*BioMetals* **15:** 153–160, 2002.



# Bisucaberin – A dihydroxamate siderophore isolated from *Vibrio salmonicida*, an important pathogen of farmed *Atlantic salmon* (*Salmo salar*)

Günther Winkelmann[1,*], Dietmar G. Schmid[2], Graeme Nicholson[2], Günther Jung[2] & Duncan J. Colquhoun[3]

[1]*Institut für Mikrobiologie und Biotechnologie, and* [2]*Institut für Organische Chemie, Universität Tübingen, Auf der Morgenstelle 28, D-72076 Tübingen, Germany;* [3]*National Veterinary Institute, Post Box 8156 Dep., 0033 Oslo, Norway;* [*]*Author for correspondence (Tel: +49-70712973094; Fax: +49-7071295002; E-mail: winkelmann@uni.tuebingen.de)*

Received 14 January 2002; accepted 22 January 2002

*Key words:* bisucaberin, iron, siderophores, *Vibrio salmonicida*

**Abstract**

A siderophore of the bacterial fish pathogen, *Vibrio salmonicida*, was isolated from low-iron culture supernatant and structurally characterized as bisucaberin by FTICR- and FAB-MS, NMR and GC-MS analysis of the hydrolysis products. Although the cyclic dihydroxamate bisucaberin has previously been isolated from a marine bacterium, *Alteromonas haloplanktis*, its involvement in cold-water vibriosis of Atlantic salmon (*Salmon salar*) is novel. Bisucaberin production in iron-limited media was highest at temperatures around and below 10 °C, correlating well with temperatures at which outbreaks of cold-water vibriosis occur. Due to the very high stability constant of $K = 32.2$, bisucaberin is a most efficient iron scavenger which may contribute to the virulence of *V. salmonicida* in Atlantic salmon.

## Introduction

The marine bacterium *Vibrio salmonicida* is the causal agent of cold-water vibriosis (Hitra disease), a fatal bacterial septicaemia of farmed Atlantic salmon (*Salmo salar*) and to a lesser extent farmed cod (*Gadus morhua*) and rainbow trout (*Oncorhynchus mykiss*). Little is known regarding the iron acquisition system of *V. salmonicida*, although previous studies have provided evidence that a siderophore mediated iron uptake exists (Colquhoun & Sørum 2001). The genus *Vibrio* contains both pathogenic and non-pathogenic species, of which the human pathogen, *V. cholerae* (serogroup O1), causes world-wide pandemics.

*V. anguillarum* has been shown to cause terminal hemorrhagic septicaemia in salmonid fish and evidence for the involvement of the siderophore, anguibactin, and a highly efficient plasmid-encoded iron transport system has been well documented (Wolf & Crosa 1986; Crosa 1989). *Photobacterium (Vibrio) damselae* causes diseases in warm and cold-water fishes such as damselfish (*Chromis punctipinnis*), brown shark (*Carcharinus plumbeus*), yellow tail (*Seriola quinqueradiata*), seabream (*Sparus auratus*) and turbot (*Scophthalmus maximus*) (Fouz *et al.* 1994). Several other marine vibrios have been shown to be opportunistic pathogens in a variety of organisms.

A great structural diversity has been observed among the various siderophores produced by *Vibrio* species (reviewed in Drechsel & Winkelmann 1997). Thus, *V. cholerae, V. vulnificus* and *V. fluvialis* produce the catecholate siderophores, vibriobactin (Griffith *et al.* 1984), vulnibactin (Okujo *et al.* 1994) and fluvibactin (Yamamoto *et al.* 1993a), while *V. parahaemolyticus* produces the carboxylate-type siderophore vibrioferrin (Yamamoto *et al.* 1992; 1993b). Anguibactin isolated from *V. anguillarum* is a mixed-type catecholate-thiazoline-hydroxamate

siderophore (Jalal *et al.* 1989), structurally related to acinetobactin (Yamamoto *et al.* 1994). There are also reports on the occurrence of the citrate-based hydroxamate aerobactin and cloning of the iutA gene for ferric aerobactin uptake in some *Vibrio* species (Haygood *et al*. 1993; Murakami *et al.* 1998, 2000).

Although other virulence factors such as haemolysin, cytolysin and hydrogen peroxide production have been implicated in fish pathogenic *Vibrio* species, the production of siderophores and their cognate uptake systems are crucial in overcoming the iron-withholding capacity of iron-binding proteins of the host (Wertheimer *et al.* 1999). The present finding of bisucaberin as a siderophore of *V. salmonicida* is further proof of the role of siderophores as virulence factors.

