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Rhizobactin, a Structurally Novel Siderophore from *Rhizobium meliloti*

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Abstract: The nitrogen-fixing Gram-negative bacterium *Rhizobium meliloti* DM4 when cultured on a low-iron media forms a siderophore which corrects iron starvation in the microorganism. The compound N^2-[2-[(1-carboxyethyl)amino]ethyl]-N^6-(3-carboxy-3-hydroxy-1-oxopropyl)lysine, designated rhizobactin, contains ethylenediaminedicarboxyl and α-hydroxycarboxyl moieties as metal-coordinating groups. The ethylenediamine group is novel as a natural product and is unprecedented as a ligand in the siderophore series, which characteristically contain catechol or hydroxamate functional groups.

When the iron concentration is less than about 1.0 μM or when the iron is bound in a form unavailable to the cell, a high affinity assimilation system has been found to be expressed in virtually all aerobic and faculative anaerobic microorganisms carefully examined for its presence.[1] This system consists of two parts, namely, soluble, relatively low molecular weight, virtually ferric-specific ligands, generically termed siderophores (Gr: sider = iron; phore = bearer), and the cognate membrane receptor and transport system for the iron-laden form of the siderophore.[2,3] Both components of the system are coordinately induced and repressed, according to the iron concentration. The high affinity system has evidently been retained through evolutionary time as a device for assuring survival of microorganisms under conditions where the iron supply is limiting, such as within host tissue, in certain soils and in aqueous environments where the pH approaches, or exceeds, neutrality. In the presence of atmospheric oxygen, iron(II) is rapidly oxidized to iron(III); the latter forms insoluble ferric oxyhydroxide precipitates, $-\log K_s = 38$.[4]

The agronomically important bacterium *Rhizobium meliloti* is generally capable of fixing atmospheric dinitrogen when symbiotically associated with certain legumes. These microbial symbionts are thereby forced to acquire iron from the plant. It is pertinent to note that *Rh. meliloti* DM4 excretes and utilizes the siderophore rhizobactin, whereas several other wild-type *Rh. meliloti* strains do not.[5]

The isolation of rhizobactin became possible once the colorimetric assays used to detect catechol[6] and hydroxamate[7] functional groups were replaced with an equally sensitive but less restrictive EDDA–Luria broth (LB) bioassay.[5] This report on the structure elucidation of rhizobactin as an aminopoly(carboxylic acid) (i.e., a complexone[8]) extends the types of microbial siderophores into three distinct chemical classes.

Experimental Section

Materials. 1-Fluoro-2,4-dinitrobenzene (DNFB) was procured from the Eastman Kodak Co. *N*-(2,4-Dinitrophenyl)-ϵ-L-lysine (DNP = *N*-(2,4-dinitrophenyl)) was acquired from Mann Research Laboratories. The disodium salt of 4,4′-[3-(2-pyridyl)-1,2,4-triazine-5,6-diyl]bis(benzenesulfonic acid) (ferrozine)[9] was obtained from the Hach Chemical Co. Sequanol grade trifluoroacetic acid (TFA) was purchased from the Pierce Chemical Co. Potassium hydrogen phthalate, $HKC_8H_4O_4$ (acidimetric standard, Mallinckrodt analytical grade, >99.95%) was dried at 130 °C prior to use. All other materials were of the highest available purity.

Production of Rhizobactin. *Rh. meliloti* DM4 was obtained from K. T. Shanmugam, University of California (U.C.), Davis, and maintained on LB agar plus chloramphenicol (Cm; 25 μg/mL). The bacterium was grown on various low iron media to find conditions under which cell division time could be reduced by the simple expedient of adding iron. Supplementing the defined media[5] with trace elements[10] other than iron did not enhance cell growth. Moreover, an aqua-blue bioactive substance from trace element supplemented cultures copurified with golden yellow-green ferrated rhizobactin and contained ~7.5% copper by atomic absorption spectroscopic (AAS) analysis. Consequently, trace elements were omitted from the media. The stationary-phase cell density for iron deficient cultures (i.e., <1 μM iron) was typically one-half that of iron sufficient cultures, based on spectrophotometric measurements at 650 nm. The purification scheme for rhizobactin has been reported in detail elsewhere.[5]

Microelemental analyses were made by the Microanalytic Laboratory in the Chemistry Department, U.C., Berkeley.

Chromatography. Thin-layer chromatography (TLC) was carried out on silica gel F-254 plates (EM Laboratories) in one of the following solvent systems: (A) methanol/water, 9:1; (B) methanol/dichloromethane, 1:1; (C) *n*-propyl alcohol/34% ammonia, 7:3.

