SIDEROCHELIN, A NEW FERROUS-ION CHELATING AGENT PRODUCED BY *NOCARDIA*

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A new ferrous-ion chelating agent, siderochelin, was isolated from fermentation broths of *Nocardia* sp. SC 11,340. Siderochelin was produced by conventional submerged culture and purified by solvent extraction and recrystallization. The antibiotic was crystallized from acetonitrile as a mixture of diastereoisomers. The molecular formula of siderochelin was determined as $C_{11}H_{13}N_3O_3$ on the basis of elemental analysis and mass spectrometry, and the structure was elucidated by X-ray crystallography. The compound shows a broad spectrum of antimicrobial activity, being active against bacteria, fungi and protozoa.

During a screening program designed to detect inhibitors of lipopolysaccharide biosynthesis, we detected a novel compound with ion-chelating properties produced by *Nocardia* sp. SC 11,340. We have named this compound siderochelin. This paper deals with the isolation, characterization and biological properties of siderochelin.

Screening of Soil Actinomycetes

Cultures of aerobic actinomycetes were isolated from soil samples by stamping air dried soils onto a selective medium of the following composition—0.5 % glucose, 0.1% KNO_3, 0.05% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% NaCl, 0.001% $FeSO_4 \cdot 7H_2O$ and 2% agar (pH 7.0). Immediately before use, actidione (Calbiochem) was added to the medium to give a final concentration of 100 μg/ml. After 10~14 days incubation at 28°C, colonies were picked and transferred to agar slants containing 0.1% yeast extract, 0.1% beef extract, 0.2% NZ amine A (casein hydrolysate), 1% glucose and 1.5% agar (pH 7.3). Submerged culture was carried out at 28°C for 5~6 days in 250 ml flasks containing 50 ml of liquid medium of the following composition: 0.4% yeast extract, 1% malt extract and 0.4% glucose.

Assay

Fermentation samples (50 μl) were added to 10 mm paper discs that after drying were placed on agar plates (3.5% BBL seed agar and 0.5% NaCl) containing 10 μg/ml diumycin[1] and seeded with *E. coli* SC 10,888. Plates were incubated overnight at 37°C and zones of activity recorded.

Taxonomy of the Producing Strain

The siderochelin producing strain SC 11,340 was isolated from a soil sample collected in Virginia.

Morphology

Nocardia sp. SC 11,340 produces a vegetative mycelium which fragments into rod and coccal forms within 5 days. The cells are non-acid fast and Gram-positive.

Colonies growing on a solid medium are smooth to doughy in consistency and are whitish gray

in color. On glycerol asparagine agar there are no distinguishing characteristics; on tomato paste oatmeal agar, a soluble purple pigment is produced that permeates throughout the solid medium. No aerial mycelium is formed.

Acid hydrolysates of whole cell walls analyzed by the method of BECKER *et al.*[2], indicate the presence of meso-diaminopimelic acid, with galactose and arabinose as the major sugar components. There is some ribose present but no madurose. This cell wall analysis represents a Type IVA pattern[3], and is diagnostic for the genus *Nocardia*.

To determine whether acid products were formed from various carbohydrates in the presence of *Nocardia* sp. SC 11,340, the microorganism was cultured for 10 days on the basal medium of AYERS *et al.*[4] in the presence of each of the following carbohydrates, with the following results:

Basal Medium					
(Control)	−	Melibiose	−	Maltose	+
Adonitol	+	Raffinose	−	Mannitol	−
Arabinose	−	Rhamnose	−	Mannose	−
Cellobiose	−	Trehalose	−	Xylose	−
Erythritol	−	Glycerol	+	Fructose	−
Glucose	+	Inositol	+	Sucrose	+
Melezitose	−	Lactose	−	Sorbitol	−

Legend: +: acids formed, −: acids not formed

Nocardia sp. SC 11,340 decomposes casein and hypoxanthine, but not tyrosine, xanthine and guanine. The organism hydrolyzes gelatin, but not starch.

Fermentation

A 4-liter flask containing 1,500 ml of production medium was inoculated with 10 ml of seed culture washed from an agar slant with 0.01% Duponol.

