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UNIVERSITY OF CALIFORNIA
Santa Barbara

Structures and Metal-Binding Characteristics of Siderophores
from the Marine Bacteria *Deleya aquamarina*,
Alteromonas luteoviolacea and a *Vibrio* Species

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in Chemistry

by Pamela Diane Holt

March, 1998

Committee in Charge:

Professor Alison Butler, Chair

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"For I know the plans I have for you, says the Lord. Plans for good and not for evil, to give you a future and a hope." Jeremiah 29:11.

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Lewis, Brent L.; Holt, Pamela D.; Taylor, Steven W.; Wilhelm, Steven W.; Trick, Charles G.; Butler, Alison; Luther, George W. III. "Voltammetric estimation of iron(III) thermodynamic stability constants for catecholate siderophores isolated from marine bacteria and cyanobacteria." *Mar. Chem.* (1995) 50:179-188.

Haygood, Margo G.; Holt, Pamela D.; Butler, Alison. "Aerobactin production by a planktonic marine *Vibrio* sp." *Limnol. Oceanogr.* (1993) 38:1091-1097.

ABSTRACT

Structures and Metal-Binding Characteristics of Siderophores from the Marine Bacteria *Deleya aquamarina*, *Alteromonas luteoviolacea* and a *Vibrio* Species

by

Pamela Diane Holt

Surface seawater iron levels of 0.1-1 nM are one to four orders of magnitude lower than the estimated bacterial growth requirement of 0.05-1 μM Fe^{3+} . Therefore, marine bacteria use specialized iron(III) transport mechanisms in order to obtain the iron(III) they require. One iron(III) uptake mechanism is the production of siderophores: low-molecular weight, strongly iron(III)-chelating organic molecules secreted by many microorganisms to solubilize and transport iron. To date, only a few marine siderophore structures have been determined. The focus of this project is the determination of the structures and metal-binding properties of marine bacterial siderophores.

The structures of four closely related tri-hydroxamate siderophores, the aquachelins, from the marine bacterium *Deleya aquamarina* have been determined using amino acid analysis and peptide sequencing by tandem mass spectrometry. NMR spectroscopy and chiral GC analysis of partially hydrolyzed peptide fragments completed the structure characterization. Aquachelins A, B, C and D consist of a heptapeptide L-threo- β -hydroxy aspartate, D-serine, L-serine, D-glutamine, D-N⁶-acetyl, N⁶-hydroxy-ornithine, D-serine, L-N⁶-acetyl, N⁶-hydroxy-ornithine amide-bonded through the N-terminus to one of four fatty acids: (Z)-5-dodecenoic acid, dodecanoic acid, (Z)-7-tetradecenoic acid or tetradecanoic acid.

The siderophore from a marine *Vibrio* species was found to be aerobactin, a citrate-dihydroxamate siderophore that is also produced by several terrestrial species. This is the first instance of the production of the same siderophore by organisms from widely disparate environments and genetic classifications.

The coordination chemistry of two previously discovered marine siderophores, alterobactins A and B, from *Alteromonas luteoviolacea* was investigated. The stability constant for iron(III) coordination to alterobactin A was estimated using a square wave voltammetric method to be 10^{49-53} . This independent electrochemical technique confirms the stability constant previously estimated by spectrophotometric competition with EDTA. Additionally, the stability constant for the complex of Fe(III) and alterobactin B was determined at pH 6 and 8.2, in the two pH-dependent coordination modes of the iron(III) complex.

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List of Abbreviations

Alt-A = Alterobactin A
Alt-B = Alterobactin B
APCI = atmospheric pressure chemical ionization
ASM = Artificial seawater medium
ASW = Artificial seawater
ATCC = American Type Culture Collection
Bis-Tris = bis(2-hydroxyethyl)iminotris-(hydroxymethyl)methane
CAA = casamino acids media
CAS = Chrome Azurol S
cat = catechol
CDTA = *trans*-1,2-cyclohexylenedinitrilotetraacetate
COSY = COrrrelation SpectroscopY
CV = cyclic voltammetry
DABITC = dimethylaminoazobenzene isothiocyanate
DABTH = dimethylaminoazobenzenethiohydantoin
DF = desferrioxamine B
DMS = dimethyl sulfide
DMSO = dimethyl sulfoxide
DQF-COSY = Double Quantum Filtered COrrrelation SpectroscopY
DSS = 4,4 dimethyl 4-silapentane sodium sulfonate
EDDA = ethylenediamine-di-(*o*-hydroxyphenylacetic acid
EDTA = ethylenediamine tetraacetic acid
EPR = electron paramagnetic resonance
ESI-MS = Electrospray ionization mass spectrometry
FAB-MS = Fast atom bombardment mass spectrometry
FAC = ferric ammonium citrate
Fur = ferric uptake regulatory protein
GC = gas chromatography
HEPES = *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid
HMBC = Heteronuclear Multiple Bond Correlation
HMDE = hanging mercury drop electrode
HMQC = Heteronuclear Multiple Quantum Correlation
HNLC = High-nitrate, low-chlorophyll
HP = Hewlett-Packard
HPLC = high-performance liquid chromatography
IR = infrared spectroscopy
iuc = iron uptake chelate
iut = iron uptake transport

LySta = 4,8-diamino-3-hydroxyoctanoic acid
Marfey's reagent = *N*- α -(2,4-dinitro-5-fluoro-phenyl)-L-alaninamide
MS = Mass spectrometry
MES = 2-*N*-(morpholino)ethanesulfonic acid
NBA = 3-nitrobenzyl alcohol
NMR = Nuclear Magnetic Resonance
NSM = Natural seawater medium
NSW = Natural seawater
PVC = polyvinyl chloride
RNA = ribonucleic acid
rRNA = ribosomal ribonucleic acid
RP-HPLC = reverse-phase high-performance liquid chromatography
SCE = Saturated calomel electrode
SWV = square wave voltammetry
TEP = transparent exopolymer particles
TFA = trifluoroacetic acid
TOCSY = T^Otal C^Orrelation S^Pectroscop^Y
UV-Vis = ultraviolet-visible

Chapter 1: Introduction

Iron is the most abundant transition metal in the earth's crust, but it is often biologically unavailable because of the low solubility of iron(III) hydroxides. Although Fe(II) is soluble, it is readily oxidized to Fe(III) by atmospheric oxygen, which then forms a complex mixture of iron(III) oxyhydroxides, or rust. The solubility product of $\text{Fe}(\text{OH})_3$ is 10^{-38} , so the calculated concentration of $\text{Fe}^{3+}(\text{aq})$ at pH 7 is 10^{-17} M.

Iron is an extremely versatile element. It is used in electron transport, oxygen transport, and a wide variety of enzymes with diverse functions. Iron is a good electron transfer agent in enzymes because of its accessible reduction potential ($\text{Fe(III)/Fe(II)} = 0.770$ V), which can be tailored by the coordination environment of an enzyme to achieve a wide range of reduction potentials (Crichton, 1991). It is found in the active site of electron transport proteins such as the cytochromes, ferredoxins and iron-sulfur proteins (2Fe-2S, and 4Fe-4S(SR)₄ are common forms of Fe-S centers) (Cotton and Wilkinson, 1988). Iron is also responsible for oxygen transport in hemoglobin and myoglobin where the oxygen binds to Fe(II)-heme (ibid.). Many other enzymes incorporate iron at the active site, such as peroxidases and catalases which are both heme-containing proteins (Lippard and Berg, 1994). The non-heme iron enzymes

ribonucleotide reductase and methane monooxygenase both contain oxygen-bridged diiron(III) centers at their active sites (Lippard and Berg, 1994). The nitrogenase system contains both an iron protein (a 4Fe-4S cluster, Georgiadis et al., 1992) and a molybdenum-iron protein (containing 1Mo:3Fe:3S and 4Fe:3S clusters, Kim and Rees, 1992) which are of great importance in biological nitrogen fixation. Consequently, iron is an essential element for practically all living organisms (Cotton and Wilkinson, 1988). Terrestrial bacteria typically require approximately 1 μ M iron for growth (Lankford, 1973).

The biological usefulness of iron is in stark contrast to its potential toxicity. Iron(II) in the presence of hydrogen peroxide forms Fenton complexes which have cytotoxic activity because they can catalytically oxidize organic substrates (Sawyer et al., 1993). In order to avoid free iron in mammalian systems, both because of the insolubility of Fe(III) and the potential for toxic reactions with Fe(II), iron is coordinated to transfer proteins, such as transferrin and lactoferrin, and excess iron is stored in ferritin (Crichton, 1982). In some bacterial strains iron is stored in a related protein known as bacterioferritin, distinguished particularly by the presence of heme (Yariv et al., 1981). The only organisms which apparently do not require iron are the lactobacilli which do not contain more than a few atoms of iron per cell. These bacteria can tolerate environments high in H₂O₂ and acid, which could potentially lead to

undesirable Fenton chemistry in the presence of iron (Archibald, 1983).

Iron in seawater: The elemental composition of the ocean is quite different in several important respects from terrestrial, or even lacustrine environments. The concentrations of transition metal ions in seawater are listed in Table 1-1. Transition metals play an important role as trace elements for microorganisms. However, iron levels are extremely low in surface seawater, (20 pM - 1 nM) (Rue and Bruland, 1995; Gordon et al., 1982; Martin et al., 1989, 1991; Landing and Bruland, 1987) and the lack of iron limits phytoplankton growth in certain areas of the ocean which otherwise contain excess nutrients (Martin and Fitzwater, 1988). High-nitrate, low-chlorophyll (HNLC) regions occur in large portions of the world's oceans, especially the Southern Ocean. When iron(III) is added to seawater samples from HNLC regions, the phytoplankton grow until they consume the available nitrate (Martin and Fitzwater, 1988).

Recently, iron addition experiments were performed in the equatorial Pacific Ocean to observe the effect on parameters such as phytoplankton growth, nutrient depletion, and utilization of carbon dioxide (IronEx I, Fall 1993: Martin et al., 1994; IronEx II, Summer 1995: Coale et al., 1996). Carbon dioxide flux in and out of the ocean has important effects on global climate change. Phytoplankton in surface ocean waters take up carbon dioxide and fix it as

Transition Metal	Concentration (nM)
Ti ^a	0.004-0.03
V	20-35
Cr	2-5
Mn	0.2-3
Fe ^b	0.02-1
Co	0.1
Ni	2-12
Cu	0.5-1.2
Zn	0.05-9
Mo	100
W	0.6
Re	0.014-0.045

Table 1-1. Concentrations of transition metal ions in surface seawater. All values taken from Bruland (1983) unless otherwise noted. ^aOrians et al. (1990); ^bRue and Bruland (1995).

organic carbon. As they are grazed by zooplankton, organic carbon detritus sinks into the deep ocean, thus effectively removing carbon from the atmosphere unless the organic material is recycled. Bacterial consumption of organic carbon is a source of CO₂ flux out of the ocean.

In the IronEx II experiment in 1995, three separate additions of ferrous sulfate were made to a 72 km² patch of ocean water over a period of a week. This strategy simulated the natural addition of iron via dust deposition. The first infusion led to an overall concentration of 2 nM iron. The two subsequent inputs each added the equivalent of 1 nM iron. Within one week of the last iron addition, the system reverted to its original state. (Coale et al., 1996).

Because of the sudden supply of iron, a massive phytoplankton bloom occurred which was correlated with a drop in surface water CO₂ levels (Cooper et al., 1996) and an increase in dimethyl sulfide (DMS) production (Turner et al., 1996). DMS has been associated with cloud formation and climate cooling. The suggestion was therefore made that iron levels in the ocean may affect global climate change by affecting both CO₂ and DMS levels in the atmosphere.

Marine bacterial growth was not measured in either of the IronEx experiments. Bottle experiments from the Antarctic in October of 1995 (Pakulski et al., 1996) support the theory that oceanic heterotrophic bacteria are iron-limited, just as the phytoplankton are. Marine bacteria must compete with

with phytoplankton for the limited quantity of available iron. Tortell and co-workers (1996) measured iron levels in marine heterotrophic bacteria in the subarctic Pacific in the fall of 1995 and reported that oceanic bacteria account for 20 to 45% of biological iron uptake. They also calculated that bacteria, which compose approximately 40% of the biogenic carbon in the open ocean, compose ~50% of the biogenic iron pool. Understanding iron uptake by marine bacteria is critical to understanding the biogeochemical iron cycle, which in turn has great impact on the carbon cycle. Hence, it is important to understand the iron uptake mechanisms of marine bacteria.

One strategy for iron uptake by bacteria is the production of iron(III) chelators, known as siderophores. Two classes of iron(III) chelators have been shown to exist in seawater (Rue and Bruland, 1995) but their identities have not yet been determined. Work in both the Pacific (Rue and Bruland, 1995) and Atlantic (Wu and Luther, 1995) Oceans measured the concentrations of iron(III) chelators using electrochemical techniques.

In the North Pacific Ocean, the total concentration of ferric binding ligands is 2 nM, which is much larger than the dissolved iron concentration in surface waters of 0.2 nM. Two classes of ligands were identified and named L_1 and L_2 . L_1 ligands have a conditional stability constant, K^{cond} , of $10^{23.08} \text{ M}^{-1}$, while for L_2 ligands, $K^{\text{cond}} = 3 \times 10^{21} \text{ M}^{-1}$, assuming 1:1 complexation in both

cases. L_1 exists in surface waters at a concentration of 0.44 nM and L_2 at 1.5 nM. The authors estimate that 99.9% of the dissolved iron(III) is complexed in the North Pacific (Rue and Bruland, 1995).

In the Atlantic Ocean, by comparison, the strong Fe(III)-binding class was found to occur at a concentration of 0.45-0.60 nM with a conditional stability constant of $10^{23.22}$ (Wu and Luther, 1995). The weaker class was not detectable by the methods used. However, in the Atlantic Ocean samples the total ferric ion concentration was close to that of the strong Fe-binding ligands, approximately 0.45-0.60 nM (Wu and Luther, 1995). In the Pacific Ocean samples, the iron concentration was approximately half that of the L_1 concentration, 0.17 - 0.27 nM (Rue and Bruland, 1995).

Additional work on IronExII seawater samples (Rue and Bruland, 1997) showed an increased concentration of both classes of ligands after the iron additions. Iron(III) binding ligands are produced rapidly in response to increased iron availability, and appear to be biologically available for uptake (Rue and Bruland, 1997). This is consistent with the hypothesis that small amounts of iron cause rapid growth and hence iron(III) chelate production by the multiplying cells.

Bacterial iron uptake: Iron availability can be critical to the virulence of

pathogenic microorganisms (Wooldridge and Williams, 1993). If organisms are unable to obtain sufficient iron from the host, then they are not able to multiply. Many mechanisms exist for bacterial iron(III) uptake from mammalian systems. Some organisms use ferritin, hemoglobin or free heme directly. Others reduce iron(III), for example from transferrin, and take it up in the ferrous form (Otto et al., 1992). Many microbes produce siderophores which compete with the host's iron carriers, and may additionally be responsible for inhibition of the host immune systems by suppression of T cell formation (Autenrieth et al., 1991).

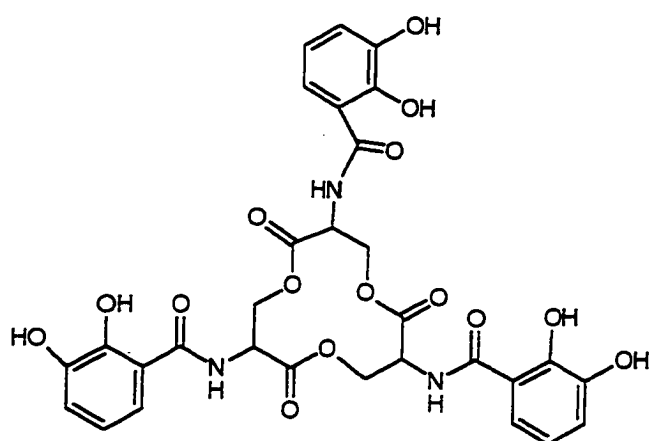
In order to supply their iron requirements, many terrestrial bacteria produce siderophores, small molecules which chelate and transport iron(III), (Raymond et al., 1984). It was hypothesized that iron uptake systems of marine bacteria, particularly free-living strains, might be different from those of terrestrial strains because of the extremely low concentration of iron in seawater. Marine bacteria may require less iron than their terrestrial counterparts or they may have extremely effective methods of obtaining iron(III) from the marine environment.

Siderophores: The term siderophore is derived from the Greek *sideros phoreue* meaning iron carrier (Lankford, 1973). Siderophores are a class of small

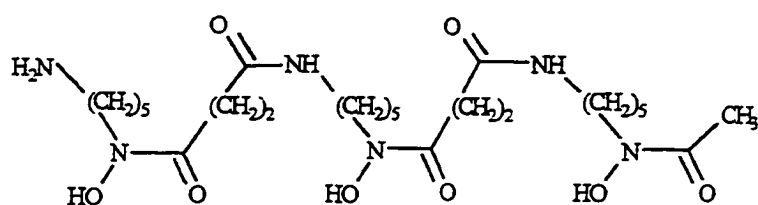
molecules designed to function as iron(III) transport agents for a variety of organisms, from bacteria to plants. Siderophores have been defined as "low molecular weight, virtually iron(III) specific ligands induced by growth of microorganisms at sub-optimal levels of iron" (Neilands, 1984). They are excreted for solubilization and transport of iron(III) into the organisms. Thus, in times of iron stress, siderophore synthesis is turned on; in times of iron surplus, siderophore production is repressed.

Siderophore structures vary widely, and include ferric coordination moieties such as catecholate, hydroxamate and α -hydroxy carboxylic acids, among others. Enterobactin, a tri-catecholate; desferrioxamine B, a tri-hydroxamate; and rhizobactin, containing an α -hydroxy carboxylic acid and two carboxylic acids; illustrate the three main classes of siderophore structures discovered to date (Figure 1-1). Usually, iron(III) is coordinated by oxygen atoms, however, in a few cases, nitrogen or sulfur coordination is also found.

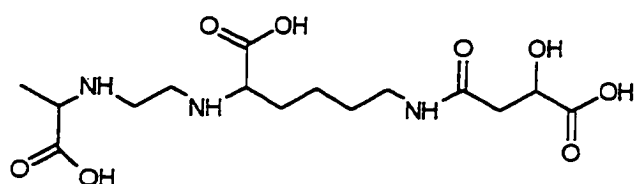
Most siderophores are hexadentate chelators which bind iron(III) stoichiometrically, forming an octahedral coordination sphere around the metal center. However, a few siderophores like bisucaberin (Takahashi et al., 1987) and rhodotorulic acid (Atkin and Neilands, 1968) are only tetradentate and bind in a 3:2 ratio of iron(III):siderophore (Carrano et al., 1979). The X-ray crystal structure of the 3:2 complex of alcaligin to Fe(III) was recently reported by



Enterobactin



Desferrioxamine B



Rhizobactin

Figure 1-1. Structures of enterobactin (O'Brien and Gibson, 1970), desferrioxamine B (Bickel et al., 1960) and rhizobactin (Smith et al., 1985) demonstrating different iron(III) coordinating ligands found in siderophores.

Raymond and co-workers (Hou et al., 1996). A 1:1 metal-ligand ratio is more entropically favorable than a 3:1 or 3:2 ratio.

Siderophores are characteristically very selective and effective chelators of ferric ions. Stability constants for iron(III)-coordination (Table 1-2) range from $10^{22.5}$ (e.g. aerobactin) (Harris et al., 1979) to 10^{49} (e.g. enterobactin) (Loomis and Raymond, 1991). Siderophores coordinate iron(II) with much lower affinity. Comparison of equilibrium constants for the reaction of $\text{Fe}^{3+}(\text{aq})$ with fully deprotonated siderophore ligands does not satisfactorily indicate the relative iron-binding strengths of these ligands, since the pK_a values for deprotonation vary widely. To get around this problem, a pM value has been defined for specific conditions ($10\ \mu\text{M}$ ligand; $1\ \mu\text{M}$ Fe(III) ; $\text{pH } 7.4$) which can be used to compare siderophore Fe(III) -binding capabilities (Table 1-2) under the same conditions. However, the pM value is defined for conditions which may not be physiologically relevant even for enteric bacteria, much less for marine bacteria which live in an environment with sub-nanomolar iron levels and a pH of approximately 8.3. Therefore, comparisons based on pM values do not indicate how well siderophores function in their natural environments.

Open-Ocean Siderophores: In this work, marine bacterial siderophores were investigated to determine their structures and their binding affinities to iron(III)

Siderophore	log K	pM
Enterobactin	49	35.6
Alterobactin A ^a	49-53	34
Coprogen	30.2	27.5
Desferrioxamine B	30.99	26.6
Ferrichrome	29.1	25.2
Aerobactin	22.5	23.3
Rhodotorulic acid	62.2 ^b	21.9

Table 1-2. Stability constants and pM values for iron(III) coordination by selected siderophores. See text for explanation. Values obtained from the review by Matzanke et al.,(1989) except as noted. ^aValues from Reid et al.(1993). ^bRhodotorulic acid forms a 3:2 metal:ligand complex with Fe(III).

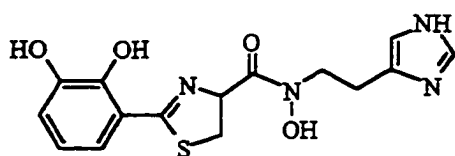
and other transition metal ions of marine importance. Siderophores may be important ligands for chelation of iron in seawater, which in turn affects the growth of other microorganisms such as phytoplankton. It is possible that siderophores are responsible for the presence of the strong ferric ion binding ligand class, L_1 . However, it is necessary to measure the conditional stability constants of marine siderophores under the same conditions in order to compare them to the natural ligands (Rue and Bruland, 1995).

Several independent researchers have shown that siderophores are produced by marine microorganisms. In an early screening experiment, 43 % of 182 bacterial isolates from the Sargasso Sea produced iron(III)-binding compounds (Gonye and Carpenter, 1974). Later, Trick (1989) used a growth assay with EDDA (ethylenediamine-di-(*o*-hydroxyphenylacetic acid)) and an iron(III) addition assay to test 30 known strains of open-ocean bacteria for siderophore production. Trick determined that hydroxamate siderophores were widely produced, but he found no catecholate siderophores in the strains he tested. However, several of the marine strains produced siderophores which were neither hydroxamate nor catecholate (Trick, 1989).

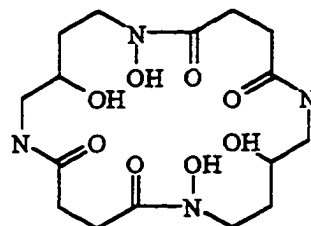
Haygood et al. (1993) used the Chrome Azurol S (CAS) assay in agar plate form (Schwyn and Neilands, 1987) to test for the production of siderophores by planktonic marine bacteria. The CAS method selects only the

subset of the microbial population that is capable of growing on the agar medium, so it is quite possible that large numbers of siderophore-producing marine bacteria were not detected. From the 40 meter samples, 82% of the colonies were positive for siderophore production. In the 100 meter seawater samples however, only 8% of the colonies developed halos which indicate the presence of siderophores.

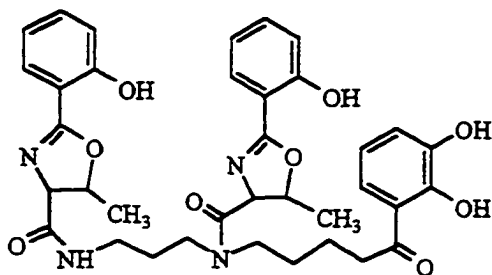
Clearly many strains of marine bacteria produce iron(III)-reactive (CAS-positive) compounds. However, only a few marine siderophores have been isolated and structurally characterized. The marine siderophores whose structures have been determined to date, including those from this work, are pictured in Figure 1-2. Bisucaberin and alcaligin were isolated from sediment-dwelling bacteria while vulnibactin and anguibactin were isolated from pathogenic bacteria, two environments which should have greater iron availability than open-ocean seawater. On the other hand, *Alteromonas luteoviolacea*, which produces alterobactins A and B, was isolated from surface seawater. In this work, aerobactin will be shown to be produced by *Vibrio* sp. DS40M5 (Chapter 4) and the aquachelin siderophore structures will be elucidated and shown to be produced by *Deleya aquamarina* strain DS40M3 (Chapter 2). DS40M3 and DS40M5 were both isolated from the same area of open-ocean surface seawater north of the Cape Verde islands off the coast of



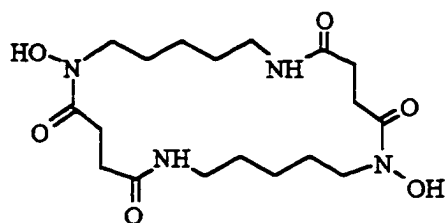
Anguibactin



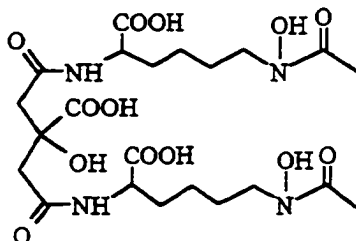
Alcaligin



Vulnibactin



Bisucaberin



Aerobactin

Figure 1-2. Structures of known marine siderophores: anguibactin from *Vibrio anguillarum* (Jalal et al., 1989); alcaligin from *Alcaligenes xylosoxidans* subsp. *xylosoxidans* (Nishio and Ishida, 1990); vulnibactin from *V. vulnificus* (Okujo et al., 1994); bisucaberin from *Alteromonas haloplanktis* (Takahashi et al., 1987); aerobactin from a *Vibrio* sp. strain DS40M5 (Chapter 4 of this work).

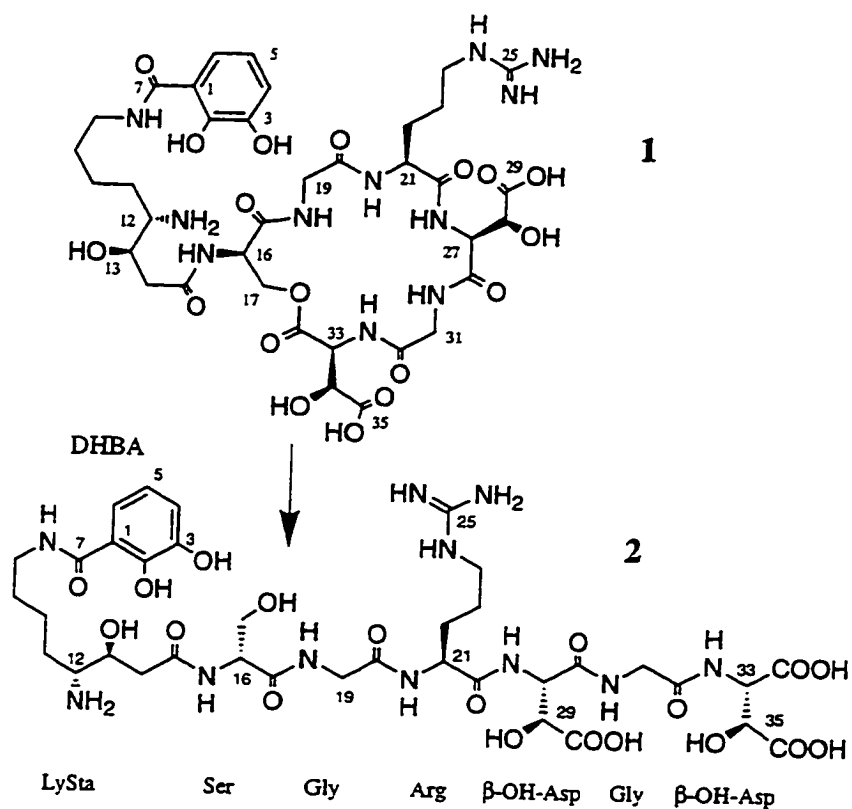


Figure 1-2. (cont.) Alterobactins A (1) and B (2) from *A. luteoviolacea* (Reid et al., 1993). LySta stands for lysine-statine (4,8-diamino-3-hydroxyoctanoic acid) after Salituro et al. (1987) and DHBA stands for 2,3-dihydroxybenzoic acid.

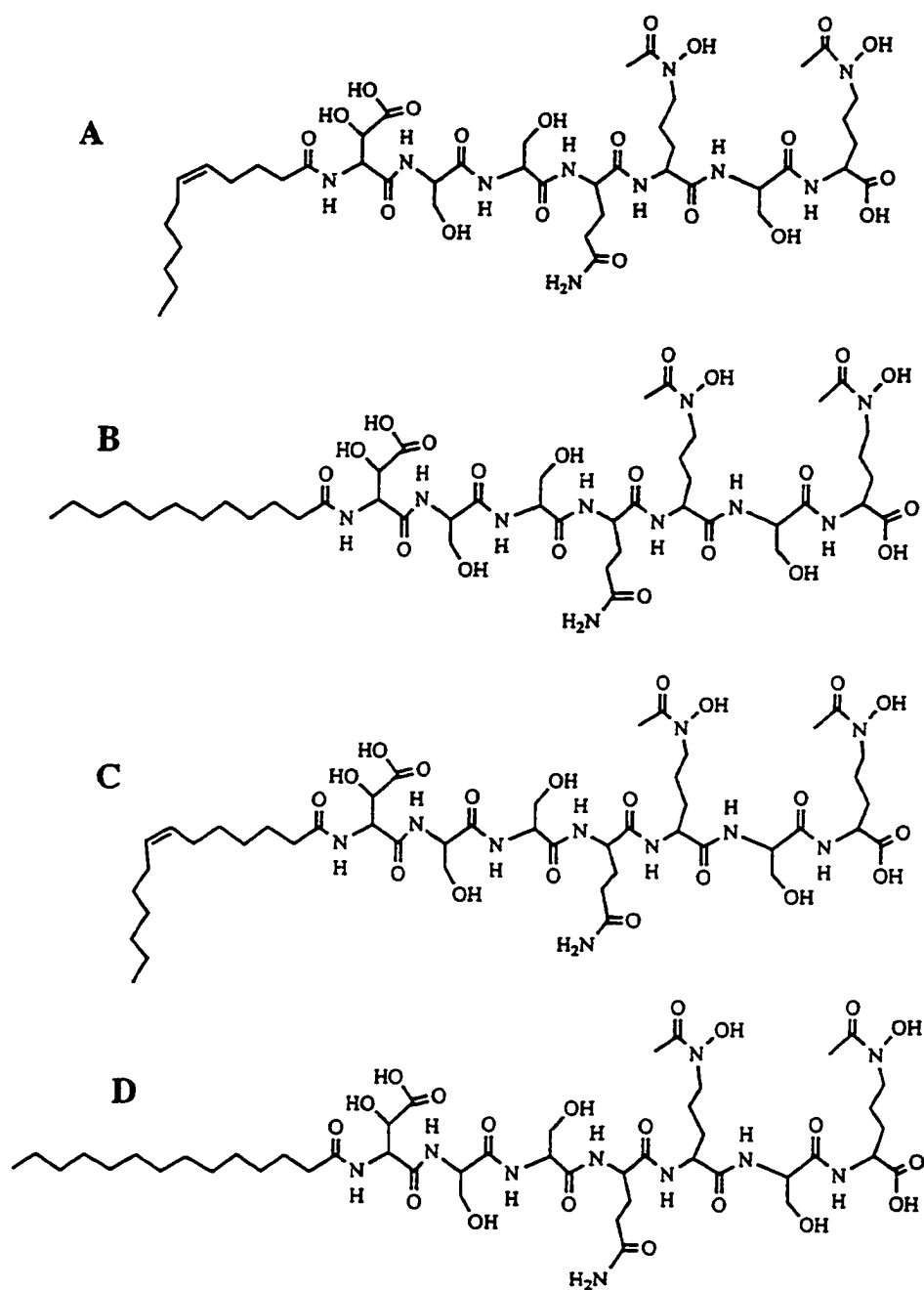
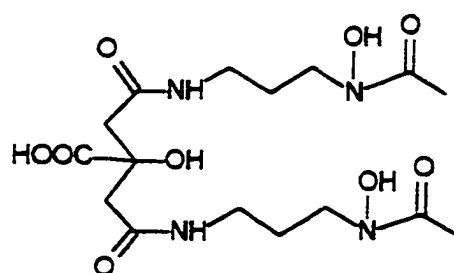


Figure 1-2. (cont.) Aquachelins A-D from *Deleya aquamarina* strain DS40M3
(Structure identification will be described in Chapter 2).

West Africa.

Marine cyanobacteria (blue-green algae) have also been shown to produce siderophores. Schizokinen (Figure 1-3), isolated from fresh-water algae of the genus *Anabaena*, is the only structurally characterized siderophore from a cyanobacterium identified to date (Simpson and Neilands, 1976). However, both catechol and hydroxamate compounds have been identified in cyanobacterial culture supernatants and many strains appear to produce more than one CAS-reactive compound (Wilhelm and Trick, 1994). Clearly, more work remains to be done on algal siderophore production.

Medical applications: Iron in mammals is distributed in a wide variety of enzymes, and storage and transport proteins such as ferritin, transferrin and lactoferrin (Martinez et al., 1990). Levels of iron in the human body are regulated in order to prevent infection by adjusting the concentrations of iron storage proteins (Weinberg, 1984). Excess iron in tissues has been correlated with diseases ranging from heart disease (Salonen et al., 1992) to cancer (Weinberg, 1984) and therefore maintaining a proper iron balance is critical. Iron accumulates in the brain during the course of some diseases such as Parkinson's, Huntington's and Alzheimer's (Ward et al., 1995). Iron(III) in the brain is expected to be involved in reactions with dopamine, a critical brain



Schizokinen

Figure 1-3. Schizokinen from cyanobacteria of the genus *Anabaena* (Simpson and Neilands, 1976)

compound which contains catechol moieties (Ward et al., 1995).

Siderophores are used as drugs to chelate and remove excess iron from patients with iron-overload diseases. As strong, selective iron(III) chelators, siderophores are obvious potential drugs for iron removal. In fact, the mesylate salt of desferrioxamine B (Figure 1-1) is marketed as Desferal™ by Ciba-Geigy, as part of the treatment of Cooley's anemia and other diseases whose treatment ironically leads to iron overload (Martell et al., 1981). Other compounds have since been synthesized and tested in an on-going effort to produce improved drugs for iron overload. Siderophore structures provide a natural starting point for the development of synthetic iron(III) chelators.

In a recent study, the Fe(III) complexes of desferrioxamine B and diethylenetriaminepentaacetic acid were shown to protect mice against septic shock (Kazmierski et al., 1996). The mechanism was proposed to be catalytic reduction of nitric oxide to N₂O. In this case, the iron(III) complexes of the chelators are the effective agents, not the free ligands.

Also, siderophore-antibiotic conjugates are being developed as a strategy to fight bacterial pathogens (Miller and Malouin, 1993). One strategy to combat bacterial pathogens is to exploit siderophore uptake proteins as receptors for antibiotics. Natural iron(III)-binding antibiotics such as the albomycins (Benz et al., 1982) were an initial clue leading to the development of synthetic

conjugates. Making antibiotics that are recognized by siderophore receptor proteins and taken into the cell as though they were siderophores provides some protection from bacteria that develop antibiotic resistance. The siderophore uptake system is so necessary for cell survival that the receptor needs to function in spite of the antibiotic treatment. Although mutations take place that allow the bacteria to survive in culture in the presence of the antibiotic by shutting down the receptors, the mutated strains do not multiply *in vivo*. In the animal, the mutation is presumably reversed, which restores sensitivity to the siderophore antibiotic (Miller and Malouin, 1993).

In other work, siderophores have been shown to be effective against the malaria parasite. The parasite takes up iron(III) from infected red blood cells. Synthetic lipophilic siderophore analogs were used to successfully inhibit parasite multiplication *in vitro*, and the method has promise for therapeutic treatment of malaria (Lytton, et al., 1994).

Research summary: In this dissertation, the structure determination of the aquachelins, four new marine siderophores, will be described in Chapter 2. Also, a known terrestrial siderophore, aerobactin, will be shown to be produced by a marine bacterial strain in Chapter 4. In addition, the coordination chemistry of two previously discovered marine siderophores, alterobactins A and

B, is investigated further in Chapter 3.

The marine bacterial strain DS40M3 will be shown to produce a series of four siderophores, aquachelins A, B, C and D (Chapter 2). Isolate DS40M3 will be demonstrated to be a member of the species *Deleya aquamarina*. The siderophores produced by *Deleya aquamarina* strain DS40M3 consist of a heptapeptide L-threo- β -hydroxy aspartate, D-serine, L-serine, D-glutamine, D-N⁶-acetyl, N⁶-hydroxy-ornithine, D-serine, L-N⁶-acetyl, N⁶-hydroxy-ornithine amide-bonded through the N-terminus to one of four fatty acids: (Z)-5-dodecenoic acid, dodecanoic acid, (Z)-7-tetradecenoic acid or tetradecanoic acid.

Aerobactin, a known terrestrial siderophore, will be demonstrated to be produced by from the marine bacteria *Vibrio* spp. DS40M5 (Chapter 4). This is the first instance of the production of the same siderophore by organisms from widely disparate environments and genetic classifications. A subsequent report by Okujo and Yamamoto (1994) identified the production of aerobactin by two species of *Vibrio*, *V. hollisae* and *V. mimicus*, both of which are human pathogens of clinical interest.

The ferric stability constants of both alterobactin A and alterobactin B (Figure 1-2) determined by electrochemistry will be reported in Chapter 3. The results confirm the stability constant reported earlier (Reid et al., 1993). Vanadium(V) interactions with the alterobactins were also investigated and

preliminary results will be described in Chapter 3.

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Chapter 2: Siderophores from *Deleya aquamarina* strain DS40M3

Introduction

Siderophores often contain modified amino acids such as N^{δ} -acetyl, N^{δ} -hydroxy-ornithine in the ferrichromes (Neilands, 1984); N^{δ} -acetyl, N^{δ} -hydroxy-lysine in aerobactin (see Chapter 4); or β -hydroxy aspartate in the alterobactins (see Chapter 3). These modified amino acids frequently serve as ligands to iron(III). In addition to modified amino acids, both D and L amino acids are often found in bacterial siderophores, which has been proposed to be a strategy to avoid peptidase digestion (Teintze et al., 1981), since most peptidases only attack peptides with L amino acids at the C terminus, and react slowly if there are D amino acids next to the C terminus (Lipscomb, 1970).

For structure determination of siderophore peptides, both amino acid sequence and chirality are important. Typical peptide analysis techniques such as Edman degradation are often not applicable for determination of the modified amino acids, so other methods are required to determine the amino acid sequence. Sometimes long-range NMR techniques such as the HMBC (Heteronuclear Multiple Bond Correlation) experiment can provide sequence data across the peptide bonds (Griesinger et al., 1994). Alternatively, mass spectrometry, which requires much less material than NMR, can be used to

sequence small peptides (Papayannopoulos, 1995).

In recent years, the mass spectrometry of biological molecules has come of age with the advent of techniques such as electrospray ionization. Electrospray is a "soft" ionization technique which enables large, non-volatile molecules such as proteins and oligonucleotides to be placed in the gas phase without fragmentation. It also often creates ions with multiple charges such that their mass to charge ratios can be determined with a benchtop quadrupole mass spectrometer. Large proteins and nucleic acids have been analyzed and their molecular weights determined by considering the envelope of charged species created in the experiment (Fenn et al., 1989).

For small molecules such as the siderophores, tandem mass spectrometry (MS/MS) gives most or all of the fragments needed to piece together the primary sequence. In the MS/MS experiment, a single ion is selected from an initial mass spectrum and dissociated further by collisional activation with an inert gas such as nitrogen. The selected parent ion thus gives rise to a mass spectrum of daughter ions.

Under low energy collision conditions, peptide bond cleavage products dominate the spectrum, along with water, ammonia and carbon monoxide losses from those ions. Peptides are typically cleaved at the amide bond to give fragments from both the N-terminus and the C-terminus. Fragments cleaving

from the N-terminus are labelled B fragments. Peptide fragments cleaved from the C-terminus are labelled Y fragments and pick up two extra protons to maintain the charge on the free amine nitrogen (Roepstorff, 1984). If two amide bonds are broken, internal fragments can also be generated. Another common result of the low-energy collision MS/MS experiment is the production of immonium ions ($\text{HN}=\text{CH-R}$) from the constituent amino acids (Papayannopoulos, 1995).

Once the amino acid sequence of a particular peptide has been elucidated, the stereochemistry of each residue must be determined. Chiral amino acid analysis can be performed in different ways. Two common methods are HPLC analysis using Marfey's chiral reagent, *N*- α -(2,4-dinitro-5-fluorophenyl)-L-alaninamide, (Marfey, 1984) and GC analysis using a chiral column such as Chirasil-Val (Demange et al., 1988).

If a single amino acid occurs in both D and L forms in the molecule, special efforts must be made to assign the correct chirality for each residue. Bacteria are known to incorporate both D and L amino acids into siderophores (Neilands, 1984). Partial hydrolysis of the siderophore can generate useful fragments which contain only a few amino acids. These fragments can then be derivatized, separated by HPLC, hydrolysed and individually analyzed by chiral amino acid analysis so that the stereochemical sequence of the entire peptide can

be resolved (Demange et al., 1990).

This chapter describes the isolation and structure determination of a series of four siderophores produced by marine bacterial strain DS40M3. This open-ocean isolate was shown to belong to the species *Deleya aquamarina* by Margo Haygood of Scripps Institution of Oceanography, University of California, San Diego (see Discussion section).

Experimental

Siderophore isolation: Bacterial strain DS40M3 (obtained from Margo Haygood of Scripps Institution of Oceanography) was maintained on natural seawater agar plates containing 15 g/L agar, 5 g/L Bacto peptone and 0.5 g/L yeast extract, a modification of Zobell's medium (Zobell, 1941). Single colonies were streaked as lawns and inoculated after 24 hours of growth into batch cultures consisting of 2 liters of artificial seawater media in 4 L Erlenmeyer flasks. Artificial seawater (ASW) contained 15.5 g/L NaCl, 0.75 g/L KCl, 12.35 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 2.9 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. The salts were dissolved individually and filtered through Chelex membranes (BioRad), then brought up to volume with Nanopure water. Artificial seawater medium (ASM) was made from ASW with 2 g/L casamino acids (Difco), 1 g/L NH_4Cl and 0.1 g/L glycerophosphate (Haygood and Nealson, 1985).

On some occasions a sodium chloride medium was used (without Chelex filtration) which still produced good quantities of the siderophores. The NaCl media contained 15 g/L NaCl, 100 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 4 g/L casamino acids, 2 g/L NH_4Cl , 0.2 g/L glycerophosphate and 1 g/L KH_2PO_4 , pH adjusted to 7 with NaOH.

Growth of DS40M3 with added L-ornithine - Ornithine medium contained 5 mM L-ornithine (Stephan et al., 1993) added to a medium consisting of 15 g/L NaCl, 0.2 g/L $\text{MgSO}_4 \cdot \text{H}_2\text{O}$, 5 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 4 g/L casamino acids, 2 g/L NH_4Cl , and 2.5 g/L K_2HPO_4 , adjusted to pH 7.5 with NaOH. Succinate medium contained 15 g/L NaCl, 0.2 g/L $\text{MgSO}_4 \cdot \text{H}_2\text{O}$, 4 g/L succinic acid, 1 g/L $(\text{NH}_4)_2\text{SO}_4$, and 2.5 g/L K_2HPO_4 , adjusted to pH 7.5.

Production of siderophores - The chrome azurol S (CAS) assay was used to measure siderophore production (Schwyn and Neilands, 1987). Samples of culture media were obtained via sterile pipet and analyzed for cell density by measuring the optical density at 600 nm (Brock et al., 1994). The compounds of interest were found in the supernatant, so the sample was centrifuged and mixed 1:1 (v/v) with CAS assay solution and allowed to react for 30 minutes. The presence of siderophores was determined by the CAS response measured as a decrease in absorbance at 620 nm. Culture flasks were shaken on a rotary shaker (New Brunswick) at 120-130 rpm for approximately 3 days or until the

CAS response of the supernatant was maximal.

Purification of siderophores - The culture medium was centrifuged (8000 rpm, 10 min, GS-3 (Sorvall) rotor) and the supernatant was decanted from the cell pellet. The supernatant was shaken with approximately 150 g of Amberlite XAD-2 (Aldrich) at 110 rpm for one hour to adsorb organic compounds. The loaded XAD-2 resin was poured into a 2.5 x 40 cm column and washed sequentially with one column volume of Nanopure water, 50% methanol and 100% methanol. The DS40M3 siderophores eluted from XAD-2 with 100% methanol, although a small amount was also eluted with 50% methanol. This procedure separated the siderophores from the majority of the other compounds which eluted from XAD-2 in the 50% methanol step.

CAS reactive fractions (10:1 CAS:eluent) from the XAD-2 column were dried and redissolved in water with 0.1% trifluoroacetic acid (TFA) (Solvent A) for HPLC purification. The high-performance liquid chromatography system (Waters) consisted of two 510 solvent pumps, a Rheodyne injector, and a photodiode array 996 detector or a 486 variable-wavelength detector. A semi-prep Vydac C-4 column (10 x 250 mm) was employed to separate the four siderophore fractions and to remove any remaining contaminants. The original HPLC program began with 5 minutes at 100% A, followed by a linear gradient to 100% B (80% CH₃CN, 20% H₂O, 0.1% TFA) in 90 minutes. The

siderophores initially eluted as four fractions with retention times between 47 and 56 minutes and were given the common names M3-47, M3-50, M3-53 and M3-56. The fractions were later designated aquachelins A, B, C and D, respectively. A second HPLC separation on the same column used a gradient from 100% A to 60% B in 30 minutes for final purification of each individual siderophore. The CAS-reactive fractions from the HPLC separation were dried in a Speedvac and stored in a vacuum desiccator.

Growth with added Fe(III): *D. aquamarina* DS40M3 was grown in cultures with various amounts of added iron(III) to determine the effect of iron(III) concentration on siderophore production. Ferric ammonium citrate (FAC) stock solution was made at 6 mg/mL. It was sequentially diluted 1:10 five times to generate stocks for addition to each flask. One milliliter of each diluted iron(III) solution was sterile filtered with 0.22 μ m syringe filters (Nalgene) into 6 separate 4 L flasks containing 2 L of ASM. Final concentrations of FAC ranged from 0.3 ng/mL to 3 μ g/mL. The ASM was inoculated with *D. aquamarina* DS40M3 from 24 hour lawns. Cultures were assayed twice daily for cell density and CAS reactivity as described above for a total of 6 days.

Qualitative chemical analyses: The siderophores were tested for the presence

of catecholate and hydroxamate functional groups. The presence of catechol in the siderophores was evaluated according to Arnow (1937) using 3,5 di-*tert*-butyl catechol as a standard. Blanks were run on distilled water. Approximately 0.5 mg of each siderophore was dissolved in 1 mL of water. One milliliter of 0.5 M HCl, 1 mL of nitrite/ molybdate reagent (100 g/L NaNO₂; 100 g/L NaMoO₄), 1 mL of 1 M NaOH and 1 mL of water were added sequentially to each sample. The presence of a red color ($\lambda_{\text{max}} = 510 \text{ nm}$) in the final mixture indicates the presence of catechol (Arnow, 1937).

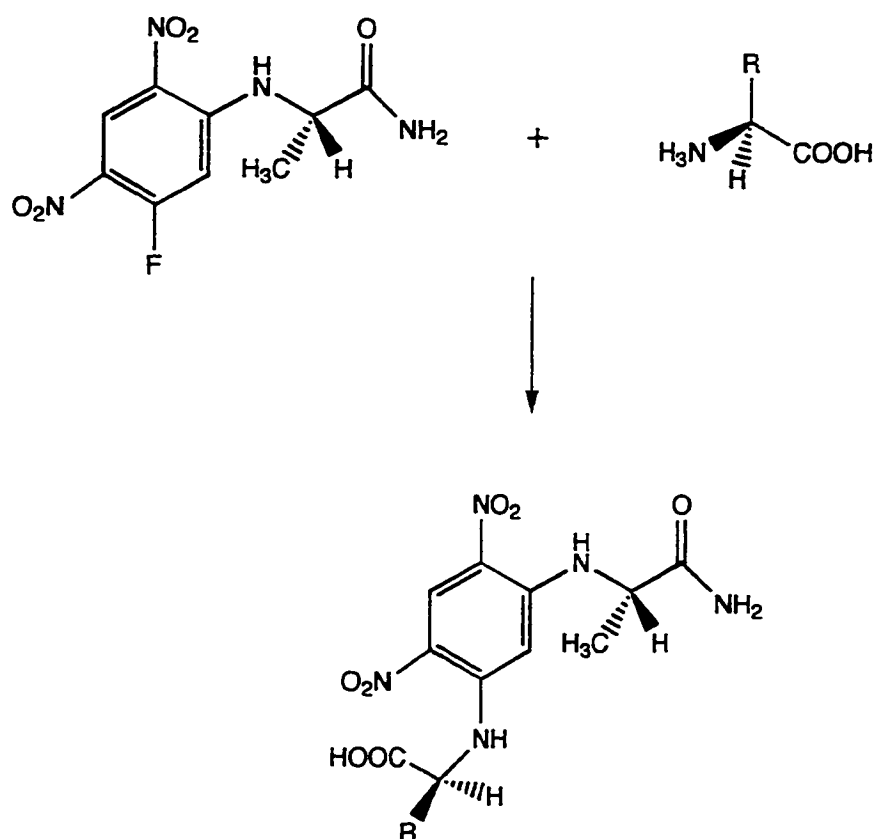
The Csaky test was used as a qualitative test for the presence of the hydroxamate functional group according to the procedure of Gillam et al. (1981). Desferrioxamine B (Desferal™, Ciba-Geigy) was used as a positive standard for hydroxamate. One milligram samples were placed in 15 mL screw-cap vials and dissolved in 3 mL H₂O, then 1 mL 12 M HCl was added. The resulting 3 M HCl solution was capped and heated at 100 °C overnight to release bound hydroxylamine. Two milliliter aliquots of each solution were placed in 50 mL volumetric flasks, and 10 mL of 2 M sodium acetate was added, bringing the pH to 5.5. Then, 2 mL of 1% sulfanilamide in 30% acetic acid and 2 mL I₂ (0.65% I₂ in 1% KI in H₂O) were added and the mixture was allowed to react for 5 minutes. Excess iodine was reduced by reaction with 2 mL 1.5% NaAsO₃. Then, 2 mL of 0.05% *N*-(1-naphthyl)ethylenediamine was

added and the solution incubated for 30 minutes for color development. Finally, the solutions were brought up to 50 mL in the volumetric flasks, and the absorbance was measured at 543 nm.

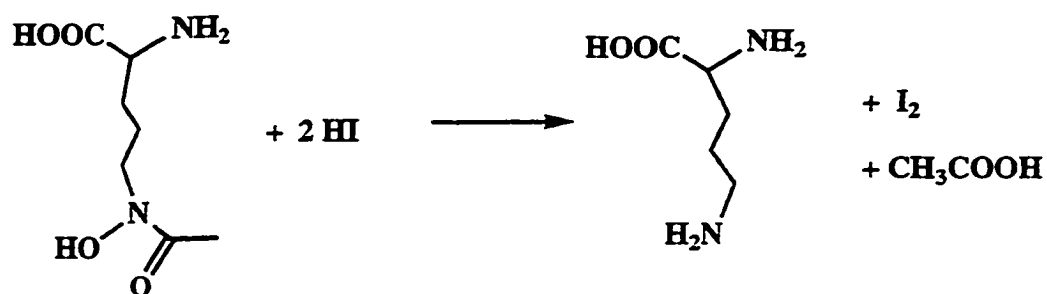
HPLC amino acid analysis: In order to identify the stereoisomers of the amino acids present, chiral amino acid analysis of the aquachelins was performed using Marfey's reagent (*N*- α -(2,4-dinitro-5-fluoro-phenyl)-L-alaninamide; Scheme 2-1) (Marfey, 1984). Aquachelin samples (~0.5 mg each) were hydrolyzed at 90-110 °C for 12-24 hours in 6 M hydrochloric acid or 50% hydriodic acid. Hydrolysis with hydriodic acid was used to reduce *N*⁶-hydroxy, *N*⁶-acetyl-ornithine to ornithine (Emery and Neilands, 1961; Scheme 2-2). The hydrolysates were then derivatized by Marfey's procedure as described below.

Excess acid was removed under vacuum and the samples repeatedly resuspended in water and dried to remove as much acid as possible. The dried material was resuspended in 100 μ L water and 200 μ L of 1.0% Marfey's reagent in acetone was added. Forty microliters of 1 M NaHCO₃ were added to make the solution slightly alkaline. The HI hydrolysed samples were adjusted with dilute NaOH to pH ~8. Then the samples were heated at 40 °C for one hour, cooled, and neutralized with 20 μ L 2 M HCl.

Derivatized amino acids were separated by C-18 analytical HPLC (Vydac



Scheme 2-1. Reaction of Marfey's reagent, *N*-α-(2,4-dinitro-5-fluoro-phenyl)-L-alaninamide with an amino acid to form a diastereomer (Marfey, 1984).

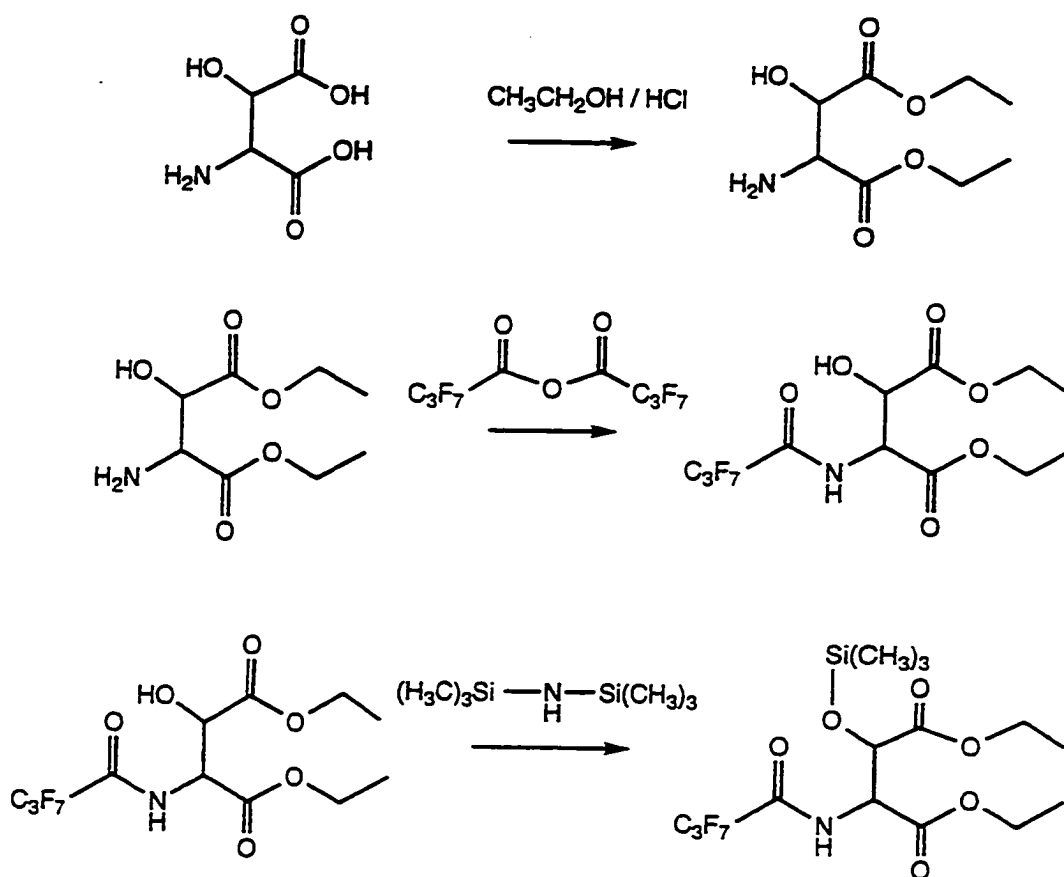


Scheme 2-2. Hydrolysis of *N*⁶-hydroxy, *N*⁶-acetyl-ornithine by hydriodic acid.

or YMC ODS-AQ, 4.6 x 250 mm) using a gradient from 10% B (100% CH₃CN) to 40% B in 45 minutes. Solvent A was 50 mM triethylamine phosphate, pH 3.0. The flow rate was 0.8 mL/min and the absorbance was monitored at 340 nm. Derivatized L and D,L amino acid standards (Sigma) were analyzed to identify the retention times of each isomer. Standard additions to the hydrolyzed siderophore samples verified the identity of the amino acids present. To verify the presence of *L-threo*- β -hydroxy aspartate, a sample of alterobactin B was hydrolyzed and derivatized, then co-injected with a sample of aquachelin C.

Chiral GC amino acid analysis: Samples of the aquachelins were also hydrolyzed, derivatized (Scheme 2-3) and analyzed by Chirasil-Val gas chromatography according to Demange et al. (1988) to determine the retention times of each of the amino acids. Approximately 0.5 mg of each siderophore was hydrolyzed overnight at 110 °C in 6 M HCl in a sealed ampoule under argon. After hydrolysis, the ampoules were opened and their contents transferred to 1.5 mL screw-cap vials with teflon cap liners. The samples were dried under a gentle stream of nitrogen or argon with mild heating by sand baths in heating mantles set at approximately 40-60 °C.

Ethyl esters: Acetyl chloride (Aldrich) was added slowly to 200 proof



Scheme 2-3. Derivatization scheme for GC amino acid analysis (Demange et al., 1988) using β -hydroxy aspartate as a model amino acid.

ethanol (1:3 v/v) in an ice bath under a gentle stream of argon in order to make 5 M HCl in ethanol. Ethyl esters were made by adding 200 μ L of the 5 M HCl in ethanol to each sample, sealing the vials, and reacting the mixture at 90 °C for 30 min in the oven. The samples were cooled and dried as above.

Pentafluoropropionyl esters: The ethyl esters were dissolved in 100 μ L of pentafluoropropionic anhydride (Aldrich) and incubated at room temperature for 30 min., then dried as above.

Trimethylsilylation - The pentafluoropropionic ethyl esters were incubated at room temperature in 200 μ L methanol for 30 minutes and the methanol was removed as before. The samples were then reacted with 100 μ L of hexamethyldisilazane at 80 °C for 30 minutes to form trimethylsilyl derivatives of hydroxyl groups, such as those on β -hydroxy aspartate and serine. Excess reagents were removed under a stream of nitrogen or argon as described above.

GC conditions - The gas chromatograph (Hewlett Packard, Model 5890) was fitted with a splitless inlet and a Chirasil-Val column (Alltech Associates) for chiral separations. The injector and flame ionization detector (FID) were heated to 250 °C (Reid, 1993). The carrier gas was H₂ at 20 mL/min. The temperature gradient began at 80 °C, held constant for 3 min., then increased to 200 °C at 5 °C/ min.

The derivatized samples were dissolved in 20-100 μ L of toluene and 0.2-

2 μ L was injected (Demange et al., 1988).

Mass spectrometry: FAB-MS was performed by the Southern California Mass Spectrometry facility at the University of California, Riverside. Exact mass determination was completed in 1993 for a sample of aquachelin C on a ZAB instrument in a matrix of 3-nitrobenzyl alcohol (NBA). Another exact mass determination using the same instrument and matrix was obtained in 1997 of the sodium adduct of aquachelin B.

Electrospray mass spectrometry was performed using a Micromass Platform II single quadrupole mass analyzer in the Chemistry Department at UCSB. Both standard electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) sources were used. The liquid chromatography system attached to the MS probe inlet was a Michrome Ultrafast Microprotein Analyzer with a reverse phase cartridge trap to absorb, concentrate and wash samples. The samples were loaded and washed with 98% H₂O, 2% CH₃CN and eluted with 80% B (A = 0.1% TFA/ 2% CH₃CN/ 98% H₂O; B = 0.1% TFA/ 90% CH₃CN/ 10% H₂O) at 50 μ L/min. All samples were run in positive ion mode. Mass ranges covered from 200 to 1500 m/z and the cone voltage was typically set to 45 V, except when deliberately increased to 90 or 110 V to encourage fragmentation. Approximately 1 μ M solutions (0.5 - 1 mL each) of

each siderophore and their respective iron(III) complexes were prepared for infusion. Aquachelin C was also complexed with gallium chloride and aluminum nitrate to prepare the respective metal complexes which were purified by C-4 HPLC prior to ESI-MS analysis. One set of siderophore samples was analyzed by APCI (at 200 $\mu\text{L}/\text{min}$) to encourage fragmentation and therefore locate the fatty acid fragments.

Tandem Mass Spectrometry: Tandem mass spectrometric analysis of each of the aquachelins was performed in 1996 by Brian Arbogast at Oregon State University on a Perkin Elmer API III Sciex triple quadrupole mass spectrometer. Parameters included orifice plate voltage = 80 V, ion spray voltage = 4700 V. Argon was the collision gas and the collision energy was 35 eV. Samples ($\sim 1 \mu\text{M}$) were introduced via the attached LC through a salt trap which was washed with 2% acetic acid in water. The compounds were eluted with 65% $\text{CH}_3\text{CN}/ 35\% \text{H}_2\text{O}/ 0.2\% \text{HCOOH}$ at 25 $\mu\text{L}/ \text{min}$.

Microderivatizations: In order to determine the number of carboxylic acid, hydroxyl and amine groups present in each aquachelin molecule, small-scale ($\sim 0.1 \text{ mg}$) derivatizations of the siderophores and their iron(III) complexes were performed according to Sharman and co-workers (1995) and analyzed by

ESI-MS.

Approximately 0.5 mg of each siderophore was freshly purified by C-18 analytical HPLC using the short gradient in the siderophore isolation section above. The CAS-reactive eluent was divided into 3 test tubes and dried, thus preparing derivatization substrates. In addition, iron(III) was added to another 0.5 mg of each siderophore in order to make the iron(III) complexes which were also divided into three test tubes and dried for derivatization. Methyl esters of the carboxylic acids were prepared by incubation of a sample of each siderophore for 24 hours in 50 μ L of methanol acidified with HCl (1 drop of concentrated HCl in 10 mL methanol). A mild acetylation intended to acetylate amine groups (Sharman et al., 1995) was done by addition of 50 μ L of 1:1 acetic anhydride/ 0.1 M ammonium acetate (pH 8) to a sample of each siderophore, followed by 2 hours of ultrasound. A more rigorous acetylation to derivatize both amines and hydroxyls (Sharman et al., 1995) was accomplished by adding 50 μ L of 2:1 v/v pyridine/ acetic anhydride to each siderophore and incubating for 4 hours at room temperature. Reagents from each derivatization reaction were removed *in vacuo* and the samples were analyzed by ESI-MS as described above.

Deuterated samples - To measure the number of exchangeable protons, deuterated samples were prepared by incubation in D₂O, and then injected via

a 50 μ L loop into the ESI-MS system.

NMR: NMR experiments were performed using several different instruments. One dimensional ^1H and ^{13}C spectra in D_2O were done using a GN 500 MHz (General Electric) instrument. ^1H spectra in d^6 -DMSO were run on a Gemini 200 MHz instrument. ^1H NMR standard parameters include a pulse width of 30 μsec , acquisition delay of 1.5 sec and block size of 16K points. ^{13}C NMR standard parameters include a pulse width of 8 μsec , acquisition delay of 1 sec and block size of 16K points. Two-dimensional Double-Quantum Filtered COrrrelation SpectroscopY (DQF-COSY), TOtal Correlation SpectroscopY (TOCSY), and inverse-detected ^1H - ^{13}C Heteronuclear Multiple Quantum Correlation (HMQC) experiments with $J = 150$ Hz for short-range and $J = 4$ Hz for long-range multiple bond detection, were performed on 10 mg of aquachelin A in 80% H_2O / 20% D_2O by Dr. Janet Cheetham of Amgen Inc. (Newbury Park, CA) using a Bruker 500 MHz instrument in the College of Engineering at UCSB. The same suite of experiments, except replacing the $J = 4$ Hz HMQC with a Heteronuclear Multiple Bond Correlation experiment (HMBC), was performed on a 7.5 mg sample of aquachelin C in d^6 -DMSO by Carole Bewley at Scripps Institute of Oceanography in collaboration with Professor John Faulkner on a Varian 500 MHz instrument. Standard instrument

software and parameters were employed for all experiments. Parameters for the set of experiments from Scripps were not sent with the spectra.

Stereochemical sequence determination: To determine the positions of the two D-serines, the L-serine and the D- and L-ornithines, chiral amino acid analysis of peptide fragments was carried out. Various attempts to determine the sequence are described below.

Growth of DS40M3 with d₃-L-serine and d₆-L-ornithine - If the D and L amino acids are synthesized independently, then feeding the bacteria with labelled L amino acids would incorporate the label only in specific positions. Then, tandem mass spectrometry could be used to determine the stereochemical sequence by sequencing the labelled peptide. Deuterium labelled 3,3,4,4,5,5-D₆-ornithine·HCl and 2,3,3-D₃-serine were obtained from Cambridge Isotope Labs. Solutions of 50 mg d₆-ornithine in 5 mL H₂O and 200 mg d₃-serine in 10 mL H₂O were sterile filtered into 500 mL flasks containing 250 mL of NaCl media. The cultures were inoculated with a 24 hour bacteria lawn and shaken at 220 rpm for 3-4 days. The siderophores were isolated as described above, except the XAD-2 resin was treated batch-wise with 50% methanol and then 100% methanol. The isolated siderophores were separated in one pass on an analytical C-4 HPLC column (Vydac, 4.6 x 250 mm) and the separated

compounds were analyzed by ESI-MS.

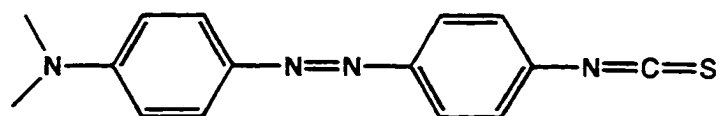
Partial hydrolysis in dilute HCl and HI - Samples of aquachelin C (0.5 mg) were placed in 1 M or 0.1 M HCl in ampoules at 110 °C overnight to partially hydrolyze the peptide and release the C-terminal amino acid. The hydrolyzed samples were dried and subjected to Marfey's amino acid analysis as described above. Both HI and HCl cleave peptide bonds, but HI also removes the N-OH functional group from *N*^δ-hydroxy, *N*^δ-acetyl-ornithines (Scheme 2-2).

Partial hydrolysis for peptide separation - Small peptide fragments were generated from the original molecules by partial hydrolysis according to Demange et al. (1990) in order to isolate peptides containing only one serine or one ornithine and thus determine the stereochemical sequence. Samples of aquachelin B and aquachelin C (~0.5 mg each) were dissolved in water and 50 or 100 µL aliquots, respectively, were placed in ampoules. An equivalent volume of 12 M HCl was added to make 6 M HCl and the ampoules were sealed under argon. In order to determine the optimal hydrolysis time, the ampoules were placed in the oven at 90 °C for 5 to 30 minutes. At five minute intervals, an ampoule was removed from the oven and the partially hydrolysed sample was transferred to a 1.5 mL microcentrifuge tube and bubbled with argon or nitrogen for an hour to remove HCl. The samples were then dried in

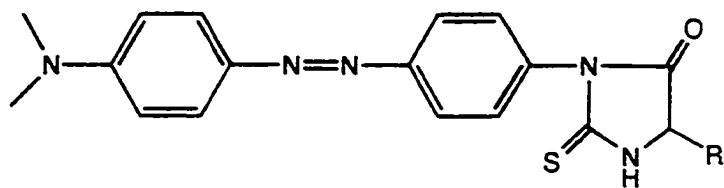
a Speedvac (Savant). Each sample was dissolved in 100 μ L 2% CH₃CN/ 98% H₂O and 5 or 10 μ L of each sample was injected into the electrospray mass spectrometer for analysis.

For full-scale analysis of peptide fragments, five 1 mg samples of aquachelin C and five 2 mg samples of aquachelin B were hydrolyzed as described above for 20 minutes at 90 °C. After acid removal and drying *in vacuo*, each sample was derivatized as described below.

DABITC peptide derivatization - The hydrolyzed peptide mixtures were derivatized according to Chang (1981a) using dimethylaminoazobenzene isothiocyanate (DABITC) (Scheme 2-4). The dried peptide mixtures were dissolved in 100 μ L of water, placed in 1.5 mL microcentrifuge tubes, and 600 μ L DABITC solution (30 mg in 3 mL of sequencing grade pyridine (Fluka)) was added to each tube. The mixtures were vortex mixed, then heated at 70 °C for 1-2 hours in the microcentrifuge tube heating block. Then the samples were extracted 10 times with 1 mL aliquots of 2:1 (v/v) heptane: ethyl acetate. After each aliquot of solvent was added, the samples were vortex mixed, centrifuged and the organic layer removed with a 1 mL syringe. After the samples were extracted 5 times, 200 μ L of water was added and they were extracted 5 additional times until the organic layer was essentially colorless, indicating removal of excess DABITC. The aqueous layers were frozen at -80 °C and



DABITC



DABTH derivative

Scheme 2-4. Structure of DABITC, dimethylaminoazobenzene isothiocyanate, and the DABTH, dimethylaminoazobenzenethiohydantoin, derivative formed by acid hydrolysis of DABITC derivatized peptides (Chang, 1981a).

lyophilized overnight.

HPLC separation of DABITC peptides - Derivatized peptide fractions were separated on a reverse phase C-18 (YMC ODS-AQ) HPLC column (4.6 x 250 mm) using 10 mM $\text{NH}_4\text{CH}_3\text{CO}_2$, pH 5 as solvent A and CH_3CN as solvent B. For the aquachelin C samples, the gradient program used a linear gradient from 20% B to 75% B in 15 minutes at 1 mL/ min. For aquachelin B peptides, the gradient was from 20% B to 50% B in 30 minutes, then to 75% B by 45 minutes at 1 mL/ min. The variable-wavelength detector (Waters) was set to measure the absorbance at 436 nm. The lyophilized samples were dissolved in 50 μL of 50% CH_3CN / 50% H_2O , centrifuged to remove particulates, then injected. Fractions were collected every 0.2 min from 12 to 32 minutes. Combined fractions were dried in the SpeedVac (Savant).

The combined DABITC peptide fractions were re-purified if the peaks on the initial separation were overlapped. The re-purification employed the same HPLC conditions as above, using gradients modified to spread out the appropriate portion of the chromatogram as follows. Early eluting fractions were purified using a gradient from 20 to 50% B in 30 minutes. Late-eluting fractions were re-purified with a gradient from 20 to 50% B in 15 minutes, then to 75% B by 30 minutes.

Chiral amino acid analysis of DABITC peptides - The collected DABITC

peptide samples were redissolved in 100 μ L of water and transferred to ampoules. After adding 100 μ L of 12 M HCl to each ampoule, the contents were bubbled with argon for 1 minute, then the ampoules were sealed and placed in an oven at 110 $^{\circ}$ C for 24 hours. [Note that this completely destroys the N-terminal amino acid because it rearranges to form a dimethylaminoazobenzenethiohydantoin (DABTH) amino acid (Scheme 2-4) which is not stable under prolonged hydrolysis conditions (Chang, 1981b).] After hydrolysis, the samples were derivatized and analyzed by Chirasil-Val gas chromatography as described above.

Ozonolysis: Double bonds are cleaved cleanly by ozonolysis followed by reduction to aldehydes. Analysis of the aldehyde products identifies the original location of unsaturation. Samples of 4 mg each of aquachelin A and aquachelin C in 4:1 methanol: CH_2Cl_2 were reacted with O_3 at -78°C until a blue color persisted. Excess ozone was removed by bubbling with N_2 . Approximately three equivalents of dimethyl sulfide or one equivalent of triphenyl phosphine were added to cleave the ozonide and produce aldehydes. The solution was stirred at -78°C for 10-20 minutes, then allowed to warm to room temperature while stirring for one hour. The samples were extracted with petroleum ether/water or pentane/water. The organic layers were analyzed by GC/MS

while the aqueous layers were analyzed by electrospray mass spectrometry. A sample of aquachelin B was also analyzed as a control, since it contains a saturated C₁₂ fatty acid.

GC/MS conditions - The organic extracts from ozonolysis of aquachelins A and C (and aquachelin B as a control), containing the cleaved aldehyde products, were analyzed by Paul Mollard, a graduate student in Dr. B.H. Lipshutz' group at UCSB, using a Hewlett-Packard 5972 mass selective detector coupled to an HP 5890 series II gas chromatograph fitted with an HP-5 capillary column (5% cross-linked phenylmethylsilicone, 30 m x 0.25 mm i.d.). Helium was used as the carrier gas. The temperature gradient started at 30 °C for 5 minutes, followed by a ramp at 20 °C/min to 280 °C.

UV-Visible spectrophotometry: Spectra were obtained on a Kontron Uvikon 860 or a Varian Cary 3E. Fe(ClO₄)₆·6H₂O was used to prepare stock solutions of 24.4, 11.2, or 1.1 mM iron(III) in 10mM HClO₄. Stock solutions were standardized against ferrozine (Stookey, 1970). See Chapter 3 Experimental section for details.

Spectra of the iron(III) complexes of aquachelin B and aquachelin C were obtained at different pH values between 1.5 and 8. Ten milliliter samples of 0.1 mM ferric aquachelin were made and then 500 µL samples were removed, pH

adjusted to the desired value by addition of 10 mM KOH, 1 M HCl or 0.1 M HCl, then the volume of each sample was made up to 1 mL with water and the spectrum obtained. The samples were referenced against water in a matched blackened cuvette.

Siderophores from ATCC strains: Based on the 16S ribosomal RNA characterization (see Discussion), the type strains of *Deleya aquamarina* (ATCC #14400) and *Halomonas meridiana* (ATCC #49692) were purchased from the American Type Culture Collection (ATCC). Batch cultures were grown as described above to test siderophore production. Siderophores were isolated as described for the deuterium-labelled amino acid growth experiments with *D. aquamarina* DS40M3 and analyzed by electrospray mass spectrometry.

Results and Interpretation

Deleya aquamarina strain DS40M3 (see Discussion section for bacterial characterization) produces four siderophores designated aquachelins A, B, C, and D. The compounds are white powders which are somewhat soluble in water and alcohols, but quite soluble in methanol/water mixtures. Aqueous solutions of the aquachelins produce bubbles and foam when agitated. Upon addition of iron(III) the solutions turn orange and the iron complexes may

precipitate as dark red solids. The red precipitates are soluble in base, and dissolve to form yellow solutions.