Ion-pair reversed-phase liquid chromatography (RPLC) was performed by the method of Mahoney and Hermodson,[11] using a Supelco LC-18 column (250 mm × 10 mm i.d.) and the equipment described previously.[5] Rhizobactin specimens (0.25 mL, 5 mg/mL) were injected and run in 0.1% (0.013 M) TFA for 6 min isocratically and eluted with a 15-min linear gradient into 0.1% TFA–10% methanol. Fractions were monitored spectrophotometrically at 230 nm, tested for activity with the bioassay, and lyophilized to dryness.

High-Voltage Paper Electrophoresis. The ionophoretic mobilities of compounds were determined by using a flat-bed device in one of the following buffers: (A) glacial acetic acid/88% formic acid/water, 25:72:903, pH 1.8; (B) 4% sodium formate, pH 2.8; (C) 25 mM potassium phthalate, pH 5.0; (D) 100 mM sodium phosphate, pH 6.6. Samples were spotted on Whatman No. 1 paper, subjected to electrophoresis (ca. 40 V/cm, 40–60 min) by using a Savant 0–2-kV constant-voltage power supply, and detected with the bioassay or by one of the following indicator sprays: (1) 0.25% ninhydrin in acetone and 0.2 M sodium acetate/95% ethanol, 1:9. (2) 3% $FeCl_3 \cdot 6H_2O$ in 95% ethanol, 10% sodium dithionite in 0.1 M phosphate buffer, pH 7.0 (freshly prepared), and 0.1% ferrozine (iron analysis reagent)[9] in methanol. Glutathione (reduced form), glutamic acid, ethylenediaminetetraacetic acid and *N*(2,4-dinitrophenyl)-ϵ-L-lysine were used as reference standards.

Spectroscopy. Atomic absorption spectroscopic measurements were made with a Perkin-Elmer 372 spectrophotometer in the flame mode. Solution absorbancies were measured by using a Beckman Model 25 spectrophotometer. Angles of ellipticity (θ), in degrees, were recorded directly with a Cary Model 60 spectropolarimeter.

Nuclear magnetic resonance (NMR) spectra were recorded on 300- and 400-MHz instruments at Varian, on 180-, 200-, and 250-MHz instruments in the Chemistry Department, U.C., Berkeley, all of custom design, and on a 300-MHz Bruker instrument in the Pesticide Chemistry

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and Toxicology Laboratory, U.C., Berkeley. The predicted ^{13}C chemical shifts were calculated by the method of Grant and co-workers[12] by using the equation for substituted hydrocarbons: $\delta_k\ B + \sum A_l n_{kl} + C$, where δ_k is the ^{13}C chemical shift (δ) of the kth carbon, B is a constant (−2.6 ppm) about equal to the δ of methane, A_l is the additive δ parameter of the lth position relative to carbon k, and C is a correction factor for branched hydrocarbons. The empirical increments of −NHC(O)R, namely $A_\alpha = 28.1$, $A_\beta = 10.4$, $A_\gamma = -3.1$, and $A_\delta = 0.7$, noted as having been used in calculations for the corresponding arginine carbons by Horsley, Sternlicht, and Cohen,[13] were obtained by reverse computation. All other increments are listed elsewhere.[12,14] The relative acidities of D_2O solutions (pD) were calculated by the method of Glasoe and Long.[15]

High-resolution (HR) electron impact (EI) and secondary ion (SI) mass spectra (MS) were recorded in the Space Sciences Laboratory, U.C., Berkeley. Fast atom bombardment (FAB), chemical ionization (CI), and additional EIMS were recorded in the Chemistry Department, U.C., Berkeley. Several preliminary FAB spectra were recorded in the Biochemistry Department, Imperial College, London. Infrared (IR) solid-state spectra were recorded with a Perkin-Elmer Model 710B spectrophotometer calibrated with polystyrene film prior to use.

Potentiometric Titration. Measurements were taken with an aqueous 40 mM solution of the HCl salt of rhizobactin at room temperature, using a Metrohm Model 645 dositometer and a Corning Model 125 pH meter, calibrated with buffer standards prior to use. The 0.1 N NaOH titration solution was standardized with potassium hydrogen phthalate. Tracings were made with a Kipp and Zonen BD41 recorder. The compound to be titrated was prepared by adding a molar excess of HCl to a rhizobactin solution (on ice) and lyophilizing it to dryness. The material was then dissolved in absolute ethanol and precipitated by using diethyl ether to remove the excess HCl.

Oxidative Degradation, Acid Hydrolysis, and Organic Acid Analysis. $KMnO_4$ was added to a rhizobactin solution until no further precipitation occurred. The suspension was adjusted to 0.1% NaOH and refluxed for several hours. The supernatant fluid was collected by centrifugation, divided into aliquots, and lyophilized to dryness. Rhizobactin was hydrolyzed by using 6 N HCl in a sealed tube, previously flushed with nitrogen and evacuated, for 24 h at 110 °C. The hydrolysate was concentrated to dryness and dissolved in water repeatedly to remove the residual HCl.

