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Isolation, Characterization, and Synthesis of Pyrimine, an Iron(II)-Binding Agent from *Pseudomonas* GH*

Ross Shiman and J. B. Neilands

ABSTRACT: An iron(II)-binding agent from a soil isolate, designated *Pseudomonas* GH, was crystallized, characterized, and synthesized. The substance may be named L-5(2-pyridyl)-2-amino-5-ketopentanoic acid or L-2(2-pyridyl)-Δ^1-pyrroline-5-carboxylic acid, respectively, depending on whether the side chain is open or cyclized. Pyrimine is suggested as the trivial name.

A stable 3:1 complex (ferropyrimine) is formed with ferrous iron at pH values greater than about 2.0. Pyrimine was synthesized by Claisen condensation of methyl picolinate with *N*-trityl-L-glutamic acid dimethyl ester.

Over the past few years, it has become apparent that the path of iron in microbial metabolism is controlled and directed by the coordination of this element to specific organic structures. Obviously, in order to understand the biochemical mechanisms involved in iron metabolism in living cells, it will be necessary to elucidate the chemical nature of the organic compounds which are found associated with iron within the living cell.

It is a reasonable assumption that naturally occurring binding agents which form *very stable* complexes with iron will play a role in the metabolism of the metal. Most such compounds hitherto studied in this laboratory have, by virtue of the presence of phenolic (Ito and Neilands, 1958) or hydroxamate (Emery and Neilands, 1959) functional groups, displayed a pronounced preference for ferric iron. Thus, in the case of the ferrichrome compounds, six oxygen atoms engage a single metal ion in a spin-five, octahedral complex in which ferric iron is held with a stability constant of about 10^{30} (Neilands, 1964).

Recently our colleagues in the Department of Bacteriology of this University, Dr. George Hegeman and Dr. R. Y. Stanier, brought to the laboratory a microbial culture which they obtained in the course of plating a soil extract on agar medium. The organism and the agar on which it was growing had acquired a brilliant magenta color. Since Hegeman and Stanier had obtained some indication that the pigment contained iron, we concluded that the substance was worthy of investigation. The compound was quickly established to be a ferrous iron coordination derivative and because of the rather unique property of binding divalent iron we set about its isolation and characterization.

The purpose of this paper is to describe the crystallization, characterization, and synthesis of a substance which may alternatively be named L-5(2-pyridyl)-2-amino-5-ketopentanoic acid or L-2(2-pyridyl)-Δ^1-pyrroline-5-carboxylic acid. We suggest the trivial name pyrimine. At all pH values greater than about 2, the compound affords a stable ferrous complex (ferropyrimine).[1]

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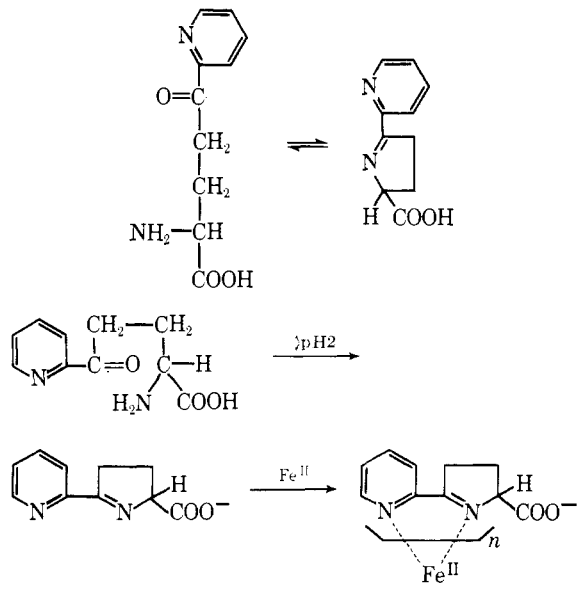
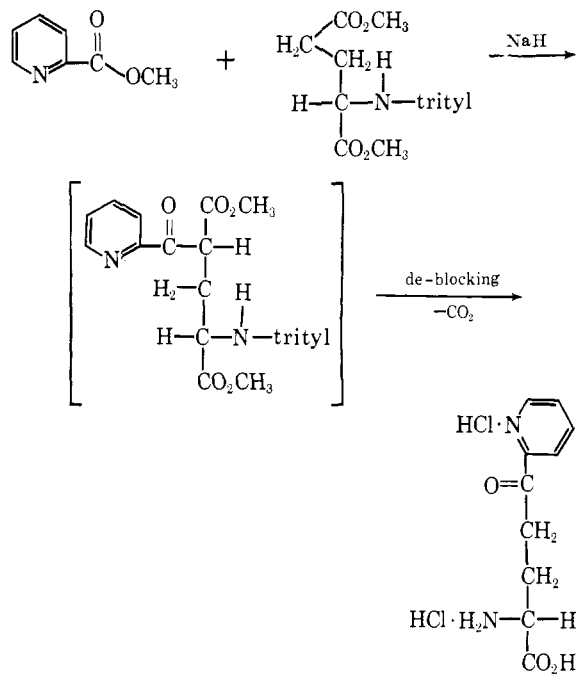
[1] The metal-binding characteristics will be the subject of future investigations; it is anticipated that other divalent ions, such as Cu^{II}, are also strongly bound.