Siderophore isolation: The aquachelins are relatively hydrophobic compounds and do not elute from XAD-2 in the 50% methanol fraction where the majority of the organic compounds which adsorb to XAD-2 are found. Once 100% methanol is applied as the eluent, the siderophores elute cleanly. Thus, use of XAD-2 resin separates the aquachelins from salts which do not stick to the resin and the majority of the other organic compounds in the media. Separation of the siderophores by C-4 HPLC (Figure 2-1) elutes four CAS-reactive fractions which have been designated aquachelins A, B, C, and D, respectively (see below). HPLC retention times for metal complexes of the aquachelins are about 2 minutes shorter than for the free ligands.

Siderophore yields from ASM cultures range from 0.2 to 5 mg/L of pure material: approximately 0.2 mg/L of both aquachelins A and D, 2 mg/L of aquachelin B and 5 mg/L of aquachelin C. The yield was dramatically increased the first time cultures were grown in ornithine medium (see Experimental section). One 2 L culture yielded 35 mg of aquachelin C. However, this result is not reproducible, and there must be other factors such as flask treatment which influence the yield. Ornithine addition has some effect

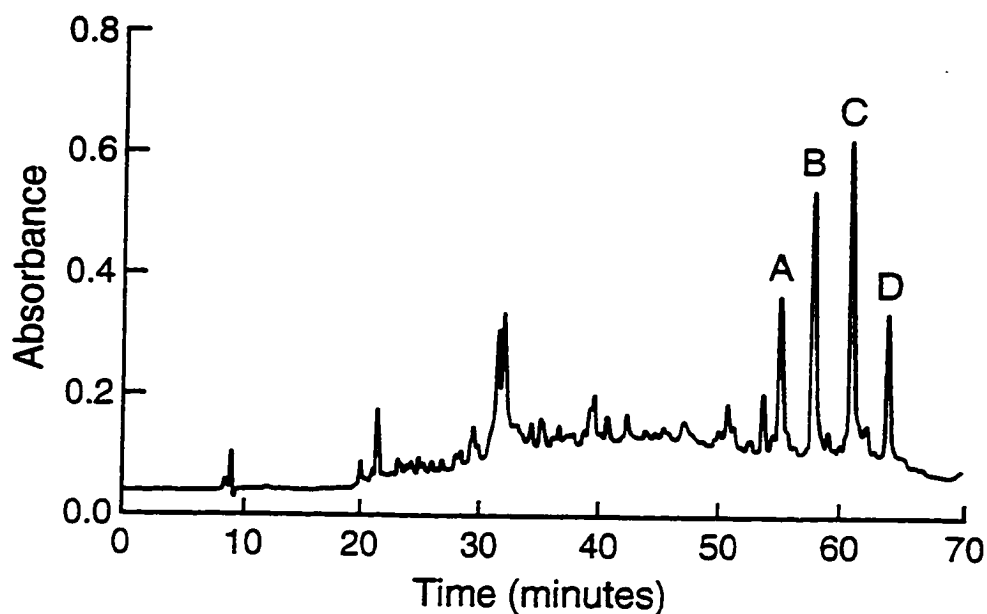


Figure 2-1. HPLC spectrum showing the separation of aquachelins A-D after their initial isolation using an Amberlite XAD-2 column. A preparative Vydac C-4 RP-HPLC column (24 x 250 mm) was used with a gradient starting with 5 minutes at 100% A (0.1% TFA), followed by a linear gradient to 60% B (80% CH₃CN, 20% H₂O, 0.1% TFA) in 60 minutes. The eluent was monitored at 230 nm. The siderophores elute as four fractions (A-D) with retention times between 55 and 65 minutes.

on siderophore yields, but not as great as the initial result indicated.

In the presence of 0.3 $\mu\text{g/mL}$ or more ferric ammonium citrate (FAC) (Figure 2-2), no aquachelin production is observed. The CAS absorbance at 620 nm drops when siderophore is present because iron(III) is removed from the Fe(III)-CAS dye complex. Shut-down of aquachelin production in the presence of iron(III) indicates inhibition of the siderophore production system by Fe(III).

The cultures grow to much denser levels in the presence of additional iron(III) (Figure 2-3), consistent with the hypothesis that iron(III) is a limiting nutrient in these cultures. Cell pellets isolated from the two flasks with the highest concentration of iron(III) appear reddish in color, which may indicate the presence of bacterioferritin (Yariv et al., 1981). Cell pellets from the other cultures are white or cream-colored.

Qualitative chemical tests: The aquachelins are negative for catechol according to the Arnow assay. No color is observed in the aquachelin samples, while the controls are distinctly red. All of the aquachelins test positive for hydroxamate by the Csaky assay, giving a pink color that is visibly distinct from the pale pink of the water blank. The blank absorbance was 0.049 at 543 nm compared to 0.074 to 0.158 for the aquachelin samples. The absorbance depends on the quantity used for the assay. The desferrioxamine B control gave an absorbance

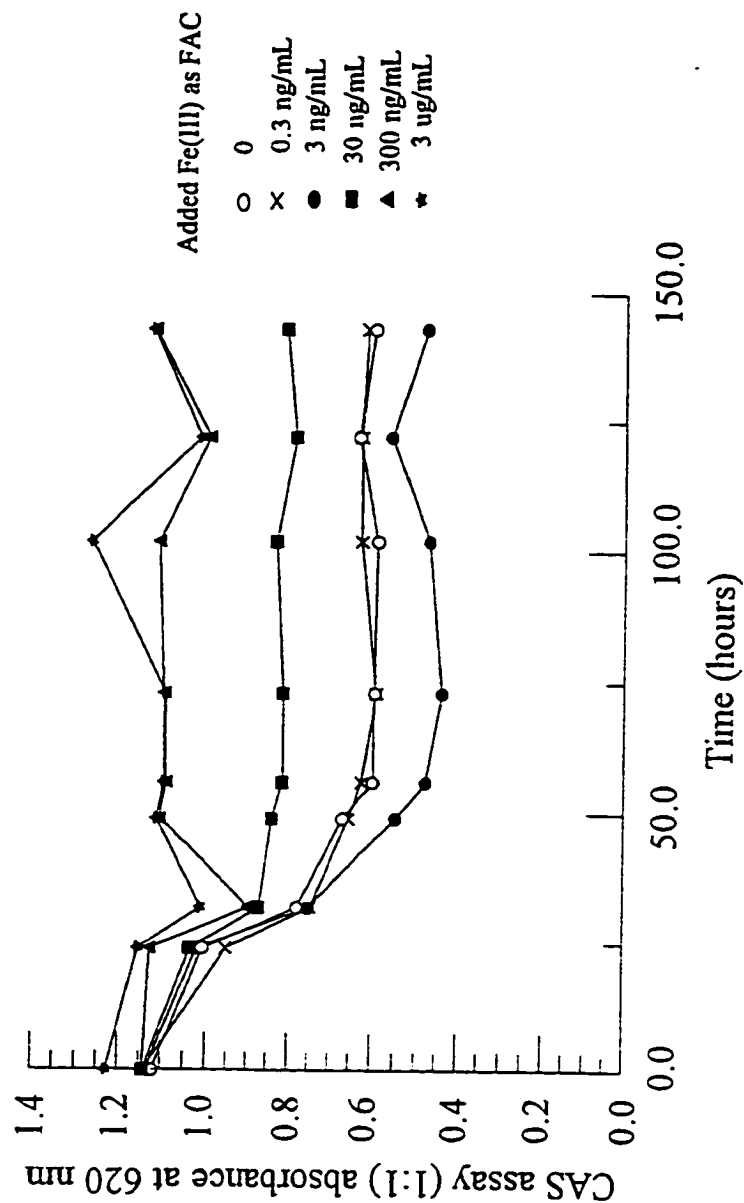


Figure 2-2. Siderophore production measured by CAS assay response from the supernatant of cultures of *Deleya aquamarina* strain DS40M3 in ASM with various concentrations of added Fe(III) as ferric ammonium citrate from 0.3 ng/mL to 3 μ g/mL FAC.

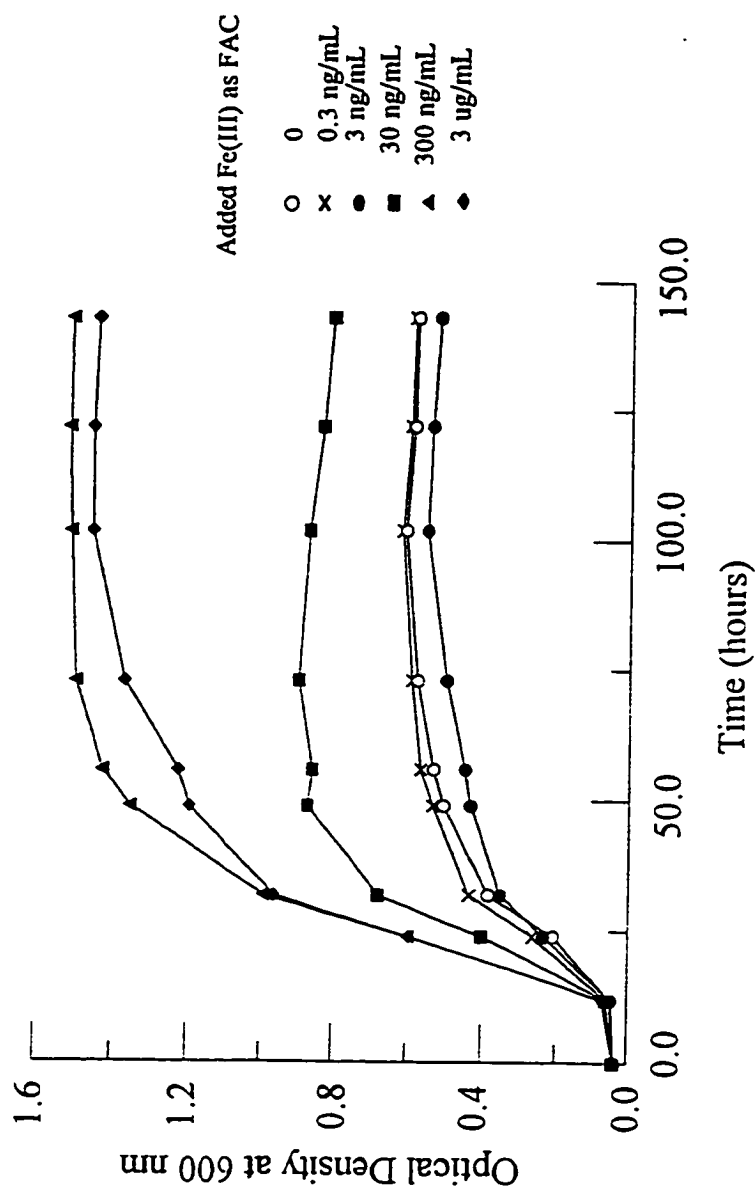


Figure 2-3. Growth of cultures of *Deleya aquamarina* strain DS40M3 in ASM with various concentrations of added Fe(III) as ferric ammonium citrate from 0.3 ng/mL to 3 µg/mL FAC.

of 0.107 at 543 nm.

Amino acid analysis: Two D-serines, one L-serine, one D-glutamic acid (arising from D-glutamine, see below), one each of D- and L-ornithine and one L-threo- β -hydroxy aspartate were identified by HPLC amino acid analysis with Marfey's (*N*- α -(2,4-dinitro-5-fluoro-phenyl)-L-alaninamide) derivatization. The identity of each peak was confirmed by co-elution with its corresponding derivatized amino acid standard (Sigma). The retention times and their assignments are given in Table 2-1. HCl hydrolysis gives essentially the same HPLC chromatogram for each siderophore. Figure 2-4 shows the chromatogram from amino acid analysis of aquachelin C from the Millenium system. See Appendix figures A-1 to A-4 for the chromatograms of all four aquachelins run on a Waters Baseline HPLC system, producing the retention times reported in Table 2-1.

Hydriodic acid hydrolysis confirms the presence of *N*-hydroxy-ornithine, which supplies two hydroxamates to the molecules. Both D and L isomers of ornithine are present (Figure 2-5; Table 2-1). HI hydrolysis is required in order to see the ornithine peaks because of the need to reduce the hydroxylated side-chain amine (Scheme 2-2). Each isomer of ornithine gives rise to 3 peaks (Figures 2-5, A-5 and A-6): one peak for Marfey's reagent attached to the

Aquachelin Figure	A, HCl A-1	B, HCl A-2	C, HCl A-3	D, HCl A-4	B, HI A-5	C, HI A-6
L-threo-β-OH-aspartic acid	19.68	19.64	19.38	19.72	20.62	20.68
L-ornithine	-	-	-	-	21.47	21.54
D-ornithine	-	-	-	-	22.08	22.18
D,L-orn, N ^δ -M ^a	-	-	-	-	25.88	26.05
L-serine	25.21	25.21	24.88	25.27	26.08	26.22
D-serine	26.63	26.63	26.33	26.68	27.71	27.88
D-glutamic acid ^b	31.76	31.80	31.46	31.81	32.64	32.83
Marfey's reagent	34.88	34.88	34.52	34.98	36.18	36.32
L-ornithine, 2M ^c	42.78	42.90	42.61	-	43.34	43.43
D-ornithine, 2M ^c	45.63	45.74	45.42	-	46.31	46.38

Table 2-1. HPLC amino acid analysis retention times (minutes) for aquachelins A-D after HCl hydrolysis, and aquachelins B and C after HI hydrolysis. Samples (5-10 μL each) were injected onto an analytical C-18 column (Vydac, 4.6 x 250 mm) using a gradient from 10% B (100% CH₃CN) to 40% B in 45 minutes. Solvent A was 50 mM triethylamine phosphate, pH 3.0. The flow rate was 0.8 mL/min and the absorbance was monitored at 340 nm. Differences in retention times are attributed to the samples being run on different days. With the exception of small peaks identifiable as the doubly derivatized ornithines, the ornithines do not appear in the HCl hydrolyzed samples. ^aThe ornithine residues derivatized at the N^δ position only, which gives one peak for both isomers. ^bGlutamic acid is observed because glutamine is converted to glutamic acid during hydrolysis. ^c2M refers to the doubly derivatized ornithines.

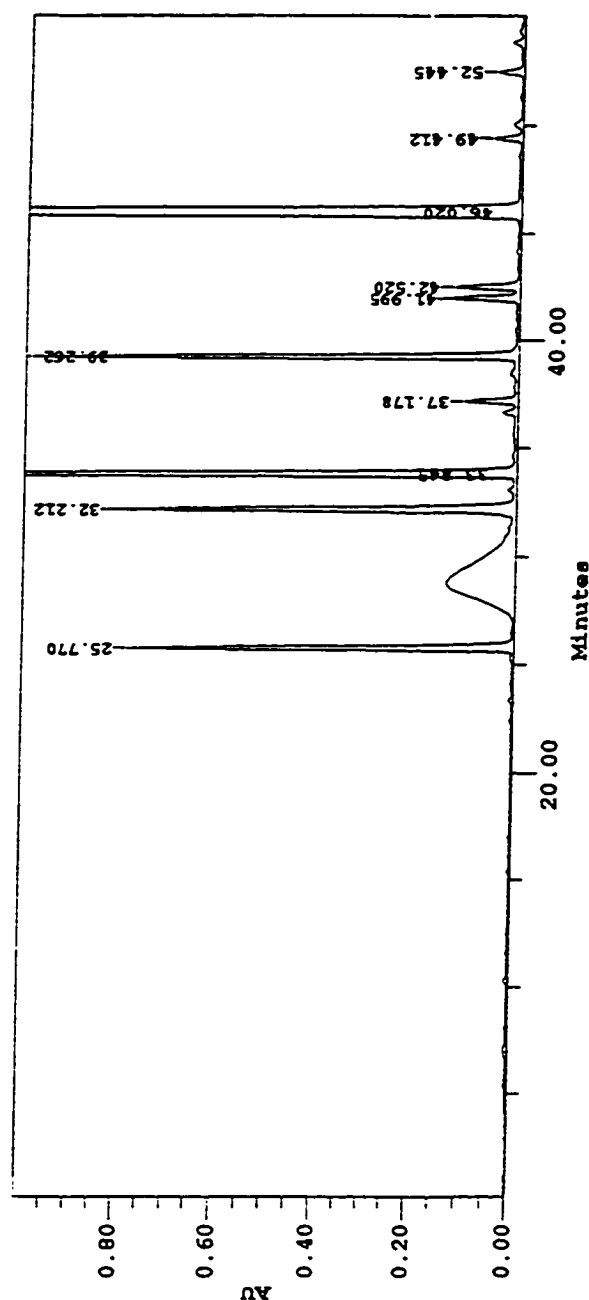


Figure 2-4. Amino acid analysis of 6 M HCl hydrolyzed aquachelin C with Marfey's derivatization. Derivatized amino acids (5 μ L samples) were separated by C-18 analytical HPLC (Waters Millennium system) (Vydac or YMC ODS-AQ, 4.6 x 150 mm) using a gradient from 90% 50 mM triethylamine phosphate, pH 3.0 (solvent A), 10% CH₃CN (solvent B) to 40% B in 45 minutes at a flow rate of 0.8 mL/min. The absorbance was monitored at 340 nm.

Retention times are assigned as follows: 25.770 min = *L-threo*- β -hydroxy aspartate; 32.212 min = *L*-serine; 33.845 min = *D*-serine; 39.262 min = *D*-glutamic acid (arising from *D*-glutamine, see text); 46.020 min = excess Marfey's reagent. Small peaks which match the doubly derivatized *L*- and *D*-ornithines appear at 49.412 and 52.445 minutes, respectively, although the singly derivatized peaks are not visible without HPLC hydrolysis (Figure 2-5). The other small peaks are unknown contaminants.

Appendix Figures A-1 to A-4 show the chromatograms of all four aquachelins obtained on a Waters Baseline system. The retention times in Table 2-1 are derived from the appendix figures.

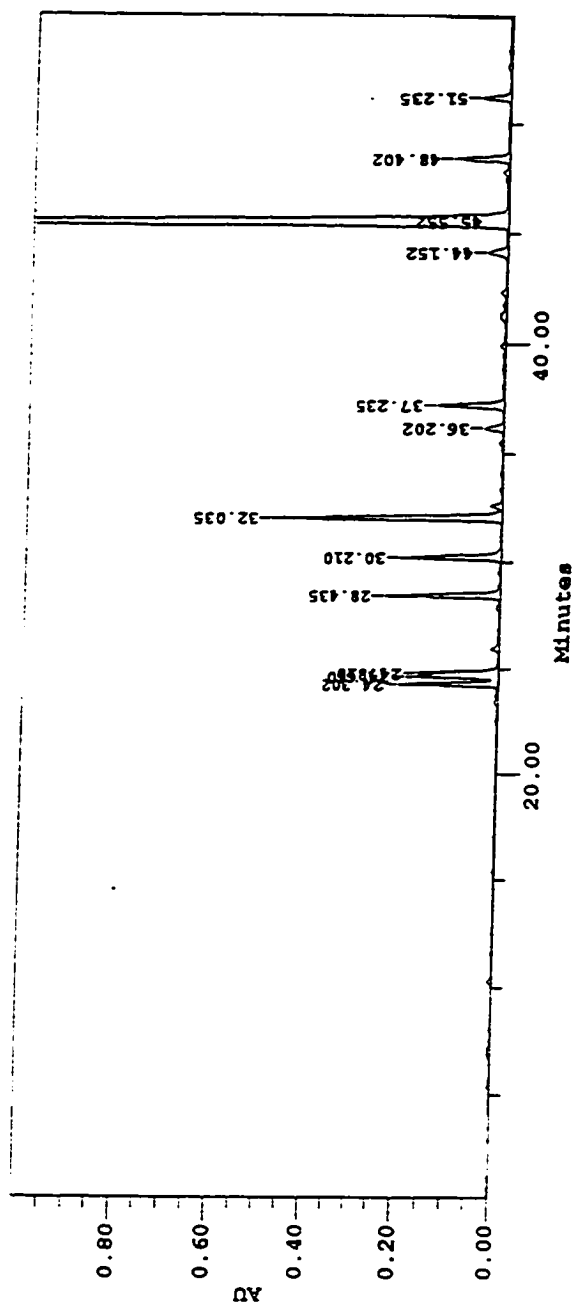


Figure 2-5. Marfey's amino acid analysis of 57% HI hydrolyzed aquachelin C. The HPLC column was an analytical C-18 (Vydac or YMC ODS-AQ, 4.6 x 150 mm) using a gradient from 90% 50 mM triethylamine phosphate, pH 3.0 (solvent A), 10% CH₃CN (solvent B) to 40% B in 45 minutes at a flow rate of 0.8 mL/min. Ten microliters of sample were injected. The absorbance was monitored at 340 nm.

Retention times are assigned as follows: 24.302 min = D-ornithine; 24.660 min = L-threo- β -hydroxy aspartate; 24.818 min = L-ornithine; 28.435 min = singly-derivatized D and L-ornithine; 30.210 min = L-serine; 32.035 min = D-serine; 37.235 min = D-glutamic acid (arising from D-glutamine, see text); 45.552 min = excess Marfey's reagent. Doubly derivatized L- and D-ornithines appear at 48.402 and 51.235 minutes, respectively.

See Appendix Figures A-5 and A-6 for the chromatograms of aquachelins B and C from the Baseline HPLC system, which give the retention times in Table 2-1.

amino acid amine (24.302 min = D-ornithine; 24.818 min = L-ornithine), one peak for Marfey's attached to the terminal amine of the side chain (28.435 min), and one peak for the double modification at both amines (48.402 and 51.235 min for L- and D-ornithine respectively). A total of 5 peaks are observed in the D,L-ornithine samples, instead of 6, because of the coincidence of the side-chain (N^{δ}) derivatized peaks. For the side-chain derivatives, the chiral centers of the amino acid and Marfey's reagent are separated by the full length of the side chain and the diastereomers are not resolved. Two D-serines and one L-serine are present (32.212 min = L-serine; 33.845 min = D-serine; Figure 2-4). The glutamine (see NMR results below) is in the D configuration, based on its identity with D-glutamic acid, since glutamine is converted to glutamic acid under hydrolysis conditions. β -Hydroxy aspartate from the aquachelins (25.770 min) co-elutes with the peak derived from hydrolysis of alterobactin B in 6 M HCl, thus confirming that the β -hydroxy aspartate residue is S,S or L-*threo* (Reid et al., 1993). The retention times and identities of each peak from HCl and HI hydrolysis and Marfey's derivatization (corresponding to Appendix Figures A-1 to A-6) are listed in Table 2-1.

Chiral GC analysis of fully hydrolyzed siderophores shows the same ratios of amino acids as determined by the HPLC method (Figure 2-6 and A-7 to A-9, Table 2-2). However, HI hydrolysis does not seem to be required since

Aquachelin	A	B	C	D	A + Si ^a	B + Si ^a
Figure	2-6	A-7	A-8	A-9	A-10	2-7
L-threo-β-OH-aspartic acid	15.414	15.405	15.395	15.396	11.400	11.604
D-serine	6.306	6.309	6.308	6.292	6.150	6.330
L-serine	6.538	6.541	6.537	6.533	6.309	6.499
D-glutamic acid	14.332	14.302	14.313	14.278	14.094	14.320
D-orithine	19.101	19.085	19.090	19.081	18.324	19.076
L-ornithine	19.408	19.394	19.395	19.388	18.540	19.38

Table 2-2. Retention times (minutes) for Chirasil-Val GC amino acid analysis of aquachelins. Between 0.2 and 2 µL were injected onto the Hewlett Packard 5890 GC (using a splitless injector port). The temperature gradient initially held constant at 80 °C for three minutes, then increased to 200 °C at 5 °C/ min. Carrier gas was H₂ at 20 mL/ min and the samples were detected using a flame ionization detector at 250 °C.

^a Aquachelins A and B were run both with and without silylation with TMS. ^b Glutamic acid is observed because glutamine is converted to glutamic acid during hydrolysis.

the ornithine residues are visible after HCl hydrolysis. Some samples were silylated with trimethylsilane (Scheme 2-3) in order to improve the stability of the β -hydroxy aspartate to GC conditions (Demange et al., 1988). If the hydroxyl groups are silylated (Figure 2-7 and A-10), the retention time of the β -hydroxy aspartate peak moves from 15 to 11 minutes and the peak is much larger. The retention times of the serines are essentially unchanged by silylation (Table 2-2).

Mass spectrometry: The exact mass of the molecular ion of aquachelin C was measured by fast-atom bombardment mass spectrometry to be 1091.5488. The elemental composition, $C_{46}H_{79}N_{10}O_{20}$, yields a calculated mono-isotopic mass of 1091.547211, which is only -1.5 ppm away from the measured exact mass. The exact mass of the sodium adduct of aquachelin B was measured to be 1087.5084 and the calculated mass is 1087.513506, a difference of 4.7 ppm. The other aquachelins did not give strong enough signals to be used for exact mass determinations.

Electrospray chromatograms of the aquachelins (Figures 2-8 to 2-11) show multiple peaks. Typically, the major species are the siderophore (MH^+), its aluminum adduct ($M_{Al}H^+$) and its iron(III) adduct ($M_{Fe}H^+$) (Table 2-3). Iron(III) and aluminum are not added to the siderophores, but are picked up in

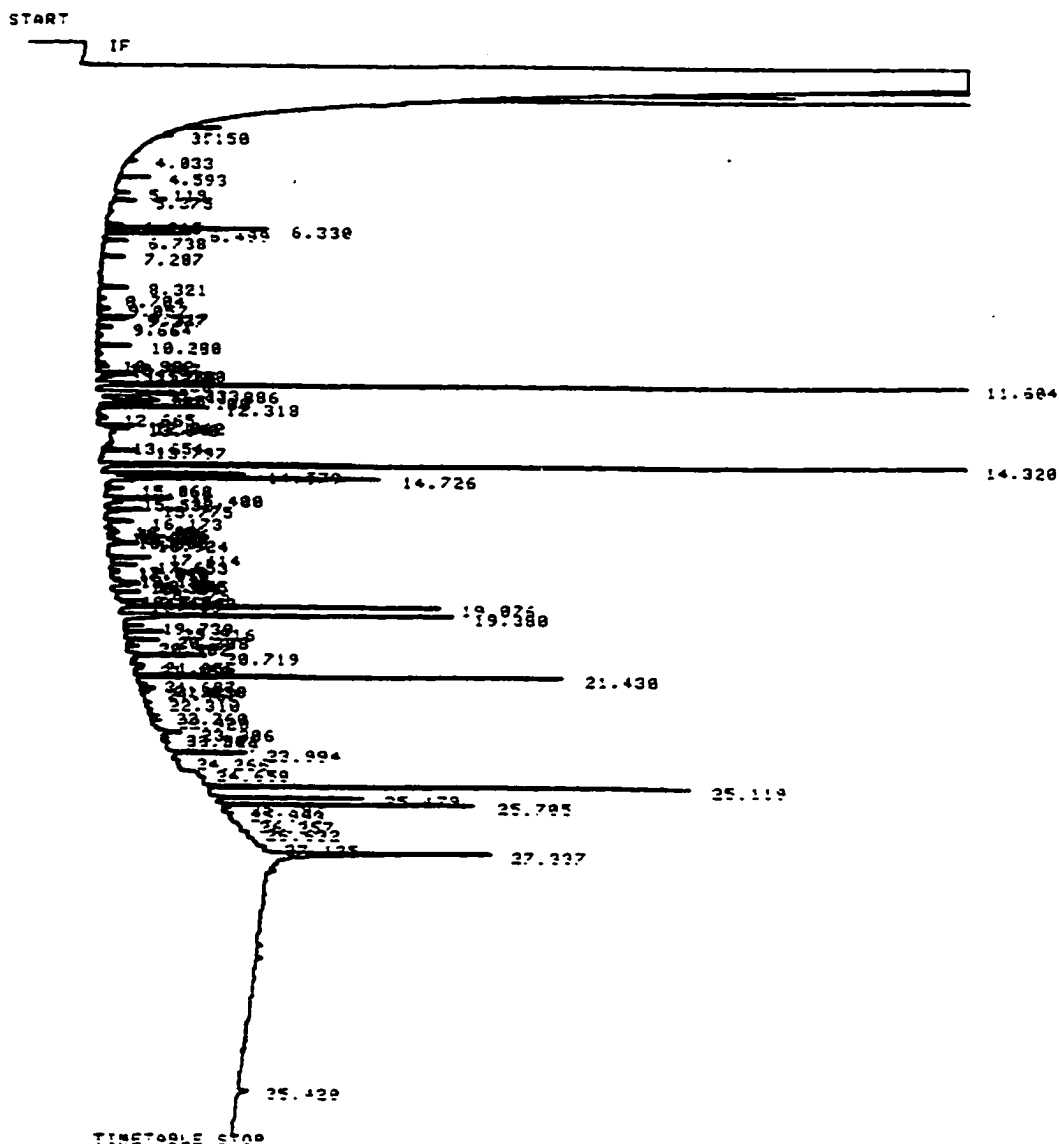


Figure 2-7. GC chromatogram of fully hydrolyzed aquachelin B after silylation showing the ratios of each amino acid present. See Table 2-2 for assignments and Appendix for data for aquachelin A (Figure A-10). GC conditions are as described in Figure 2-6.

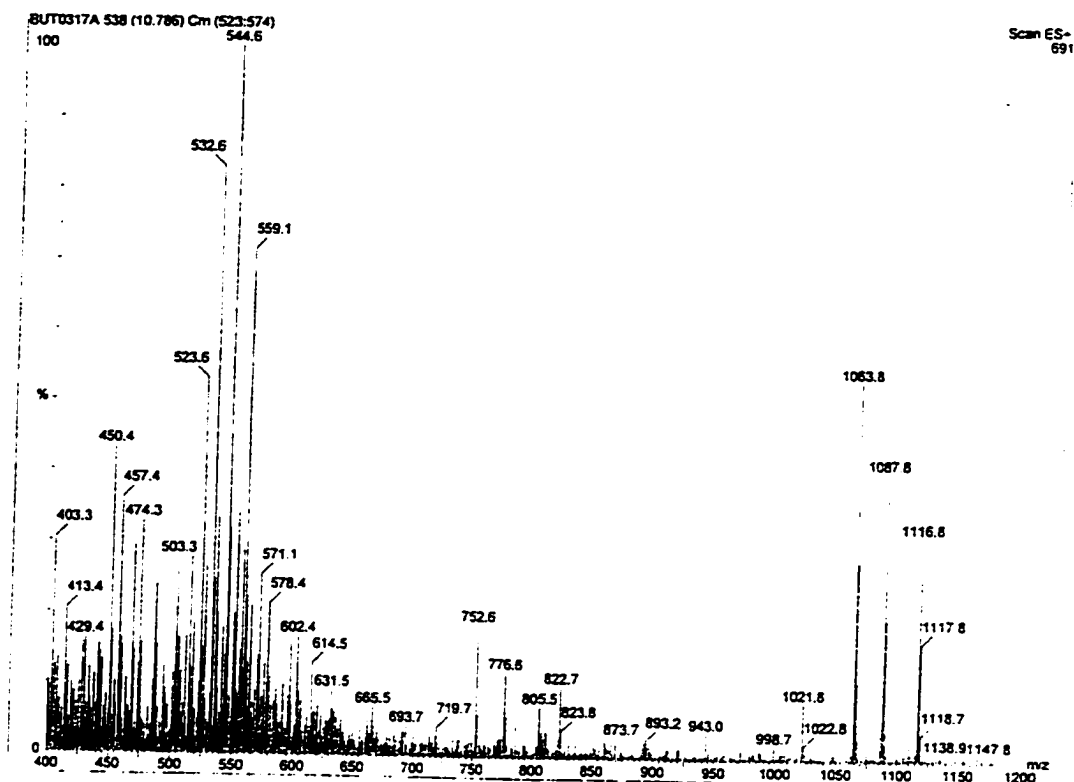


Figure 2-8. Electrospray mass spectrum of aquachelin A, m/z 1063.8.

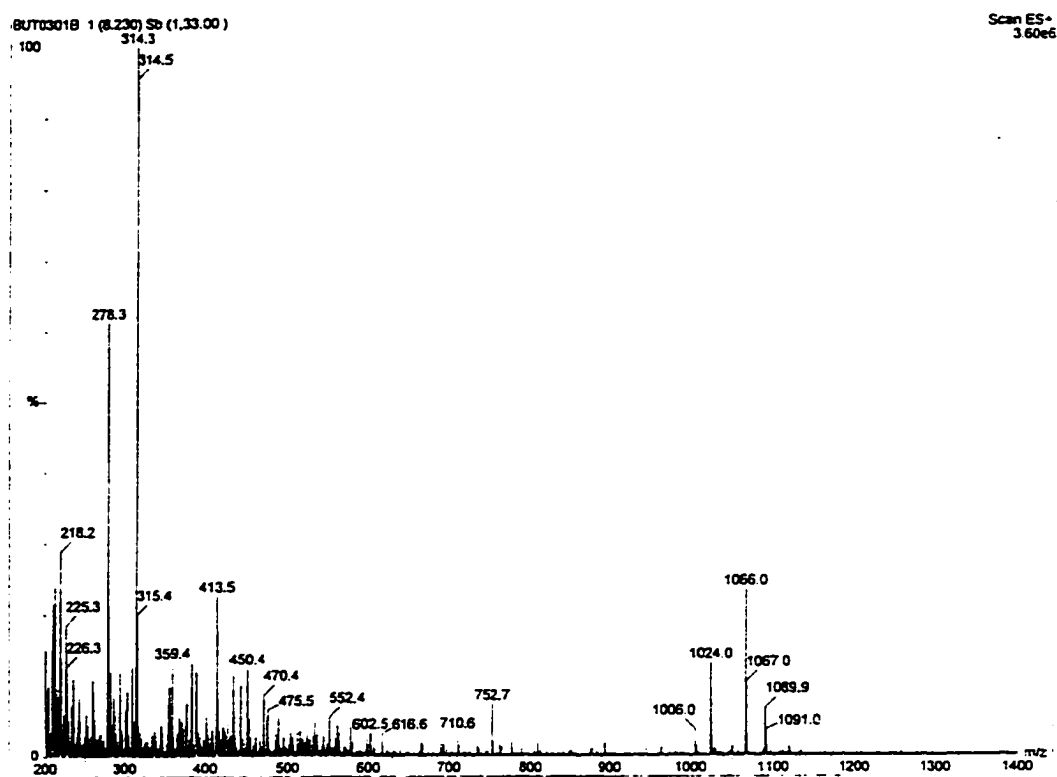


Figure 2-9. Electrospray mass spectrum of aquachelin B, m/z 1066.0.

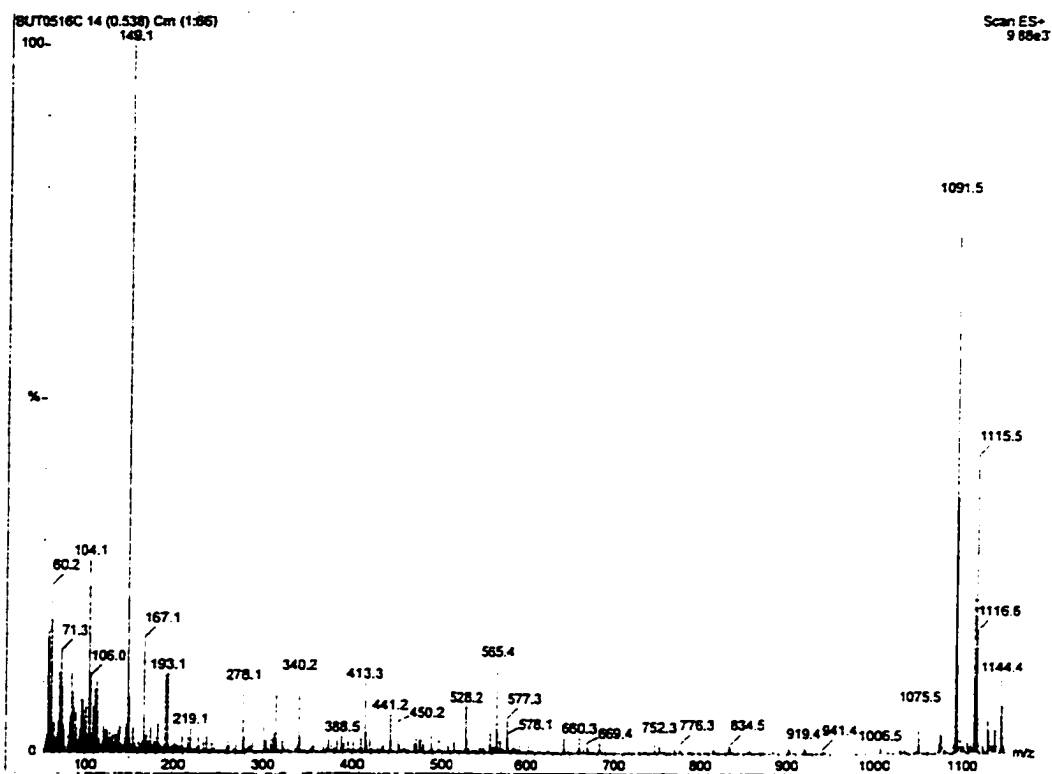


Figure 2-10. Electrospray mass spectrum of aquachelin C, m/z 1091.5.

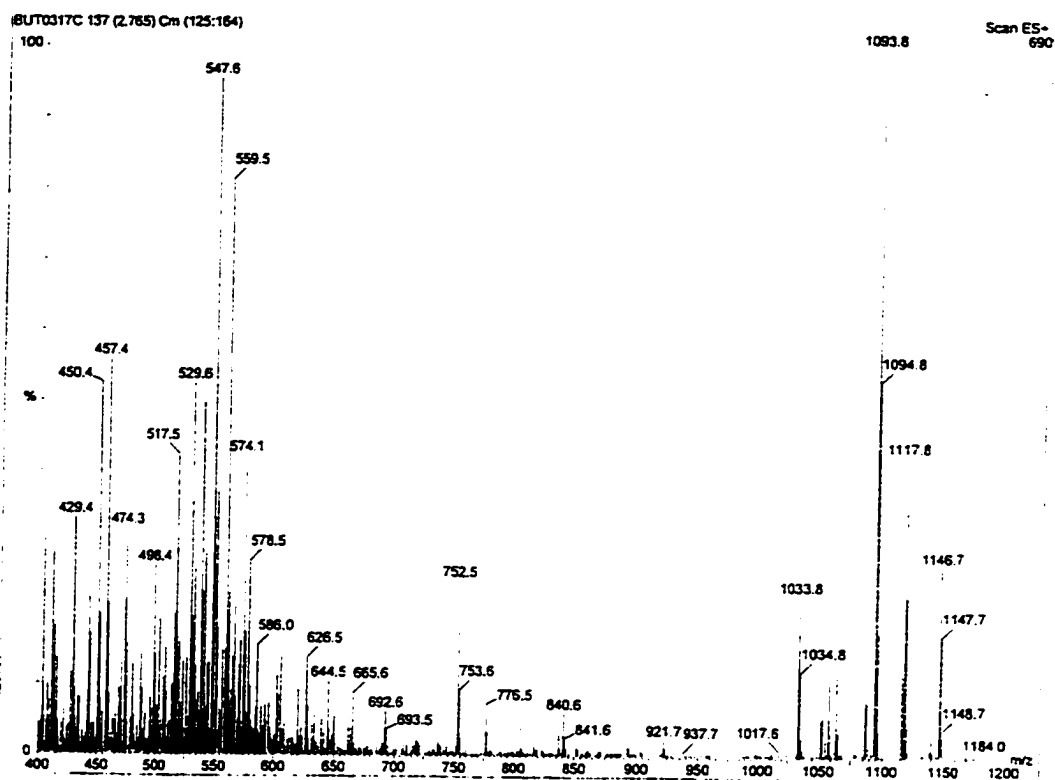


Figure 2-11. Electrospray mass spectrum of aquachelin D, m/z 1093.8.

Aquachelin	MH ⁺	M _{Al} H ⁺	M _{Fe} H ⁺
A (Figure 2-8)	1063.8	1087.8	1116.8
B (Figure 2-9)	1066.0	1089.9	1118.8
C (Figure 2-10)	1091.5	1115.5	1144.4
D (Figure 2-11)	1093.8	1117.8	1146.7

Table 2-3. ESI-MS m/z values for the aquachelins, and their Fe(III) and Al(III) adducts. M = siderophore, M_{Al} = Al(III) + siderophore - 3 H⁺, M_{Fe} = Fe(III) + siderophore - 3 H⁺.

varying amounts during isolation. Only 1:1 complexes of siderophore to aluminum or iron(III) are observed. The iron(III) complexes show an increase of 53 mass units over the MH^+ m/z because of the addition of 56 amu for Fe^{3+} and the loss of three protons from the siderophore to keep the adduct monoprotonated. The presence of aluminum adducts was tested by making and purifying the aluminum adduct of aquachelin C and analyzing it by ESI-MS. The exclusive appearance of a peak at m/z 1115 demonstrates that the peaks at 24 mass units above the molecular ion are aluminum adducts, which also lose 3 H^+ when formed (Table 2-3). Iron(III) readily displaces aluminum, as ESI-MS chromatograms of the iron(III)-aquachelins show essentially no aluminum adducts (Figures 2-12 to 2-15). ESI-MS of the gallium complex of aquachelin C shows peaks at m/z 1157.4 and m/z 1159.4 in a 3:2 ratio as expected for the isotopes ^{69}Ga and ^{71}Ga at 60% and 40% natural abundance, respectively.

Close examination of the siderophore and iron(III)-aquachelin ESI-MS results shows not only the molecular ions, but also fragments which can be assigned to portions of the siderophore structure. Addition of iron(III) gives iron(III) adducts for the portions of the molecules which still contain both N^5 -hydroxy-ornithines.

Fragmentation in the tandem MS experiments (Figures 2-16 to 2-19) generates primarily Y (Table 2-4) and B (Table 2-5) fragments (Roepstorff,

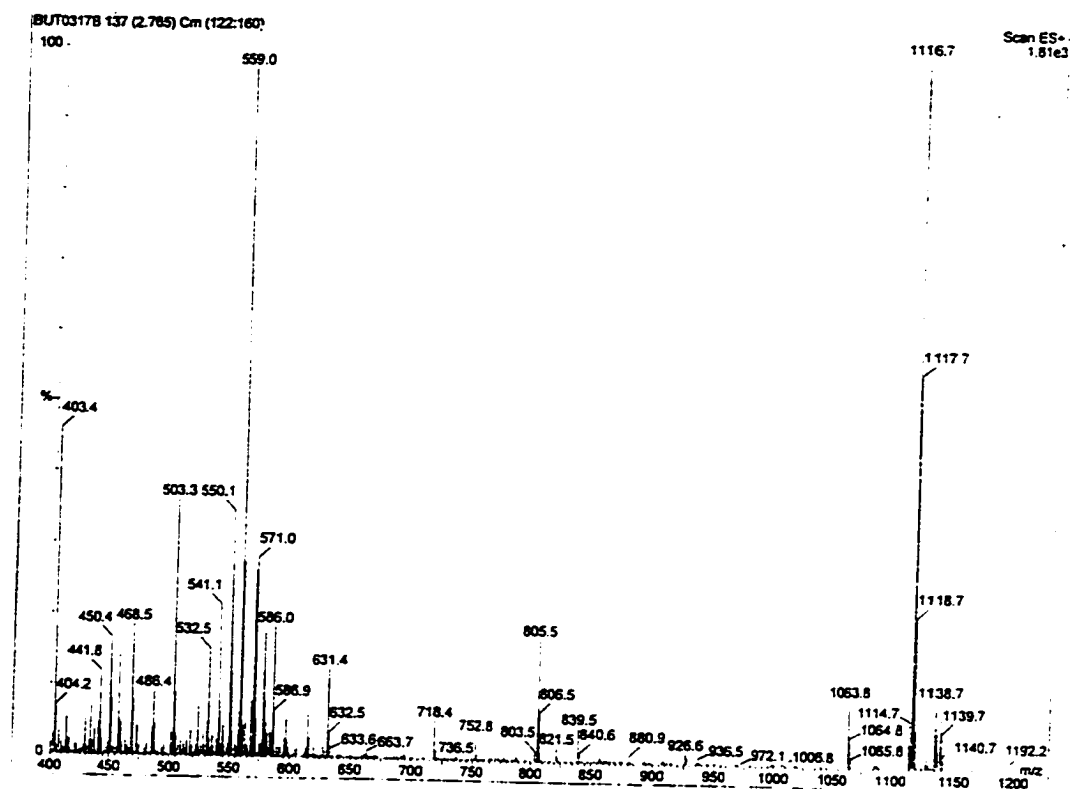


Figure 2-12. Electrospray mass spectrum of Fe(III)-aquachelin A, m/z 1116.7.

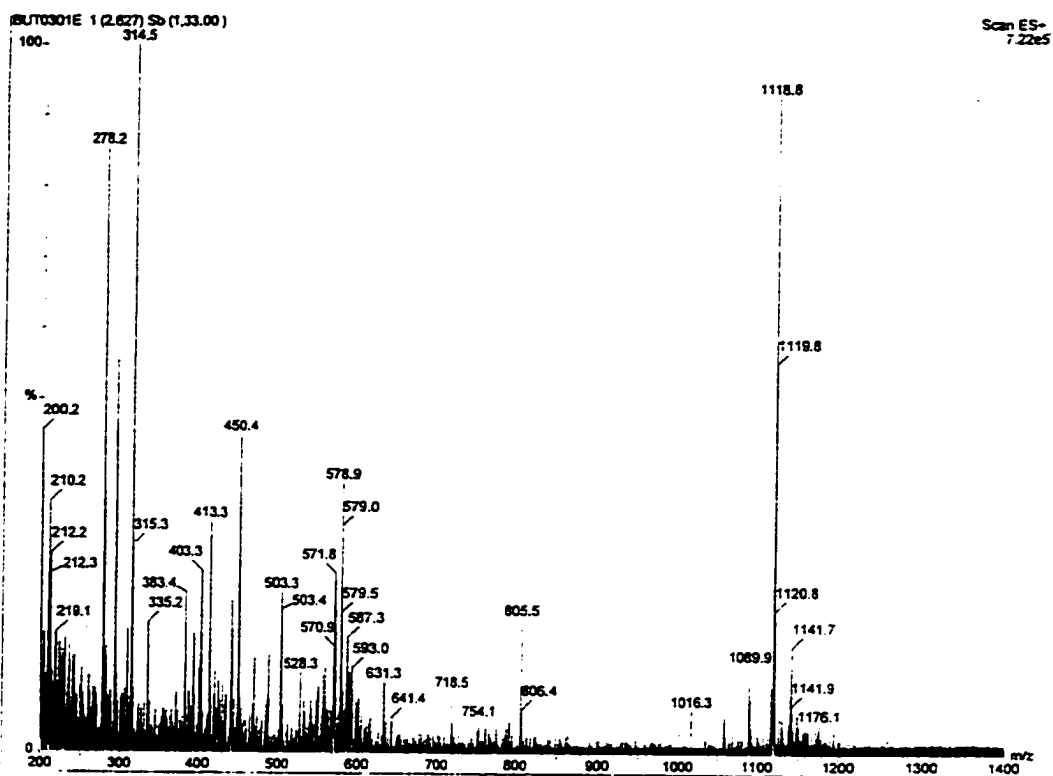


Figure 2-13. Electrospray mass spectrum of Fe(III)-aquachelin B, m/z 1118.8.

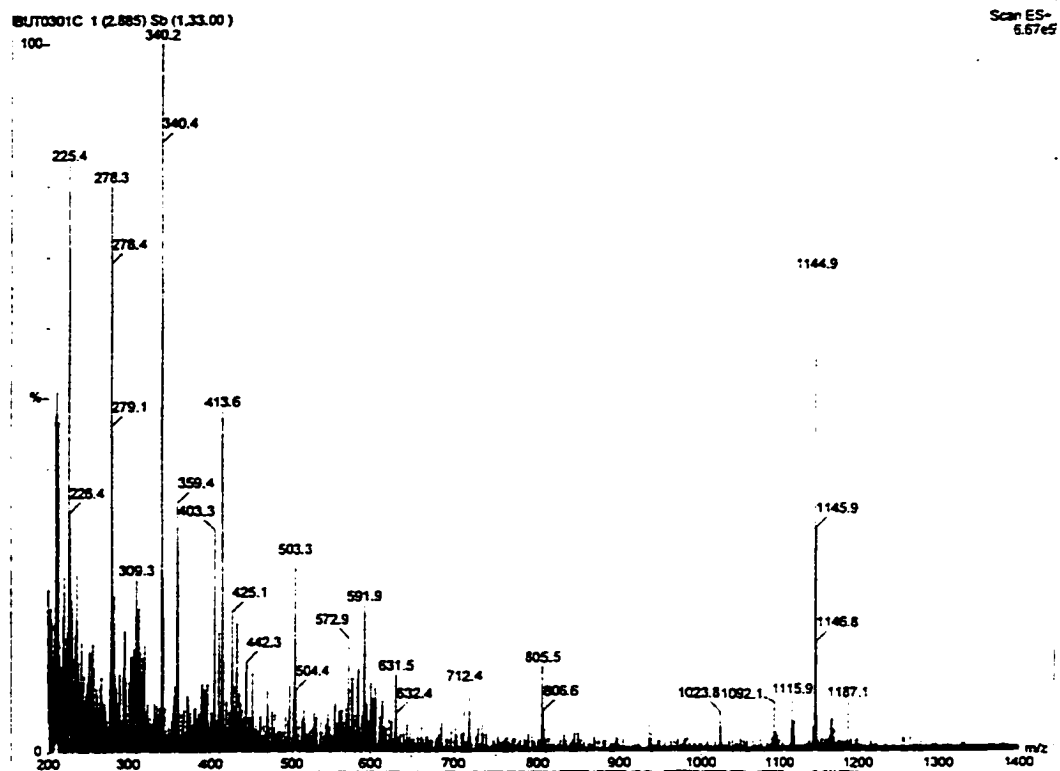


Figure 2-14. Electrospray mass spectrum of Fe(III)-aquachelin C, m/z 1144.4.

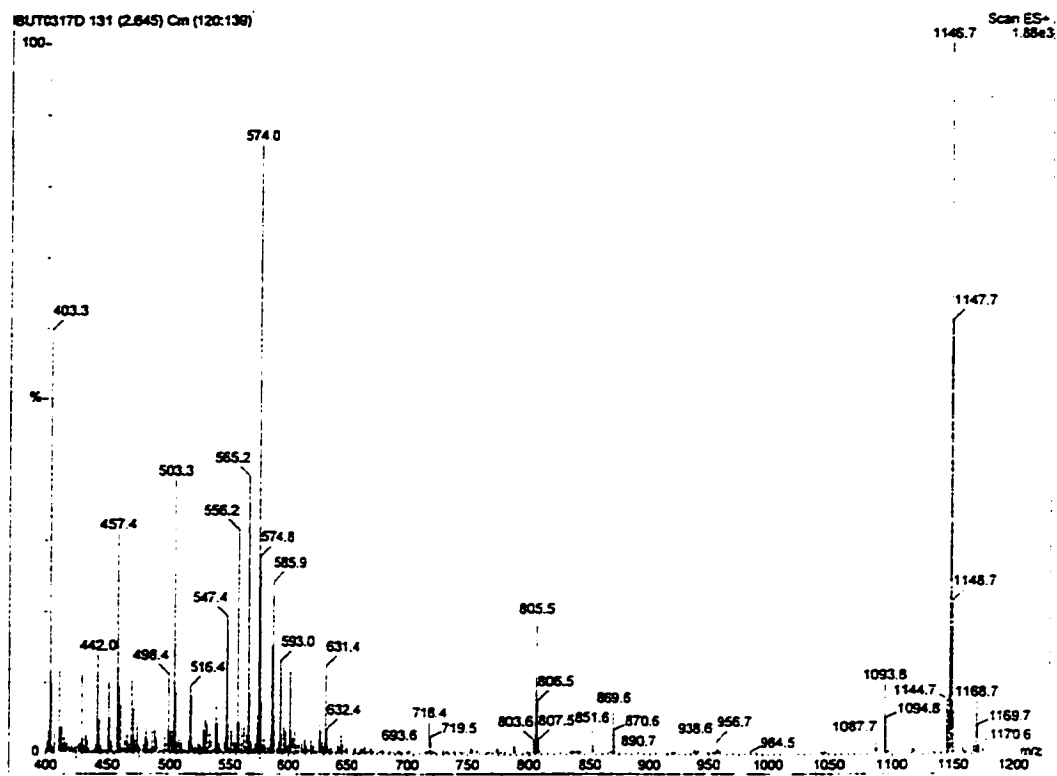


Figure 2-15. Electrospray mass spectrum of Fe(III)-aquachelin D, m/z 1146.7.

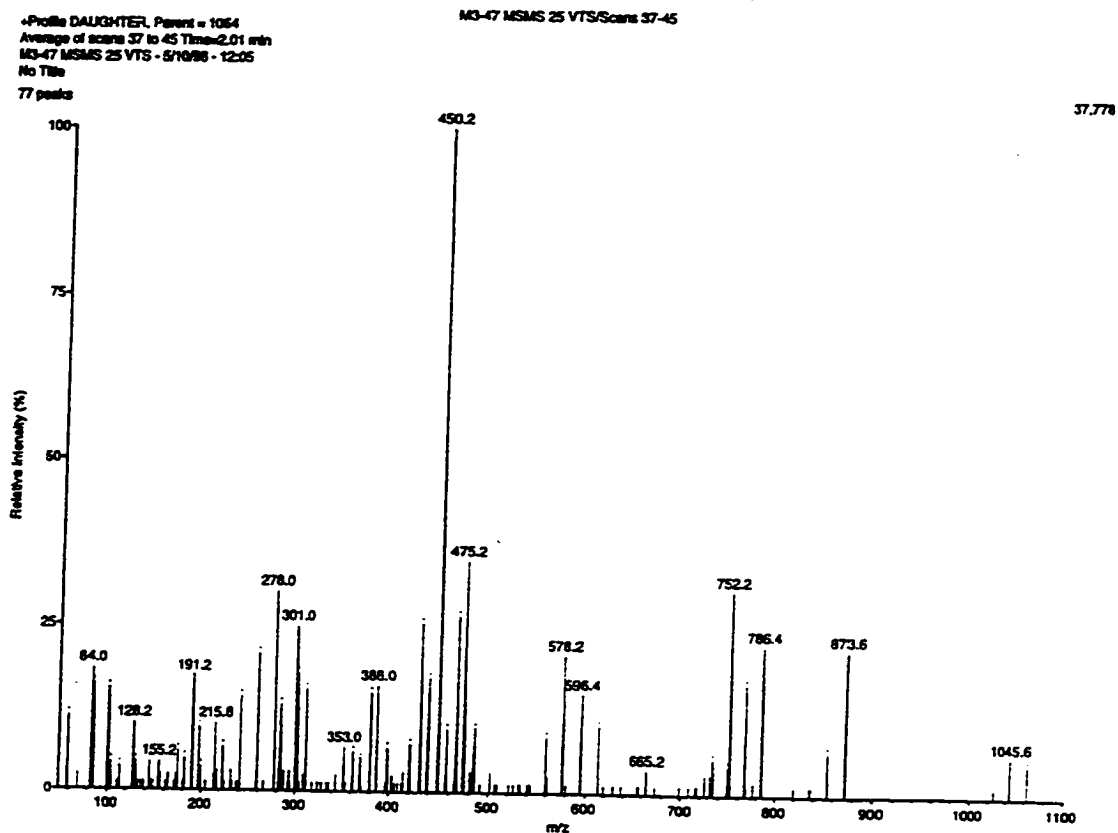


Figure 2-16. Tandem mass spectrum of aquachelin A obtained by Brian Arbogast of the Oregon State University, Department of Agriculture Mass Spectrometry Facility. See Figure 2-20 for analysis of the observed fragmentation.

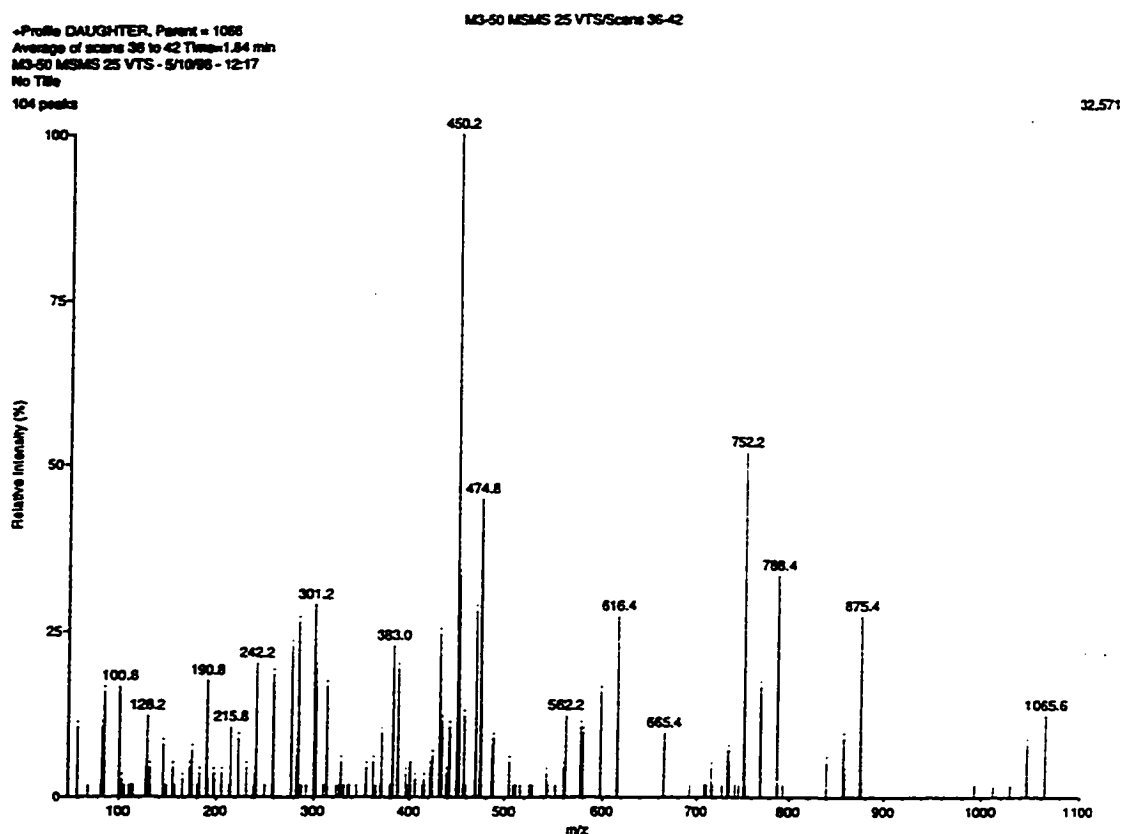


Figure 2-17. Tandem mass spectrum of aquachelin B obtained by Brian Arbogast of the Oregon State University, Department of Agriculture Mass Spectrometry Facility. See Figure 2-21 for analysis of the observed fragmentation.

+Profile DAUGHTER, Parent = 1092
 Average of scans 35 to 50 Time=2.01 min
 MS-ES MSMS 25 VTS - 5/10/96 - 12:24
 No Title

MS-ES MSMS 25 VTS/Scan 35-50

3675

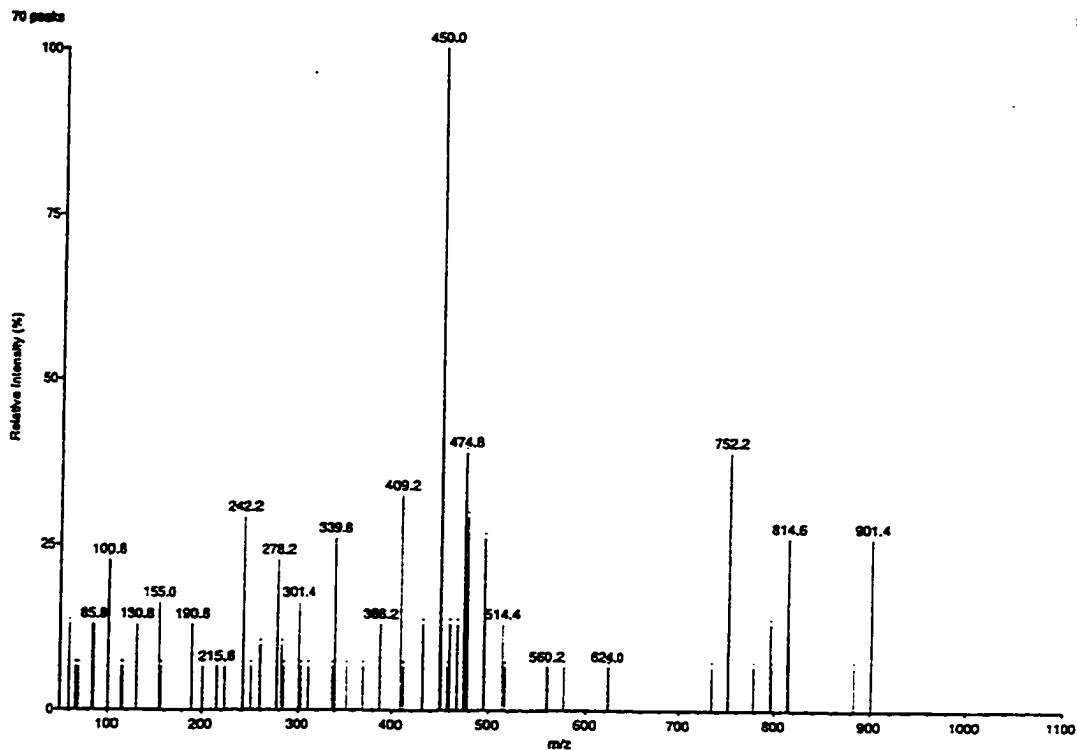


Figure 2-18. Tandem mass spectrum of aquachelin C obtained by Brian Arbogast of the Oregon State University, Department of Agriculture Mass Spectrometry Facility. See Figure 2-22 for analysis of the observed fragmentation.

+Profile DAUGHTER, Parent = 1094
Average of scans 30 to 58 Time=2.31 min
M3-56 MSMS 25 VTS - 5/10/96 - 12:37
No Title
55 peaks

M3-56 MSMS 25 VTS/Scan 30-58

22,138

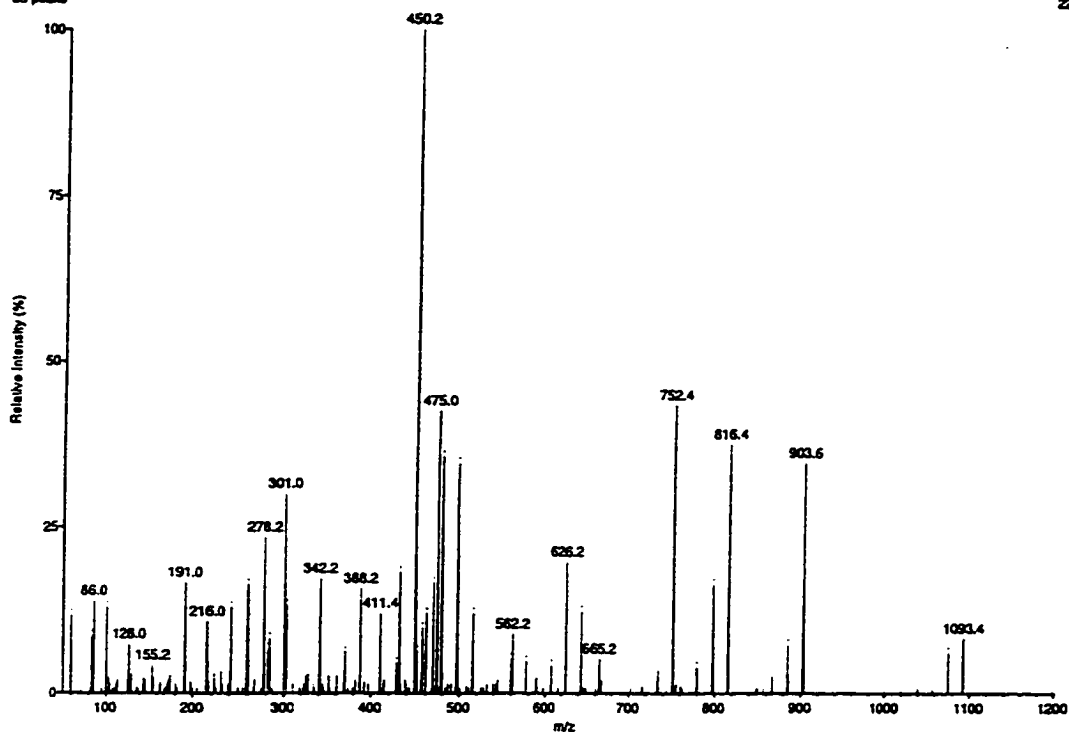


Figure 2-19. Tandem mass spectrum of aquachelin D obtained by Brian Arbogast of the Oregon State University, Department of Agriculture Mass Spectrometry Facility. See Figure 2-23 for analysis of the observed fragmentation.

Assignment	Aquachelin A	Aquachelin B	Aquachelin C	Aquachelin D
Y ₆	752	752	752	752
Y ₆ - H ₂ O	734	734	734	-
Y ₅	665	665	-	665
Y ₅ - H ₂ O	-	-	-	-
Y ₄	578	578	578	578
Y ₄ - H ₂ O	560	560	560	560
Y ₃	450	450	450	450
Y ₃ - H ₂ O	432	432	432	432
Y ₂	278	278	278	278
Y ₂ - H ₂ O	260	260	260	260
Y ₁	191	191	191	191
Y ₁ - H ₂ O	-	173	-	-

Table 2-4. Y fragment (Roepstorff, 1984) m/z values observed by tandem mass spectrometry of the aquachelins. All fragments include addition of two protons to create a positive charge on the amine. No Y₇ fragments were observed under the conditions employed (see Experimental section). Most of the Y fragments show loss of water which may arise from the serine side-chains as discussed (Discussion section).

Assignment	Aquachelin A	Aquachelin B	Aquachelin C	Aquachelin D
B ₇	873.6	875.4	901.6	904.2
B ₇ - H ₂ O	855.4	857.4	883.6	885.6
B ₆	786.4	788.4	814.6	816.4
B ₆ - H ₂ O	768.2	770.2	796.4	798.4
B ₅	614.4	616.4	-	644.4
B ₅ - H ₂ O	596.4	598.2	624.6	626.2
B ₄	486.2	488.2	514.4	516.2
B ₄ - H ₂ O	467.8	470.4	496.2	498.0
B ₃	398.8	-	-	429.0
B ₃ - H ₂ O	381.0	383.0	409.2	411.4
B ₂	312.0	314.2	340.2	342.2

Table 2-5. B fragment (Roepstorff, 1984) m/z values observed by tandem mass spectrometry of the aquachelins.

1984) from C- and N-terminal amide bond cleavage, respectively. Figures 2-20 through 2-23 show the Y/B cleavage patterns¹ for each of the aquachelins. No fragmentation between the β -hydroxy aspartate and the fatty acid tail is observed by MS/MS (see APCI below). However, all of the amide bonds in the remainder of the heptapeptide cleave to produce Y and B fragments and internal fragments (y_i, b_i) and immonium ions (Table 2-6) as described by Papayannopoulos (1995).

The N^6 -hydroxy ornithine residues are both acetylated as demonstrated by the mass difference of 42 amu. The resulting modified ornithine residues contain the hydroxamate functionalities needed for iron(III) chelation.

APCI mass spectrometry To analyze the variable portion of the siderophore structures, Atmospheric Pressure Chemical Ionization (APCI) was employed to fragment the molecules further. Peaks for the fatty acid portion of each aquachelin (B_1 fragments in Figures 2-20 to 2-23), are observed at m/z 181, 183, 209 and 211 respectively (see Appendix Figures A-11 to A-14). These masses correlate with the expected masses for dodecenoic acid, dodecanoic acid, tetradecenoic acid and tetradecanoic acid.

Microderivatizations - Derivatization reactions are useful in pinpointing

¹ B fragments: Cleavage products from the N-terminus of a peptide.

Y fragments: Cleavage products from the C-terminus of a peptide, which must pick up two extra protons to maintain the charge on the free amine nitrogen (Roepstorff, 1984).

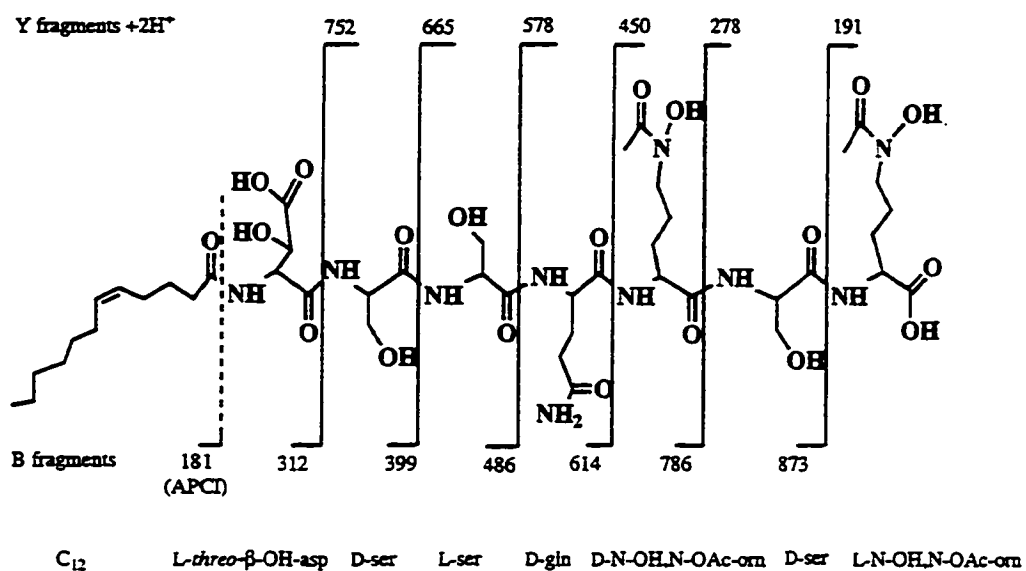


Figure 2-20. Y and B fragment analysis of aquachelin A. The B₁ fragment (*m/z* 181) was only observed using APCI (see Results and Interpretation).

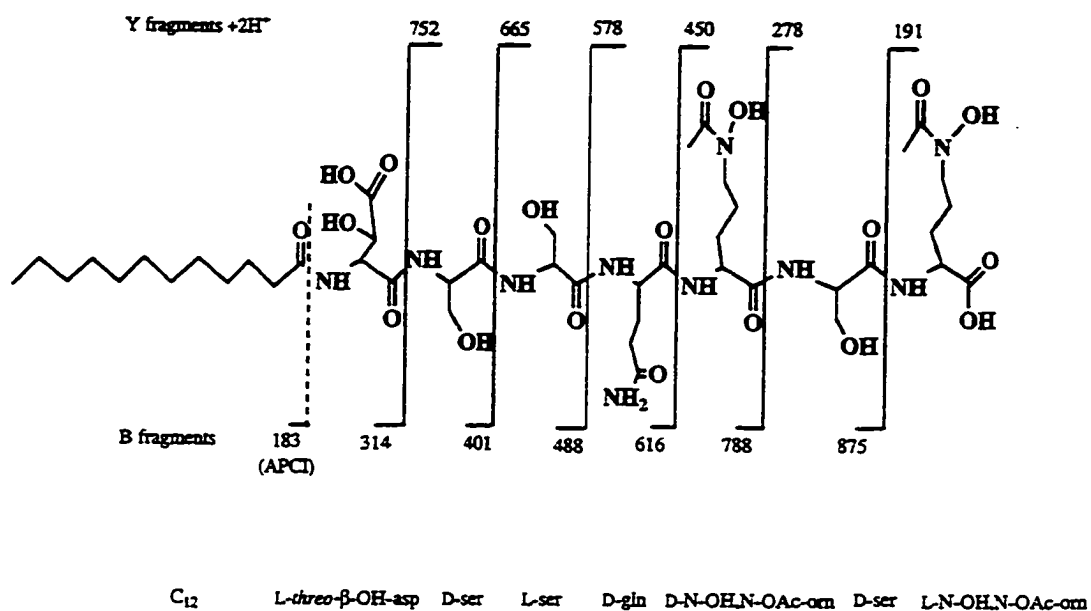


Figure 2-21. Y and B fragment analysis of aquachelin B. The B₁ fragment (*m/z* 183) was only observed using APCI (see Results and Interpretation).

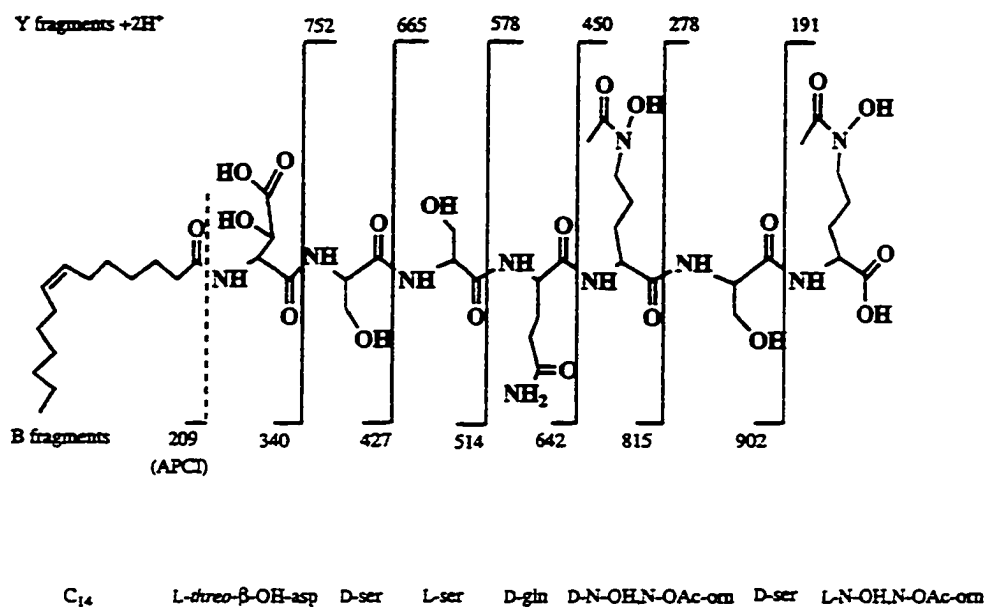


Figure 2-22. Y and B fragment analysis of aquachelin C. The B₁ fragment (*m/z* 209) was only observed using APCI (see Results and Interpretation).