Malic dehydrogenase (L-malate/nicotinamide adenine dinucleotide oxidoreductase, E.C. 1.1.1.37)[16] was used as an enzymatic assay to detect L-malate in the 6 N HCl hydrolysate. The NADH formed in the assay reaction was quantitated spectrophotometrically at 340 nm. The composition of the reaction mix was 1.6 mL of 0.1 M glycine buffer, pH 10, 0.2 mL of 10 mM NAD anion, 2 units of enzyme (Sigma Chemical Co., product M7508), and 0.5–1.5 μmol of L-malate, or an aliquot of the hydrolysate, diluted to a final reaction mix volume of 2 mL.

The ether-soluble organic acids were extracted from a mixture of the hydrolysate and celite by using diethyl ether, as outlined by Elsden et al.,[17] and analyzed by MS.

Derivatives. The 2,4-dinitrophenyl (DNP) derivatives were made by the method of Sanger.[18]

The methyl ester derivative was prepared by dissolving rhizobactin in 5% HCl–methanol and stirring the solution (on ice) for 12 h; the derivative was concentrated to dryness and dissolved in water repeatedly to remove the residual methanol.

Results and Discussion

Composition and Properties. Ferrated rhizobactin preparations typically showed one spot after TLC on silica in solvents A and B that was ninhydrin positive (after heating) and iodine staining and had respective R_fs of 0.69 and 0.45. Nonetheless, one deferrated preparation gave two characteristic molecular ion (M + H) peaks at 378 and 406 amu, by positive SIMS employing Cs^+ and glycerol,[19] and the correlating (M − H) peaks by negative SIMS; FABMS using thioglycerol showed analogous (M + H) peaks.

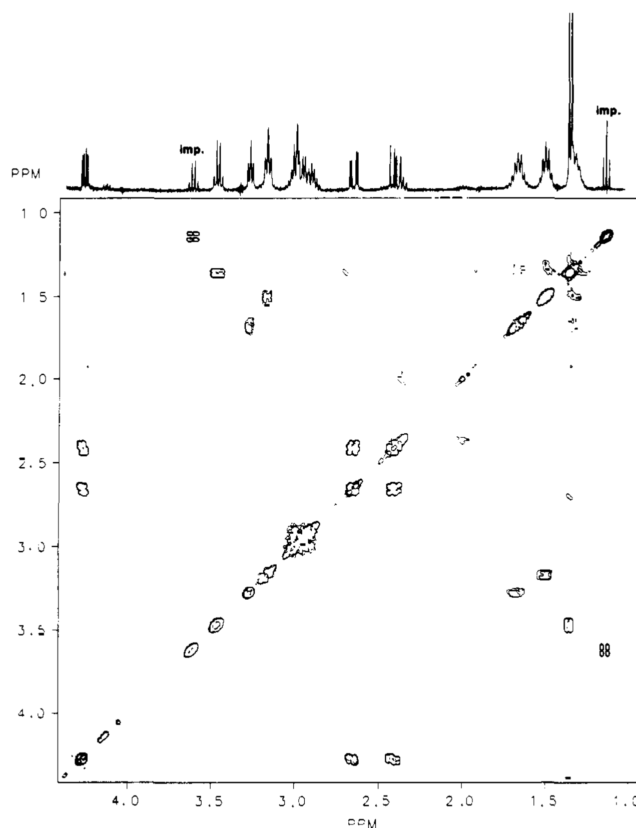

Figure 1. ^1H–^1H HOMCOR spectrum (400 MHz, pD 8.4): imp. denotes an ethanol impurity.

Consequently, ion-pair RPLC in 0.1% TFA was implemented and resolved the preparation into a major bioactive component [retention time (t_R) = 15.4 min; FABMS (M + H) peak at 378 amu], a minor bioactive component [t_R = 19 min; FABMS (M + H) peak at 360 amu], plus three traces (t_Rs = 29, 34.4, and 40.4 min). Moreover, reinjecting a specimen of the major peak yielded two peaks whose retention times corresponded to the major and minor components. HRSIMS (378.1940, M + H, $C_{15}H_{28}N_3O_8$ requires 378.1876, Δ6.4 mmu) and microelemental analyses, (C, 47.44; H, 7.08; N, 10.69. Anal. Calcd for $C_{15}H_{27}N_3O_8$: C, 47.74; H, 7.21; N, 11.13) established the molecular formula of rhizobactin as $C_{15}H_{27}N_3O_8$.

Electrically neutral rhizobactin yielded crystals from water–ethanol as a colorless compound soluble in alkaline water and virtually insoluble in any conventional organic solvent. Rhizobactin crystals decomposed without melting above 240 °C. The electronic absorption spectrum is featureless save for end absorption in the ultraviolet. $[\theta]^{22}{}_D$ −58 °C (10 mg/mL, pH 6.9).

