Bioorganic Chemistry **29,** 387–397 (2001)
doi:10.1006/bioo.2001.1225, available online at http://www.idealibrary.com on IDEAL®

4-Hydroxy-2-nonylquinoline: A Novel Iron Chelator Isolated from a Bacterial Cell Membrane

Paulette W. Royt,*,[1] Robert V. Honeychuck,† Vadim Ravich,* Padma Ponnaluri,* Lewis K. Pannell,‡ Jeffrey S. Buyer,§ Vikas Chandhoke,¶ Wayne M. Stalick,† Leo C. DeSesso,† Sean Donohue,† Rashmi Ghei,*,† Joseph D. Relyea,† and Raphael Ruiz*

**Biology Department, †Chemistry Department, and ¶Shared Research Instrumentation Facility, George Mason University, Fairfax, Virginia 22030; ‡Structural Mass Spectrometry Facility, NIDDK, NIH, Bethesda, Maryland 20892; and §Sustainable Agricultural Systems Laboratory, USDA, Beltsville, Maryland 20705*

Received June 28, 2001

The membrane associated iron chelator of *Pseudomonas aeruginosa* has been extracted from membranes of iron-rich cells with ethanol and purified by reverse phase HPLC. Using ^{13}C NMR and FAB mass spectroscopy, the structure of the chelator has been determined to be 4-hydroxy-2-nonylquinoline. This compound has been previously isolated and named pseudan IX, a name which we use here. We synthesized pseudan IX and show that the spectral properties of the synthesized compound and the purified compound are nearly identical. Also purified from the ethanol extract of membranes is 4-hydroxy-2-heptylquinoline, i.e., pseudan VII. Bacterially purified pseudan IX binds iron as indicated by the incorporation of radiolabeled iron into the chelator and by the formation of pink micelles in a concentrated ethanol extract. The formation of pink micelles upon addition of iron to the synthesized compound indicates that it binds iron.

Key Words: 4-hydroxy-2-nonylquinoline; iron chelator; pseudan; *Pseudomonas aeruginosa*; siderophore; iron metabolism; iron transport.

INTRODUCTION

Microorganisms synthesize a variety of iron chelators that are secreted by the cells and serve to solubilize external iron before transport into the cell. Called siderophores, these ligands typically form six-coordinate octahedral complexes with iron. Catecholates, phenolates, and hydroxamates are chelating groups most often identified in microbial siderophores. However, novel functional binding groups including oxazoline nitrogen, alpha-hydroxy carboxylates, complexone-like structures, and hydrazide have more recently been identified in microbial siderophores (*1*). Some microorganisms

[1] To whom correspondence and reprint requests should be addressed. Fax: (703) 993-1046. E-mail: proyt@gmu.edu.

0045-2068/01 $35.00

use siderophores to store iron following entry into the cell (*2*), protecting the cell from oxidative damage caused by free iron. Bacteria also use haem-containing bacterioferritins and haem-free ferritins to store iron. These proteins have a hollow core in which up to 2000 iron atoms can be stored as ferric-hydroxyphosphate (*3*).

A study of iron transport and metabolism in *Pseudomonas aeruginosa* led to the isolation of an iron chelator associated with the cytoplasmic membrane of iron-rich cells (*4*). Known as the membrane associated iron chelator (MAIC), the compound is extracted from membranes with ethanol, and in earlier studies, isolated on thin-layer chromatograms. These chromatograms revealed a purple band and a brown band, both of which contained bound Fe. This was evident from radiolabeled experiments in which $^{55}FeCl_3$ was added to the growth medium. The ethanol extract of membranes of these cells revealed radiolabeled brown and purple bands on TLC plates. Also, addition of $^{55}FeCl_3$ to the complete ethanol extract of cells resulted in the appearance of radiolabeled bands on a thin layer chromatogram. A role of the chelator in iron transport or in iron storage was suggested.

The current investigations have shown that the purple and brown bands are two forms of the chelator. The compounds have been purified from the ethanol extract of iron-rich *P. aeruginosa* by high-pressure liquid chromatography (HPLC). The structure of the brown compound, determined by NMR and FAB mass spectroscopy, is that of 4-hydroxy-2-nonylquinoline. The purple compound has been identified as 4-hydroxy-2-heptylquinoline. We have synthesized 4-hydroxy-2-nonylquinoline and compared its ^{13}C NMR spectrum, FAB mass spectrum, and UV spectrum with those of the compound purified from the bacterium. Previously isolated from the filtrates of 4- to 6-week-old cultures of *P. aeruginosa*, the C-9 compound was originally called Pyo Ic and shown to exhibit antibiotic activity (*5*). The name pseudan was applied later (*6*) and is the name we use here. The pseudans have been identified as having alkyl chains of C_7–C_{12}, e.g., pseudan IX (*7*).