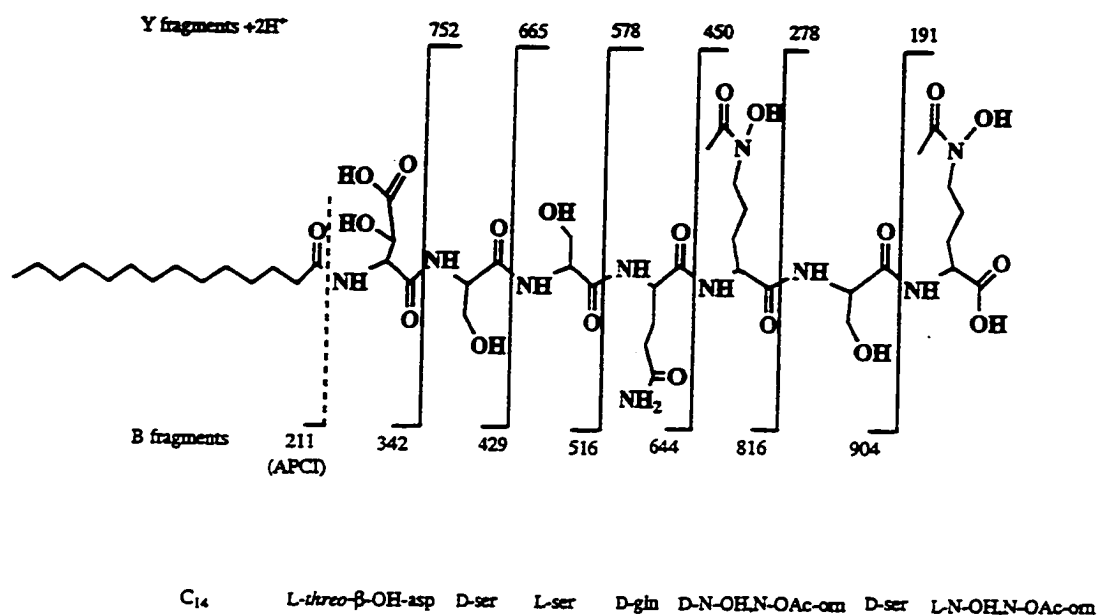


Figure 2-23. Y and B fragment analysis of aquachelin D. The B₁ fragment (*m/z* 209) was only observed using APCI (see Results and Interpretation).

Assignment	Aquachelin A	Aquachelin B	Aquachelin C	Aquachelin D
ser-immonium	60.0	60.0	60.2	60.0
gln-side chain	84.0	84.2	-	84.0
gln-immonium	101.0	100.8	100.8	101.2
gln	128.2	128.2	-	128.0
gln-H ⁺	129.0	128.8	-	-
β-hydroxy-aspartate	130.8	131.0	130.8	-
y ₅ b ₅ - H ₂ O	198.0	198.0	-	-
y ₅ b ₅ (SQ)	215.8	215.8	216.2	216.0
y ₃ b ₇ - H ₂ O	242.2	242.2	242.2	242.2
y ₄ b ₆ (QO)	301.0	301.2	301.4	301.0
y ₆ b ₅ (SSQ)	303.0	303.0	303.6	303.0
y ₅ b ₆ (SQQ) +	388.0	388.2	388.2	388.2
y ₄ b ₇ (QOS)				
y ₆ b ₆ (SSQO) +	475.2	474.8	475.2	475.0
y ₅ b ₇ (SQOS)				

Table 2-6. Selected m/z from tandem mass spectra of the aquachelins with their assignments. Internal fragments are labelled according to Papayannopoulos, 1995 and the peptide fragments are identified according to the amino acid abbreviations (S = serine, O = ornithine, Q = glutamine).

the number of free acids, amines and hydroxyls. Using electrospray mass spectrometry to analyze the results allows these derivatizations to be performed on a very small scale (0.1 mg or less) (Sharman et al., 1995). For esterification in methanol, the methyl ester will add 14 mass units per carboxylate. Mild acetylation conditions add 42 units for each amino group. More vigorous acetylation should add 42 units for both the free amino and hydroxyl groups. Sharman et al. (1995) observed that the hydroxylamine groups are also acetylated under both sets of conditions.

The esterification conditions appear to have hydrolyzed the material, because a large number of peaks are obtained for all esterification samples, but the peaks are all smaller than the molecular ion. Mild acetylation conditions give a mixture of products with up to four additions of acetyl groups. Figure 2-24 shows major peaks at m/z 1217.5 and 1259.5 for the addition of three and four acetyl groups, respectively, to aquachelin C. The two N^{δ} -hydroxy ornithines, the glutamine and possibly the β -hydroxy aspartate are acetylated under these conditions, although the yield is low for the quadruply acetylated material. Strong acetylation conditions added 6 acetyl groups (Figure 2-25; m/z 1343.4), because the three serine hydroxyls are acetylated in addition to the amino acids acetylated under mild conditions. Only a small peak at m/z 1385, corresponding to addition of seven acetyl groups, is observed for aquachelin C

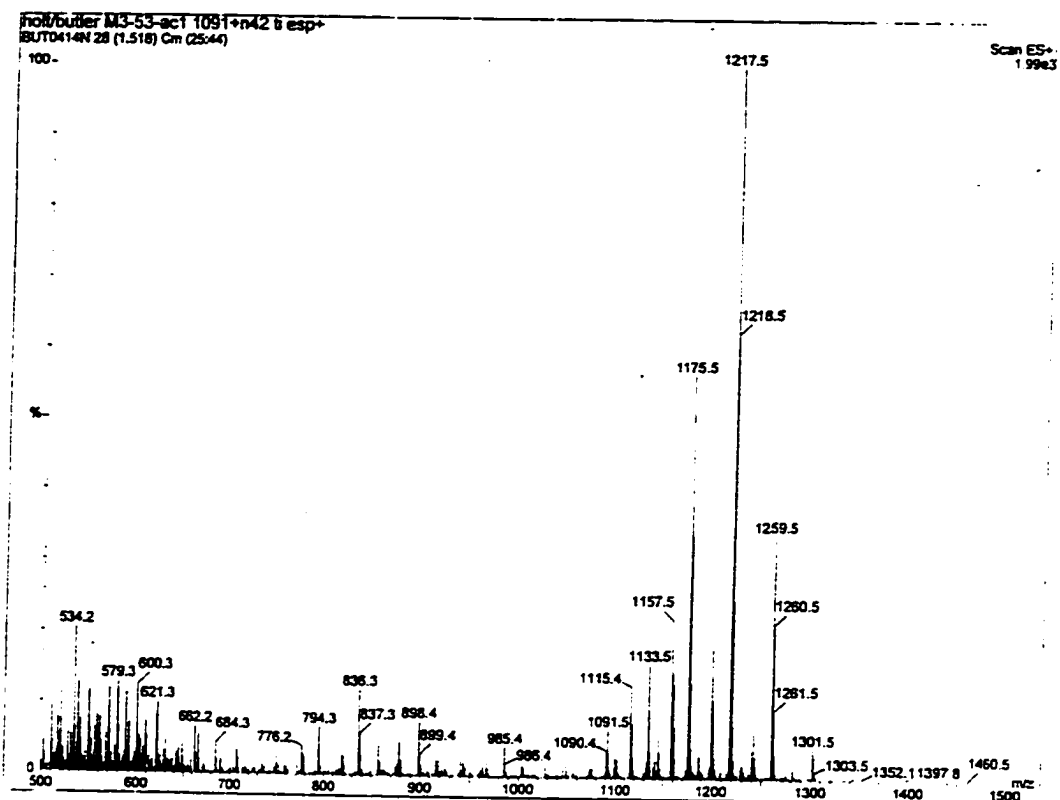


Figure 2-24. Electrospray mass spectrum of the microderivatization products from mild acetylation of aquachelin C. See appendix figures A-15, A-16 and A-17 for aquachelins A, B and D, respectively.

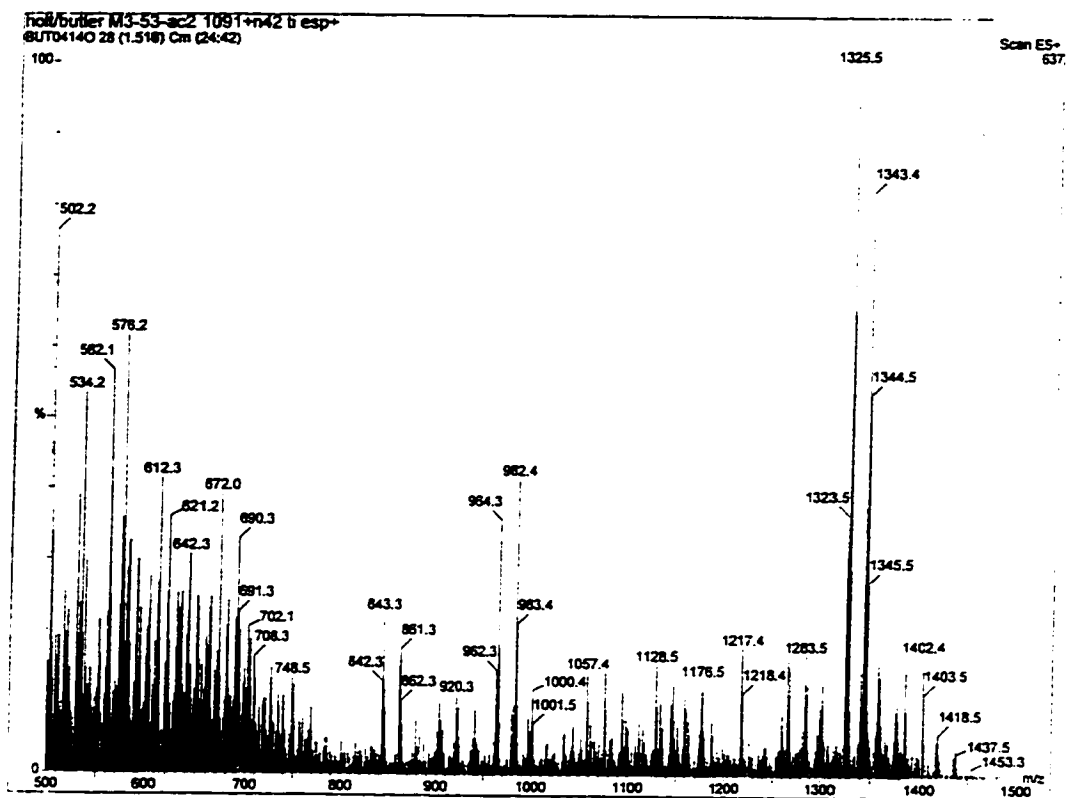


Figure 2-25. Electrospray mass spectrum of the microderivatization products from strong acetylation of aquachelin C. See appendix figures A-18, A-19 and A-20 for aquachelins A, B and D, respectively.

(Figure 2-25). Apparently seven acetyl groups are quite difficult to add. When the ferric siderophore is acetylated, the two *N*⁶-hydroxy ornithines are blocked, and therefore under the mild acetylation conditions addition of one acetyl group is dominant (*m/z* 1186.3 in Figure 2-26) although a small amount of material has two acetyl groups (*m/z* 1228.3 in Figure 2-26). Although the β -hydroxy aspartate would also be blocked by coordination to iron(III), it is likely to be more easily displaced than the *N*⁶-hydroxy, *N*⁶-acetyl-ornithines. Therefore, the β -hydroxy aspartate residue is the most likely second site for acetylation of the ferric complex. Under strong acetylation conditions the iron(III) is displaced, and addition of six acetyl groups is observed, just as in the iron-free aquachelins. Table 2-7 lists the results for microderivatization of all the aquachelins and the chromatograms for aquachelins A, B and D are presented in the Appendix.

Deuterated samples - Another method of obtaining information about an unknown molecule is to measure the number of exchangeable protons, such as carboxylic acid protons, and amide and hydroxyl hydrogens. Exchange with deuterium adds one mass unit per deuterium added to the molecule. Deuteration of the siderophores yields an envelope of peaks in the electrospray mass spectrum resulting from varying extents of deuteration. In the case of aquachelin B, peaks corresponding to 10-18 sites of deuterium exchange are

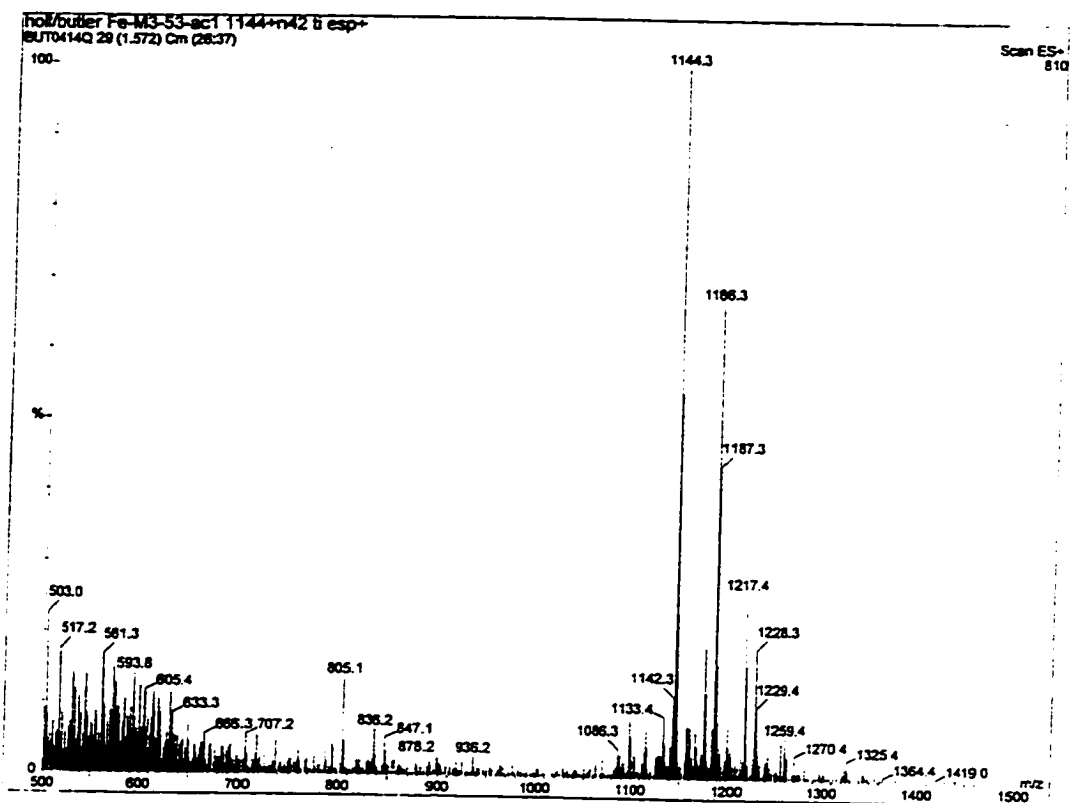


Figure 2-26. Electrospray mass spectrum of the microderivatization products from mild acetylation of Fe(III)-aquachelin C. See appendix figures A-21, A-22 and A-23 for the Fe(III) complexes of aquachelins A, B and D, respectively.

Mild acetylation:

Acetylations	Aquachelin A	Aquachelin B	Aquachelin C	Aquachelin D
1	1105.4	-	1133.5	1135.5
2	1147.4	1149.8	1175.5	1177.4
3	1189.4	1191.8	1217.5	1219.5
4	1231.4	1233.8	1259.5	1261.4

Acetylations	Fe-Aquachelin A	Fe-Aquachelin B	Fe-Aquachelin C	Fe-Aquachelin D
1	1158.2	1160.5	1186.3	1188.3
2	1200.3	1202.5	1228.3	1230.3

Strong acetylation:

Acetylations	Aquachelin A	Aquachelin B	Aquachelin C	Aquachelin D
6	1315.4	-	1343.4	1345.4
6 - H ₂ O	1297.4	1299.4	1325.5	1327.4

Acetylations	Fe-Aquachelin A	Fe-Aquachelin B	Fe-Aquachelin C	Fe-Aquachelin D
6	-	-	1343.4	1345.4
6 - H ₂ O	1297.3	1299.6	1325.4	1327.4

Table 2-7. Microderivatization results from electrospray mass spectrometry of the aquachelins and their iron(III) complexes under conditions of mild or strong acetylation (see Experimental).

observed. However, in the case of aquachelins C and D there are large increases in mass corresponding to 20-30 deuteriums, or perhaps addition of aluminum along with the deuteration. Therefore, no additional information was obtained by using this method.

NMR: The NMR results verify the presence of the amino acids identified above by amino acid analysis and mass spectrometry:

¹H NMR - The one-dimensional ¹H NMR spectrum of aquachelin A in 80% H₂O/ 20% D₂O obtained by Dr. Janet Cheetham on a Bruker 500 MHz instrument located in the College of Engineering at UCSB is shown in Figure 2-27. The side-chain NH₂ protons of glutamine have resonances at 6.7 and 7.3 ppm, thus identifying that glutamine is present (appearing as glutamic acid in the HPLC amino acid analysis above).

Resonances from the amide NH protons appear between 7.7 and 8.4 ppm in ¹H spectra taken in 80% H₂O/ 20% D₂O. The proton resonances in the 4.4-4.5 ppm range arise from the α protons of the 7 amino acids. The ¹H resonances of the serine β protons are at 3.6-3.9 ppm. The glutamine β and γ protons appear at 1.9 and 2.2 ppm, respectively. The ornithine δ protons resonances occur at 3.5 ppm, while the ornithine β and γ proton resonances appear around 1.5 ppm but overlap with resonances from the fatty acid

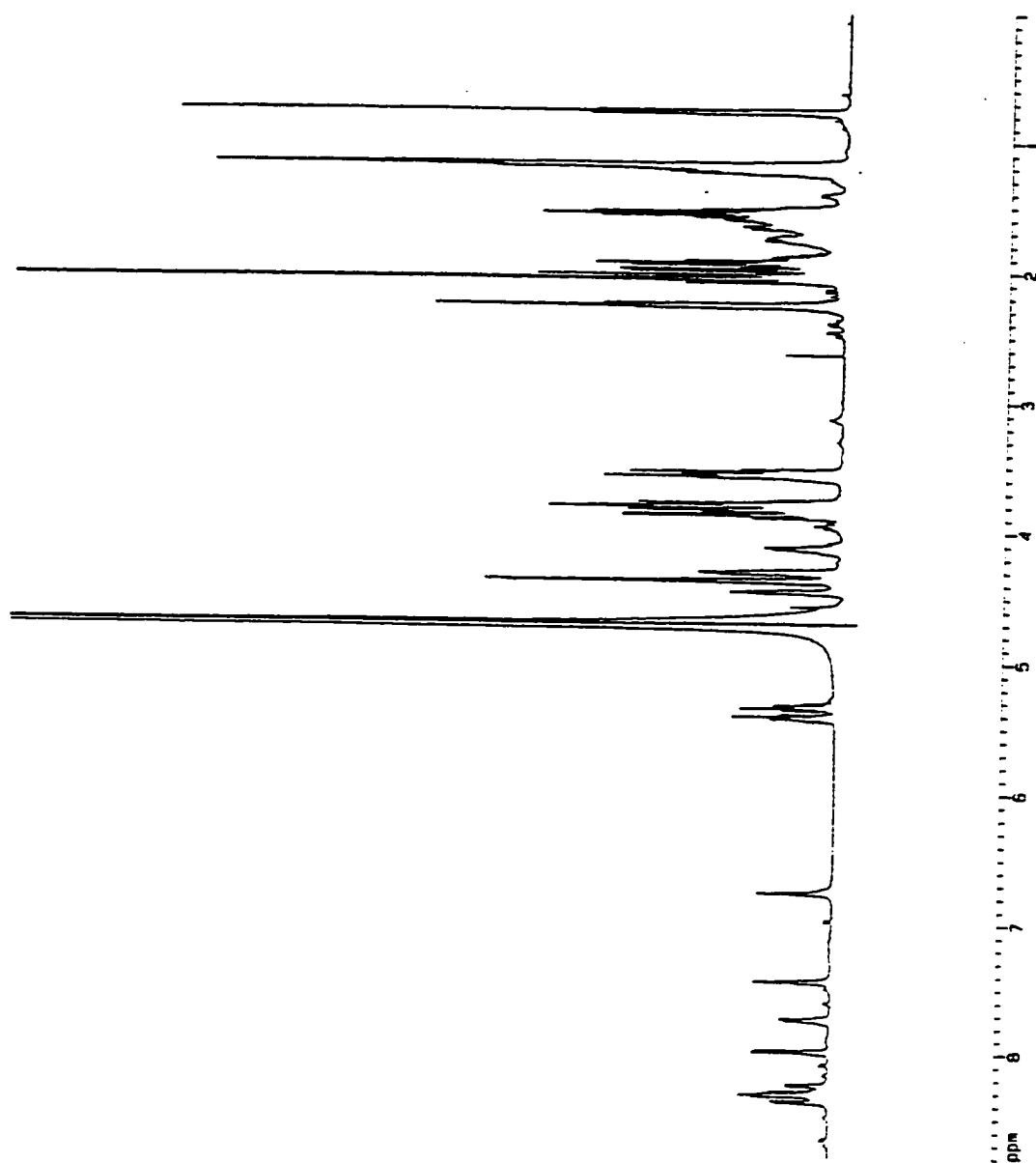


Figure 2-27. ^1H NMR spectrum of 10 mg aquachelin A in 80% H_2O / 20% D_2O obtained by Janet Cheetham using the 500 MHz Bruker instrument in the College of Engineering at UCSB. Solvent H_2O is presaturated and appears at 4.75 ppm. Spectrum referenced to DSS as an external standard, $\delta(\text{DSS}) = -0.076$ ppm. Pulse width = 30 μsec .

methylenes.

^1H resonances for the fatty acid chain methylenes occur between 2.5 and 1.0 ppm. The terminal CH_3 protons of the fatty acid moiety give rise to a triplet at 0.8 ppm. The acetyl CH_3 protons of the two N^{δ} -acetyl, N^{δ} -hydroxy-ornithines appear as a strong singlet at 2.0 ppm. Additionally, in the spectra of aquachelins A and C (Figures 2-27 and 2-28) multiplets are observed at 5.4 to 5.5 ppm which arise from the protons of the double bonds in those fatty acids. No resonances above 5.2 ppm are observed in the spectra of aquachelins B and D (Figures 2-29 and 2-30), as expected for the saturated fatty acids. Otherwise, the spectra are essentially identical.

The ^1H -NMR spectrum of aquachelin C in d_6 -DMSO (Figure 2-28) shows additional resonances at 9.7 and 12.7 ppm which are ascribed to the N^{δ} -hydroxy- ornithine N-OH protons, and the carboxylic acid protons respectively.

^{13}C NMR - The ^{13}C spectra of the aquachelins A, B and C were all obtained at 125 MHz on the General Electric GN500 instrument in the Chemistry Department at UCSB. The ^{13}C spectrum of aquachelin D was obtained at 125 MHz using the Varian Inova instrument in the Chemistry Department at UCSB. ^{13}C resonances for aquachelin A (Figure 2-31) between 170 and 180 ppm result from the carbonyl carbons including the carboxyl terminus, the β -hydroxy aspartate COOH and the seven amide carbonyl carbons

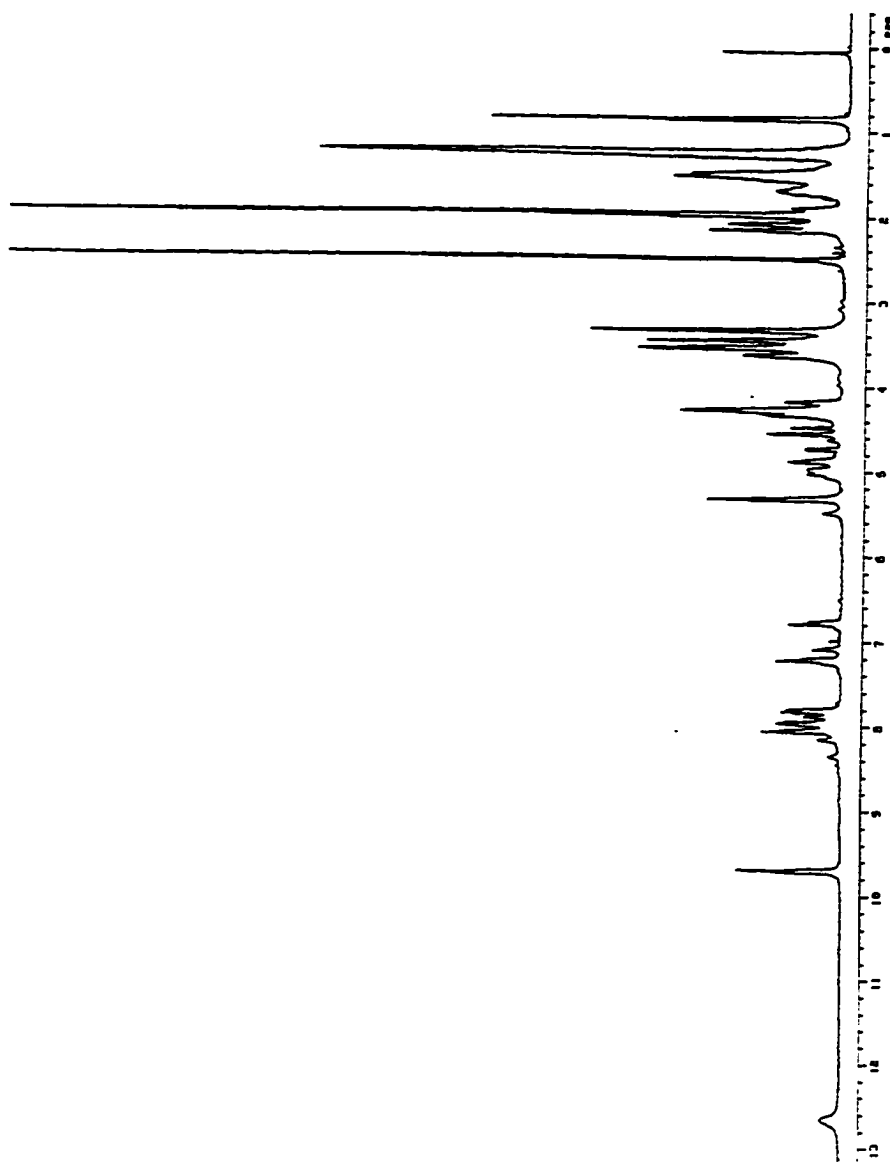


Figure 2-28. ^1H NMR spectrum of 7.5 mg aquachelin C in d_6 -DMSO obtained by Carole Bewley in Dr. John Faulkner's laboratory at Scripps Institution of Oceanography in San Diego, CA, using a 500 MHz Varian instrument. Some protonated solvent DMSO appears at 2.5 ppm, and residual water appears at 3.3 ppm.

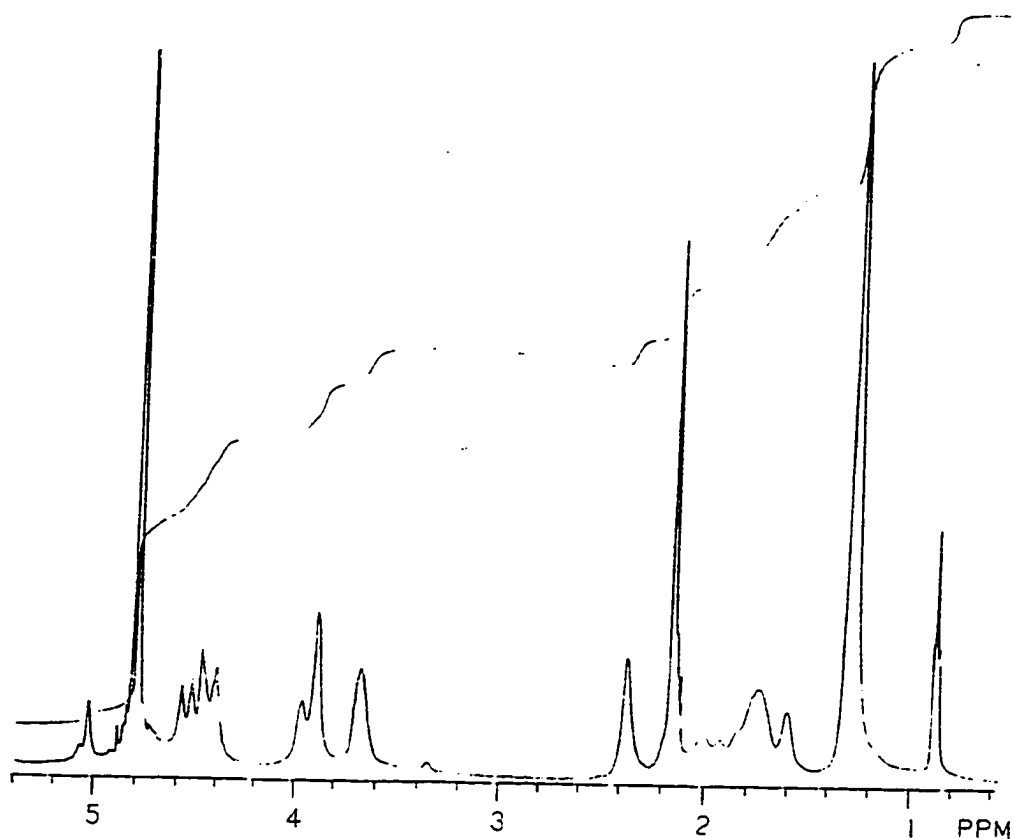


Figure 2-29. ¹H NMR spectrum of 10 mg aquachelin B in D₂O at 500 MHz on a General Electric instrument in the Chemistry Department at UCSB. The spectrum is referenced to solvent HDO at 4.8 ppm. Pulse width = 30 μsec; acquisition delay = 1.5 sec; sixteen transient acquisitions of 16,384 points.

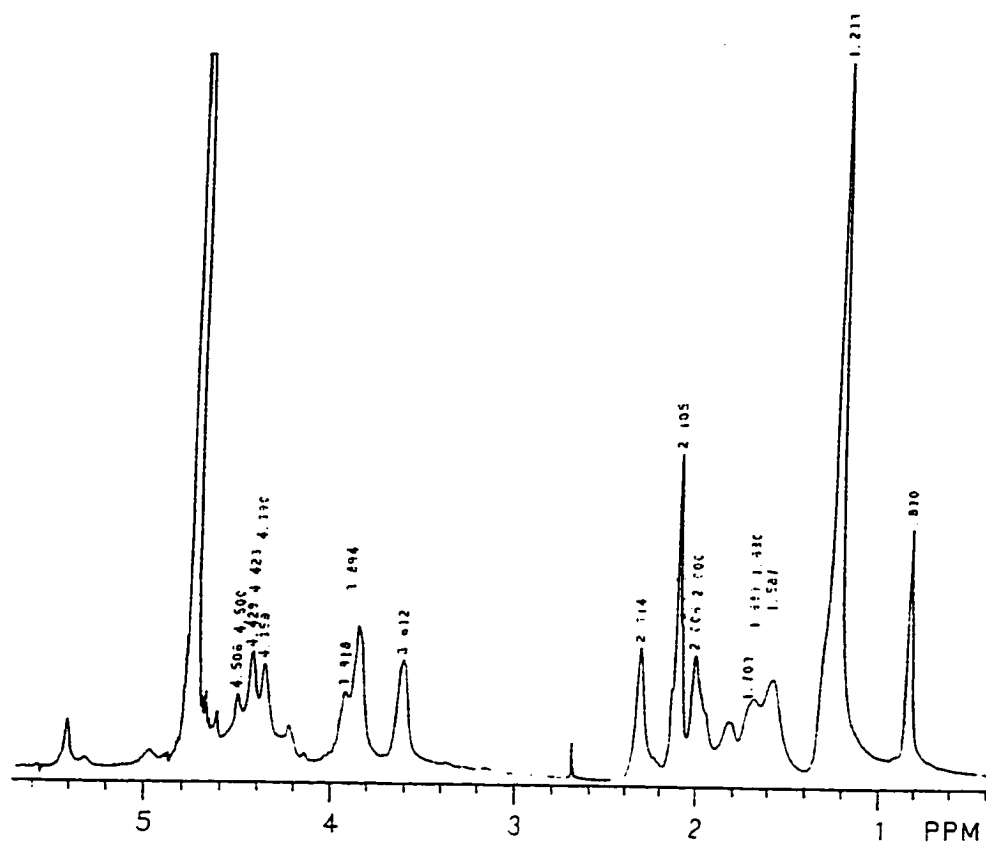


Figure 2-30. ¹H NMR spectrum of 8 mg aquachelin D in D₂O at 500 MHz on a General Electric instrument in the Chemistry Department at UCSB. The spectrum is referenced to solvent HDO at 4.8 ppm. Pulse width = 30 μsec; acquisition delay = 1.5 sec; 32 transient acquisitions of 16,384 points.

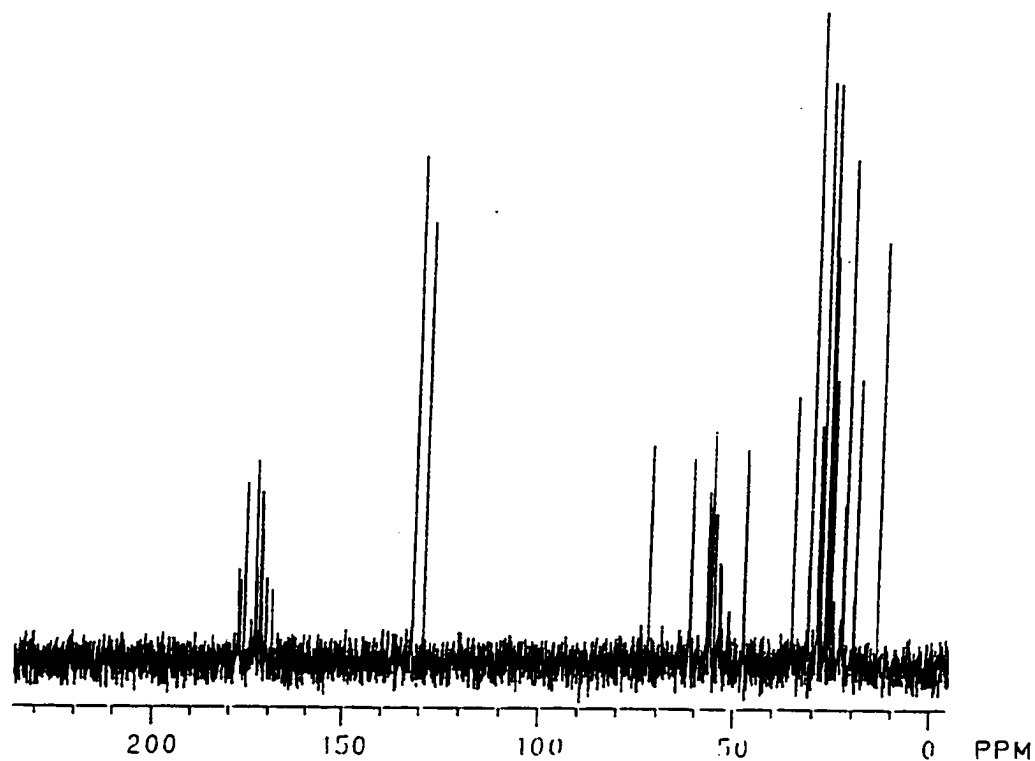


Figure 2-31. ^{13}C NMR spectrum of 10 mg aquachelin A in D_2O at 125 MHz on a General Electric instrument in the Chemistry Department at UCSB. 30,488 transient acquisitions of 16,384 points. Pulse width = 8 μsec ; acquisition delay = 1 sec; line broadening = 1 Hz; referenced to TMS as an external standard.

(six from the amide bonds and one from the glutamine side chain). Two carbon resonances at 129 and 131 ppm in aquachelin A appear as a result of the double bond. The carbon resonance at 72 ppm is from the β carbon of β -hydroxy aspartate. The resonances of the β carbons of the three serine residues are seen at 65-67 ppm. The ^{13}C resonances of the seven amino acid α carbons occur around 50 ppm. The δ carbon resonances of the N^{δ} -hydroxy, N^{δ} -acetyl-ornithines appear at 48 ppm. Methylene carbon resonances from the fatty acid chain and the β and γ methylene side chains of ornithine and glutamine are present between 20 and 35 ppm. The terminal methyl ^{13}C resonance appears at 13 ppm.

In aquachelin C, the double bond carbons occur at 130 ppm (Figure 2-32). No carbon resonances occur near 130 ppm in aquachelins B (Figure 2-33) and D (Figure 2-34), which is consistent with the absence of double bonds in those molecules.

Two-dimensional NMR - The configurations of the double bonds were determined to be *cis* by analyzing the chemical shifts of the carbon atoms adjacent to the double bond. DQF-COSY (Figure 2-35) and HMQC ($J = 150$ Hz) (Figure 2-36) connectivities obtained by Dr. Janet Cheetham confirm that the chemical shifts for aquachelin A are 27.3 and 26.7 ppm for the neighboring carbons ($\text{CH}_2\text{CH}=\text{}$). Calculations of the chemical shifts for *cis* and *trans*

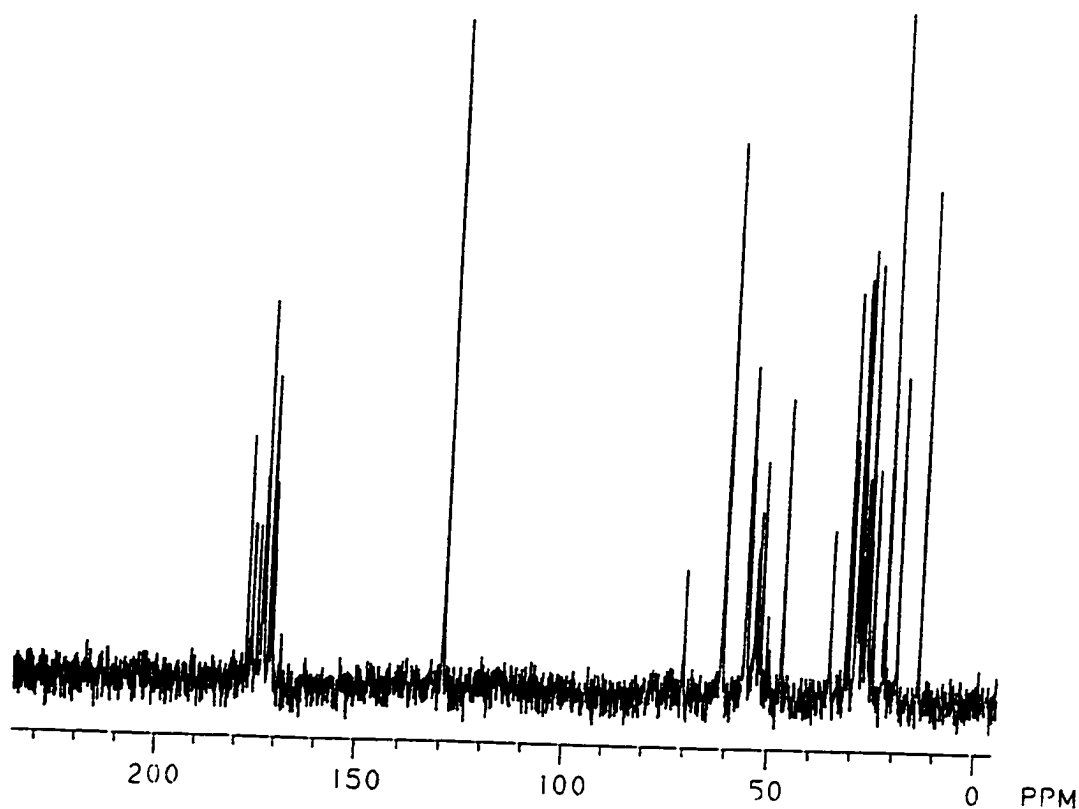


Figure 2-32. ¹³C NMR spectrum of 20 mg aquachelin C in D₂O at 125 MHz on a General Electric instrument in the Chemistry Department at UCSB. 30,012 transient acquisitions of 16,384 points. Pulse width = 8 μsec; acquisition delay = 1 sec; line broadening = 1 Hz; referenced to TMS as an external standard.

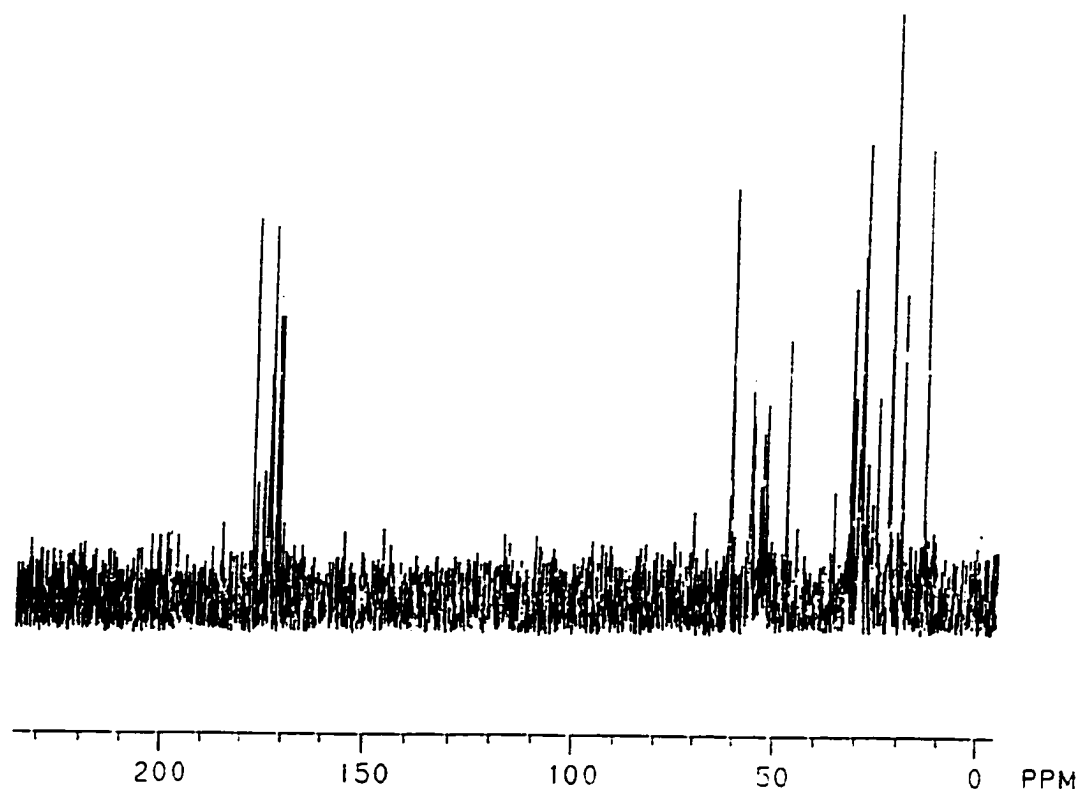


Figure 2-33. ¹³C NMR spectrum of 10 mg aquachelin B in D₂O at 125 MHz on a General Electric instrument in the Chemistry Department at UCSB. 29,636 transient acquisitions of 16,384 points. Pulse width = 8 μsec; acquisition delay = 1 sec; line broadening = 1 Hz; referenced to TMS as an external standard.

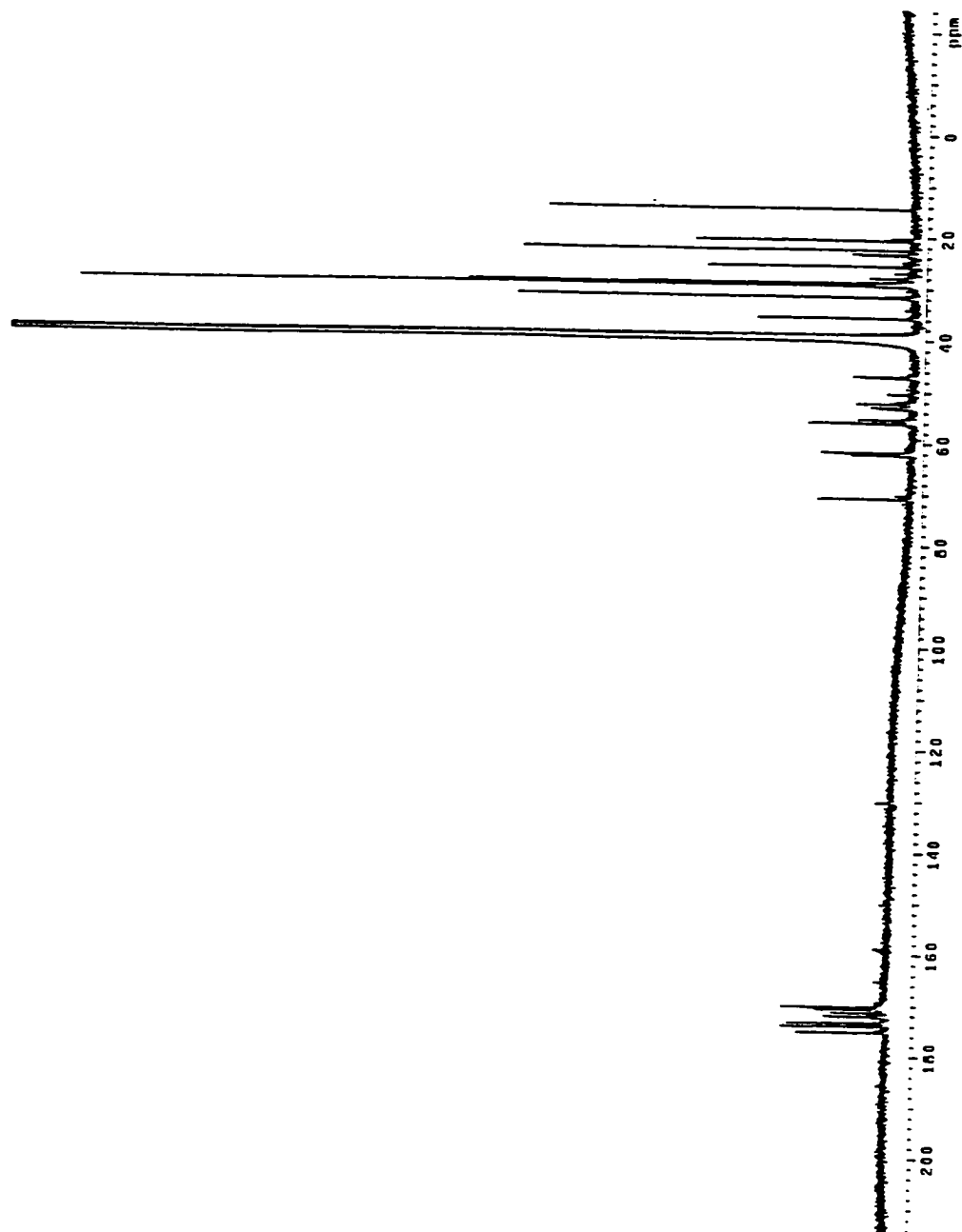


Figure 2-34. ¹³C NMR spectrum of 8 mg aquachelin D in d₆-DMSO at 125 MHz on a Varian instrument in the Chemistry Department at UCSB. Pulse width = 8 μsec; acquisition delay = 5 sec; referenced to TMS as an external standard.

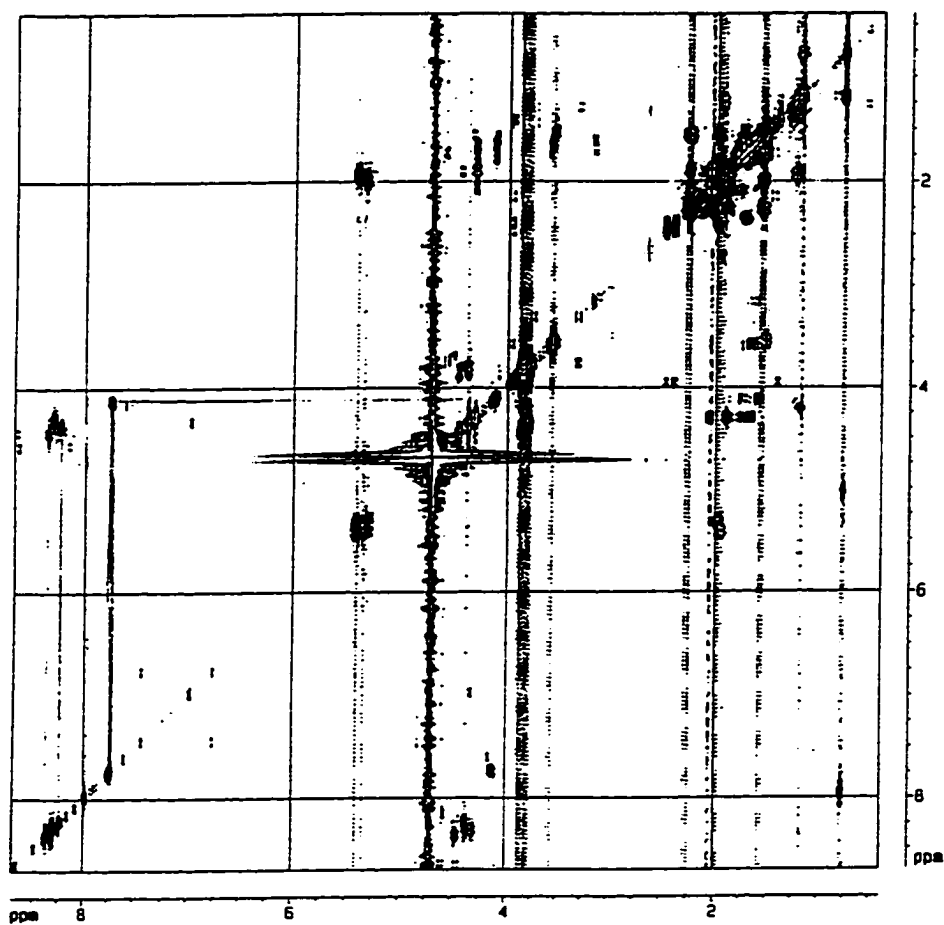


Figure 2-35. ^1H - ^1H DQF-COSY of 10 mg aquachelin A in 80% H_2O / 20% D_2O obtained by Janet Cheetham using the 500 MHz Bruker instrument in the College of Engineering at UCSB. Matrix size = 512 x 2048 points.

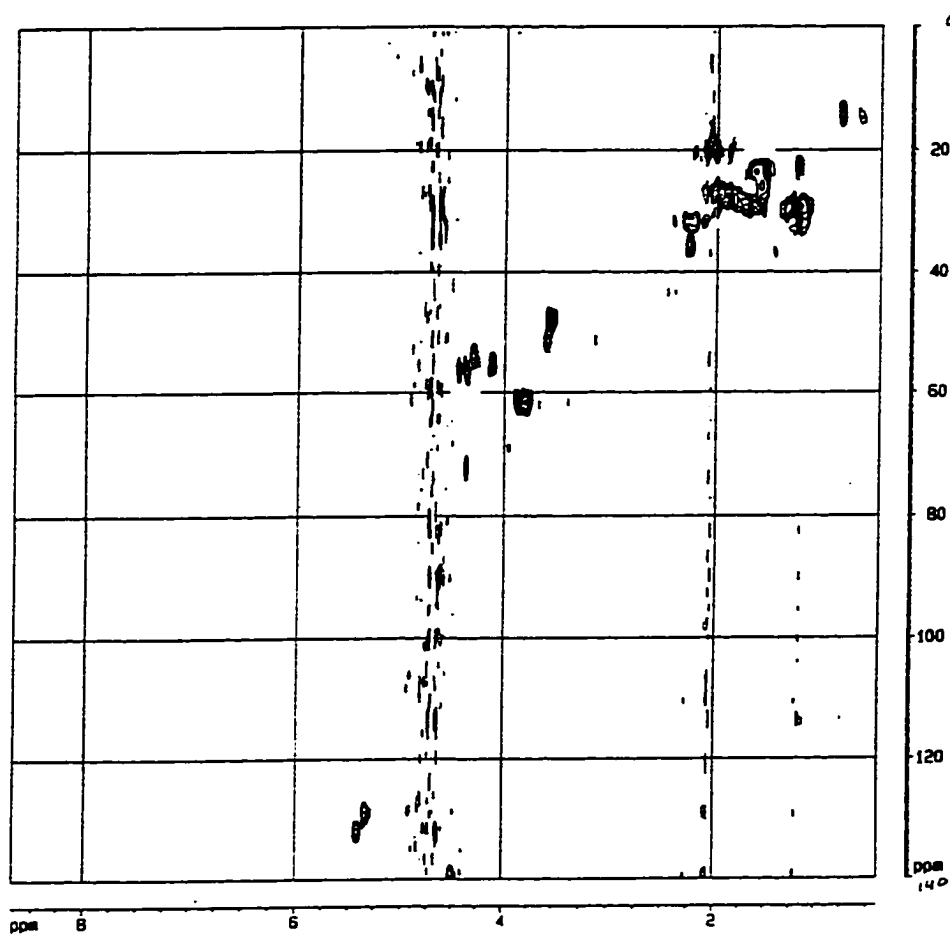


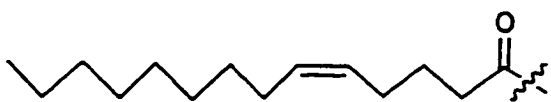
Figure 2-36. HMQC of 10 mg aquachelin A in 80% H₂O/ 20% D₂O obtained by Janet Cheetham using the 500 MHz Bruker instrument in the College of Engineering at UCSB. Optimized for $J_{\text{H-C}} = 150$ Hz; matrix size = 128 x 2048 points.

isomers of the fatty acids in aquachelins A and C were performed by Dr. John Faulkner of Scripps Institution of Oceanography using equations for prediction of carbon chemical shifts (Silverstein et al., 1981). The observed chemical shifts are consistent with a *cis* configuration (calculated to be 27.3 and 26.7 ppm), and not *trans* (calculated to be 33.3 and 32.7 ppm).

Aquachelin C is calculated to have ^{13}C chemical shifts of 27.5, 130.1, 130.1 and 28.0 ppm for the $\text{CH}_2\text{CH}=\text{CHCH}_2$ portion of the fatty acid chain if the configuration is *cis*. It is similar to myristoleic acid, *cis*-14:1(5)², (Scheme 2-5). According to the Aldrich Library of ^{13}C NMR spectra (Pouchert and Behnke, 1993) myristoleic acid has chemical shifts at 26.96, 129.72, 129.95 and 27.19 ppm. The *trans* isomer of 14:1(7)² has chemical shifts for the same carbons at 33.2, 131.1, 131.1 and 33.7 ppm. Again, the carbons adjacent to the double bond are quite distinctive. The DQF-COSY (Figure 2-37) and HMQC (Figure 2-38) obtained by Carole Bewley in Dr. John Faulkner's laboratory at Scripps Institution of Oceanography show that the experimental values for these carbons are 27.018, 129.636, 129.636 and 26.899 ppm, respectively. Therefore, the double bond in aquachelin C is also *cis*.

The spin systems for each amino acid are distinguishable on the basis of

²The nomenclature for fatty acids indicates the number of carbons, the number of double bonds and the location of the double bonds (counting from the carbonyl carbon). e.g. #C:# of double bonds (location of double bonds)



Scheme 2-5. Myristoleic acid, *cis*-14:1(5) or (*Z*)-5-tetradecenoic acid.

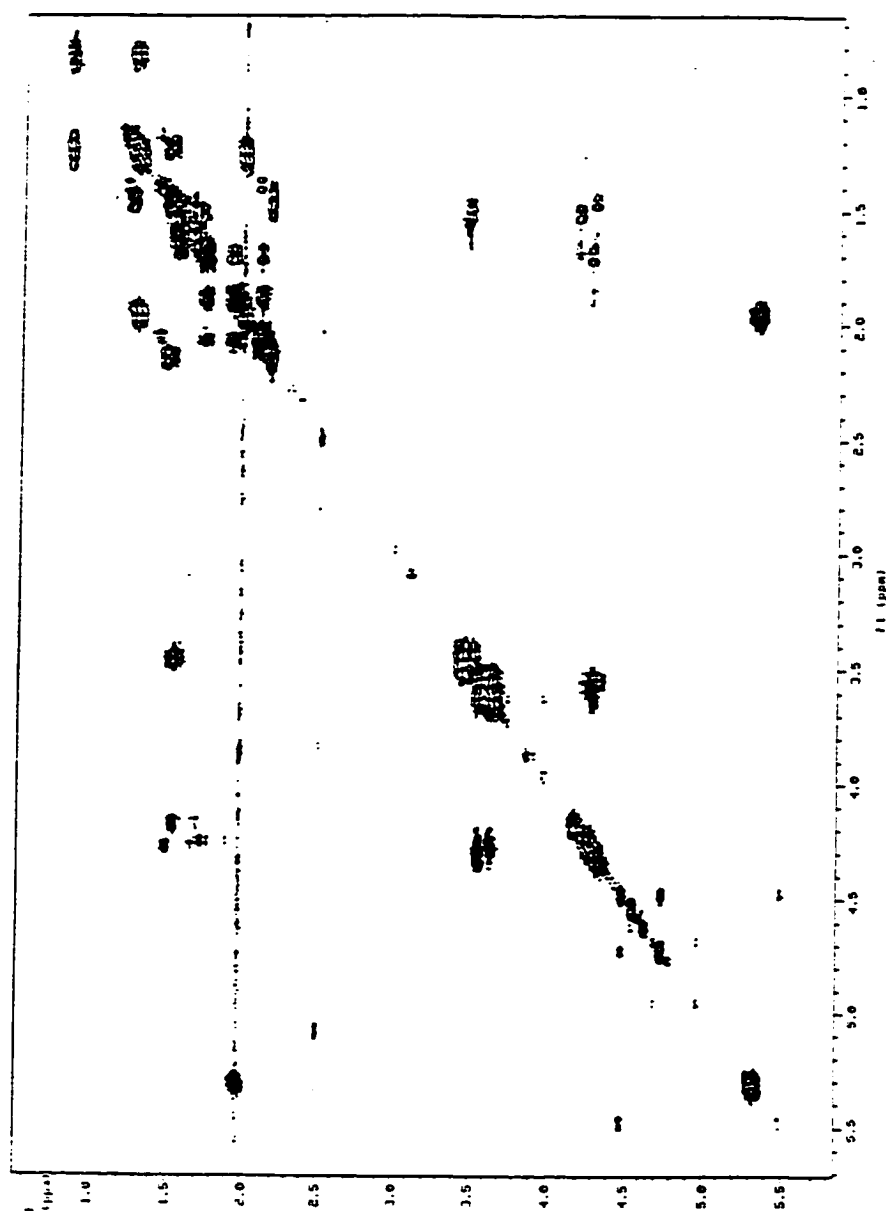


Figure 2-37. ^1H - ^1H DQF-COSY of 7.5 mg aquachelin C in d_6 -DMSO obtained by Carole Bewley in Dr. John Faulkner's laboratory at Scripps Institution of Oceanography in San Diego, CA, using a 500 MHz Varian instrument.

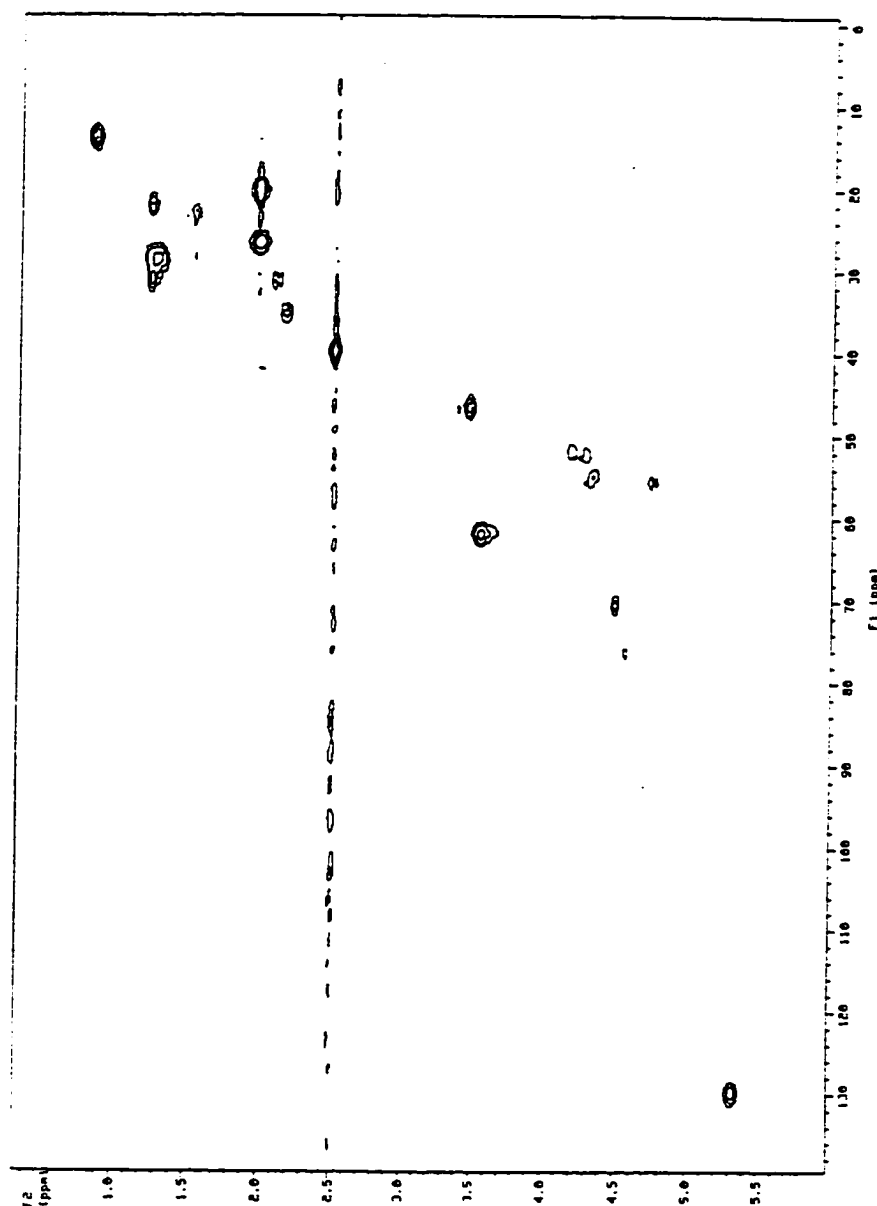


Figure 2-38. HMQC of 7.5 mg aquachelin C in d_6 -DMSO obtained by Carole Bewley in Dr. John Faulkner's laboratory at Scripps Institution of Oceanography in San Diego, CA, using a 500 MHz Varian instrument.

the TOCSY experiment. The TOCSY spectra for aquachelin A (obtained by Dr. Cheetham) and aquachelin C (obtained by Carole Bewley) are shown in Figures 2-39 and 2-40. Both short- and long-range ^1H - ^1H couplings are observed within amino acid side-chains. Only continuous chains of coupling are observable, however, which means that the individual amino acids are distinct. Long-range proton-carbon interactions are observable using the inverse-detected HMQC ($J = 4$ Hz) or HMBC experiment. Spectra for aquachelins A and C are shown in Figures 2-41 and 2-42. Because of the near overlap of the serine and ornithine resonances, however, the sequence of amino acids was not obtained from this experiment.

In summary, the NMR data indicate that the glutamic acid observed in the amino acid analysis is derived from glutamine. Also, the chemical shifts of the carbon atoms adjacent to the double bonds in aquachelins A and C are characteristic of a *cis* double bond.

Configuration of the amino acids in sequence: Although the mass spectra enabled the determination of the amino acid sequence, and chiral amino acid analysis provided the relative ratios of each isomer of each amino acid residue, there remained the unanswered question of the stereochemistry of the D- and L-serines and ornithines. At least one of each amino acid isomer is present in the

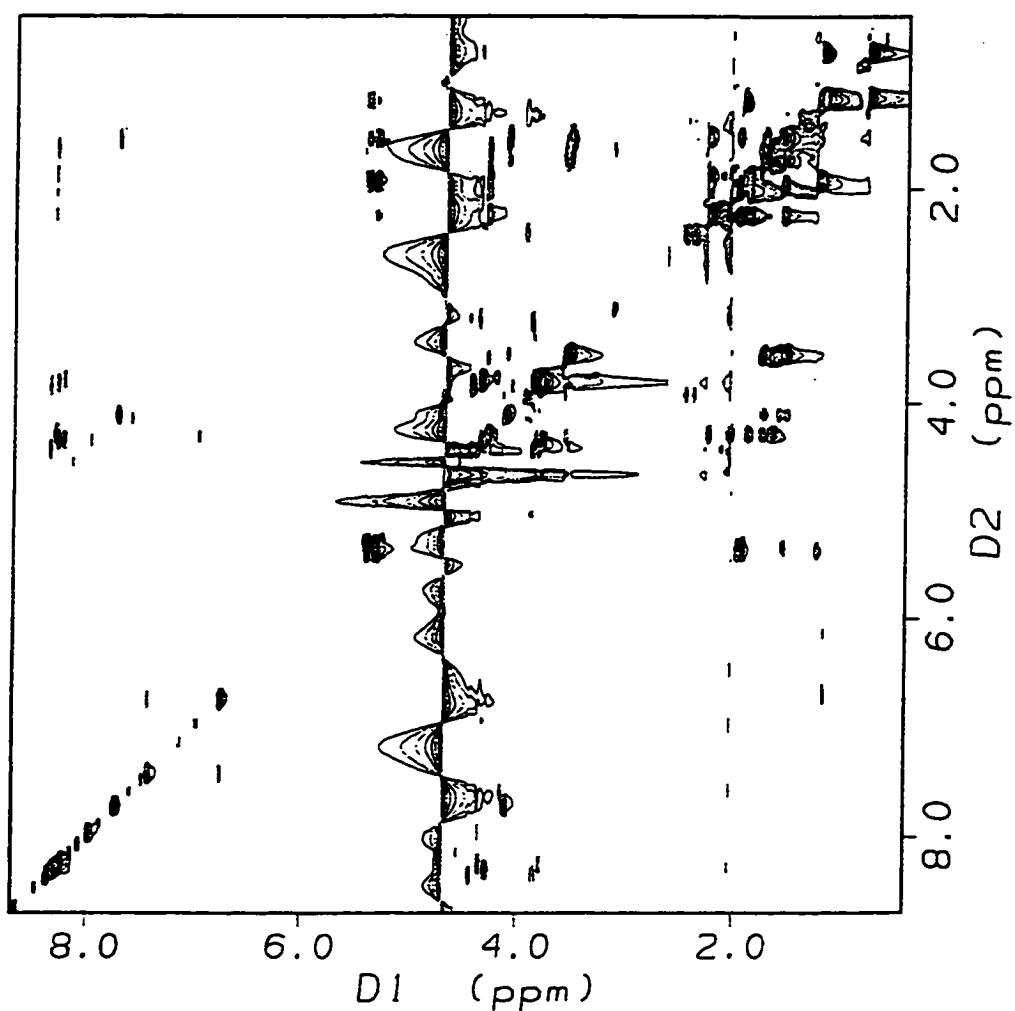


Figure 2-39. TOCSY of 10 mg aquachelin A in 80% H₂O/ 20% D₂O obtained by Janet Cheetham using the 500 MHz Bruker instrument in the College of Engineering at UCSB. Matrix size = 200 x 2048 points.

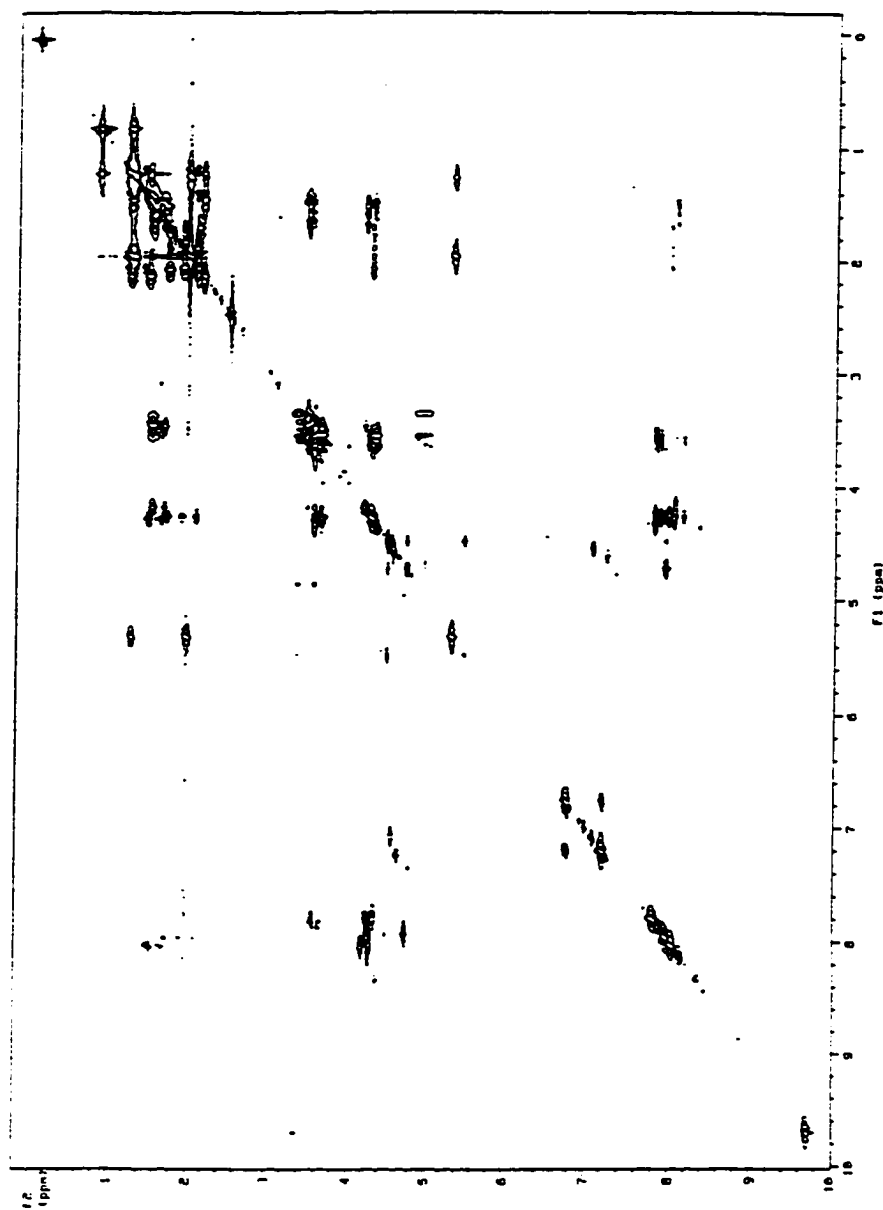


Figure 2-40. TOCSY of 7.5 mg aquachelin C in d_6 -DMSO obtained by Carole Bewley in Dr. John Faulkner's laboratory at Scripps Institution of Oceanography in San Diego, CA, using a 500 MHz Varian instrument.

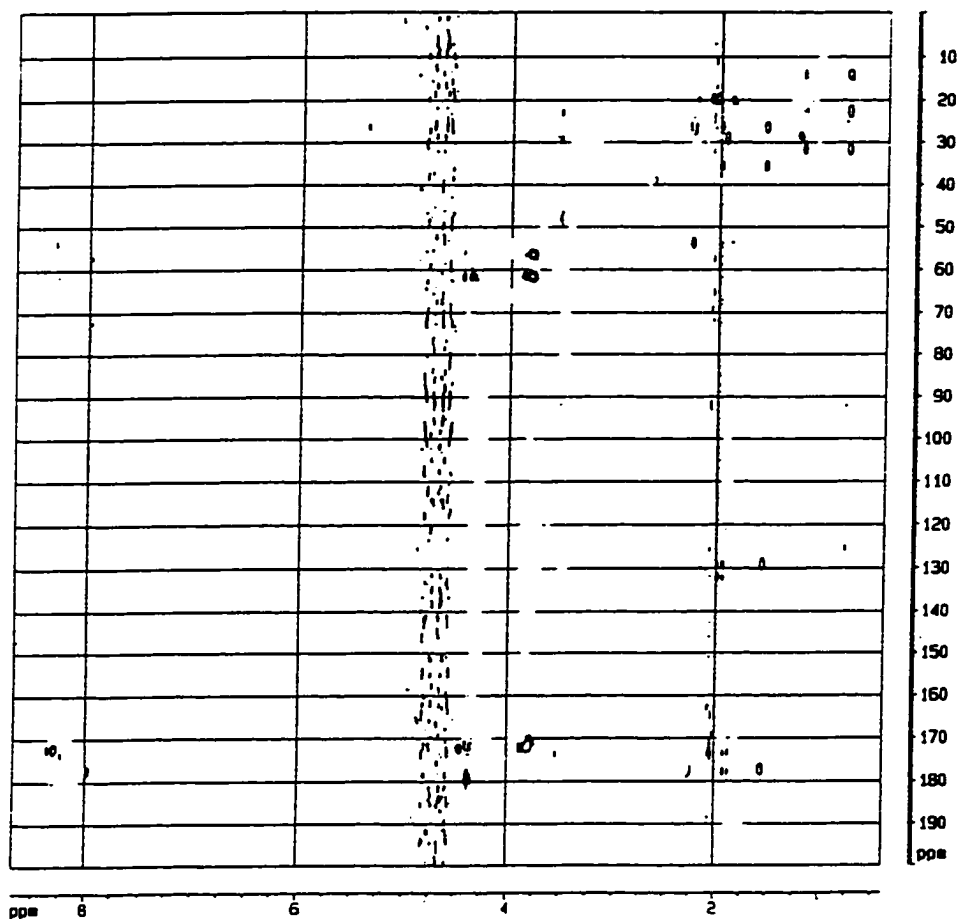


Figure 2-41. HMQC of 10 mg aquachelin A in 80% H₂O/ 20% D₂O obtained by Janet Cheetham using the 500 MHz Bruker instrument in the College of Engineering at UCSB. Optimized for $J_{H-C} = 4$ Hz. Matrix size = 200 x 2048 points.

