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Isolation, Characterization, and Properties of Fusarinine, a δ-Hydroxamic Acid Derivative of Ornithine*

Thomas Emery

ABSTRACT: Several previously undescribed hydroxamic acids have been isolated from *Fusarium* sp. and *Fusarium roseum* (ATCC 12822). Iron at concentrations as low as 4×10^{-7} M severely depresses the hydroxamate formation. The amount of hydroxamic acids reaches a maximum after 5–6 days of growth and then rapidly declines. All of the hydroxamates appear to contain δ-*N*-hydroxyornithine as the hydroxylamino moiety. No other amino acids were found. The compounds are all intensely ninhydrin positive. The most abundantly produced of these substances, fusarinine, has been characterized as δ-*N*-(*cis*-5-hydroxy-3-methylpent-2-enoyl)-δ-*N*-hydroxy-L-ornithine. No growth factor or antibiotic properties could be demonstrated for this compound. The finding that *N*-hydroxyornithine-δ-hydroxamates can occur at the amino acid level suggests that such hydroxamates are precursors of the hydroxamate functions of the ferrichrome compounds.

Approximately two dozen hydroxamic acids of microbial origin have now been described, and there is increasing evidence that hydroxamic acids are common to many, if not most, microorganisms. Although the structural diversity of these compounds is enormous these substances all have a common feature: the hydroxylamino group participating in the hydroxamate linkage is always donated by an hydroxylamino acid or a close derivative thereof. The most commonly found hydroxylamino acid is δ-*N*-hydroxyornithine, $HONH(CH_2)_3CH(NH_2)COOH$. Although this amino acid derivative has not been found as such in nature, it is the fundamental subunit of albomycin (Turková *et al.*, 1962), the ferrichromes (Emery and Neilands, 1961), ferrichrysin, ferrirhodin, ferrirubin, and ferricrocin (Zähner *et al.*, 1963). In all of these compounds the α-amino and α-carboxyl groups of the ornithine residues are linked by amide bonds in a cyclic hexapeptide, and the hydroxylamino groups are acylated to yield the hydroxamic acids.

This paper describes the occurrence of a number of new hydroxamic acids synthesized by the fungus *Fusarium*. All of these compounds appear to contain δ-*N*-hydroxyornithine. One of these compounds, fusarinine, has been identified as δ-*N*-(*cis*-5-hydroxy-3-methylpent-2-enoyl)-δ-*N*-hydroxy-L-ornithine and is thus the first ornithine hydroxamate described in which the amino and carboxyl groups of the ornithine moiety are not bound in peptide linkage. The significance of this finding to the problem of the biosynthesis of hydroxamic acids is discussed.

Experimental

Isolation and Purification of Hydroxamic Acids of Fusaria. Fusarium roseum (ATCC 12822) or *Fusarium* sp., isolated as a laboratory contaminant, was grown in 2-liter Fernbach flasks containing 500 ml of modified Grimm-Allen medium (Garibaldi and Neilands, 1955). The inoculum was 5–10 ml of a 48-hour culture of the organism grown on the same medium. The flasks were incubated at 26° on a reciprocal shaker with a 3.8-cm stroke at 80 strokes per minute. After 5–6 days the mycelia were removed by suction filtration and the clear yellowish filtrate was concentrated to dryness *in vacuo* at 40°. The residue was triturated several times with methanol. The combined methanol extracts

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were filtered and the filtrate was cooled to −30°. A crystalline material of nonhydroxamate nature was removed by centrifugation. The solution was then concentrated to yield a golden-yellow syrup.

To the syrup was added about two volumes of water and the *p*H was adjusted to 5.2 with glacial acetic acid. The solution was subjected to high voltage electrophoresis at 5° using water-washed Geon 427 (B. F. Goodrich Chemical Co., Cleveland, Ohio) as an inert support. The buffer was acetic acid–pyridine–water, 14:10:930, *p*H 5.2. The electrophoresis was allowed to continue for 16 hours at a potential of 10 v/cm (50–60 ma). The electrophoretically neutral compound, which could be located by its slightly yellow color, was eluted from the Geon block with water and concentrated *in vacuo*. High voltage paper electrophoresis (32 v/cm) was carried out on an aqueous solution of the compound using the same buffer or 3.5% formic acid. The compound was detected on the dried paper by spraying with 10% ferric chloride or with 0.5% ninhydrin in acetone. Ascending paper chromatography on Whatman No. 1 paper was carried out with the following solvents systems: (a) 1-butanol-ethanol-H_2O, 4:1:5 (upper phase); (b) pyridine-water, 65:35; (c) methanol-water-diethylamine, 80:20:4; (d) butanol–acetic acid–water, 100:12:25; (e) water-saturated 1-butanol. Paper chromatography on the first solvent system was used to purify the compound further. The eluted compound could be lyophilized to yield a noncrystalline, slightly yellow powder which was extremely hygroscopic. The iron chelate was obtained by the addition of 10% ferric chloride to an aqueous solution of the compound until the maximum absorbancy at 480 mμ was reached. A brick-red precipitate was obtained by the addition of acetone. The precipitate was dissolved in water and lyophilized. Although this substance could not be crystallized, it was less hygroscopic than the iron-free material and was suitable for analysis. Elemental analysis was performed by Schwarzkopf Analytical Laboratories, Woodside, N.Y.

