Isolation and Characterization of a New Hydroxamic Acid from *Pseudomonas mildenbergii*†

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ABSTRACT: A low molecular weight hydroxamic acid was produced by *Pseudomonas mildenbergii* in iron-deficient media associated with green fluorescent peptides. The chemical structure of this hydroxamic acid has been investigated for comparison to known iron-binding siderophores. The hydroxamic acid was extracted from lyophilized culture media with ethanol and methanol and crystallized as the hydrochloride. The product had a molecular weight of 202.6 and an empirical formula of $C_9H_{11}O_2N$·HCl and contained hydroxylamine nitrogen. The infrared, nuclear magnetic resonance, and mass spectral data suggested that the chemical structure was *N*-methylphenylacetohydroxamic acid. *N*-Methylphenylacetohydroxamic acid was synthesized, and its melting point, elemental analysis, and molecular weight were identical with those of the natural product. The compound chelated ferric iron, producing a distinctive iron chelate absorption band at 470 nm. Its relationship to the green fluorescent peptides is discussed.

During studies with green fluorescent peptides from iron-deficient *Pseudomonas mildenbergii*[1] (Hulcher, 1968a,b; Love & Hulcher, 1964), a low molecular weight hydroxamic acid was found to be associated with or bound to a green fluorescent peptide (Hulcher, 1968a). Further studies have revealed that this hydroxamate is accumulated in a free form in culture media of *P. mildenbergii* when grown on a simple, chemically devised medium devoid of iron but with vigorous aeration. In the present work, the hydroxamate has been isolated and characterized as an iron-binding (ferric) compound with the structure, *N*-methylphenylacetohydroxamic acid. The hydroxamate was isolated from six different cultures of iron-deficient cells, but in three batches of cells grown with iron, none of the compound could be isolated.

Naturally occurring hydroxamic acids with a variety of different structures have been described and reviewed by Neilands (1966, 1967). These compounds play important roles in iron binding, transport, and metabolism in microorganisms. Most of the ferric hydroxamates previously reported were obtained from fungi, yeast, streptomycetes, and bacteria other than pseudomonads (Neilands, 1967). Neilands (1981a) has reviewed the chemical structures of iron-binding compounds of microbial origin. Iron absorption and transport compounds in microorganisms also have been reviewed recently (Neilands, 1981b). The high-affinity, ferric-specific ligands of molecular weight 500–1000 are defined as siderophores and include ferrichrome and ferric enterobactin. Ferrichrome is a ferric trihydroxamate. The pseudomonads are known to produce siderophores of the hydroxamic class. Norcardamine, a trihydroxamate, was recently identified in iron-deficient media from *Pseudomonas stutzeri* and shown to be identical with desferrioxamine E (Meyer & Abdallah, 1980).

Materials and Methods

Cell Cultures. Cells of *P. mildenbergii* (Love & Hulcher, 1964) were grown at 25 °C with vigorous aeration on iron-deficient medium containing glucose (3 μmol/mL), L-serine, L-proline, L-lysine, and L-phenylalanine (5 μmol each per 1 mL), KH_2PO_4 (6 mmol), KNO_3 (3.4 mmol), KCl (2.1 mmol), and $MgSO_4$ (2.87 mmol) per L of deionized water as previously described (Love & Hulcher, 1964). Cells were removed by centrifugation of 18-h cultures, and the cell-free supernatant was lyophilized or dried by flash evaporation below 40 °C. The product contains a copious amount of green fluorescent peptides which add to the bulk of the product.

Analytical Methods. The visible and ultraviolet spectra were determined by using a Cary Model 14 recording spectrophotometer. The infrared spectra were produced by a Perkin-Elmer Model 21 infrared spectrophotometer. Elemental analyses for C, H, and N were performed by the Schwarzkopf Microanalytical Laboratory, Woodside, NY. The titrations for p*K* and neutral equivalent were performed by using a Beckman Zeromatic pH meter, Model 9602. The nuclear magnetic resonance spectrometry was performed in a Varian Model 60 NMR spectrometer. The mass spectra were obtained from the Morgan-Schaffer Corp., Montreal, Canada. The test for the detection of hydroxylamine derivatives was that described by Emery & Neilands (1960). The procedure for estimating bound hydroxylamine was described by Csáky (1948). *N*-Methylhydroxylamine was purchased from Aldrich Chemical Co., Milwaukee, WI. Triethylamine, acetone, and other chemicals were from Fisher Scientific Co., Pittsburgh, PA. The ninhydrin procedure to test for α-amino acids was that described by Spies (1957).