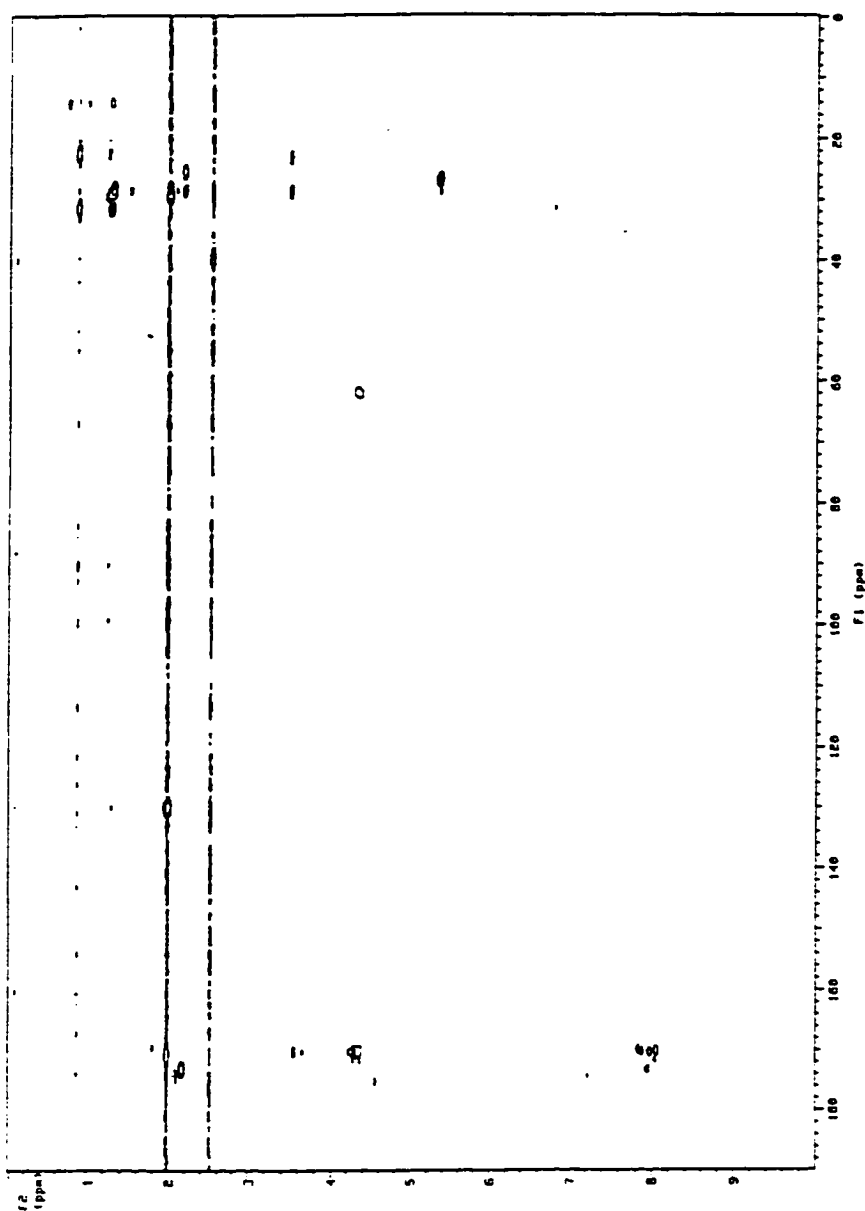


Figure 2-42. HMBC of 7.5 mg aquachelin C in d_6 -DMSO obtained by Carole Bewley in Dr. John Faulkner's laboratory at Scripps Institution of Oceanography in San Diego, CA, using a 500 MHz Varian instrument.

peptide.

Growth of DS40M3 with added ornithine - Deuterated L amino acids were fed to the bacteria in an attempt to distinguish the chiralities of the two serine and ornithine sites. In the presence of 5 mM d₃-L-serine, the bacteria did not produce as much siderophore as in the presence of d₆-L-ornithine. Isolation of the siderophores from both experiments through a single HPLC separation resulted in small samples for ESI-MS analysis. Only aquachelins B and C were isolated from the serine culture, and only the aluminum and iron(III) adducts were clearly observable. The mass spectra show increases in mass of 3, 6 or 9 (Table 2-8) over the non-deuterated iron(III)-aquachelin C (m/z 1144). Therefore, up to 3 d₃-L-serines are included in the molecule, implying conversion of L to D by the biosynthetic process. Analogously, growth in the presence of d₆-L-ornithine gives aquachelins with mass increases of 12 units (Table 2-8) over the non-labelled molecules (see Table 2-3). Addition of 12 mass units indicates incorporation of the labelled ornithine at both available positions in the peptide.

Partial hydrolysis in dilute HCl and HI - Partial hydrolysis of aquachelin C by dilute acid overnight followed by Marfey's derivatization (see Experimental) was attempted in order to determine the chirality of the amino acids released. Terminal amino acids are expected to be released more readily

d₆-L-ornithine:

Species	Aquachelin A	Aquachelin B	Aquachelin C	Aquachelin D
MH ⁺ + 12	-	1077.3	-	1105.3
Al-MH ⁺ + 12	1099.0	1100.8	1127.5	1129.5
Fe(III)-MH ⁺ + 12	1128.3	1130	1156.4	1158.5

d₃-L-serine:

Species	Aquachelin B	Aquachelin C
Al-MH ⁺ + 3	-	1118.5
Al-MH ⁺ + 6	1095.5	1121.6
Al-MH ⁺ + 9	1098.7	1124.4
Fe(III)-MH ⁺ + 3	-	1146.7
Fe(III)-MH ⁺ + 6	1124.2	1149.5
Fe(III)-MH ⁺ + 9	1127.5	1152.8

Table 2-8. Electrospray mass spectrometry results for deuterium-labelled aquachelins produced by growth of *Deleya aquamarina* strain DS40M3 in the presence of d₆-L-ornithine or d₃-L-serine. Molecular ions were not observed for the serine samples, but the aluminum and iron(III) complexes were observed as shown with the indicated increase in mass from deuterium substitution.

than internal ones. Only a few amino acids can be identified from the hydrolysis results. *L-threo-β*-hydroxy aspartate (25.672 min in Figure 2-43) is quickly released even at the lowest acid concentrations. Also, D-serine is readily visible at 33.030 min., while the other amino acid peaks are small or absent (Figure 2-43). Excess Marfey's appears at 45.947 min, and a new peak at 42.313 minutes is most likely from the remaining un-hydrolyzed peptide. From this evidence, the serine adjacent to the *β*-hydroxy aspartate is most likely a D-serine. However, because of uncertainty in the interpretation of the results and the possibility of multiple cleavage products, this line of investigation did not yield sufficient information to identify the stereochemical amino acid sequence. Separation of individual peptides after partial hydrolysis, as described below, is a preferable approach.

DABITC: The final method used to determine the stereochemical sequence of the aquachelins proceeded as follows: partial hydrolysis with brief heating to 90 °C; derivatization with DABITC and separation of the derivatized peptides; then total hydrolysis and chiral GC amino acid analysis of the peptides.

Partial hydrolysis ESI-MS results - Five to 30 minute hydrolyses in 6 M HCl were compared to determine the optimal time for production of small peptides. Hydrolysis for 20 minutes is optimal for production of the greatest

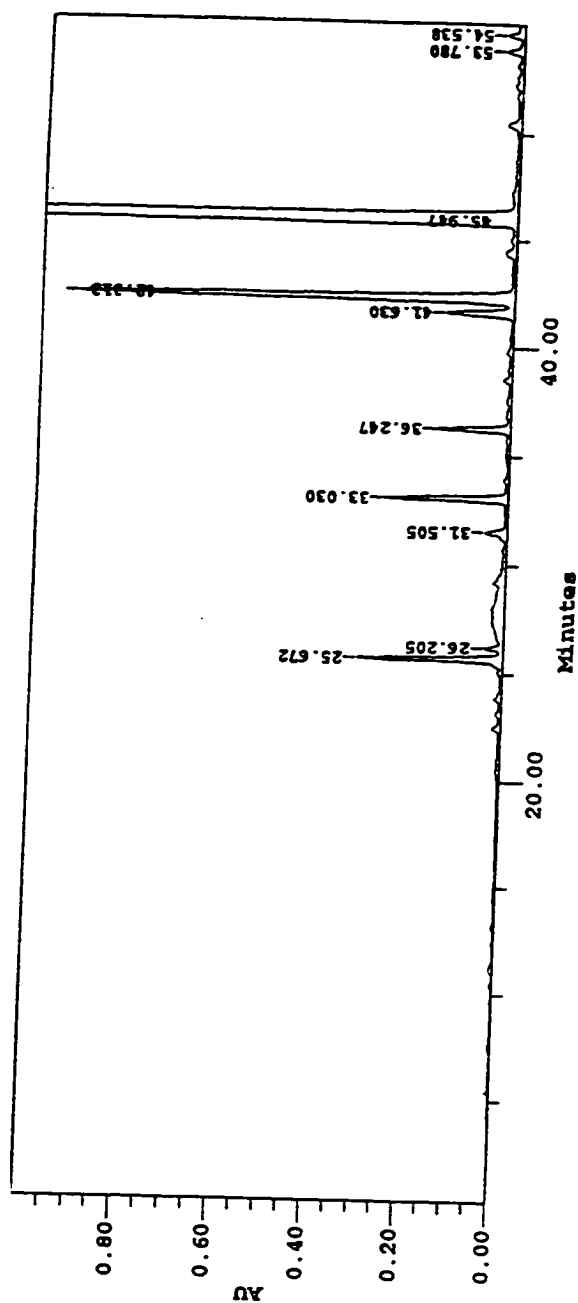


Figure 2-43. Partial hydrolysis of aquachelin C in 0.1 M HCl with Marfey's derivatization showing the release of L-threo- β -hydroxy aspartate at 25.672 min and D-serine at 33.030 min in 1:1 ratios. The HPLC column was an analytical C-18 (Vydac or YMC ODS-AQ, 4.6 x 150 mm) using a gradient from 10% B (B = CH₃CN) to 40% B in 45 minutes at a flow rate of 0.8 mL/min. Solvent A = 50 mM triethylamine phosphate, pH 3.0. Ten microliter samples were injected and the absorbance was monitored at 340 nm.

number of useful fragments. Shorter hydrolysis times lead to smaller amounts of the interesting fragments, and a significant fraction of the initial molecule still present. Longer times give fewer, smaller fragments. This result parallels that reported by Demange et al. (1990) for pyoverdins Pt and Pf who also found 20 minutes to be the optimal hydrolysis time.

Derivatization and HPLC separation of DABITC peptides - The orange DABITC peptides are only partially soluble in 50% acetonitrile/ water. The more hydrophobic peptides are soluble in pyridine, but are not suitable for HPLC analysis using an acetonitrile buffer system (Chang, 1981a). Three different categories of peaks were observed. Initial peaks may be non-derivatized material containing the fatty acid tail. Several peaks are fairly well-separated from each other in the central portion of the gradient. A number of strong peaks were observed close together at the end of the gradient, including excess DABITC. Twelve peaks were separated after partial hydrolysis of aquachelin C. The sixteen DABITC-derivatized peptides obtained from partial hydrolysis of aquachelin B were separated by HPLC as shown in Figure 2-44.

Chiral GC amino acid analysis of the DABITC peptides - The amino acid sequence of aquachelin B is identified by examination of the chiral GC amino acid analysis of the peptide fragments from partial hydrolysis of the siderophore (Table 2-9) The position of the L-serine was deduced from peak 4

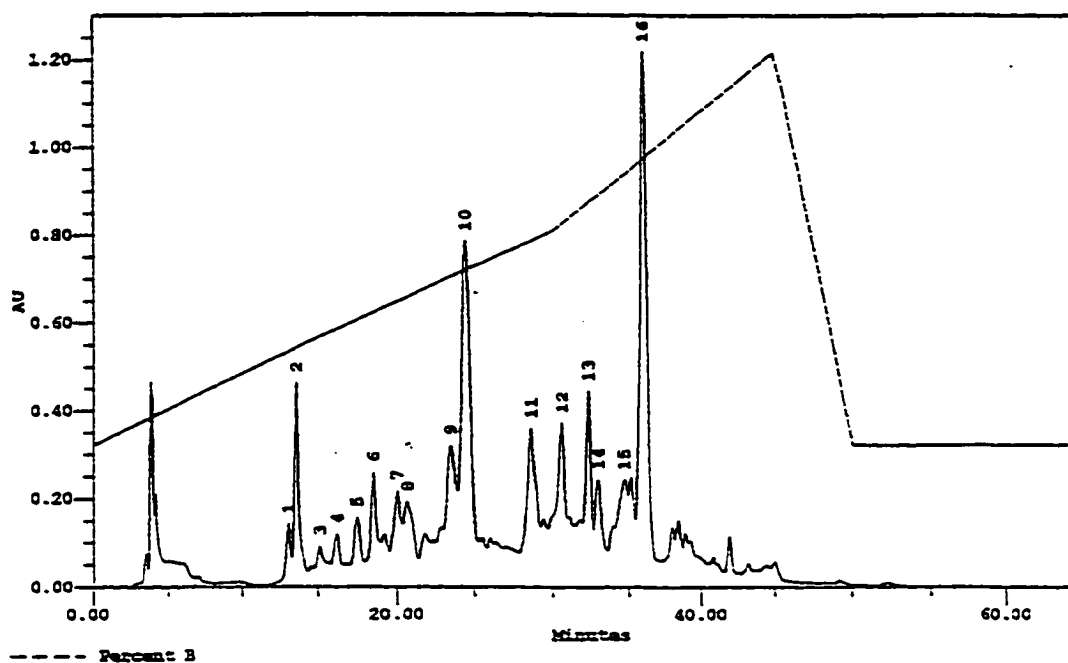


Figure 2-44. DABITC HPLC separation for partially hydrolyzed aquachelin B. Derivatized peptides were separated on a reverse phase C-18 (YMC ODS-AQ) HPLC column (4.6 x 150 mm) using 10 mM $\text{NH}_4\text{CH}_3\text{CO}_2$, pH 5 as solvent A and CH_3CN as solvent B. The gradient program went from 20% B to 50% B in 30 minutes, then to 75% B by 45 minutes at 1 mL/min. The Waters 486 detector was set to measure the absorbance at 436 nm.

Peptide fragment	Amino acids determined by Chirasil-Val GC
1	D-ser, L-ser, D-gln, D-orn, L-orn
2	2 D-ser, L-ser
3	n.d.
4	L-ser, D-gln
5	D-ser, L-ser, D-gln, D-orn
6	n.d.
7	n.d.
8	L-orn
9	2 D-ser, L-ser, D-gln, D-orn, 3 L-orn
10	L-orn
11	D-ser, L-ser, D-gln, D-orn
12	L-ser, D-gln, D-orn
13	D-gln, D-orn
14	D-gln, D-orn
15	D-ser, L-ser, D-gln, D-orn, L-orn
16	3 D-ser, 2 L-ser, D-gln, 2 D-orn, L-orn

Table 2-9. Chiral gas chromatographic amino acid analysis of DABITC peptides from aquachelin B. HPLC peaks 3, 6 and 7 were small or poorly separated and were not analyzed.

(Figure 2-45) which demonstrates that L-serine must be next to D-glutamine. The C-terminal residue must be L-ornithine, as shown by peaks 5 (Figure 2-46) and 11 which contain the same amino acids but no L-ornithine, and therefore must be cleaving to include one or the other of the D-serines, but not both. Also, a large peak for L-ornithine alone (peak 10) is observed, indicating it is likely to be a terminal residue.

The amino acid analysis of aquachelin C is consistent with the results from aquachelin B, but the samples are not as clean and the results are somewhat ambiguous. The presence of side-products which complicate the GC analysis and decrease the signal to noise ratio is probably a result of oxidation during hydrolysis. Argon bubbling of the peptide solutions for one minute before hydrolysis in 6 M HCl avoids oxidation and produces cleaner GC results.

Thus, by analyzing the stereochemistry of the amino acids in the peptides generated by partial hydrolysis of aquachelin B, it was possible to determine the positions of the D- and L-serine and ornithine residues. Since the amino acid composition and sequence of each of the siderophores is identical according to chiral HPLC amino acid analysis and tandem mass spectrometry, respectively, the sequence stereochemistry is presumed to be identical in all four aquachelins. The only structural differences occur in the fatty acid chains.

The structures of the aquachelins consist of the heptapeptide *L-threo-β*-

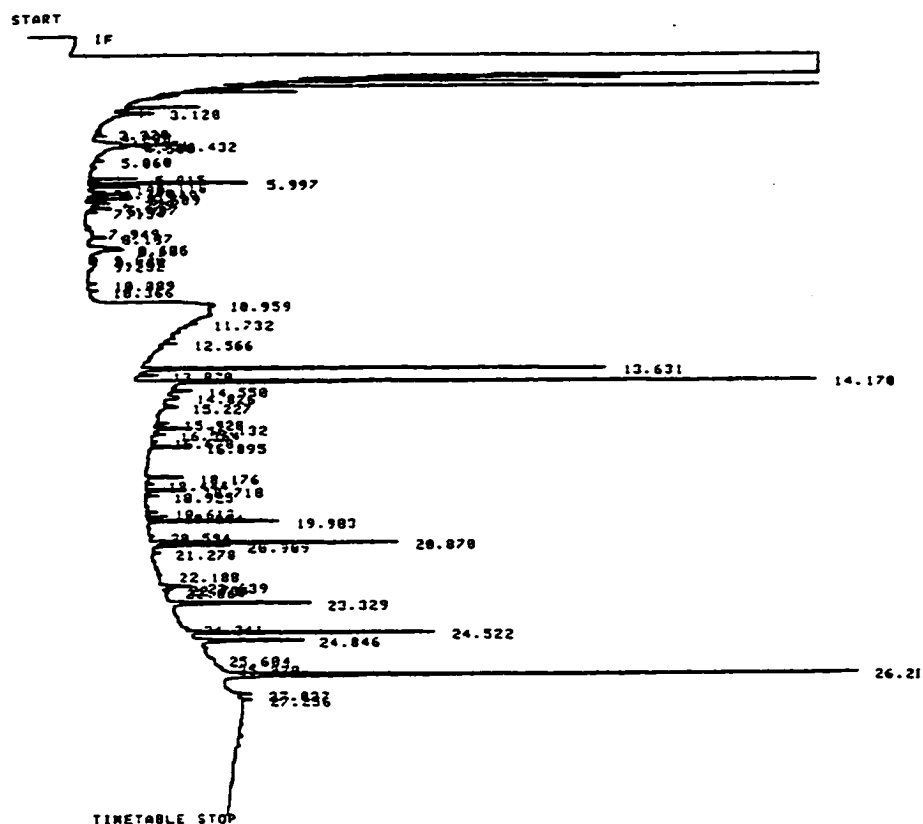


Figure 2-45. GC chromatogram of DABITC peptide 4 from aquachelin B showing the presence of L-serine at 5.997 min and D-glutamic acid (from glutamine - see Results) at 13.631 min.

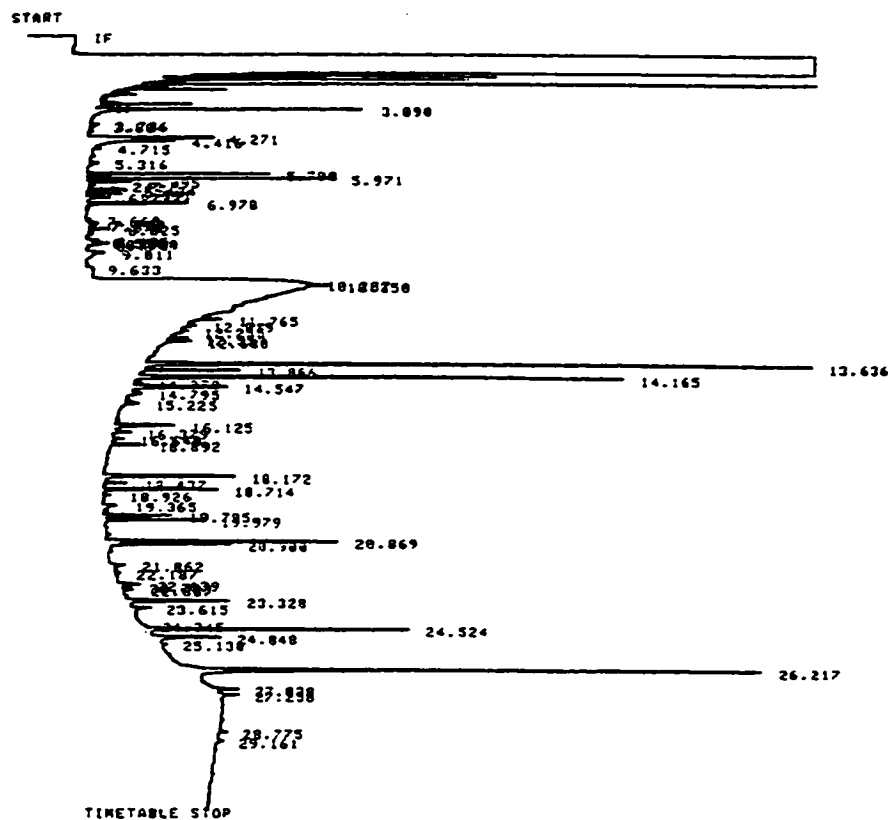


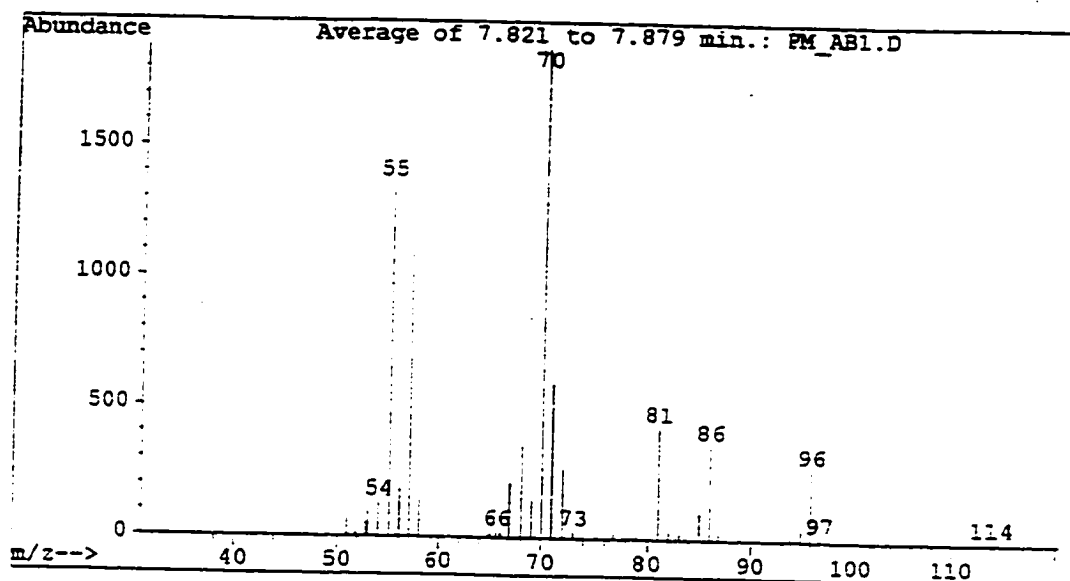
Figure 2-46. GC chromatogram of DABITC peptide 5 from aquachelin B showing the presence of D-serine at 5.790 min, L-serine at 5.971 min, D-glutamic acid (from glutamine - see Results) at 13.636 min, and D-ornithine at 18.172 min.

hydroxy aspartate, D-serine, L-serine, D-glutamine, D-*N*⁶-acetyl, *N*⁶-hydroxy-ornithine, D-serine, L-*N*⁶-acetyl, *N*⁶-hydroxy-ornithine attached at the N-terminus to a saturated or mono-unsaturated fatty acid, C₁₂ or C₁₄.

Identification of the position of the double bonds in aquachelins A and C:

Ozonolysis of the two siderophores which contain double bonds (aquachelins A and C) shows the production of heptanal (*m/z* 114) by GC/MS (Figure 2-47). Therefore, the fatty acids are 12:1(5) and 14:1(7) in aquachelins A and C respectively. The central double bonds explain the chemical similarity observed in the ¹³C NMR, where the double bond carbon resonances are close together (129 and 131 ppm, Figure 2-31) in aquachelin A, and overlapped (at 130 ppm, Figure 2-32) in aquachelin C.

Electrospray mass spectrometry of the aqueous layers shows a mixture of species fourteen mass units apart (see Appendix Figures A-24 and A-25). This could be interpreted to mean that a number of different double-bond isomers are present in the siderophores. However, the control on aquachelin B, which contains a saturated fatty acid, also showed a series of ions 14 and 28 mass units apart (Appendix Figure A-26), indicating that side reactions are taking place in the aqueous layer. Also, the GC/MS results give no indication of a mixture of aldehyde products.



114
Heptanal

$C_7H_{14}O$

$CH_3(CH_2)_5CHO$



Figure 2-47. GC/MS analysis of heptanal from ozonolysis of aquachelin C. GC/MS was performed by Paul Mollard in Professor Bruce Lipshutz' lab in the Chemistry department at UCSB. The standard spectrum of heptanal is from the EPA/NIH database (Heller and Milne, 1978). The mass range of the instrument was set to collect only above m/z 50, so the smaller fragments in the standard are not observed in the sample.

UV-Visible Spectrophotometry: At pH 7.46 a shoulder of the iron(III)-aquachelin C spectrum appears at approximately 400 nm with an extinction coefficient of $1880 \text{ M}^{-1}\text{cm}^{-1}$ (Figure 2-48). At pH 2.07 the λ_{max} is 426 nm with an extinction coefficient of $1558 \text{ M}^{-1}\text{cm}^{-1}$. Below pH 2, the λ_{max} shifts even further to 475 nm by pH 1.11. The spectrophotometric behavior of Fe(III)-aquachelin B as the pH is lowered is very similar to that of Fe(III)-aquachelin C (Figure 2-49).

As the acid concentration increases, the aquachelins become partially protonated and iron(III) coordination begins to change from bis-hydroxamate toward mono-hydroxamate. The shift occurs between pH 3 and 2 in Figures 2-48 and 2-49. See the Discussion section for further explanation. Also see Chapter 4 for the analogous spectra of aerobactin, another bis-hydroxamate with an α hydroxy acid functionality which coordinates to iron(III).

Production of aquachelins by *D. aquamarina* (ATCC #14400) and *H. meridiana* (ATCC #49692): Two species, *Deleya aquamarina* and *Halomonas meridiana*, were determined by Professor Margo Haygood of Scripps Institution of Oceanography at the University of California, San Diego to genetically match DS40M3 (see below). The type strains of both species also produce the

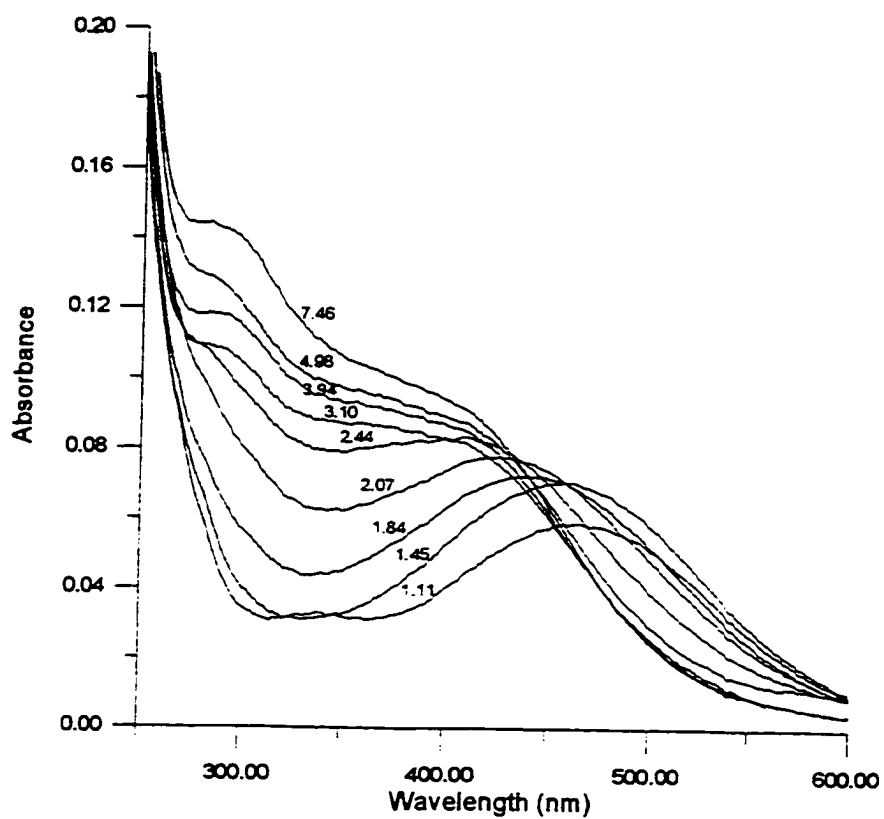


Figure 2-48. UV-visible spectra of 50 μM Fe(III)-aquachelin C at various pH values, obtained in a 1 cm cell.

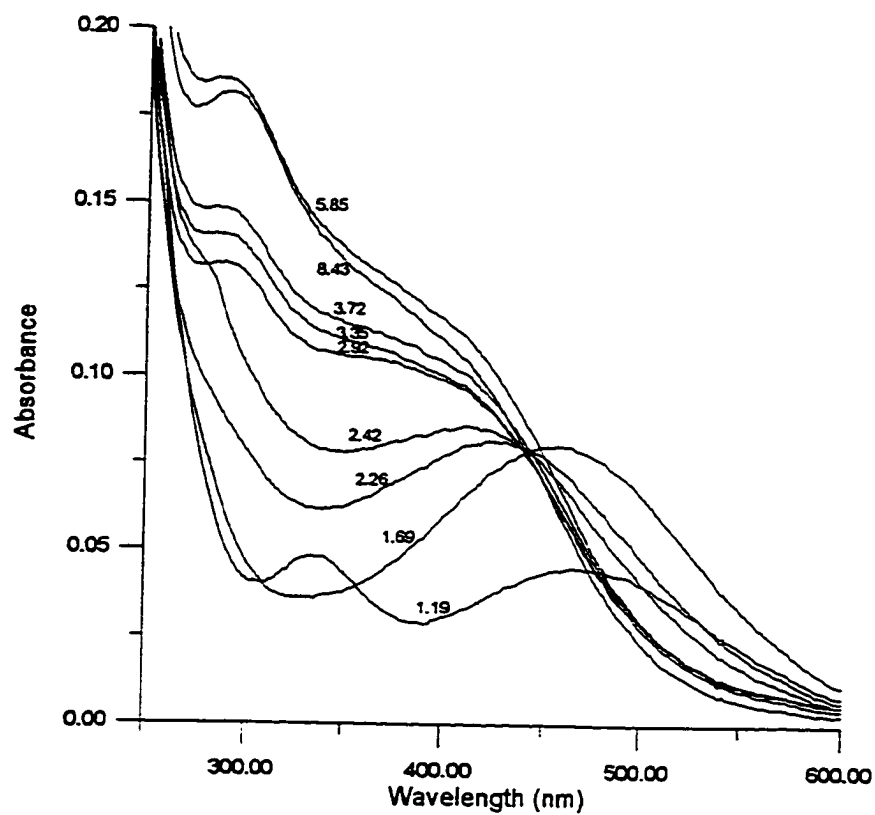


Figure 2-49. UV-visible spectra of 50 μM Fe(III)-aquachelin B at various pH values, obtained in a 1 cm cell.

aquachelins. After isolation of the siderophores by the method described in the Experimental section, both *D. aquamarina* and *H. meridiana* yield small amounts of the same siderophores produced by DS40M3. The HPLC retention times (Figure 2-50) and the ESI-MS masses of the siderophores (Table 2-10) correlate with the four aquachelin fractions. *H. meridiana* (ATCC #49692) produces significantly more total siderophore than does *D. aquamarina* (ATCC #14400), but neither species produces as much as *D. aquamarina* DS40M3.

Discussion

A series of four new siderophores, aquachelins A-D (Figure 2-51), were isolated from the marine bacterium *Deleya aquamarina* DS40M3. The biosynthesis of the aquachelins is turned on in low iron(III) and off in high iron(III) environments (Figure 2-2), indicating that they are used as siderophores by the bacterium. Siderophores are defined as low molecular weight, iron(III) specific ligands whose synthesis is induced by growth in iron-deficient conditions (Neilands, 1984). The aquachelins consist of a fatty acid chain of 12 or 14 carbons in length, saturated or mono-unsaturated, attached to a heptapeptide containing β -hydroxy aspartate and two *N*⁶-acetyl, *N*⁵-hydroxy-ornithine residues as coordination sites for iron(III).

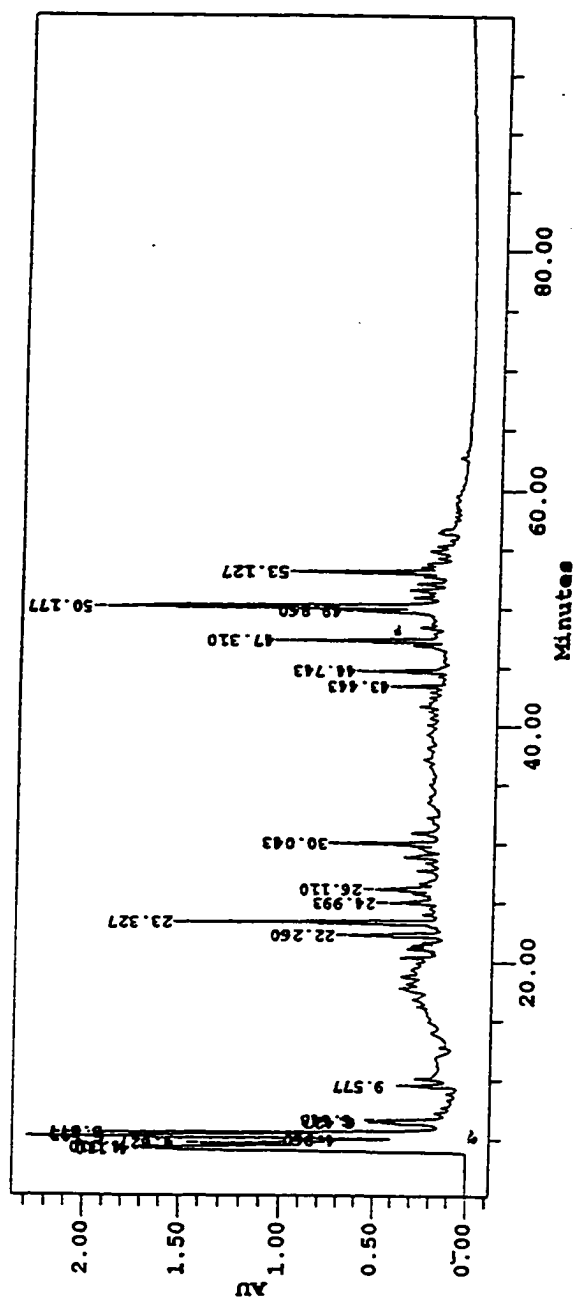


Figure 2-50. HPLC chromatogram of the aquachelins isolated from *Halomonas meridiana* (ATCC #49692) on an analytical C-4 HPLC column (Vydac, 4.6 x 150 mm) using a gradient beginning with 5 minutes at 100% A (0.1% TFA), followed by a linear gradient to 100% B (80% CH₃CN, 20% H₂O, 0.1% TFA) in 90 minutes.

Aquachelin	DS40M3 <i>m/z</i>	<i>H. meridiana</i> <i>m/z</i>	<i>D. aquamarina</i> <i>m/z</i>
A	1063.8	(1063.4) ^a	1063.3
B	1066.0	1065.4	1065.3
C	1091.9	1091.4	1091.4
D	1093.8	1093.4	1093.3

Table 2-10. Electrospray mass spectrometry masses for the siderophores from strain DS40M3, *H. meridiana* (ATCC #49692) and *D. aquamarina* (ATCC #14400).

^aCalculated from the aluminum adduct at *m/z* 1087.4 because the molecular ion was not observed. See Table 2-3 for the masses of the DS40M3 aluminum and iron adducts.

Iron coordination: The β -hydroxy aspartate moiety is most likely involved in complexing iron(III). Teintze et al. (1981) showed coordination of iron(III) by β -hydroxy aspartate in the X-ray crystal structure of ferric pseudobactin. The aquachelins form 1:1 iron(III):ligand complexes. Only 1:1 complexes were observed in the electrospray mass spectra (Figures 2-12 to 2-15).

The UV-visible behavior of the aquachelins is slightly different from that of aerobactin (Figure 4-14; Harris et al, 1979). It appears that the α -hydroxy acid function of β -hydroxy aspartate gives a more stable iron(III) complex than the same structure from citric acid. The ferric aquachelin absorbance maxima shift from 426 to 475 nm between pH 3 and 1.1 (Figures 2-48 and 2-49) as the pH is lowered. The spectrum changes from the orange color characteristic of a bis- or tris-hydroxamate iron(III) complex toward the purple of a monohydroxamate iron(III) species. Loss of the isosbestic point under the most acidic conditions (approaching pH 1) indicates that more than two species are present, and it may be interpreted as a partial loss of iron(III) coordination.

Fatty acids: The production of four related siderophores is relatively uncommon. Series of siderophores differing only in the identity of fatty acid chains have previously been isolated only from mycobacteria. Mycobacteria produce mycobactins, membrane-bound siderophores which contain long-chain

fatty acids (C_{14} to C_{20}) (Snow, 1970). The chloroform-soluble class of exochelins, also produced by mycobacteria, are nearly identical to the mycobactins, but contain short-chain fatty acid esters (C_1 to C_7) and are released to the supernatant (Gobin et al., 1995). The only other siderophores so far found to contain fatty acids are palmitoylcoprogen with a saturated C_{16} alkyl chain (Anke et al., 1991), and rhizobactin 1021, which has (*E*)-2-decenoic acid as the acyl group on the nitrogen of one of its two hydroxamates (Persmark et al., 1993).

Deleya aquamarina has been reported to produce fatty acids including saturated C_{12} (between 0.5 and 1%), saturated C_{14} (3-4%) and mono-unsaturated C_{14} (<0.5%) (Akagawa and Yamasato, 1989). Percentages are reported relative to total nonhydroxylated acid content. The fatty acid content was determined by gas chromatographic analysis of the fatty acid methyl ester derivatives extracted from a 20 mg cell pellet (Akagawa and Yamasato, 1989). Bacteria typically produce either saturated or *cis*-mono-unsaturated fatty acids. The introduction of *cis* double bonds during fatty acid synthesis generally proceeds by one of two known mechanisms, the aerobic and anaerobic pathways (Schweizer, 1989). Both pathways act on acyl-CoA substrates. In the aerobic mechanism, desaturation is both oxygen and NAD(P)H dependent but the mechanism is not fully understood. In the anaerobic mechanism, a β,γ

dehydratase enzyme produces a double bond which is not subsequently reduced as are the usual α,β dehydration products in the fatty acid chain elongation cascade (Schweizer, 1989). It is also possible that a desaturating enzyme reacts with fatty acids to generate double bonds at various locations (Fulco, 1974).

The presence of the fatty acids may be a method to anchor the siderophore to the outer membrane to prevent diffusion away from the cell, as siderophores are usually released to the environment outside of the bacterial cell where they would be free to diffuse away in the ocean. Also, lipopeptides which act as biosurfactants are known to be produced by some bacteria, particularly ones living in petroleum reservoirs (Yakimov et al., 1995). The aquachelins are lipopeptides with some surfactant properties such as foaming. It is conceivable that lipopeptide siderophores could provide an advantage in the competition for iron(III) by increasing the access of the siderophores to fecal pellets or other micro-environments in the water column where iron(III) can be found and solubilized. The variety of fatty acids used in aquachelin synthesis may arise from the available fatty acids ordinarily synthesized by the bacterium, but the biological source and function of the fatty acid tails is unknown.

Genetic characterization: Several strains of marine bacteria were isolated by Dr. Margo Haygood of the Scripps Institute of Oceanography during a cruise

off the Canary Islands in September, 1990. Genetic analysis of the bacteria was undertaken by Dr. Haygood's laboratory. DS40M3 is most closely related to *Deleya aquamarina* and *Halomonas meridiana* as shown by comparison of their 16S ribosomal RNA, which generated the relationship tree in Figure 2-52. *D. aquamarina* (ATCC #14400) and *H. meridiana* (ATCC #49692) have identical 16S rRNA sequences, similar fatty acid profiles and identical polar lipid patterns (Dobson et al., 1993). The type strains of *Halomonas meridiana* and *Deleya aquamarina* both produced the aquachelins, though in smaller quantities than strain DS40M3.

Halomonas meridiana was isolated from an Antarctic saline lake and can tolerate a range of salt concentrations (haloversatile) (James et al., 1990). However, this species designation may be premature, since it is likely that strains of *D. aquamarina*, a common marine species, were trapped in the lake when it was formed about 10,000 years ago. This time span is quite short compared to bacterial mutation rates, and therefore *Halomonas meridiana* is probably not justifiably a separate species (Margo Haygood - personal communication). Only DNA-DNA hybridization can distinguish between these strains to determine whether or not they are actually the same species. The current definition of a bacterial species requires DNA-DNA hybridization levels of greater than 70%. That experiment has not yet been done on these bacteria.

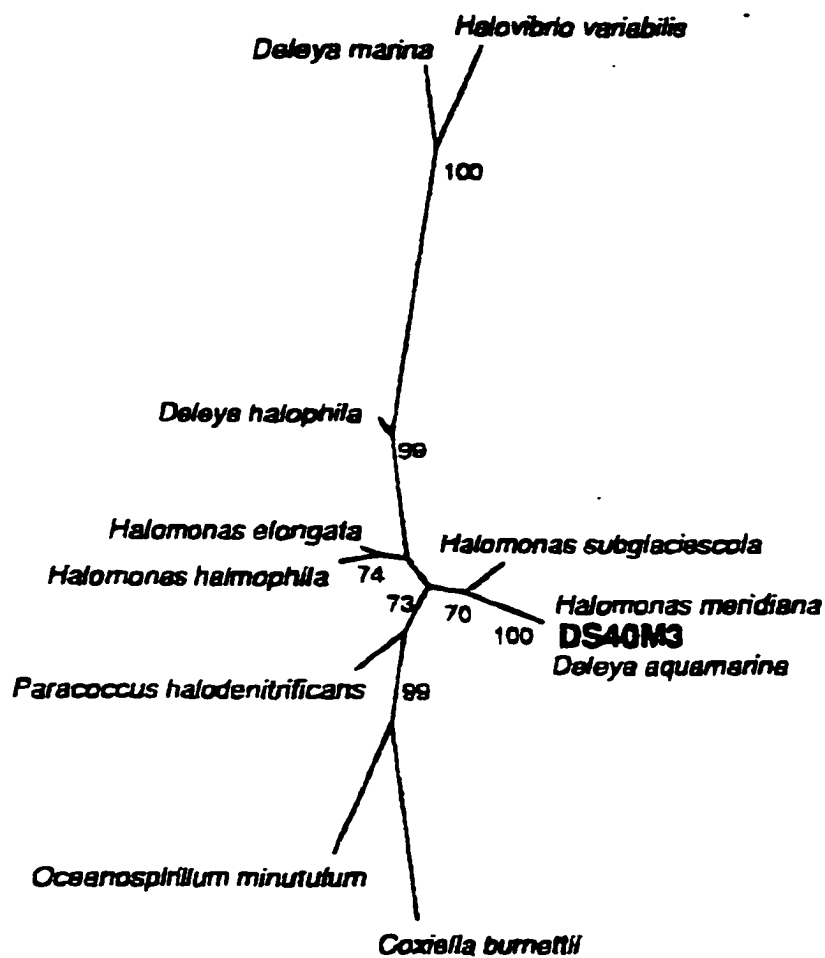


Figure 2-52. Results of 16S-rRNA analysis of DS40M3 showing its relationship to *Halomonas meridiana* and *Deleya aquamarina*. The data were obtained by Professor Margo Haygood at Scripps Institution of Oceanography, San Diego CA. Figure used by permission.

Therefore, isolate DS40M3 has been provisionally assigned to the species *Deleya aquamarina* based on its location of isolation.

Screening studies by Trick (1989) on a variety of marine bacteria showed that strain *Alcaligenes aestus*, subsequently reclassified as *Deleya aquamarina* (Akagawa et al., 1989), produced siderophores. Trick (1989) tested the siderophores with the Arnow assay for catechol and a colorimetric assay involving iron(III) addition for hydroxamates. He concluded that the siderophores from *Deleya aquamarina* contained hydroxamates but not catecholates. Now, the full structures of these hydroxamate siderophores have been determined as described above.

Conclusions

Aquachelins A, B, C and D (Figure 2-51), the siderophores produced by *Deleya aquamarina* strain DS40M3, consist of a heptapeptide *L-threo*- β -hydroxy aspartate, D-serine, L-serine, D-glutamine, D-*N*⁵-acetyl, *N*⁵-hydroxy-ornithine, D-serine, L-*N*⁵-acetyl, *N*⁵-hydroxy-ornithine amide-bonded through the N-terminus to one of four fatty acids. The only structural differences among the four siderophores are the presence of a C₁₂ or C₁₄ fatty acid chain, saturated or mono-unsaturated at the 5 or 7 position, respectively.

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Chapter 2: Appendix

This appendix contains additional data for chapter 2, primarily consisting of parallel data for all of the aquachelin siderophores. For some experiments, only one representative figure is presented in chapter 2 and the other figures are in the appendix.

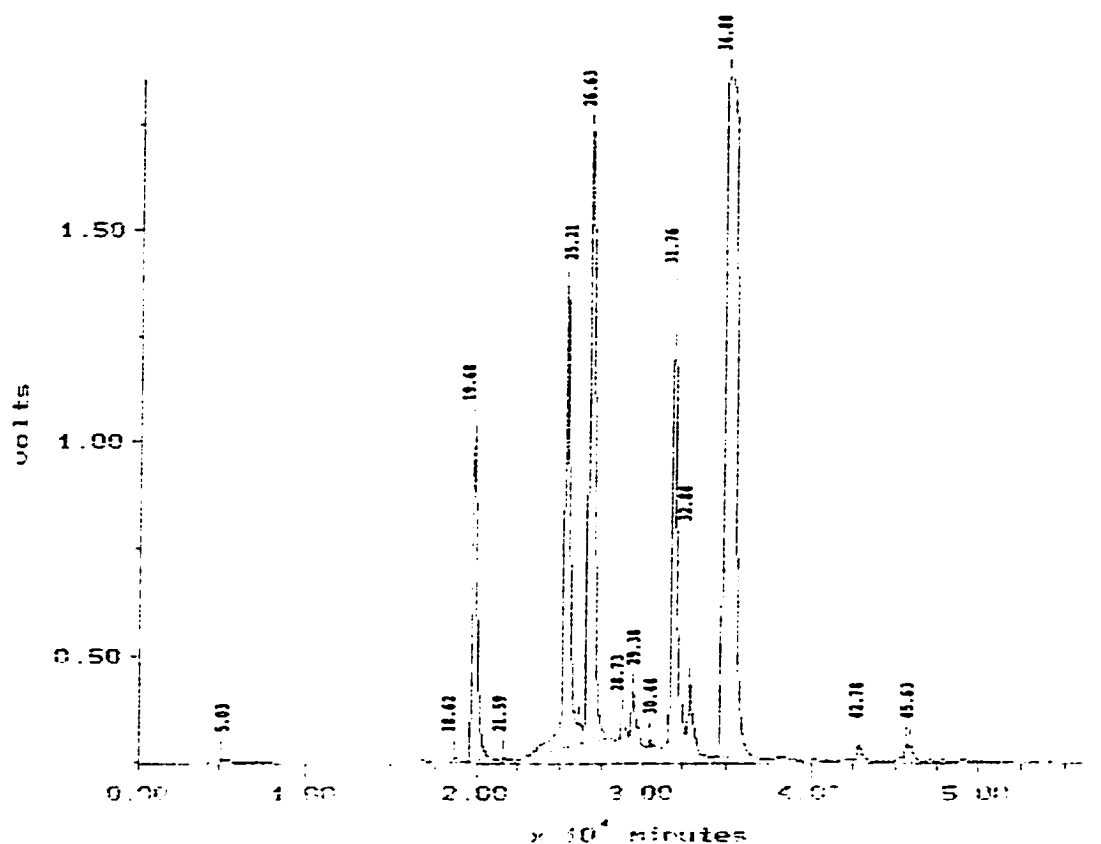


Figure A-1. Amino acid analysis of 6 M HCl hydrolyzed aquachelin A with Marfey's derivatization. Derivatized amino acids (5 μ L samples) were separated with a Waters Baseline HPLC system using a C-18 analytical column (Vydac or YMC ODS-AQ, 4.6 x 150 mm). The gradient was from 10% B to 40% B in 45 minutes at a flow rate of 0.8 mL/min. Solvent A was 50 mM triethylamine phosphate, pH 3.0 and solvent B was CH₃CN. The absorbance was monitored at 340 nm. See Figure 2-4 for the chromatogram of aquachelin C on a Waters Millennium system.

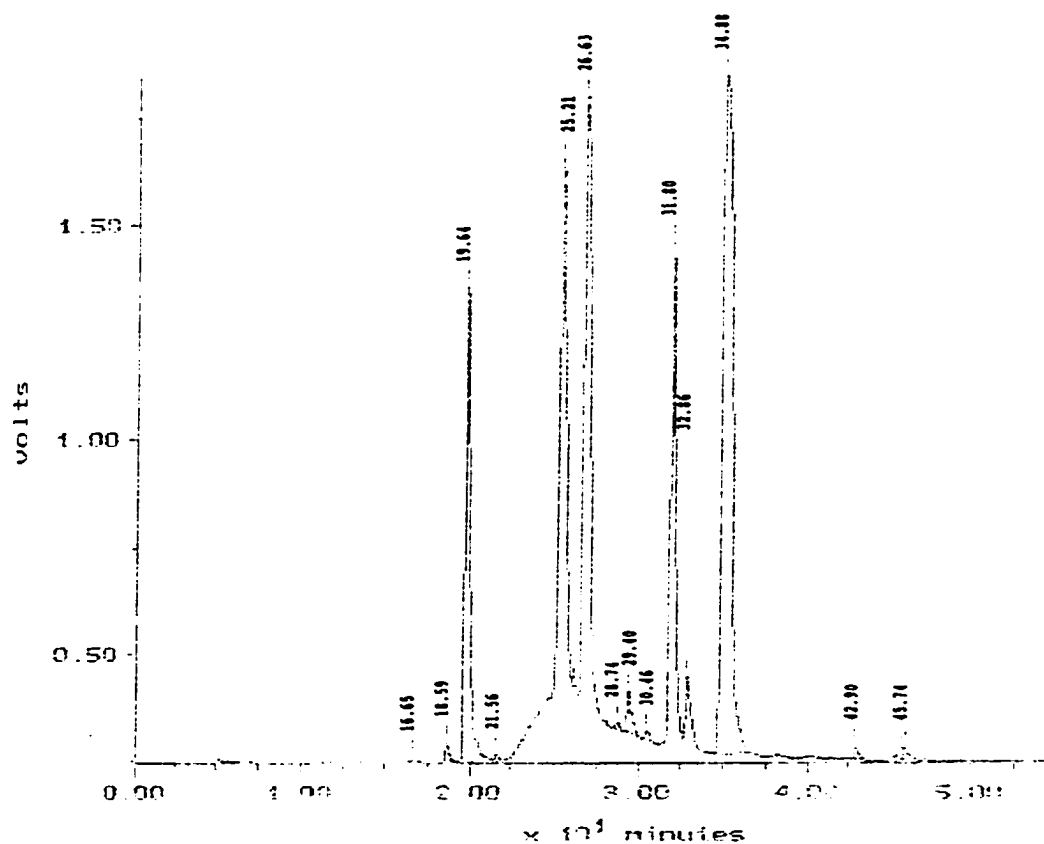


Figure A-2. Amino acid analysis of 6 M HCl hydrolyzed aquachelin B with Marfey's derivatization. Derivatized amino acids (5 μ L samples) were separated with a Waters Baseline HPLC system using a C-18 analytical column (Vydac or YMC ODS-AQ, 4.6 x 150 mm). The gradient was from 10% B to 40% B in 45 minutes at a flow rate of 0.8 mL/min. Solvent A was 50 mM triethylamine phosphate, pH 3.0 and solvent B was CH₃CN. The absorbance was monitored at 340 nm. See Figure 2-4 for the chromatogram of aquachelin C on a Waters Millennium system.

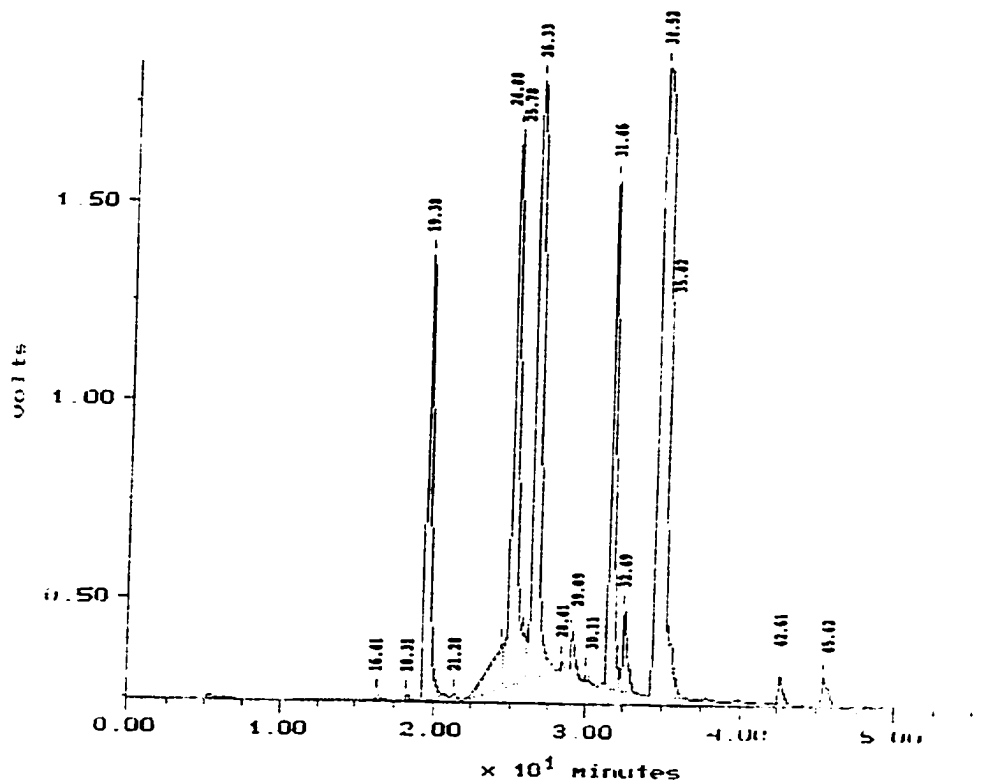


Figure A-3. Amino acid analysis of 6 M HCl hydrolyzed aquachelin C with Marfey's derivatization. Derivatized amino acids (5 μ L samples) were separated with a Waters Baseline HPLC system using a C-18 analytical column (Vydac or YMC ODS-AQ, 4.6 x 150 mm). The gradient was from 10% B to 40% B in 45 minutes at a flow rate of 0.8 mL/min. Solvent A was 50 mM triethylamine phosphate, pH 3.0 and solvent B was CH₃CN. The absorbance was monitored at 340 nm. See Figure 2-4 for the chromatogram of aquachelin C on a Waters Millenium system.

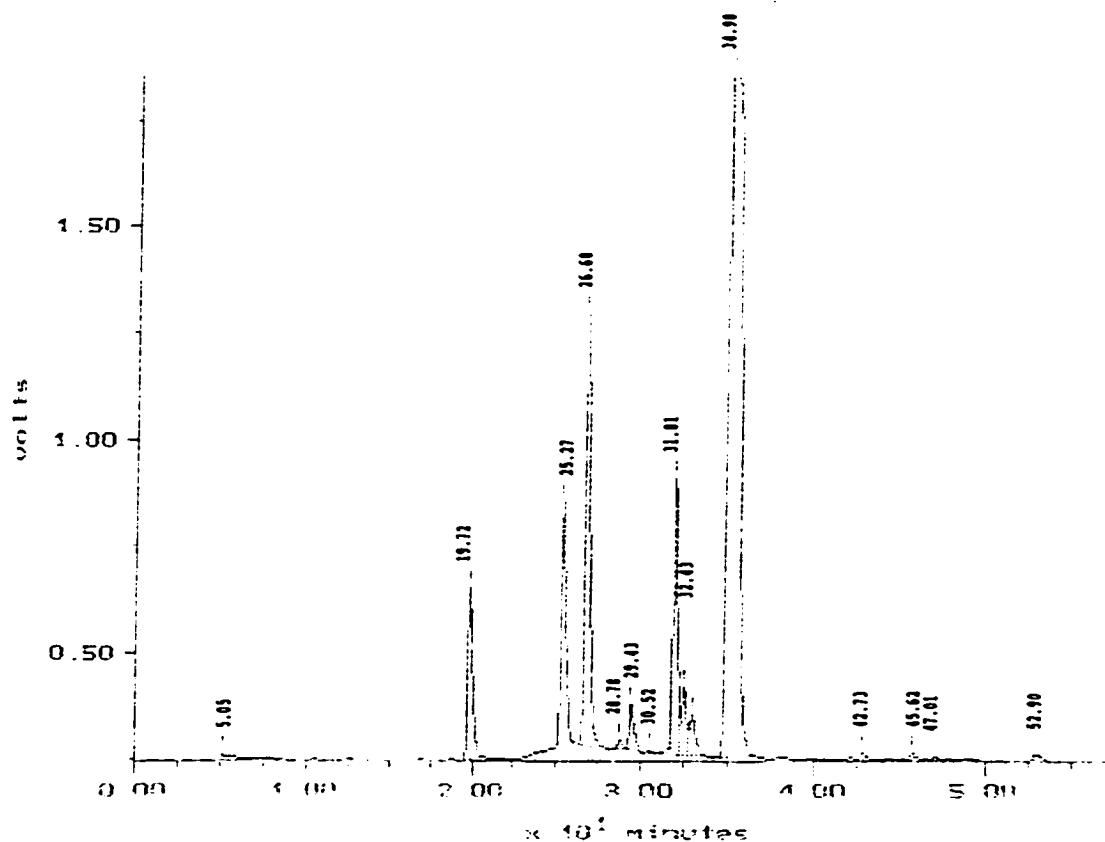


Figure A-4. Amino acid analysis of 6 M HCl hydrolyzed aquachelin D with Marfey's derivatization. Derivatized amino acids (5 μ L samples) were separated with a Waters Baseline HPLC system using a C-18 analytical column (Vydac or YMC ODS-AQ, 4.6 x 150 mm). The gradient was from 10% B to 40% B in 45 minutes at a flow rate of 0.8 mL/min. Solvent A was 50 mM triethylamine phosphate, pH 3.0 and solvent B was CH₃CN. The absorbance was monitored at 340 nm. See Figure 2-4 for the chromatogram of aquachelin C on a Waters Millenium system.

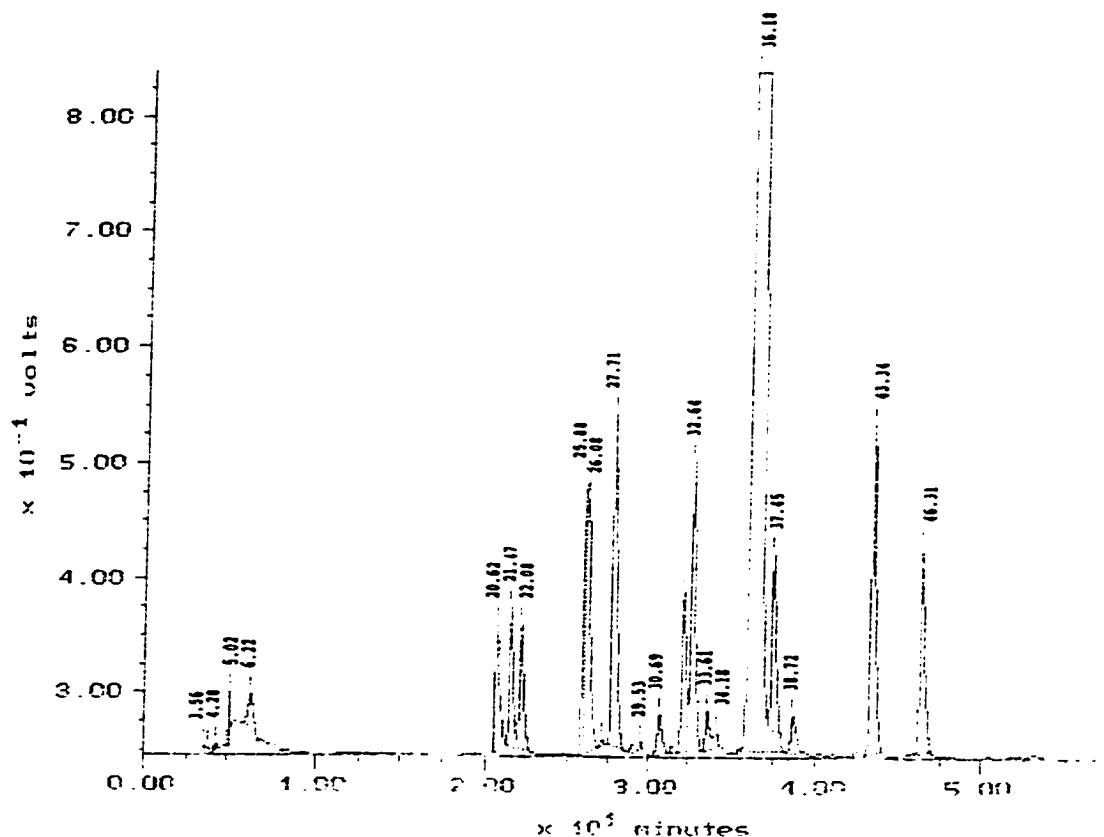


Figure A-5. Marfey's amino acid analysis of 57% HI hydrolyzed aquachelin B. Derivatized amino acids (5 μ L samples) were separated with a Waters Baseline HPLC system using a C-18 analytical column (Vydac or YMC ODS-AQ, 4.6 x 150 mm). The gradient was from 10% B to 40% B in 45 minutes at a flow rate of 0.8 mL/min. Solvent A was 50 mM triethylamine phosphate, pH 3.0 and solvent B was CH₃CN. The absorbance was monitored at 340 nm. See Figure 2-5 for the chromatogram of aquachelin C on a Waters Millennium system.

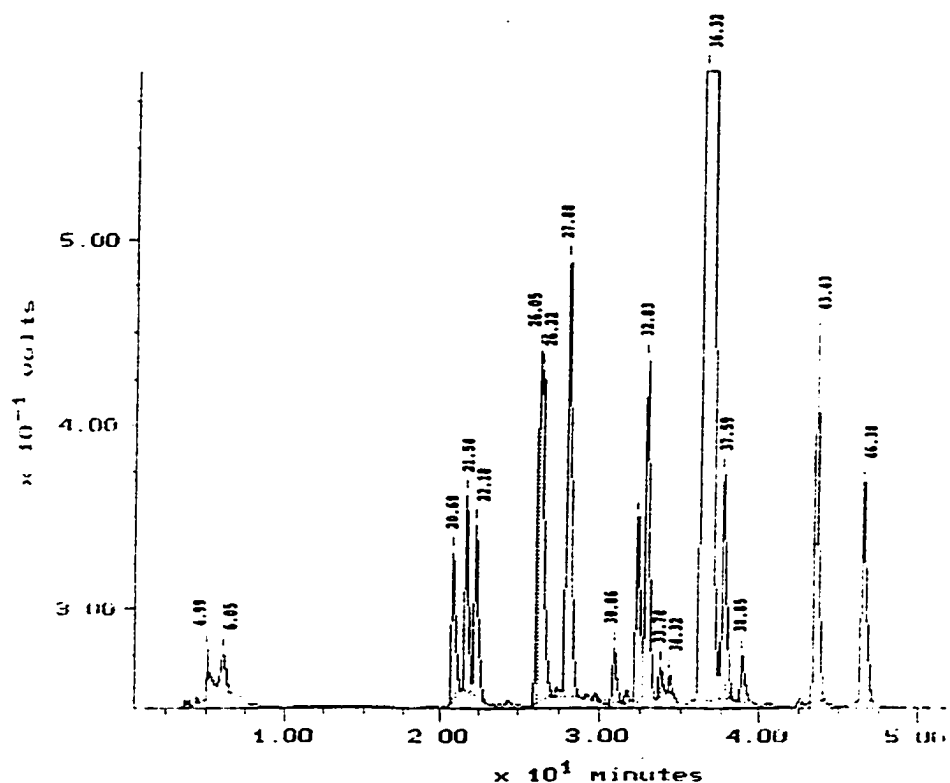


Figure A-6. Marfey's amino acid analysis of 57% HI hydrolyzed aquachelin C. Derivatized amino acids (5 μ L samples) were separated with a Waters Baseline HPLC system using a C-18 analytical column (Vydac or YMC ODS-AQ, 4.6 x 150 mm). The gradient was from 10% B to 40% B in 45 minutes at a flow rate of 0.8 mL/min. Solvent A was 50 mM triethylamine phosphate, pH 3.0 and solvent B was CH₃CN. The absorbance was monitored at 340 nm. See Figure 2-5 for the chromatogram of aquachelin C on a Waters Millennium system.

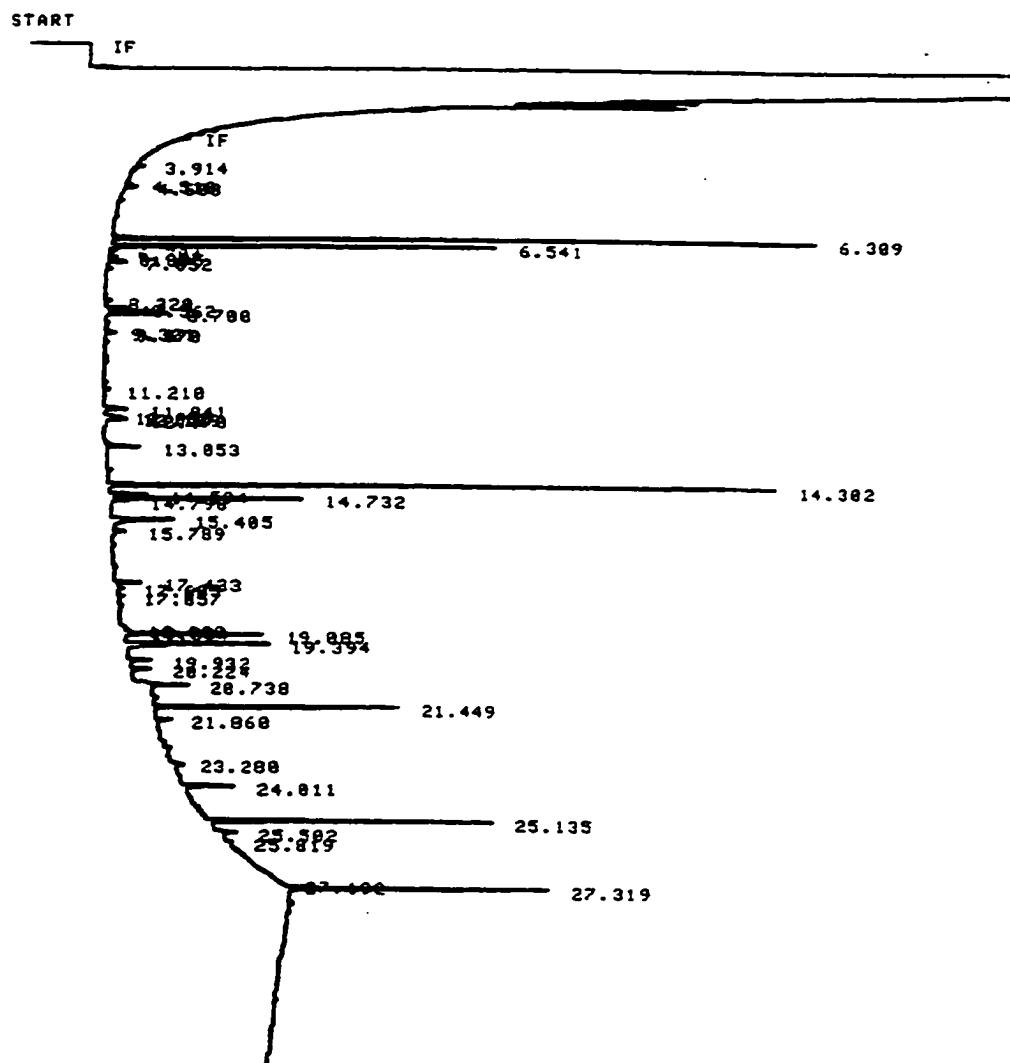


Figure A-7. GC chromatogram of fully hydrolyzed aquachelin B derivatized as described in the Experimental section, but without silylation. Each amino acid appears as in the HPLC chromatograms. See Table 2-2 for assignments. See Figure 2-6 for GC results for aquachelin A. GC conditions were as follows: a Hewlett Packard, Model 5890 gas chromatograph was fitted with a splitless injector inlet and a Chirasil-Val column (Alltech Associates). The injector and flame ionization detector (FID) were heated to 250 °C (Reid, 1993). The carrier gas was H₂ at 20 mL/min. The temperature gradient began at 80 °C, held constant for 3 min., then increased to 200 °C at 5 °C/ min. Samples were dissolved in 20-100 µL of toluene and 0.2-2 µL was injected.

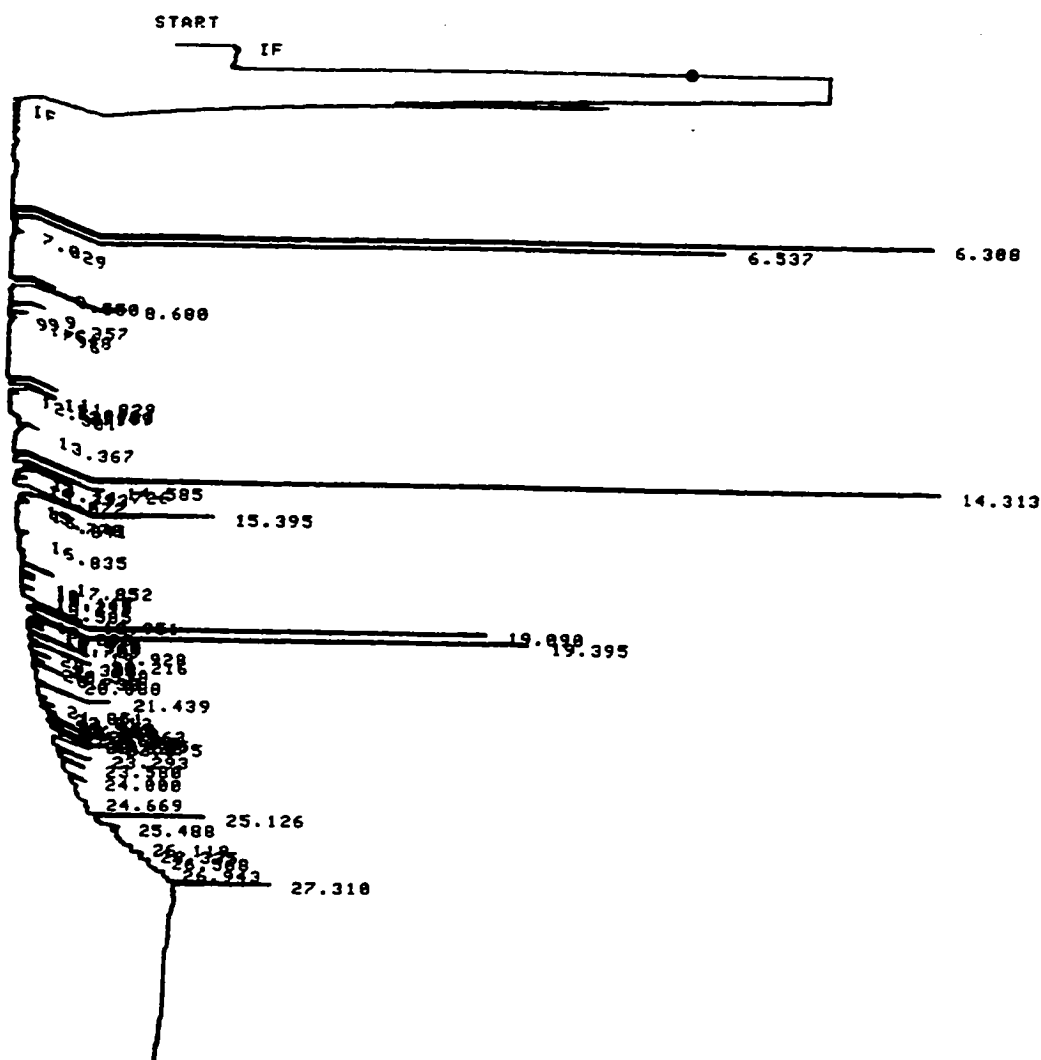


Figure A-8. GC chromatogram of fully hydrolyzed aquachelin C derivatized as described in the Experimental section, but without silylation. Each amino acid appears as in the HPLC chromatograms. See Table 2-2 for assignments. See Figure 2-6 for GC results for aquachelin A. GC conditions were as described in Figure A-7. Note that the plotter was mildly malfunctioning in this instance and therefore the peaks appear bent.

