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N^{α}-Acetylfusarinines: Isolation, Characterization, and Properties†

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ABSTRACT: The isolation, purification, and structural elucidation of a new group of naturally occurring hydroxamic acids are described. These compounds are produced by an unidentified species of *Penicillium* when grown on iron deficient medium. They are closely related structurally to the fusarinines, whose basic structure contains N^{δ}-(*cis*-5-hydroxy-3-methylpent-2-enoyl)-N^{δ}-hydroxy-L-ornithine, but the α-amino groups in the new compounds are protected by N^{α}-acetyl groups. The most complex of the compounds is N,N',N''-triacetylfusarinine C, or N,N',N''-triacetylfusigen, a cyclic triester composed of three molecules of N^{α}-acetylfusarinine. The trimer has growth factor activity for *Arthrobacter* JG-9 about equal to fusarinine C itself. The monohydroxamate, N^{α}-acetylfusarinine, is more active than the trimer. The greater chemical stability of these compounds compared to the unacetylated fusarinines reconciles the utilization of otherwise unstable amino acid esters as iron transport agents.

The fusarinines are a group of hydroxamic acid derivatives of ornithine produced by several species of fungi (Diekmann and Zähner, 1967; Sayer and Emery, 1968; Neilands, 1973). These compounds are unique among the naturally occurring hydroxamic acids in that the subunit, fusarinine, or N^{δ}-(*cis*-5-hydroxy-3-methylpent-2-enoyl)-N^{δ}-hydroxy-L-ornithine, is polymerized into dimers and trimers via ester bonds between the carboxyl group of the ornithine and the 5-hydroxy group of the acyl moiety. This is in contrast to ferrichromes and ferrioxamines, in which the hydroxamic acid subunits are joined via amide bonds (Neilands, 1973). The aminoacyl ester bonds of the fusarinines are extremely labile, which leads one to question the utility of these compounds for their postulated role as extracellular iron sequestering agents.

We have now discovered a new fusarinine type compound in the culture supernatant of an unidentified species of *Penicillium*. This paper describes the isolation of this compound and related compounds and their identification as N^{α}-acetylfusarinines. The most complex of them is a cyclic trimer made up of three molecules of N^{α}-acetylfusarinine joined by head-to-tail ester bonds exactly analogous to the unacetylated fusarinine polymers. However, in contrast to the latter substances, the new siderochrome is very stable by virtue of the acetylation of the α-amino groups.

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Materials and Methods

Isolation of Hydroxamates. The *Penicillium* sp. was isolated from a garden variety eggplant. It was subsequently cultured on Saubaroud-dextrose medium. For hydroxamate production, the organism was grown on a minimal iron medium as previously described for *Fusarium roseum* (Emery, 1965). After 4–5 days of growth, the mycelia were removed by suction filtration and the filtrate, pH 6–7, concentrated to about $\frac{1}{10}$ the original volume. Hydroxamates were extracted by phenol–chloroform (1:1, v/v) and back into water after the addition of five volumes of ether. Ferric chelates were made by addition of excess ferrous sulfate and aeration for 2 h followed by phenol–chloroform extraction. The ferric chelate of the major component, triacetylfusarinine C (triacetylfusigen), could be separated from the other chelates by extraction of either the concentrated culture supernatant, or the aqueous extract after phenol–chloroform extraction, with 1-butanol. Similarly, the major component could be selectively extracted as the free ligand by extraction with benzene–chloroform (1:1, v/v). In either case, the other iron chelating components left behind in the aqueous phase could be further purified by paper electrophoresis. The free ligands or ferric chelates were easily separated by paper electrophoresis with pyridine–acetate buffer (Emery, 1965). Compounds were eluted from the paper with deionized water.