While this work was in progress we became aware of an earlier description of a pigment elaborated by *Bacillus roseus fluorescens* (Marchal, 1956). The spectral properties of this pigment seem to be identical with those of the ferrous complex of the compound reported here. In a recent personal communication Prof. Marchal has informed us that *B. roseus fluorescens* should properly be classified as a *Pseudomonas* species. The organism isolated by Hegeman and Stanier, used in the present work, also appears to be a member of the genus *Pseudomonas*. It is motile and is an obligate aerobic, Gram-negative rod. The organism has been given the tentative designation *Pseudomonas* GH. Professor Marchal has generously undertaken a comparison of our culture with the species described by him. So far as the pigment is concerned, the French workers purified the substance to some extent, but they did not obtain crystals and hence did not carry out an extensive structural examination.

Optically active pyrimine was synthesized in reasonable yield by Claisen condensation of methyl picolinate with *N*-tritylglutamic acid dimethyl ester, the latter prepared from L-glutamic acid.

Our work does not provide definitive information on the amino–imino equilibrium. Presumably the dihydrochloride which we isolate is in the open chain form, else the neutralization equivalent of the thoroughly dried material would be 263 rather than 281 (see below). Furthermore, the infrared spectrum shows absorption bands in the correct position for an α-amino acid hydrochloride.

Future work will be concerned with the physiological role of pyrimine in *Pseudomonas* GH. The chelation characteristics are regarded to be of special interest. It is a remarkable fact that the special metal binding activity of substances such as hydroxamic acids and biheterocyclic ring compounds, which have been used by coordination chemists in the past half century for detection and study of ferric and ferrous ion, respectively, have been "discovered" and exploited throughout antiquity by microorganisms. Actually, the coordination characteristics of systems containing 2-ketopyridine or pyridine aldehyde, a primary amine, and ferrous iron have been studied rather extensively in recent years (Krumholz, 1965; Green *et al.*, 1964). However, compounds of the type reported here, wherein the Schiff base–forming primary amine is covalently anchored to the 2-ketopyridine, have not previously been reported in nature. Pyridines substituted in the 3 position are abundant in nature, especially in plants; 2-substituted pyridines are less commonly encountered (Klingsberg, 1960).

Experimental Procedures and Results

Isolation. Stock cultures of *Pseudomonas* GH were maintained at 5° on slants containing the liquid medium used for production, plus 1.5% agar. For production of pyrimine the organism was inoculated into 10 liter batches of sterile medium containing per liter: 14 g of ammonium citrate, 4 g of sucrose, 1.38 g of $NaH_2PO_4 \cdot H_2O$, 2.28 g of $K_2HPO_4 \cdot 3H_2O$, 1.0 g of NH_4Cl, 0.5 g of $MgSO_4$, and 0.01 g of $CaCl_2$. The flask was incubated for 3 days at 20° with vigorous aeration; higher incubation temperatures gave unsatisfactory yields.

The cells were removed in the Sharples centrifuge and the supernatant concentrated on the flash evaporator (37°) to about 800 ml. The pH was adjusted to 3.7–3.9. After the addition of saturating quantities of NaCl the solution was extracted with benzyl alcohol until all of the ferrous-binding activity had been removed. At this stage it was necessary to centrifuge in order to separate the phases. The pooled benzyl alcohol extract was twice extracted with 0.25 volume of 1 N HCl. The combined extracts were concentrated to 40 ml and the pH was adjusted to 3.8; extraction with benzyl alcohol and

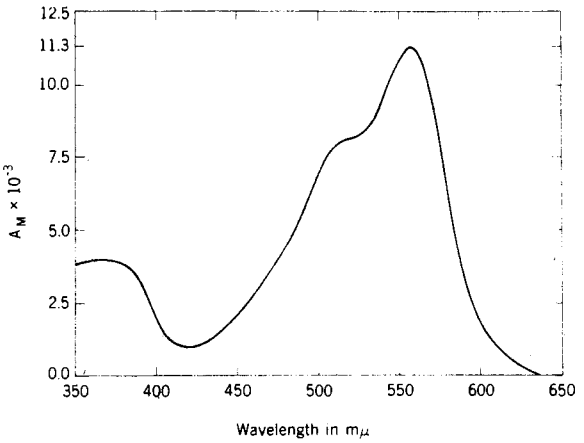


FIGURE 1: Absorption spectrum of the 1:3 ferro-pyrimine complex at pH 4.0 in 0.05 M potassium phthalate buffer. The spectrum was measured with the Cary Model 15 spectrophotometer. The millimolar absorbancy coefficient at 558 mμ is 11.3 ● 0.1.