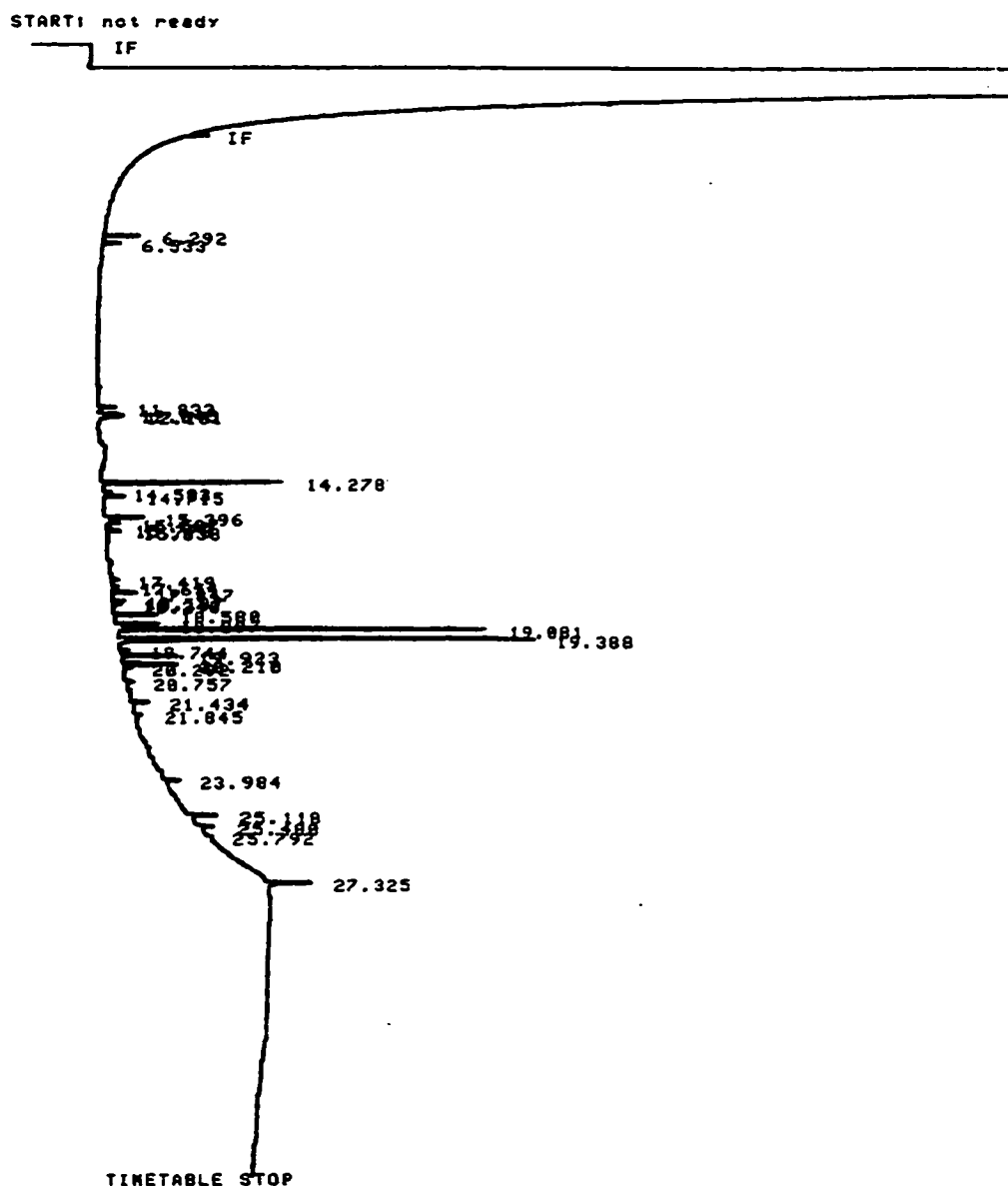


Figure A-9. GC chromatogram of fully hydrolyzed aquachelin D derivatized as described in the Experimental section, but without silylation. Each amino acid appears as in the HPLC chromatograms. See Table 2-2 for assignments. See Figure 2-6 for GC results for aquachelin A. GC conditions are as described in Figure A-7.

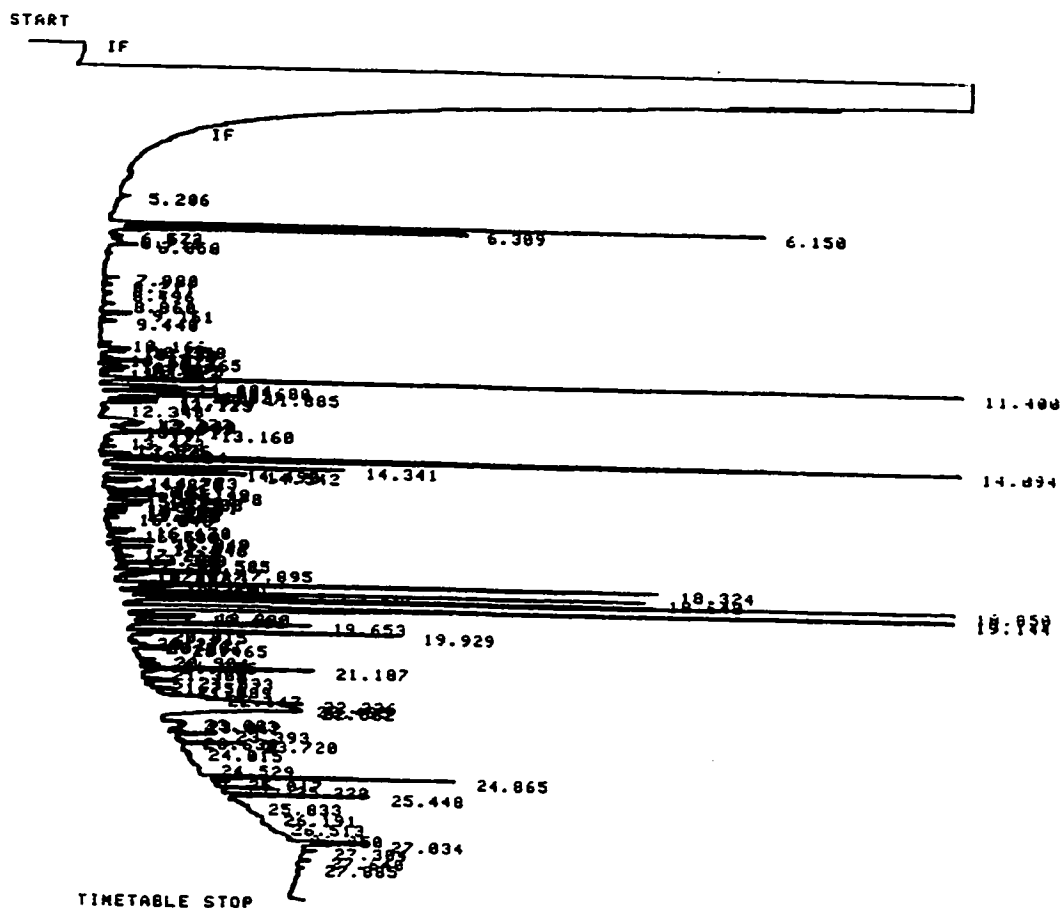


Figure A-10. GC chromatogram of fully hydrolyzed aquachelin A after silylation showing the ratios of each amino acid present. See Table 2-2 for assignments and Figure 2-7 for data for aquachelin B. GC conditions are as described in Figure A-7.

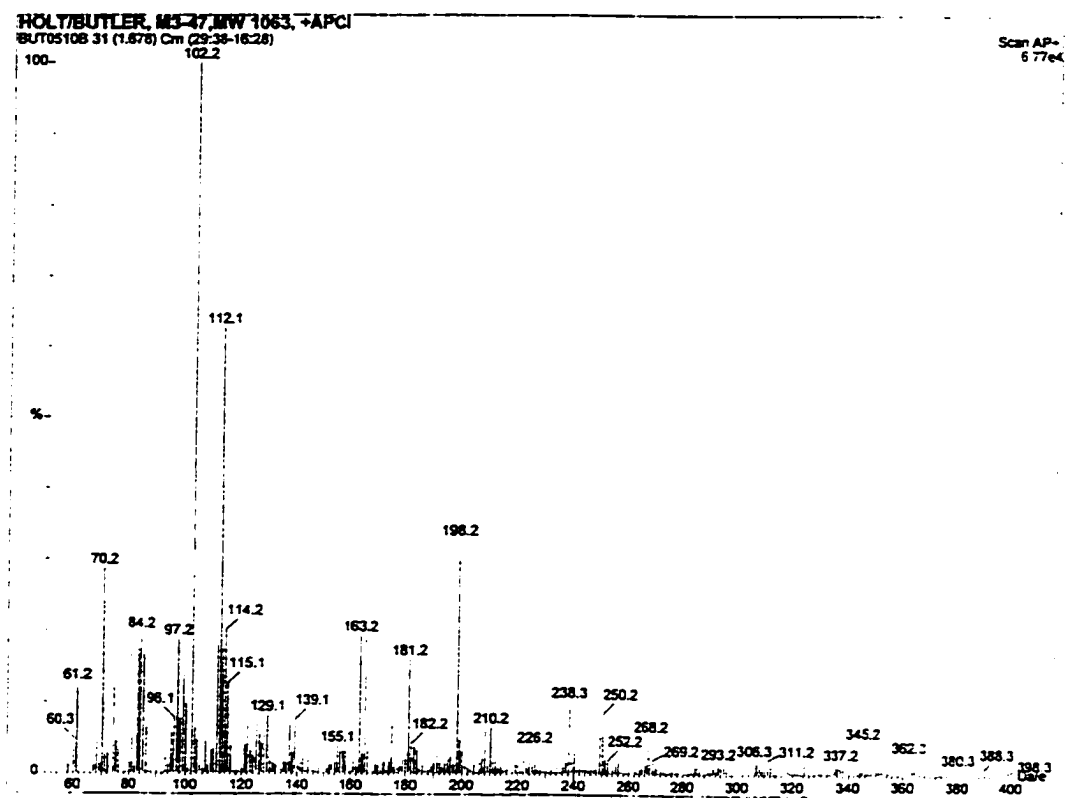


Figure A-11. Atmospheric Pressure Chemical Ionization (APCI) mass spectrum of aquachelin A, showing the C₁₂ (12:1(5)) fatty acid released at 198 *m/z*.

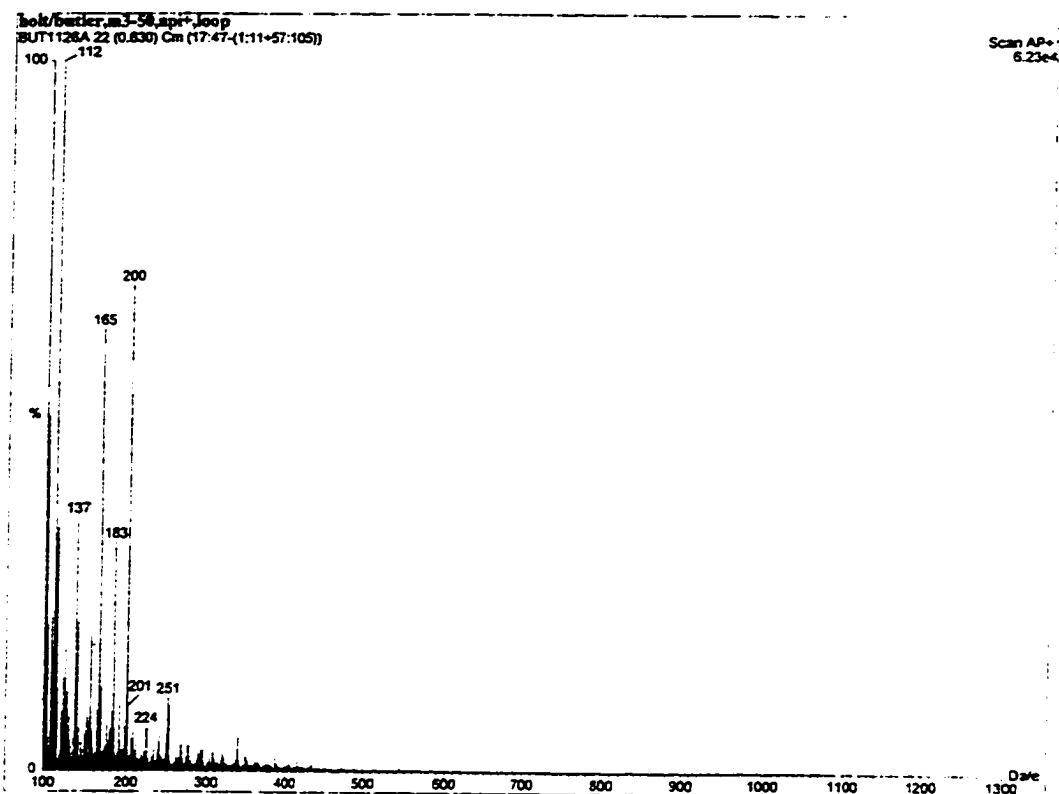


Figure A-12. Atmospheric Pressure Chemical Ionization (APCI) mass spectrum of aquachelin B, showing the C₁₂ fatty acid released at 200 *m/z*.

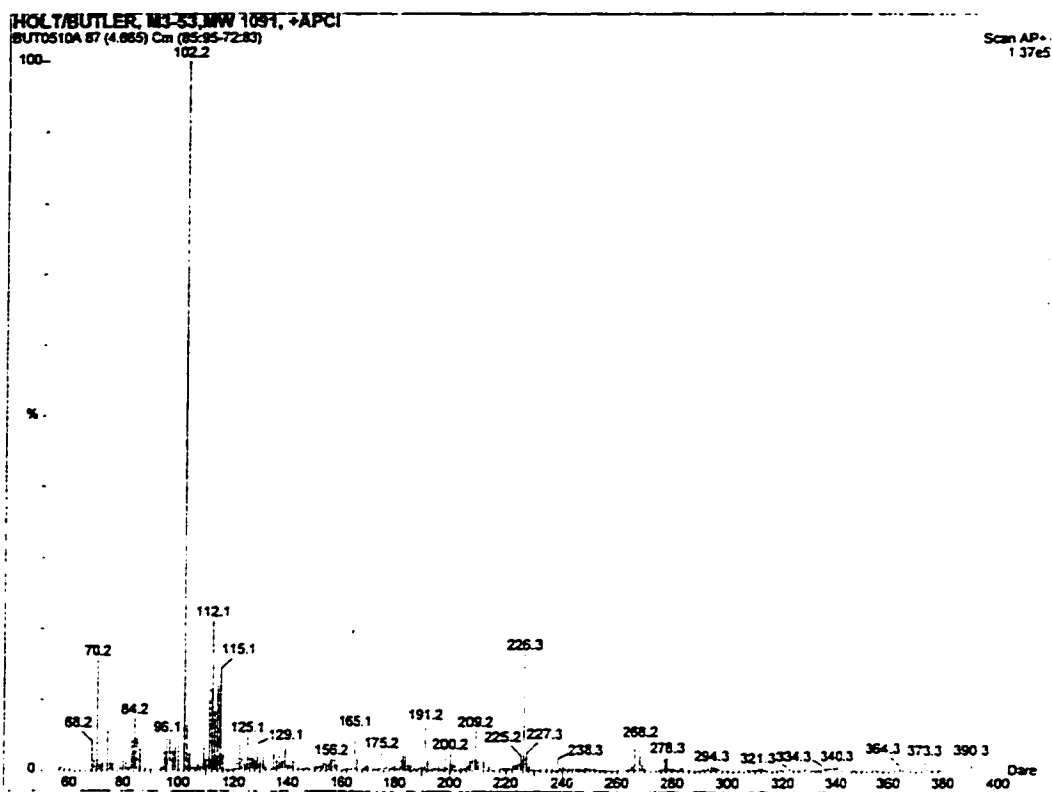


Figure A-13. Atmospheric Pressure Chemical Ionization (APCI) mass spectrum of aquachelin C, showing the C₁₄ (14:1(7)) fatty acid released at 226 *m/z*.

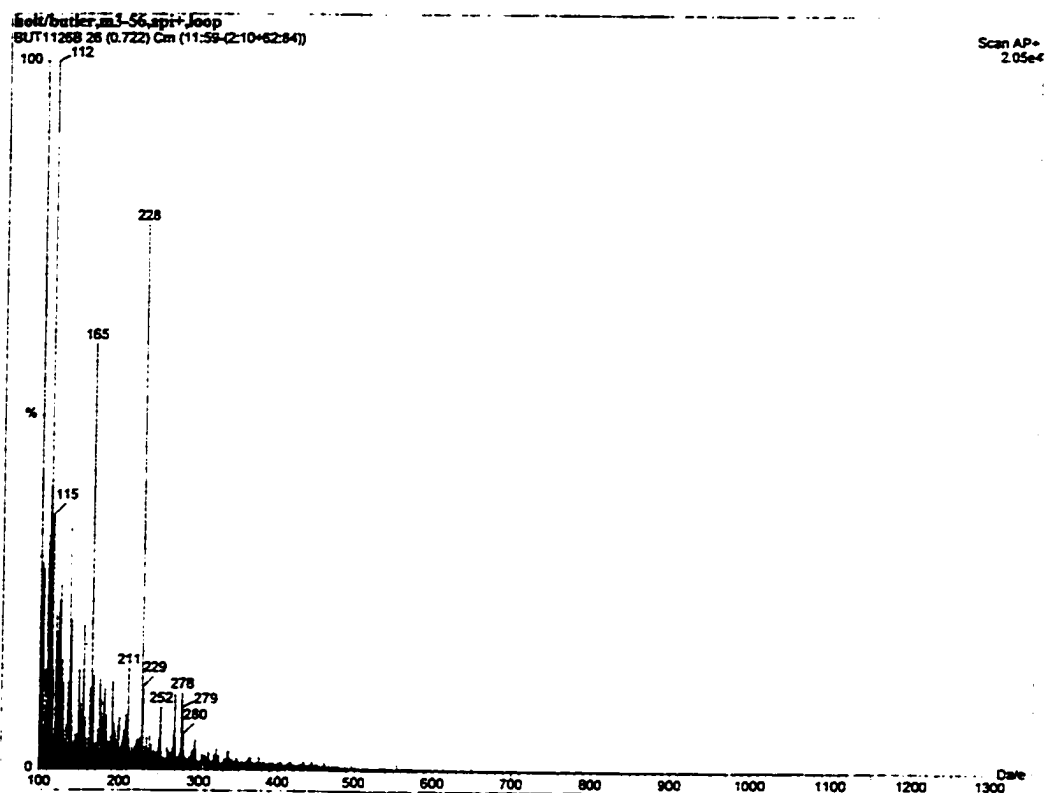


Figure A-14. Atmospheric Pressure Chemical Ionization (APCI) mass spectrum of aquachelin D, showing the C_{14} fatty acid released at 228 m/z .

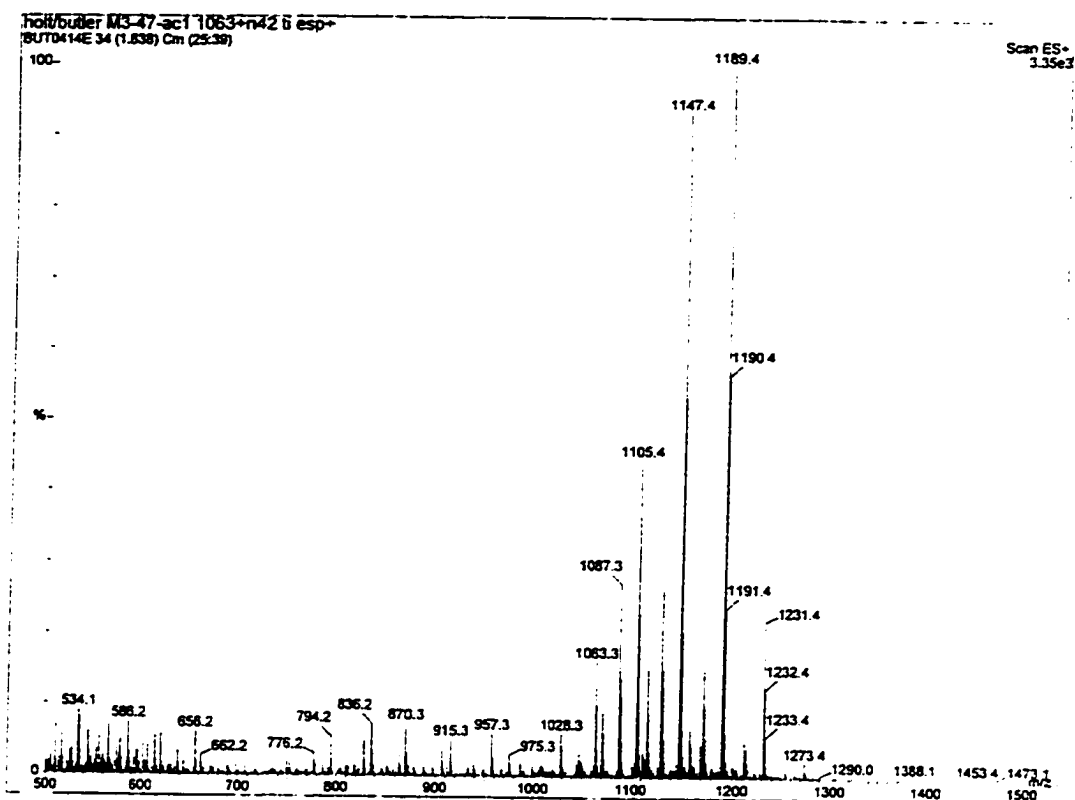


Figure A-15. Electrospray mass spectrum of the microderivatization products from mild acetylation of aquachelin A. See Figure 2-24 for aquachelin C and Table 2-7 for assignments.

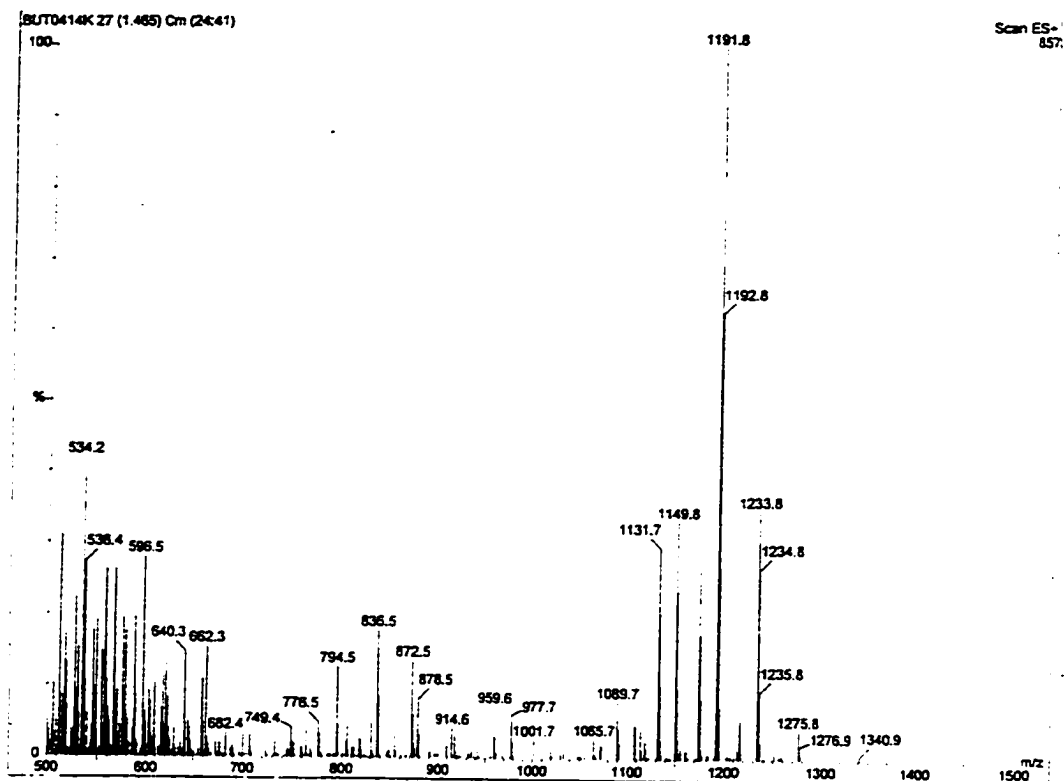


Figure A-16. Electrospray mass spectrum of the microderivatization products from mild acetylation of aquachelin B. See Figure 2-24 for aquachelin C and Table 2-7 for assignments.

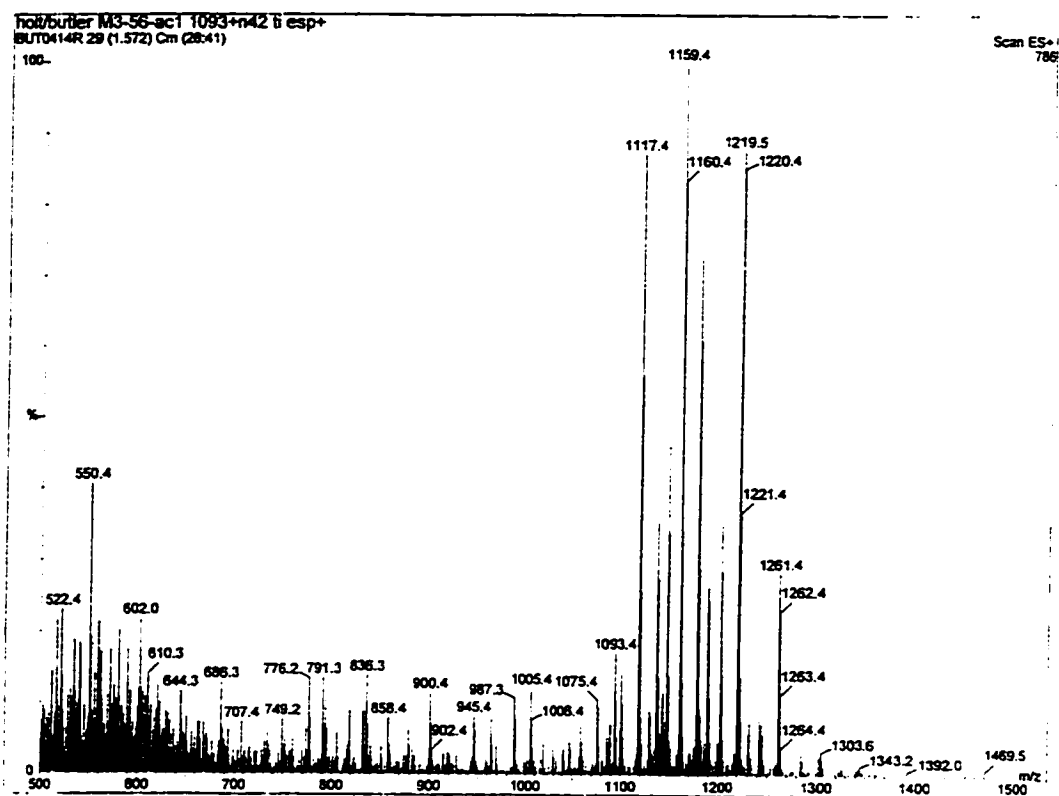


Figure A-17. Electrospray mass spectrum of the microderivatization products from mild acetylation of aquachelin D. See Figure 2-24 for aquachelin C and Table 2-7 for assignments.

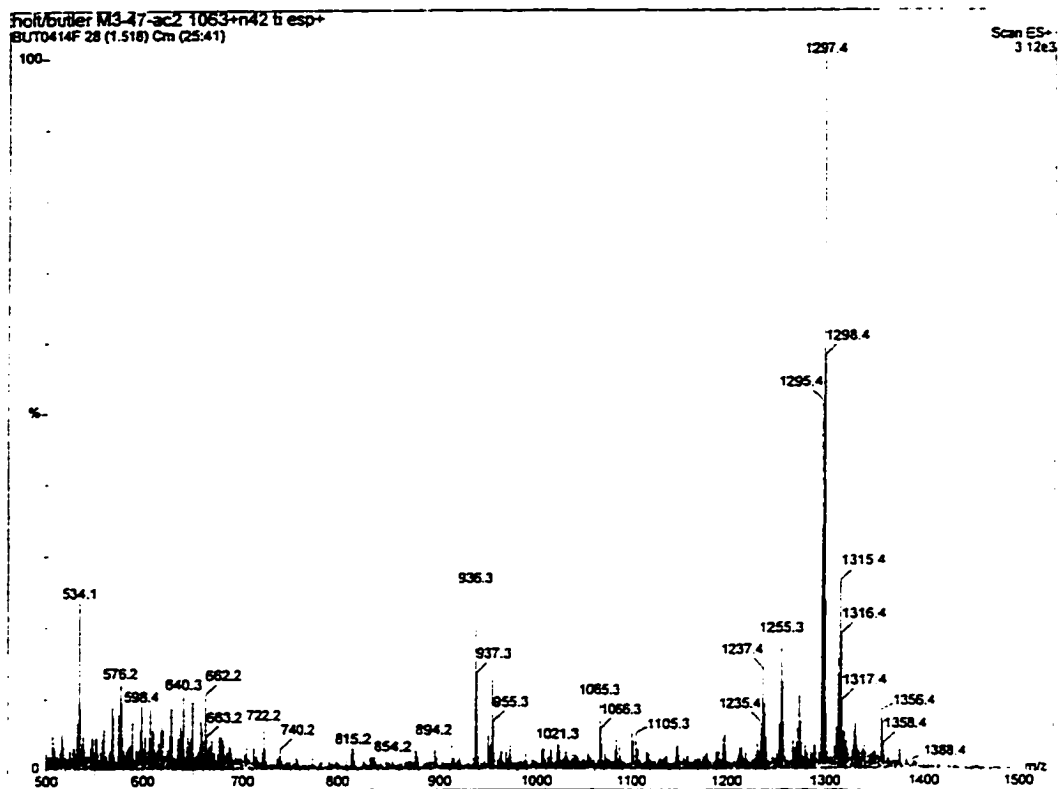


Figure A-18. Electrospray mass spectrum of the microderivatization products from strong acetylation of aquachelin A. See Figure 2-25 for aquachelin C and Table 2-7 for assignments.

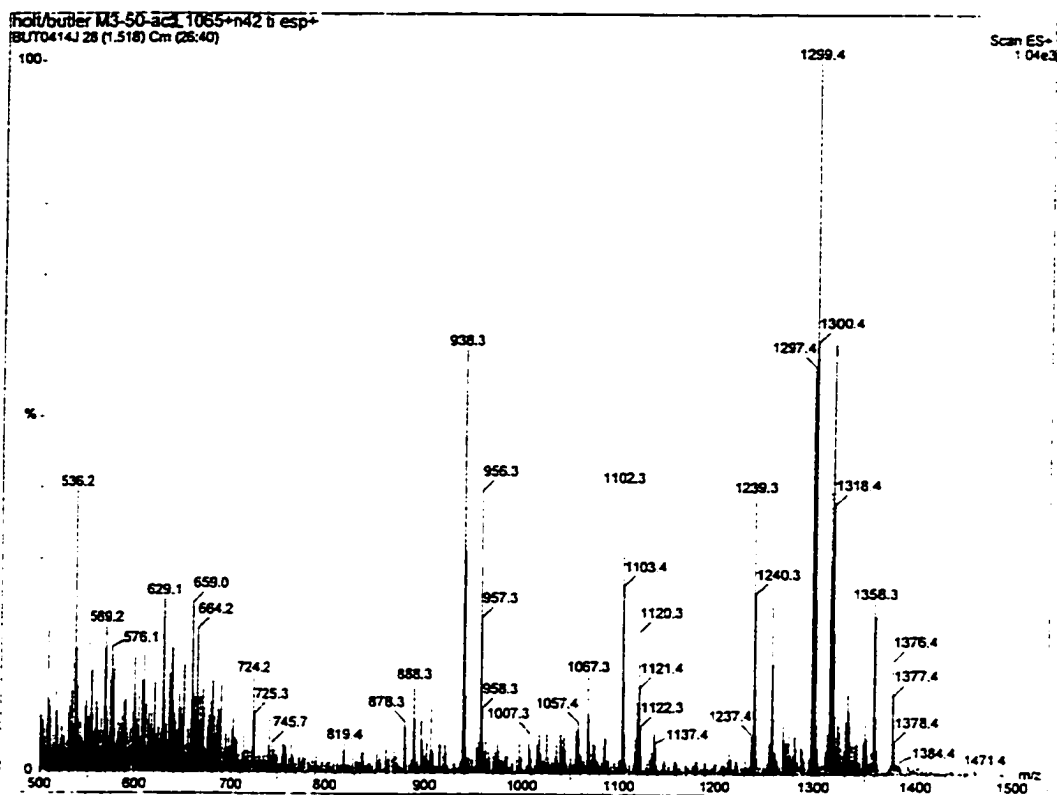


Figure A-19. Electrospray mass spectrum of the microderivatization products from strong acetylation of aquachelin B. See Figure 2-25 for aquachelin C and Table 2-7 for assignments.

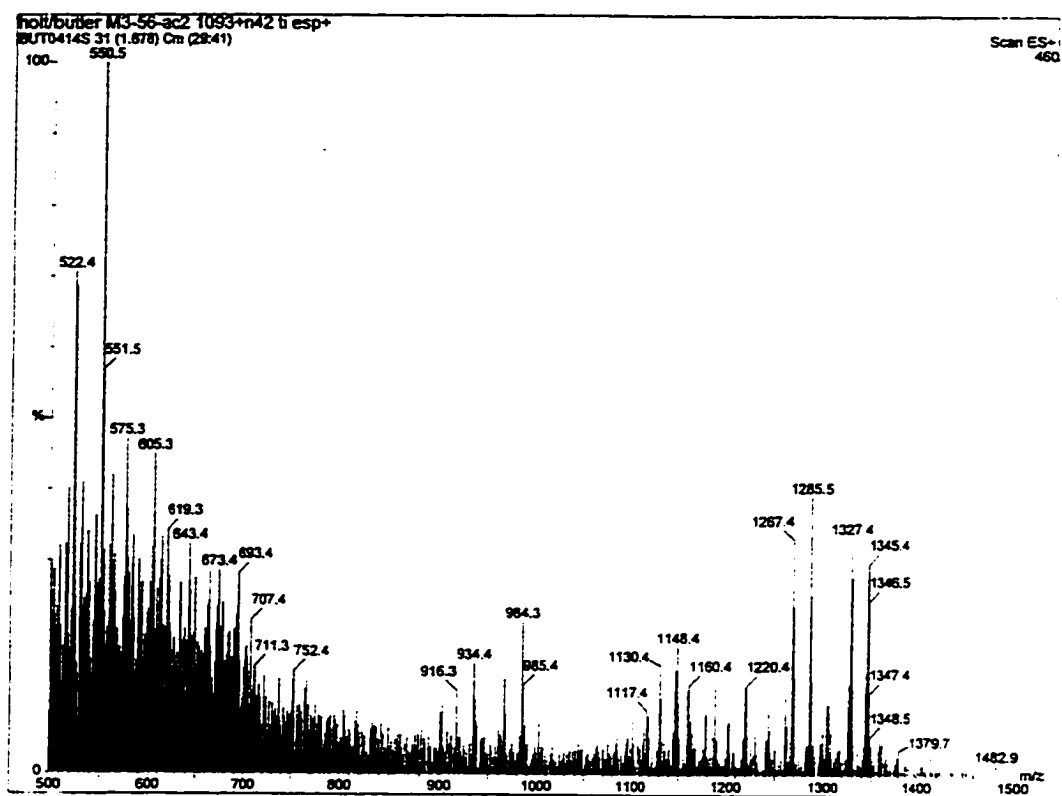


Figure A-20. Electrospray mass spectrum of the microderivatization products from strong acetylation of aquachelin D. See Figure 2-25 for aquachelin C and Table 2-7 for assignments.

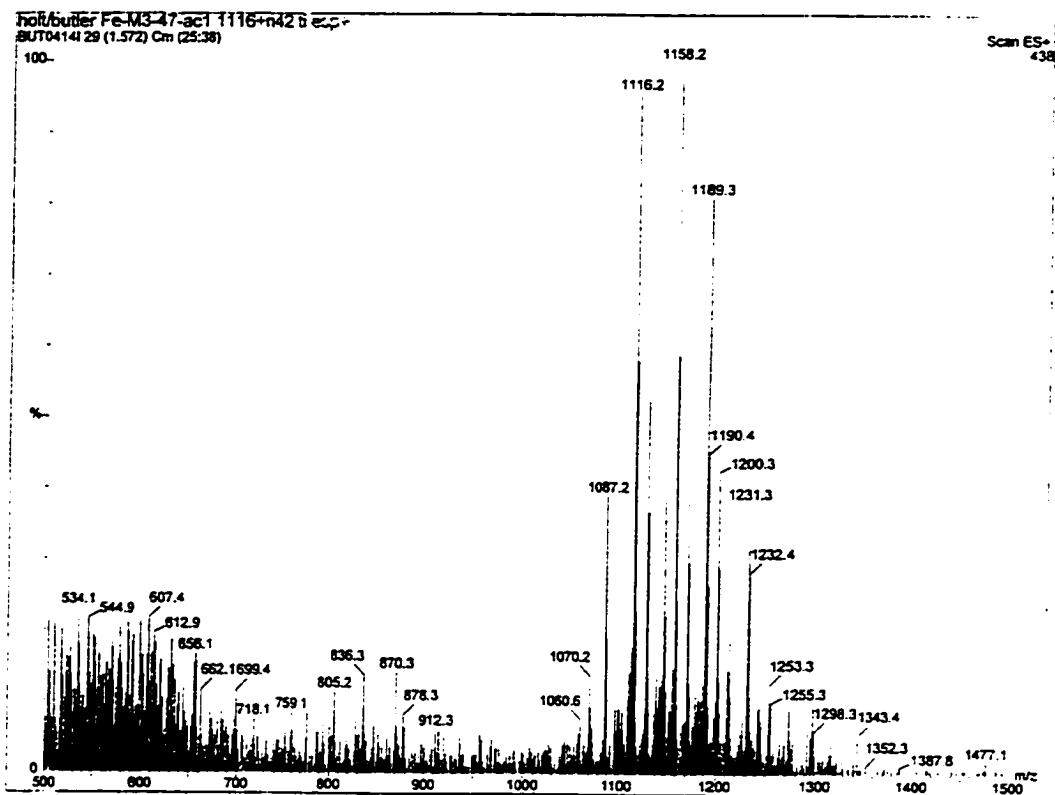


Figure A-21. Electrospray mass spectrum of the microderivatization products from mild acetylation of Fe(III)-aquachelin A. See Figure 2-26 for the Fe(III) complex of aquachelin C and Table 2-7 for assignments.

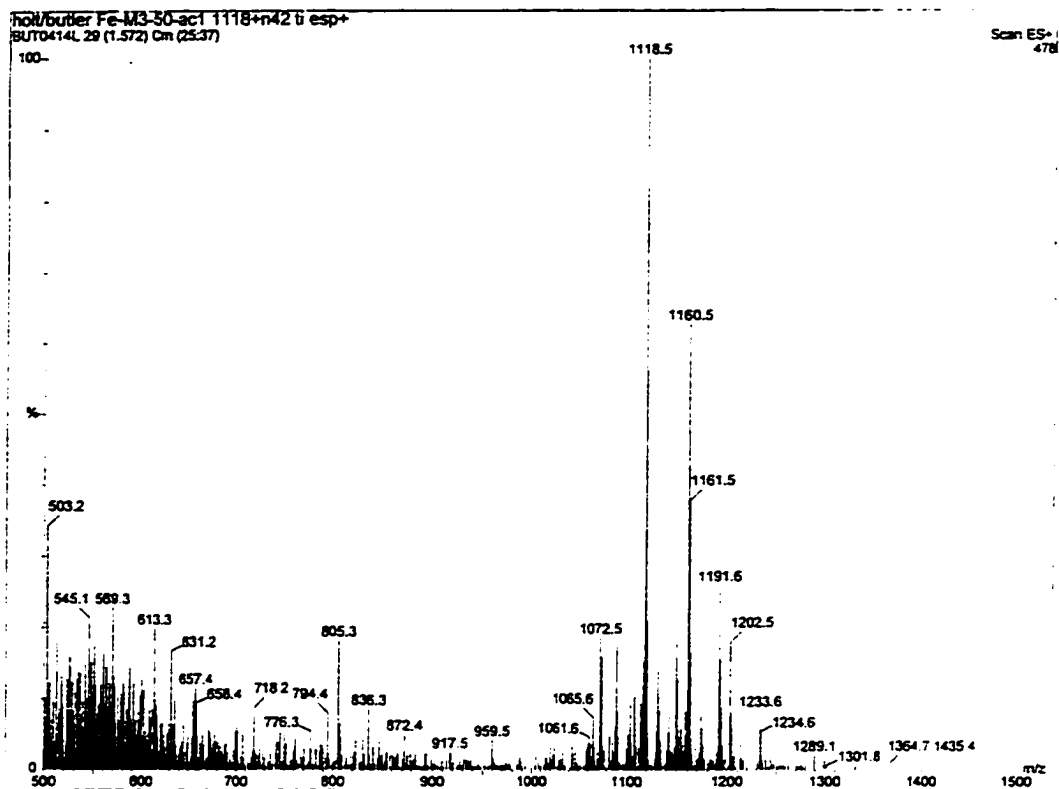


Figure A-22. Electrospray mass spectrum of the microderivatization products from mild acetylation of Fe(III)-aquachelin B. See Figure 2-26 for the Fe(III) complex of aquachelin C and Table 2-7 for assignments.

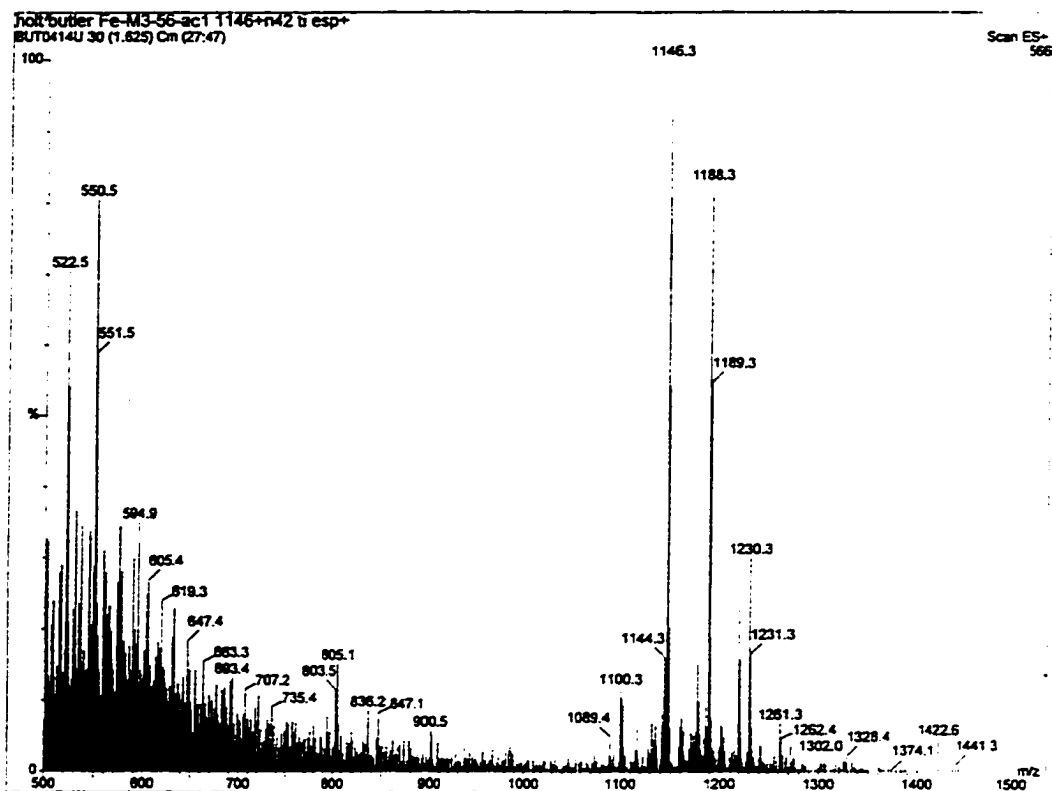


Figure A-23. Electrospray mass spectrum of the microderivatization products from mild acetylation of Fe(III)-aquachelin D. See Figure 2-26 for the Fe(III) complex of aquachelin C and Table 2-7 for assignments.

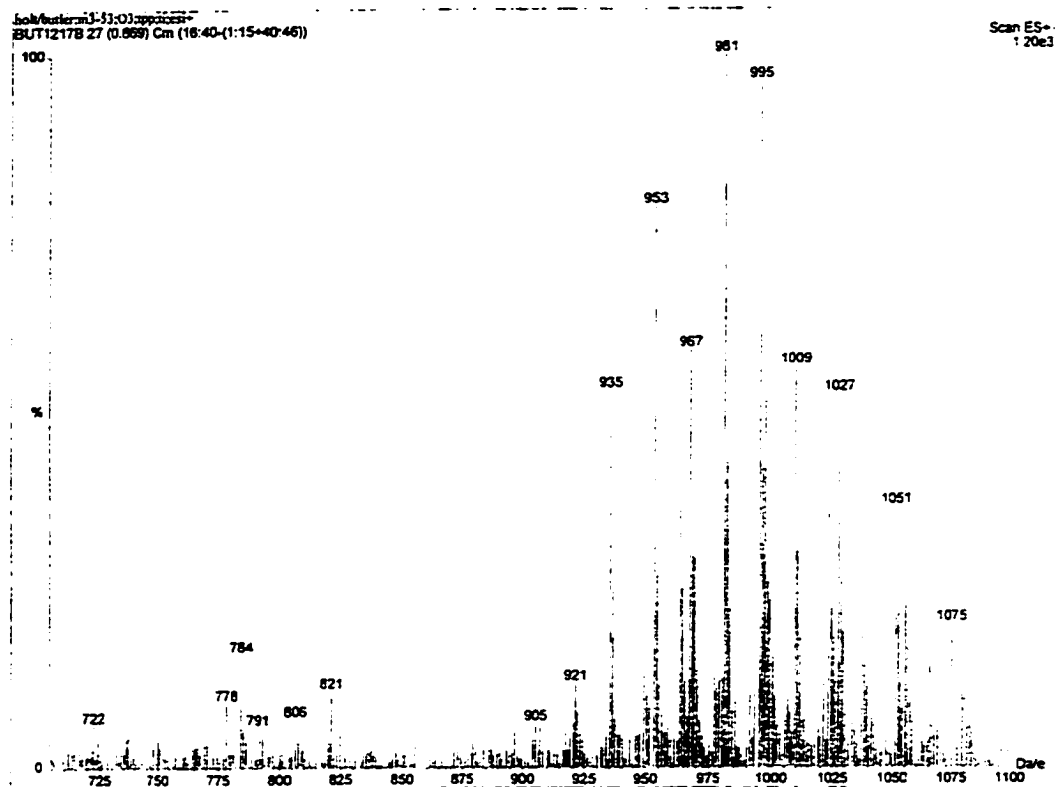


Figure A-24. Electrospray mass spectrum of the aqueous layer following ozonolysis of aquachelin C, workup with triphenylphosphine and extraction with pentane.

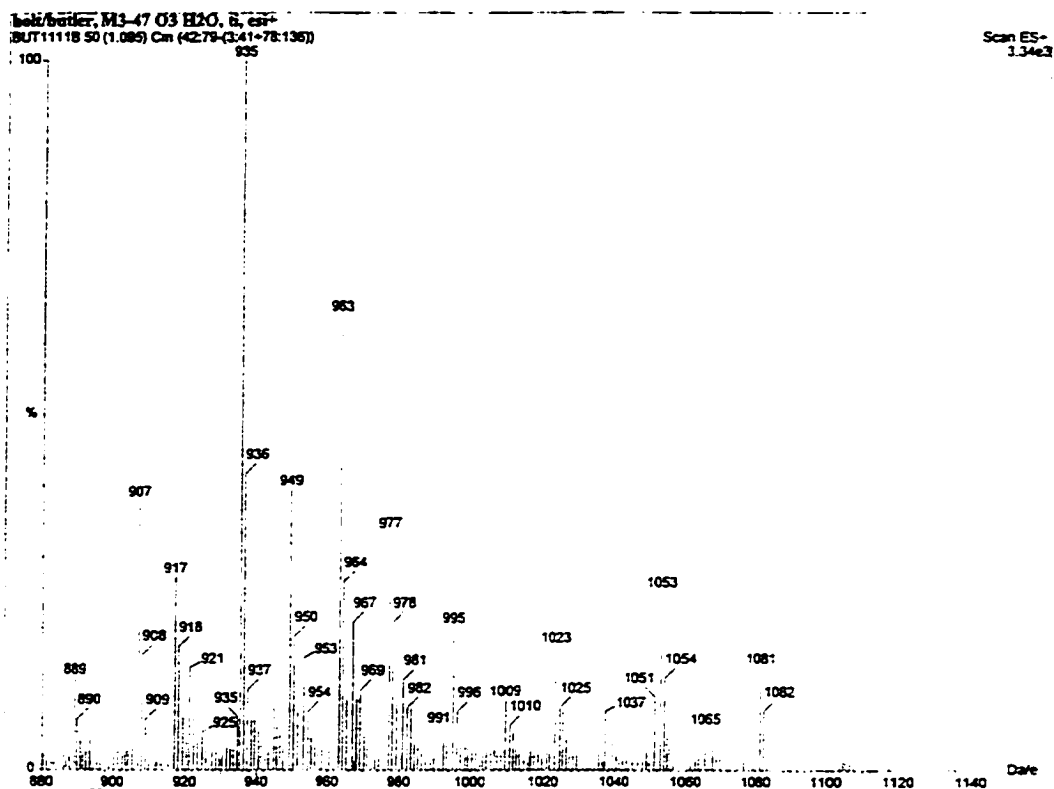


Figure A-25. Electrospray mass spectrum of the aqueous layer following ozonolysis of aquachelin A, workup with triphenylphosphine and extraction with pentane.

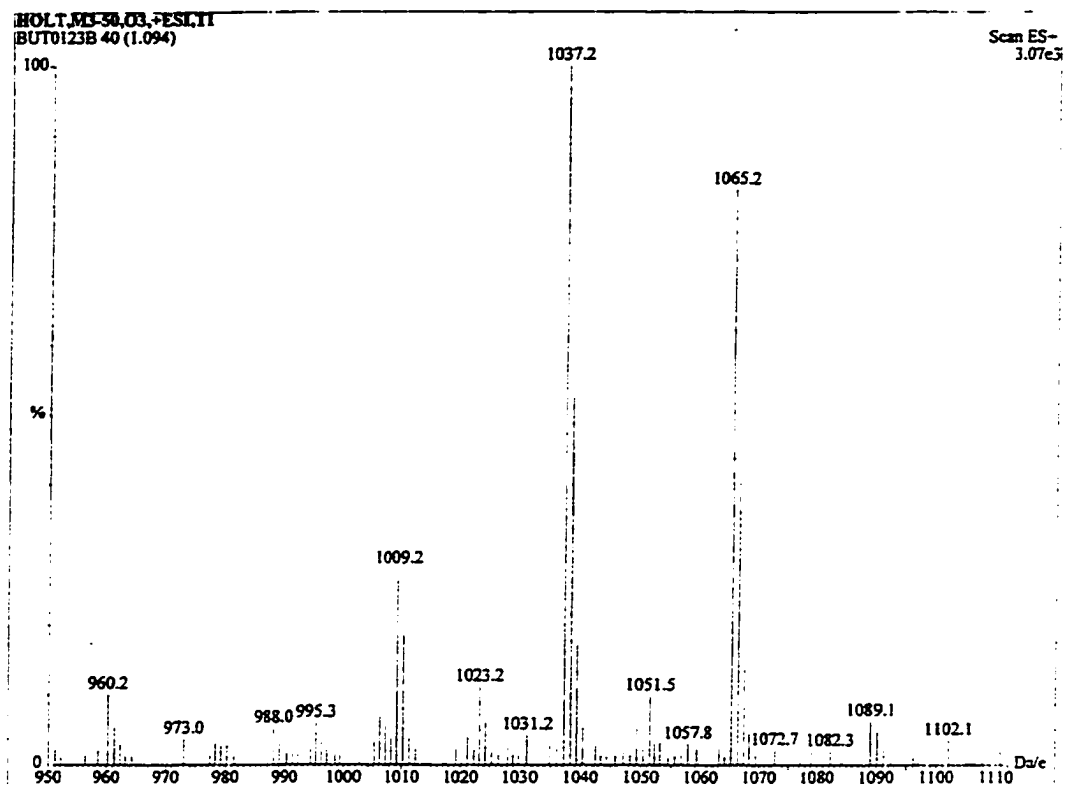


Figure A-26. Electrospray mass spectrum of the aqueous layer following ozonolysis of aquachelin B (which does not contain a double bond) workup with triphenylphosphine and extraction with pentane.

Chapter 3: Inorganic Chemistry of Alterobactins A and B

Introduction

The marine bacterium *Alteromonas luteoviolacea* strain S2 produces two siderophores, alterobactins A and B (Figure 3-1) (Reid et al., 1993). Alterobactin A is a cyclic depsipeptide with a 4,8-diamino-3-hydroxyoctanoic acid tail which is linked to 2,3-dihydroxybenzoic acid by an amide bond. The linear alterobactin B molecule is the product of ester hydrolysis of the depsipeptide ring of alterobactin A (Reid, 1993). The total synthesis of alterobactin A was recently reported, and the synthetic material was found to be structurally identical to the natural product (Deng et al., 1995).

The alterobactins contain one catechol and two β -hydroxy aspartate residues available for iron(III) coordination. All three of those moieties are expected to be used by alterobactin A in order to coordinate Fe(III) in a hexadentate fashion. The stability constant of ferric alterobactin A was calculated from the results of spectrophotometric competition with EDTA to be in the range of 10^{49} - 10^{53} , depending on the protonation constants chosen for the β -hydroxy aspartate hydroxyl groups (Reid et al., 1993). Although alterobactin A binds Fe^{3+} with 1:1 stoichiometry, alterobactin B binds in a 2:1 ligand to metal ratio (Reid, 1993). Spectrophotometric competitions cannot be used to

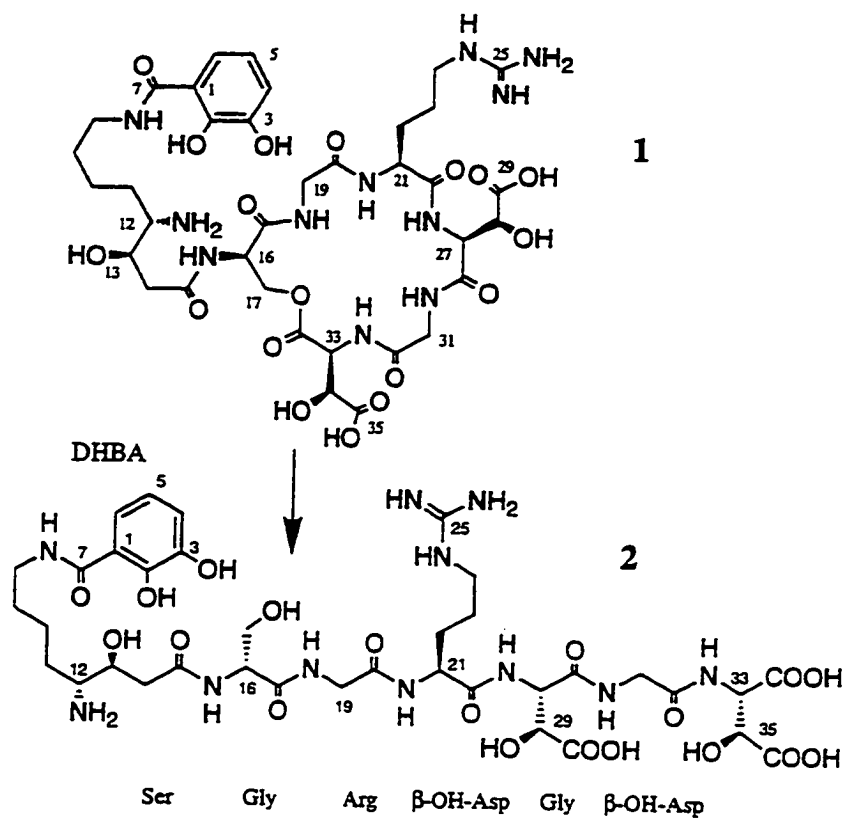


Figure 3-1. Structures of alterobactins A (1) and B (2).
DHBA: 2,3-dihydroxy benzoic acid.

determine the stability constant of iron(III) binding by alterobactin B, because the structure of the Fe(III)-(alterobactin B)₂ complex is not yet known.

Stability constants by electrochemistry: Siderophores characteristically bind iron(III) both tightly and selectively. However, they bind iron(II) much less strongly. For example, the proton-independent stability constant for Fe(II)-enterobactin is 10^{19} (Taylor et al., 1994; Loomis and Raymond, 1991b; Lee et al., 1985) while the proton-independent stability constant for Fe(III)-enterobactin is 10^{49} (Loomis and Raymond, 1991b). According to the Nernst equation (see Equation 15; Discussion section), the reduction potentials of complexes are related to their binding affinities. If the stability constant for Fe(II) coordination by a particular ligand is known, the formation constant for Fe(III) coordination can be determined directly from the reduction potential of the Fe(III) complex. If the stability constant with Fe(II) is not known, as is more often the case, then the "chelate-scale" approach can be employed. The original idea of Hoyle and West (1959), who developed a chelate scale for copper(II) ligands, has now been extended to zinc (Lewis et al., 1995) and iron(III) (Taylor et al., 1994). Taylor et al. (1994) obtained a linear plot of the stability constants of known iron(III) chelators (e.g. NTA, catechol and enterobactin; see the caption of Table 3-1 for a full list) versus their reduction potentials (Figure 3-2 and Table

Complex	E_p (V vs. SCE)	log K	Reference
[FeCDTA]	-0.145	30.0	Smith and Martell, 1977a
[FeNTAtiron] ⁴⁺	-0.182	31.7	Morin and Scharff, 1973
[FeNTAcat] ²⁻	-0.211	32.9	Taylor et al., 1993
[Fe(cat) ₂]	-0.354 ^a	34.7	Avdeef et al., 1978
[Fe(4Ncat) ₃] ³⁻	-0.440 ^a	40.0	Avdeef et al., 1978
[Fe(cat) ₃] ³⁻	-0.680 ^{a,b}	43.7	Avdeef et al., 1978
[Fe(ent)] ³⁻	-0.924	49.0	Loomis and Raymond, 1991

Table 3-1. Electrochemical and stability constant data for complexes in the chelate scale of Taylor et al., 1994. Electrochemical data was obtained in 5 mM Bistris buffer pH 7.0, 0.1 M NaCl with 20 μ M iron(III) except as noted. Error bars are $\pm$ 10 mV. Stability constants are reported at 25 $^{\circ}$ C, I = 0.1. ^a 10 μ M iron(III) ^b pH = 10.0.

CDTA: *trans*-1,2-cyclohexylenedinitrilotetraacetate; NTA: nitrilotriacetate; tiron: 4,5-dihydroxy-1,3-benzenedisulfonic acid; cat: catechol (1,2-dihydroxy benzene); 4Ncat: 4-nitrocatechol; ent: enterobactin (cyclic triester of 2,3-dihydroxybenzoylserine).

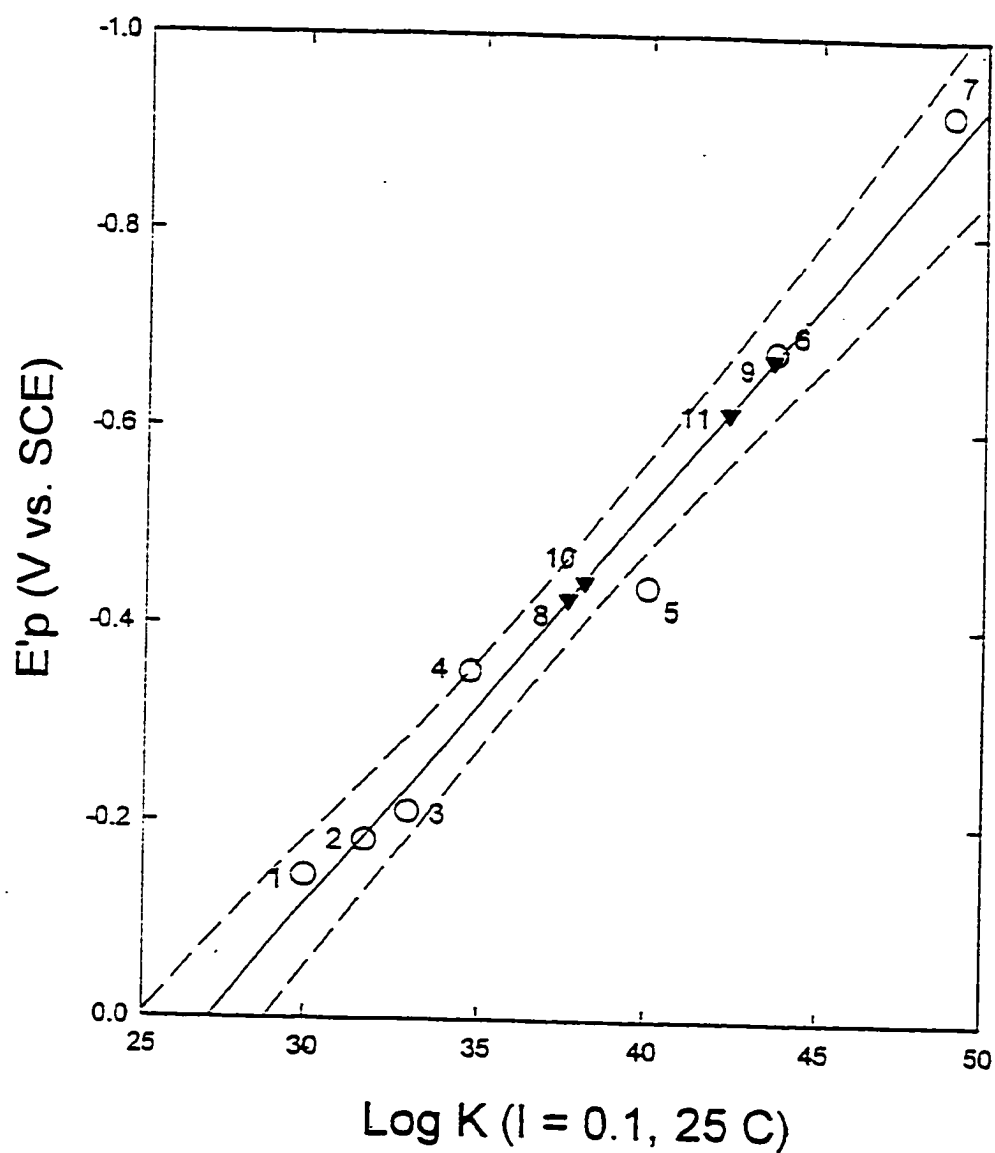


Figure 3-2. Chelate scale based on the standard compounds in Table 3-1 (Taylor et al. 1994; figure used by permission of Professor J. Herbert Waite, University of Delaware).

3-1). The stability constants for other iron(III)-complexes can thus be derived from their reduction potentials, obtained under the same conditions as the standards. Ferric siderophore complexes have very high stability constants and at neutral pH their reduction potentials range from approximately -0.5 to -1 V relative to the saturated calomel reference electrode (Raymond, 1984). Square wave voltammetry (SWV) at a hanging mercury drop electrode (HMDE) can be used to measure the very negative reduction potentials of ferric siderophore complexes in aqueous solution. In addition, SWV is very sensitive, so small quantities of siderophore are sufficient for analysis.

Siderophores in the ocean: Siderophores are typically isolated from culture media supernatants, which implies that the molecules are released outside of the cells in order to obtain iron from the environment. In the case of oceanic siderophores, loss of the chelators to ocean waters would be a serious drawback to this system. However, since the bacteria are likely to live in colonies, siderophores produced by one cell could be taken up by another in the same colony. Alternatively, some marine bacteria may be able to use siderophores produced by other species. Such a strategy has been seen in terrestrial systems such that bacteria produce membrane receptors for uptake of siderophores that they do not produce. *E. coli* for example, produces receptors for ferrioxamine

B (Nelson et al., 1992), ferrichrome (Hantke and Braun, 1975), coprogen (Hantke, 1983) and rhodotorulic acid (Hantke, 1983) although it does not synthesize any of those compounds. Thus, competitive siderophore uptake by bacteria of different species could allow more efficient use of freely diffusible siderophores. Another potential strategy for siderophore recovery is the chemotactic response of bacteria (Brock and Madigan, 1988). Certain chemicals, particularly food sources, induce bacteria to swim toward a location with a higher concentration of the chemical. Such a mechanism could lead bacteria to areas with a higher concentration of ferric siderophore.

A different mechanism for iron(III) uptake via siderophores in the ocean could be to tether the siderophores to the cell. Theoretically, such a tether would be analogous to a fishing line with an iron chelator attached to it, providing a means for the cell to retrieve the Fe(III)-bound siderophore. Although siderophores are typically found in culture supernatants, the attachment might be fragile enough to be broken during isolation. For example, hydrophobic interactions between a bacterial slime layer and siderophore molecules could retain the siderophores in the immediate vicinity of the cell.

Gauthier (1976) reported that there was no visible glycocalyx (a network of sugars that forms a distinct layer outside of a cell (Brock and Madigan, 1988) around *A. luteoviolacea*. However, it appears that the cells' purple pigment is

associated with a type of slime layer, since a dark purple material separates from a white cell pellet when liquid culture colonies are centrifuged. This apparent slime could be involved in tethering siderophores, or in production of marine snow particles.

Marine snow: Divers often see particulate matter in the ocean, called marine snow, which is derived from fecal pellets and other organic detritus falling from surface waters down through the water column. Alldredge and co-workers (1993) discovered a new form of marine snow that had not previously been recognized, transparent exopolymer particles (TEP). Many types of bacteria actually grow on TEP, a sticky slime layer which is produced by algae and perhaps also by bacteria (Passow et al., 1994). If "free-living" marine bacteria are in fact associated with particles, (even if the particles are invisible to normal microscopic techniques, like TEP) the bacteria could inhabit a special niche in the ocean. A stable population of bacteria could develop on the TEP and both siderophores and iron sources could be incorporated into the substrate web. Purple bacteria have been observed growing from colonies of marine snow (Edward F. DeLong, personal communication) and it is possible that *A. luteoviolacea* and its alterobactin siderophores are associated with such polymer networks.

Chromobacterium violaceum siderophores: *Alteromonas luteoviolacea* produces the purple pigment violacein, which is responsible for part of the species name (Gauthier, 1976). The terrestrial aquatic bacterium *Chromobacterium violaceum* also produces violacein, and therefore could be genetically similar in other ways, such as siderophore production. *A. luteoviolacea* was originally called a *Chromobacterium* but was reclassified based on its similarity to *Alteromonas* (Gauthier, 1976). *C. violaceum* was investigated for siderophore production in 1968 by Müller and Zähler who reported that it produced ferrioxamine E (Figure 3-3), a trihydroxamate related to desferrioxamine B (see Figure 1-1).

This chapter describes the electrochemical determination of the ferric stability constants of both alterobactin A and alterobactin B. The results confirm the stability constant reported earlier for Fe(III)-Alt-A (Reid et al., 1993) estimated from spectrophotometric competition experiments with EDTA.

Vanadium is the second most abundant transition metal in the ocean (see Table 1-1) and shows a nutrient-like profile in the water column, such that its concentration is somewhat lower at the surface where there is increased biological activity (Bruland, 1983). Marine bacteria encounter a large excess of vanadium over iron(III) in seawater (see Table 1-1) and siderophore-vanadium interactions could be important. Preliminary studies by Reid (1993)

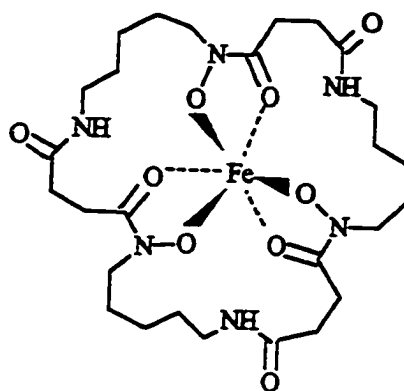


Figure 3-3. Structure of ferrioxamine E (Müller and Zähner, 1968).

indicated that at pH 6 vanadate could compete with iron(III) for alterobactin A, but at pH 8 the ferric siderophore complex remained intact. In this chapter, vanadium(V) additions to alterobactin A are described. Additionally, competitions between iron(III) and vanadium(V) were carried out to determine if excess vanadate can displace iron(III) from ferric alterobactin A or vice versa.

Purple bacterial strains in seawater were collected from aggregates of marine snow to determine whether or not *A. luteoviolacea* is potentially particle-associated. The slime layer of *A. luteoviolacea* was tested for formation of TEP. Finally, the purple bacterial strain *Chromobacterium violaceum* which is sometimes found in freshwater environments was tested for production of the alterobactins.

Experimental

Isolation: Alterobactins A and B were isolated according to the procedure of R. Reid (1993). Cultures of *Alteromonas luteoviolacea* strain S2 were grown as 2 L batches in 4 L flasks. The culture medium was natural seawater medium containing 2 g/L casamino acids (Difco), 1 g/L NH₄Cl and 0.1 g/L glycerophosphate (modified from Haygood and Nealson, 1985). Separate additions of 2 mL 0.2 M phosphate buffer, pH 8 and 200 mg L-arginine were

made to two 2 L batch cultures, with minimal effect on siderophore yields. Natural seawater (NSW) was obtained from the UCSB pipeline. The seawater was pumped through a PVC (polyvinyl chloride) 1.5 ft. diameter pipe located 1500 ft. offshore of the Goleta Point, in 30 feet of water. It was filtered through a bed of sand and gravel. NSW was stored in carboys at 4 °C for 1 to 4 months until needed. The flasks were inoculated with lawns of *A. luteoviolacea* S2 grown for 12-24 hours. The cultures were shaken on a New Brunswick rotary shaker at 110 rpm for 2 days. Sometimes the flasks were shaken at higher speeds, up to 200 rpm, which gave higher yields of alterobactin A. Although siderophore production varied considerably from batch to batch, the optimal growth time for *A. luteoviolacea* in 2 L culture in 4 L flasks was determined to be two days regardless of shaker speed. Growth for two days maximized siderophore yield over production of waste products which can interfere with siderophore isolation. The culture medium was then centrifuged at 8000 rpm for 10 minutes in a GS-3 (Sorvall) rotor to pellet the cells. Approximately 150 g of Amberlite XAD-2 (Aldrich) was added to the two liters of supernatant, then shaken at 110 rpm for one hour in a 4 L flask. The loaded resin was poured into a 2.5 x 40 cm column and washed sequentially with approximately one column volume each of doubly deionized water (Barnstead Nanopure), 50% methanol/ water and 100% methanol. The eluent from the

XAD-2 column was tested with the Chrome azurol S (CAS) assay (Schwyn and Neilands, 1987) in a 1:10 ratio of eluent to assay solution, in order to locate the siderophores.

CAS reactive fractions were dried and redissolved in water with 0.1% trifluoroacetic acid (TFA) for HPLC purification. Solvent A was 0.1% TFA solution, pH 2; solvent B was 80% CH₃CN, 20% H₂O, 0.1% TFA. A semi-preparative Vydac C-4 column (10 x 250 mm) was employed to separate the siderophores from most of the other material. The gradient stayed at 100% A for five minutes, followed by a linear change to 50% B in 60 minutes.

The semi-purified alterobactins were separated from each other using an Ultrahydrogel 120 gel filtration HPLC column (4.6 x 250 mm, Waters) in 10 mM acetate buffer, pH 5, or in pure water to avoid the subsequent desalting step and concomitant loss of material. If needed, the siderophores were purified by passage over the semi-prep C-4 HPLC column a second time with a gradient from 100% A to 30% B in 30 minutes.

The best HPLC purification was obtained by a single pass on a preparative C-4 column (25 x 250 mm, Vydac) using a gradient from 100% A to 30% B in 30 minutes, then to 50% B in 5 minutes. The flow rate was 7 mL/min and the detector wavelength was set to monitor the catechol absorbance band at 310 nm.

Electrospray mass spectrometry: Mass spectrometry was performed using a Micromass Platform II single quadrupole mass analyzer in both positive and negative ion modes. Iron(III) complexes of the alterobactins were prepared by addition of $\text{Fe}(\text{ClO}_4)_3$ solution to a solution of siderophore in water, and purified by HPLC on a semi-preparative C-4 column as above, except in the absence of TFA. Samples of the alterobactins and their ferric complexes were dissolved in 50% CH_3CN / 50% H_2O and introduced at 50 $\mu\text{L}/\text{min}$ via syringe infusion. Cone voltage was set to 40 V.

NMR of gallium-Alt-A: NMR spectra were obtained with a GN (General Electric) 500 MHz instrument. The gallium(III) complex of alterobactin A was made by addition of 50 mM gallium chloride in 0.03 M HCl / 1 M NaCl . The gallium stock solution was made by R. Reid by boiling a gallium ingot (Johnson Matthey) in concentrated HCl for several days (Reid, 1993), partially neutralizing the resulting solution with NaOH to pH 1.5, and dilution to final volume with water. A more effective preparation procedure for the gallium(III) solution would be to boil the ingot in 5:2 v/v HCl/HNO_3 for several hours (Loomis and Raymond, 1991a). $\text{Ga}(\text{III})$ -Alt-A was neutralized with KOH and centrifuged to remove $\text{Ga}(\text{OH})_3$ precipitate. Some samples of $\text{Ga}(\text{III})$ -Alt-A were prepared originally by Richard Reid (1993) and stored in a vacuum

desiccator. These complexes were redissolved in H_2O , dried and then taken up in D_2O for ^1H NMR.

Potentiometry: Potentiometric titrations were performed using a Radiometer Copenhagen pH meter (Model PHM 84) and GK2321C combined glass electrode with an internal calomel reference. Solutions were kept at constant temperature using water-jacketed beakers attached to a water bath (Lauda RM6, Brinkman). Samples were degassed with argon (purified by passing through a column of NaOH pellets and Drierite) and kept under an argon atmosphere by bubbling throughout the titration. The argon was saturated with water by bubbling it through a solution of the same ionic strength as the titration solution. Stock solutions of 1.000 M KNO_3 made from oven-dried KNO_3 (Fisher) were used to make ionic strength adjustments.