Extraction of the Hydroxamic Acid. Eight liters of dried culture media was extracted with ethanol, and after evaporation or addition of ethyl ether, a precipitate of 244.7 mg of preparation A was obtained. However, when the dried culture media were acidifed to pH 2 with HCl solution and dried, exhaustive extraction with methanol yielded 1.607 g/4 L (preparation B). Again precipitation with ether gave a white solid. Although the acidification and methanol extraction gave the greatest yield, a small amount of fluorescent peptide was extracted into methanol, but not into ethanol. For removal of the trace of fluorescent peptide and amino acids, the dried precipitate (B) was acidified and dried as before and extracted with absolute ethanol in which the peptides and amino acids were insoluble. Upon addition of ethyl ether, a copious white crystalline material was precipitated. The dried product was extracted with benzyl alcohol and crystallized with ethyl ether. The crystals were washed with ethyl ether several times and dried. The yields of white crystalline (needles) hydrochloride

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[1] Abbreviation: *P. mildenbergii*, *Pseudomonas mildenbergii*.

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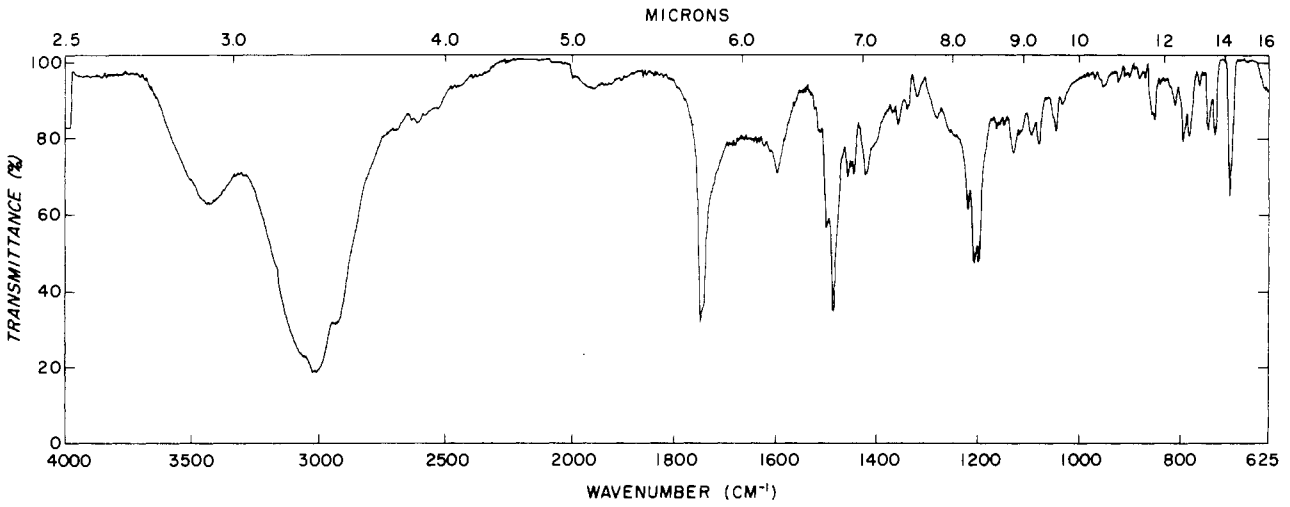


FIGURE 1: Infrared absorption spectrum of the crystalline hydroxamic acid hydrochloride extracted from iron-deficient culture media of *Pseudomonas mildenbergii*. The samples were examined in KBr crystals.

ranged from 188 to 230 mg/L. The pH of a solution containing 5.4 mg of the crystallized product in 20 mL of deionized water was 2.8.