^{13}C and ^1H NMR. The broad band decoupled ^{13}C NMR spectra at pD 3.1 and 8.4 exhibited 11 non-carbonyl and 4 carbonyl carbons. The relative integrals of the coupled ^1H NMR peaks at pD 3.1 and 8.4 established that 20 protons were bonded to carbon. A ^1H–^1H homonuclear correlation (HOMCOR) spectrum showed four isolated spin systems (Figure 1) that define four structural sections: (1) CH_2CH_2; (2) $CHCH_3$; (3) $CHCH_2CH_2CH_2CH_2$; and (4) CH_2CH (ABX). Moreover, the coupled ^1H NMR spectrum at pH 3.1 resembled the cumulative spectra of an ethylenediamine group, plus alanine, lysine, and L-malic acid.[20] A ^{13}C–^1H heteronuclear correlation (HETCOR) spectrum (Figure 2) gave the ^{13}C and ^1H assignments listed in Tables I and II.

Long-range HOMCOR and HETCOR methods repeatedly failed to provide any valid couplings between the structural sections. However, the shift of most signals showed a marked pD dependence (data compiled in Tables I and II). As noted by

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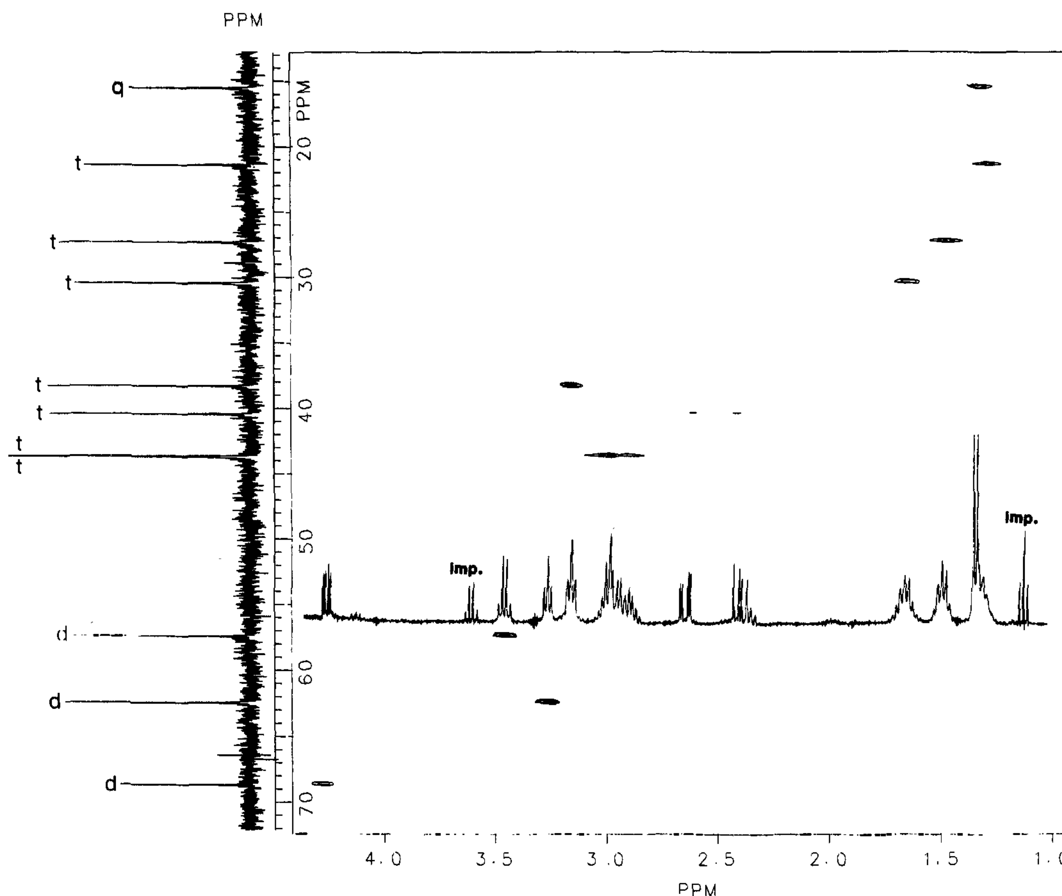

Figure 2. ^{13}C–^{1}H HETCOR spectrum (100 MHz, pD 8.4).

Table I. ^{13}C NMR Data (75.47 MHz)

pD atom	pD 3.1 δ	pD 8.4 δ
C-1	174.0[a]	177.6[c]
C-2	62.9	62.4
C-3	29.1	30.3
C-4	21.2	21.4
C-5	27.9	27.3
C-6	38.7	38.3
C-1′	42.9[b]	43.6[d]
C-2′	42.0[b]	43.5[d]
C-1″	58.2	57.4
C-2″	14.8	15.4
C-3″	172.9[a]	176.8[c]
C-1‴	172.3[a]	172.4[c]
C-2‴	40.3	40.5
C-3‴	68.3	68.7
C-4‴	177.4[a]	179.1[c]

[a–c]Chemical shifts with the same superscript may be transposed within a column.