Analytical Methods. All chemicals were analytical or reagent grade. N^{δ}-Hydroxy-L-ornithine was obtained by hydrolysis of rhodotorulic acid (Atkin and Neilands, 1968). The fusarinine compounds were obtained from the culture supernatant of a low iron culture of *Fusarium roseum* (Emery,

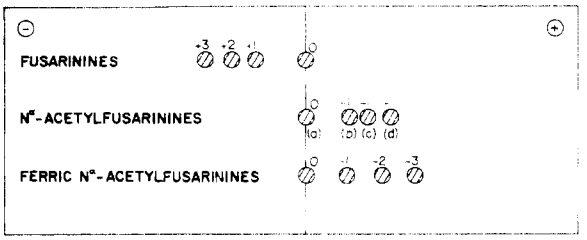


FIGURE 1: Paper electrophoresis at pH 5.2 on pyridine–acetic acid–water (14:10:930), Whatman No. 1 paper, at 28 V/cm for 45 min. The numbers above the spots are the net charge(s) on the molecules.

1965). Acetylation of fusarinine C (fusigen) was done by the method of Diekmann and Zähner (1967).

Hydrolyses were carried out in sealed tubes in vacuo, unless otherwise stated. Ornithine was quantitatively determined by the method of Chinard (1952). Bioassay for L-ornithine was done using *Escherichia coli* AR 3923-R2 by the method of Piperno and Oxender (1968). The organism showed no growth in controls with D-ornithine at concentrations as high as 1000 times the concentrations of L-ornithine used in the standard assay curve. *Arthrobacter* JG-9 bioassays were by the method of Luckey et al. (1972).

Visible and ultraviolet spectra were taken on a Beckman Acta V spectrophotometer. Infrared spectra were taken on a Beckman IR-20. NMR spectra were taken with a Varian XL 100 spectrometer at 100 MHz, in $CDCl_3$ for triacetylfusarinine C, D_2O for acetylfusarinine, and neat for 3-methylpent-2-eno-5-lactone. Tetramethylsilane was used as internal standard.

Elemental analysis was performed by Galbraith Analytical Laboratories, Inc. The data presented are the average of three separate determinations.

Results

Isolation and Purification of N^{α}-Acetylfusarinines. The hydroxamates are found in the culture supernatant after growth of *Penicillium* sp. under iron deficient conditions (Methods). Isolation of the compounds was initially accomplished by extraction into phenol–chloroform and reextraction into water. However, solubility studies indicated that the major component was quite soluble in benzene and chloroform, in contrast to most other siderochromes. Therefore, sufficient quantities of this metal-free ligand for structural determination were purified by benzene–chloroform extraction. The ferric chelate of this compound could also be readily purified by selective extraction with 1-butanol. Final purification of the other components was achieved by paper electrophoresis (Methods). Iron free ligands were obtained from the chelates by the 8-hydroxyquinoline method (Diekmann and Zähner, 1967). All attempts to crystallize any of the compounds as either the free ligands or ferric chelates were unsuccessful. The latter could be obtained as red-brown, hygroscopic powders by lyophilization. In a typical preparation, about 300 mg of the major, electrophoretically neutral compound was obtained from 1 l. of the culture supernatant medium. About 20–50 mg of each of the other compounds were obtained.

Paper Electrophoresis. Four ferric chloride positive compounds are present in the culture supernatant of *Penicillium* sp., all of which are extractable by phenol–chloroform. One of the substances is neutral on paper electrophoresis at pH 5.2 and the other three are separated as anions. At pH 1.9, all four substances are neutral, indicating protonation of the anionic groups(s) and absence of free amino groups. A comparison of the electrophoretic behavior of these compounds to the fusarinines is striking for the mirror image symmetry (Figure 1). It has been established that fusarinine is a neutral zwitterion, and that polymerization by ester bond formation leads to increasing charge, and hence electrophoretic mobility, because of the increase of the number of free amino groups per molecule. The fastest moving substance, fusarinine C, is thus a trication with three free amino groups (Sayer and Emery, 1968).

The electrophoretic pattern of the *Penicillium* hydroxamates at first suggested that they also differed by one charge unit. However, it was soon found that the neutral substance yields the other three substances upon partial acid hydrolysis, suggesting that, in contrast to the fusarinines, the neutral substance is the most complex. It was subsequently found that the α-amino groups are substituted so that the only charge is that of the α-carboxylate group. With a fixed charge of -1, the three anions are separable because of the increase of the charge to size ratio, the ratio being greatest for the fastest moving monomer. Because of its cyclic nature, the neutral compound possesses no ionizable group.