Preparation of Potassium N-Methylphenylacetohydroxamate. *N*-Methylhydroxylamine hydrochloride (24 mmol) was dissolved in a minimal volume (20 mL) of absolute ethanol, and 26 mmol of triethylamine was added in acetone (10 mL). The triethylamine hydrochloride crystals were removed by centrifugaiton, and more acetone was added to remove remaining hydrochloride. The supernatant containing *N*-methylhydroxylamine was evaporated to remove triethylamine and ethanol. The *N*-methylhydroxylamine was dissolved in 5.0 mL of dry chloroform, and after being cooled in an ice–salt bath, 10.56 mmol of phenylacetyl chloride in 5 mL of dry chloroform was added dropwise with stirring. After 30 min, the mixture was allowed to warm to room temperature. The chloroform mixture was extracted with two 5-mL portions of 2 N hydrochloric acid to facilitate separation of *N*-methylphenylacetohydroxamic acid hydrochloride in the aqueous phase from phenylacetic acid and *N*-methylhydroxylamine in the chloroform phase. The hydrochloric acid extracts were combined and washed with ethyl ether. The aqueous solution was prepared and titrated with 0.1 N KOH to pH 9; the titration was continued slowly to avoid excess KOH until there was a sharp pH change. The solution was evaporated at 40 °C. The solid was extracted with absolute ethanol, precipitated with ethyl ether, and collected by centrifugation. Crystallization was from ethanol and ether. The yield obtained was 38%.

Phenylacetohydroxamic Acid. This compound, which differs from the above-described hydroxamate only by the absence of an *N*-methyl group, was synthesized as described above except that hydroxylamine was used in place of *N*-methylhydroxylamine.

Results

Properties and Characterization of the Natural Hydroxamic Acid. The crystalline HCl salt was soluble in water, methanol, ethanol, 1-propanol, benzyl alcohol, acetic acid, dimethyl sulfoxide, and pyridine but insoluble in chloroform, ether, benzene, and cyclohexane. It gave a negative reaction with ninhydrin (Spies, 1957), indicating it to be free of amino acids. An aqueous solution of the natural compound in hydrochloride form (5.4 mg/20 mL of water, 1.3 mM) gave a pH of 2.8 and upon titration with 0.1 N NaOH displayed an estimated pK of 8.9 for the neutral to anionic form. Titration of 5 mg/5 mL from pH 2 to 10.6 again gave a pK of 8.9 with a neutral equivalent of 200. The neutral form predominates at pH 5.4, and the end point for the anionic form was pH 10.6. The ultraviolet absorption maximum was observed at 258 nm ($E^{1\%}_{1cm}$ = 1.005 at pH 7.0). The compound began to melt and disappear from 180 to 205 °C. The molecular weight was determined to be 202 (eubilliscopic). The elemental analysis found for the hydrochloride form was 52.8% C, 6.8% H, 7.1% N, and 18.0% Cl, indicating an empirical formula of $C_9H_{11}O_2N{\cdot}HCl$. The theoretical analysis was 53.7% C, 6.0% H, 7.0% N, 15.9% O, and 17.6% Cl. This result would give a molecular weight of 202.6, in keeping with the neutral equivalent and eubilliscopic data. The compound was subjected to the periodic acid procedure for detection of alkyl-substituted hydroxylamine compounds (Emery & Neilands, 1960) producing ultraviolet absorption at 264 nm. This indicated the secondary hydroxamic nature of the compound. Analysis by the diazotization procedure of Csáky (1948) for free and bound hydroxylamine nitrogen gave an estimated 10.1 μg of hydroxylamine nitrogen per 200 μg of compound.

The infrared spectrum was taken in a Nujol and KBr crystal (Figure 1) on the hydrochloride salt of the compound. A prominent band was displayed at 5.72 μm for a carbonyl group (C=O stretching vibration). A band occurring at 6.25 μm showed the C–C aromatic stretching vibrations, and a band at 7.05 μm indicated the O–H stretching vibration. Thus, these data indicated that the compound contained a benzene or pyridine ring and a carbonyl group of an ester, or a similar bonding group and an –OH group. Five milligrams of the compound was hydrolyzed at 200 °C for 2 h in 80% H_2SO_4, and the isolated product gave an absorption band at 233 nm, indicating a benene ring structure. The *p*-toluenesulfonyl chloride derivative gave infrared bands for SO_2 (8.5–9.0 μm) and a *p*-methyl group (12.25 μm), thus indicating again the presence of a hydroxyl group.

The NMR spectrum of the compound (10%) was performed in deuterium-labeled dimethyl sulfoxide by using a trace of tetramethylsilane in a Varian A-60 nuclear magnetic resonance spectrometer at 32 °C (Figure 2). Methyl protons appeared at 3.2 ppm, methylene protons at 4.05 ppm, and aromatic protons at 9.25 ppm, and a shielded proton peak occurred at 11.25 ppm. The shielded proton at 11.25 ppm appeared to be either a hydrogen-bonded alcohol or an aldehyde group. All peaks were referred to that of tetramethylsilane.