Wüthrich for the common amino acids,[21] successive deprotonation of the weak carboxylic acid and the amino groups of rhizobactin resulted in sizeable downfield shifts for most ^{13}C signals and a range of upfield shifts for the ^{1}H signals.

In particular, the ethylenediamine four-proton singlet (observed with resolution enhancement as two peaks with nearly the same δ) collapsed into an asymmetric fold upon removing one amine proton ($pK_4' \sim 6.8$; see Potentiometric Titration Data below); removing a second amine proton ($pK_5' \sim 9.7$) resulted in an overall amine deprotonation signal shift (Δ_b = pD 12.0δ – pD 5.9δ) for the 1′- and 2′-methylene protons of ca. –0.9 and –0.7 ppm. Likewise, the 1″- and 2-methine protons, substituents of the second and third sections, had comparable Δ_bs of –0.65 and –0.70 ppm, respectively; the 2″- and 3-β-methylene protons had Δ_bs of –0.32 and –0.40, correspondingly. It follows that C-1″ and C-2 are bonded to ethylenediamine nitrogens.

The comparable ^{13}C and ^{1}H signals of the ethylenediamine group indicate that the carboxyethyl group of the second section is substituted on $N^{2'}$ instead of N^2. Accordingly, the $\Delta_b/\Delta pD$ values for the 2- and 3-protons are larger near amino pK_4', whereas the corresponding values for the 1″- and 2″-protons are larger near amino pK_5'; (pK_a' values for the common amino acids in D_2O are within ±0.1 pH units of the corresponding pK_a' values in H_2O).[22] Analysis of the DNP derivatives below validates this specific assignment.

On the other hand, the 4-, 5-, 6-, 2‴, and 3‴-protons had small Δ_bs. Nevertheless, the Δ_b and δ of each hydrocarbon indicate that an amide bond is situated between the third and fourth sections and that C-6 is bonded to the amidic nitrogen. Further, deprotonation of a weak carboxylic acid nearby (Δ_a = pD 5.9δ – pD 3.1δ) resulted in sizeable Δ_as for the 2‴A-, 2‴B-, and 3‴-protons of –0.15, –0.05, and –0.21 ppm, respectively. Therefore, the 2‴-protons are proximal to the amidic carbonyl, whereas the 3‴-proton is nearer the weak acid (see Degradation Products below).

Esterifying the carboxylic acids present with 5% HCl–methanol and integrating the three resultant methyl ester peaks [^{1}H NMR (300 MHz, D_2O, internal HDO; δ 4.8) 3.9, 3.9, and 3.8] showed that rhizobactin contains three carboxylic acids (see Potentiometric Titration Data below); FABMS of the compound gave a (M + H) peak at 420 amu, indicative of the trimethyl ester derivative.

Moreover, the predicted ^{13}C δs for the structure presented here (Figure 3), based on empirical additivity calculations (Table III), agree well with the measured values. The minor deviation for C-5 might result from residual inductive effects or structural

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Table II. ^{1}H NMR Data (200 MHz; internal DSS,[a] δ 0)

	soln pD									
protons	3.07[b] δ signal	5.93 δ	6.61 δ	6.99 δ	7.45 δ	8.40[c] δ signal	8.66 δ	9.62 δ	10.55 δ	12.0 δ
H-2	3.75 t	3.71	3.59	3.47	3.40	3.33 t	3.27	~3.2	3.05	3.01
H-3	1.91 m	1.90	1.83	1.78	1.74	1.72 m	1.69	~1.6	~1.5	~1.5
H-4	1.40 m	1.39	1.39			1.36 m			1.32	1.32
H-5	1.56 m	1.56	1.54	1.54	1.54	1.54 m	1.54	~1.5	~1.5	~1.5
H-6	3.21 m	3.21	3.21	~3.2	3.20	3.19 t	3.19	~3.2	~3.2	3.18
H-1′[d]	3.47 s	3.44	3.32	~3.2	~3.1	~3.1 m	~3.0	~2.8	~2.7	~2.7
H-2′[d]	3.47 s	3.44	3.32	~3.2	~3.1	~3.0 m	~2.9	~2.8	~2.6	~2.5
H-1″	3.79 q	3.74	3.67	3.60	3.55	3.51 q	3.46	3.32	~3.2	3.09
H-2″	1.52 d	1.50	1.47	1.44	1.42	1.40 d	1.37	1.30	1.21	1.18
H-2‴A	2.62 dd	2.48	2.47	2.47	2.44	2.44 dd	2.43	2.43	2.43	2.43
H-2‴B	2.73 dd	2.68	2.68	2.68	2.68	2.68 dd	2.68	2.68		
H-3‴	4.51 dd	4.30	4.30	4.30	4.30	4.29 dd	4.29	4.29	4.29	4.29