Production Medium		
Yeast extract	4 g	Distilled water to 1 liter
Malt extract	10 g	Adjusted to pH 7.3
Dextrose	4 g	

The culture was incubated for 72 hours on a rotary shaker at 28°C using an agitation rate of 300 rpm.

A 1.5-liter portion of seed culture was used to inoculate a 38-liter stainless steel fermentor containing 28.5 liters of production medium and the culture was grown for 72 hours at 28°C using an agitation rate of 220 rpm and an air flow of 65.1 liter/minute.

A 12.5-liter portion of second stage seed culture was used to inoculate a 380-liter stainless steel fermentor containing 250 liters of production medium. The fermentor was operated for 6 days at 28°C using an agitation rate of 155 rpm and an air flow of 283.2 liter/minute.

The production of siderochelin was followed with the bioassay procedure.

Isolation

The isolation of siderochelin from fermentations of *Nocardia* sp. SC 11,340 is outlined in Fig. 1. The mycelium was removed by centrifugation and the broth filtrate (185 liters) concentrated under reduced pressure at or below 45°C to approximately 3 liters. The concentrate was poured into 25 liters of methanol slowly with stirring for 30 minutes. The mixture was centrifuged and the inactive precipitate discarded. After concentrating the supernatant to 1.8 liters, it was first extracted with an equal volume of hexane followed by an equal volume of toluene and finally three extractions with an equal volume MeOH - $CHCl_3$ (1 : 9). The hexane and toluene extracts were inactive leaving most of the acti-

Fig. 1. Isolation of siderochelin A and siderochelin B.

250 liters fermentation of *Nocardia* sp. SC11,340.
centrifuge.
concentrate supernatant *in vacuo*.
3 liters of concentrate.
add to 25 liters of methanol.
centrifuge and concentrate supernatant *in vacuo*.
1.8 liters of concentrate.
extract with 1.8 liters of hexane and with 1.8 liters of toluene, discarding the organic phase.
extract antibiotics from the aqueous phase into methanol - chloroform, 1: 9.
concentrate extract and crystallize antibiotics from acetonitrile
Siderochelin A, 1.8 g
Siderochelin B, 0.1 g
Mixture of siderochelins A and B, 1.4 g

Table 1. Properties of siderochelin A and siderochelin B.

	Siderochelin A	Siderochelin B
Crystalline form	Platelets	Needles
Melting point	165~168°C	200~201°C
UV (MeOH): λ_{max} ($E^{1\%}$)	315(220)	315(220)
Mass spectrometry—mol. ion	235	235
Empirical formula	$C_{11}H_{13}N_3O_3$	$C_{11}H_{13}N_3O_3$
Elemental analysis		
Calcd. for $C_{11}H_{13}N_3O_3$	Found	Found
C=56.22 %	56.14	56.38
H= 5.58	5.44	5.60
N=17.88	17.66	17.78
Optical rotation $[\alpha]_D^{22}$	(*c* 0.30, MeOH)	(*c* 0.21, MeOH)
λ 589 nm	+1.7°	−1.9°
578	+2.3	+2.4
546	+2.3	+1.4
436	+43.9	−23.0
TLC (silica gel MeOH - $CHCl_3$, 1 : 19)	Rf=0.40	Rf=0.40

vity in the MeOH - $CHCl_3$ extract. Concentration of the combined MeOH - $CHCl_3$ extracts yielded 4.1 g of crude crystalline siderochelin. The crystals contained two diastereoisomers that could be separated on the basis of shape, into platelets (A) and needles (B). The two isomers are distinguished by proton NMR spectroscopy: siderochelin A has a C–CH_3 resonance at 1.70 ppm and siderochelin B shows a C–CH_3 resonance at 1.60 ppm. The 4.1 g of siderochelin isolated from the 250-liter fermentation contained 80% siderochelin A and 20% of siderochelin B. Separation of the isomers was carried out by fractional crystallization from acetonitrile.

Physical and Chemical Properties

Both siderochelin A and B are soluble in methanol, ethanol, ethylacetate, acetone and chloroform but insoluble in hexane and water. Their physical and chemical properties are listed in Table 1.

The UV and IR (Fig. 2) spectra for the two diastereoisomers are indistinguishable. The NMR spectra are shown in Figs. 3 and 4.