Samples were analyzed in the mass spectrometer. The spectrum was obtained at 120 °C at voltages of V_I = 70 V,

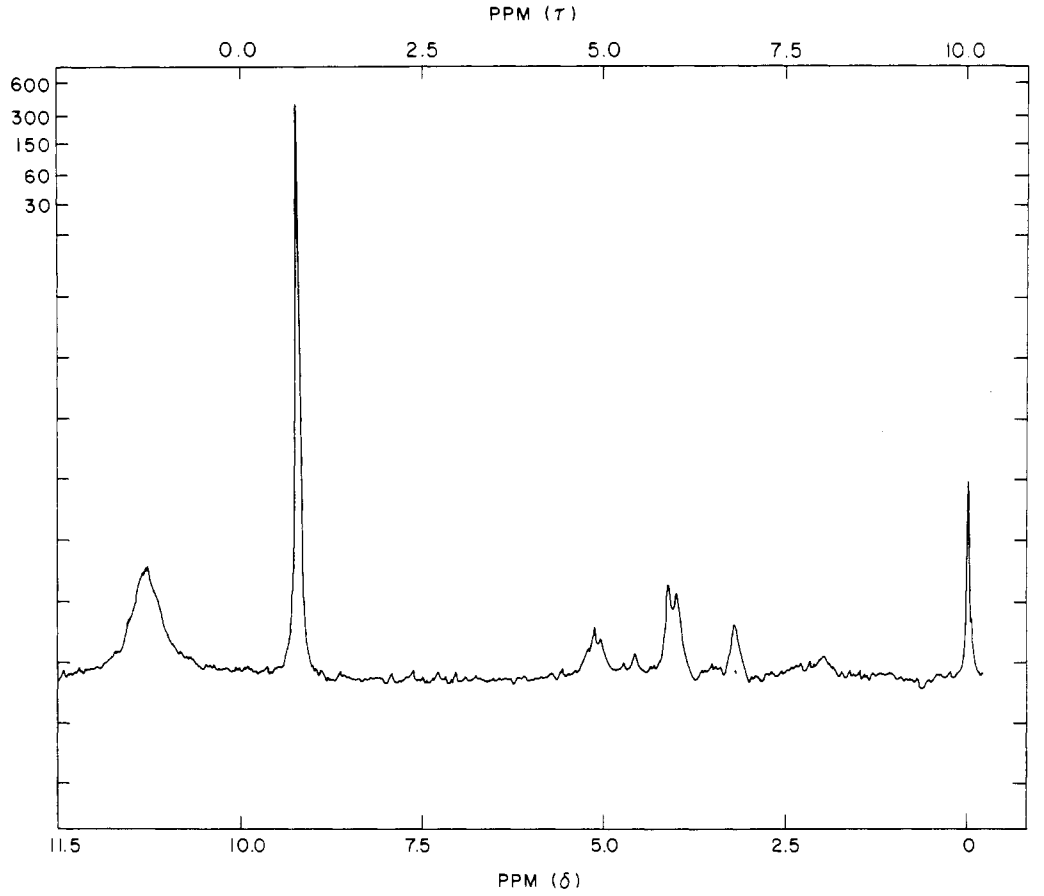


FIGURE 2: Nuclear magnetic resonance spectrum of the crystalline hydroxamic acid hydrochloride. The sample (10%) was prepared in deuterium-labeled dimethyl sulfoxide with tetramethylsilane reference at 32 °C.