## Materials and methods

### *Bacterial strain and growth conditions*

*V. salmonicida* (strain NVI 1193/87, plasmid profile 21, 3.4, 2.8 MDa), isolated from an Atlantic salmon suffering from cold-water vibriosis, was pre-cultured at 15 °C on blood agar plates (Heart infusion agar base, Difco) containing 5% bovine blood and 2% NaCl. For siderophore production bacteria were grown in an iron-poor, semi-defined medium containing per litre: $Na_2HPO_4$, 6.78 g, $KH_2PO_4$, 3.0 g, NaCl 15.0 g, $NH_4Cl$, 1.0 g, $MgCl_2$ 0.017 g, casamino acids 3.0 g, and glucose 4.0 g. Initial cultures (30 ml), incubated at 6 °C with shaking (200 rpm) on an orbital shaker, were supplemented with 70 ml, 125 ml and 275 ml fresh media following 24 h, 48 h, and 72 h of incubation, respectively. The final medium (500 ml) was incubated for a further 72 h before harvest. Siderophore production was monitored daily using the Chrom-Azurol-S (CAS) universal siderophore assay (Schwyn & Neilands 1987). All glassware was treated with 6 M HCl to remove contaminating surface iron, and rinsed 20 times with de-ionized water (Purite select, Oxon, U.K.) before use.

### *Siderophore isolation and purification*

Siderophores were isolated from iron-limited cultures (8 l) of *V. salmonicida* grown at 6 °C. After removing the cells by centrifugation, desferri-siderophores were absorbed by passing the supernatant down a column (70 × 40 mm) of XAD-4 resin (Supelco, PA), at approximately 2 bed volumes $h^{-1}$. The column was then washed with de-ionized water and the desferri-siderophores eluted with approximately two bed volumes of methanol. Purification was achieved by gel-filtration on LH20 (Amersham Pharmacia, Freiburg) and Biogel P2 (BioRad, Munich). CAS-positive fractions were collected, combined, concentrated by rotary evaporation and lyophilized.

### *Preparative and analytical HPLC*

The crude siderophores were further purified by preparative HPLC (Shimadzu LC20 pumps) on a preparative reversed-phase column (Nucleosil C18, 5 $\mu$m, 8 × 250 mm) using a gradient (0–60%) of water (0.1% formic acid)/acetonitrile (0.1% formic acid) over 50 min (flow rate 3 ml min, detection 220 nm). The collected fractions were checked for purity by analytical HPLC (Shimadzu LC10 pumps) on a reversed-phase column (Nucleosil C18, 5 $\mu$m, 4 × 250 mm) using a gradient increasing from 6–60% water/acetonitrile (0.1% trifluoroacetic acid), for 20 min and isocratic at 60% for additional 5 min with detection at 220 nm. In order to obtain highly purified siderophores for mass spectrometric determination, contaminating peaks were removed by preparative HPLC on an analytical column (4 × 250 mm) and the peak-fractions of several runs collected.

### *Mass spectrometry*

Fast atom bombardment (FAB) mass spectra were recorded on a Varian MAT 711 instrument coupled to an SS 200 data system. The FAB spectra were measured from a glycerol/p-toluenesulfonic acid matrix at an ion source temperature of 323 K. High resolution electrospray ionisation Fourier transform ion cyclotron resonance mass spectrometry (ESI-FTICR-MS) (Marshall *et al.* 1998) was carried out with a passively shielded 4.7 Tesla APEX II-ESI/MALDI-FTICR mass spectrometer (Bruker Daltonik, Bremen, Germany). The mass spectrometry software XMASS version 5.0.10 (Bruker Daltonik) running on a Silicon Graphics O2 Workstation was used for data acquisition and processing. Mass calculation was performed with the standard elemental mass compilation of Audi and Wapstra (1995) .

Within the superconducting magnet of the FTICR mass spectrometer a cylindrical ‘infinity’ cell is integrated. The mass range measured was m/z 200–2000 and broadband excitation took place from m/z 150–2000. Instead of pulsed cooling gas the ‘sidekick’ cell component of the ‘infinity’ cell was used to trap

ions (Caravatti & Alleman 1991). In general 512k data points were acquired. To yield the highest mass accuracy for the unequivocal determination of the elemental formula, an internal four point calibration using the maximum number of data points (1024 k) acquirable was performed. In this way a mass resolving power of $m/\Delta m_{50\%} \approx 150{,}000$ and a mass accuracy of 0.2 ppm could be obtained.