[a] DSS denotes 2,2-dimethyl-2-silapentane-5-sulfonic acid. [b] Coupling constants: $J_{2'''A,2'''B}$ = 14.9, $J_{2'''A,3'''}$ = 7.6, $J_{2'''B,2'''A}$ = 14.9, $J_{2'''B,3'''}$ = 5.0, $J_{3''',2'''A}$ = 7.6, $J_{3''',2'''B}$ = 5.0 Hz. [c] Measurements taken at 300 MHz. Coupling constants: $J_{2'''A,2'''B}$ = 14.6, $J_{2'''A,3'''}$ = 9.4, $J_{2'''B,2'''A}$ = 14.6, $J_{2'''B,3'''}$ = 3.6, $J_{3''',2'''A}$ = 9.4, $J_{3''',2'''B}$ = 3.6 Hz. [d] Chemical shifts for H-1′ and H-2′ may be transposed within a column.

Table III. Predicted ^{13}C Chemical Shifts (ppm; 75.47 MHz, pD 3.1)

C-2 δ		C-3 δ		C-4 δ		C-5 δ		C-6 δ	
B	−2.6	B	−2.6	B	−2.6	B	−2.6	B	−2.6
$A_\alpha i\text{-COO}^-$	20	$A_\alpha^{CH_3}$	9.1	$A_\alpha^{CH_3}$	9.1	$A_\alpha^{CH_3}$	9.1	$A_\alpha CH_3$	9.1
$A_\alpha^{NR_3^+}$	30.5	$A_\alpha^{CH_3}$	9.1	$A_\alpha^{CH_3}$	9.1	$A_\alpha CH_3$	9.1	A_αNHC(O)R	28.1
$A_\alpha^{CH_3}$	9.1	$A_\beta^{i\text{-COO}^-}$	3	$A_\beta CH_3$	9.4	A_βNHC(O)R	10.4	$A_\beta CH_3$	9.4
$A_\beta^{CH_3}$	9.4	$A_\beta^{NR_3^+}$	5.5	$A_\beta^{CH_3}$	9.4	$A_\beta^{CH_3}$	9.4	$A_\gamma CH_3$	−2.5
$A_\gamma^{CH_3}$	−2.5	$A_\beta^{CH_3}$	9.4	$A_\gamma^{COO^-}$	−2.5	$A_\gamma CH_3$	−2.5	$A_\delta CH_3$	0.3
$A_\delta^{CH_3}$	0.3	$A_\gamma^{CH_3}$	−2.5	$A_\gamma^{NR_3^+}$	−7.0	$A_\delta^{COO^-}$	0	$A_\epsilon NR_3^+$	−0.5
$A_\epsilon^{NHC(O)R}$	0	$A_\delta^{NHC(O)R}$	−0.7	$A_\gamma^{NHC(O)R}$	−3.1	$A_\delta NR_3^+$	−0.5	$A_\epsilon COO^-$	0
(3°)2°	−3.7	(2°)3°	−2.5	(2°)2°	0	(2°)2°		(2°)2°	0
pred	60.5	pred	27.8	pred	21.8	pred	32.4	pred	41.3
meas	62.9	meas	29.1	meas	21.2	meas	27.9	meas	38.7

C-1′,2′ δ		C-1″ δ		C-2″ δ		C-2‴ δ		C-3‴ δ	
B	−2.6	B	−2.6	B	−2.6	B	−2.6	B	−2.6
$A_\alpha CH_3$	9.1	$A_\alpha i\text{-COO}^-$	20	$A_\alpha CH_3$	9.1	$A_\alpha CONH_2$	22	$A_\alpha i$-COOH	16
$A_\alpha NR_3^+$	30.5	$A_\alpha CH_3$	9.1	$A_\beta NR_3^+$	5.5	$A_\alpha CH_3$	9.1	A_αOH	49
$A_\beta NR_3^+$	5.5	$A_\alpha NR_3^+$	30.5	$A_\beta i\text{-COO}^-$	3	$A_\beta i$-COOH	2	$A_\alpha CH_3$	9.1
(2°)2°	0	(3°)1°	0	(1°)3°	−1.1	A_βOH	10	$A_\beta CONH_2$	2.5
pred	42.5	pred	57.0	pred	13.9	(2°)3°	−2.5	(3°)2°	−3.7
meas	42.9, 42.0	meas	58.2	meas	14.8	pred	38.0	pred	70.3
						meas	40.3	meas	68.3

constraints. Discrepant values for the analogous δ carbon of lysine residues have been reported.[23,24]

It is pertinent to note that a new isolated spin system arose if rhizobactin was subjected to excessive heat (~50 °C) or remained at a pH below 3 for several hours: ^{1}H NMR (300 MHz, pD 3.1) δ 4.3, 2.5, 2.4.