Reductive Hydrolysis: Detection of Ornithine. Several mg of the purified iron-free material was dissolved in 0.5 ml of 48% hydriodic acid and sealed in a glass tube *in vacuo*. After hydrolysis at 110° for 16 hours the acid was removed in a vacuum desiccator over sodium hydroxide flakes. Paper chromatography was carried out on the residue dissolved in a small amount of water. Ornithine was assayed chemically by the method of Chinard (1952), and microbiologically using *E. coli* 39A-23R3 as follows: 5 ml of sterile Davis medium (Davis and Mingioli, 1950) containing 0–1000 μg of L-ornithine monohydrochloride (Nutritional Biochemicals Corp.) was inoculated with 1 drop of a 24-hour culture of the organism. The tubes were incubated at 32° with shaking for 18 hours and the absorbance at 600 mμ was determined with a Bausch and Lomb Spectronic 340 spectrophotometer. Optical density values ranged from 0.070 for 10 μg of ornithine to 0.98 for 1000 μg of ornithine.

Nonreductive Hydrolysis: Detection of δ-N-Hydroxyornithine. The hydrolysis was carried out as described above using glass-distilled 6 N hydrochloric acid instead of hydriodic acid. Paper electrophoresis of the dried hydrolysate dissolved in water was performed using the pyridine–acetic acid buffer described above. Ornithine and authentic δ-*N*-hydroxyornithine prepared from ferrichrome A (Rogers and Neilands, 1963) were used as markers. Catalytic hydrogenation was carried out using Adams' catalyst and hydrogen at 1 atm.

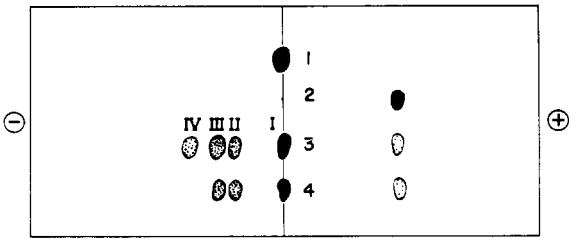


FIGURE 1: Paper electrophoresis at *p*H 5.2 on pyridine–acetic acid–water, 14:10:930, Whatman No. 1 paper at 32 v/cm for 0.5 hour. (1) Ferrichrome; (2) ferrichrome A; (3) medium from 6-day *Fusarium* sp. culture; (4) medium from 6-day *Fusarium roseum* (ATCC 12822) culture. The spots from the *Fusaria* cultures were developed by spraying with 10% $FeCl_3 \cdot 6\ H_2O$ or 0.5% ninhydrin in acetone.

Isolation of 3-Methypent-2-eno-5-lactone. To 1.0 ml of an aqueous solution containing about 10 mg of the hydroxamate was added 0.2 ml of 0.1 N periodic acid. After standing for 5 minutes at room temperature the solution was extracted five times with ether. The combined ether extracts were concentrated to about 0.1 ml and subjected to analysis using a Perkin-Elmer Model 810 gas chromatograph equipped with a dual flame ionization detector. The column was 180 × 0.3 cm with a liquid phase of silicone oil DC-710 (20%) on Anakrom ABS, 90/100 mesh. Authentic *cis*-5-hydroxy-3-methylpent-2-enoic acid was prepared by the method of Cornforth *et al.*, (1958). The acid spontaneously lactonized upon standing. Samples of the acids for infrared spectroscopy (Beckman IR 5 spectrophotometer) were isolated from the gas chromatograph using a gas stream splitter. Ultraviolet and visible spectra were taken on a Bausch and Lomb Spectronic 505 spectrophotometer.

Results

Purification and Properties of Fusarium Hydroxamic Acids. During studies on the biosynthesis of the ferrichrome compounds by the smut fungus *Ustilago sphaerogena* a fungal contaminant was observed in one of the culture flasks. After growth of the contaminant, the culture fluid gave a strongly positive ferric chloride test typical of that observed for other hydroxamic acid–producing fungi. The organism was isolated in pure culture and continued to exhibit this striking hydroxamate formation when grown on an iron-deficient medium. The organism was identified as a

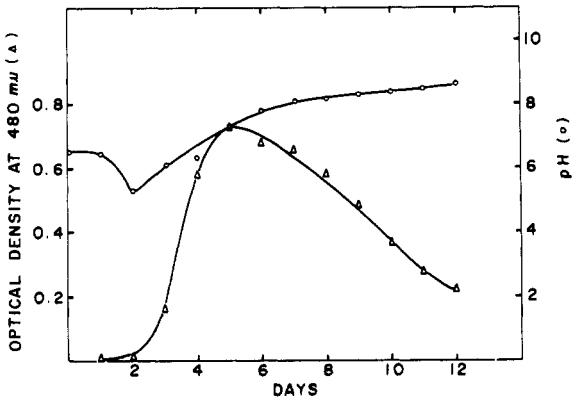


FIGURE 2: Formation of hydroxamates (—△—) and *p*H changes (— O —) of a *Fusarium roseum* (ATCC 12822) culture. See Experimental for growth conditions.