The siderophore was dissolved in acetonitrile/water (1:1 v/v) containing 0.5% acetic acid for positive ion determinations or 1% triethylamine for negative ion determinations. For direct infusion determinations using a syringe pump, ESI (Analytica of Branford, Branford, USA) was performed with a grounded capillary sprayer needle mounted 60 ° off-axis. No supporting nebulizer gas was used for direct infusion measurements. The flow rate was 1 $\mu$l/min. The vacuum with a base pressure of $2.5 \times 10^{-10}$ mbar was provided by turbomolecular pumps (Edwards, Sussex, UK). For fragmentation studies, high resolution FTICR-MS/MS was acquired by sustained off-resonance irradiation (SORI) (Gauthier *et al.* 1991) and collision induced dissociation (CID) using argon as collision gas. Before adding the collision gas, correlated sweep isolation was used to isolate the precursor ion of the siderophore molecule at m/z 401 and eject all other ions from the analyzer cell.

*Hydrolysis and GC/MS*

Purified samples were hydrolyzed with 6 M HCl at 110 ° C for 12 h. followed by derivatization to the ethyl ester/trifluoroacetyl derivatives. These were analysed by GC-MS (HP 6890/5973-Agilent) on a fused silica Chirasil-$\gamma$-Dex capillary and with electron impact ionization as described previously (Reissbrodt *et al.* 1990).

*Biotests*

Growth promotion assays with the ferric-hydroxamate auxotroph *Microbacterium flavescens* JG9 (formerly classified as *Arthrobacter* or *Aureobacterium*) were performed according to Rabsch and Winkelmann (1991) with slight modification. From a preculture grown for 2 d in a low-iron JG9-medium, 50 $\mu$l was used to inoculate freshly prepared JG9-soft agar (0.6%). Samples (5 $\mu$l) of the isolated siderophore fractions and reference compounds (sterilized in a microwave oven for 45 s) were pipetted directly onto the agar surface. Growth zones were read after 48 h incubation at 27 °C.

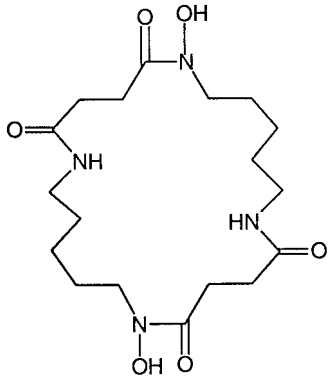


*Fig. 1.* Structure of the cyclic dihydroxamate siderophore bisucaberin (iron-free).

A corresponding biotest was established also with *Staphylococcus carnosus* TM 300 which proved suitable for the detection of a variety of siderophores, including hydroxamate, catecholate and carboxylate siderophores. Sterilized siderophore samples (5 $\mu$l) were pipetted on freshly prepared LB-soft agar (0.6%) incoculated with 10 $\mu$l of an overnight culture of *S. carnosus* TM 300 and containing 150 $\mu$M each of the iron chelators, 2,2-bipyridyl and ethylenediamine di(o-hydroxyphenylacetic acid) (EDDHA) to reduce the available iron in the medium. Growth zones were read after 12 h incubation at 37 °C. As a control, a bioassay with *Escherichia coli* AN311 (Young & Gibson 1979) was performed which is *fep*$^-$ (and also *fhuA*$^-$) but also *foxA*$^-$, unable to recognize ferrioxamine-type siderophores like other *E. coli* strains (Deiss *et al.* 1998)

## Results

Isolation of bisucaberin (Figure 1) was achieved from iron-limited cultures of *V. salmonicida* (1193/87) grown at 6 °C for several days. Crude siderophore extracts were obtained after adsorption onto XAD-4 and elution with methanol and were subsequently purified by gel-filtration and preparative HPLC on reversed-phase columns. Two HPLC peaks were isolated, one of which ($R_t$ =10.5 min) showed CAS-activity and siderophore activity in growth promotion tests while a second peak ($R_t$ = 12.5 min), had low CAS activity but good growth promotion activity. Comparison with a reference sample from our siderophore collection indicated the presence of iron-free and iron-containing bisucaberin (Figure 2).