Ferric ion accommodates three hydroxamate groups to form a chelate in which the three charges of the metal are neutralized by the three hydroxylamino anions. The fact that there is no change of electrophoretic mobility of compounds a and b of Figure 1 upon chelation of iron is compatible with their being trihydroxamic acids. Compounds c and d have significantly greater migration rates when electrophoresed as the ferric chelates. This is as expected for mono- and dihydroxamic acids with a net charge before chelation of -1. Thus, the monomer would exist as ML_3^{3-} and would move more rapidly than the singly negatively charged ligand.

Reductive and Nonreductive Hydrolysis. The majority of naturally occurring hydroxamic acids of fungal origin are derivatives of N^{δ}-hydroxyornithine. Hydrolysis of each of the compounds isolated from *Penicillium* with 6 N HCl yields only one amino acid, which is detectable with either ninhydrin or tetrazolium, the latter reagent indicating an hydroxylamino group (Snow, 1954). The amino acid was identified as N^{δ}-hydroxyornithine by chromatographic comparison with an authentic sample. Reductive hydrolysis of the compounds with 48% HI yields ornithine as sole amino acid.

The number of *N*-hydroxyornithine residues per mole of the neutral metal-free species was determined by reductive hydrolysis with HI followed by quantitative ornithine determination by the method of Chinard (1952). A value of 2.7 ornithines/mol was obtained. Hydrolysis of deferriferrichrome A as a control gave 2.9 ornithines/mol (theoretical = 3). We concluded that the neutral species is a trihydroxamic acid, and this was confirmed by other tests (below). The somewhat low ornithine value for the *Penicillium* siderochrome is possibly attributable to incomplete hydrolysis of the amide bond, which is more stable than the ester and hydroxamate bonds to HI hydrolysis. A free α-amino group is required to give a positive reaction by the Chinard method.

Although N^{δ}-hydroxy-L-ornithine is the most frequently found amino acid occurring in fungal hydroxamates, the enantiomer of this compound has also been reported in ferribactin, a product of *Pseudomonas fluorescens* (Maurer et al., 1968). To determine the configuration of the *N*-hydroxyornithine in the *Penicillium* siderochrome, an L-ornithine requiring mutant of *E. coli* was utilized. The HI hydrolysate was found to contain only L-ornithine.

Identification of the Acyl Group of the Hydroxamate Function. Derivatives of mevalonic acid are often found as the

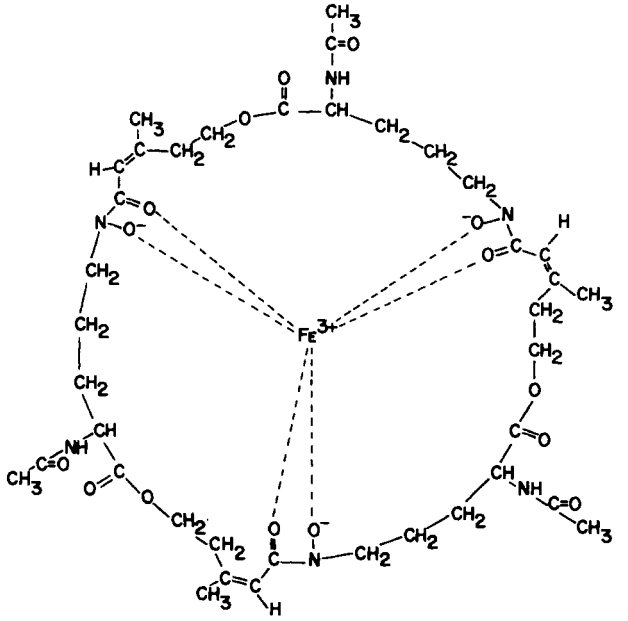


FIGURE 2: Structure of ferric *N,N',N''*-triacetylfusarinine C (*N,N',N''*-triacetylfusigen).