Fig. 2. IR spectrum of siderochelin A in KBr.

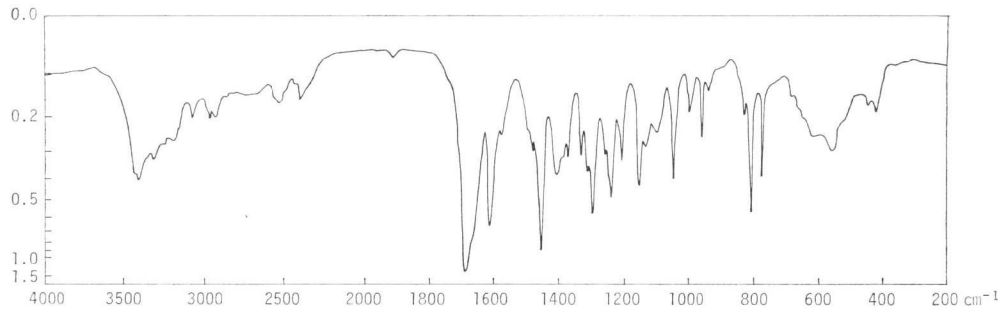


Fig. 3. ^{1}H-NMR spectrum of siderochelin A in $CDCl_3$.

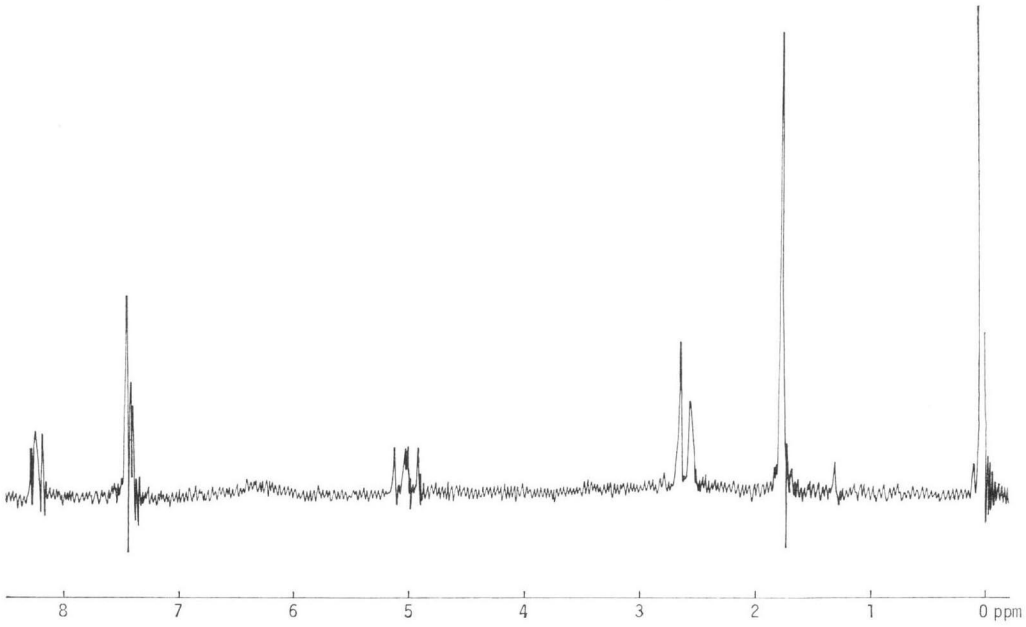
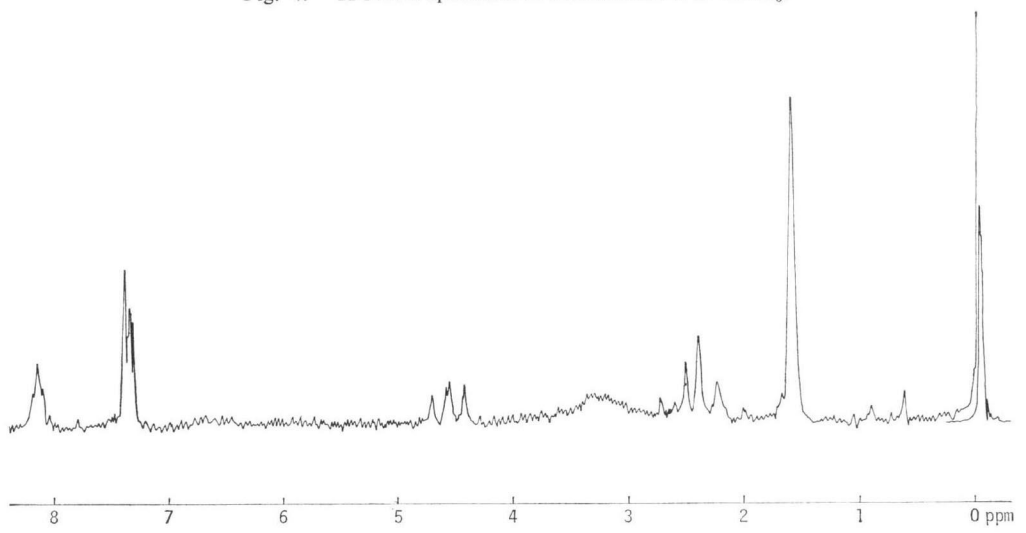
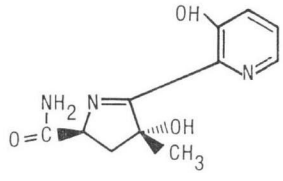