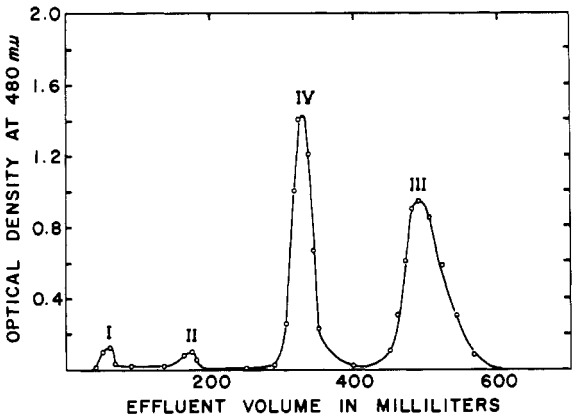


FIGURE 3: Separation of the iron chelates of *Fusarium* sp. hydroxamates on CM-cellulose. Filtered culture medium (150 ml) was extracted with phenol-chloroform (1:1) after the addition of ferric chloride until the maximum OD at 480 mμ was reached. The chelates were extracted into water by the addition of 10 volumes of ether to the organic phase. The aqueous extract was concentrated to 4 ml and placed on a 28- × 205-mm column of CM-cellulose (Bio-Rad). After washing with water the colored bands were eluted with pyridine–acetic acid–water, 14:10:930. The flow rate was 4 ml/min.

species of *Fusarium*, a common air-borne fungus. *Fusarium roseum* (ATCC 12822) exhibited equally impressive hydroxamate formation when grown under the same conditions. A sample of the medium after 6 days growth was subjected to high voltage paper electrophoresis at *p*H 5.2. When sprayed with ferric chloride, hydroxamic acids give purple spots on a yellow background. A number of ferric chloride–positive spots were observed (Figure 1). The most intense spot was neutral at this *p*H but moved as a cation when the electrophoresis was performed at *p*H 2 using 3.5% formic acid. This component was thus not identical with ferrichrome, which remains uncharged at the lower *p*H, and the presence of a free carboxyl group appeared likely. The *Fusarium* isolated as a contaminant originally produced an hydroxamate completely lacking in *Fusarium roseum* cultures; on continued subculturing, however, this most positively charged species (compound IV, Figure 1) disappeared and the two organisms showed identical patterns of hydroxamate formation. All subsequent work was performed with *Fusarium roseum*. At *p*H 5.2 ferrichrome A moves as an anion because of the presence of three free carboxyl groups. *Fusarium* consistently produced a species of hydroxamate with an electrophoretic mobility at this *p*H identical with ferrichrome A. However, this material was produced in such poor yield that no further work was done on it.

It was obvious that the principal hydroxamic acids produced by *Fusarium* were not of the ferrichrome type, since all known compounds related to ferrichrome are neutral or acidic substances, nor was the electrophoretic behavior of these new compounds attributable to any other known hydroxamates. An additional observation that distinguished these substances from almost all other naturally occurring hydroxamic acids was that they exhibited an intense reaction with ninhydrin typical of α-amino acids. Even when the electrophoresis was carried out on the iron chelates, the reaction with ninhydrin was so intense as to develop over the red-brown color of the chelates. The ferrichrome compounds give no reaction with ninhydrin, and it was apparent that we were in fact dealing with a group of hitherto undescribed compounds.

Figure 2 shows the time course of hydroxamate formation. The maximum production is reached after 5–6 days, which is typical of hydroxamate formation in other organisms. However, the steady decrease after this time was not expected and is undoubtedly a consequence of the instability of these compounds, as will be discussed below. Hydroxamates were routinely isolated after 6 days of growth. The initial decrease in *p*H of the culture medium is typical of other hydroxamate-producing organisms. However, in other cases studied the *p*H remains relatively constant at *p*H 5–6. The increase of *p*H in the case of *Fusarium* is probably a reflection of the formation of the basic (cationic) hydroxamates. Fungi produce copious amounts of hydroxamic acids only when cultured in iron-deficient media. This effect was likewise observed with *Fusarium*, concentrations of iron as low as 4×10^{-7} M depressing hydroxamate formation by over 50%. The iron feedback inhibition of hydroxamate synthesis originally described by Neilands (1957) is thus also operative in this organism. The nature of this inhibition remains unknown.