Infrared Spectrum. The IR spectrum of rhizobactin possessed the characteristic features of an aminocarboxylic acid:[25,26] IR KBr_{max} 3600–2050 (m br), 3340 (m), 3100 (m), 2980 (w), 2900 (m), 1747 (s) [(NH^+CHCOOH)CO stretch (st)], 1630 (s)/1595 (s)/1575 (m) shoulder [(NH^+CHCOO^-) NH bend, CO st], 1500 (w), 1450 (w), 1415 (s), 1360 (m), 1320 (w), 1300 (w), 1260 (m), 1214 (w), 1100 (m), 1065 (m), 1038 (w), 915 (w), 865 (w), 800 (m), 780 (w), 740 (w), 652 cm^{-1} (w).

Electron Impact Mass Spectra. A molecular ion peak for rhizobactin was absent in the HREIMS; HREIMS, m/z (rel intensity) (the ten most significant peaks) 341 (13), 323 (8), 268 (20), 171 (24), 143 (35), 127 (32), 113 (100), 97 (43), 84 (58), 70 (86), 56 (98). These peaks are indicative of the following characteristic fragments: M − $2H_2O$, $C_{15}H_{23}N_3O_6$ requires 341.1587, 341.1598, Δ1.1 mmu; M − $3H_2O$, $C_{15}H_{21}N_3O_5$ requires 323.1481, 323.1502, Δ2.1 mmu; a pentanolactam[27] base peak derived from the N^2-[2-[(1-carboxyethyl)amino]ethyl moiety, $C_5H_9N_2O$ requires 113.0715, 113.0717, Δ0.2 mmu; $C_5H_9N_2O$ + CH_2, $C_6H_{11}N_2O$ requires 127.0871, 127.0871; $C_5H_9N_2O$ +

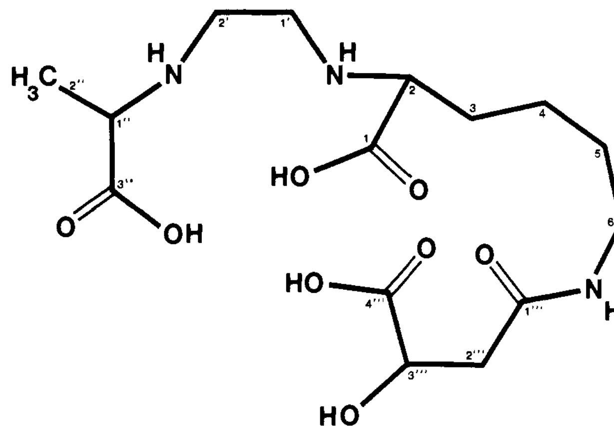

Figure 3. Rhizobactin.

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$CHCO_2H$, $C_7H_{11}N_2O_3$ requires 171.0770, 171.0779, Δ0.9 mmu; several lysine fragments,[28] C_4H_8N requires 70.0657, 70.0662, Δ0.5 mmu; $C_5H_{10}N$ requires 84.0813, 84.0813; $C_6H_{11}N$ requires 97.0891, 97.0863, Δ–2.8 mmu. In addition, fragments indicative of the alanine and malamic acid substituents were evident: $C_3H_5O_2$ requires 73.0290; 73.0289, Δ–0.1 mmu; and $C_3H_5O_3$ requires 89.0239, 89.0241, Δ0.2 mmu.

Potentiometric Titration. The hydrochloric salt of rhizobactin contained, per proton equivalent at $pK_4' \sim 6.8$, 2.9 ± 0.2 acidic protons with pK_as <5, and 1.3 ± 0.2 protons at $pK_5' \sim 9.7$. Titration in water–methanol (1:1) only slightly depressed the group at $pK_4' \sim 6.8$; this group is an amine inasmuch as carboxylic acids are weakened drastically by titration in aqueous–alcohol solution.[29] The ethylenediamine moiety accounts for the two groups with pK_a's near 6.8 and 9.7. Ethylenediamine-*N,N'*-diacetic acid contains analogous groups with pK_a's near 6.5 and 9.6.[30]

Degradation Products. Permanganate oxidation of a 15 μmol specimen afforded 17 nmol of ethylenediamine, as determined with the amino acid analyzer. The approximate yields in nanomoles of ninhydrin-positive compounds eluting similar to lysine, alanine, glutamic acid, threonine, and glycine were 60, 57, 57, 41, and 40, respectively.