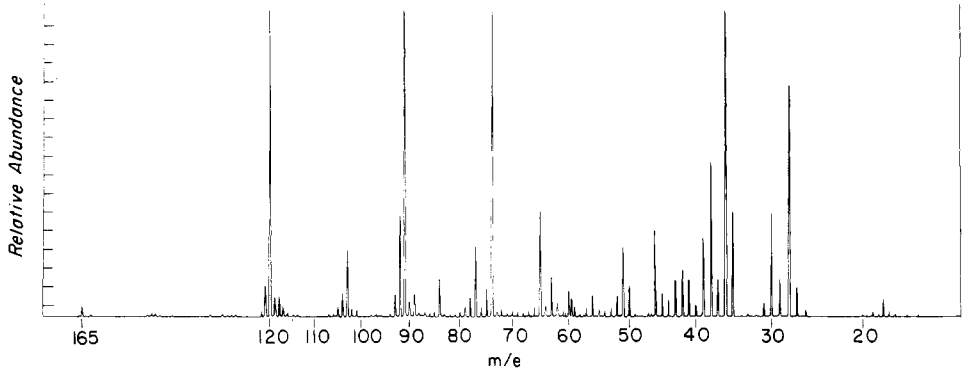
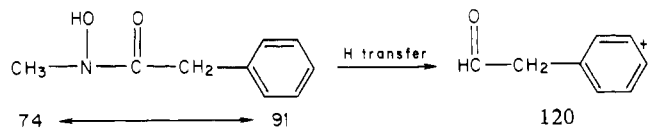
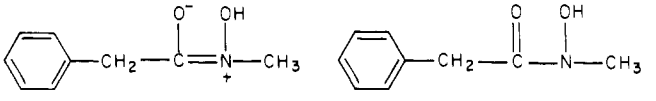


FIGURE 3: Mass spectrum of crystalline hydroxamic acid hydrochloride. The analyzer settings are described under Results.

V_A = 1750 V, and V_M = 2000 V with the analyzer set at $TIC_i = 9 \times 10^{12}$ and $TIC_f = 9 \times 10^{-12}$. The mass spectrum obtained is given in Figure 3. A parent peak was established at *m/e* 165. Thus, it must contain an odd number of nitrogen atoms. No evidence was found for a pyridine structure. The large mass abundance at *m/e* 91 suggested a group with the structure $C_6H_5CH_2$ and the *m/e* 77 peak was consistent with the structure $C_6H_5^+$. The base peak of *m/e* 74 was interpreted as $CH_3^+N(OH)=C=O$ or as $CH_3N(OH)^+C=O$. The spectrum fragmentation pattern was rationalized as



The hydrochloride was also evident from the masses at *m/e* 36 and 38. The structure of the hydroxamic acid was very probably *N*-methylphenylacetohydroxamic acid. This compound can exist in the following two resonance forms:



Repeated mass spectrum analyses as an unidentified compound gave identical results and interpretation.

Synthetic Potassium N-Methylphenylacetohydroxamic Acid. This compound was first prepared in very small yield by the procedure of Blatt (1943) using methylphenyl acetate, sodium ethoxide, and *N*-methylhydroxylamine. Much better yields were obtained when *N*-methylhydroxylamine hydrochloride was freed of hydrochloride with triethylamine in methanol and the separated *N*-methylhydroxylamine reacted in chloroform at 0 °C with half the stoichiometric amount of phenylacetyl chloride. The potassium salt of the product was obtained in 38% yield, and the elemental analysis of the neutral

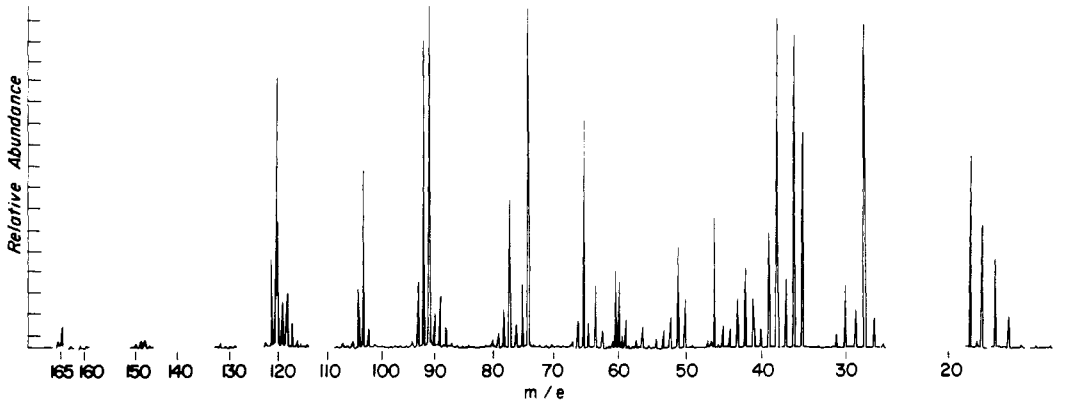


FIGURE 4: Mass spectrum of crystallized synthetic *N*-methylphenylacetohydroxamic acid hydrochloride. Analyzer settings are described under Results.