Purification of these compounds at first proved unexpectedly difficult. The alcohol extraction method used so successfully by Garibaldi and Neilands (1955) for the isolation of the ferrichromes was unsuccessful. An attempt was made to extract the iron chelates using phenol-chloroform by the method of Zähner *et al.*

(1963). Initially this procedure met with some success. An aqueous solution of the highly colored extract was subjected to column chromatography on carboxymethylcellulose (Figure 3). Each peak appeared pure when subjected to high voltage paper electrophoresis. The intensity of the peaks in the elution diagram is not representative of the actual amounts of the components in the culture fluid since the extraction procedure was not quantitative, and it is probable that the neutral component (I), which always appeared to be the most abundant species by electrophoresis, was much less efficiently extracted than the others. It is interesting to note that the order of elution does not follow the basicity of the compounds as judged by electrophoresis, components III and IV emerging in reverse order. The phenol-chloroform extraction method subsequently proved to be impracticable, however, because of the formation of unbreakable emulsions. It was finally observed that the compounds could be extracted directly from the dried supernatant culture fluid (see Experimental) to yield the iron-free materials. This proved to be fortunate as it was also observed that removal of iron from the chelates by the standard techniques was not possible because of the instability of these substances. Final purification was achieved by electrophoresis and paper chromatography. Since the neutral material was produced in greatest yield, subsequent work was done with this compound, henceforth called fusarinine.

Purified fusarinine yielded a viscous syrup when concentrated *in vacuo*. The syrup did not crystallize upon standing at $-15°$ for extensive periods of time. Efforts to crystallize the compound as the hydrochloride or as various salts were unsuccessful. The compound could be precipitated from aqueous or alcoholic solution by the addition of acetone to yield a white material which formed an intractable gum upon brief exposure to air. This behavior is not unusual with naturally occurring hydroxamic acids. All efforts to crystallize the iron-free ferrichromes have been unsuccessful, although the iron chelates are beautifully crystalline. The iron chelate of fusarinine could not be crystallized, although it was obtained in a form suitable for elemental analysis. However, since fusarinine yielded only one ferric chloride- or ninhydrin-positive spot upon paper electrophoresis at two different *p*H values and upon paper chromatography on five different solvent systems, the compound was considered pure enough for structural work, and the results to be presented are indicative of its purity. The R_F values of fusarinine chromatographed using the solvent systems described in the experimental section are as follows: (a) 0.36; (b) 0.88; (c) 0.85; (d) 0.32; (e) 0.20. Qualitative tests for phosphorous, sulfur, and halogen were negative.

Spectral Properties of Fusarinine–Iron Chelate. Figure 4 shows the absorption spectrum of fusarinine in the range 300–600 mμ in the presence of a stoichiometric amount of iron added as ferric chloride. At *p*H 8 there is a broad absorption peak with a maximum at 440 mμ. Such a peak is typical of 3:1 hydroxamate–iron chelates at *p*H values near neutrality. This peak is in fact

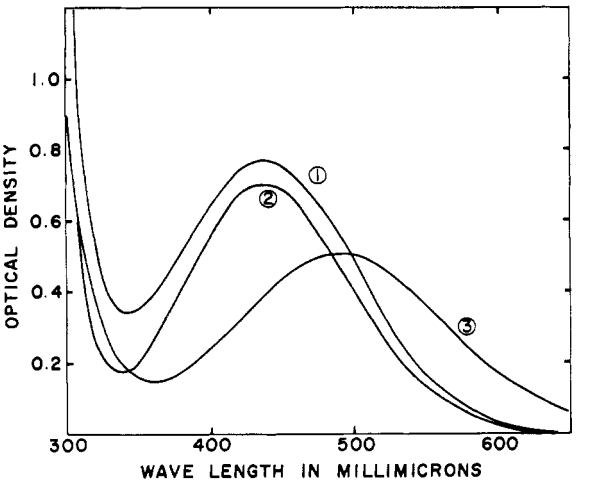


FIGURE 4: Absorption spectra of ferrichrome A and fusarinine–iron chelate. Ferrichrome A at *p*H 8.0 and 2.5 (1); fusarinine–iron chelate at *p*H 8.0 (2), at *p*H 2.5 (3).

qualitatively identical with that of ferrichrome A whose spectrum is also shown in Figure 4. Addition of excess sodium hydrosulfite causes the immediate disappearance of the peak as the iron is reduced to the ferrous form. An excess of ethylenediaminetetraacetate (EDTA) also bleaches the compound as the iron is removed to form the colorless EDTA–iron chelate. Ferrichrome A and other naturally occurring trihydroxamic acids are much more resistant to bleaching by these reagents because of the greatly enhanced chelate effect provided by the presence of three hydroxamate functions on the same molecule. These results therefore indicated the monohydroxamic acid nature of fusarinine.

Another property useful in distinguishing monohydroxamate- and trihydroxamate–iron chelates is the change in absorption spectra with increasing acidity. At a *p*H value near neutrality a simple (mono) hydroxamic acid forms a 3:1 chelate with ferric ion. As the *p*H is lowered to about 3, protons compete with iron for the hydroxamate groups and a 2:1 chelate is favored. At a *p*H value of 1 or less the 1:1 chelate is favored. The changes are accompanied by a shift of the visible absorption spectrum to higher wavelengths and a decrease in absorbance. The absorption spectrum of fusarinine at *p*H 2.5 clearly shows this effect, while the spectrum of ferrichrome A at this lower *p*H is identical with that at *p*H 8. This difference is a useful qualitative test to distinguish monohydroxamic acids, such as acetohydroxamic acid, from the naturally occurring trihydroxamates with their much greater affinity for iron. Fusarinine is clearly a monohydroxamic acid by this criterion.