The pH meter was calibrated against 0.1 M HCl (Baker Dilut-It) by titrating a 5 mL acid sample with 1 mL aliquots of freshly prepared 0.1 M KOH. Base solutions were made from boiled water and standardized in triplicate against potassium hydrogen phthalate (Aldrich, primary standard grade) to a phenolphthalein endpoint. The carbonate level in the base solution was determined by a Gran's plot of the calibration curve (Gran, 1952; Martell and Motekaitis, 1992).

The $\text{Fe}(\text{ClO}_4)_3$ stock solution (nominally 24.2 mM) was standardized against ferrozine (Aldrich; Stookey, 1979). The ferrozine assay was performed by taking 800 μL aliquots of each diluted sample and adding 500 μL NH_2OH HCl to reduce $\text{Fe}(\text{III})$ to $\text{Fe}(\text{II})$. After equilibration for 5 minutes with the reductant, 100 μL ferrozine (10 mg/ mL) and 100 μL 5 M $\text{NH}_4\text{CH}_3\text{CO}_2$ buffer pH 5.5 were added and the mixture's absorbance was measured at 562 nm (ϵ 27,900 $\text{M}^{-1}\text{cm}^{-1}$; Stookey, 1979). Dilutions of the stock solution were made into 10 mM HClO_4 using acid-washed volumetric glassware to avoid precipitation of iron hydroxides or adventitious iron contamination of the samples. Three separate dilutions (1:1000, 1:500 and 1:333) were made in triplicate, and assayed with ferrozine in triplicate. The acid content of the iron stock solution was measured by titration in the presence of excess EDTA according to Harris et al. (1979b).

Titration of alterobactin A and alterobactin B were performed to measure pK_a values. During the sample titrations, titrant was added with Pipetman pipettors or with a Gilman microburet in 10 or 20 μL aliquots. Each solution was titrated from an initial acidic pH to approximately pH 10 with 0.1 M KOH . The data were entered into the computer programs BEST (Martell and Motekaitis, 1992) and MINQUAD (Sabatini et al., 1974) for calculation of the protonation constants.

Ferric alterobactin A solutions were prepared by addition of stoichiometric amounts of stock 26.5 mM $\text{Fe}(\text{ClO}_4)_3$ in HClO_4 to 10 μmol Alt-A in 0.1 M KNO_3 . The solution was brought up to an initial volume of 10 mL and titrated with standardized KOH as above to determine the number of protons released from alterobactin A upon coordination to iron(III).

Electrochemistry: Experiments were performed in Professor George W. Luther III's laboratory at the University of Delaware, College of Marine Studies, in Lewes, Delaware. The electrochemical apparatus consisted of an EG&G Princeton Applied Research (PAR) model 384-B polarographic analyzer equipped with a PAR model 303A static mercury drop electrode (SMDE). Measurements were taken in square-wave voltammetry mode using a 200 mV/sec scan rate, 20 mV pulse amplitude, and 100 Hz pulse frequency. Potentials were recorded relative to a saturated calomel electrode (SCE) with mercury drop size set to large. Solutions were made in 5 mM bis(2-hydroxyethyl)iminotris-(hydroxymethyl)methane (Bis-Tris) buffer, with 0.1 M NaCl for ionic strength control. Stock ferric chloride solutions were made by dilution of atomic absorption standards into dilute HCl. Aliquots of the iron(III) stocks were diluted to 3-20 μM in 5 mL 5 mM Bis-Tris at pH 6, 7 or 8.2. After removal of dissolved oxygen by bubbling with argon, the solutions were

titrated with 1 mM siderophore solution until the potential of the reduction peak stopped shifting.

Cyclic voltammograms (CV) and square wave voltammetry with the DLK-100 system (which provides the forward, reverse and resultant current voltammograms) were utilized to assess reversibility of the electrochemical processes (Bond, 1980; Taylor et al., 1994; Lewis et al, 1995).

Alterobactin A competition with Desferrioxamine B: Competitions for iron(III) between alterobactin A and desferrioxamine B were used to measure the stability constant of ferric alterobactin A.

Stock solutions - Weighed samples of desferrioxamine B mesylate (DF) (Desferal™, Ciba-Geigy) were dissolved in deionized water (Barnstead Nanopure) to make stock solutions. Iron(III)-DF was made by adding aliquots of 26.5 mM $\text{Fe}(\text{ClO}_4)_3$ in 10 mM HClO_4 to solutions of desferrioxamine B. The concentration of Fe(III)-DF and DF was verified by measuring the absorbance of the Fe(III)-DF sample at 430 nm [ϵ 2800 $\text{M}^{-1}\text{cm}^{-1}$ (Matzanke, 1991)].

Alterobactin A was dissolved in H_2O and the concentration determined by measuring the absorbance of the ferric alterobactin A sample [ϵ = 2050 $\text{M}^{-1}\text{cm}^{-1}$ at 476 nm (Reid, 1993)]. Ferric alterobactin A was made by adding aliquots of 11.2 mM $\text{Fe}(\text{ClO}_4)_3$ stock solution to alterobactin A solutions. The

absorbance of an aliquot of the ferric alterobactin A solution (diluted 1:9) was determined at 476 nm to determine the concentration of Fe(III)-Alt-A.

All competition samples were prepared and equilibrated in 0.1 M MES at pH 6.

HPLC - Samples were analyzed by analytical C-4 (Vydac; 4.6 x 250 mm) HPLC on a Waters Millennium system using 510 pumps and a Waters 996 photodiode array detector. The detector was set to record from 200 to 500 nm every 2 sec. The HPLC gradient changed linearly from 100% A to 50% B in 7 minutes after a 1 minute wash with 100% A; held at 50% B for 2 minutes and returned to 100% A in 2 minutes. Solvent A was 10 mM MES (2-*N*-(morpholino)ethanesulfonic acid), pH 6. Solvent B was 10 mM MES pH 6, in 20% H₂O/ 80% CH₃CN. Samples were collected for 17-19 minutes each. For the competitions, the column was equilibrated at 100% A for one to three minutes between injections to ensure retention of alterobactin A which was quite sensitive to residual acetonitrile.

Stock solutions were prepared as follows : desferrioxamine B at 2.1 mM (1.4 mg/mL); Fe(III)-DF at 3.2 mM (2.1 mg DF in 868 μ L H₂O plus 132 μ L 26.5 mM Fe(ClO₄)₃ in 10 mM HClO₄) at 3.2 mM; Alt-A at 1.32 mM; and ferric alterobactin A at 1.1 mM (108 μ L of 11.2 mM Fe(ClO₄)₃ stock solution added to 550 μ L 1.32 mM alterobactin A solution).

Each standard solution above was measured to obtain a calibration curve on the HPLC. Triplicate 10, 20, 30, 40 and 50 μL samples were injected. Two-dimensional HPLC chromatograms at individual wavelengths were extracted from the 3-D data obtained by the photodiode array detector. For each standard, an optimal wavelength (e.g. the λ_{max}) was chosen for integration. DF was measured at 215 nm, Alt-A at 310 nm (the catechol absorption maximum), Fe-DF at 450 nm and Fe-Alt-A at 476 nm. Although the computer could automatically assign and integrate the peaks, each 2-D chromatogram was checked manually for retention time and integration consistency. Samples with anomalous peaks were eliminated from the calibration calculation. One-point calibrations with 50 μL of each stock solution were obtained on the day of the competition.

For the 1:1:1 DF:Alt-A:Fe(III) competition, stock samples of 0.97 mM Alt-A and 1.3 mM DF were freshly prepared. Equimolar amounts of 11.2 mM $\text{Fe}(\text{ClO}_4)_3$ were added to aliquots of each siderophore and the samples were allowed to equilibrate for 30 minutes before one equivalent of the competing siderophore was added. Thus, the competition was performed by approaching the equilibrium from both directions, e.g. starting with ferric DF or ferric Alt-A and adding the other siderophore as the free ligand. HPLC samples (50 μL injections) were taken of the initial mixture within 5 minutes of mixing and

approximately every hour for 6 hours, then at 24 and 30 hours.

UV-Visible Spectrophotometry - Spectra were obtained using a Cary 3E (Varian) scanning from 200 to 800 nm. In addition, 50 μ L aliquots of the samples were analyzed using the HPLC method described above at the 24 and 48 hour time points. The competition samples approached equilibrium from both directions and were run in duplicate. Control solutions of the starting materials (Alt-A, DF, Fe(III)-Alt-A and Fe(III)-DF) were prepared at the concentrations used in the competitions and measured by both HPLC and UV-Visible spectrophotometry.

For the 1:1 equilibrium, fresh stocks of 2.85 mM DF and 0.333 mM Alt-A were prepared. For 2:1 and 5:1 equilibrium samples, the stock solutions of DF, and Alt-A were freshly prepared at 4.8 mM and 1.5 mM, respectively. To make ferric siderophore complexes, equimolar amounts of 5.53 mM $\text{Fe}(\text{ClO}_4)_3$ in 10 mM HClO_4 were added to siderophore solutions and equilibrated for 30 minutes. The concentration of the $\text{Fe}(\text{ClO}_4)_3$ stock solution in 10 mM HClO_4 was determined by the ferrozine method described above (Potentiometry section) (Stookey, 1979). The competing ligand was added to the ferric siderophore in the appropriate ratio and spectra were taken immediately, then at various time points up to 48 hours. Samples of 1:1:1 alterobactin A, desferrioxamine B and $\text{Fe}(\text{ClO}_4)_3$ were prepared such that each constituent was

present in the initial sample at 66 μM . For 2:1 and 5:1 equilibria, one equivalent was 50 μM .

Vanadium(V) and alterobactin A: Vanadate was added to alterobactin A both aerobically and anaerobically for UV-visible analysis and competition with iron(III). All samples were prepared using 1.9 mM NH_4VO_3 , 2.04 mM Alt-A (15 mg/5 mL) and 11.2 mM $\text{Fe}(\text{ClO}_4)_3$ in 10 mM HClO_4 . Samples were made to 0.3 mM concentration of metal and ligand in 0.1 M buffer using MES or phosphate at pH 6 and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid) or phosphate at pH 8. The total volume of each sample was 3 mL and spectra were measured in matched 1 cm pathlength quartz cells.

Anaerobic samples were prepared by capping the cells with rubber septa and attaching them to small vials via Teflon cannulae. Solutions of metal and ligand were placed in separate vials, degassed with argon for 30 minutes to an hour and then combined through the cannula into the cell. For competition experiments, the initial complex was made and incubated for an hour before the competing metal ion was added. Spectra were obtained at regular intervals, but between scans cells were initially kept in the dark under black cloths and stored in a drawer to avoid photolysis from room light. No difference was observed between cells in the dark and cells in the light, however. Scans were taken

from 200 to 800 nm on a Kontron 860 spectrophotometer at 600 nm/min. ^{51}V NMR spectra were obtained on a GN300 MHz instrument and referenced to a standard of VOCl_3 , $\delta(\text{VOCl}_3) = 0.0$ ppm.

Each experiment was performed at pH 6 and 8, both aerobically and anaerobically. Competition spectra were taken at 10 minute intervals initially, while the incubations of alterobactin A with vanadium(V) were measured at intervals of fifteen minutes to several hours.

Chromobacterium violaceum: A freeze-dried sample of *Chromobacterium violaceum* was purchased from the American Type Culture collection (ATCC #12472) and revived according to their instructions. Because this bacterium is a Class II human pathogen, all manipulations involving the bacteria were done in a Class II Biosafety cabinet (LabConco). After use, cultures and all glassware and surfaces were decontaminated with a freshly prepared 10% bleach solution. Initial screening for siderophores and salt tolerance was done on agar plates. CAS and non-CAS plates were made in either natural seawater or pure water using nutrient agar (BBL). Cultures were grown in different liquid media in order to optimize siderophore production: casamino acids media (2g/L casamino acids; 1 g/L NH_4Cl ; 0.1 g/L glycerophosphate) supplemented with 5 ml/L artificial seawater (ASW, see chapter 2) to add salts; and a rich broth

made with 5 g/L yeast extract and 5 g/L Bacto peptone. The supernatant of the casamino acids media were worked up according to alterobactin A and alterobactin B isolation procedures as described above except that 0.2% sodium azide was added to the supernatant after centrifugation to kill the cells, and the solution was also sterile-filtered through 0.22 μm GS filters (Millipore) to remove any remaining cells. Reactive samples purified by HPLC were dissolved in 0.5 mL D_2O for ^1H NMR analysis. NMR spectra were taken on a GN 500 MHz instrument using standard parameters.

Marine snow: Marine snow was collected by Dr. Uta Passow in collaboration with Professor Alice Alldredge, from the Marine Science Institute at UCSB. Samples of marine snow and surrounding seawater were obtained via syringe by divers on two occasions from water in the Santa Barbara Channel. Samples collected on six days in December 1994 were of dinoflagellate snow dominated by *Gonyaulax* species. Samples from June 19, 1995 (the only day of the cruise when marine snow was observed) were very fresh large flocs consisting of diatoms from *Rhizosolenia* species. All samples were collected from the chlorophyll *a* maximum, at approximately 20 m deep.

A drop of each sample was then spread on a NSW agar plate (Zobell, 1941) and incubated at 12 °C for several weeks. Purple colonies were

subcultured in an effort to obtain single colonies. Pure cultures were grown in various NSW-based liquid media. CAA = Casamino acids media as described above. YP-1X stands for 0.5 g/L yeast extract (Difco) and 0.5 g/L Bacto peptone. YP-10X stands for 1 g/L yeast extract and 5 g/L Bacto peptone. YE stands for 2 g/L yeast extract and 0.1 g/L glycerophosphate. Samples were also grown on CAS agar plates (Schwyn and Neilands, 1987) to determine whether or not they produced siderophore halos.

Cultures of *A. luteoviolacea* were analyzed for production of TEP using the alcian blue dye procedure of Passow et al. (1994). Three 200 mL cultures in 500 mL flasks were analyzed at 3, 7 and 24 hours, respectively. Aliquots of 250 μ L to 1 mL of culture medium were filtered through 0.2 μ m filters to collect cells. The filters were stained with alcian blue (0.02 in 0.06% acetic acid) and digested with 5 mL 80% H₂SO₄ for 20 hr. The amount of stain retained on the filter was determined by measuring the absorbance of each digested sample at 215 nm. Blank media samples and an unstained sample of 24 hour culture were also tested to measure background interferences.

Results and Interpretation

Isolation using the preparative C-4 HPLC column (Vydac, 25 x 250 mm) was the best method to obtain large (6 mg/ 4 L) quantities of pure alterobactin

A. Attempts to increase production of the alterobactins by adding phosphate or L-arginine to the growth medium did not have significant effects on the yields. Alterobactin A converts to alterobactin B over time in the presence of base or acid (Reid, 1993), as a result of hydrolysis of the ester bond, therefore stored samples should be tested by analytical HPLC to guarantee purity.

Electrospray mass spectrometry: Electrospray mass spectrometry results (Table 3-2) show the masses of the alterobactins and their 1:1 Fe(III) complexes in positive and negative ion modes. The ferric complexes show a mass increase of 53 as a result of addition of iron(III) and loss of three protons to neutralize the charge. The 2:1 complex for alterobactin B: Fe(III) was not observed, which could mean that the complex was unstable under the electrospray conditions. However, a 1:1 Fe(III):Alt-B adduct did appear in negative mode at m/z 997. Fe(III)-Alt-A in negative ion mode gave both a singly charged and a doubly charged peak at m/z 979 and m/z 489 respectively. In positive ion mode, the iron(III)-Alt-A complex (m/z 981) also appeared with replacement of 2 additional hydrogens by two potassiums (m/z 1057.1) or a combination of sodium and potassium (m/z 1041.1). Therefore, up to 5 proton losses are observed by electrospray mass spectrometry.

Ionization mode	Alterobactin A	Fe(III)-Alt-A	Alterobactin B	Fe(III)-Alt-B
+esi (M+H) ⁻	928.3/ 464.8	981.1 (1057.1)*	946.1/ 473.7	999.2
-esi (M-H) ⁻	926.2	979.2/ 489.2	944.4	997.2/ 498.7

Table 3-2. Electrospray mass spectrometry results in terms of m/z where z is 1 or 2. Where two values of m/z are given, the second number is for the doubly ionized species. Only 1:1 complexes were observed for both Fe(III)-alterobactins.

* The value in parentheses represents the $K_2Fe\text{-Alt-A}^+$ ion, which implies replacement of 5 protons from alterobactin A by 2 K^+ and 1 Fe^{3+} .

NMR of gallium-Alt-A: ^1H NMR of Ga(III)-Alt-A shows three resonances, initially observed in D_2O at 9.3, 10.9, and 11.7 ppm, from the glycine amide protons and the amide proton of the LySta (4,8-diamino-3-hydroxyoctanoic acid) fragment. These protons slowly exchange with deuterium and become NMR silent after one or more days in solution (Figure 3-4). The slow exchange is likely to arise from hydrogen bonding interactions. Hydrogen bonding between the proton of an amide (formed from a dihydroxybenzoate moiety and an amine) and the *ortho* oxygen of catechol is well established (Garrett et al., 1992). The predicted conformation of Fe(III)-Alt-A also indicates hydrogen bonding between the glycine amide proton (NH between C_{30} and C_{31}) to the C_{20} carbonyl oxygen and between the glycine amide proton (NH between C_{18} - C_{19}) to the C_{12} amide nitrogen.

Potentiometry: Potentiometric titrations of alterobactins A and B are shown in Figures 3-5 and 3-6 respectively. Fitting the results with the computer programs BEST (Martell and Motekaitis, 1992) and MINQUAD (Sabatini et al., 1974) gave the pK_a values tabulated in Table 3-3. Both programs produced good fits for 5 and 6 protons released from Alt-A and Alt-B, respectively. The only reasonable possibilities for protons that can be observed by a titration of alterobactins A and B are from the two carboxylic acids, the two catechol

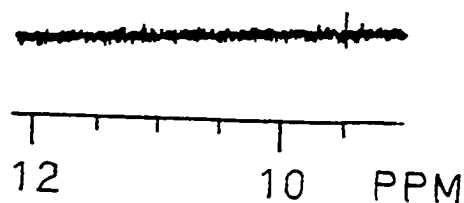
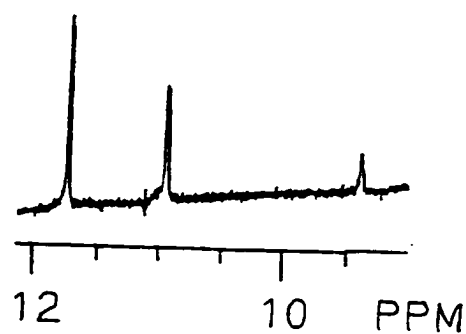


Figure 3-4. ¹H NMR of Ga-alterobactin A in D₂O at 500 MHz on a General Electric GN500. Pulse width = 30 μ sec; acquisition delay = 1.5 sec; 16 transient acquisitions of 16384 points; referenced to HDO at 4.8 ppm. Spectra are shown for the sample immediately after dissolving in D₂O and 5 days later showing the disappearance of the hydrogens in hydrogen bonds by slow exchange with deuterium.

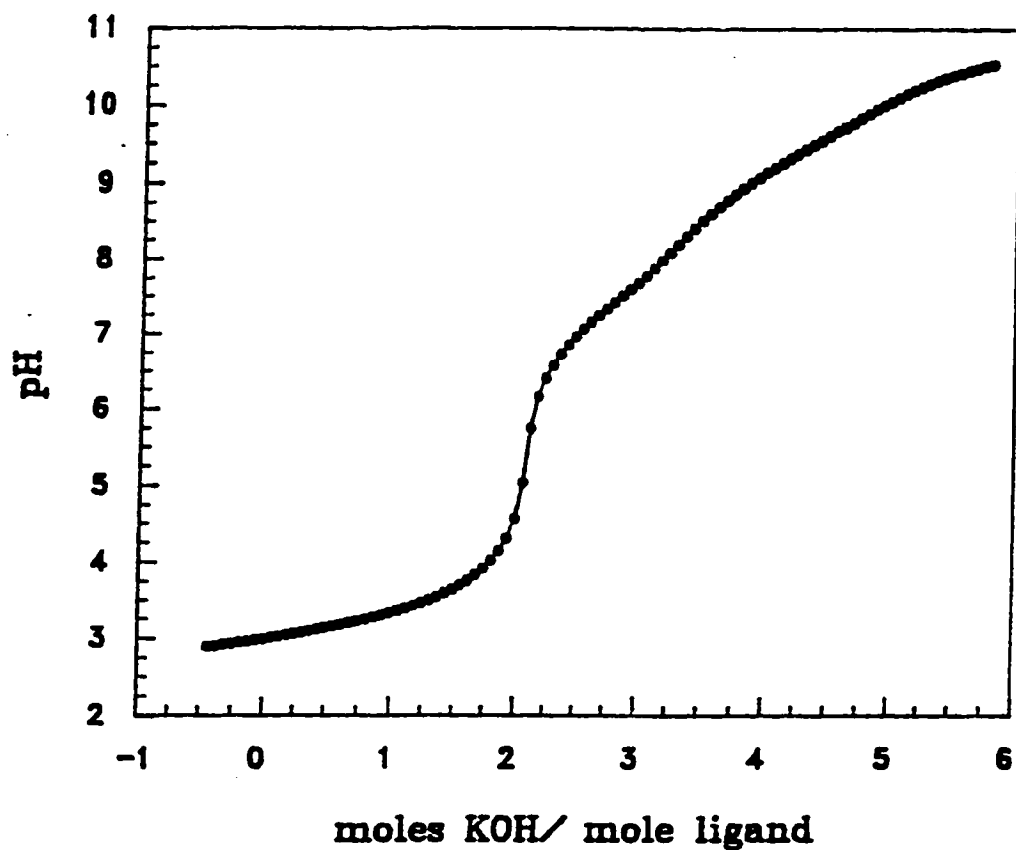


Figure 3-5. Potentiometric titration of 17.2 μmol alterobactin A with 0.1071 M KOH in the presence of 0.5 M KNO_3 .

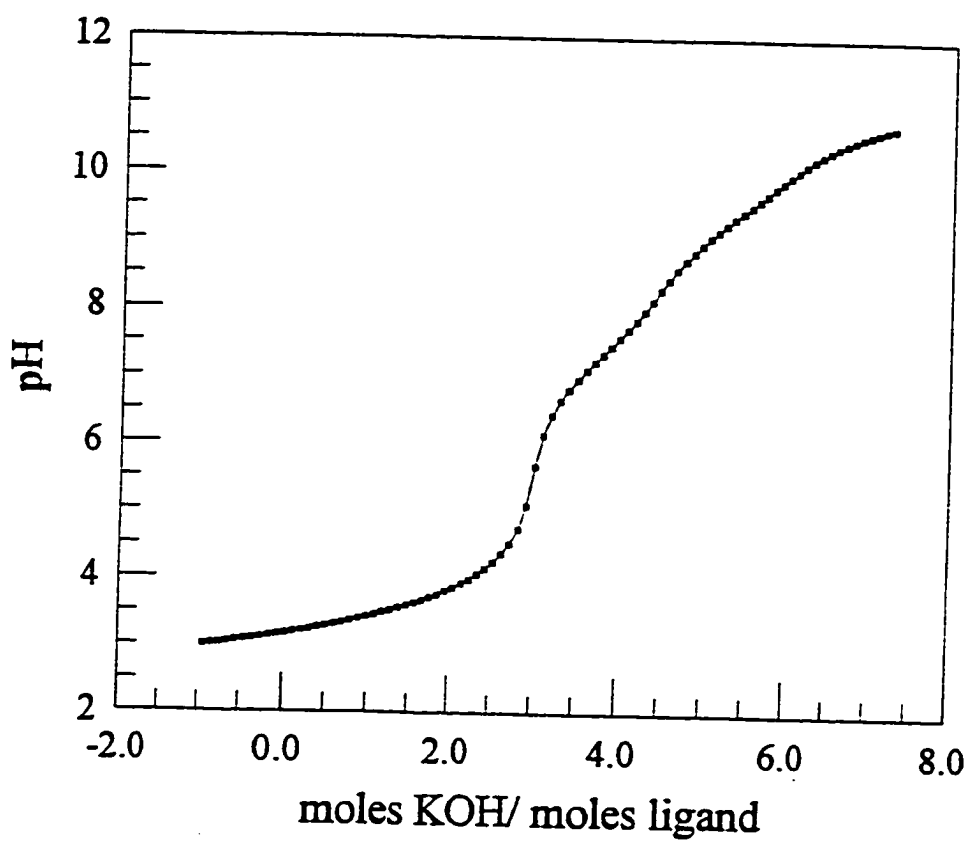


Figure 3-6. Potentiometric titration of 11.1 μmol alterobactin B with 0.1071 M KOH in the presence of 0.5 M KNO_3 .

pK	Alt-A (BEST)	Alt-A (MINIQUAD)	Alt-B (BEST)	Alt-B (MINIQUAD)
1	9.67	9.66	9.68	9.54
2	8.78	8.83	8.36	8.32
3	7.08	7.07	6.98	6.86
4	3.23	3.23	3.94	3.82
5	2.6	2.59	3.13	3.16
6	-	-	3.13	3.03

Table 3-3. Formation constants (as $-\log K$) for protonation of alterobactins A and B. Potentiometric titration curves were analyzed with the programs BEST (Martell and Motekaitis, 1992) and MINIQUAD (Sabatini et al., 1974).

protons and the hydroxyamine. The guanidinium proton of arginine has a pK_a of 12.48 and is not likely to be a factor. Both Alt-A and Alt-B gave the expected pK_a s of around 3.5 for the two and three carboxylic acids, respectively (Table 3-3). Although Alt-A is hydrolyzed to Alt-B at the high pH range, the three high pK values obtained for Alt-A match the three highest pK_a values of Alt-B quite well, so hydrolysis is not responsible for the results. The pK_a s of Alt-A and Alt-B at 6.8, 8.3 and 9.5 are somewhat lower than expected. The pK_a of 6.8 is assigned to the first catechol proton, in analogy to enterobactin (Figure 1-1) which has pK_a values of 6.0, 7.5 and 8.55 for the *ortho*-catechol protons (Loomis and Raymond, 1991b). For comparison, the pK_a values of *N,N*-dimethyl-2,3-dihydroxybenzamide are 8.42 and 12.1 (Smith and Martell, 1977). In the alterobactins, the pK_a of the *ortho*-catechol proton may be lowered because of interactions with the many charged functionalities in the molecule. The *meta*-catechol proton pK_a is expected to be beyond the range of a glass electrode (i.e. ~ 12), but it could also be lower and is assigned to the pK_a of 9.5 or 10. The remaining pK_a of 7.5 to 8 is assigned to deprotonation of the amine of 4,8-diamino-3-hydroxyoctanoic acid. By comparison, ethanolamine has a pK_a of 9.62 (Smith and Martell, 1977). The high pK_a protons are more readily removed from the alterobactins than from the simple model complexes.

Potentiometric titration of the iron complex of alterobactin A was also performed. An $\text{Fe}(\text{ClO}_4)_3$ stock solution was standardized against ferrozine as described in the Experimental section. The results are plotted in Figure 3-7, and the concentration was determined to be 26.5 mM Fe(III). To measure excess acid in the iron(III) stock solution, a titration was performed in the presence of excess EDTA and a Gran's fit (Gran, 1952) was made to the titration curve (Figure 3-8). The acid in the titration vessel is derived from EDTA chelation of iron(III) which releases two equivalents of protons for each equivalent of iron(III) present, plus the excess acid in the $\text{Fe}(\text{ClO}_4)_3$ solution (Schwarzenbach, 1957; Harris, 1979b). Gran's function, ϕ , (Gran, 1952) is essentially a calculation of H^+ concentration according to Equation 1:

$$\phi = (V_i + V_{\text{KOH}})10^{-\text{pH}} \quad (1)$$

V_i = initial volume

V_{KOH} = Volume of KOH added

Proton concentration changes rapidly as the acid is neutralized, and the Gran function becomes extremely small. Plotting ϕ vs. V_{KOH} generates a curve which approaches zero as the endpoint of a titration is reached. The amount of base needed to neutralize all of the acid in the solution can be calculated from the x-

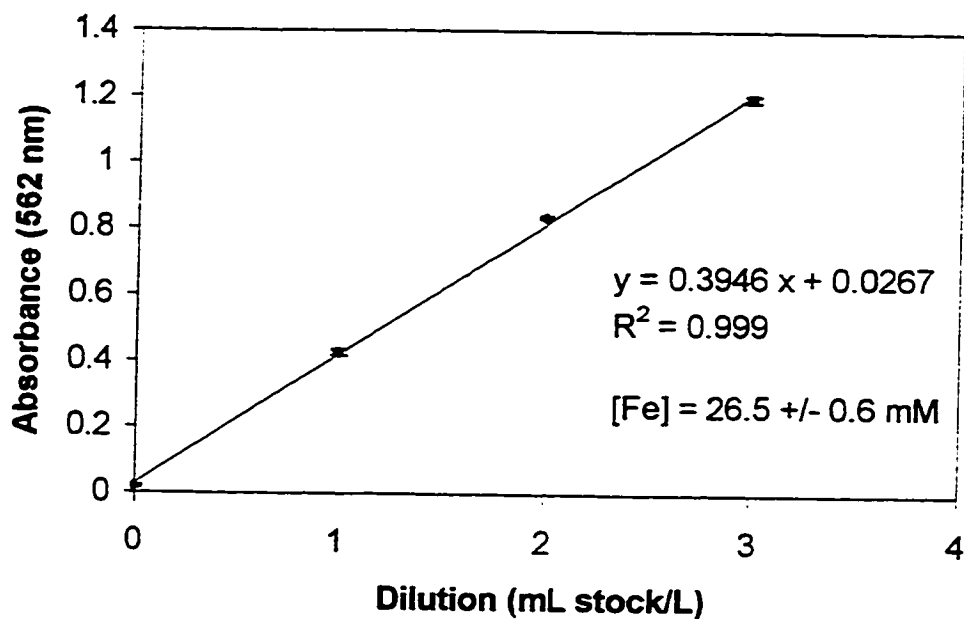


Figure 3-7. Determination of the concentration of $\text{Fe}(\text{ClO}_4)_3$ stock solution by the ferrozine method (Stookey, 1970). Each point is the average of nine different measurements at that concentration. Error bars indicate the standard deviation.

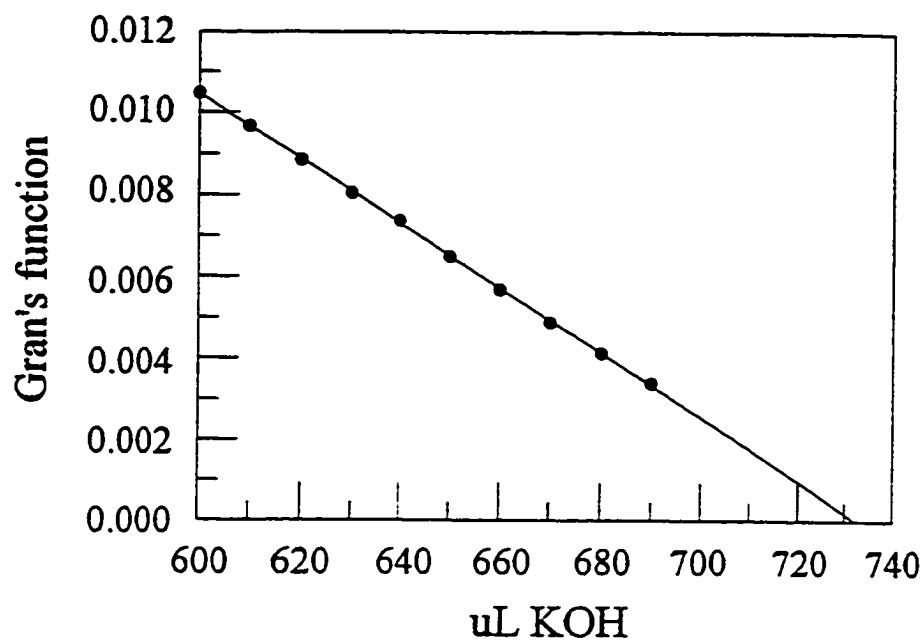


Figure 3-8. Gran's fit to the EDTA titration of the $\text{Fe}(\text{ClO}_4)_3$ stock solution in order to determine the amount of excess acid present. From the intercept at 732 μL of KOH added, the concentration of H^+ in the Fe(III) stock solution is calculated to be 32.75 μM .

intercept of a fit to the line as it approaches neutrality (Figure 3-8). However, the points selected for the line can affect the calculated endpoint somewhat as the points actually fall along a curve which reaches an asymptote at the axis.

Potentiometric titration of ferric alterobactin A shows the number of protons released per mole of complex (Figure 3-9). Upon chelation of iron(III), six protons are released. These protons are assigned to the two catechol protons, the two carboxylic acid protons and the two β -hydroxy aspartate hydroxyl protons of alterobactin A. Because acid is present in the $\text{Fe}(\text{ClO}_4)_3$ stock solution, the amount of excess added acid was subtracted from the total amount of added base. Thus, the x-axis represents the amount of base used to titrate the protons released from the ligand as it binds iron(III). A Gran's plot (Gran, 1952) was used to accurately define the endpoint of the titration (Figure 3-10). The data in Figure 3-10 show 5.97 protons released, based on the ratio of moles of hydroxide consumed to moles of ligand titrated. The exact choice of points used for the Gran's plot can shift the final result by ± 0.1 equivalent. A second experiment using 10.4 μmol ferric alterobactin A was calculated to release 5.8 protons per alterobactin A molecule. Overall, the release of 6 protons from the catechol and the two β -hydroxy aspartate moieties upon chelation of iron(III) is well-supported.

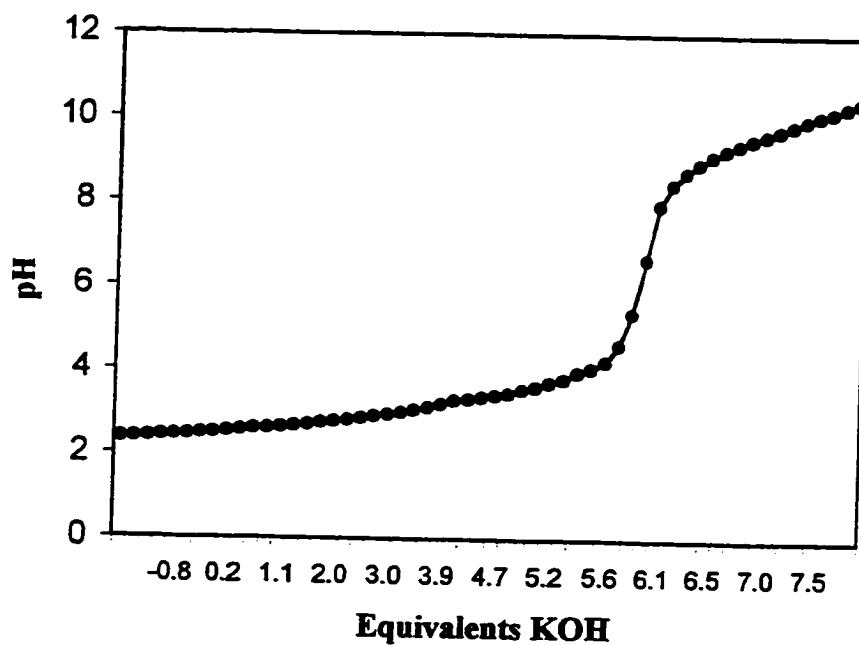


Figure 3-9. Potentiometric titration of 9.98 μmol Fe(III)-alterobactin A with 0.1156 M KOH in the presence of 0.1 M KNO_3 to maintain constant ionic strength.

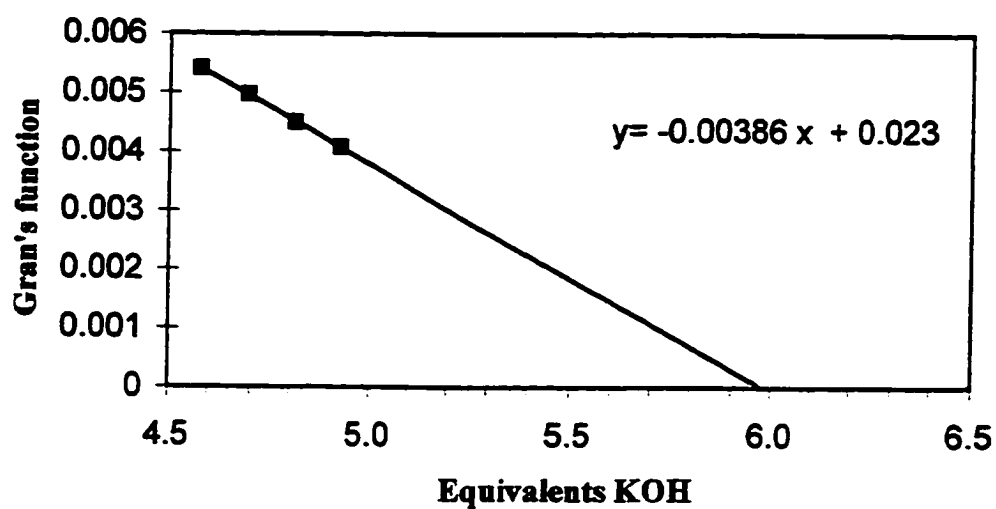


Figure 3-10. Gran's plot of the titration of Fe(III)-Alt-A in Figure 3-9 showing the endpoint after 5.97 equivalents of KOH have been added, therefore 6 protons are released from alterobactin A upon coordination of Fe(III).

Electrochemistry: Using 5 mM Bis-Tris buffer to keep iron(III) in solution, the reduction potentials of ferric Alt-A at pH 7 (Figure 3-11) and ferric Alt-B at pH 6 (Figure 3-12a) and pH 8.2 (Figure 3-12b) were determined. Ferric alterobactin B has two pH-dependent forms as described in the Discussion section, so the electrochemistry was performed at pH values where only one form is present.

Upon initial addition of alterobactin A (40 μ M) to the solution of 10 μ M Fe(III) in Bis-Tris, a complex with a peak at -0.614 V forms. Over time, the peak at -0.614 V decreases in current while the current of the -0.972 V peak increases (Figures 3-11 and 3-13). The reaction appears to be complete in approximately 15 min (Figures 3-11 and 3-13). The data was fit to a power function ($y = ax^b$) and does not fit a typical exponential function ($y = ae^{bx}$), so no rate constant was obtained.

Cyclic voltammetry of ferric Alt-A (Figure 3-14) shows reversibility of the wave at -0.972 V. Although the SWV peak appears to have a shoulder, possibly from absorption effects, the CV trace is clean. The reduction of Fe(III)-(Alt-B)₂ was also shown to be reversible by cyclic voltammetry at both pH 6 (Figure 3-15) and pH 8.2 (Figure 3-16). Reversibility is required for application of the chelate scale (see Discussion section). Table 3-4 lists the reduction potentials versus SCE for each of the ferric alterobactin species and

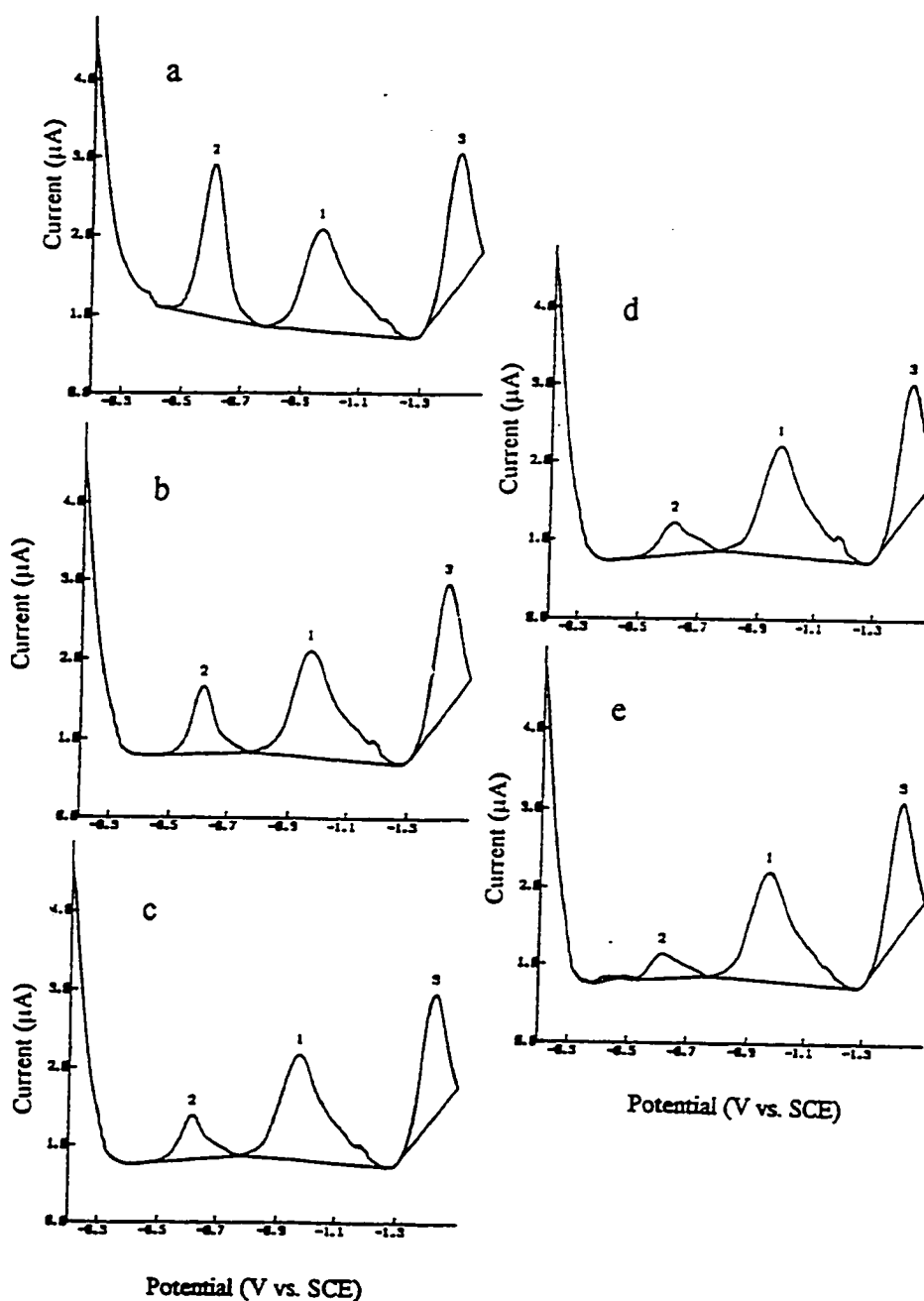


Figure 3-11. Square wave voltammetry of $10 \mu\text{M Fe}^{3+} + 40 \mu\text{M Alt-A}$ in $5 \text{ mM BisTris pH } 7$ over time showing the decrease in the peak at -0.614 V (peak 2) and the increase in the peak at -0.971 V (peak 1). a) Initial scan. b) to e) scans taken every 2 minutes. Peak 3 is the Fe(III)/Fe(II) reduction wave.

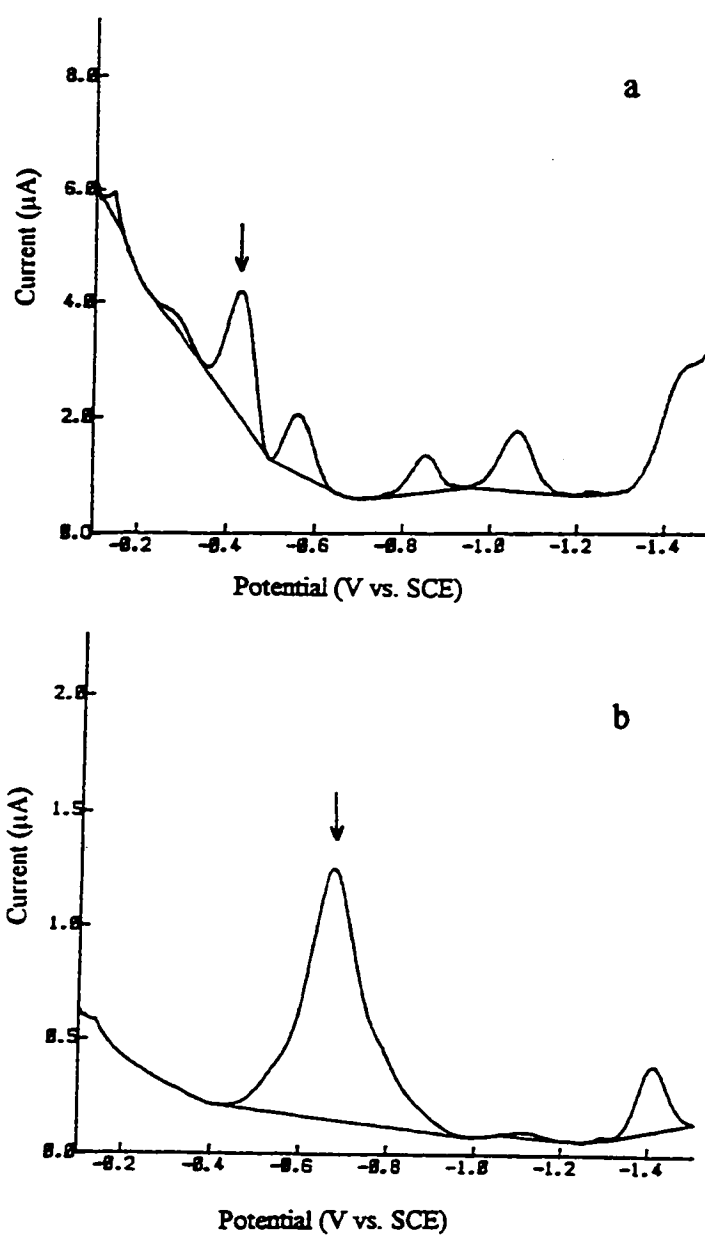


Figure 3-12. Square wave voltammetry of $10\ \mu\text{M}\ \text{Fe}^{3+} + 20\ \mu\text{M}$ Alterobactin B in 5 mM BisTris at a) pH 6 and b) pH 8.2.

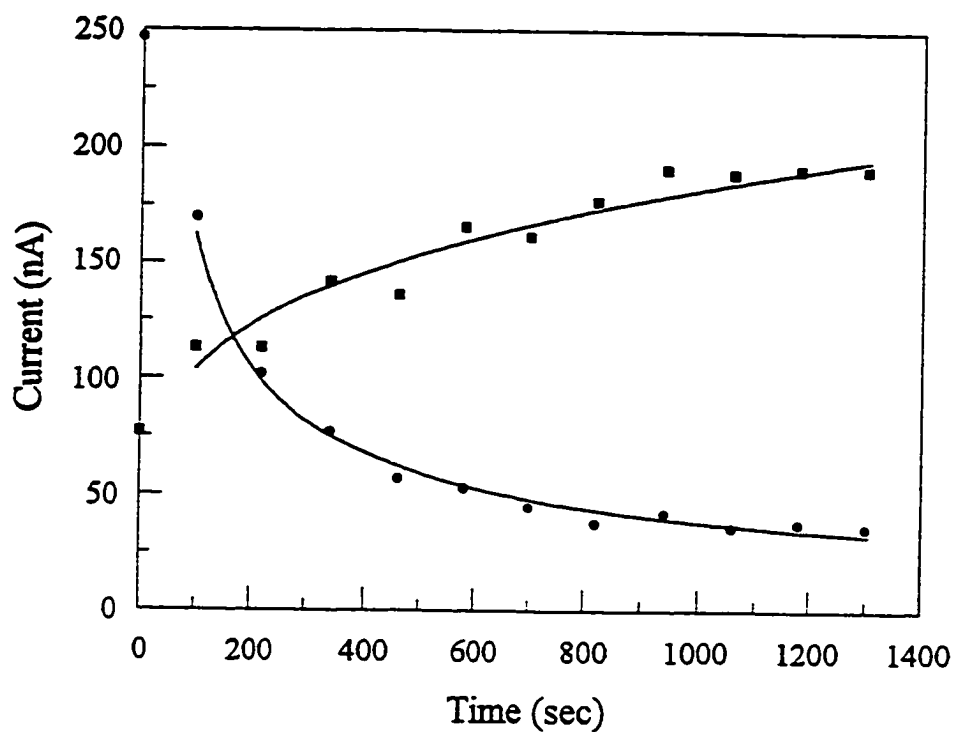


Figure 3-13. Increase in height of the -0.971 V reduction peak and decrease in height of the -0.614 V peak for Fe(III)-Alt-A over time as the complex is formed. The lines are power fits to the data points shown.

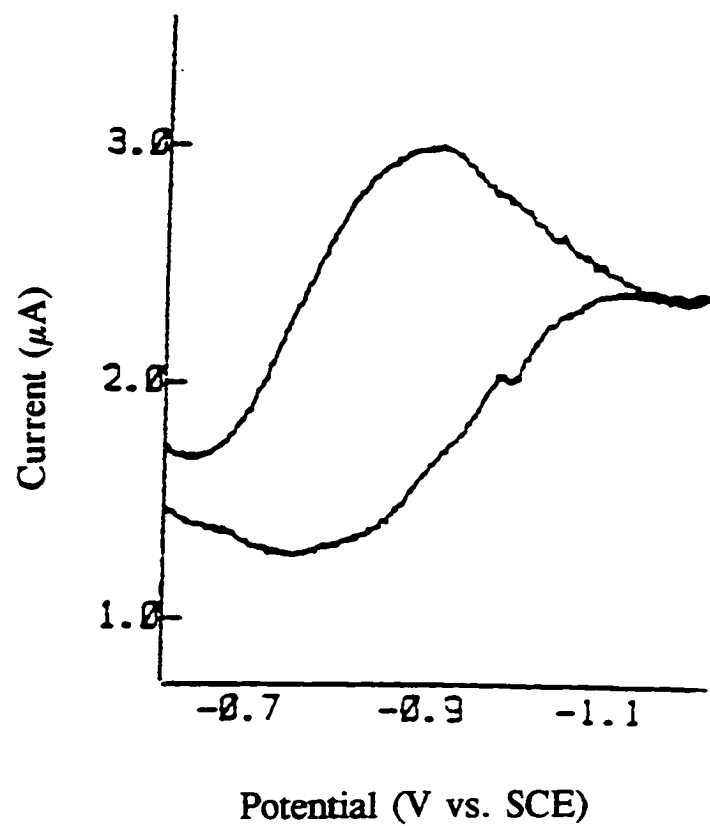


Figure 3-14. Cyclic voltammetry at 50 mV/ sec of $10\ \mu\text{M Fe}^{3+}$ + $40\ \mu\text{M Alt-A}$ in 5 mM BisTris at pH 7.

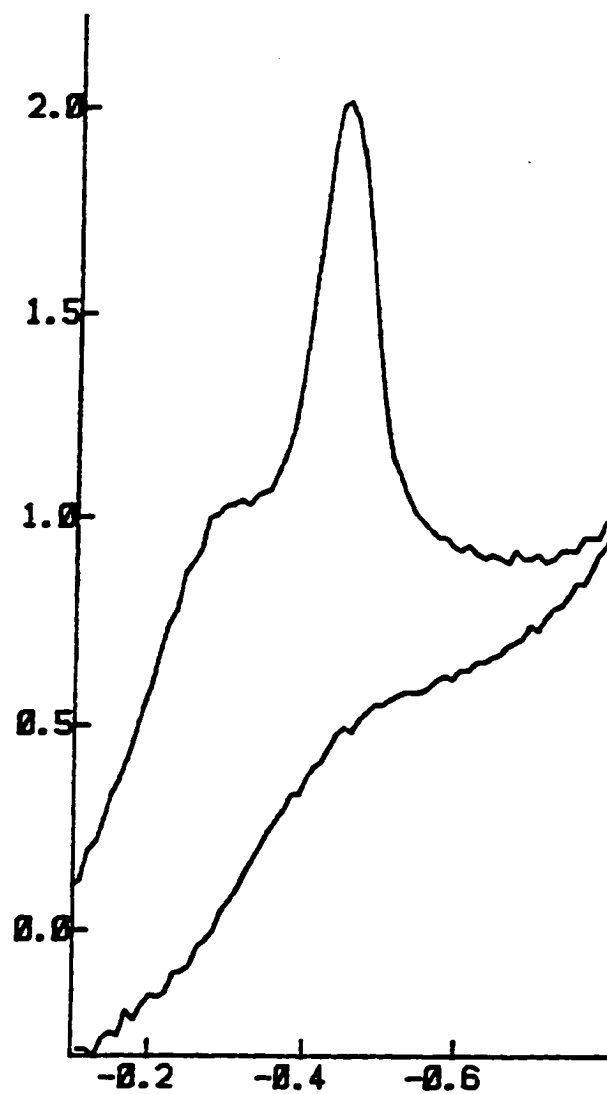


Figure 3-15. Cyclic voltammetry at 100 mV/sec of 10 μM Fe^{3+} + 60 μM Alterobactin B in 5 mM BisTris, pH 6.

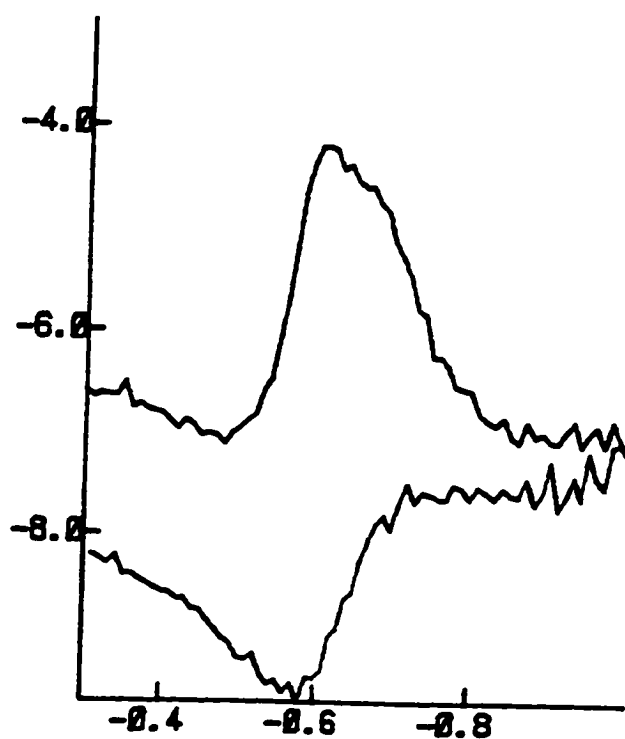


Figure 3-16. Cyclic voltammetry at 100 mV/sec of 10 μM Fe^{3+} + 60 μM Alterobactin B in 5 mM BisTris, pH 8.2.

Siderophore	Reduction potential (V vs. SCE)	Stability Constant
Fe ^{III} -Alterobactin A (pH 7)	-0.972	51 ± 2
Fe ^{III} -(Alterobactin B) ₂ (pH 6)	-0.428	37.6 ± 1.2
Fe ^{III} -(Alterobactin B) ₂ (pH 8)	-0.672	43.6 ± 1.5

Table 3-4. Stability constants derived from the chelate scale (Taylor et al., 1994) from reduction potentials measured by square wave voltammetry.

their calculated stability constants based on the chelate scale of Taylor et al. (1994) (Figure 3-2).

Alterobactin A competitions with desferrioxamine B:

HPLC - The HPLC chromatogram of the competition of Fe-Alt-A with DF is shown in Figure 3-17. The four different components are clearly separated at 215 nm, and use of the diode array detector also allows simultaneous measurement at other wavelengths, such as the $\lambda_{\max}$ values of the ferric complexes.

The calibration curves for standard solutions were calculated based on peak area at the $\lambda_{\max}$ for each compound. The calibrations give straight lines, which indicate linear correlations between amount injected and peak area detected. However, the siderophores are capable of extracting iron(III) from the system wherever it is present, so any excess iron in or from the stainless steel tubing or on the column alters the measured amounts of each iron containing species, and therefore the concentrations of free ligand. Also, injection of each sample potentially contaminates the system with any excess iron present, which can interfere with subsequent samples.

In the competition samples, data for all but the 1:1 case were difficult to interpret because the equilibrium was pushed so far to one side that little of

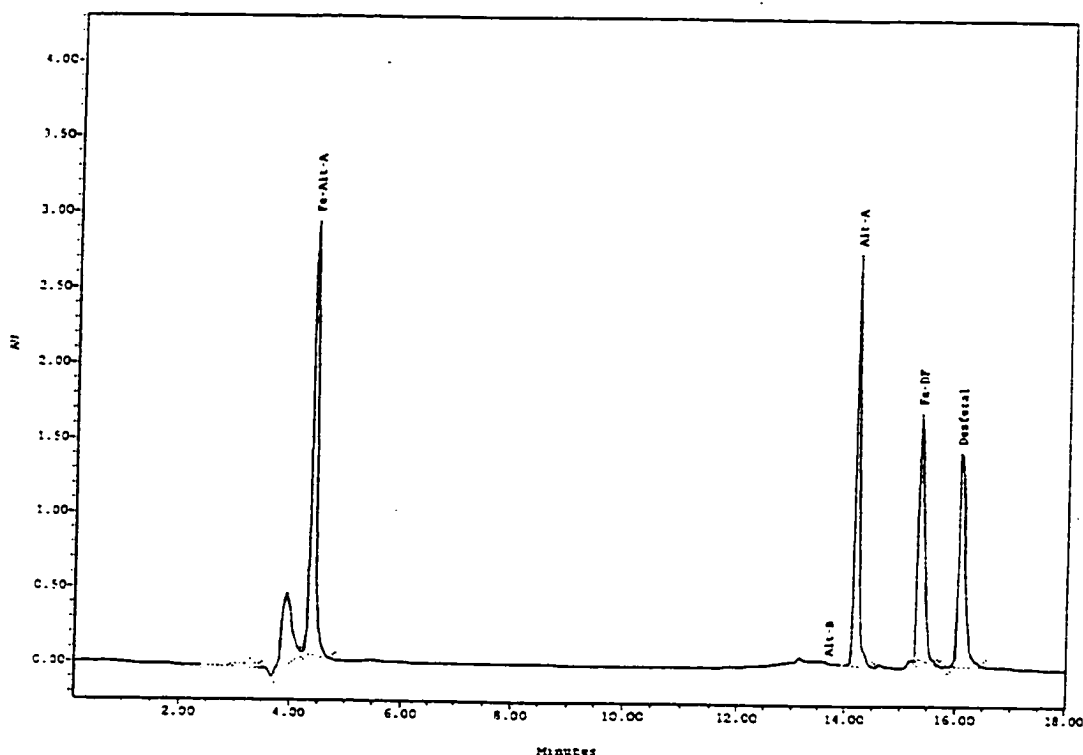


Figure 3-17. HPLC chromatogram at 215 nm showing the separation of Fe(III)-Alt-A, Alt-A, Fe(III)-DF and DF. The HPLC gradient changed linearly from 100% A to 50% B in 7 minutes after a 1 minute wash with 100% A; held at 50% B for 2 minutes and returned to 100% A in 2 minutes. Solvent A was 10 mM MES (2-*N*-(morpholino)ethanesulfonic acid), pH 6. Solvent B was 10 mM MES pH 6, in 20% H₂O/ 80% CH₃CN. Samples were collected for 18 minutes.

the other species was present. Because of difficulties with iron(III) contamination and accurate measurement of all species present, no values of the stability constant were obtained from the HPLC competition experiments.

UV-Visible Spectrophotometry - Competitions between alterobactin A and desferrioxamine B reached equilibrium within approximately 24 hours (see figure captions for exceptions). The 1:1 competition between DF and Alt-A reached equilibrium at approximately 50:50 Fe(III)-DF: Fe(III)-Alt-A, regardless of the direction of approach (Figures 3-18 and 3-19).

Competitions at 2:1 and 5:1 ratios of FeL to L' (Figures 3-20 to 3-23) were dominated by the iron(III) complex originally in excess, and in the 5:1 cases very little change was observed.

Calculations were based on the equilibria:



or



such that total iron and total ligand can be determined as follows:

$$[\text{Fe(III)}]_{\text{tot}} = [\text{Fe(III)-Alt-A}] + [\text{Fe(III)-DF}] \quad (4)$$

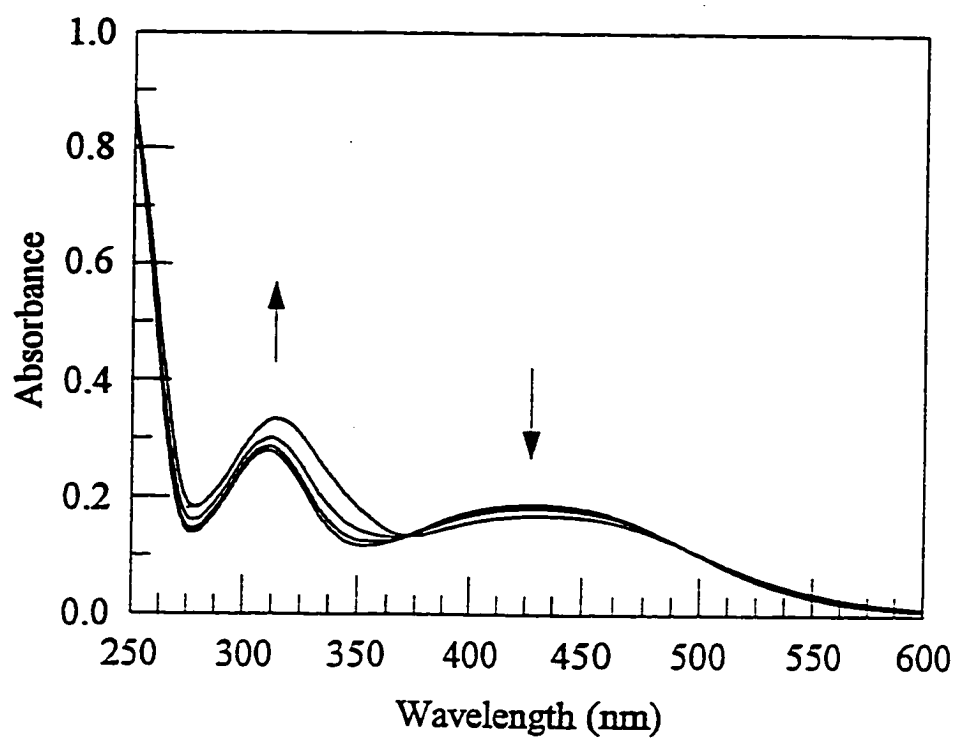


Figure 3-18. UV-Visible competition of 66 μM 1:1 Fe-DF + Alt-A at pH 6 in 0.1 M MES. Spectra were obtained in a 1 cm cell at 0, 1, 7, 24 and 48 hours.

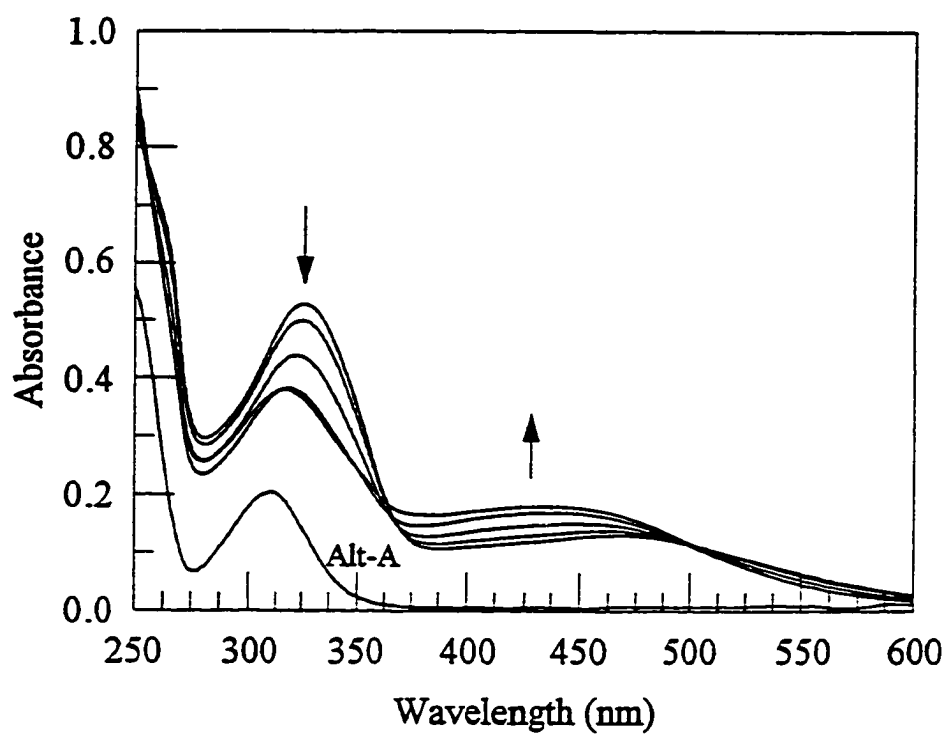


Figure 3-19. UV-Visible competition of 66 μM 1:1 Fe-Alt-A + DF at pH 6 in 0.1 M MES. Spectra were obtained in a 1 cm cell at 0, 1, 7, 24 and 48 hours.

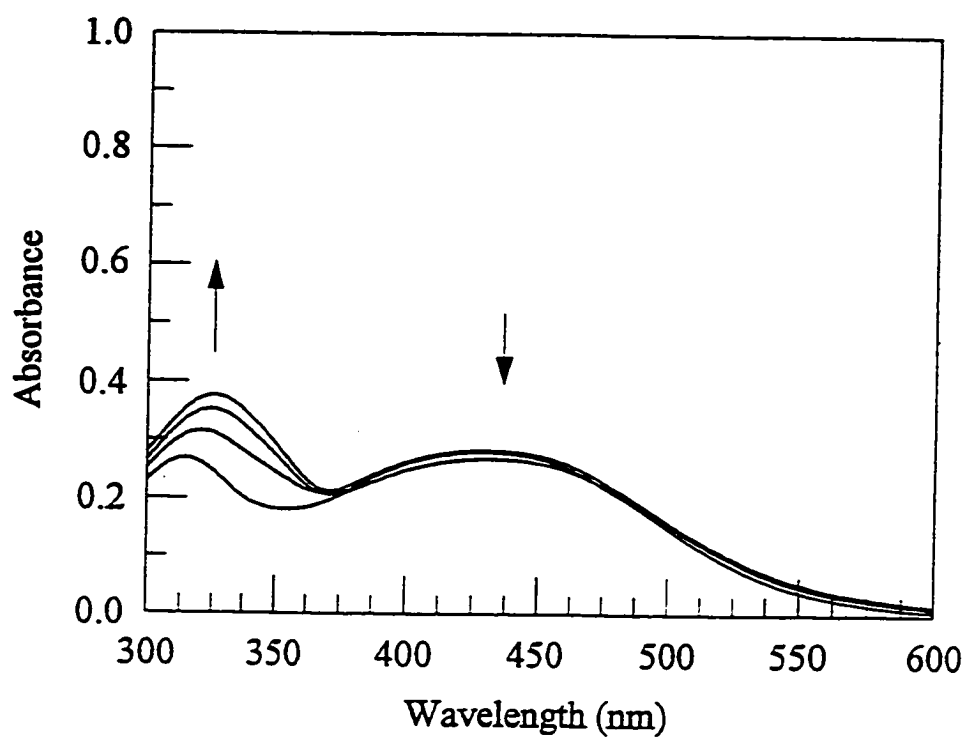


Figure 3-20. UV-Visible competition of 2:1 Fe-DF + Alt-A at pH 6 in 0.1 M MES. Spectra were obtained in a 1 cm cell at 0, 6, 24 and 48 hours. One equivalent is 50 μ M.

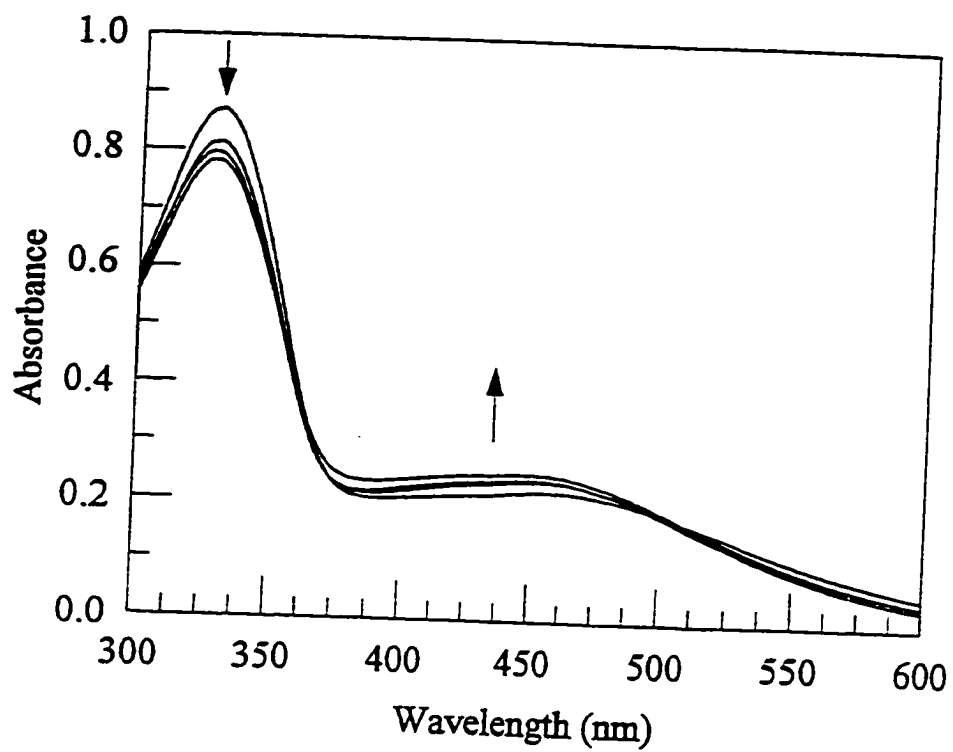


Figure 3-21. UV-Visible competition of 2:1 Fe-Alt-A + DF at pH 6 in 0.1 M MES. Spectra were obtained in a 1 cm cell at 0, 6, 24 and 48 hours. One equivalent is 50 μ M.

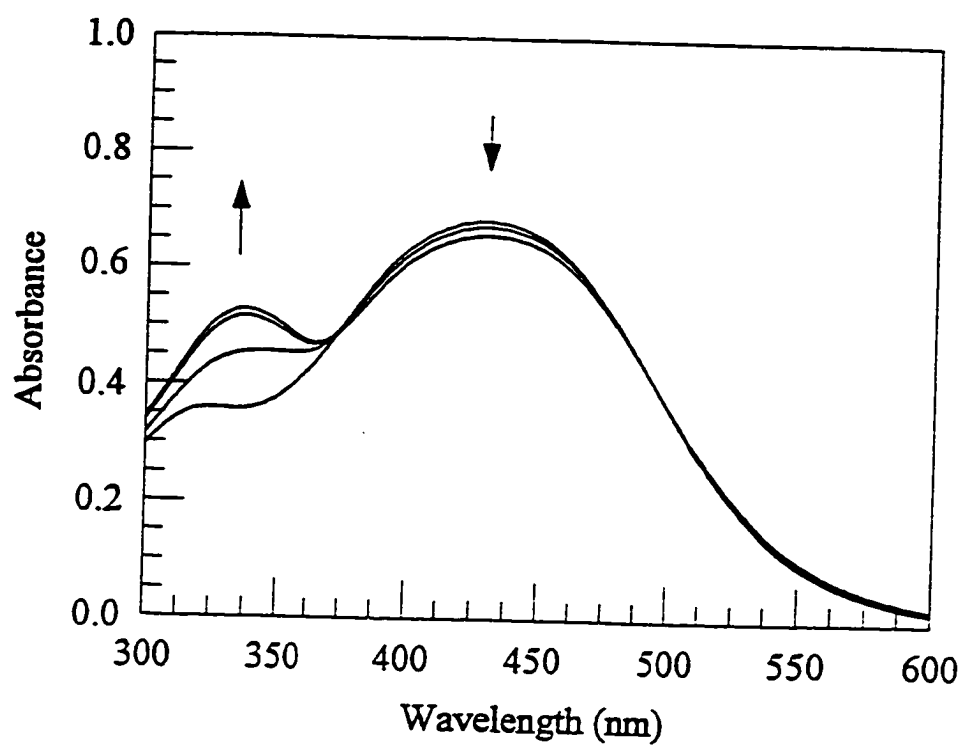


Figure 3-22. UV-Visible competition of 5:1 Fe-DF + Alt-A at pH 6 in 0.1 M MES. Spectra were obtained in a 1 cm cell at 0, 6, 24 and 48 hours. One equivalent is 50 μ M.