Fig. 4. ^{1}H-NMR spectrum of siderochelin B in $CDCl_3$.



Siderochelin A was shown to be (*trans*)-3,4-dihydro-4-hydroxy-5-(3-hydroxy-2-pyridinyl)-4-methyl-2*H*-pyrrole-2-carboxamide (Fig. 5) by X-ray crystallography. The spectroscopic properties of siderochelin B indicate that it is a diastereoisomer of siderochelin A. The absolute stereochemistry was not determined for either diastereoisomer.

Fig. 5. Structure of siderochelin A (relative configuration).



X-Ray Crystallography

The monoclinic unit cell parameters $a=11.739\,(2)$, $b=7.256\,(1)$, $c=29.809\,(5)$ Å, $\beta=108.65\,(2)°$ were determined diffractometrically from fifteen high angle reflections. The crystal density (1.308 g cm^{-3}) measured by flotation in hexane-carbon tetrachloride mixtures suggested the presence of eight molecules of $C_{11}H_{13}N_3O_3$ per unit cell. In view of the systematic extinctions noted on Weissenberg photographs of this chiral structure, space group C2 with two crystallographically independent molecules per asymmetric unit was assumed. This assignment was confirmed through the complete structural analysis described below.

The intensities of 1371 reflections were measured diffractometrically using the $\theta-2\theta$ variable scan rate technique (monochromatic CuKα; $\lambda=1.5418$ Å, $2\theta_{max}=115°$). Of these, the 1266 reflections with $I>2.5\,\sigma\,(I)$ were used in the solution of structure by direct methods, and subsequent full matrix least squares refinements. All coordinates (except Y of N1), and anisotropic temperature parameters for the non-hydrogen atoms were refined. Hydrogen atoms were located in difference maps at final stages

Table 2. Fractional atomic coordinates.