Acid Hydrolysis of Fusarinine. In order to determine the amino acid composition of fusarinine, it was hydrolyzed with 48% hydriodic acid. This procedure quantitatively reduces all hydroxylamino groups to

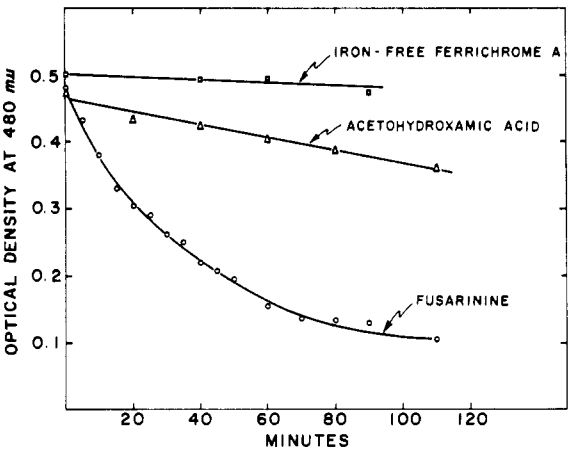


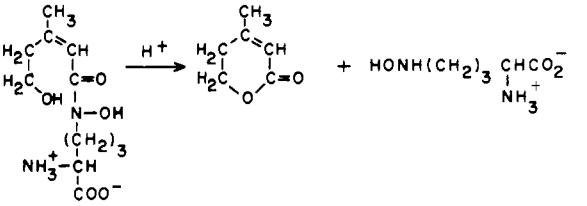
FIGURE 5: Relative rates of hydrolysis of iron-free ferrichrome A, acetohydroxamic acid, and fusarinine at 50° in 0.5 N HCl. One-tenth ml of 10% $FeCl_3 \cdot 6 H_2O$ was added to aliquots and the optical density was determined after dilution to 5 ml with water.

amino groups (Emery and Neilands, 1961). When the hydrolysate of fusarinine was examined by paper electrophoresis and paper chromatography only one ninhydrin-positive spot was seen. This product was chromatographically identical with ornithine and gave a positive Chinard reaction, which is specific for ornithine and proline (Chinard, 1952). The configuration of the ornithine in the hydrolysate was examined by means of a microbiological assay with *E. coli* 39A-23R3 (Baich and Vogel, 1962). This organism shows no growth response to D-ornithine, and DL-ornithine gives exactly one-half the growth response of an equal amount of the L isomer. The microbiological assay showed 7.4 ± 0.2 mg of L-ornithine/ml of the fusarinine acid hydrolysate. Total ornithine by the Chinard chemical assay yielded 7.5 ± 0.2 mg/ml, and it can be concluded that the ornithine of fusarinine is exclusively of the L configuration, as for the ornithine derived from the ferrichrome compounds.

When fusarinine was subjected to mild hydrochloric acid hydrolysis and the hydrolysate examined by paper electrophoresis, one ninhydrin- and tetrazolium-positive spot (Snow, 1954) was found; its electrophoretic mobility was identical with that of δ-*N*-hydroxyornithine obtained by hydrolysis of iron-free ferrichrome A. The product reacted with periodic acid to yield a compound with an intense ultraviolet absorption peak at 267 mμ. This reaction with periodate is characteristic of *N*-alkylhydroxylamino compounds (Emery and Neilands, 1960b, 1962). Finally, the product of the hydrolysis could be hydrogenated catalytically to yield ornithine as the sole product. No ornithine could be detected in the hydrolysate before reduction. At this point it was apparent that fusarinine was a δ-*N*-acyl derivative of δ-*N*-hydroxyornithine, and it only remained to identify the acyl moiety.

Identification of the Acyl Moiety of Fusarinine. δ-*N*-Hydroxyornithine has been found in a number of hydroxamates of microbial origin. Table I lists the acyl groups taking part in the hydroxamate function in these compounds. The close structural relationship of the acids to mevalonic acid is quite remarkable. Certain chemical and physical properties of fusarinine led us to suspect that the acyl group of this substance was in fact identical with that of ferrirhodin, namely, *cis*-5-hydroxy-3-methylpent-2-enoic acid. Fusarinine shows an intense ultraviolet absorption with a peak at 223 mμ, similar to that shown by the *trans*-β-methylglutaconic acid isolated from ferrichrome A. This absorption is characteristic of the —RC=CH—CO— group, and it seemed highly probable in analogy to other known hydroxamates that R would prove to be methyl. A dicarboxylic acid such as methylglutaconic acid was ruled out because of the neutrality of fusarinine at *p*H 5.2 on paper electrophoresis.