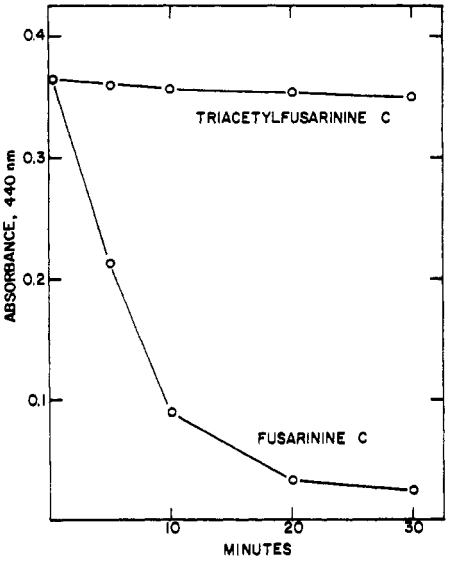


FIGURE 3: Comparison of acid stability of fusarinine C and *N,N',N''*-triacetylfusarinine C. The compounds purified by paper electrophoresis were dissolved in 0.5 N HCl and heated at 60 °C in capped tubes. Aliquots of 0.5 ml were mixed with 0.1 ml of 10% $FeCl_3 \cdot 6H_2O$ and made to 5.0 ml for absorbance measurements.

acyl group of the hydroxamate function in siderochromes. The ultraviolet absorption spectrum indicated an α,β unsaturation in our hydroxamates (see below). Ether extraction of the HCl hydrolysate of our neutral compound yielded a substance with ultraviolet and infrared spectra identical to the lactone, 3-methylpent-2-eno-5-lactone, obtained by hydrolysis of fusarinine (Emery, 1965). Formation of the lactone established the double bond in the parent compound as cis. Nuclear magnetic resonance confirmed this assignment of structure: δ 1.86 (s, 3 H), 2.27 (t, 2 H), 4.20 (t, 2 H), 5.58 (s, 1 H).

Partial Hydrolysis. The occurrence of N^{δ}-hydroxyornithine in an electrically neutral compound indicated that the α-amino groups of the amino acid must be blocked. This was confirmed by the observation that a tetrazolium positive, ninhydrin-negative substance was formed under conditions of partial acid hydrolysis that are known to hydrolyze ester and hydroxamate bonds, but not amide bonds (Emery and Emery, 1973). The electrophoretic properties of the compound are compatible with its being N^{α}-acetyl-N^{δ}-hydroxy-L-ornithine:

$$HONH_2^+CH_2CH_2CH_2\underset{\displaystyle NHCOCH_3}{\underset{|}{C}}HCOO^-$$

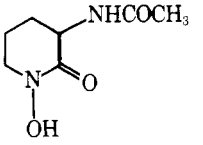
Identification of the acyl group as acetyl was accomplished by elemental analysis and NMR (below).

The data above are compatible with the neutral compound's being *N,N',N''*-triacetylfusarinine C, or *N,N',N''*-triacetylfusigen (Figure 2). The greater stability of amide and hydroxamate bonds compared to ester bonds under basic conditions predicts that this compound should yield N^{α}-acetylfusarinine upon partial base hydrolysis:

$$HOCH_2CH_2\underset{\displaystyle CH_3}{\underset{|}{C}}=CH\underset{\displaystyle OH}{\underset{|}{C}}-NCH_2CH_2CH_2\underset{\displaystyle NHCOCH_3}{\underset{|}{C}}HCOO^-$$

Treatment of the metal-free neutral compound with 1 N NH_4OH or 0.1 N KOH yielded the most rapidly moving component (d of Figure 2) as sole product. Treatment of a mixture of hydroxamates gave the identical product. Spectrophotometric titration of this compound showed an isosbestic point at 256 nm attributable to ionization of the hydroxamic acid group adjacent to α,β unsaturation, identical with that of fusarinine (Emery, 1965).

