., 1980). Other previously characterized siderophores and related compounds have been reviewed (Nielands, 1973).

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



II



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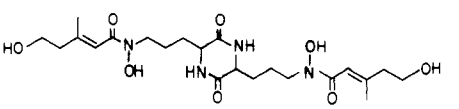
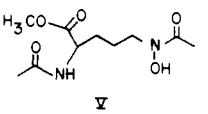


IV



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.

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