An unusual property of fusarinine, distinguishing it from other synthetic and naturally occurring hydroxamic acids, is its marked acid lability. Hydrolysis with 0.5 N hydrochloric acid at 100° led to complete destruction, in less than 5 minutes, of the hydroxamic acid linkage, as determined by the ferric chloride color reaction. The rate of hydrolysis at 50° is shown in Figure 5. The first-order rate constant was calculated to be 0.02 min^{-1}. Iron-free ferrichrome A and acetohydroxamic acid are far more stable under these conditions. As stated above, δ-*N*-hydroxyornithine was released during the hydrolysis and it was apparent that the acid lability of fusarinine was a consequence of the nature of the acyl group of the hydroxamate function. *cis*-5-Hydroxy-3-methylpent-2-enoic acid is known to lactonize spontaneously (Cornforth *et al.*, 1958). Under acid conditions, such lactone formation would be a driving force for the hydrolysis of the hydroxamate linkage and explain the acid lability of fusarinine:

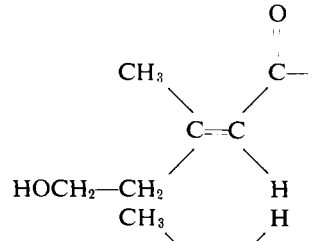
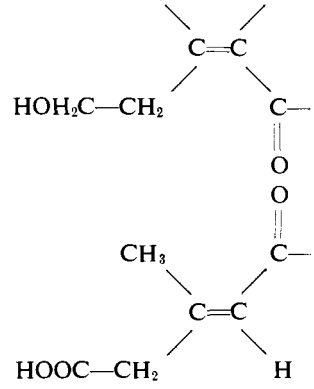


This structure of fusarinine is in agreement with the elemental analysis obtained for the 3:1 fusarinine–iron chelate.

Anal. Calcd for $C_{33}H_{57}O_{15}N_6Fe$ (834): C, 47.53; H, 6.89; N, 10.08; Fe, 6.70. Found: C, 47.44; H, 7.63; N, 9.88; Fe, 6.99.

The acyl moiety of fusarinine was released by mild acid hydrolysis and extracted into ether. Alternatively, the acyl group could be released by reaction of fusarinine with periodic acid. Synthetic *cis*-5-hydroxy-3-methylpent-2-enoic acid was prepared by dehydration of mevalonic acid lactone followed by saponification of the unsaturated lactone (Cornforth *et al.*, 1958). Upon standing, the free acid reverted to the lactone, which was compared by gas-phase chromatography to the compound isolated from fusarinine. Both the natural

TABLE I: Acyl Groups of Some Naturally Occurring Hydroxamic Acids.

Acyl Group	Compound	Reference
	Ferrichrome	Emery and Neilands (1960a)
	Ferrichrysin	Zähner *et al.* (1963).
	Ferricrocin	Zähner *et al.* (1963)
	Albomycin	Poddubnaya and Krysin (1962)
	Ferrirubin	Zähner *et al.* (1963)
	Coprogen	Zähner (personal communication)
	Ferrirhodin	Zähner *et al.* (1963)
	Ferrichrome A	Emery and Neilands (1960a)

and synthetic compounds gave symmetrical peaks with identical retention times using a number of different column temperatures and flow rates. A mixture of the natural and synthetic compounds gave one symmetrical peak under all conditions. As a matter of interest, *trans*-5-hydroxy-3-methylpent-2-enoic acid was prepared from iron-free coprogen by reaction with periodate, followed by ether extraction. The methyl ester, prepared by reaction of the acid with diazomethane in ether, moved quite close to the *cis* lactone but nevertheless was clearly separated, having a somewhat greater retention time, and gave a double peak when mixed with either the synthetic or natural *cis* lactone. Acid hydrolysis of coprogen was not satisfactory for the isolation of the *trans* acid because the drastic conditions (6 N HCl, 100°, 1 hr.) necessary to cleave the hydroxamate bonds in coprogen resulted in extensive isomerization of the acid, as evidenced by the double peak observed in gas-phase chromatography. Recently, Snow reported that the *cis*-octadec-2-enoic acid moiety of the hydroxamic acid, mycobactin P, is also extensively isomerized when isolated by acid hydrolysis, and this acid could be obtained pure only when released from mycobactin P by the periodate reaction (Snow, 1965).

Several mg of the acyl moiety of fusarinine and synthetic *cis*-3-methylpent-2-eno-5-lactone was collected by splitting the gas stream from the chromatographic column. The infrared spectra of the two compounds are shown in Figure 6 and leave little doubt as to the identity of the substances. The ultraviolet spectra were identical with a peak at 223 mμ and a molar extinction coefficient of about 8.6×10^3 in aqueous solution.