Although retention times of bisucaberin and ferrioxamine G were identical in our HPLC system, TLC separation on silica-gel plates using dichloromethane-MeOH-water (70:30:4) clearly distinguished the two siderophores. While ferric bisucaberin remained at the start, ferrioxamine G revealed a retention value of $R_f = 0.1$.The molecular mass of the protonated iron-free compound as determined by FAB was 401 amu. High resolution FTICR-MS revealed an exact mass-to-charge ratio (m/z) of 401.23953 ($[M+H]^+$-ion), consistent with the elemental composition of $C_{18}H_{33}N_4O_6$ (theoretical m/z 401.23946) (Figure 3). The relative mass error between the theoretical and experimental m/z is thus only 0.2 ppm. The elemental formular of the neutral species is thus $C_{18}H_{32}N_4O_6$which corresponds with that of bisucaberin. SORI-CID revealed one major fragment at m/z 201 (Figure 4), indicating that the molecule is decomposed into two identical $[M+H]^+$fragments which confirm the presence of a symmetrical molecule. A reference compound of bisucaberin gave nearly the same fragment ion pattern as the isolated sample. In addition, investigations in the negative-ion mode showed no mass signals, an indication of the absence of strongly acidic functional groups.

Hydrolysis with 6 M HCl and subsequent GC-MS analysis of the trifluoroacetyl/ethyl ester derivatives revealed no amino acids but a major amine peak at 31.7 min. The mass spectra of this derivative showed fragmentation (Figure 5) consistent with a trifluoroacetylated pentanediamine. N-hydroxypentanediamine is a characteristic constituent of both bisucaberin and the ferri-oxamines. A detailed GC-MS analysis of the diaminopentane residue of ferrioxamine G has previously been published (Reissbrodt *et al.* 1990). Although the fragment ion at m/z 154 indicated a linear pentane backbone, possible isomers possessing methyl groups could be excluded by $^{13}C$ NMR spectroscopy (data not shown).

Although not tested in the producing strain, the isolated bisucaberin showed good growth promotion activity in biotests with *M. flavescens* JG9 and even more pronounced activity in biotests with *S. carnosus*, confirming its function as a siderophore (Table 1). *Staphylococcus* strains are known to accept a variety of hydroxamate siderophores (Sebulsky *et al.* 2000). Although a receptor for bisucaberin has not been reported so far, the present study is evidence that bisucaberin functions as a siderophore in *S. carnosus.* The negative result of a control bioassay with *E. coli* AN311 indicates the absence of