MATERIALS AND METHODS

Bacteria and Culture Conditions

P. aeruginosa ATCC 15692 was used in all experiments. Cells were grown in tryptic soy broth (DIFCO Laboratories) or in succinate synthetic medium (SSM) as described by Meyer and Abdallah (*8*). One liter of SSM contains 4 g succinic acid, 1 g $(NH_4)_2SO_4$, 0.2 g $MgSO_4 \cdot H_2O$, 6 g K_2HPO_4, and 3 g KH_2PO_4 per liter deionized water. The pH was adjusted to 7.0. After sterilization, the medium was supplemented with iron to 30 μM using $FeCl_3$. A 1-liter aliquot of media was inoculated with 1 ml midlogarithmic phase culture and the culture shaken for 40 h at 150 rpm at 30°C in a New Brunswick rotatory shaker. Upon harvesting, cells were washed two times with 0.1 M Tris–HCl (pH 7.8) (Tris–HCl buffer). When indicated, $^{55}FeCl_3$, 20 μCi (New England Nuclear), was added to 500 ml SSM.

Membrane Preparation and Chelator Extraction

Cells were harvested and washed two times with cold Tris–HCl buffer as above. The cells of 1 liter of media were resuspended in 10 ml Tris–HCl buffer containing 2 mg each of DNase, RNase, and $MgCl_2$, and the suspension passed one time through a precooled Carver pressure cell (Fred S. Carver Inc., Menomonee Falls, WI) at

20,000 lb/in^2. The membranes were collected by centrifugation at 38,000*g* for 1 h and washed twice with Tris–HCl buffer. Ten milliliters of ethanol was added to the membranes of cells of 1 liter of culture. The suspension was mixed and incubated at room temperature for 1 h with occasional mixing. Following centrifugation at 38,000*g* for 45 min, the ethanol extract was removed.

Purification of the Chelator

In earlier studies, the ethanol extract was applied to a silica gel G plate that was then developed using chloroform/ethanol/acetic acid (90:5:5, v/v) (*4*). The unferrated chelator was identified as a yellow band (R_f 0.60) on the plate. A purple band (R_f 0.77) and brown band (R_f 0.85) on a chromatogram were identified as ferrated forms of the chelator. These spots were scraped from the plate and the compounds eluted from the matrix with ethanol.

In the present study, a Waters 600E HPLC system controlled by Millenium software was used to purify the chelator. Following centrifugation at 137,000*g* for 1 h, the ethanol extract was applied to a preparative C-18 reverse phase HPLC column (250 $\times$ 10 mm, 5 μm, 100 A, Columbus, Phenomonex). The injection volume was 0.5 ml. The mobile phase, containing water (A) and acetonitrile (B), consisted of a linear gradient of initial concentration of 60% A/40% B to 100% B over 40 min at 2 ml/min. The detector wavelength was set at 247.5 nm, and the samples scanned from 190 to 600 nm. Peaks of the chelator were determined by comparing the UV/Vis spectra with those of the brown and purple spots scraped and eluted from the thin layer plate. The solvent from HPLC fractions was removed under vacuum and the residue dissolved in appropriate solvent. The HPLC-purified chelator was analyzed by FAB mass spectroscopy and ^{13}C NMR.

Synthesis of 4-Hydroxy-2-nonylquinoline

To a 500 ml three-necked round-bottomed flask, equipped with a mechanical stirrer and a Dry-Ice condenser, was added 250 ml of anhydrous liquid ammonia and 0.2 g of $Fe(NO_3)_3 \cdot 9H_2O$, with stirring, to produce an orange solution. Freshly cut sodium (1.5 g, 0.06 mol) was added to the stirred solution, in small pieces, over a 25-min period, producing the blue solvated electron; stirring was continued until the blue color disappeared indicating the complete formation of $NaNH_2$. To the stirred sodium amide mixture, was added 5 g 0.03 mol of 4-hydroxy-2methylquinoline over a 5-min period, producing a dark red color. Freshly distilled *n*-octyl bromide (5.2 ml, 0.03 mol) dissolved in 70 ml of toluene was added over a 10-min period. The solution was stirred for 2 h and the ammonia allowed to evaporate overnight. Cautious addition of distilled water (100 ml) was made to stirred solution to discharge any remaining sodium amide. The reaction mixture was extracted with 75 ml of methylene chloride followed by two subsequent extractions of 75 ml of methylene chloride. The combined methylene chloride layers were washed with three 100 ml portions of water and dried over $MgSO_4$. The solution was suction filtered and evaporated *in vacuo*. The crude brownish powder was washed with acetone to remove colored impurities and dried to yield 1.1 g (14,4%) 4-hydroxy-2-nonylquinoline as a light tan solid, mp 143–145°C.

Incorporation of ^{55}Fe into the Chelator

The ethanol extract of membranes of cells grown in the presence of $^{55}FeCl_3$ was reduced to half volume with a nitrogen bubbler. Following refrigeration at −20°C, 0.5 ml of the extract was applied to a LH-20 Sephadex column for gel filtration in organic solvents (1 × 13 cm: Sigma) equilibrated with ethanol. Fractions, 350 μl, were collected. The chelator was identified by its UV/Vis spectrum, and its absorbance at 240 nm was determined. The ^{55}Fe content of each fraction was determined by scintillation counting.