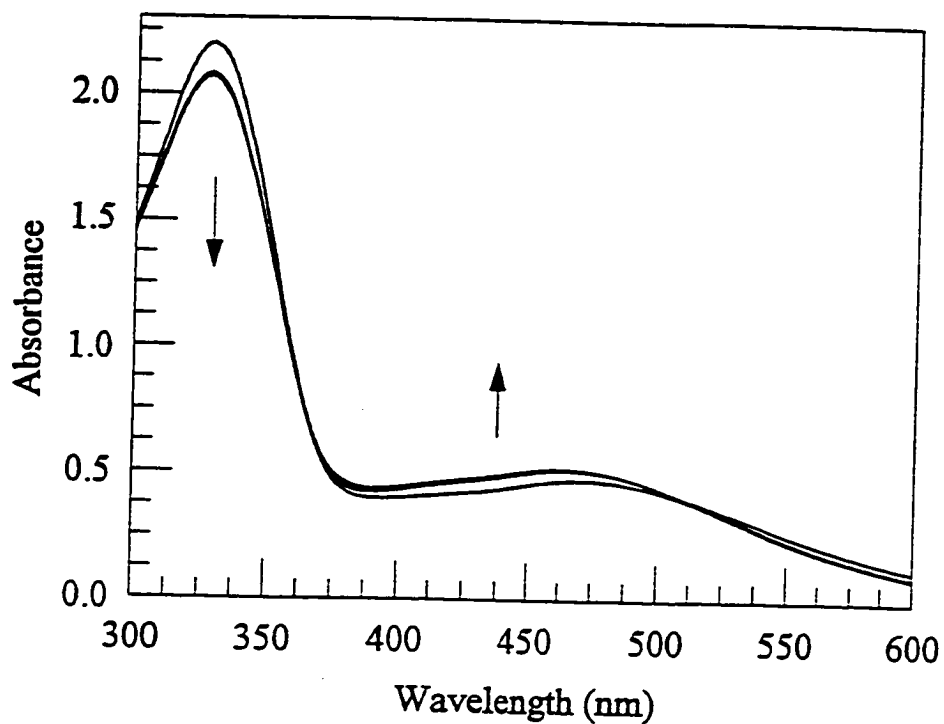


Figure 3-23. UV-Visible competition of 5:1 Fe-Alt-A + DF at pH 6 in 0.1 M MES. Spectra were obtained in a 1 cm cell at 0, 6, 24 and 48 hours. One equivalent is 50 μ M.

$$[\text{Alt-A}]_{\text{tot}} = [\text{Fe(III)-Alt-A}] + [\text{Alt-A}] \quad (5)$$

$$[\text{DF}]_{\text{tot}} = [\text{Fe(III)-DF}] + [\text{DF}] \quad (6)$$

The absorbance bands of Fe(III)-DF and Fe(III)-Alt-A overlap considerably, so the total absorbance at any point was considered to be the sum of the absorbances of each contributing species. For example:

$$\text{Abs}(430 \text{ nm}) = \epsilon_{\text{Fe-Alt-A}}[\text{Fe-Alt-A}]l + \epsilon_{\text{Fe-DF}}[\text{Fe-DF}]l \quad (7)$$

Therefore from equations (4) and (7):

$$[\text{Fe-DF}] = \frac{\text{Abs}(430 \text{ nm}) - \epsilon_{\text{Fe-Alt-A}}[\text{Fe}_{\text{tot}}]l}{(\epsilon_{\text{Fe-DF}} - \epsilon_{\text{Fe-Alt-A}})l} \quad (8)$$

Only wavelengths between 400 and 450 nm were considered, which avoided interference from the catechol absorbance of free alterobactin A at 310 nm. The values of the equilibrium constant were nearly the same throughout that region of the spectrum. The extinction coefficients of Fe(III)-DF and Fe(III)-Alt-A were determined by measurement of the spectra of each species independently.

The spectrophotometric data were used to obtain the measured stability

constant of the competition, K_{meas} , defined in equation 9 and tabulated in Table 3-5):

$$K_{\text{meas}} = \frac{[\text{Fe(III)-Alt-A}][\text{DF}]}{[\text{Fe(III)-DF}][\text{Alt-A}]} = \frac{K_{\text{Fe(III)-Alt-A}}}{K_{\text{Fe(III)-DF}}} \quad (9)$$

The overall proton-independent stability constant, K_b , for Fe(III)-alterobactin A is described by equation 10:

$$K_b = \frac{[\text{Fe}^{\text{III}}\text{Alt-A}]}{[\text{Fe}^{3+}][\text{Alt-A}^{6-}]} \quad (10)$$

The proton-dependent stability constant, K^* , depends on the pH as shown below in equation 11:

$$K^* = \frac{[\text{Fe}^{\text{III}}\text{Alt-A}][\text{H}^+]^6}{[\text{Fe}^{3+}][\text{Alt-A}^{6-}]} \quad (11)$$

In order to use the above equations to calculate the stability constant, the pK_a values of the ligand must be known, and they are used to calculate α , an expression which considers all of the protonation states of the ligand, as shown in equation 12 (Harris, 1982):

Experiment	K_{meas} at 24 hrs	$K_b(\text{FeAlt-A})$	$\log K_b$
1:1 Fe(III)Alt-A + DF	0.098	9.83×10^{48}	48.99
1:1 Fe(III)DF + Alt-A	0.082	8.15×10^{48}	48.91
2:1 Fe(III)Alt-A + DF	0.283	2.83×10^{49}	49.45
2:1 Fe(III)DF + Alt-A	0.806	8.06×10^{49}	49.91
5:1 Fe(III)Alt-A + DF	52.631	5.26×10^{51}	51.72
5:1 Fe(III)DF + Alt-A	-1.266	-1.26×10^{50}	-

Table 3-5. Results of the UV-Visible competition between alterobactin A and desferrioxamine B for Fe(III). K_{meas} was determined at 430 nm (the values are similar between 400 and 450 nm) and K_b was determined as described in Results and Interpretation.

$$\alpha^{6-} = \frac{K_1 K_2 K_3 K_4 K_5 K_6}{[H^+]^6 + K_1 [H^+]^5 + K_1 K_2 [H^+]^4 + K_1 K_2 K_3 [H^+]^3 + K_1 K_2 K_3 K_4 [H^+]^2 + K_1 K_2 K_3 K_4 K_5 [H^+] + K_1 K_2 K_3 K_4 K_5 K_6}$$

The first four pKs for Alt-A were determined by potentiometric titration and fitting with BEST and MINQUAD (Table 3-3). The α^{6-} values are also dependent on the pK_as of the β -hydroxy aspartate hydroxyl protons, which are not accessible by the glass electrode and therefore were estimated to be 16-18 (Reid et al., 1993). A range for the α^{6-} value between $10^{-28.5}$ and $10^{-32.5}$ is obtained for Alterobactin A at pH 6.

The proton-independent stability constant, K_b , can be defined in terms of K^* and α^{6-} for Fe(III)-Alt-A (Raymond, 1984).

$$K_{Fe(III)-Alt-A} = \frac{K_{Fe(III)-Alt-A}^*}{\alpha^{6-}} \quad (13)$$

The conditional stability constant for Fe(III)-Alt-A can be calculated from the measured competition equilibrium constant, K_{meas} , and the conditional stability constant of Fe(III)-DF as shown in equation 14:

$$K_{Fe(III)Alt-A}^* = K_{meas} \cdot K_{Fe(III)DF}^* = K_{meas} \cdot K_{DF} \cdot \alpha_{DF}^{3-} \quad (14)$$

The proton-independent stability constant of Fe(III)-DF is $10^{30.6}$ (Schwarzenbach and Schwarzenbach, 1963) and its α^3 value is calculated from an equation analogous to equation 12. The pK_a values of DF are 8.39, 9.03 and 9.70 (Schwarzenbach and Schwarzenbach, 1963) so α^3 becomes $10^{-9.1}$ and K_{Fe-DF}^* is $10^{21.5}$. For each K_{meas} obtained, a value of K_b can be calculated as shown in Table 3-5. The K_b values were all calculated using the more conservative α^6 value of $10^{-28.5}$. The values of K_b are relatively close together. The average K_b is $10^{49.8}$ which is consistent with other methods (see Discussion section). Using the α^6 value of $10^{-32.5}$ would raise the average K_b to $10^{53.8}$.

At large excess of Fe(III)-DF, the value of K_b could not be determined. In fact, the value of K_{meas} is negative because the absorbance of Fe(III)-DF actually increased slightly. The absorbance increase is probably an artifact from the removal and replacement of the cuvette after 24 hours or some other source of random error. The K_b value becomes negative for that case, which is chemically meaningless.

Vanadium(V) and Alterobactin A: Alterobactin A forms complexes with vanadate resulting in pH- and oxygen-dependent color changes. At pH 6, a green complex is initially formed which, in the presence of air, converts to a

gold solution within two hours. Addition of iron(III) to the gold material does not produce the usual absorbance spectrum of ferric alterobactin A, suggesting decomposition of the siderophore. The gold-colored, aerobic sample gives the ^{51}V -NMR signal of mono-vanadate at -584 ppm (Figure 3-24b). In the absence of oxygen, the green complex forms in about an hour and is stable (Figure 3-25). However, the green complex gives no ^{51}V NMR signal (Figure 3-24a), which indicates that the vanadium is no longer in the 5+ oxidation state. Addition of iron(III) to the green material leads to a green solution with red-brown precipitate, probably from iron hydroxide precipitation at pH 6. As air is introduced, the green solution slowly converts to gold solution and the precipitate dissolves.

Degassing the system by bubbling with argon was sufficiently effective in removing oxygen from the system, since the green solution of vanadate plus alterobactin A at pH 6 was stable for several days. However, these results are not entirely conclusive. The seals of the cells with septa were not air-tight in all cases and a better system would be required to maintain consistently anaerobic conditions.

At pH 8 in the presence of air, a pale green solution was initially observed, which turned to orange within an hour. In the absence of air, the same process occurs, but more slowly (Figure 3-26). Addition of iron(III) did

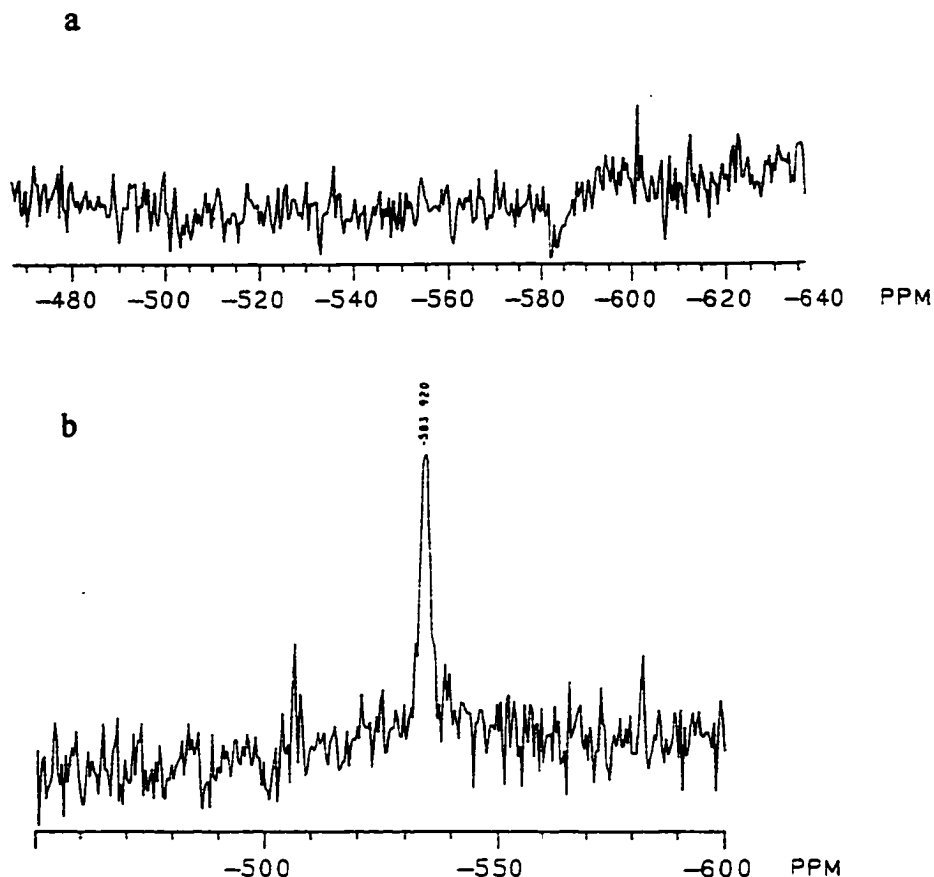


Figure 3-24. ^{51}V NMR at 79 MHz on a General Electric GN300 instrument of the complex formed when 0.3 mM vanadate was added to 0.3 mM Alt-A at pH 6 in 0.1 M phosphate buffer. a) Absence of signal from anaerobic sample. b) Appearance of vanadate resonance at -583 ppm after air is introduced. Pulse width = 20 μsec ; acquisition delay = 100 msec; 40,000 transient acquisitions of 8192 points; referenced to VOCl_3 as an external standard, $\delta(\text{VOCl}_3) = 0.0$ ppm.

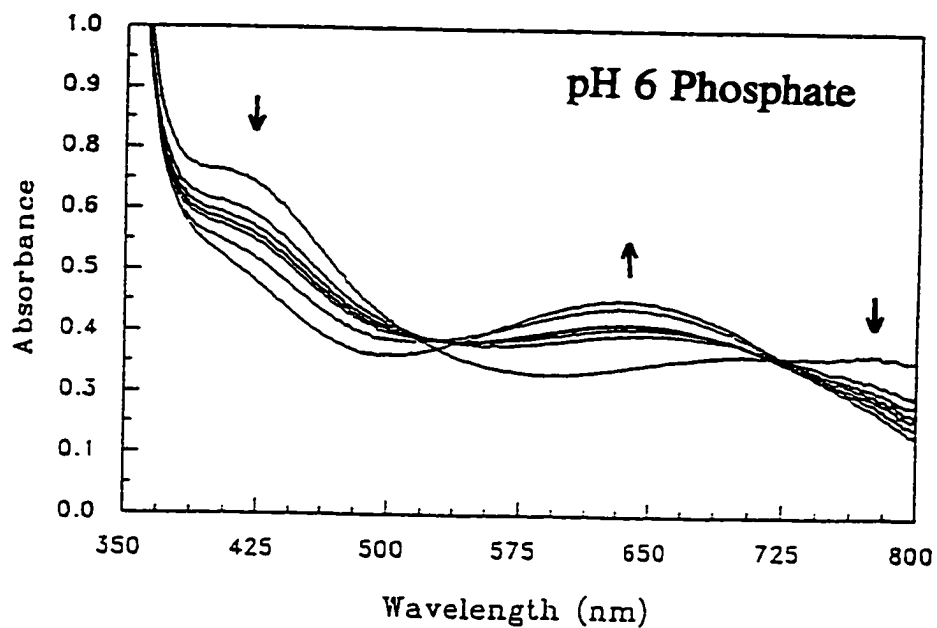


Figure 3-25. UV-visible spectrum of 0.3 mM vanadate + 0.3 mM Alt-A in 0.1 M phosphate buffer pH 6 under Argon. Spectra were obtained in a 1 cm cell every fifteen minutes for an hour, then at 5 and 22 hours.

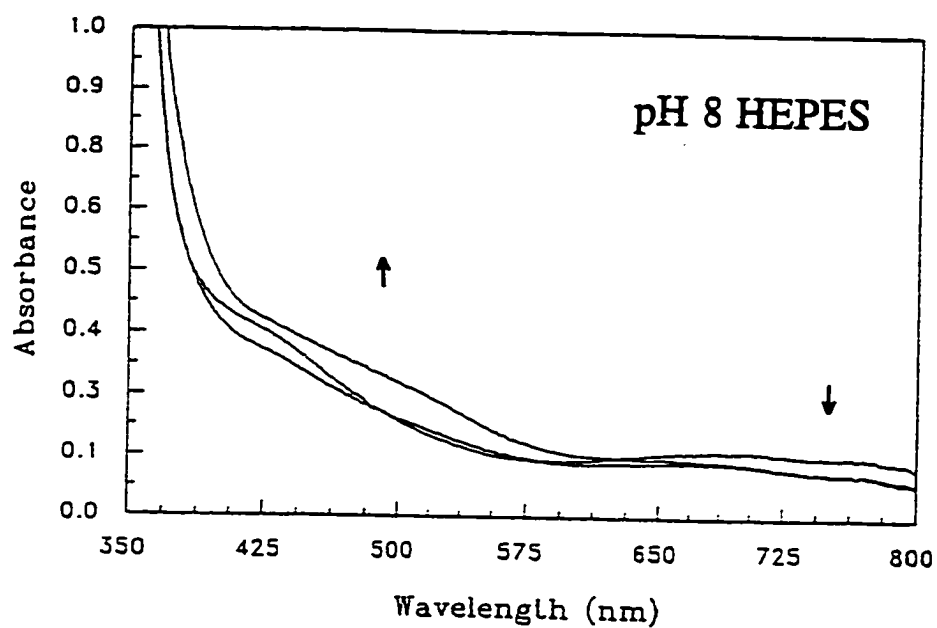


Figure 3-26. UV-visible spectrum of 0.3 mM vanadate + 0.3 mM Alt-A in 0.1 M HEPES buffer pH 8 under Ar. Spectra were obtained in a 1 cm cell at 0, 1, 1.5 and 4.8 hours.

result in the formation of the Fe(III)-Alt-A complex, indicating greater stability of the siderophore toward decomposition by vanadium(V) at pH 8. The absence of the green material may indicate the formation of a vanadium(V) complex with alterobactin A at pH 8, instead of the apparent reduction at pH 6.

All of the buffers showed the same complex formation at the two pH values investigated. At this concentration (0.3 mM) of vanadium(V), no interaction of vanadate with the buffers was observable in blank solutions either spectrophotometrically or by ^{51}V NMR. Therefore, under these experimental conditions the presence of the buffer did not interfere with the results.

Competition experiments with iron(III), vanadium(V) and Alt-A were performed anaerobically to avoid decomposition of the siderophore. At pH 6, ferric Alt-A in the presence of one equivalent of added vanadium(V) decreased in absorbance by 6.7% over 21 hours (Figure 3-27). The decrease at pH 6 is similar to the absorbance change at pH 8, which dropped by 3.7% (Figure 3-28) in 5 hours. Approaching the equilibrium from the opposite direction by adding iron(III) to vanadium(V)-Alt-A complexes at both pH values showed a slow appearance of the ferric Alt-A band at 476 nm until essentially all of the vanadium(V)-Alt-A was converted to ferric Alt-A (Figures 3-29 and 3-30).

Chromobacterium violaceum siderophores: Small halos were observed on CAS

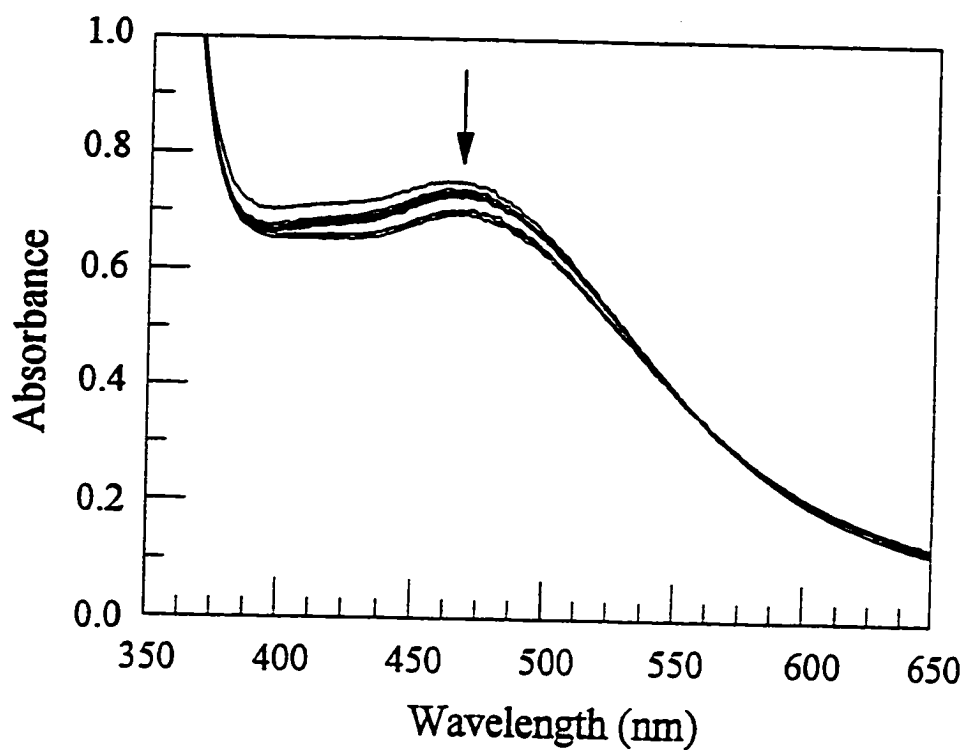


Figure 3-27. Competition of 0.3 mM Fe(III)-alterobactin A + 0.3 mM vanadate in 0.1 M phosphate buffer, pH 6 under an Ar atmosphere. Spectra were obtained in a 1 cm cell every ten minutes for an hour, then at 8, 21 and 30 hours.

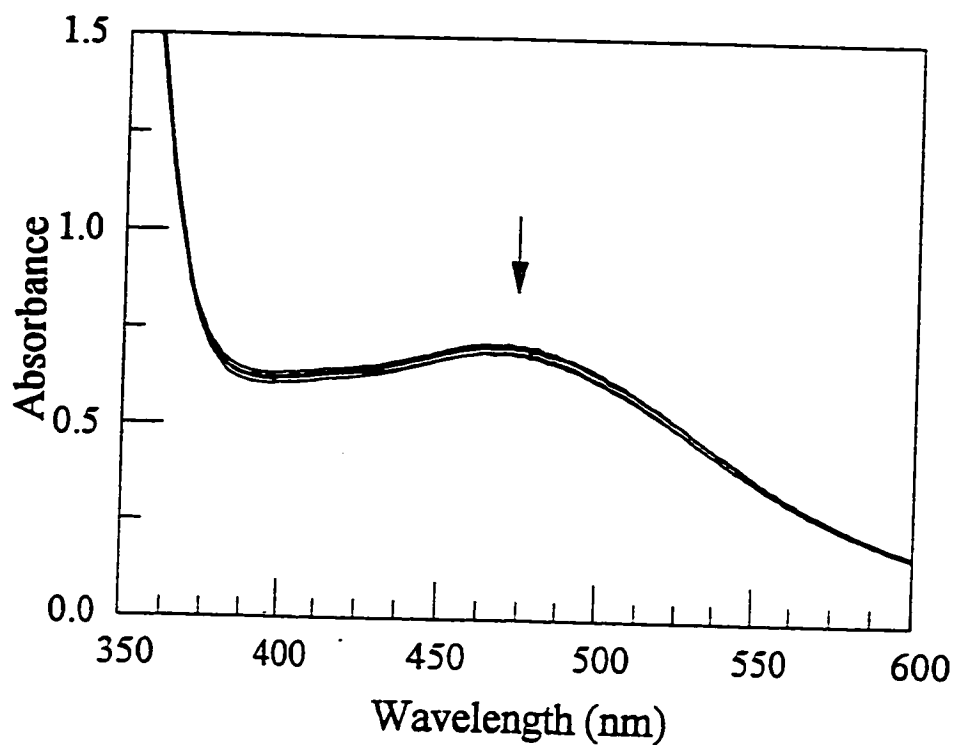


Figure 3-28. Competition of 0.3 mM Fe(III)-alterobactin A + 0.3 mM vanadate in 0.1 M phosphate buffer, pH 8 under an Ar atmosphere. Spectra were obtained in a 1 cm cell every ten minutes for an hour, then at 5, 11 and 24 hours.

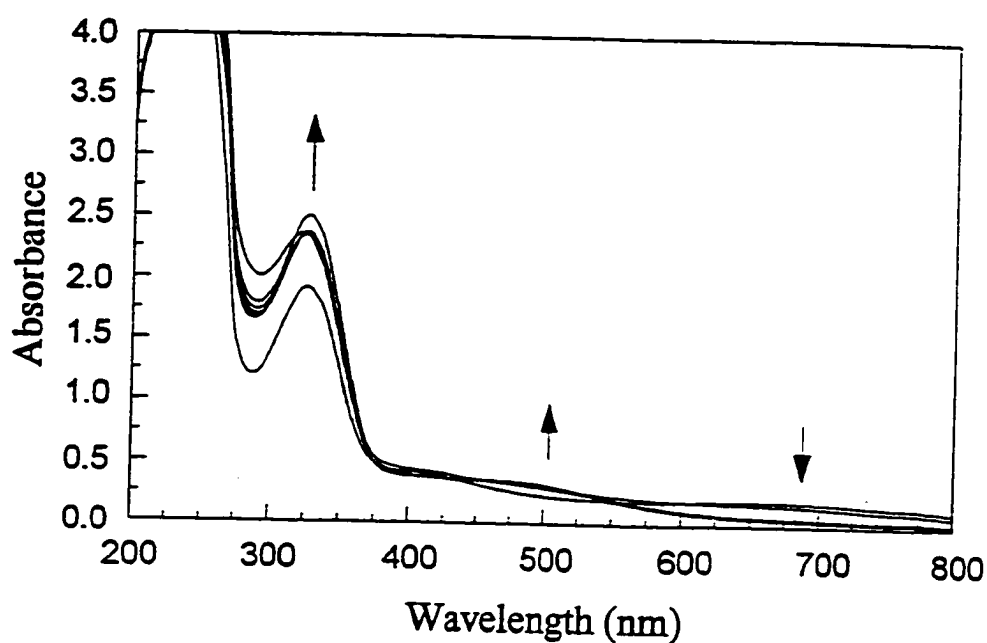


Figure 3-29. Competition of 0.3 mM V(V)-alterobactin A + 0.3 mM Fe(III), in 0.1 M phosphate buffer, pH 6 under an Ar atmosphere. Spectra were obtained in a 1 cm cell every ten minutes for an hour, then at 9 hours. The lowest spectrum is V(V)-alterobactin A alone.

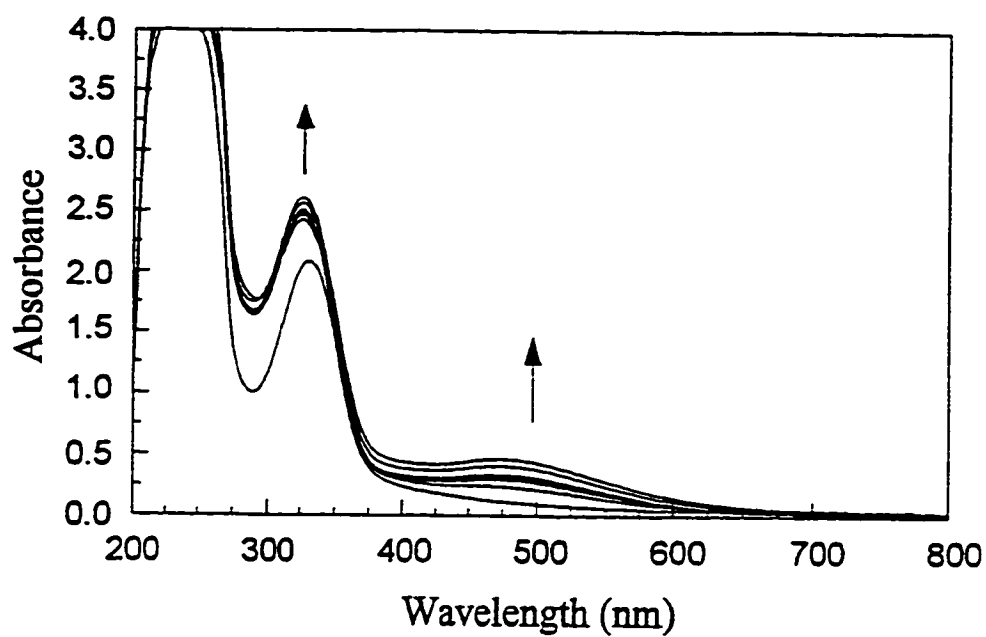


Figure 3-30. Competition of 0.3 mM V(V)-alterobactin A + 0.3 mM Fe(III), in 0.1 M phosphate buffer, pH 8 under an Ar atmosphere. Spectra were obtained in a 1 cm cell every ten minutes for an hour, then at 8 and 32 hours. The lowest spectrum is V(V)-alterobactin A alone.

agar plates, indicating siderophore production by *C. violaceum*. In liquid media, only the casamino acids media with added salts showed siderophore production. The yeast extract broth was most likely much too rich in trace metals as well as bulk nutrients for siderophore production to be observed.

Two CAS-reactive fractions were isolated from the CAA media. ^1H NMR spectra of the siderophores isolated showed catechol present in one sample based on the resonances at 6.7, 6.9 and 7.1 ppm (Figure 3-31), but it appears distinct from the spectra of alterobactin A or B. The spectrum is rather difficult to interpret, however, because of the small amount of material isolated. The other sample showed very little NMR signal, probably because of the small quantity isolated, but was not consistent with the alterobactins either. In contrast to the report by Müller and Zähler (1968), the siderophore observed did not appear to be the trihydroxamate ferrioxamine E (Figure 3-3) as it contained catechol. It is possible that the material in lower yield is ferrioxamine E, however the amount isolated was insufficient to obtain a clean ^1H NMR spectrum in order to identify the material. The siderophores isolated from *C. violaceum* in this work did not apparently correspond to either ferrioxamine E or alterobactins A and B.

Marine snow: Purple colonies were observed on the plates from marine snow

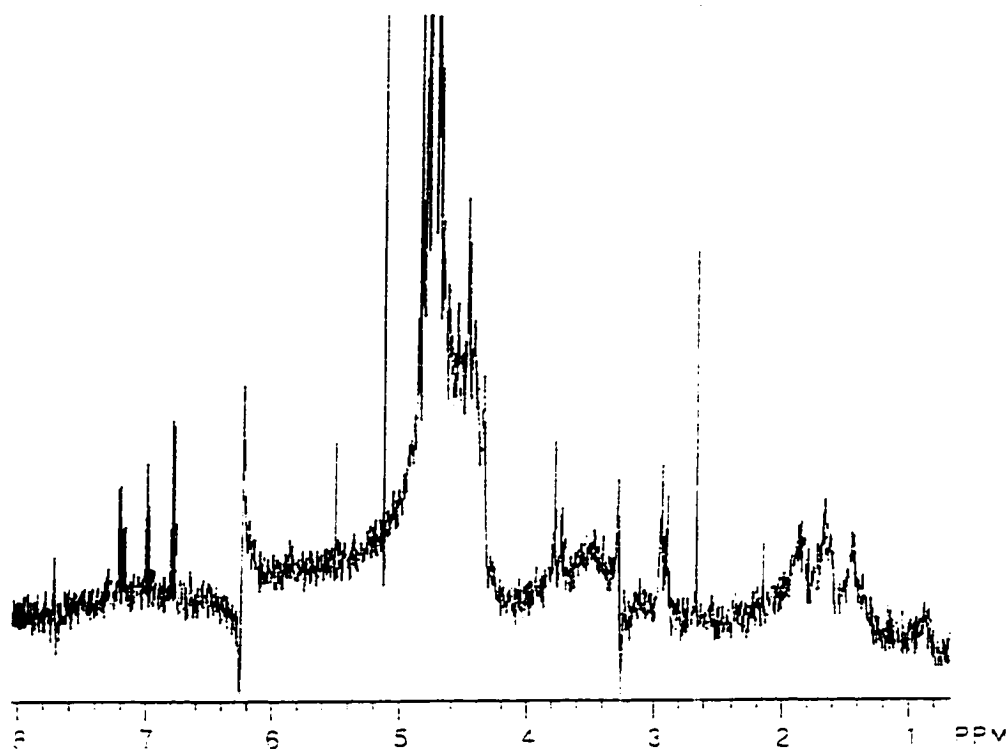


Figure 3-31. ¹H NMR at 500 MHz on a General Electric GN500 of a siderophore from *Chromobacterium violaceum*. Pulse width = 30 μ sec; acquisition delay = 1.5 sec; 16 transient acquisitions of 16384 points; referenced to H₂O at 4.8 ppm.

samples collected on June 19, 1995. Only a couple of purple colonies appeared on plates of the surrounding seawater. Given that only 2 plates of each type were made, this result is not statistically significant. The surrounding seawater samples are difficult to obtain without including some marine snow, especially since TEP is invisible to the divers. Marine snow samples from the December 1995 collection were plated onto NSW agar media and produced a few purple colonies. The purple bacteria were successfully isolated from the majority white bacteria and then grown on CAS plates where they slowly produced small halos. The purple strains grew best, if slowly, at 12 °C and did not grow well at room temperature. The first isolates (P1) grew at the surface of rich liquid cultures when they were left stationary at room temperature. Shaking the media caused them to grow more slowly. P1 did not grow at all on casamino acids media, which is the usual medium for growth of *A. luteoviolacea* strain S2. Because of difficulty in growing the bacteria under low iron conditions, no siderophore isolation was attempted.

Analysis of *A. luteoviolacea* for production of TEP showed a significantly higher alcian blue response than the blank media. Measurement of a 24 hour culture without staining showed minimal response, so the violacein pigment is not apparently interfering with the assay. However, under microscopic examination of alcian blue-stained filters, typical TEP particles

were not visible. Therefore, these bacteria are not responsible for the type of marine snow that has been called TEP, although some bacterially produced slime may contribute to the overall aggregates.

These preliminary marine snow experiments were not able to satisfactorily address the question of whether or not *Alteromonas luteoviolacea* can live on marine snow particles as opposed to simply the surrounding seawater.

Discussion

Stability constants: The Taylor chelate scale (1994) is shown in Figure 3-2. A linear relationship ($R^2 = 0.98$) is observed between the reduction potentials measured under the above conditions, and the published $\log K_b$. The equation of the line fits the Nernst equation:

$$E'_p = E_p - 2.303(RT/nF)\log K_{ox}/K_{red} \quad (15)$$

where E_p and E'_p are the reduction potentials of the free metal ion and the complex, respectively; $n = 1$ representing the number of electrons in the Fe(III) to Fe(II) reduction; and K_{ox} and K_{red} are the pH-independent stability constants of the Fe(III) and Fe(II) complexes (Heyrovsky and Kuta, 1966). It is known

that the Fe(II) complexes of enterobactin (Taylor et al., 1994) and *trans*-1,2-cyclohexylenedinitrilotetraacetate (CDTA) (Smith and Martell, 1977) have the same stability constants, $\log K = 19$. Therefore, if it is assumed that all of the complexes investigated have similar Fe(II) stability constants, then K_{red} can be incorporated into the intercept as shown in Equation 16:

$$E'_p = (E_p + 2.303(RT/nF)\log K_{\text{red}}) - 2.303(RT/nF)\log K_{\text{ox}} \quad (16)$$

For the experimental values in the chelate scale (Figure 3-2), the equation of the line becomes:

$$E'_p = 1.104 - 0.041 \log K_{\text{ox}} \quad (17)$$

The assumptions inherent to the method are: (a) there are no ion pairing or electrolyte effects; (b) there is no solvolysis of either Fe(III) or Fe(II) at high pH; (c) the diffusion coefficients for Fe(III)L and Fe(II)L are equivalent, and (d) the Fe(II)L/Fe(III)L redox couple is reversible. The potential is independent of ligand concentration because enough ligand is added to insure full complexation of the Fe(III). Ligand is added until the observed reduction potential becomes constant (constant E'_p), which implies the presence of excess

ligand with respect to Fe(III).

Alterobactin B forms a 2:1 ligand to metal complex with Fe(III) (Reid et al., 1993). Although the complete coordination environment of ferric Alt-B is not yet known, it almost certainly involves both catechols. The complex occurs in two different pH-dependent forms. Above pH 8 both catecholate oxygens bind iron(III). Below pH 6, one of the catecholate oxygen atoms is protonated, causing a shift to salicylate bonding (Figure 3-32) as described by Harris and Raymond (1979c) for enterobactin (Figure 1-1) and enterobactin analogues. Between pH 6 and 8 a mixture of the two forms is present. The log K obtained from the chelate scale for Fe(III)-(Alt-B)₂ at pH 8.2, 43.6, is six orders of magnitude larger than the estimated log K at pH 6, 37.6. The difference in reduction potentials and stability constants estimated by the chelate scale is consistent with the proposed iron binding modes. A bis-catecholate structure would be expected to bind iron(III) more tightly than a catecholate/salicylate complex. It is notable that the stability constant of the bis-catecholate form (pH 8.2, log K = 43.6) is significantly higher than that of Fe(cat)₂⁻ (log K = 34.7; Avdeef et al., 1978), indicating that the other iron(III) ligands are not simply water molecules. It is likely that the β-hydroxyaspartates are also involved in coordination, as in ferric Alt-A. In seawater, pH ~8.3, the more stable bis-catecholate form would predominate. Lowering the pH

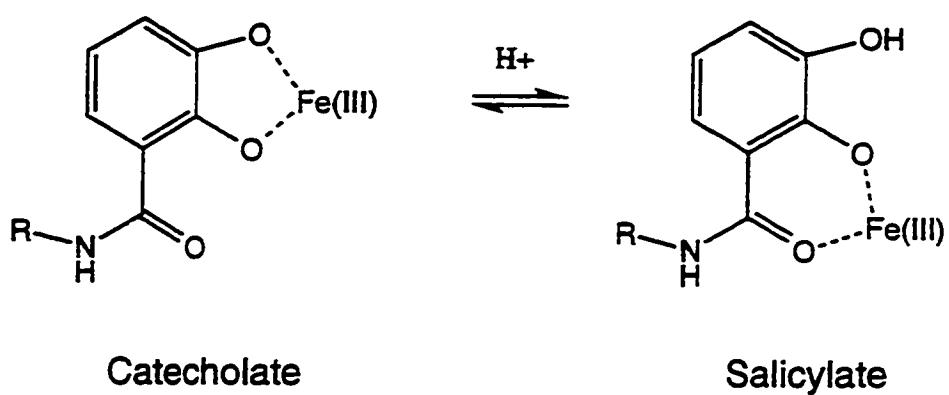


Figure 3-32. Catecholate to salicylate shift that occurs for one catechol of the $Fe(III)$ -(alterobactin B)₂ complex as the pH drops from 8 to 6.

might then provide a mechanism for iron removal once the complex is transported into the cell.

The stability constant for catecholate/ bis(β -hydroxyaspartate) coordination of iron(III) by alterobactin A was calculated by spectrophotometric competition to be $10^{49} - 10^{53}$ (Reid et al., 1993). The stability constant for Fe(III)-alterobactin A was estimated from the chelate scale to be $10^{51 \pm 2}$, which agrees closely with that determined by EDTA competition. This independent result confirms that the result from the EDTA competition was reliable and that the estimates made for the β -hydroxy aspartate hydroxyl protonation constants are in the correct range.

Upon initial addition of alterobactin A to the Fe(III) solution, a complex with a peak at -0.614 V forms. From equation 17, a log K of 42.2 ± 1.2 is obtained which is substantially smaller than that for the fully formed product. The peak at -0.614 V decreases in current, while the -0.972 V peak increases (Figure 3-11). The -0.972 V peak does show a shoulder in the SWV voltammogram which could result from absorption effects which account for the lack of exact correspondence in peak height as the reaction progresses. However, the CV shows a clean, reversible reaction with no sign of a shoulder (Figure 3-14). The conversion appears to be complete in 15 min (Figure 3-13). The initial peak at -0.614 V is attributed to the fast binding of both the

catecholate and the carboxyl groups of the aspartates in alterobactin A to Fe(III). A relatively slow deprotonation of the β -hydroxy aspartate hydroxyl protons, finally resulting in full hexadentate coordination to Fe(III), is responsible for the peak at -0.972 V. From the chelate scale, $\log K$ equals 51 ± 2 for the fully formed complex (Table 3-3).

The ring structure of alterobactin A places the β -hydroxy aspartate groups in an orientation which is ideal for complexation of iron(III). The thermodynamic stability constant, $K = 10^{51 \pm 2}$, is comparable to or greater than that of enterobactin (Figure 1-1), a tri-catechol compound with $K = 10^{49}$ (Loomis and Raymond, 1991b). The reduction potential of Fe(III)-Alt-A of -0.972 V vs. SCE is more negative than that of enterobactin, $E_p = -0.924$ V, under the same conditions (Taylor et al., 1994, Table 3-1). Lee et al. (1985) measured the Fe(III)/Fe(II) reduction potential for enterobactin at pH 7.4 to be -0.79 V vs. the normal hydrogen electrode (NHE). This potential is outside the range of biological reducing agents, however, they hypothesize that the bacteria have lower pH compartments inside the cells (e.g. the periplasmic space) where the ferric siderophore reduction potential is more positive. At a lower pH the complex would be accessible for reduction and removal of iron(II) from the siderophore. The reduction potentials become more positive as the pH is lowered because of protonation of the Fe(II)-complex (Cooper et al., 1982).

Comparisons of stability constants for different ferric complexes do not give a true measure of their relative effectiveness in their environments. In an attempt to compare chelate strength under physiological conditions, the concept of pM has been employed (Raymond, 1984). The pM value is defined as the $-\log(\text{Fe(III)}_{\text{(aq)}})$ in the presence of $10\ \mu\text{M}$ siderophore, $1\ \mu\text{M}$ iron(III) and at pH 7.4. By this definition, the pM for ferric enterobactin is 35.5 (Raymond et al., 1984) and that for ferric alterobactin A is 25.0. However, as noted above, the alterobactins are operating at around pH 8.3, unless these bacteria inhabit particles with pH gradients inside of them (Alldredge and Cohen, 1987). Therefore, a seawater pM value was defined (Reid et al., 1993) which is analogous to the above physiological pM value, except determined at pH 8.3. In this case, the pM for ferric alterobactin A becomes 30.4.

The competitions of alterobactin A with desferrioxamine B for iron(III) are also consistent with the EDTA competition results. The average K_b ranges from $10^{49.8}$ to $10^{53.8}$, depending on the values chosen for the pK_a s of the β -hydroxy aspartates. However, the UV-visible competition results are most reliable for the 1:1 situation. As the equilibrium is shifted because of an excess of one iron(III) complex over the other, the changes in the spectra become quite small. The overlap of Fe(III)-Alt-A and Fe(III)-DF is significant and therefore the ability to distinguish between the two species is inherently limited. The

calculations are actually mostly dependent on the pK_a values represented by the $\alpha_{Fe^{3+}}$ term, so although the UV-visible competition is consistent with the other results, the electrochemical technique, which does not depend on calculations involving the β -hydroxy aspartate pK_a values, is the most convincing.

These results contrast with the predictions made by Rue and Bruland (1995) who calculated the conditional stability constant of Fe(III)-alterobactin A in seawater to be $10^{10.7} \text{ M}^{-1}$. This value is actually lower than the conditional stability constants measured for natural chelators in seawater, L1 and L2 (see Chapter 1: Introduction). According to Rue and Bruland (1995), the conditional stability constant for a ligand (L) relative to total iron (Fe^*) is obtainable from the following equation:

$$K_{L/Fe^*}^{cond} = K_{L/Fe^{3+}}^{cond} / \alpha_{Fe^*} \quad (17)$$

To estimate K_{Alt-A/Fe^*}^{cond} , Rue and Bruland used the conditional stability constant ($K_{Alt-A/Fe^{3+}}^{cond} = 10^{20.7}$) obtained by EDTA competition at pH 6 (Reid et al. 1993) and the α_{Fe^*} value of $10^{10.0}$ for total iron (Fe^*) in seawater (pH ~ 8.3) (Rue and Bruland, 1995). However, their estimate is in error because of the significant effect of pH on the side-reaction coefficient of the ligand, α_L . The conditional stability constant must be determined under seawater conditions in

order to calculate a correct value of $K_{L/Fe}^{cond}$ to compare with the naturally occurring chelators.

Interaction with vanadium(V): Alterobactin A is degraded in the presence of vanadate and air, especially at pH 6. It is more stable at pH 8, however. The decomposition of the V(V)-Alt-A complex in the presence of air could be caused by a cycle of catechol oxidation/ vanadium reduction; re-oxidation of vanadium(IV) to vanadium(V) by O_2 and continued oxidative destruction of alterobactin A (Reid, 1993).

Under an argon atmosphere at pH 6 a dark green complex, $\lambda_{max} = 633$ nm, is formed (see Figure 3-25). It is possible that the green color results from reduction of vanadium(V) to form a vanadium(IV) complex. Vanadium catechol complexes have been reported in the literature, but the chemistry is quite complex and the reports often conflict. Raymond's group at UC Berkeley (Cooper et al., 1982) clarified the situation by synthesizing and characterizing a series of vanadium catechol complexes by X-ray crystallography, UV-Visible spectrophotometry and EPR spectroscopy. They found that both V(V) and V(IV) tris-catechol complexes have strong ligand to metal charge-transfer absorption bands around 550-650 nm, and appear dark blue. The $[V(IV)(cat)_3]^{2-}$ complex has two overlapping bands at 552 and 650 nm, while the $[V(V)(cat)_3]^{-}$

complex has bands at 625 and 900 nm (Cooper et al., 1982). In air, the V(IV) tris-complex converts to the V(V) complex, which occurs without significant color change. Bis-catechol complexes of vanadium(IV) have very weak bands in the visible region (Cooper et al., 1982). UV-visible spectra were not reported for the V(III)-tris catechol complex, though green crystals were prepared and the crystal structure was solved (Cooper et al., 1982). Given the above literature information, it is hypothesized that the green color observed for vanadate plus alterobactin A under argon results from the mixture of yellow decomposition product with a blue tris-catechol vanadium(IV) species. It is unlikely that a two-electron oxidation would occur to form vanadium(III), and the absence of a ^{51}V NMR signal indicates that the vanadium alterobactin A complex is no longer vanadium(V). Future work should consider the possibility of formation of a tris-complex and investigate the spectra of a 3:1 ligand to metal complex. The spectra exhibit a clean peak around 633 nm, analogous to the V(V) tris-catechol complex of Cooper et al. (1982), not the double peak of the V(IV) tris-catechol. However, the absence of a shoulder at 550 nm in the alterobactin A complex may not be sufficient evidence to eliminate the hypothesis that this is a vanadium(IV) complex.

Competitions with iron(III) and vanadium(V) show minimal effects of added vanadium(V). At either pH 6 or 8, only about 5% of the ferric-

alterobactin A complex is de-ferrated by addition of one equivalent of vanadate (Figures 3-27 and 3-28). By contrast, Reid (1993) found that vanadate at pH 6 could displace up to 50% of the iron(III) from the ferric alterobactin A complex. However, Reid's samples were incubated in air overnight before the spectra were obtained, and therefore partial destruction of the complex could have occurred. Slight changes in the absorption spectrum in the present case may result from slight excess of ligand over iron(III) or the presence of air leaks which would leave room for some vanadium(V) complexation to occur. Alternatively, vanadate may be capable of displacing iron(III), but further studies to evaluate the ability of vanadium(V) to displace iron(III) from ferric alterobactin A should employ various concentrations of excess vanadate. Future experiments should also use cuvettes designed for inert-atmosphere work in order to maintain consistently anaerobic conditions.

Conclusions

Alterobactins A and B are among the first marine bacterial siderophores whose structures and iron(III) complexation have been characterized. The stability constant of Fe(III)-Alt-A was determined by electrochemical means to be $10^{51 \pm 2}$. In the original EDTA competition experiment (Reid et al, 1993), the pK values of the β -hydroxy aspartate hydroxyl protons were estimated to be 16-

18, and the stability constant was measured to be 10^{49} to 10^{53} . Alterobactin B forms a bis-catecholate complex with iron(III) at pH 8 and above, with a stability constant of $10^{43.6}$ (Lewis et al., 1995). Therefore in seawater it would function as a strong iron(III) chelator.

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Chapter 4: Aerobactin from *Vibrio* spp. DS40M5

Introduction

Aerobactin (Figure 4-1) is a common enteric siderophore that has been isolated from several *Enterobacter* species and one species of *Pseudomonas* (Buyer et al, 1991). Okujo and Yamamoto (1994) showed that aerobactin is also produced by two species of *Vibrio*, *V. hollisae* and *V. mimicus*, both of which are human pathogens of clinical interest. Aerobactin was originally isolated by Gibson and Magrath (1969) from the species *Aerobacter aerogenes*, now reclassified as *Klebsiella pneumoniae*. It is a virulence factor in *Escherichia coli* and other bacteria of the Enterobacteriaceae family (Bindereif and Neilands, 1985).

The genetics of the aerobactin system in *E. coli* K12 are well understood (Bagg and Neilands, 1987b; Neilands, 1992). The aerobactin genes are known to be carried on plasmid DNA in some cases (e.g. pColV-K30 in *E. coli*) which may explain its occurrence in many *Enterobacter* species (Bindereif and Neilands, 1985). In cases where the genes for aerobactin production and transport are present on the chromosome, they have been shown to be located between insertion elements, suggesting introduction via transposons (Bindereif and Neilands, 1985).

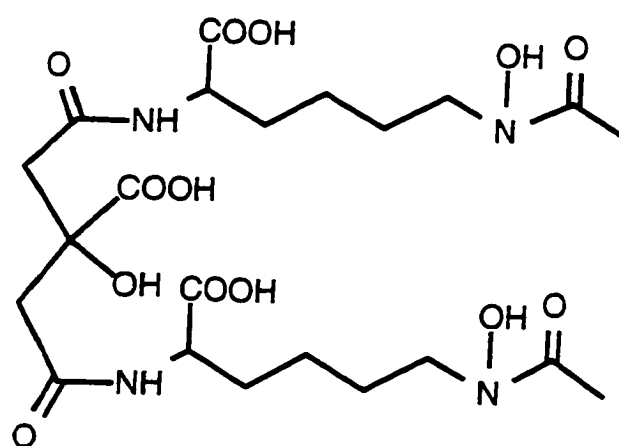


Figure 4-1. Structure of aerobactin.

There are four genes for the production of aerobactin, known as *iuc* for iron uptake chelate. An oxygenase is produced by *iucD* to hydroxylate the N^ϵ of lysine. The acetylase which acetylates N^ϵ -hydroxy lysine is generated from *iucB*. Aerobactin synthetase is coded by *iucA* and *iucC* which produce the α and β subunits, respectively. The synthetase forms amide bonds between citric acid and two molecules of N^ϵ -hydroxy, N^ϵ -acetyl lysine. One additional gene, *iutA*, is responsible for production of the membrane receptor (*iut* stands for iron uptake transport) (Bagg and Neilands, 1987b).

The production system of aerobactin in *E. coli* K12 is regulated by the Fur (ferric uptake regulatory) protein. In the presence of high iron(III) levels in the cytoplasm, synthesis of aerobactin is repressed. In the absence of iron(III), the repressor is dissociated and the synthetic sequence begins (Bagg and Neilands, 1987a). Several divalent cations, including iron(II), activate Fur to bind to the operator sequence and repress aerobactin synthesis, possibly by blocking access by RNA polymerase. Fe(II) is presumably the relevant co-repressor *in vivo*. Free iron in the reducing environment of the cytoplasm would be in the ferrous form. The binding site for the Fur dimer is a palindromic "iron-box" with the sequence 5'-GATAATGATAATCATTATC. Similar sequences are also found in the promoter regions of the ferrichrome and ferric enterobactin receptor genes of *E. coli* (Neilands, 1992).

In this chapter, the isolation and characterization of aerobactin from *Vibrio* spp. DS40M5 are reported.

Experimental

Isolation of siderophores: All water used in these experiments was purified with a Barnstead NANOpure II system which contains 2 mixed-bed ion-exchange filters and one organic-colloid filter. The purified water had a resistance of $18 \times 10^6 \Omega\text{-cm}$. Amberlite XAD-2 resin (Aldrich) was cleaned and the fines removed by repeated washing with water and ethanol. Flasks used for bacterial growth were presoaked with a dilute solution of EDTA (ethylenediamine tetraacetic acid) to remove trace metals, then rinsed extensively with water. All other chemicals were reagent grade and used without further purification.

Artificial seawater (ASW) contained 15.5 g/L NaCl, 0.75 g/L KCl, 12.35 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 2.9 g/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Haygood and Nealson, 1985). In order to dissolve the salts, each was independently dissolved and filtered through Chelex 100 membrane filters (BioRad) to remove iron. The individual solutions were then mixed and brought up to volume with water. Artificial seawater media (ASM) included 2 g casamino acids (Difco), 1 g NH_4Cl and 0.1 g glycerophosphate per liter of ASW, modified from Haygood and Nealson (1985). Two liters of media were placed in 4 L Erlenmeyer flasks,

fitted with foam plugs and aluminum foil covers, and autoclaved for at least 45 minutes at 120 °C. During autoclaving, the flasks were placed in plastic bins with about 2 cm of water in the bottom to avoid precipitation of salts. Natural seawater (NSW) was obtained from the UCSB pipeline. The seawater is pumped through a PVC (polyvinyl chloride) 1.5 ft. diameter pipe located 1500 ft. offshore of the Goleta Point, in 30 feet of water. It is filtered by passage through a bed of sand and gravel. NSW was stored at 4 °C for up to 4 months until needed. Bacteria were maintained on natural seawater agar plates containing 15 g/L agar, 5 g/L Bacto peptone and 0.5 g/L yeast extract, a modification of Zobell's medium (Zobell, 1941). Lawns of DS40M5 were made 12 to 24 hours prior to inoculation. DS40M5 was typically grown for three days in 2 L ASM in 4 L flasks on a rotary shaker at 110 rpm. The growth time was selected based on a growth experiment described below (see Figure 4-5) in which siderophore concentration reached a steady value after approximately three days.

To isolate siderophores from the supernatant, the culture medium was centrifuged (8000 rpm, 20 min) to remove the cells. Approximately 150 g of Amberlite XAD-2 (Aldrich) was added to the supernatant and the resin suspension was shaken at 110 rpm for at least one hour, after which it was poured into a column. The column was washed with one column volume of

water and eluted stepwise with 50% methanol and 100% methanol. Fractions were collected manually in 8 mL test tubes. Each fraction was tested for the presence of siderophores with a 10:1 ratio of the liquid Chrome azurol S assay (CAS) (Schwyn and Neilands, 1987) to eluent. The assay solution contained 15 μ M Fe(III) in a dye-detergent complex as described by Schwyn and Neilands (1987). CAS reactive material eluted in the 50% methanol step. Reactive fractions were pooled and dried *in vacuo*. The dried material was redissolved in 10% methanol. Insoluble material was removed by centrifugation in a table-top test tube centrifuge at 3000 rpm for 10 minutes and the supernatant was loaded onto a 100 x 2.5 cm Sephadex LH20 (Pharmacia) column and eluted in 10% methanol. The iron(III)-reactive material was then placed on a second LH20 column (35 x 1.5 cm) in 80% methanol. Final purification was achieved using a Protein C-4 reverse phase HPLC column (250 x 10 mm, Vydac) using gradient elution from 100% A (A = H₂O with 0.1 % trifluoroacetic acid (TFA), pH 2) to 75% A/ 25% B (B = 80% CH₃CN, 20% H₂O, 0.1%TFA) in 30 minutes. Peaks were detected at 230 nm with a Waters 484 variable wavelength UV detector. HPLC purification was repeated, if required, to obtain a single peak of pure aerobactin.

On one occasion aerobactin was purified according to the procedure of Neilands (1984) in order to verify that the DS40M5 siderophore would behave

in the same way as authentic aerobactin. Because the high chloride concentration in seawater would interfere with the initial ion exchange column, an XAD-2 resin step was added. After XAD-2 elution in 50% methanol, CAS reactive fractions were bound to an ion exchange column (AG 1-X2 Cl⁻ form, BioRad) and eluted with a gradient from 0.4 to 1.0 M NH₄Cl. The CAS-positive fractions were then put over a cation exchange column (AG50 W-X2, H⁺ form, BioRad) which did not bind aerobactin, but was intended to remove other impurities. The aqueous eluent was already acidic with a pH of 1. (NH₄)₂SO₄ was added to saturate the solution and then it was extracted with 50 mL portions of 1:1 phenol/ chloroform 3 times. The phenol/ chloroform layer was centrifuged in a table top centrifuge, filtered through 2 layers of Whatman No. 1 filter paper and the aerobactin was forced out of the organic layer by addition of 4-fold excess diethyl ether over phenol/ chloroform. A small amount of water was used to extract the mixture three times. The combined water layers were extracted again with diethyl ether to remove remaining phenol/chloroform and the aqueous layer was dried in a SpeedVac concentrator (Savant). The dried material was dissolved in 1.5 mL 0.2 M pyridine/ acetic acid, pH 4.8 and placed over a Biogel P-2 (BioRad) 45 x 1 cm size exclusion column in the same buffer. The column was eluted at 0.25 mL/ min and fractions were collected every two minutes. As a final purification step, the

material purified by the Biogel P-2 column was dissolved in the minimal volume of water and placed over an 8 x 2.5 cm column of Sephadex CM-25 (Pharmacia). The final product isolated from this procedure was analyzed by ^1H NMR.

Growth and siderophore production as a function of added iron(III): Various amounts of iron(III) were added to culture media to investigate its effect on growth and siderophore production. Ferric ammonium citrate (FAC) was added to 6 flasks at 1.0, 0.1, 0.01, 0.001, 0.0001 and 0 mg/L. To avoid precipitation of iron(III) salts in the autoclave, 10 or 1 mL volumes of the iron(III) stocks (200 $\mu\text{g/mL}$, 2 $\mu\text{g/mL}$ and 0.2 $\mu\text{g/mL}$ FAC) were sterile-filtered (0.2 μm syringe filters, Nalgene) into 2 L autoclaved ASM in 4 L flasks. After inoculation, the cultures were tested for growth and siderophore production as follows. Samples of the bacterial culture were taken at intervals using 2 mL sterile pipets. Typically, samples were taken every hour for the first twelve hours and every 12 to 24 hours thereafter for several days. Growth of bacteria was determined by measuring the optical density at 600nm (OD_{600}), a measure of cell density (Brock et al., 1994). One milliliter of the culture sample was centrifuged in a microcentrifuge (Eppendorf) and siderophore levels were measured by the absorbance at 620 nm of the CAS shuttle assay (CAS_{620})

(Schwyn and Neilands, 1987). In order to distinguish among the highest producing flasks, a 9:1 ratio of CAS assay solution to supernatant was measured after a 30 minute development time.

Structure determination: ^1H and ^{13}C NMR spectra were taken on a GN500 spectrometer (General Electric) at 500 and 125 MHz respectively, using the software provided. ^1H NMR standard parameters include a pulse width of 30 μsec , acquisition delay of 1.5 sec and block size of 16K points. ^{13}C NMR standard parameters include a pulse width of 8 μsec , acquisition delay of 1 sec and block size of 16K points.

A variable temperature ^1H NMR experiment was performed at 25, 35, 45 and 55 $^{\circ}\text{C}$ then back to 25 $^{\circ}\text{C}$, with a 10 minute equilibration at each temperature before the spectra were taken. Relative peak areas were determined using GEMCAP software to calculate Lorentzian fits to the spectra. Chemical shifts are reported relative to an internal standard of 4,4 dimethyl 4-silapentane sodium sulfonate (DSS).

Fast atom bombardment mass spectrometry was performed at the University of California Riverside Mass Spectrometry Facility in positive ion mode. Infrared spectra were obtained as KBr pellets on a Perkin-Elmer 1330 spectrophotometer.

Paper chromatography was performed according to the method described by Gibson and Magrath (1969) using their solvent systems B and C. Solvent B contained NH_4OH , ethanol and water in a 1:16:3 ratio. Solvent C was composed of *n*-butanol, acetic acid and water in a 60:15:25 ratio. Whatman #1 filter paper strips were inoculated with a solution of aerobactin. A staple was placed at the bottom as a weight, then the strips were attached to corks with thumbtacks and placed in test tubes containing a small amount of the solvent mixture. Each strip was eluted until the solvent neared the top and the aerobactin was visualized by spraying with an acidic 0.4% ferric chloride solution.

Iron(III) complexation: UV spectra were recorded on a Kontron Uvikon 860 spectrophotometer. Samples of aerobactin were dissolved in solutions of pH 2 or 7 to a final concentration of 0.15 mM. Solutions included 0.1% TFA or 0.1 M HCl/KCl at pH 2 and 0.2 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid) or 0.1 M phosphate at pH 7. Sequential 10 μL additions of 1.2 mM $\text{Fe}(\text{ClO}_4)_3$ in 10 mM HClO_4 were made to 800 μL of the aerobactin solution until 2 equivalents of Fe(III) had been added. Scans were recorded from 300 to 600 nm referenced against the background solution. For the titration of aerobactin with 0.78 mM $\text{Fe}(\text{ClO}_4)_3$ at pH 7 in 0.1 M phosphate

buffer, 150 μL aliquots of 0.3 mM aerobactin were mixed with 50 μL aliquots of iron(III) solution. Each sample was brought up to 1 mL with buffer, equilibrated for 2 hours and centrifuged at 13,000 rpm for 10 minutes to remove any ferric hydroxide precipitate before the scan was taken.

For the pH variation experiment, 0.84 mM ferric aerobactin was made by adding 100 μL 13.4 mM $\text{Fe}(\text{ClO}_4)_3$ to excess aerobactin (1.5 mL of 1.7 mM). Individual 100 μL samples of 0.84 mM ferri-aerobactin were added to 1 mL of buffer at each pH value. In the pH range 8.5-6.0, the buffers were 0.2 M phosphate. For the range 5.5-4.0, 0.2 M acetate was used, and between 2.5 and 3.5 a 0.2 M formate buffer was employed. UV-visible scans of the range 300-600 nm were obtained of each sample with the corresponding buffer in the reference cell. Because of the low concentrations and noise in the spectrophotometer, the curves plotted in Figure 4-14 were smoothed by the program Grapher™ for Windows. An 8th order polynomial fit produced curves which have the same shapes and absorbance features of the original data.

Vanadium(V) complexation: Vanadium(V) additions were made to aerobactin samples analogously to the iron(III) complexation experiments described above. At pH 2, 0.1 M HCl/ KCl was used, while at pH 7 the additions were made to a sample in 0.2 M HEPES, pH 7. The vanadium(V) stock solution was

prepared by dissolving NH_4VO_3 to a final concentration of 1.2 mM in the solution used for the experiment. Control spectra were obtained of vanadate alone under the same conditions.

Vanadium(V) and iron(III) competition experiments were also performed using UV-Visible spectroscopy. Spectra were taken on a Hewlett Packard 8452A diode-array spectrophotometer. The instrument blank scan was done on buffer alone. Individual samples were made of either 0.04 mM ferric-aerobactin or vanadium(V)-aerobactin in 0.1 M phosphate, pH 8. The samples were equilibrated overnight before addition of the competing metal at 0, 1, 2, 5 or 10 equivalents of Fe(III) and 0, 1, 10, 50, or 100 equivalents of V(V). The competition solutions were then equilibrated another 24 hours before the spectra were measured. The extent of iron(III) complexation was determined by the absorbance at 400 nm where the vanadium(V)-aerobactin complex has no significant absorbance.

For ^{51}V NMR experiments, aerobactin samples were prepared using 5 mg aerobactin dissolved in 2 mL 0.1 M HEPES, pH 7.4. Aliquots of 50 mM NH_4VO_3 were added sequentially to the aerobactin sample kept in a 10 mm tube. Control spectra of equal concentrations of vanadate in 0.1 M HEPES pH 7.4 were also obtained. NMR spectra were taken on the GN300 (General Electric) at 79 MHz with a 10mm broadband probe tuned to detect ^{51}V relative

to VOCl_3 as an external standard, $\delta(\text{VOCl}_3) = 0.0$ ppm. One thousand scans per control were collected. Between 10,000 and 40,000 scans were collected for each V(V)-aerobactin sample. Parameters included: $\text{P2}(90^\circ \text{ pulse}) = 20 \mu\text{sec}$; $\text{D5}(\text{acquisition delay}) = 100 \text{ msec}$; Block size = 8 K; gain = 1000; and receiver gain = 10. Peak areas were calculated using GEMCAP peak fitting software.

Genetic characterization: The identity of the bacterium was initially tested using a Biolog Inc. (Hayward, CA) carbon source utilization microtiter plate. The plate contains 95 carbon sources and tetrazolium violet as a redox indicator of usage. Bacteria that metabolize (and therefore oxidize) a given carbon source (e.g. dextrin, maltose, L-ornithine etc.) cause the indicator dye to turn purple. Cells of DS40M5 were grown on agar plates and after 24 hours suspended in 20 mL sterile-filtered ASW. Aliquots ($150 \mu\text{L}$) of the suspension were added to each well with sterile pipet tips. The pattern of usable carbon sources was determined after 25 hours using a light box to distinguish the colors more clearly. The results were sent to Biolog Inc. to check against their database.

Results and Interpretation

Isolation: Aerobactin, a white or off-white solid, was isolated as described in

the Experimental section. The yield is approximately 5 mg per 2 L culture. Based on the symmetric appearance of the peak on the HPLC chromatogram (Figure 4-2) and the lack of impurity peaks in the ^1H NMR spectrum (Figure 4-3), the material is quite pure.

Growth as a function of iron(III): Growth rates and siderophore production in the presence of varying amounts of added ferric ammonium citrate are shown in Figures 4-4 and 4-5, respectively. A typical exponential growth curve followed by stationary phase and death phase is observed for all flasks (Figure 4-4). Bacterial cell density is not greatly affected by the added iron(III). The flask with the highest optical density after 24 hours of growth is the one with the highest level of added iron(III), but the second highest density is in the flask with the lowest level of added iron(III), 0.0001 mg/L.

Siderophore production is indicated in Figure 4-5 by a drop in absorbance at 620 nm. The flask with no added iron(III) produces the most significant CAS response with a maximum drop after approximately 3 days. Each of the flasks where a drop in absorbance is observed shows a rapid initial change over the first 24 hours and a slow drop to a steady level after 2-3 days. Increasing the iron(III) concentration decreases the siderophore response. The production of aerobactin is completely repressed by the presence of 0.1 mg/L

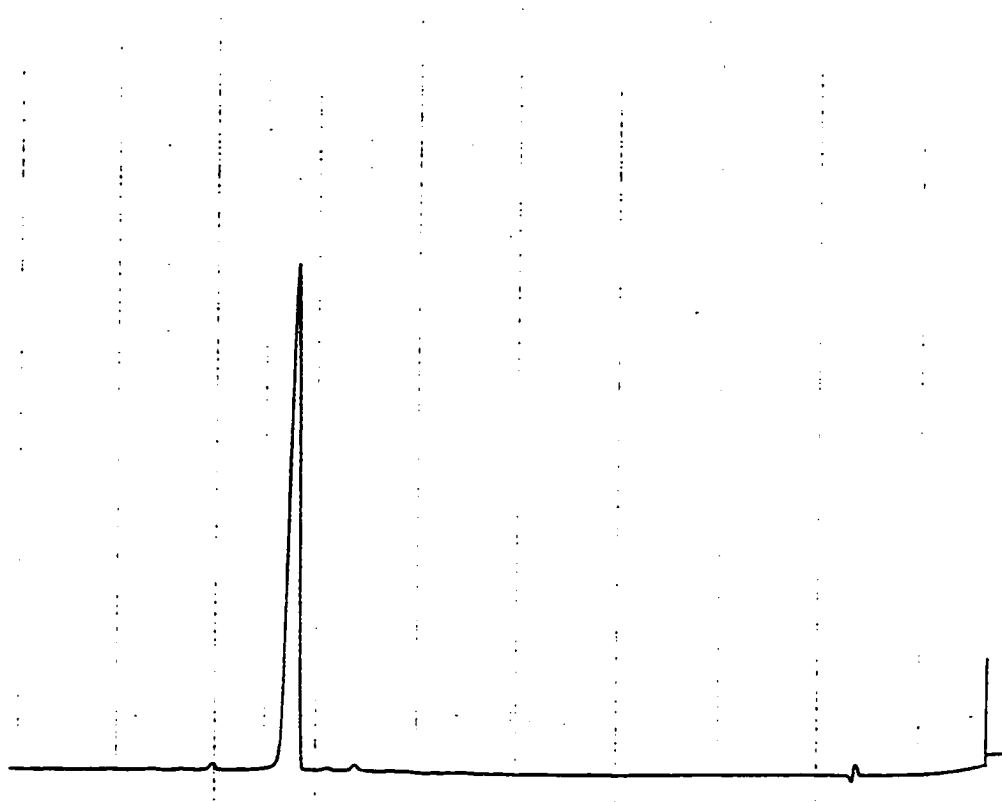


Figure 4-2. RP-HPLC chromatogram at 230 nm of purified aerobactin from an analytical C-4 column (4.6 x 250 mm, Vydac). The gradient program consisted of a linear gradient from 100% A (A = H₂O with 0.1% trifluoroacetic acid (TFA), pH 2) to 75% A/ 25% B (B = 80% CH₃CN, 20% H₂O, 0.1% TFA) in 30 minutes. Peaks were detected at 230 nm with a Waters 484 variable wavelength UV detector.

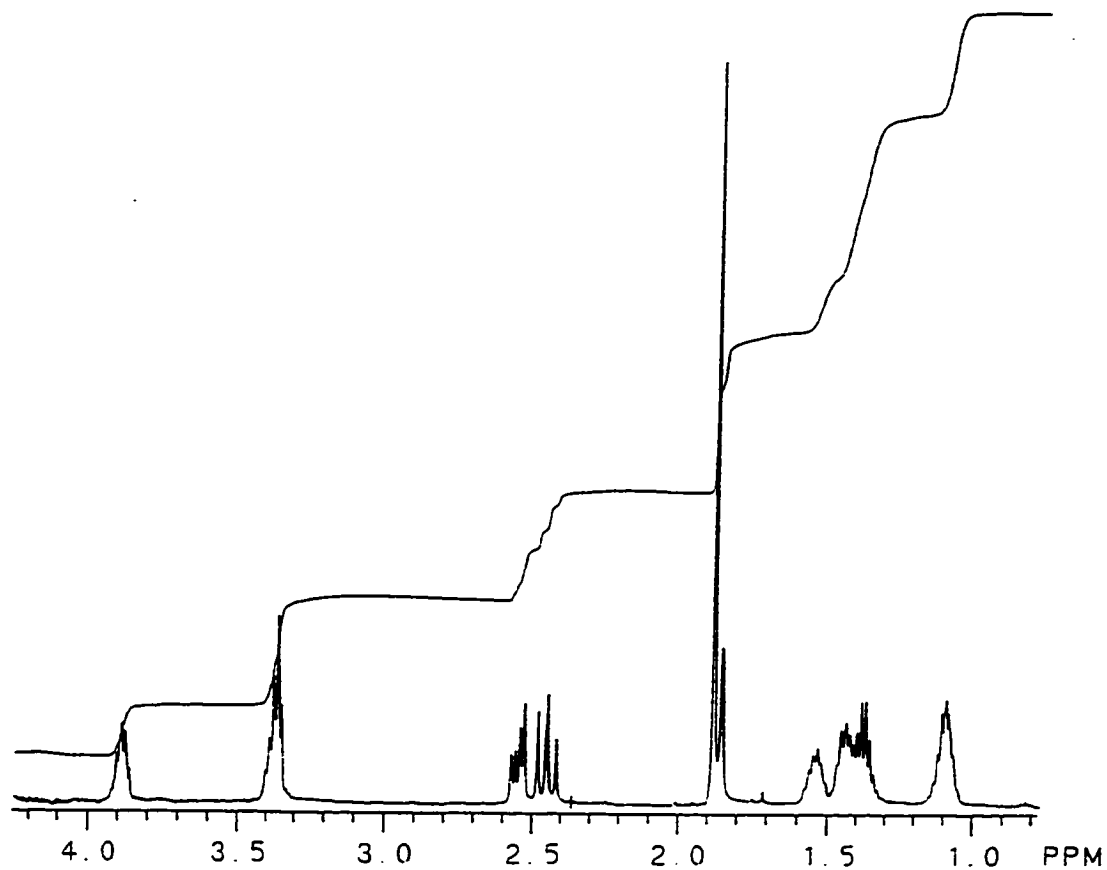


Figure 4-3. ^1H NMR spectrum of aerobactin at 500 MHz. ~ 10 mg in 0.5 mL D_2O , referenced to the HDO resonance at 4.8 ppm. Pulse width = 30 μsec ; acquisition delay = 1.5 sec; sixteen transient acquisitions of 16,384 points.

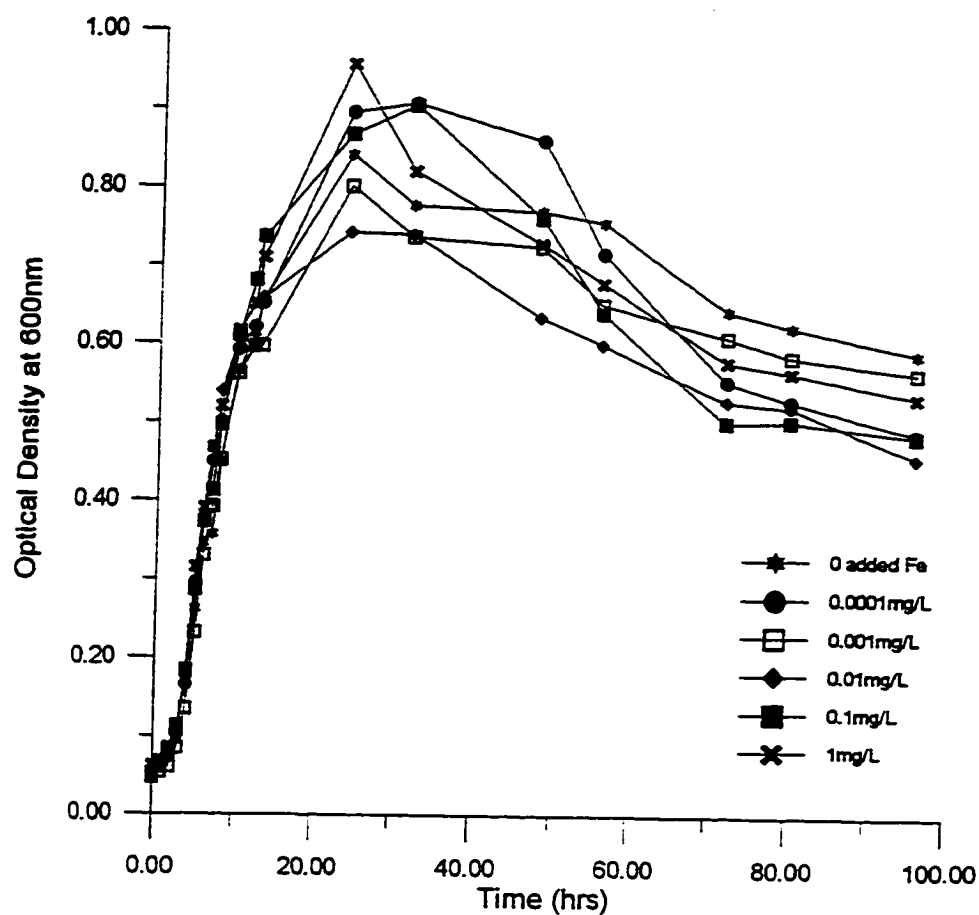


Figure 4-4. Growth curve of *Vibrio* sp. DS40M5 as a function of iron(III) concentration. Ferric ammonium citrate (FAC) was added as described in the Experimental section to produce the final concentrations shown. The optical density at 600 nm was measured at intervals for 5 days.