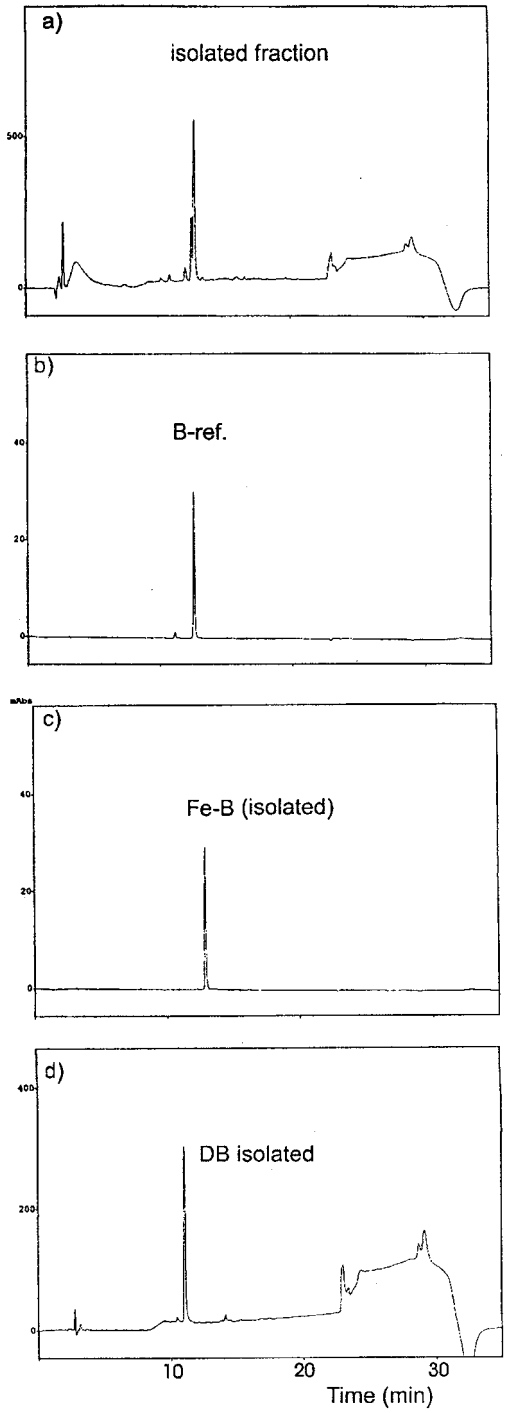


*Fig. 2.* HPLC chromatograms of a) crude siderophores after purification on Sephadex LH20 and Biogel P2 (detector wavelength 220 nm), b) reference compound of Fe-bisucaberin (435 nm), c) Fe-bisucaberin isolated by preparative HPLC (435 nm), d) isolated deferri-bisucaberin (220 nm). For HPLC conditions and gradient see *Materials and methods*.

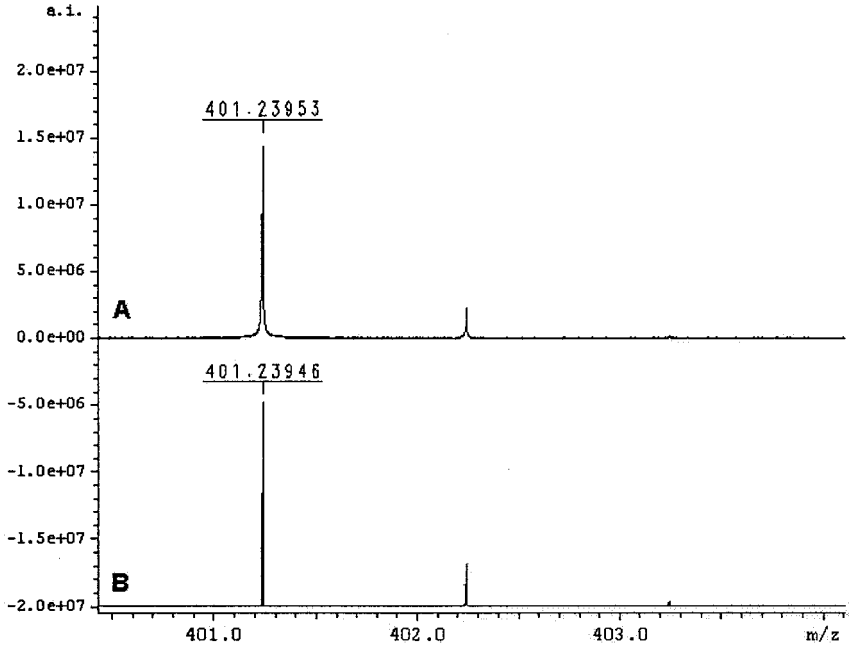


*Fig. 3.* High resolution mass spectrometry (ESI-FTICR-MS): a) Experimental mass spectrum determined from isolated deferri-bisucaberin and b) calculated spectrum from elemental composition $C_{18}H_{33}N_4O_6$ including isotope distribution.

a corresponding bisucaberin (ferrioxamine) receptor. The inability of *E. coli* to utilize bisucaberin as a siderophore corresponds well with an earlier observation of a missing ferrioxamine receptor (Deiss *et al.* 1998) as ferrioxamines and bisucaberin possess the same molecular constituents (succinic acid and N-hydroxy-diaminopentane. As the stability constant for ferric bisucaberin like many other hydroxamate siderophores (Boukhalfa & Crumbliss 2002), is higher than that of the iron chelators 2,2-bipyridyl and ethylenediamine di(o-hydroxyphenyl-acetic acid) (EDDHA) present in the assay medium, the growth promotion test is a fast and reliable bioassay for siderophore activity of bisucaberin.