Atom	X	Y	Z	Atom	X	Y	Z
O13	0.3477 (2)	0.1810 (5)	0.50380 (8)	C9′	0.2621 (4)	0.4669 (7)	0.2640 (1)
O16	−0.0539 (2)	0.3309 (5)	0.37005 (8)	C10′	0.3257 (4)	0.3193 (7)	0.2875 (1)
O17	0.1784 (2)	−0.1595 (5)	0.31109 (9)	C11′	0.3474 (4)	0.1737 (7)	0.2621 (1)
O13′	0.1920 (2)	0.4367 (5)	0.00053 (9)	C15′	0.3557 (4)	0.1342 (8)	0.1128 (1)
O16′	0.1973 (3)	−0.0387 (5)	0.12868 (9)	H2	0.178	−0.047	0.457
O17′	0.1633 (3)	0.6161 (5)	0.19097 (9)	H3A	0.139	0.314	0.464
N1	0.1721 (3)	0.0091 (0)	0.3878 (1)	H3B	0.029	0.153	0.437
N12	−0.0005 (3)	0.2594 (5)	0.3878 (1)	H9	0.087	−0.115	0.223
N14	0.4034 (3)	0.0909 (6)	0.4412 (1)	H10	−0.024	0.118	0.182
N1′	0.1470 (3)	0.4314 (5)	0.1132 (1)	H11	−0.874	0.362	0.219
N12′	0.3094 (3)	0.1643 (5)	0.2147 (1)	HN14A	0.395	0.016	0.406
N14′	0.2127 (4)	0.6623 (6)	0.0537 (1)	HN14B	0.495	0.111	0.453
C2	0.1936 (3)	0.0566 (6)	0.4385 (1)	H15A	0.211	0.437	0.400
C3	0.1022 (3)	0.2095 (7)	0.4376 (1)	H15B	0.118	0.470	0.415
C4	0.0711 (3)	0.2921 (6)	0.3879 (1)	H15C	0.110	0.516	0.360
C5	0.1056 (3)	0.1340 (6)	0.3613 (1)	HO16	−0.075	0.346	0.342
C6	0.3209 (3)	0.1138 (6)	0.4637 (1)	HO17	0.214	−0.135	0.334
C7	0.0702 (3)	0.1227 (6)	0.3096 (1)	H2′	0.039	0.418	0.046
C8	0.1081 (3)	−0.0218 (6)	0.2868 (1)	H3′A	0.156	0.149	0.036
C9	0.0699 (4)	−0.0265 (7)	0.2381 (1)	H3′B	0.077	0.098	0.060
C10	−0.0003 (3)	0.1145 (7)	0.2137 (1)	H9′	0.239	0.569	0.291
C11	−0.0325 (4)	0.2535 (6)	0.2382 (1)	H10′	0.353	0.311	0.325
C15	0.1443 (4)	0.4612 (7)	0.3864 (1)	H11′	0.396	0.074	0.282
C2′	0.1137 (4)	0.3000 (7)	0.0635 (1)	HN14′A	0.211	0.691	0.085
C3′				HN14′B	0.225	0.728	0.042
C4′	0.2276 (3)	0.1343 (6)	0.1118 (1)	H15′A	0.363	0.027	0.098
C5′	0.2043 (3)	0.2983 (6)	0.1392 (1)	H15′B	0.373	0.247	0.103
C6′	0.1778 (4)	0.4959 (6)	0.0368 (1)	H15′C	0.405	0.104	0.149
C7′	0.2472 (3)	0.3124 (6)	0.1912 (1)	HO16′	0.251	−0.024	0.156
C8′	0.2228 (3)	0.4659 (6)	0.2147 (1)	HO17′	0.153	0.586	0.160

Table 3. Average bond distances (Å), angles (°) and deviations from the average of the two independent molecules.

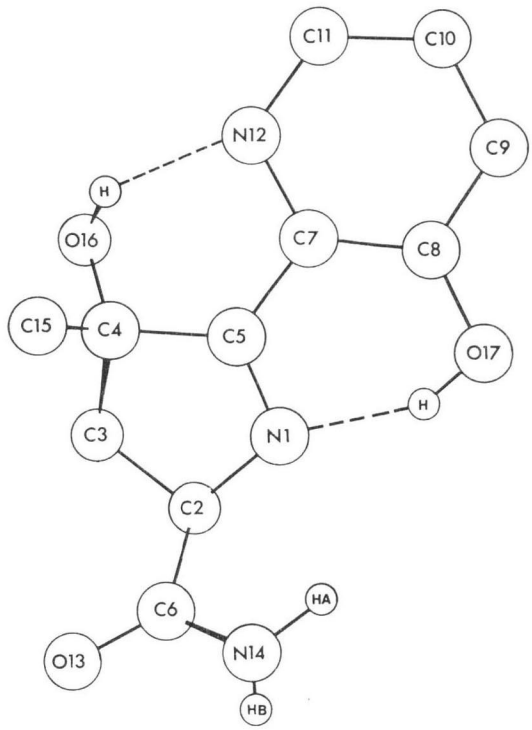
Distances

N1-C5	1.287(2)	C4-O16	1.429(10)
C5-C4	1.520(4)	C5-C7	1.468(5)
C4-C3	1.526(3)	C7-C8	1.395(3)
C3-C2	1.537(1)	C8-C9	1.385(9)
C2-N1	1.474(20)	C8-O17	1.345(4)
C2-C6	1.508(7)	C9-C10	1.367(2)
C6-N14	1.332(10)	C10-C11	1.370(1)
C6-O13	1.229(7)	C11-N12	1.336(6)
C4-C15	1.501(6)	C7-N12	1.354(7)