form found was 64.8% C, 6.66% H, and 8.53% N. The calculated theoretical analysis was 65.4% C, 6.66% H, and 8.48% N. The potassium salt had a melting point of 195–200 °C, and the hydrochloride melted at 205 °C. The neutral equivalent for the synthetic HCl salt was 201, and the pK was 8.9 for the neutral to anionic form. The potassium salt was converted to the hydrochloride, and titration of a 10 mM solution from pH 2.9 to pH 0.70 with 5 N HCl gave an approximate pK_a of 1.5–1.64 for the salt to protonated cation. The lower end point could not be shown precisely, so the pK_a may be lower. Buffering occurred from pH 1 to pH 1.8, but the pH changed sharply from pH 3 to pH 1.9. Infrared analysis of the potassium salt fit the postulated structure. The synthetic product (HCl) was analyzed with the mass spectrometer at 120 °C at voltages of V_I = 70 V, V_A = 1750 V, and V_M = 2000 V with the analyzer set at $TIC_i = 9 \times 10^{12}$ and $TIC_f = 9 \times 10^{-12}$. The mass spectrum obtained is illustrated in Figure 4. It can be seen that the parent peak occurred at m/e 165 with other mass abundance at m/e 120, 91, 77, and 74, in agreement with the pattern established for the natural compound and providing the same interpretation for the proposed structure.

Binding of Ferric Iron. the absorption spectrum of the chelate of ferric iron by the natural and synthetic compounds was obtained. After trying phthalate and acetate buffers between pH 2.0 and pH 7.0, it was found that either the hydroxamate or the iron would precipitate. However, the chelation proceeded well in 1 N acetic acid (pH 4.2), and the chelate spectra showed distinctive iron chelate bands at 460–465 nm after titration to maximal absorbance with ferric ammonium sulfate. The absorption spectra of the ferric chelates natural and synthetic *N*-methylphenylacetohydroxamic acid in 1 M acetic acid are shown in Figure 5. The maximal absorbance was about 470 nm. For comparison, the spectrum of the ferric chelate of phenylacetohydroxamic acid is included with an absorption band at 480–485 nm (purple colored). However, the absorption bands are dependent on pH. *N*-Methylbenzohydroxamic acid prepared according to Blatt (1943) (see Materials and Methods) also gave maxima at 460 nm for the ferric chelate.

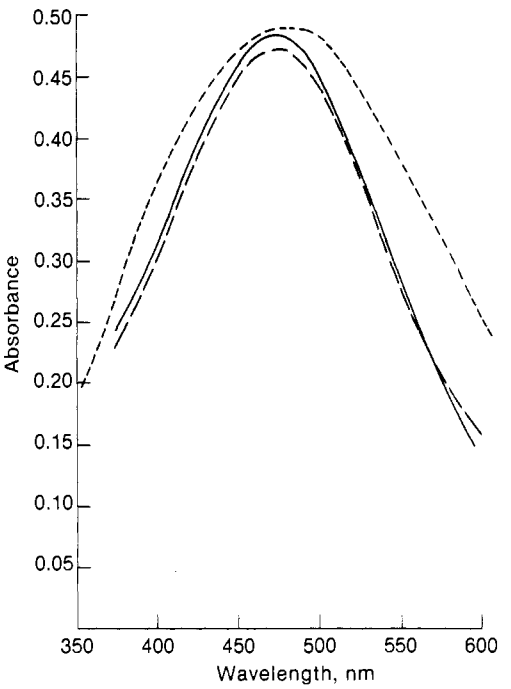


FIGURE 5: Absorption spectra of the ferric chelate of natural and synthetic *N*-methylphenylacetohydroxamic acids and phenylacetohydroxamic acid. The cuvettes contained 1.25 mM hydroxamate and 1.25 mM ferric chloride in 1 M acetic acid. The blank cuvettes contained only ferric chloride in 1 M acetic acid. The short dashed line represents the spectrum for natural *N*-methylphenylacetohydroxamate. The solid line represents the spectrum of the synthetic compound, and the long dashed line represents that for phenylacetohydroxamate.