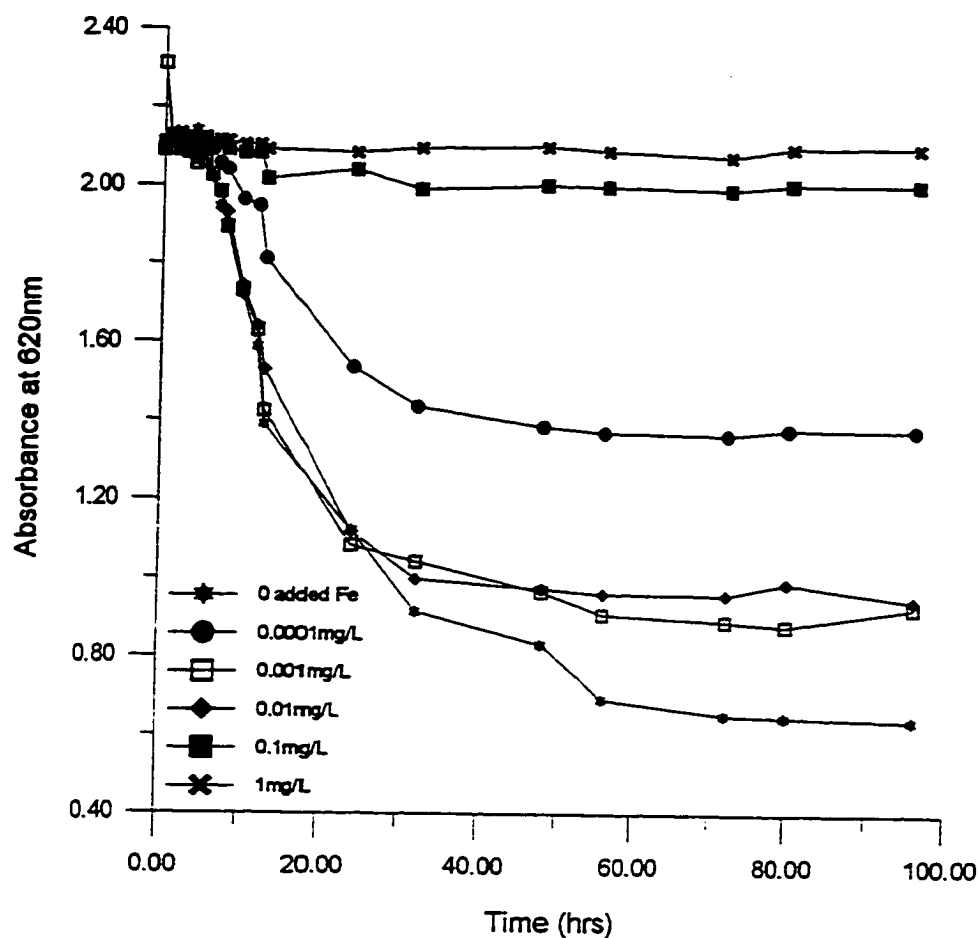


Figure 4-5. Siderophore production by *Vibrio* sp. DS40M5 measured by the CAS assay as a function of added iron(III). Ferric ammonium citrate was added as described in the Experimental section. The absorbance at 620 nm was obtained from 9:1 mixtures of CAS shuttle solution and centrifuged culture medium that were incubated for 30 minutes for full color development.

or more ferric ammonium citrate. The 0.0001 mg/L FAC flask's response (Figure 4-5) is out of sequence, and it is possible that it was contaminated with adventitious iron(III). Repression of siderophore production in the presence of available iron(III) is consistent with both the definition of a siderophore and the type of genetic regulation observed in the *E. coli* aerobactin production system, as described in the introduction to this chapter.

Structure determination: The ^1H NMR spectrum (Figure 4-3) of the DS40M5 material in D_2O is essentially identical to both of the published spectra of aerobactin (Buyer et al., 1991; Gibson and Magrath, 1969) (Table 4-1). Slight differences may be attributed to the different field strengths used. Further evidence for the structure of aerobactin is provided by the ^1H - ^1H correlation spectroscopy experiment (COSY) shown in Figure 4-6. The crosspeaks are consistent with the structure of aerobactin (Figure 4-1). Couplings are observed between the α protons of lysine at 4.1 ppm and the β protons at 1.79 ppm. The ϵ protons of lysine (3.6 ppm) are coupled to the δ protons at 1.6 ppm. The γ lysine protons are coupled to the β and δ lysine protons, and the citrate CH_2 protons are internally coupled. The ^{13}C NMR spectrum (Figure 4-7) is also consistent with the structure of aerobactin. Resonances for the carboxylic acids appear at 179 ppm; amide carbonyls at 171-173 ppm; the central citrate carbon

Assignment	<i>Aerobacter aerogenes</i>	Pseudomonad X40	<i>Vibrio</i> sp. DS40M5
Lys α	4.37 (t)	4.46 (m)	4.13 (m)
Lys ϵ	3.62 (t)	3.78 (t)	3.61 (m)
Citrate CH ₂	2.85 (2 d)	2.99 (d of q)	2.75 (q of d)
Acetyl CH ₃	2.12 (s)	2.32 (s)	2.1-2.13 (2 s)
Lys β , δ	1.0-2.0 (m)	1.75-2.1 (m)	1.63-1.79 (m)
Lys γ	1.0-2.0 (m)	1.56 (m)	1.34 (m)

Table 4-1. ¹H NMR peaks for aerobactin from three sources: *Aerobacter aerogenes* from Gibson and Magrath, 1969; Pseudomonad X40 from Buyer et al., 1991; and *Vibrio* sp. DS40M5 from this work. Multiplicity in parentheses. s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet.

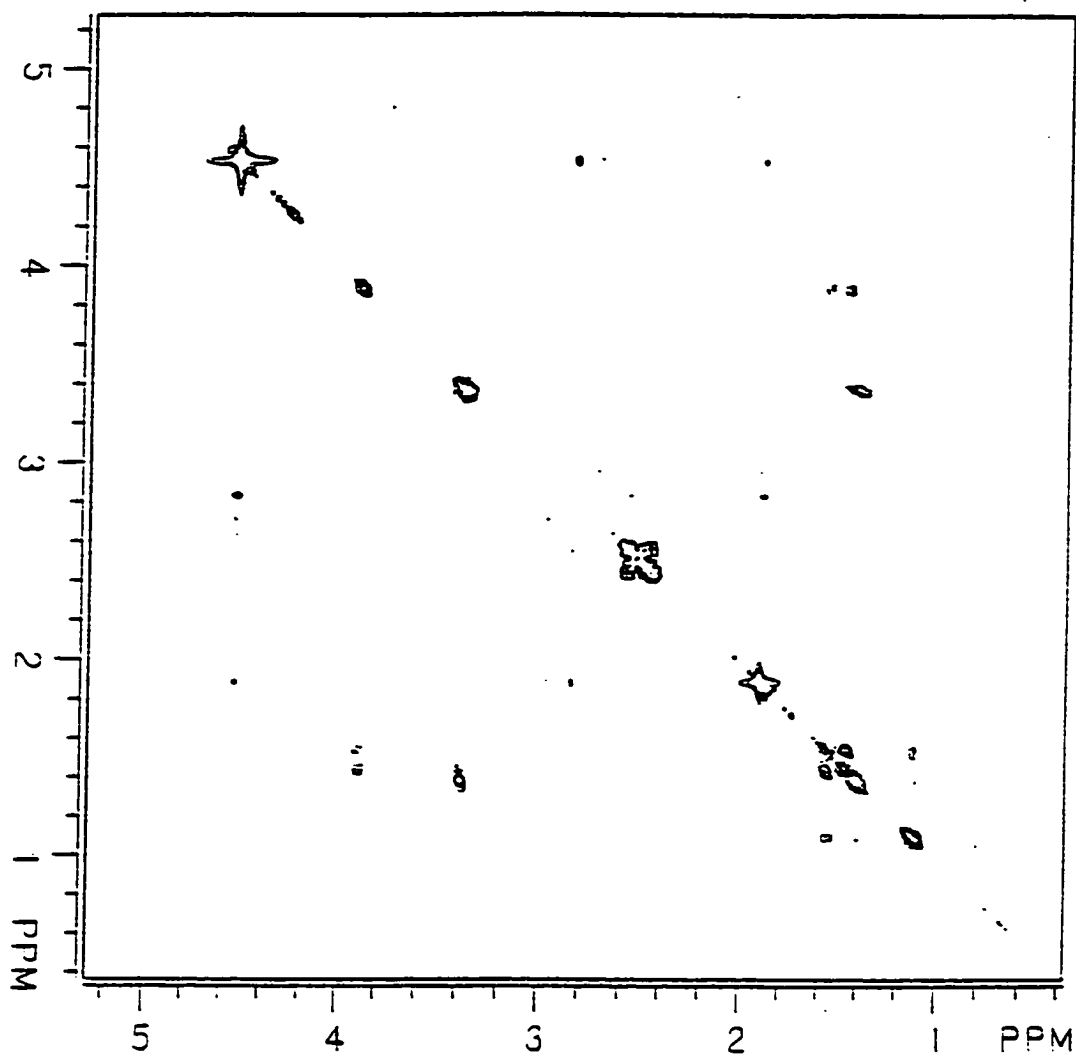


Figure 4-6. ¹H - ¹H COSY of aerobactin at 500 MHz. ~10 mg in 0.5 mL D₂O, referenced to the HDO resonance at 4.8 ppm. 128 spectra were obtained with 8 transient acquisitions of 1024 points each. Pulse width = 30 μsec; acquisition delay = 100 msec.

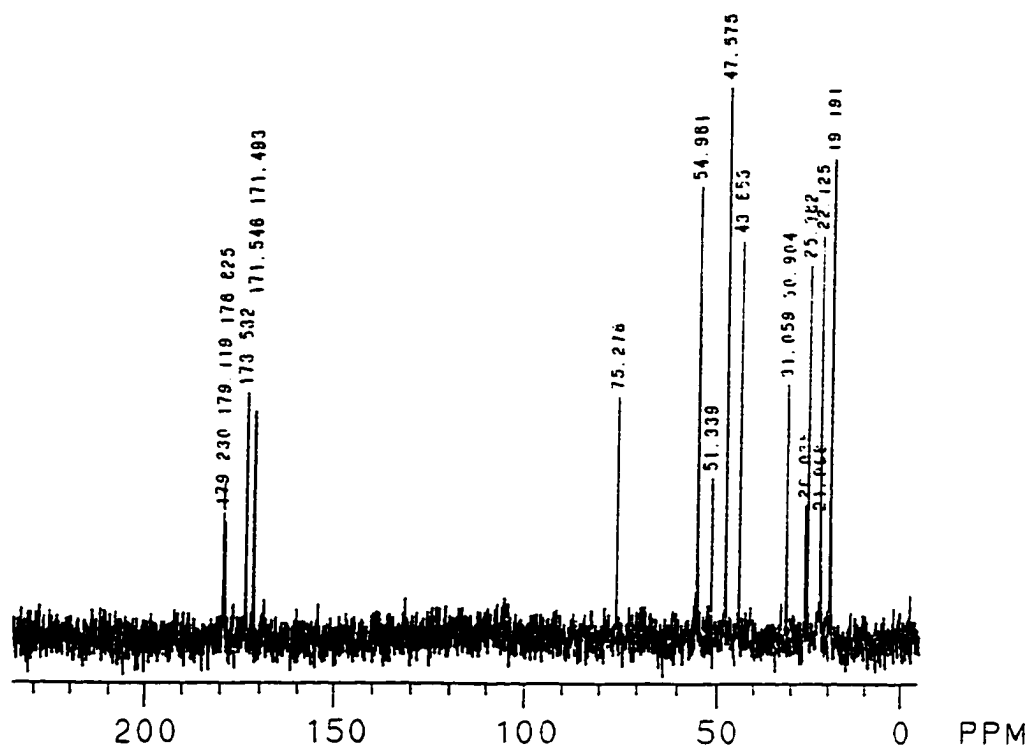


Figure 4-7. ^{13}C NMR spectrum of aerobactin at 125 MHz. ~ 10 mg in 0.5 mL D_2O . 27,652 transient acquisitions of 16,384 points. Pulse width = 8 μsec ; acquisition delay = 1 sec; line broadening = 5 Hz; referenced to TMS as an external standard.

at 75.276 ppm; lysine α carbons at 54 ppm; citrate CH_2 at 47 ppm; lysine ϵ at 43 ppm; lysine β at 30 ppm; lysine γ and δ CH_2 s at 22 and 25 ppm; and the CH_3 s of the two acetyl groups are at 19.191 ppm. Because of the symmetry of the molecule, most of the signals for the two sides overlap.

The only notable difference between the ^1H NMR spectrum of DS40M5 aerobactin and the two literature spectra (Buyer et al., 1991; Gibson and Magrath, 1969) is the presence of a double signal at 2.1 ppm for the acetyl CH_3 groups. This signal arises from rotational isomerization about the hydroxamate bond. The partial double-bond character of the carbon-nitrogen bond creates a rapidly interconverting cis-trans isomerism that can be detected on the NMR timescale. Rotamers are confirmed by a variable temperature NMR experiment in which the two signals broaden and coalesce at 55 °C (Figure 4-8). The rate of exchange and the free energy at the coalescence temperature can be calculated using the following equations (Oki, 1983):

$$\text{(Eq. 4-1)} \quad k = (\pi/\sqrt{2})\Delta\nu$$

$\Delta\nu$ = the difference in frequencies

$$\text{(Eq. 4-2)} \quad \Delta G^\ddagger = 4.57 T_c [10.32 + \log(T_c/\Delta\nu)]$$

T_c = Coalescence temperature (in Kelvin)

The free energy barrier to rotation is calculated to be 17.5 Kcal/mol. The range for formylhydroxamic acid rotational barriers is 14.2 to 16.4 kcal/mol (Kolasa,

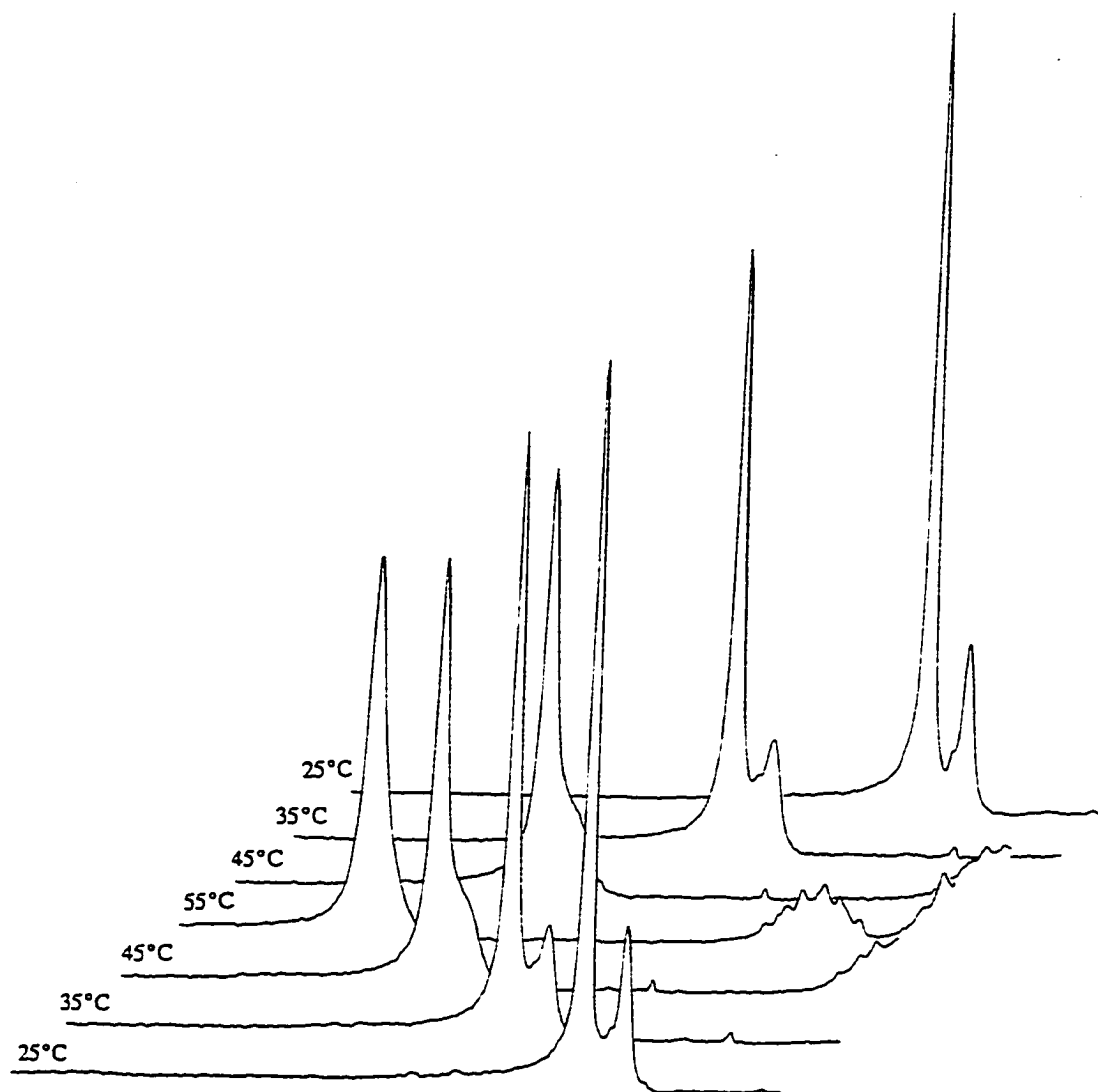


Figure 4-8. Variable temperature ¹H NMR of the amide methyl groups of aerobactin (~10 mg in 0.5 mL D₂O). Coalescence of the two signals is observed by 55 °C. Spectra are referenced to internal DSS. Pulse width = 30 μsec; acquisition delay = 1.5 sec; sixteen transient acquisitions of 16,384 points.

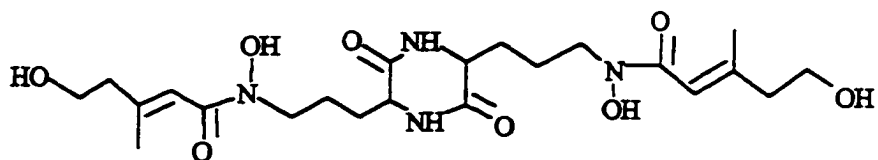
1983) while formamide has a $\Delta G^\ddagger$ of 17.8 kcal/mol (Drakenberg and Forsen, 1970). Hydroxamate rotational barriers have also been observed in the room temperature NMR spectra of dimerum acid (Figure 4-9), a di-hydroxamate siderophore produced by the fungus *Gliocladium virens* (Jalal, 1986) and desferrioxamine (Figure 4-9), a trihydroxamate siderophore that has been extensively studied (Birus et al, 1995 and references therein).

Exact mass data for aerobactin from DS40M5 gives a molecular weight for the molecular ion (MH^+) of 565.2380. The expected value for aerobactin's formula, $C_{22}H_{36}N_4O_{13}$, is 564.5216.

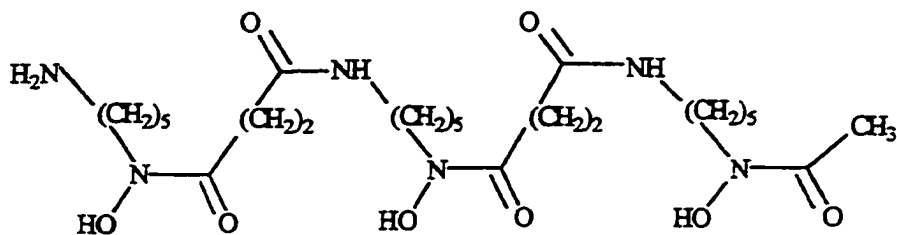
The infrared spectrum of aerobactin from DS40M5 (Figure 4-10) is virtually identical to the published spectrum. IR bands are assigned as follows: hydroxyls at $\sim 3000-3500\text{ cm}^{-1}$, carboxylic acid and amide $C=O$ stretches at 1600 cm^{-1} , C-H bends at 1400 cm^{-1} and C-O stretches at 1100 cm^{-1} (Silverstein et al., 1981).

The R_f values from paper chromatography are very similar to the literature values (Gibson and Magrath, 1969). In solvent B, Gibson and Magrath report an R_f of 0.13, while DS40M5 aerobactin has an R_f of 0.10. In solvent C the literature value is 0.63 and the experimental value is 0.62.

Neilands' (1984) procedure for the isolation of aerobactin was employed as an additional test that the DS40M5 material behaves in the same manner as



Dimerum acid



Desferrioxamine B

Figure 4-9. Structures of dimerum acid and desferrioxamine B. These hydroxamate siderophores have been shown to exhibit hydroxamate equilibrium by NMR. See Results and Interpretation.

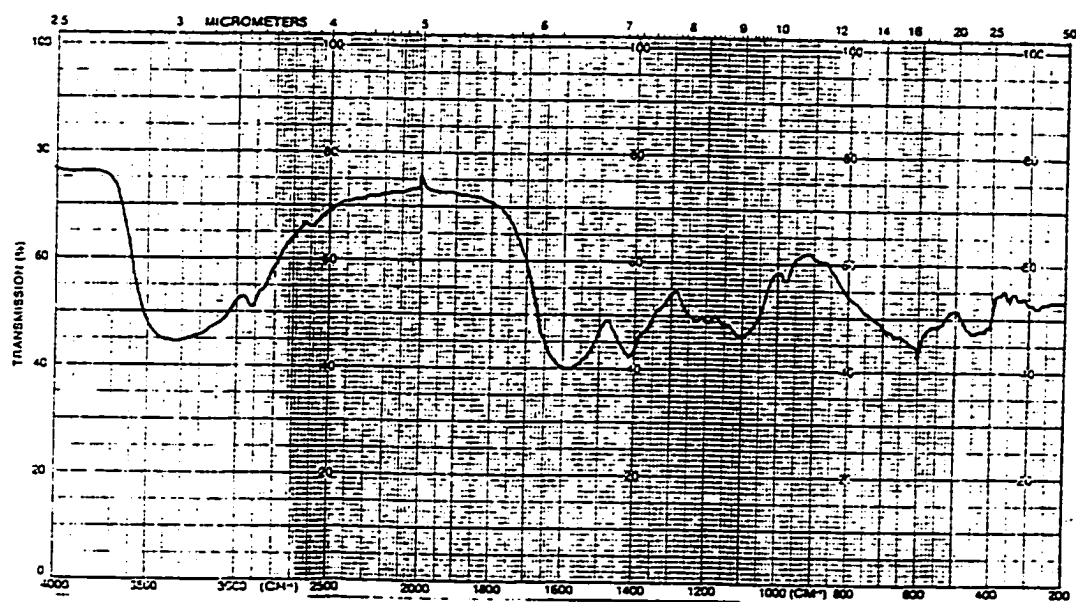


Figure 4-10. Infrared spectrum of aerobactin in a KBr pellet.

authentic aerobactin. The procedure as described in the Experimental section produced only a small amount of yellow aerobactin from DS40M5, but the ^1H NMR spectrum is consistent with the previously isolated material (Figure 4-11). A major difficulty in isolating water-soluble materials from seawater is the removal of salts. Therefore, an additional XAD-2 step is required to separate the salts from the organic material. Despite many purification steps, the CAS-reactive material remains yellow. The Sephadex LH20 and HPLC method gives much cleaner material at higher yield. However, it is clear that the siderophore from DS40M5 can be isolated by Neilands' procedure, which is additional evidence for its identity as aerobactin.

Iron(III) complexation: At pH 2 the λ_{max} of iron(III) aerobactin is 464 nm with an extinction coefficient of $2024 \text{ M}^{-1}\text{cm}^{-1}$ (Figure 4-12). At pH 7 the λ_{max} is 399 nm with an extinction coefficient of $1918 \text{ M}^{-1}\text{cm}^{-1}$ (Figure 4-13). Harris et al. (1979) report λ_{max} values for Fe(III)-aerobactin to be 450 and 399 nm at pH 2.7 and 7 respectively. At both pH values, aerobactin titrated with iron(III) gives a maximum absorbance after approximately one equivalent of iron(III) has been added, indicating formation of a 1:1 complex. Slight differences from 1:1 stoichiometry are attributed to precipitation of iron(III) hydroxides at pH 7.

Figure 4-14 shows the effect of pH on the absorbance spectrum of ferric-

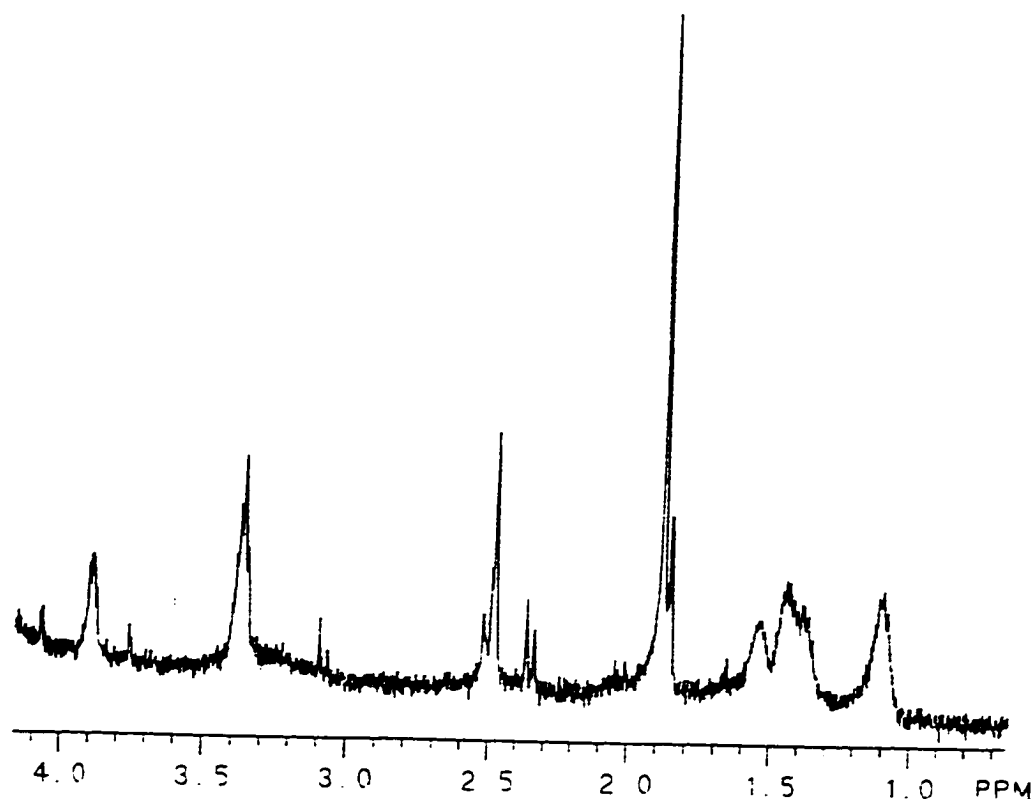


Figure 4-11. ^1H NMR spectrum in D_2O of aerobactin isolated by Neilands' (1984) procedure. Spectra are referenced to the HDO resonance at 4.8 ppm. Pulse width = 30 μsec ; acquisition delay = 1.5 sec; sixteen transient acquisitions of 16,384 points.

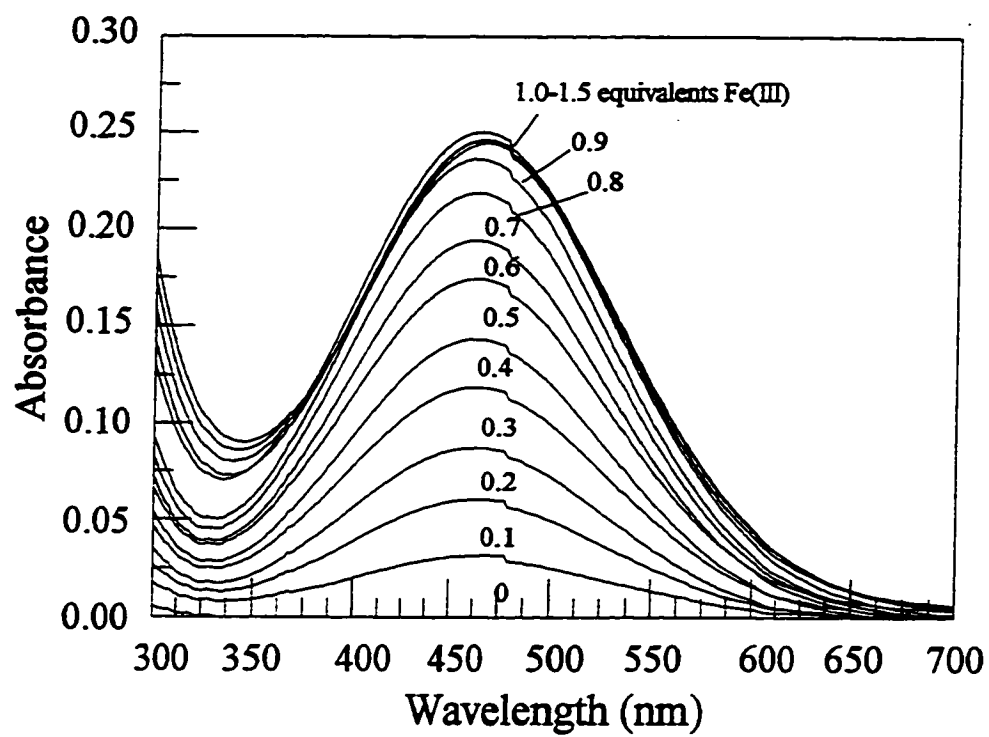


Figure 4-12. Titration of aerobactin with 1.2 mM $\text{Fe}(\text{ClO}_4)_3$ at pH 2 in 0.1% TFA. The initial sample of 0.15 mM aerobactin (800 μL) was titrated with 10 μL aliquots of the iron(III) stock solution from 0 to 1.5 equivalents.

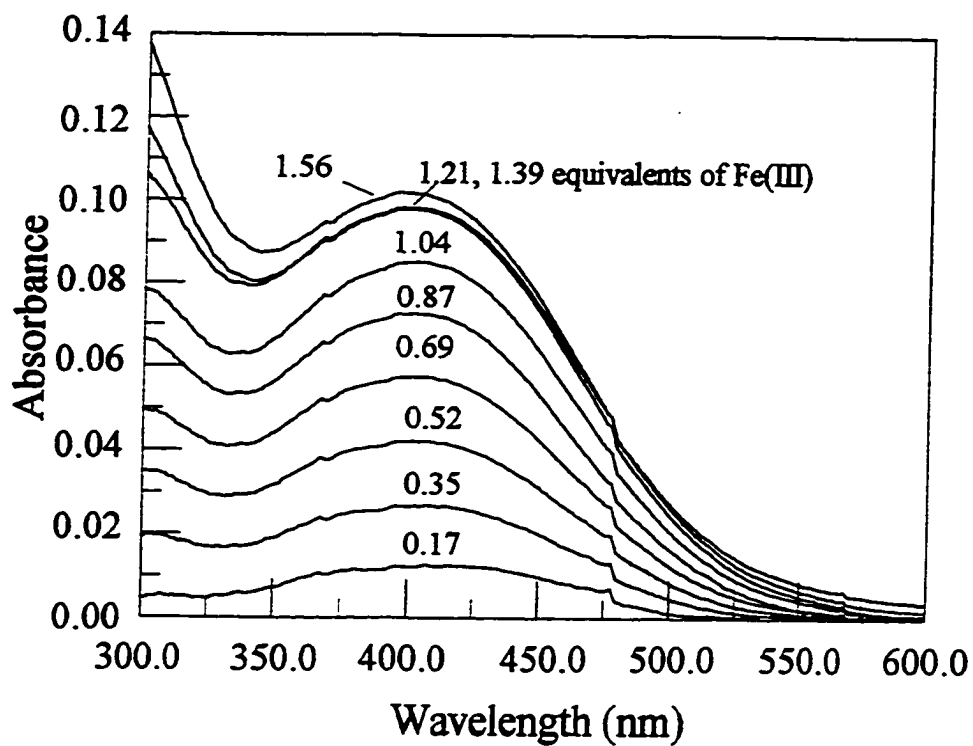


Figure 4-13. Titration of aerobactin with 0.78 mM $\text{Fe}(\text{ClO}_4)_3$ at pH 7 in 0.1 M phosphate buffer. Aerobactin samples (150 μL aliquots of a 0.3 mM solution) were mixed with 50 μL aliquots of the iron(III) solution, brought up to 1 mL with buffer, and equilibrated for 2 hours before measuring.

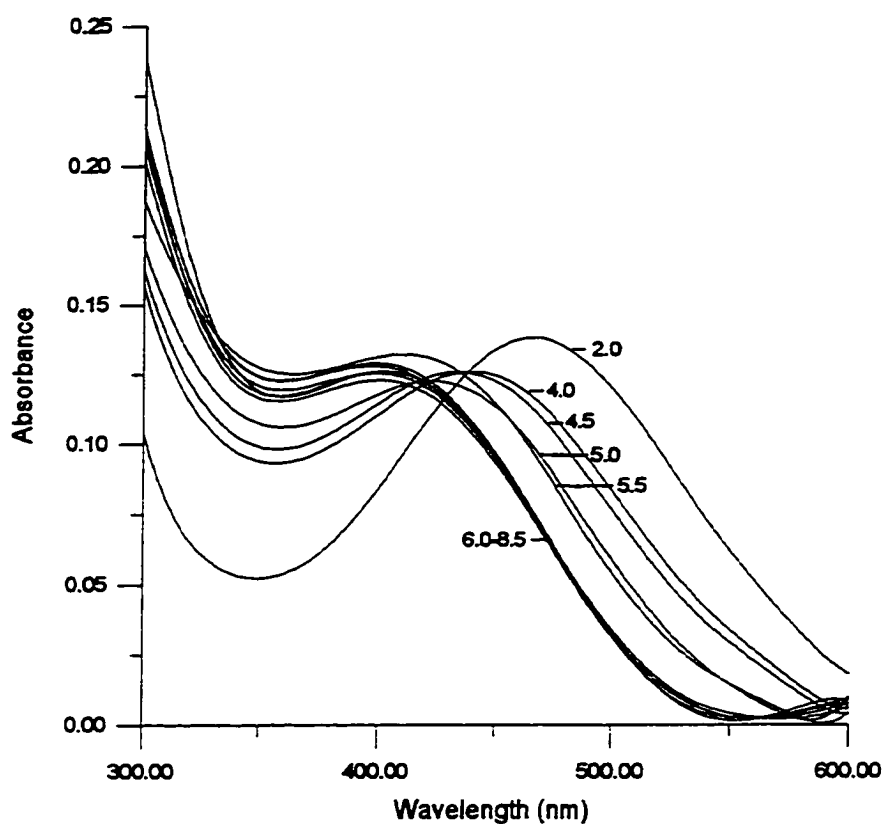


Figure 4-14. UV-Visible spectra of ferri-aerobactin as a function of pH. Samples of 76 μM ferri-aerobactin were prepared in buffers of different pH as described in the Experimental section. Plotted curves were smoothed by calculating an 8th order polynomial fit to remove noise.

aerobactin. As the pH is lowered, the complex changes color from orange to purple (i.e. $\lambda_{\max}$ shifts from 400 nm to 480 nm). As the acid concentration increases, aerobactin becomes partially protonated and iron(III) coordination changes from bis-hydroxamate to mono-hydroxamate. See the Discussion section for further explanation. The shift occurs around pH 4 in Figure 4-14, consistent with the previous work reported by Harris et al. (1979). A shift at pH 4 is indicative of a dihydroxamate ligand (Neilands, 1984).

Vanadium(V) complex: At pH 2, in 0.1 M HCl/ KCl, the pink vanadium(V)-aerobactin complex, prepared as described in the Experimental section, has a $\lambda_{\max}$ at 495 nm and an extinction coefficient of $2306 \text{ M}^{-1}\text{cm}^{-1}$. Titrating 800 μL of 1.5 mM aerobactin with vanadium(V) gives a maximum absorbance after approximately 0.8 equivalents of vanadium(V) (1.2 mM NH_4VO_3) are added (Figure 4-15). Therefore, a 1:1 complex is possibly formed between vanadium(V) and aerobactin at low pH. At pH 7 in 0.2 M Hepes, a yellow complex is formed that absorbs significantly only below 300 nm (Figure 4-16a), where vanadate alone also absorbs, although with a lower extinction coefficient (Figure 4-16b).

Vanadium(V) complexes of the tri-hydroxamate siderophore desferrioxamine B (Figure 4-9) were investigated by Luterotti and Grdinic

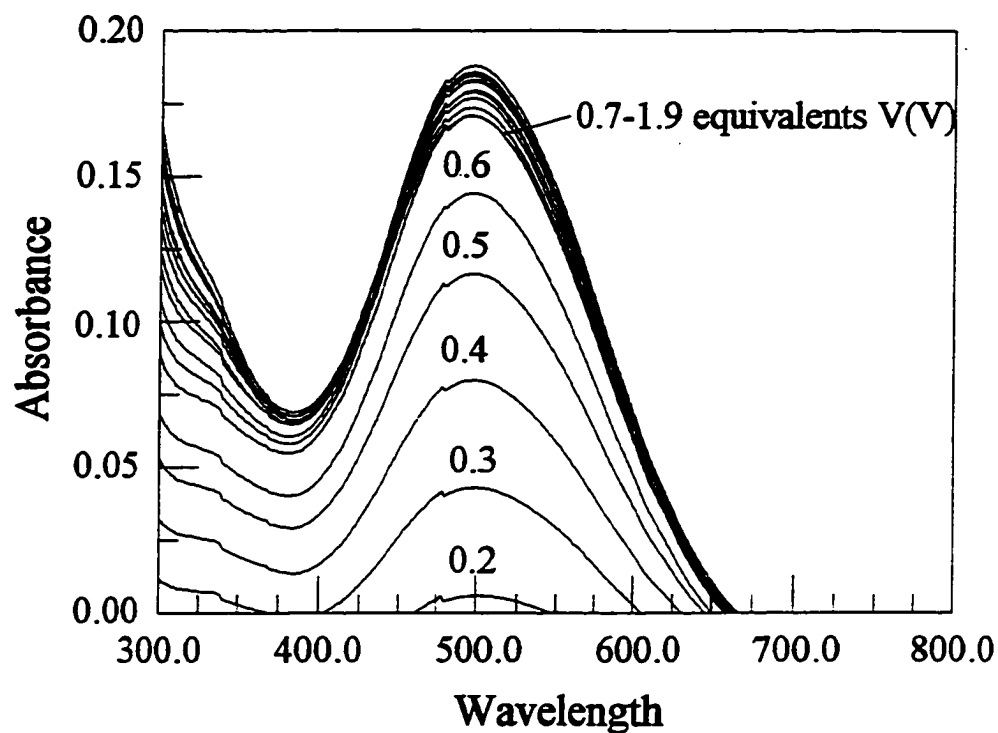


Figure 4-15. Titration of aerobactin with vanadate at pH 2 in 0.1 M HCl/ KCl. Eight hundred μL of 0.15 mM aerobactin titrated with 10 μL aliquots of 1.2 mM NH_4VO_3 up to 2 equivalents of V(V):aerobactin. Every other scan is shown.

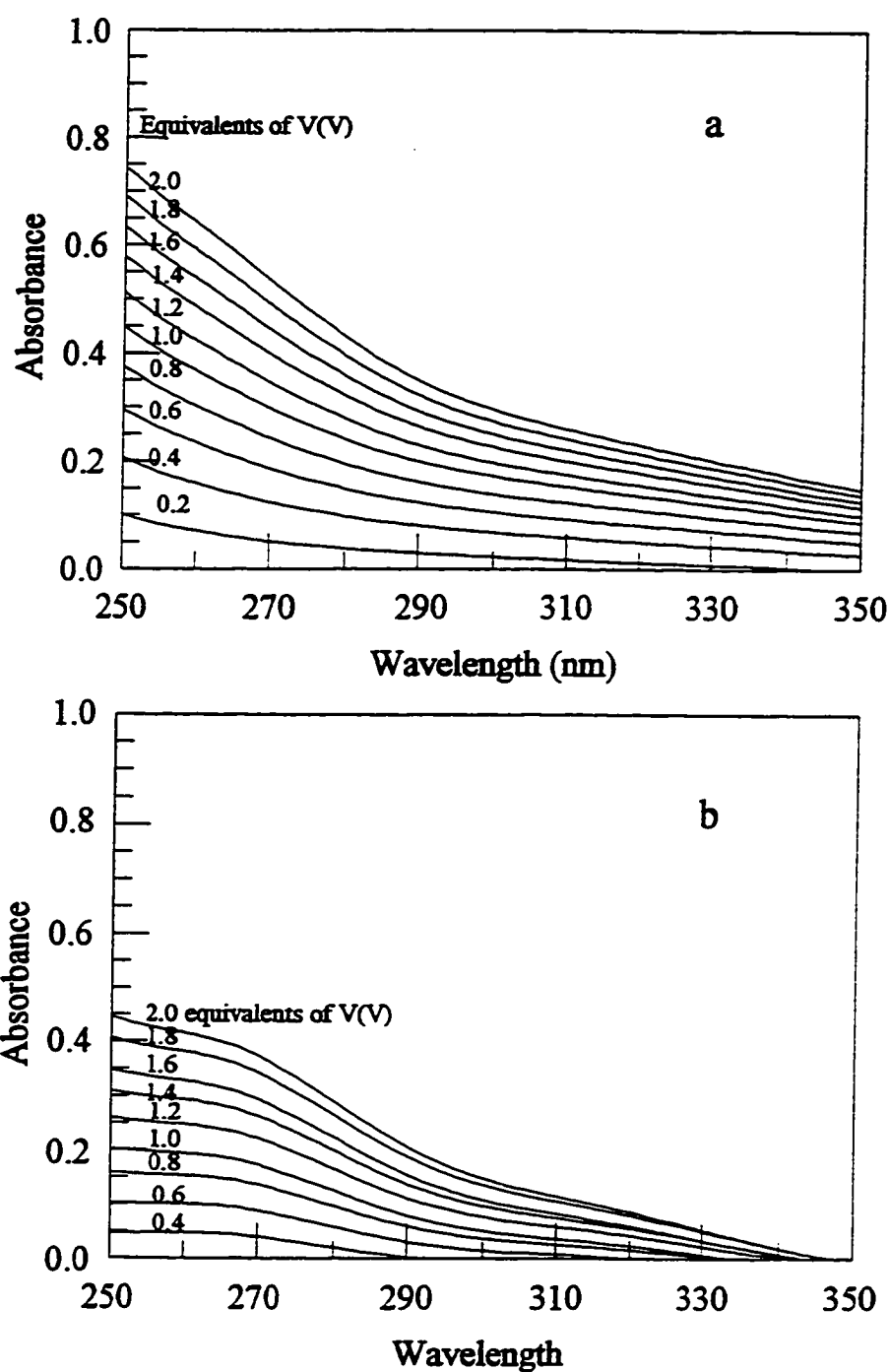


Figure 4-16. Titration of aerobactin with vanadate at pH 7 in 0.2 M HEPES. a) An 800 μ L sample of 0.15 mM aerobactin titrated with 10 μ L aliquots of 1.2 mM NH_4VO_3 until 2 equivalents of vanadium(V) were added. Every other scan is shown. b) Vanadate in pH 7 0.2 M HEPES; same conditions as in a).

(1986) by UV-visible spectroscopy. They obtained red-violet complexes at low pH ($\lambda^{\max} = 480$ nm) and yellow complexes at pH 7 ($\lambda^{\max} \sim 320$ nm). The low pH spectra appear to be quite similar to the pH 2 spectra in Figure 4-15. At pH 7, the different coordinating ligands may account for the appearance of an absorption band at 320 nm in the vanadium(V)-desferrioxamine B complex, but not in vanadium(V)-aerobactin.

UV-visible competitions between vanadium(V) and iron(III) aerobactin show that even 100-fold excess of vanadium(V) over iron(III) does not displace iron(III) from aerobactin at pH 7 (Figure 4-17). The absorbance of the ferric aerobactin complex at 400 nm is essentially unchanged by the addition of vanadate. In the other direction, addition of equimolar iron(III) to a solution of vanadium(V) and aerobactin completely displaces the vanadium(V) to form iron(III)-aerobactin (Figure 4-18). Slight absorbance increases after one equivalent of Fe(III) have been added could be from colloidal iron hydroxide precipitate which forms as the iron(III) is added to the buffered sample at pH 8.

^{51}V -NMR of the vanadium(V)-aerobactin complex at pH 7.4 (0.1 M HEPES) gives two broad resonances at -515 and -530 ppm relative to VOCl_3 (Figure 4-19). For comparison, the ^{51}V NMR spectrum of 5 mM vanadium(V) with 20 mM acetohydroxamic acid was obtained. Two resonances are observed,

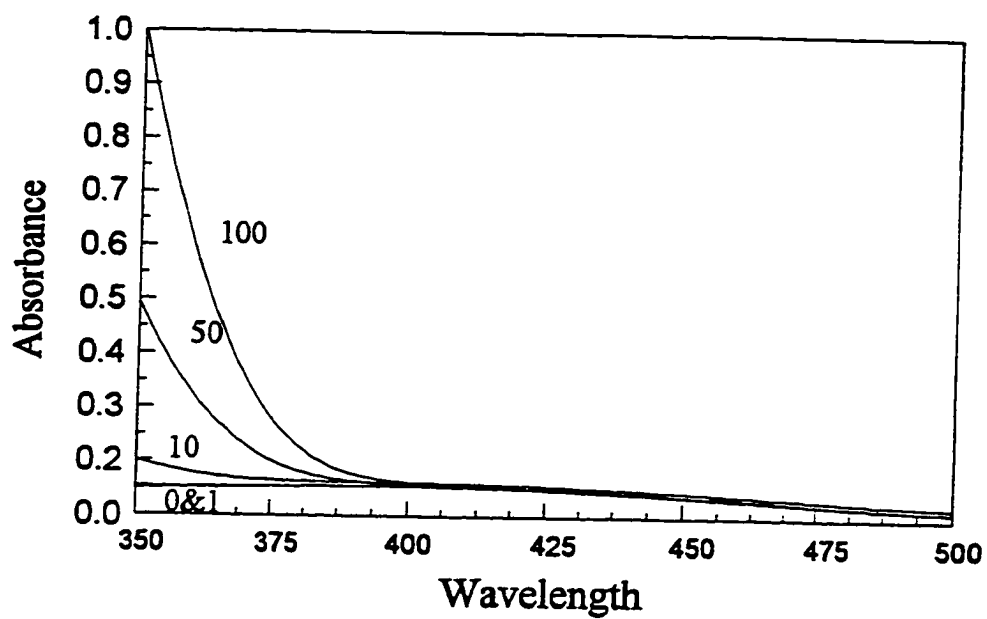


Figure 4-17. Competition of vanadate for iron(III)-aerobactin. Addition of up to 100-fold excess vanadate to 0.04 mM ferri-aerobactin in 0.1 M phosphate buffer, pH 8.

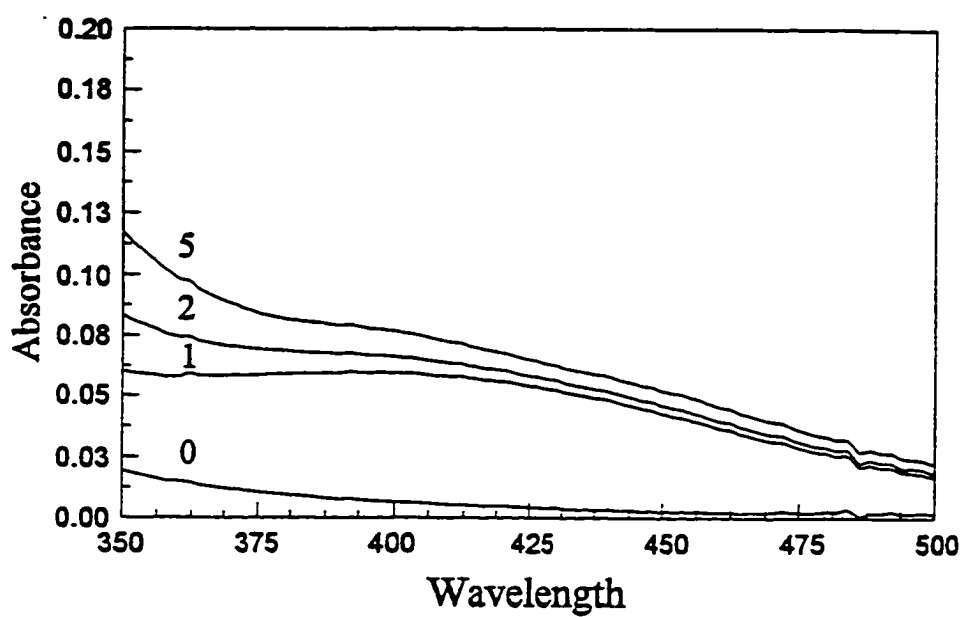


Figure 4-18. Competition of iron(III) for complexation of V(V)-aerobactin. Addition of up to 5 equivalents of iron(III) to 0.04 mM vanadium(V)-aerobactin in 0.1 M phosphate buffer, pH 8.

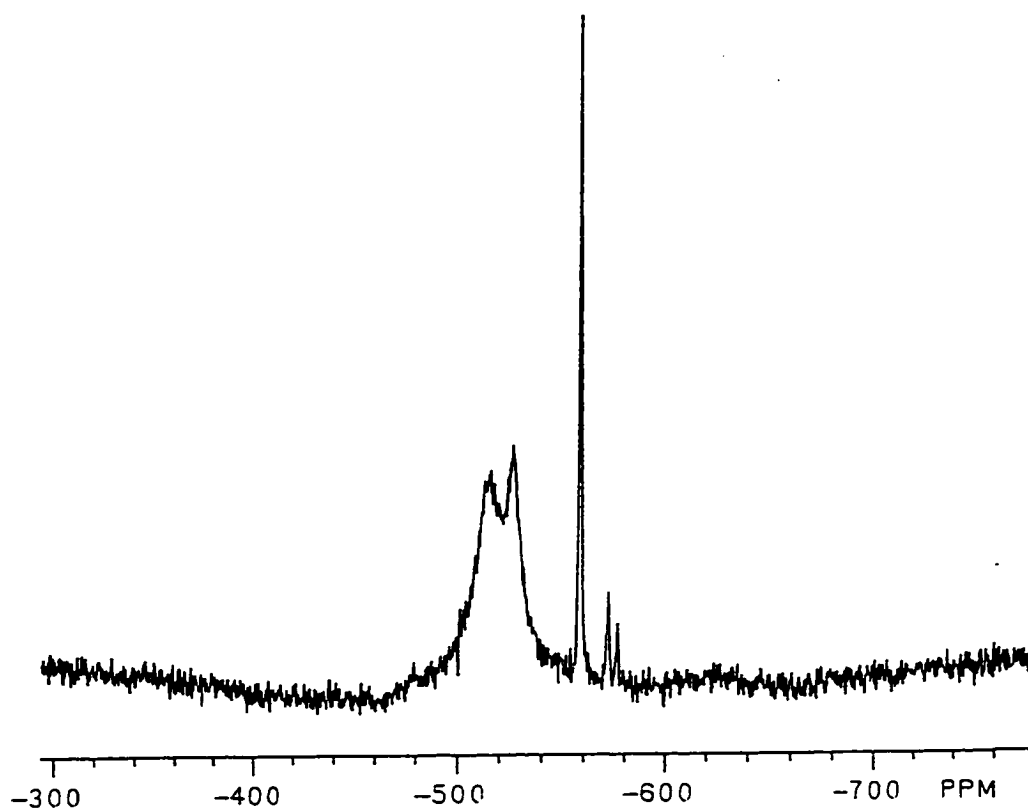


Figure 4-19. ^{51}V NMR spectrum of 4.05 mM V(V)-aerobactin in 0.1 M HEPES, pH 7.4. The spectrum is referenced to VOCl_3 as an external standard, $\delta(\text{VOCl}_3) = 0.0$ ppm. Pulse width = 20 μsec ; acquisition delay = 100 msec; 12,000 transient acquisitions of 8192 points.

at -505 and -515 ppm (Figure 4-20). Butler et al. (1989) observed a -515 ppm resonance for V(V)-desferrioxamine B at pH 7.4.

Titration of 8.8 μ moles of aerobactin with 50 mM NH_4VO_3 shows the development of the V(V)-aerobactin resonances (Figure 4-21). Free vanadate is observed even at very low ratios of vanadium(V) to aerobactin. The peak at -558 ppm appears first (corresponding to monomeric vanadate), then -571 ppm (dimer) and -576 ppm (tetramer) grow in as more vanadate is added. GEMCAP peak area determination allowed comparison of the relative peak areas for vanadate and vanadium(V)-aerobactin (Table 4-2). Given the equilibrium:



$$K_b = [\text{V}^{\text{V}}\text{-aerobactin}]/[\text{V}^{\text{V}}][\text{aerobactin}].$$

$[\text{V}^{\text{V}}]$ and $[\text{V}^{\text{V}}\text{-aerobactin}]$ were determined from peak areas using the known total vanadium(V) in each sample. Free aerobactin was calculated by subtracting vanadium(V)-aerobactin from the total amount of aerobactin. The average stability constant for formation of the vanadium(V)-aerobactin complex at pH 7 was then determined to be 2.7×10^4 ($\log K_b = 4.4$).

Genetic characterization: The Biolog Inc. (Hayward, CA) database did not give any exact matches. Table 4-3 shows the closest species identified by Biolog Inc. along with numerical scores for species similarity and distance from

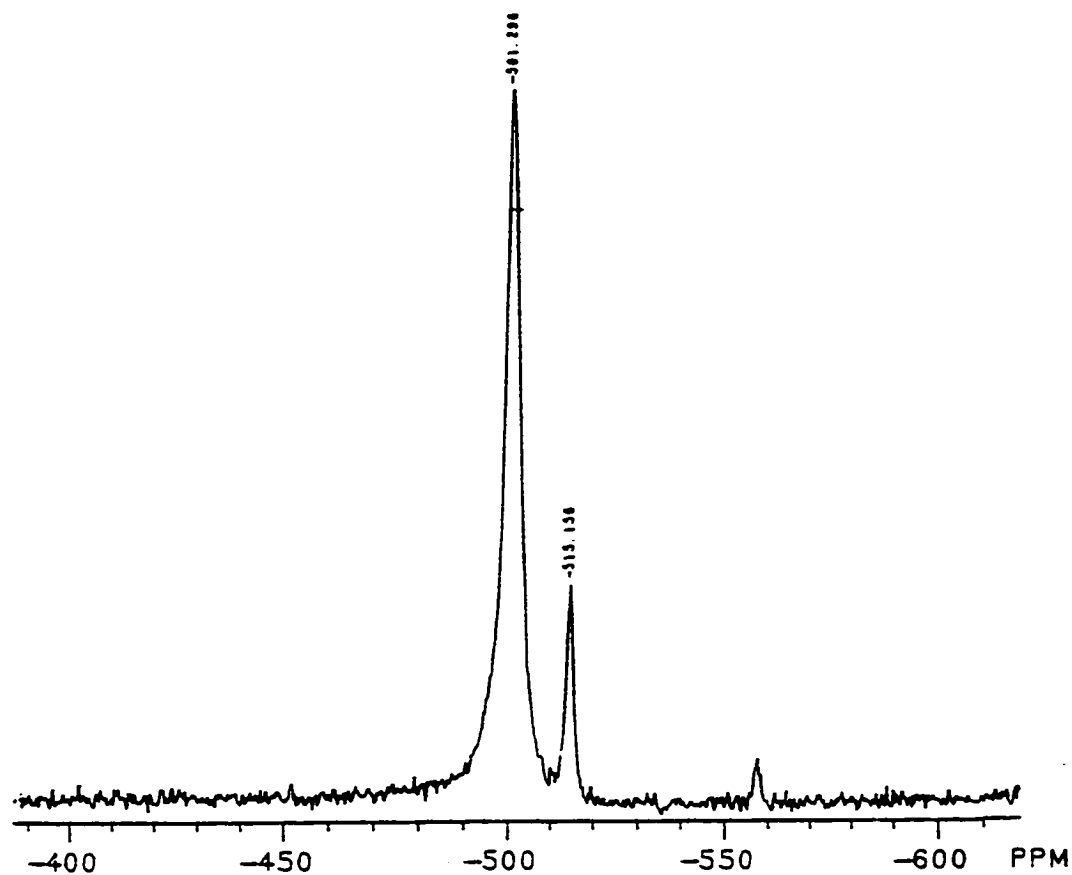


Figure 4-20. ^{51}V NMR spectrum of 5 mM NH_4VO_3 + 20 mM acetohydroxamic acid in 0.1 M HEPES pH 7.4. Total volume = 2 mL. The spectrum is referenced to VOCl_3 as an external standard, $\delta(\text{VOCl}_3) = 0.0$ ppm. Pulse width = 20 μsec ; acquisition delay = 100 msec; 12,000 transient acquisitions of 8192 points.

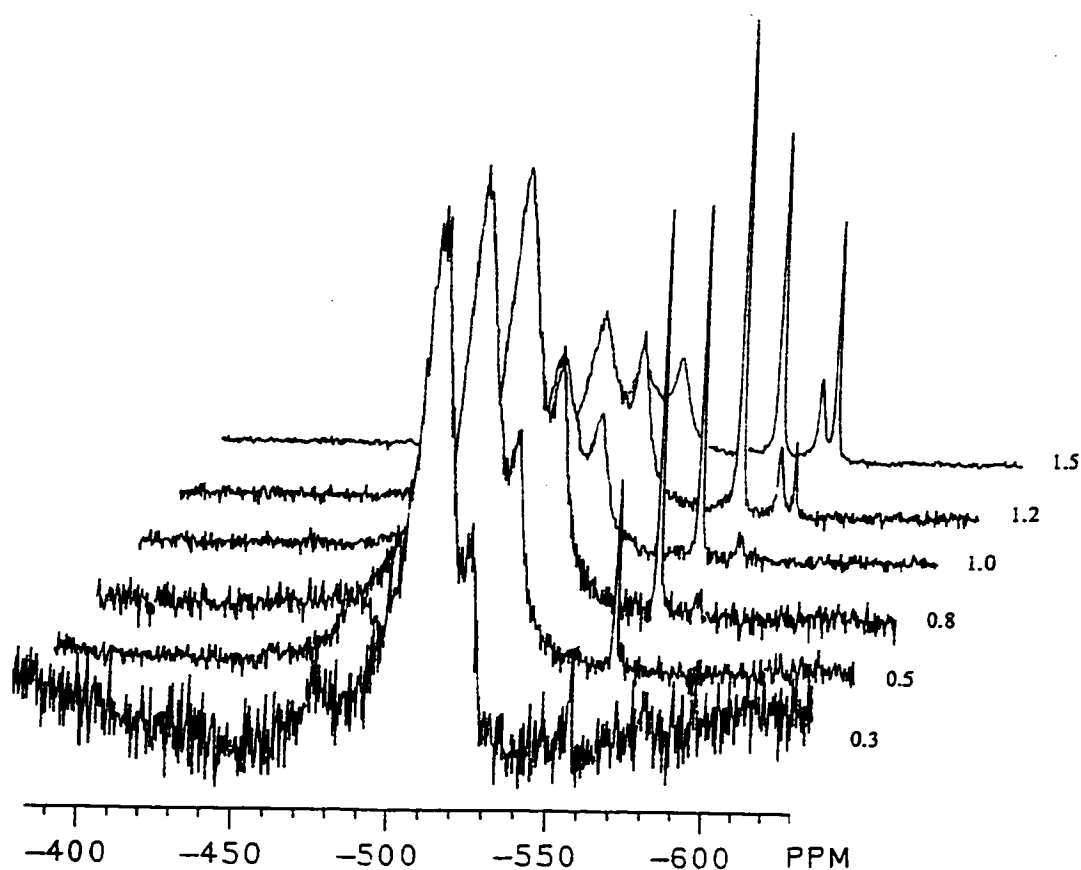


Figure 4-21. ^{51}V NMR titration of aerobactin with vanadate from 0.3 to 1.5 equivalents of vanadate relative to aerobactin. The 10 mm NMR tube contained 4.94 mg aerobactin in 2.0 mL 0.1 M HEPES, pH 7.4 and was titrated with 50 mM NH_4VO_3 . Sharp signals from free vanadate are observed at -558, -571 and -576 ppm while the vanadium(V)-aerobactin complex gives broad resonances at -515 and -530 ppm relative to VOCl_3 as an external standard, $\delta(\text{VOCl}_3) = 0.0$ ppm. Pulse width = 20 μsec ; acquisition delay = 100 msec; 10,000 to 40,000 transient acquisitions of 8192 points.

<u>Equivalents of V(V)</u>	<u>% Free V(V)</u>	<u>%Bound V(V)</u>	<u>K_b</u>
0.3	0.87	99.11	4.23 x 10 ⁴
0.5	1.1	98.9	4.93 x 10 ⁴
0.8	5.25	94.73	1.84 x 10 ⁴
1.0	11.93	88.04	1.54 x 10 ⁴
1.2	20.86	79.1	3.45 x 10 ⁴
1.5	39.65	60.32	4.11 x 10 ⁴

Table 4-2. ⁵¹V-NMR GEMCAP areas of free vs. bound vanadium (V) and the calculated K_b for V(V)-aerobactin. See Results and Interpretation section for calculations. Average K_b = 2.7 x 10⁴.

<u>Species</u>	<u>Similarity</u>	<u>Distance</u>
<i>Alteromonas haloplanktis</i>	0.410	7.273
<i>Aeromonas</i> DNA group 11	0.068	7.859
<i>Pasteurella anatis</i>	0.029	8.156
<i>Weeksella zoohelcum</i>	0.009	8.514
<i>Deleya aestiva</i>	0.000	9.677
<i>Vibrio fischeri</i>	0.000	10.558
<i>Psychrobacter immobilis</i>	0.000	10.660
<i>Shewanella putrefaciens</i> A	0.000	11.172
<i>Vibrio splendidus</i> 2	0.000	11.311
<i>Xenorhabdus nematophilus</i> SS bovienii	0.000	11.962

Table 4-3. Biolog Inc. (Hayward, CA) results for identification of DS40M5 based on carbon source utilization. No identification was made. Bacterial species are ranked in order of greatest similarity to the 25 hour pattern of oxidized substrates. See Experimental section for details.

DS40M5. The most similar species based on the 25 hour incubation was *Alteromonas haloplanktis*, another marine species.

Discussion and Conclusions

Bacterial species DS40M5 was isolated from seawater samples taken from a depth of 40 m in the Atlantic Ocean near the Canary and Cape Verde Islands as described in Haygood et al. (1993). Aliquots of seawater were plated onto artificial seawater agar containing chrome azurol S (CAS) according to Schwyn and Neilands (1987). DS40M5 is one of several CAS-positive strains that were isolated and selected for further study.

Analysis of the siderophores produced by marine bacteria led to the discovery that DS40M5 produces aerobactin (Figure 4-1), a siderophore commonly produced by enteric bacteria. It has also been reported from two human pathogenic species of *Vibrio* (Okujo and Yamamoto, 1994). This is the first report of aerobactin production by a marine isolate.

Siderophore production: From the iron(III) growth experiments in Figure 4-5, it is apparent that production of CAS reactive materials is suppressed at high iron(III) levels. Therefore, aerobactin is presumed to be acting as a siderophore for this species. Siderophore production is observed in low-iron(III) cultures

after the exponential growth phase, which is consistent with other systems (Reid, 1993; Gascoyne et al., 1991).

Structure: Aerobactin consists of citric acid amide-bonded to two molecules of *N*^ε-acetyl, *N*^ε-hydroxy lysine. The hydroxamate bonds show temperature-dependent cis-trans isomerization with a Gibbs free energy of 17.8 kcal/mol.

Iron(III) complexation: Ferric aerobactin's spectral changes with pH are characteristic of a dihydroxamate siderophore. (The prefixes di- and tri- are used to describe the number of hydroxamate moieties in a single molecule. Bis- and tris- refer to independent multidentate ligands.) As the pH is lowered, the spectrum changes from the orange of a bis- or tris-coordinated iron(III) complex to the purple of a mono-hydroxamate iron(III) species. One of the hydroxamate ligands is protonated away around pH 4. Trihydroxamate siderophores do not undergo this transition until about pH 1, where all complexing ability is rapidly lost and the solution turns colorless (Neilands, 1984).

Aerobactin is not, however, a simple dihydroxamate ligand. It has the citrate backbone which is also involved in complexing iron(III) through the central α -hydroxy carboxylate moiety. As a result, unlike other dihydroxamate siderophores, aerobactin forms a 1:1 iron(III):ligand complex, as evidenced by

the titration of aerobactin with iron(III) in Figure 4-13. Dihydroxamate siderophores which lack additional chelating functional groups, such as rhodotorulic acid (Carrano and Raymond, 1978), form 2:3 iron(III):ligand complexes at neutral pH. Raymond and co-workers (Harris et al., 1979) determined the formation constant of iron(III)-aerobactin to be $10^{22.93}$. They also observed a high pK deprotonation in the ferric complex that is likely a result of the deprotonation of the central citrate hydroxyl. Therefore, iron(III) coordination occurs through six oxygens on a single aerobactin molecule. The determined ferric affinity constant ($\log K_f = 22.93$) is lower than trihydroxamate siderophores ($\log K_f \sim 35$), but is higher than other dihydroxamates (average $\log K_f \sim 21.5$) (Harris et al., 1979).

Aerobactin is the siderophore of choice for enteric bacteria because of its ability to compete *in vivo*. Aerobactin is more effective than enterobactin in removing iron(III) from transferrin in serum, despite their vast difference in stability constants. Enterobactin is complexed by serum albumin (Konopka and Neilands, 1984); is only slightly soluble in aqueous solution; and is subject to hydrolysis of its serine ester backbone (Loomis and Raymond, 1991). By contrast, aerobactin is not complexed by serum albumin and it is both soluble and stable under physiological conditions (Konopka et al., 1982; Konopka and Neilands, 1984).

Aerobactin also has the potential to be recycled, as shown by Braun et al. (1984), since more than one equivalent of ^{55}Fe was transported into *E. coli* cells per equivalent of aerobactin. Also, tritium-labelled aerobactin did not accumulate inside of the cells as would be expected if it were only used once (Braun et al., 1984). Ferric enterobactin does accumulate inside the cell and the iron(III) is removed later, possibly by reduction to iron(II) (Ecker et al., 1986).

Vanadium(V) complexation: The complexation of vanadium(V) by aerobactin was investigated since the concentration of vanadium(V) in seawater, 20-35 nM, is vastly greater than that of iron(III), 0.02 - 1.5 nM (Bruland et al., 1994; Martin et al., 1989). Therefore, released siderophores would be much more likely to encounter vanadate than iron(III). A presumed 1:1 complex is formed between vanadium(V) and aerobactin, but the formation constant of vanadium(V)-aerobactin, determined by ^{51}V NMR to be $10^{4.4}$, is much lower than that for iron(III) aerobactin, $10^{22.93}$ (Harris et al., 1979). Even the presence of 100-fold excess vanadium(V) over iron(III) does not displace iron(III) from ferric aerobactin (Figure 4-17). Also, vanadium(V) is completely displaced from aerobactin when iron(III) is added to the complex (Figure 4-18). Therefore, vanadium(V) is not likely to interfere with the coordination of iron(III) by hydroxamate siderophores such as aerobactin.

Although hydroxamic acids have been used to analyze vanadium for many years, only a few references to structures of vanadium-hydroxamate complexes exist in the literature (Fisher et al., 1989). Crystal structures of mono and dihydroxamic acid complexes of vanadium(V) formed in the presence of HCl show pseudo-octahedral coordination of vanadium(V) by two hydroxamates, and a chloro and an oxo ligand (Fisher et al., 1989).

^{51}V NMR of complexes of vanadium(V) with desferrioxamine B (Figure 4-9) has been explored by Buglyo et al. (1995). They observed a -517 ppm resonance at near neutral pH which they assign to a mono- or bis-hydroxamate, dioxovanadium(V) complex. At pH 3, however, they observe a resonance at -447 ppm, which they attribute to a bis- or tris-chelated monooxovanadium(V) complex. The -515 ppm resonance is analogous to one of the broad signals observed in the aerobactin complex (Figure 4-19), which is therefore possibly a dioxovanadium(V) complex. The resonance at -530 is of similar area, and could be from coordination through the citrate backbone instead of one of the hydroxamates.

16S rRNA analysis: Ribosomal RNA analysis was performed by Dr. Haygood of Scripps Institute of Oceanography as described in Haygood et al. (1993). The results indicate that strain DS40M5 is most closely related to marine *Vibrio*

species, particularly *V. proteolyticus*, but is not a member of any of the species in the database. Comparison of the sequence with other bacterial sequences from the Ribosomal Database Project (Olsen et al., 1991) and computer analysis of similarities led to the relationship tree shown in Figure 4-22. The DS40M5 strain did not appear to have any plasmids, so the genes involved in aerobactin production are presumably included in the chromosomal DNA (Haygood et al, 1993).

Vibrio sp. DS40M5 was the third type of bacterium shown to produce aerobactin. The production of aerobactin by other *Vibrio* species was reported by Okujo and Yamamoto in 1994, after publication of these results (Haygood et al., 1993). The *Vibrios*, several *Enterobacter* species and the *Pseudomonad* (Buyer et al., 1991) together form a genetically diverse class of aerobactin producing bacteria.

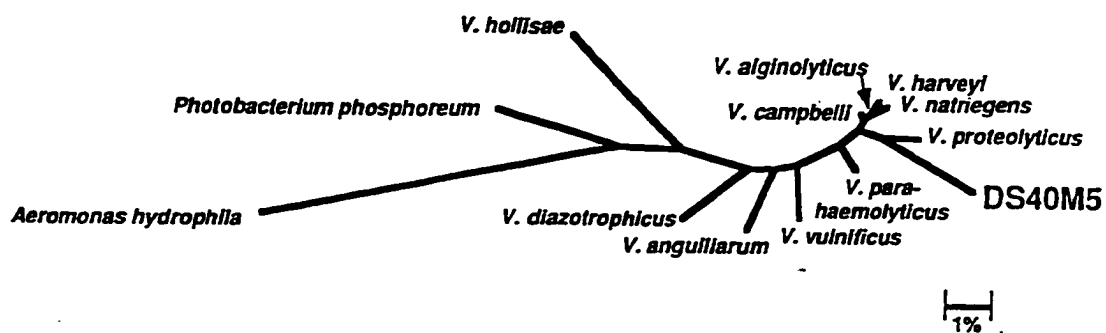


Figure 4-22. Relationship tree based on 16S ribosomal RNA analysis of DS40M5. Scale bar = 1% corrected sequence divergence. Figure adopted from Haygood et al. 1993.

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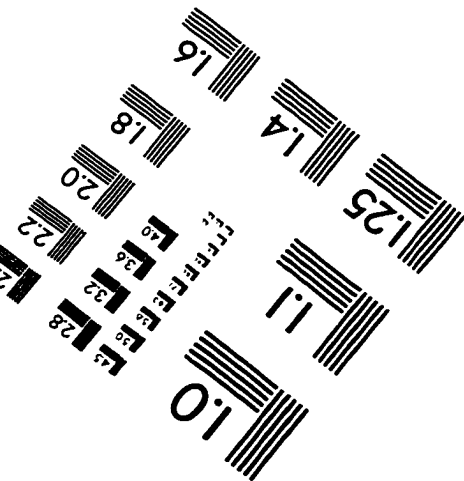
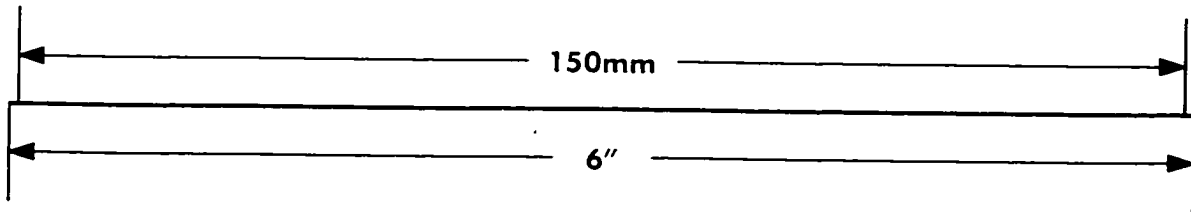
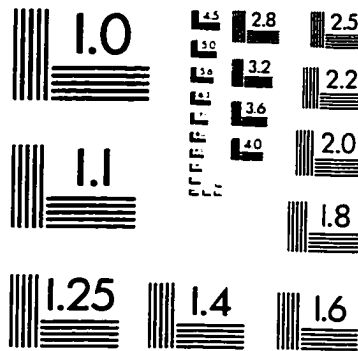
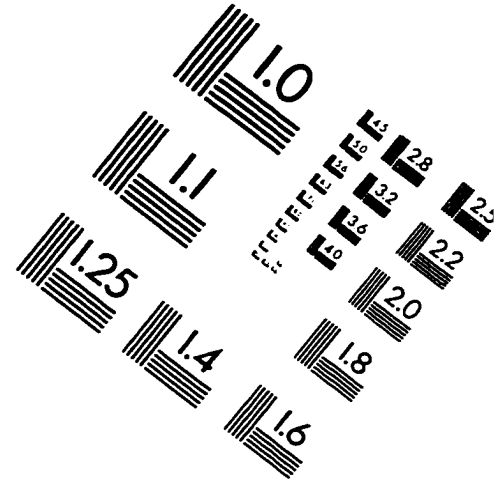
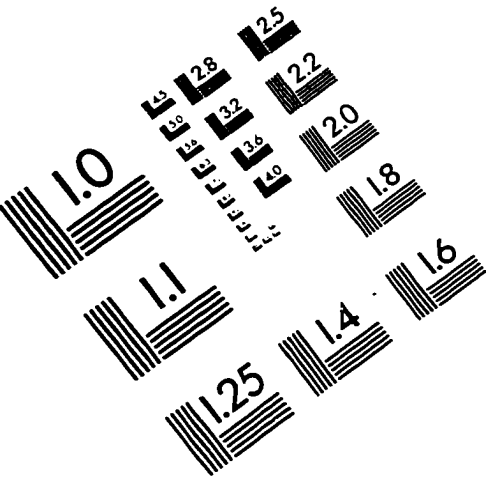
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IMAGE EVALUATION TEST TARGET (QA-3)



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