An interesting observation is that treatment of *N,N',N''*-triacetylfusarinine C with 4 N NH_4OH yields, in addition to N^{α}-acetylfusarinine, a ferric chloride positive, electrophoretically neutral material that we believe to be 1-hydroxy-3-acetylaminopiperidone:



Isowa et al. (1972) have reported that formation of this piperidone is unavoidable when working with N^{δ}-acyl derivatives of N^{α}-acetyl-N^{δ}-hydroxyornithine, and we previously observed piperidone formation during acid hydrolysis of fusarinine (Emery, 1966). It is curious that this piperidone is formed in good yield when the siderochrome is treated with 4 N NH_4OH, but not observed at all with 1 N NH_4OH or with KOH at any concentration.

NMR Spectrum. The NMR spectrum of the neutral, metal-free compound was found to be identical with the previously published spectrum of *N,N',N''*-triacetylfusarinine C obtained by exhaustive acetylation of the cyclic trimer, fusarinine C (fusigen) (Diekmann and Zähner, 1967).

Visible Spectra. It is well known that ferric trihydroxamates exhibit a much greater stability toward dissociation in acid solution than do the ferric chelates of monohydroxamates and dihydroxamates (Emery, 1971). Dissociation of the metal is accompanied by a shift in λ_{max} from 440 to 480 nm. When an aqueous solution of compound a or b of Figure 1 is acidified from pH 7 to 2, no effect is observed on the visible spectrum. In contrast, compounds c and d exhibit a pronounced bathochromic shift. Furthermore, the two former chelates are not decolorized in the presence of 0.02 M EDTA,[1] whereas the latter two compounds are rapidly bleached. These results indicate that only compounds a and b are ferric trihydroxamates.

[1] Abbreviation used: EDTA, (ethylenedinitrilo)tetraacetic acid.

Effect of N^{α}-Acyl Substituents on Acid Stability. The extreme lability of the aminoacyl ester bonds of the unsubstituted fusarinines should be suppressed by acylation of the free amino groups. The greater stability of the new trihydroxamate compared to the cyclic trimer of fusarinine itself is shown in Figure 3. In 0.5 N HCl at 60 °C, fusarinine C is 80–90% hydrolyzed in 20 min, whereas the triacetylated compound remains virtually unhydrolyzed.

Elemental Analysis. Elemental analysis of the ferric chelate of the neutral siderochrome of *Penicillium* sp. is in agreement with the structure shown in Figure 2. Anal. Calcd for ferric triacetylfusarinine C·$3H_2O$: C, 48.80; H, 5.98; N, 8.75; Fe, 5.88. Found: C, 48.70; H, 6.41; N, 8.73; Fe, 5.82.

Effect of Iron on Hydroxamate Production. The presence of iron in the growth medium suppresses the biosynthesis of hydroxamic acids in vivo (Neilands, 1973; Emery, 1971). Addition of 0.1 mM iron to the growth medium of *Penicillium* sp. causes almost complete suppression of hydroxamate excretion into the medium.

Growth Factor Activity for Arthrobacter. Arthrobacter JG-9 is an auxotrophic bacterium which responds to most naturally occurring trihydroxamic acids. Figure 4 shows the response of this organism to several siderochromes compared at the same concentrations. None of the new compounds shows growth factor activity as high as ferrichrome or coprogen. As expected, the cyclic trihydroxamate shows slightly greater activity than the linear trihydroxamate, and the dihydroxamate shows virtually no activity. The new trihydroxamates show approximately the same activity as fusarinine trihydroxamates. Most unexpected, however, was the very high activity of the monohydroxamic acid, N^{α}-acetylfusarinine, exceeding even the activity of the cyclic trimer. This high activity is unique for a monohydroxamic acid.