In hitherto known hydroxamic acids containing δ-*N*-hydroxyornithine, both the α-amino and α-carboxyl groups of the ornithine residues are bound in peptide linkage. The fact that these groups are unsubstituted in fusarinine imparts to it some additional chelating properties. It has already been pointed out that the properties of fusarinine with respect to iron chelation at neutral and acidic conditions are in all respects those expected for such a monohydroxamic acid. However, this compound shows an unexpected tenacity for ferric ion at alkaline *p*H values. The extreme insolubility of ferric hydroxide makes it impossible for all but the very strongest chelates, such as ferrichrome and ferrichrome A, to maintain a measurable amount of iron in solution at a *p*H higher than 11. Ferric ion is instantly precipitated from acetohydroxamic acid, and even such a stable chelate as EDTA-Fe(III) cannot compete with OH^- at *p*H 11. However, when the iron chelate of fusarinine is brought to this *p*H by the addition of sodium hydroxide the iron remains in solution even though the red color of the chelate is discharged. This behavior is typical of the ferrichromes

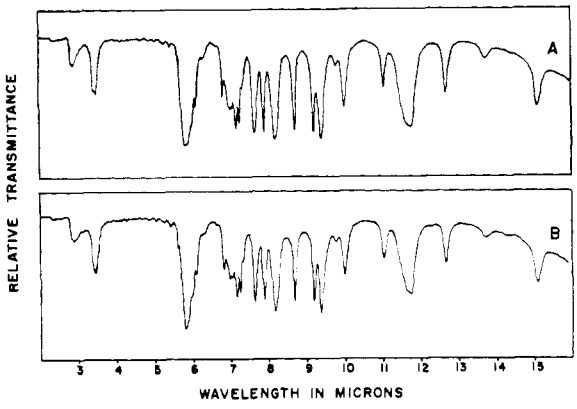
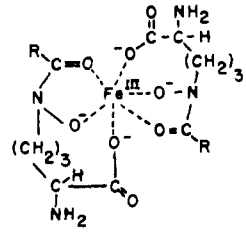


FIGURE 6: Infrared spectra of synthetic 3-methylpent-2-eno-5-lactone (A) and the compound isolated from the acid hydrolysate of fusarinine (B).

and is unusual for a monohydroxamate. Ornithine itself exhibits no unusually strong chelation of iron. A possible explanation of this phenomenon is the participation of the free carboxylate or amino group of fusarinine in the chelation. Examination of a molecular model indicates that either of these groups is capable of swinging below the plane of the iron-hydroxamate ring to coordinate a third position of the octahedral iron. A second molecule of fusarinine could occupy the other three coordination positions of the iron, thus occupying all six coordination positions of the metal. A 2:1 hydroxamate–iron chelate would thus be formed,



R = $HOCH_2CH_2C(CH_3)=CH-$

each ligand being tridentate.

Another interesting property of fusarinine is its unusual chelation of copper. Cupric ion forms insoluble green precipitates with simple hydroxamic acids, and this property is also observed with the naturally occurring hydroxamic acids. However, when a saturated solution of cupric acetate is added dropwise to an aqueous solution of fusarinine, a green color is initially formed followed by a blue-green color upon further addition of the copper. At no concentration of copper does a precipitate form. This property is undoubtedly a consequence of the solubilizing effect of the α-carboxyl and α-amino groups, which themselves form a soluble blue copper chelate.

The ultraviolet absorption spectra of fusarinine at various *p*H values are shown in Figure 7. In general, in

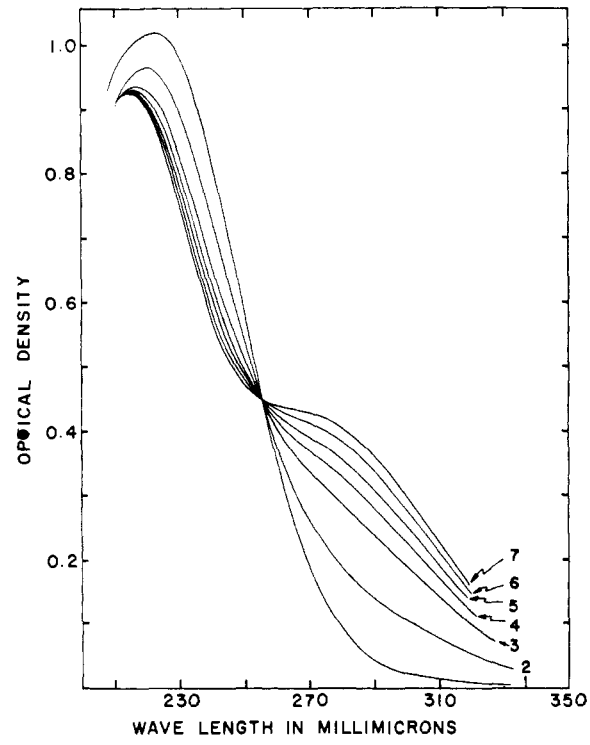


FIGURE 7: Ultraviolet absorption spectrum of fusarinine as a function of *p*H. The concentration of fusarinine was 0.032 mg/ml and the *p*H was adjusted by addition of 1 N HCl or 1 N NaOH. The *p*H values for the curves are (1) 5.8; (2) 8.5; (3) 8.9; (4) 9.0; (5) 9.4; (6) 9.8; (7) 10.1.

basic solutions hydroxamic acids show a shift of the ultraviolet end absorption toward a higher wavelength. The isosbestic point at 256 mμ is also observed in iron-free ferrichrome A, although the spectral change with *p*H is less pronounced. Iron-free ferrichrome shows no such effect. This phenomenon can be attributed to the α,β unsaturation of the acyl function of the ionized hydroxamate group. A spectrophotometric titration of fusarinine shows a pK_a' of 8.8, exactly in the range expected for this hydroxamic acid.