Hydrolysis of a 110 nmol specimen using 6 N HCl produced about 16, 25, and 30 nmol of compounds eluting like glutamic acid, histidine, and an anomalous basic compound. Approximately 10 μmol of authentic L-malic acid was detected in a 30 μmol specimen of the 6 N HCl hydrolysate, using the malic dehydrogenase assay; an ether-extracted specimen gave peaks at 117 and 135 amu, by CIMS with methane and EIMS, indicative of M – OH and M + H ions of malic acid, correspondingly.

Rhizobactin migrated as an anion during paper electrophoresis at pH values above 2.8 and had a net charge of –1 at pH 5. Ferrated rhizobactin migrated as an anion at pH values above 5.

2,4-Dinitrophenyl Derivatives. The reaction of DNFB with rhizobactin yielded a water-soluble compound with a net charge of +1 during paper electrophoresis in buffer A at pH 1.8. The derivative, tentatively identified as mono-DNP-rhizobactin, was insoluble in ethyl acetate and in acetone and had an R_f of 0.34 after TLC on silica in solvent C.

Two additional derivatives present became insoluble in water when the pH was lowered to 1.0. The second, tentatively identified as the tri-DNP derivative of hydrolyzed rhizobactin, partitioned between the ether and water layers during centrifugation at 6700*g* for 5 min; the third had separated as a pellet. The two compounds were soluble in ethyl acetate and in acetone, had net charges of zero during paper electrophoresis at pH 1.8, and exhibited respective R_fs of 0.60 and 0.55 after TLC on silica in solvent C. The relative integrals of the DNP protons in the third derivative [(300 MHz, acetone-d_6, internal acetone; δ 2.05): 8.7, 8.4, and 7.8 ppm], and the doubling of resonances of the 7.8 and 8.4 ppm signals, led to its assignment as *N,N'*-bis(2,4-dinitrophenyl)rhizobactin. It is pertinent to note three additional properties of this derivative which prove that its precursor, rhizobactin, contains two 2° amines: an absorbance maxima at 390–395 nm in 1 N NaOH ($\epsilon_M = 2.09 \times 10^4$)[31] with an A_{350}/A_{390} ratio of 0.66[32] and a corresponding FABMS (M + H) peak at 710 amu.

Summary

Separation of rhizobactin as its stable iron chelate facilitated the isolation of this water-soluble siderophore.[5] Following deferration, the molecule was observed to undergo a chemical rearrangement if subjected to excessive heat or to a pH below 3; hydrolysis of the malamic acid substituent and lactam formation, via dehydration of the ethylenediaminedicarboxyl moiety, are both indicated by the data. Analogous lactam rearrangements have been detected in preparations of other unusual amino acids.[33-36]

The ethylenediamine group is novel as a natural product[37] and is unprecedented as a ligand in the siderophore series.[38] Two larger polyamines, namely spermidine[39] and norspermidine,[40] act as structural backbones in three catechol-type siderophores. *N,N'*-Ethylenediaminedisuccinic acid has also been isolated from the culture filtrate of an actinomycetes recently and shown to be a specific inhibitor against phospholipase C.[36]

The α-hydroxycarboxylate ligand, present as a β-malamic acid substituent, has been found in two additional types of siderophores. The three members of a mixed catechol–hydroxamate type contain β-hydroxyaspartic acid;[41] one member appears to contain a malamide substituent that is not a ligand. It is also present as citrate in the three citrate–hydroxamate-type siderophores.[38]

Besides the above microbial products, five homologues of *N*-[(*N*-(3-hydroxy-3-carboxypropyl)-3-amino)-3-carboxypropyl]azetidine-2-carboxylic acid, amino acids from graminaceous plants regarded as possible "phytosiderophores", contain α-hydroxycarboxylate and α-aminocarboxylate ligands.[42] A sixth homologue, containing three aminocarboxylate ligands instead, has been isolated from diverse vascular plants.[43,44] Notable amongst such plants is alfalfa, a symbiotic host of the bacterium which excretes rhizobactin.

Further, the phytosiderophores and many other complexones exhibit strong coordination properties toward dissimilar divalent and trivalent metals.[36,45-47] Rhizobactin's corresponding features thus modify the notion that siderophores contain virtually ferric-specific ligands. Indeed, the biological function of these compounds may ultimately extend beyond iron transport.[33,36,48]

Accordingly, an investigation of the coordination chemistry of rhizobactin, when correlated to a molecular analysis of the requisite genes, would elucidate the relevance of the system to *Rhizobium*, in planta and ex planta. The configuration at the two remaining unknown chiral centers and the metal-binding properties of rhizobactin, as well as its chemical synthesis, are under investigation.

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Registry No. Rhizobactin, 92308-51-5; rhizobactin trimethyl ester, 94732-86-2; mono-DNP-rhizobactin, 94732-89-5; *N,N'*-bis(2,4-dinitrophenyl)rhizobactin, 94732-87-3; tri-DNP-rhizobactin, 94732-88-4; iron, 7439-89-6.

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