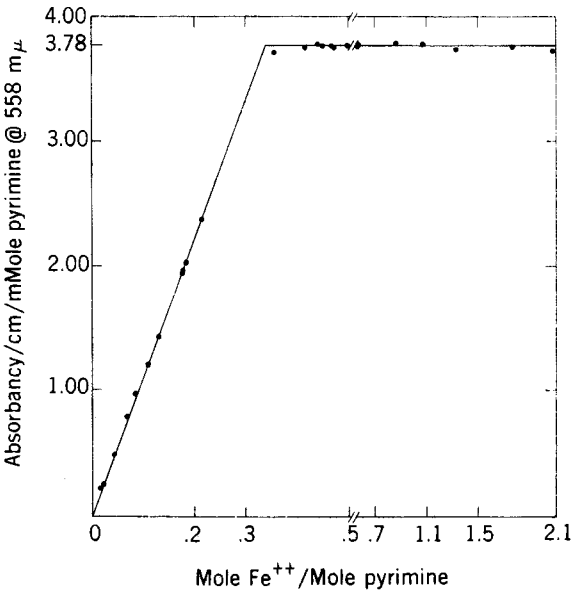


FIGURE 2: Spectrophotometric titration of pyrimine with ferrous ion. Varying amounts of standard ferrous ammonium sulfate solution were added to a constant amount of pyrimine in 0.05 M potassium phthalate buffer at 25°. Measurements were made with the Beckman-Gilford spectrophotometer (slit width 0.04 mm). All samples were measured 4 hr after mixing. All solutions were made with deoxygenated, glass-distilled water and kept under N_2.

1 N HCl was then repeated. The latter extract was concentrated to about 5 ml on the flash evaporator, 2 ml of ethanol added, and acetone then introduced slowly until incipient turbidity. Recrystallization was effected from 0.5 N HCl–acetone, using about 1.0 g of crude material in 3 ml of 0.5 N acid. The yield of recrystallized material was about 1.0 g of colorless needles.

An alternate procedure, based on direct batchwise absorption onto Dowex 50W(H^+, 50–100 mesh), gave crude crystalline material with very little effort. Approximately 100 g of moist resin was stirred for 1 hr with the acidified (pH 1.0) supernatant solution from 10 liters of culture. The resin was collected and washed with water and 1 N HCl, and the pyrimine was eluted with 6 N HCl. The eluate was evaporated to dryness and the crude pyrimine dihydrochloride precipitated in the usual way. These preparations were invariably contaminated with salt and to be effective the procedure just described should be combined with an extraction step.

The initial addition of iron to this medium at a level of 5 mg/liter resulted in the production of the ferrous derivative. This was never done in practice, however, since iron appeared to reduce the yield, and the intact complex was more difficult to purify.

Properties and Characterization. The compound could be obtained as a hydrochloride (see above) or a sodium salt. The latter was secured by extraction of the zwitterion form from benzyl alcohol with the aid of very dilute NaOH. Concentration and addition of acetone yielded colorless plates. In either form it was extremely soluble in water and insoluble in nearly all organic solvents. The recrystallized hydrochloride salt sintered at 165° and decomposed at 177° (uncor). Qualitative tests were positive for N and negative for S and P.

Anal. Found: C, 40.60; H, 5.38; N, 9.55; Cl, 23.32.

These values are compatible with the empirical formula $C_{10}H_{14}O_3N_2Cl_2 \cdot H_2O$. The presence of the water molecule was established by allowing a 28.01 mg (0.1 mmole) of anhydrous sample, prepared by desiccation under vacuum over Anhydrone, to spontaneously rehydrate for 2 hr in air. The sample gained in weight 1.68 mg (0.09 mmole of H_2O).

A substantial amount of information could be gleaned from the character of the reaction with ferrous iron. The magenta solution displayed a form (Figure 1), stoichiometry, and intensity (Figure 2) reminiscent of that of the ferrous coordination compounds of *o*-phenanthroline, bipyridyl, terpyridine, and other derivatives bearing a set of conjugated N atoms in the 1–4 positions (Green *et al.*, 1964; Krumholz, 1965). Unlike ferrous bipyridyl, however, our complex was not formed to any extent in 0.1 N HCl. Treatment of the compound with $NaBH_4$ at pH 7 or hydrogenation in the presence of palladium chloride in acidic media destroyed the characteristic iron-binding properties; a positive ninhydrin reaction developed after reduction. The infrared spectrum of the sodium salt gave further support for the presence of an imine.