Burnham and Neilands (1961) have shown that growth of *Arthrobacter* JG-9 is dependent upon ferrichrome or other naturally occurring hydroxamic acids. Synthetic monohydroxamic acids show no growth-factor activity for this organism. It was of interest to determine if fusarinine, a naturally occurring monohydroxamic acid, would be active. When tested with *Arthrobacter* JG-9 by the agar plate diffusion method of Zähner *et al.* (1960), fusarinine showed no growth factor activity at a concentration of 0.1 mg/ml. Ferrichrome gave a definite growth response at a concentration of 0.005 mg/ml. A number of naturally occurring hydroxamic acids show antibiotic activity. However, fusarinine at a concentration of 0.1 mg/ml showed no antibiotic activity against *E. coli* or *B. subtilis* when tested by the plate diffusion method.

Discussion

Prior to the isolation of fusarinine, work was in progress in our laboratory on the biosynthesis of hydroxamic acids. δ-*N*-Hydroxyornithine, the most commonly found hydroxyamino acid, had only been observed as a component of cyclic hexapeptide hydroxamates such as ferrichrome. Since cyclic peptides containing ornithine are common products of microbial metabolism, it seemed not unreasonable to postulate that the route of biosynthesis of a compound such as ferrichrome would proceed via synthesis of cyclotriglycyltriornithine. The three free δ-amino groups of the ornithine residues could then be oxidized to hydroxylamino groups and acetylated to form the three hydroxamate functions. This route of biosynthesis seemed attractive not only because it invoked the synthesis of a cyclic peptide but also because it obviated the formation of free δ-*N*-hydroxyornithine, a compound that is very unstable and whose chemical reactivity might be incompatible with more than a transitory existence within the cell.

Fusarinine is a structural subunit of ferrirhodin, which contains three residues of fusarinine in cyclic peptide linkage with one molecule of glycine and two of serine (Zähner *et al.*, 1963). The isolation of fusarinine demonstrates that an hydroxamic acid derivative of δ-*N*-hydroxyornithine occurs at the amino acid level, and shows that in this case incorporation of the amino acid into a peptide is not requisite for the synthesis of the hydroxamate group. Hadacidin, *N*-formyl-*N*-hydroxyglycine, is another example of an hydroxamic acid derivative of a nonpeptide-bound *N*-hydroxyamino acid. It now seems possible that hydroxamate formation in general occurs at the *N*-hydroxyamino acid level. According to this hypothesis, δ-*N*-acetyl-δ-*N*-hydroxyornithine should be a precursor of ferrichrome, and the very reactive hydroxyamino group would be protected throughout the biosynthetic reaction sequence by its initial acetylation to form the stable hydroxamate group. Experiments are now in progress to test this hypothesis.

The acylation of the δ-*N*-hydroxyornithine to form the hydroxamate must be a very specific reaction. Although a number of different acyl groups have been found in trihydroxamic acids (Table I) only one is ever found in a given compound. For example, the acyl groups of ferrichrome are acetyl groups, and *trans*-β-methylglutaconyl are the acyl groups of ferrichrome A, but no hydroxamate containing mixed acyl groups is known. In view of the great reactivity of the hydroxyamino group, the enzyme or enzymes responsible for the acylation of δ-*N*-hydroxyornithine must not only be extremely specific for the acyl group but also very active; otherwise nonspecific acylation and other side reactions would be expected to occur at the hydroxyamino group. A search for such enzymes is under way. It is striking that the acyl groups of all trihydroxamic acids of the ferrichrome type are close derivatives of mevalonic acid. Ferrichrome itself contains acetate, a mevalonic acid precursor. Ferrichrome type compounds have been demonstrated in a multitude of microorganisms, and it can hardly be coincidental that the acyl group of the hydroxamates are always mevalonic acid derivatives.

The amounts of hydroxamates formed by fungi are surprisingly high. Up to 1 g of the ferrichrome compounds have been found per liter of a *Ustilago* culture. More than 800 mg of δ-*N*-hydroxyornithine have been found in the extracellular hydroxamates of a liter of *Fusarium* culture fluid, representing well over 1 g of hydroxamic acids. This is even more remarkable when one considers that the cellular dry weight is only about 2 g per liter. It is obvious that δ-*N*-hydroxyornithine, in the form of hydroxamic acid derivatives, must be considered as the quantitatively most important amino acid in these fungi under appropriate growth conditions.

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