Angles

N1-C5-C4	114.7(2)	O13-C6-N14	122.5(5)
C5-C4-C3	100.8(1)	N1-C5-C7	121.2(0)
C5-C4-C15	110.2(1)	C4-C5-C7	124.0(1)
C5-C4-O16	112.3(6)	C5-C7-C8	121.9(1)
C15-C4-O16	110.8(1)	C5-C7-N12	116.1(4)
C3-C4-C15	113.1(4)	N12-C7-C8	112.0(3)
C3-C4-O16	109.3(6)	C7-C8-O17	121.8(2)
C2-C3-C4	102.2(2)	C7-C8-C9	118.9(1)
N1-C2-C3	105.4(4)	C9-C8-O17	119.2(3)
C2-N1-C5	109.6(2)	C8-C9-C10	118.8(0)
N1-C2-C6	112.5(4)	C9-C10-C11	119.2(0)
C3-C2-C6	113.6(8)	C10-C11-N12	124.0(4)
C2-C6-N14	117.5(7)	C11-N12-C7	117.0(6)
C2-C6-O13	120.0(1)		

Fig. 6. Crystal structure of siderochelin A.



in the analysis. They were assigned isotropic temperature factors and included, though not refined, in the final cycles of refinements. Least squares weights $w=\sigma^{-2}(F_0)$ were calculated assuming $\sigma^2(I)=\varepsilon^2+p(I)^2$ where ε is the statistical counting error and $p=0.02$.

The final R factor was 0.045 with weighted $R=0.05$. Fractional atomic coordinates and errors are given in Table 2. The structures and conformations of the two crystallographically independent molecules are identical within experimental error. The average bond distances, angles and deviations from the average values for the two molecules are given in Table 3.

The four atoms C2, N1, C5, C4 of the 1-pyrroline ring are planar within experimental error with the fifth atom, C3, displaced from this plane by 0.37 Å. The torsional angle N1–C5–C7–C8 is only 1° (3° in the other molecule) so that the planar pyridine ring and its hydroxyl substituent also essentially lie in this plane (displacement of O17 from this plane is 0.03; 0.06 Å in the other molecule). The two chelating atoms N1 and O17 in this conformation are separated by 2.615 Å. It is of interest that this conformation of siderochelin A (and presumably also siderochelin B) may also simultaneously present a secondary chelation site through interactions with N12 and O16 (*intra*molecular N—O distance= 2.842 Å). These potential ligands are joined by two *intra*molecular OH—N hydrogen bonds in the crystal structure (Fig. 6).

Table 4. Antimicrobial activity of siderochelin *vs.* aerobic bacteria.

Organism	SC Number	MIC (μg/ml)	
		Siderochelin A	Apoferrosamine
Acinetobacter calcoaceticus	8,333	50.0	25.0
Enterobacter cloacae	10,459	>100.0	100.0
Escherichia coli	10,404	100.0	100.0
Escherichia coli	10,857	50.0	50.0
Escherichia coli	10,896	25.0	25.0
Klebsiella aerogenes	10,436	100.0	100.0
Klebsiella pneumoniae	11,066	50.0	25.0
Proteus morganii	9,774	50.0	50.0
Pseudomonas aeruginosa	8,754	>100.0	100.0
Pseudomonas aeruginosa	9,330	50.0	50.0
Pseudomonas aeruginosa	9,545	25.0	25.0
Salmonella typhimurium	9,201	50.0	50.0
Serratia marcescens	9,782	100.0	50.0
Serratia marcescens	1,111	>100.0	100.0
Shigella sonnei	8,449	50.0	50.0
Staphylococcus aureus	2,400	>100.0	>100.0
Staphylococcus aureus	10,165	50.0	100.0
Staphylococcus aureus	11,239	>100.0	>100.0
Streptococcus faecalis	9,011	>100.0	>100.0

Inoculum level=10^4 CFU.
Medium=Antibiotic agar #1 (BBL).