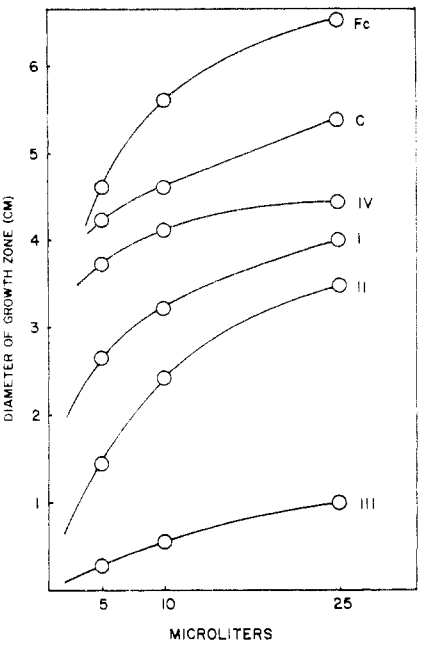


FIGURE 4: *Arthrobacter* JG-9 bioassay of growth factor activity. The acetylfusarinine compounds were purified by paper electrophoresis. All solutions were adjusted to an absorbance of 0.311 at 440 nm. Bioassay by the penicillin disk method was done according to Luckey et al. (1972). Volumes placed on the disks are shown on the abscissa. (Fc) ferrichrome; (C) coprogen; (I) *N,N′,N″*-triacetylfusarinine C; (II) *N,N′,N″*-triacetylfusarinine B; (III) *N,N′*-diacetylfusarinine A; (IV) *N*-acetylfusarinine.

Discussion

Cyclic trihydroxamic acids are commonly found as excretory products of fungi grown under iron deficient conditions. They are believed to serve as ferric ionophores in these organisms. The extreme lability of the fusarinine hydroxamates produced by *Fusarium roseum* has prevented investigation of the possible physiological role of these compounds as ionophores, and, in fact, gave reason to wonder if such unstable compounds could serve such a role. The triacetylated fusarinine C produced by *Penicillium* sp. is very stable by virtue of protection of the α-amino groups of the ornithine residues. Evidence presented in the following paper suggests that this compound is indeed a ferric ionophore for this organism. It may be that small amounts of this stable derivative of fusarinine C are also produced by *Fusarium roseum*, but have escaped detection.

It is curious that the culture fluids of both *Fusarium roseum* and *Penicillium* contain, in addition to the siderochrome, sizeable amounts of the monomeric hydroxamic acid, the dimer, and the linear trimer. We previously suggested that in the case of the fusarinines this might be due to the spontaneous hydrolysis of these unstable compounds. This cannot be the case of the stable triacetylated fusarinines. We have found that extracts of *Penicillium* sp. contain an enzyme capable of hydrolyzing *N,N′,N″*-triacetylfusarinine into the linear trimer, dimer, and finally monomer (Emery, 1976). Therefore, it is not unreasonable to assume that the substances result from enzymatic hydrolysis.

It is interesting to speculate on the biosynthetic origin of the N^{α}-acetyl groups. Does this occur by acetylation of fusarinine C, or is the acetyl group introduced at an earlier stage of synthesis? Acetylation at the level of ornithine would have the advantage of circumventing the very labile aminoacyl esters as intermediates during synthesis.

Added in Proof

While this manuscript was in preparation, a report appeared describing the isolation of several siderochromes, including *N,N′,N″*-triacetylfusarinine C (triacetylfusigen), from the culture medium of several species of *Aspergillus* (Diekmann and Krezdorn, 1975). Most interesting is their finding that triacetylfusarinine C and a siderochrome of the peptide type, ferricrocin, can both be produced by a single species.

Acknowledgments

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Fungal Ornithine Esterases: Relationship to Iron Transport†