Discussion

The hydroxamate siderophores, mostly from fungi, have molecular weights from 500 to 1000. This paper describes the characteristics of a small hydroxamate, *N*-methylphenylacetohydroxamic acid, which is produced in iron-deficient media by *Pseudomonas mildenbergii*. Previously, this compound was found associated with purified yellow-green fluorescent peptides in iron-deficient media after treatment with 6 N HCl at 100 °C for 3 h. It was thought to be bound covalently to the peptide. However, the present work shows that this hydroxamate is synthesized as a free molecule by this bacterium. The compound could not be detected in dried supernatant from iron-grown cells by using the same extraction procedure described for the hydroxamic acid. However, quantitative studies are needed to clarify this point. It would be of interest to determine if this small hydroxamate functions as a siderophore or if it is a precursor of the yellow-green fluorescent peptide which also chelates iron and contains hydroxylamine nitrogen. It is emphasized that the hydroxamic acid is only one of the products accumulated under these growth conditions since copious amounts of green fluorescent peptides are produced also.

The *N*-methyl substitution on the hydroxamate nitrogen may contribute to enhanced complexation with iron. Monzyk & Crumbliss (1979) suggested that *N*-alkyl groups do enhance

complexation with ferric iron and render it to be a stronger base than those with H substitution. This would also promote formation of the cation tautomer by transfer of the lone electron pair from N to the carbonyl function. This may account for formation of the hydrochloride salt. Generally, the free acid or chelate is extracted, but in this study, the hydrochloride was extracted. Little knowledge is available about the cationic tautomer of hydroxamates. Protonation of hydroxamic acids by excess acid has been claimed (Dutta & Ghosh, 1967) with p*K*s of 1.45–1.85. The estimated p*K* for the protonated to dissociated form of synthetic *N*-methylphenylacetohydroxamic acid was 1.5–1.64. The true value may be lower. The neutral form predominates at pH 5.4. The p*K* for the neutral to anionic form was 8.9, typical of hydroxamates. The occurrence of the hydrochloride form was supported by elemental analysis and the mass spectrum. The absorption band at pH 4.2 of *N*-methylphenylacetohydroxamate (470) is shifted by 10–15 nm from that of phenylacetohydroxamate. This can be attributed to *N*-methyl substitution also.

Shiman & Neilands (1965) characterized a ferrous iron-binding compound, pyrimine, form pseudomonads GH which is quite different from this compound. A compound with some similarities was reported as 2,3-dihydroxybenzoylglycine from *Bacilus subtilis* (Ito & Neilands, 1958). Further studies on the ferric chelate of this compound are needed, and the relationship of this hydroxamate accumulation to that of the green fluorescent peptides is required to understand the role of these compounds in the iron metabolism of pseudomonads.

Acknowledgments

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References

Blatt, A. H. (1943) in *Organic Syntheses*, Collect. Vol. II, p 67, Wiley, New York.

Csâky, T. Z. (1948) *Acta Chem. Scand. 2*, 405–545.

Dutta, R. L., & Ghosh, S. (1967) *J. Indian Chem. Soc. 44*, 820–827.

Emery, T. F., & Neilands, J. B. (1960) *J. Am. Chem. Soc. 82*, 4903.

Hulcher, F. H. (1968a) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 27*, 787.

Hulcher, F. H. (1968b) *Biochem. Biophys. Res. Commun. 31*, 247–251.

Ito, T., & Neilands, J. B. (1958) *J. Am. Chem. Soc. 80*, 4645–4647.

Love, S. H., & Hulcher, F. H. (1964) *J. Bacteriol. 87*, 39–45.

Meyer, J. M., & Abdallah, M. A. (1980) *J. Gen. Microbiol. 118*, 125–129.

Monzyk, B., & Crumbliss, A. L. (1979) *J. Am. Chem. Soc. 101*, 6203–6213.

Neilands, J. B. (1966) *Struct. Bonding (Berlin) 1*, 59–108.

Neilands, J. B. (1967) *Science (Washington, D.C.) 156*, 1443–1447.

Neilands, J. B. (1981a) *Annu. Rev. Biochem. 50*, 715–731.

Neilands, J. B. (1981b) *Annu. Rev. Nutr. 1*, 27–46.

Shiman, R., & Neilands, J. B. (1965) *Biochemistry 4*, 2233–2236.

Spies, J. S. (1957) *Methods Enzymol. 3*, 468.