A spot test for pyridine derivatives was positive (Feigl, 1956). Oxidation with nitric acid (Gilman and Blatt, 1941) gave a fragment which by paper chromatography gave an identical R_F and co-chromatographed with standard picolinic acid (R_F of 0.5 in 1-butanol–

acetic acid–water, 4:1:1). The n.m.r. spectrum of a 10% solution of the hydrochloride salt in D_2O indicated the presence of a monosubstituted pyridine ring. The infrared spectrum of the sodium salt was characteristic of a 2-substituted pyridine.

The nature of the side chain was deduced in the following way. The n.m.r. spctrum in the Varian A-60 instrument provided evidence for the arrangement $-CH_2-CH_2-CH-$; the terminal methylene exhibited slow hydrogen exchange in acidic aqueous media, suggestive of an adjacent carbonyl group. An optical rotatory dispersion measurement, with the Cary Model 60 spectropolarimeter in acidic solution, revealed a simple dispersion curve similar to those given by L-amino acids (Djerassi, 1960). The anhydrous compound gave $[\alpha]_D^{20}$ +26° in 1 N HCl. The compound was isoelectric on paper electrophoresis at about pH 4. At this pH the residual positive charge on the heterocyclic nitrogen is apparently balanced by a unit negative charge on a carboxyl group. Titration of the isoelectric species from pH 4 to 7.5 in the automatic titration apparatus (Neilands and Cannon, 1955) gave a monovalent curve with $pK = 5.2$, corresponding to a neutralization equivalent of 281 (anhydrous form). No additional functional groups were titrated between pH 7.5 and 11; in this range the compound behaved on paper electrophoresis as an anion.

Synthesis. A 50-ml round-bottom flask was charged with 7.0 g (45 mmoles) of methyl picolinate (Engler, 1894), 2 ml of dry *n*-butyl ether, and 2.0 g (40 mmoles) of oil suspension of NaH. After placing a small stirring bar in the flask, the latter was then fitted with a Claisen adapter bearing a dropping funnel over the opening and a calcium chloride drying tube in the side arm. After purging the assembly with dry N_2, the flask was lowered into an oil bath at 100°.

Exactly 5.5 g (13 mmoles) of *N*-trityl-L-glutamic acid dimethyl ester (Zervas and Theodoropoulous, 1956) dissolved in 10 ml of dry *n*-butyl ether was added in ten portions over a period of 1 hr. Vigorous stirring was maintained during this period and agitation was continued for 45 min after all of the ester had been added. The reaction mixture was allowed to cool to room temperature and was then chilled in an ice bath. The product was carefully scraped out into a beaker and the lumps were crushed under 20 ml of dibutyl ether. While the mixture was kept cold and vigorously stirred, the excess NaH was decomposed by the addition of small pieces of ice. After bubbling had ceased, a 30-ml volume of cold water was added and the mixture was stirred until complete solution had been effected. Another 10 ml of butyl ether was added, the two phases were separated, and the organic half was discarded.

The aqueous solution was brought to pH 7 in the ice bath by addition of 6 N HCl, resulting in the appearance of a tacky precipitate. The supernatant was decanted and discarded, the residue dissolved in 10 ml of chloroform, and the chloroform solution washed twice with 3 ml of water.

After the addition of 15 ml of 3 N HCl, the chloroform solution was heated on the steam bath for 2 hr. The resulting solution was extracted three times with an equal volume of benzyl alcohol in order to remove the bulk of the black color. The residual solution was evaporated to about 3 ml and, after addition of 2 ml of ethanol, the product was crystallized by addition of acetone in the usual way. Recrystallization was achieved from 0.5 N HCl–acetone (1 g/3 ml of 0.5 N HCl). The sample was dried overnight at 0.01 mm in the presence of magnesium perchlorate.

The yield was 1.35 g (4.5 mmoles, 35%). The rotation was 30% of that found for the natural compound, thus indicating some racemization. The infrared spectra of natural and synthetic pyrimine were identical. Similarly, the two specimens gave identical mobility on paper electrophoresis in acidic, neutral, and basic solvents. The spectra of the 3:1 ferrous complex (Figures 1 and 2) for the synthetic and natural compounds were superimposable throughout the visible and ultraviolet regions. Likewise the synthetic sample afforded the two characteristic salt forms (dihydrochloride and sodium salt), the same pK_a (5.2), and the same neutralization equivalent (300, monohydrate).

Anal. Calcd for $C_{10}H_{14}O_3N_2Cl_2$: C, 42.68; H, 5.03; N, 9.96. Found: C, 42.5; H, 5.2; N, 10.2.

Acknowledgment

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