Thomas Emery

ABSTRACT: Extracts of *Fusarium roseum* (ATCC 12822) contain an enzyme which hydrolyzes the ornithine ester bonds of fusarinine C, a cyclic trihydroxamic acid produced by this organism. The methyl ester of N^{δ}-dinitrophenyl-L-ornithine is also a substrate for the enzyme, and an assay was devised using this substrate. The enzyme exhibits a sharp maximum of activity at pH 7.5 and is extremely temperature sensitive. It is strongly inhibited by $HgCl_2$ and *p*-chloromercuribenzoate, and it is competitively inhibited by N^{δ}-dinitrophenyl-D-ornithine methyl ester (K_i = 0.3 mM). Methyl esters of glycine, L-alanine, dinitrophenyl-L-alanine, dinitrophenyl-β-alanine, and N^{δ}-dinitrophenyl-N^{α}-acetyl-L-ornithine are not substrates, although N^{ϵ}-dinitrophenyl-L-lysine methyl ester is as effective as the ornithine derivative. Nonspecific lipases do not hydrolyze ornithine esters, nor does trypsin. The three ester bonds of fusarinine C are progressively hydrolyzed by the enzyme to eventually yield the monomer, fusarinine. The ferric chelate of fusarinine C is not hydrolyzed. An enzyme from *Penicillium* sp. was isolated with identical properties toward N^{δ}-dinitrophenyl-L-ornithine methyl ester as substrate. It also hydrolyzes *N,N′,N″*-triacetylfusarinine C, a cyclic trihydroxamate containing N^{α}-acetylornithine ester bonds, which is produced by this organism. This substrate is hydrolyzed to N^{α}-acetylfusarinine. In contrast to the *Fusarium* enzyme, this enzyme is fully active toward the ferric trihydroxamate chelate. However, replacement of iron by aluminum leads to a completely inactive substrate. Production of the enzyme is severely suppressed by iron in the growth medium. It is proposed that these specific ornithylesterases provide a mechanism of cellular iron release by hydrolysis of the ferric ionophores, and that an iron-exchange step occurs prior to, and is a prerequisite for, hydrolysis of the ester bonds.

It is now well established that microbial iron metabolism is dependent upon low-molecular-weight iron chelating agents, called siderochromes (Neilands, 1973; Emery, 1971a). Under conditions of iron-deficient growth, the ligand is excreted into the medium in large quantities where it solubilizes and chelates iron with an affinity constant of approximately 10^{30}. Active transport systems have been described which carry the siderochrome into the cell (Emery, 1971b; Brown and Ratledge, 1975). Iron is required for many cellular processes, and an unresolved problem is the mechanism by which the organism can dissociate the metal from chelates with such high binding constants. It has frequently been suggested that dissociation depends upon reduction of the iron to the ferrous state, for which siderochromes have little affinity (Neilands, 1973; Brown and Ratledge, 1975). On the other hand, evidence has been presented that in *Escherichia coli* iron release from the siderochrome, ferric enterochelin, is dependent upon a cellular esterase (O'Brien et al., 1971). Enterochelin contains three dihydroxybenzoylserine molecules joined in ester linkage. Hydrolysis of the ester bonds yields dihydroxybenzoylserine, a bidentate ligand which is a much poorer chelator of ferric ion than the hexadentate enterochelin.

Fusarium roseum and other fungi produce siderochromes of the trihydroxamic acid type consisting of three molecules of fusarinine, N^{δ}-(*cis*-5-hydroxy-3-methylpent-2-enoyl)-N^{δ}-hydroxy-L-ornithine, joined by head-to-tail ester bonds. The resulting cyclic trimer, in which the three hydroxamate groups form a hexadentate ligand with ferric ion, is called fusarinine C, or fusigen (Diekmann and Zähner, 1967; Sayer and Emery, 1968). Fusarinine C is believed to be a ferric ionophore in *Fusarium roseum,* but investigation of this system has been hampered by the extreme lability of the aminoacyl ester bonds in the fusarinine compounds. We have now found that *Penicillium sp.* produces a stable derivative of fusarinine C, *N,N′,N″*-triacetylfusarinine C (Moore and Emery, 1976). The purpose of this paper is to describe specific esterases of *Fusarium roseum* and *Penicillium* sp. which hydrolyze ester type siderochromes to the monomeric subunits, fusarinine and N^{α}-acetylfusarinine, respectively, and to consider the role of these unique aminoacylesterases in the release of iron from the siderochromes.

Materials and Methods

Substrates. Fusarinine C was isolated from cultures of *Fusarium roseum* as previously described (Sayer and Emery, 1968). Fusarinine C labeled with ^{14}C was prepared by growth of the organism for 3 days with 10 μCi of DL-[U-^{14}C]ornithine and 20 μCi of [2-^{14}C]acetic acid added to the medium. *N,N′,N″*-Triacetylfusarinine C was obtained from the culture supernatant of *Penicillium* sp. (Moore and Emery, 1976). The linear trimer of N^{α}-acetylfusarinine was obtained by extraction of the culture supernatant with phenol–chloroform and purification by paper electrophoresis.

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