It is of further interest that the preferred conformation of the carboxamide group in both molecules defines an approximately eclipsed orientation of the C6–N14 and C2–N1 covalent bonds, with one of the primary amide hydrogens directed *intra*molecularly toward the pyrroline nitrogen atom (N1—N14 distance = 2.727 Å). This hydrogen atom is also close to O16 of a symmetry-related neighbor (*inter*molecular N14—O16 distance = 3.004; 3.161 Å for the other molecule). The other primary amide hydrogen atoms is *inter*molecularly hydrogen bonded to O13 of a symmetry-related neighbor (N14—O13 distance = 2.940 Å; 2.998 Å for the other molecule). No hydrogen bonds connect the two crystallographically independent molecules.

Biological Properties

Siderochelin A is weakly active against a range of aerobic and anaerobic microorganisms (Tables 4 and 5) and shows a similar level of spectrum of activity to that of apoferrosamine, a ferrous-ion chelating agent produced by a strain of *Pseudomonas fluorescens*[5]. The compound appears to be less active than apoferrosamine against the dermatophytes but both compounds show activity *vs. T. vaginalis*. Siderochelin and apoferrosamine are inactive against *C. albicans* (Table 6).

Mode of Action

Iron uptake studies using *E. coli* SC 10,995 revealed a 75% inhibition of uptake of $^{55}Fe^{++}$ by siderochelin A at the MIC concentration. In addition, the activity of siderochelin A is antagonized in the presence of Fe^{++}, Fe^{+++}, Co^{++} and Zn^{++} ions. These results are highly suggestive that the mode of action of siderochelin is related to its ability to chelate metal ions. Detailed mode of action studies will be reported elsewhere.

Table 5. Antimicrobial activity of siderochelin *vs.* anaerobic bacteria.

Organism	SC Number	MIC (μg/ml)	
		Siderochelin A	Apoferrosamine
Bacteroides fragilis	9,005	50.0	25.0
Bacteroides fragilis	9,844	12.5	100.0
Bacteroides fragilis	10,277	50.0	50.0
Bacteroides fragilis	10,278	50.0	50.0
Bacteroides fragilis	10,279	50.0	50.0
Bacteroides fragilis	10,281	50.0	100.0
Bacteroides fragilis	11,085	100.0	25.0
Bacteroides fragilis	11,086	50.0	50.0
Bifidobacterium dentium	11,260	>100.0	100.0
Clostridium histolyticum	8,572	12.5	50.0
Clostridium perfringens	11,256	50.0	50.0
Clostridium septicum	1,780	12.5	50.0
Clostridium sporogenes	2,372	12.5	50.0
Eubacterium lentum	11,261	12.5	25.0
Fusobacterium necrophorum	11,338	12.5	25.0
Haemophilus vaginalis	8,568	100.0	100.0
Haemophilus vaginalis	9,640	100.0	100.0
Peptococcus variabilis	11,264	50.0	25.0
Peptostreptococcus anaerobius	11,263	50.0	12.5
Propionibacterium acnes	4,020	50.0	50.0

Inoculum level = 10^5 CFU.
Medium = MUELLER HINTON agar (Difco) + 5% whole sheep blood + 0.2% lysed blood.

Table 6. Activity of siderochelin against yeasts, fungi and protozoa.

Organism	SC Number	MIC (μg/ml)	
		Siderochelin A	Apoferrosamine
*Candida albicans**	5,314	>100	>100
*Trichophyton mentagrophytes**	2,637	>100	25
*Epidermophyton floccosum**	9,185	>100	25
*Trichophyton rubrum**	9,199	50	25
*Microsporum canis**	9,237	100	25
*Trichomonas vaginalis***	8,560	6.2	6.2

* Inoculum: 10^4 CFU; Medium: Malt extract - tryptone - glucose - yeast extract agar.
** Inoculum: 10^4 cells/ml; Medium: DIAMOND's medium.

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