NMR Spectroscopy

NMR spectra were collected in chloroform-d and referenced to internal tetramethylsilane at 0 ppm for ^{13}C, on a Bruker DPX300 instrument, at 75 MHz. Polarization transfer sequences (DEPT 90 and DEPT 135) were used for ^{13}C assignments.

FAB Mass Spectroscopy

FAB mass spectra were obtained on a JEOL SX102 mass spectrometer operated at an accelerating voltage of 10 kV. Samples were desorbed from a nitrobenzyl alcohol matrix using 6 keV xenon atoms. Mass measurements were performed at 10,000 resolution using electric field scans and the sample peak bracketed by two polyethylene glycol reference ions. Linked scan analysis of the parent ion (MH^+) was performed to positively identify the compound.

Micelle Formation

Micelles formed naturally in a condensed ethanol extract of membranes and were observed by brightfield microscopy. Also, synthesized pseudan IX, 0.2 M in ethanol, was mixed with 0.2 M $FeCl_3$ in ethanol in a 3/1 ratio. Following reduction to half the volume using a nitrogen bubbler, micelles were observed as above. An Olympus BX-60 microscope and an Optronics DEI 750 camera were used.

RESULTS

An HPLC chromatogram of the elution of the chelator from the reverse phase column is shown in Fig. 1. The major peak at 33.2 min, compound 1, coeluted with the brown compound isolated from the TLC plate. The peak at 26.6 min, compound 2, coeluted with the purple compound seen on the TLC plates. Minor peaks with retention times greater than 33.2 min are also seen on the chromatogram.

The UV/Vis spectra of compounds 1 and 2 (Fig. 2), eluted from the HPLC column, reveal strong absorption in the UV, and minimal detectable absorption in the visible (insert). The doublet at 315 and 327 nm is characteristic of these compounds. Compound 1 has more absorption at 259 nm and 267 nm than does compound 2. The UV/Vis spectra of the minor peaks eluted from the HPLC before and after 33.2 min are similar to those of compounds 1 and 2. The absorption of those peaks increases at ∼260 and ∼270 nm as does time of elution.

Preliminary mass spectroscopy and proton NMR data (data not shown) suggested that compounds 1 and 2 were 4-hydroxy-2-alkyl quinolines (trivial name, pseudan). In order to confirm this, we synthesized 4-hydroxy-2-nonyl quinoline (pseudan IX,

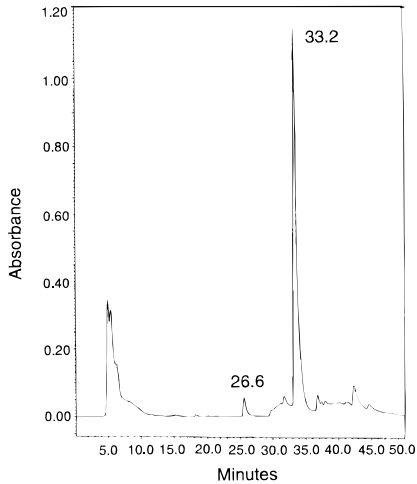

FIG. 1. HPLC analysis of the ethanol extract of membranes of iron-rich *P. aeruginosa* using a C18-reverse phase column and an acetonitrile/water gradient as described in the text. The peak at 26.6 min is that of compound **2**, and that at 33.2 min is of compound **1**.

Fig. 3) and compared its properties to those of compounds 1 and 2. The synthesis of 4-hydroxy-2-nonyl quinoline has been reported previously (*9*). That synthesis appeared to be low yielding and indirect, so a new pathway was devised. This method started with the commercially available (Aldrich) 4-hydroxy-2-methylquinoline. Reaction of this compound with two equivalents of sodium amide in liquid ammonia solvent gave the dianion where both aromatic substituents were deprotonated. Reaction of the dianion with one equivalent of the appropriate alkyl halide gave selective alkylation

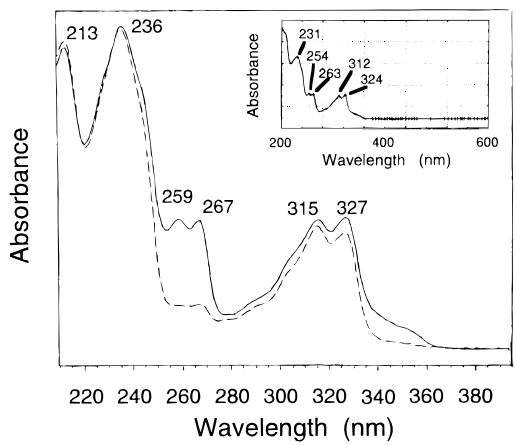

FIG. 2. UV/Vis spectra of (—) compound 1 and (----) compound **2**, eluted from the HPLC column, in acetonitrile/H_2O. Insert is spectrum, 200–600 nm, of purified compound **1** in ethanol.