*Table 1.* Growth promotion tests on ferri-bisucaberin, deferri-bisucaberin, and some other hydroxamate siderophores as reference compounds using *M. flavescens* JG9, *S. carnosus* TM300 and *E. coli* AN311 as indicator strains.

| Siderophore (1 mM) | *M. flavescens* JG9 | *S. carnosus* TM300 | *E.coli* AN311 |
|---|---|---|---|
| Fe-bisucaberin | + | + | − |
| Deferri-bisucaberin | + | + | − |
| Ferrioxamine E | + | + | − |
| Coprogen | + | + | + |
| Ferrichrome | + | + | − |

## Discussion

Cold-water vibriosis of Atlantic salmon caused by *V. salmonicida* is highly dependent on the prevailing temperature. The disease is most frequently observed around or below 10 °C (Colquhoun, et al. 2002). As shown in a previous report, siderophore production by *V. salmonicida in vitro*, as determined by the CAS assay, is also temperature dependent and highest at temperatures around 10 °C or lower (Colquhoun & Sørum 2001). In the present paper, the isolated siderophore of *V. salmonicida* is identified as bisucaberin, a symmetric cyclic dihydroxamate previously isolated from a marine bacterium *Alteromonas haloplanktis* (Takahashi *et al.* 1987). The molecular constituents of bisucaberin are identical to those of the cyclic trihydroxamates ferrioxamine E and G and similar to some other ferrioxamines (B, $D_1$) containing succinic acid and N-hydroxy-1,5-diaminopentane residues (Dhungana *et al.* 2001) which may suggest that uptake of both siderophores might require recognition of these molecular moieties in a FoxA-like receptor (Deiss *et al.* 1998).

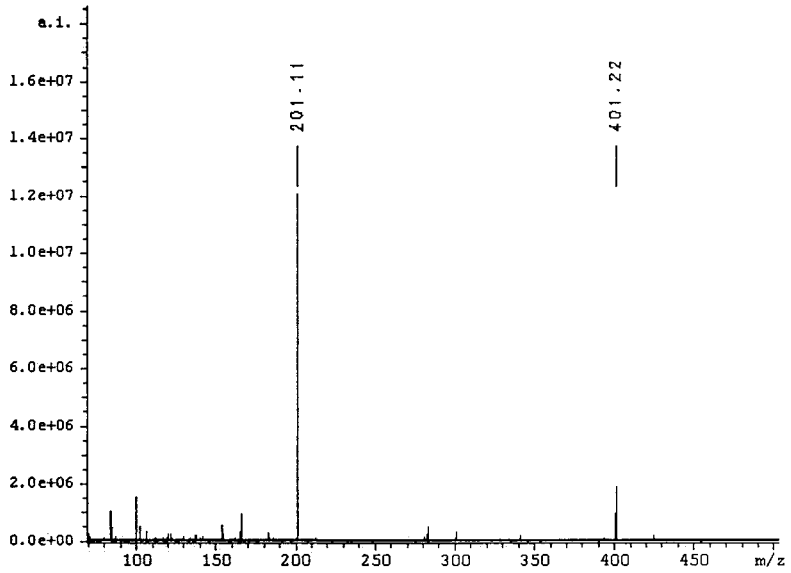


*Fig. 4.* Fragmentation of deferri-bisucaberin by FTICR-MS/MS showing a dominant fragment of m/z 201 $[M + H]^+$.

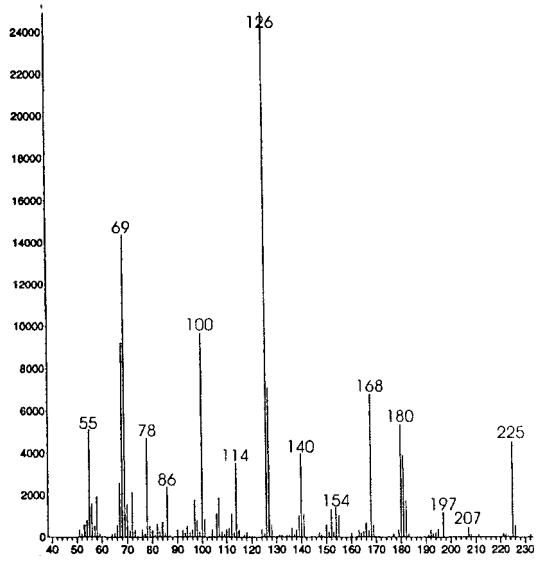


*Fig. 5.* GC-MS analysis of the trifluoroacetylated hydrolysis product (main peak, average 31.65 to 31.73 min).

A vibrio producing ferrioxamine G has recently been reported by the group of Alison Butler (Martinez *et al.* 2001). Bisucaberin is also structurally related to alcaligin, isolated from *Alcaligenes (denitrificans) xylosoxidans* (Nishio *et al.* 1988; Nishio & Ishida 1990) and from two taxonomically close pathogenic bacteria, *Bordetella pertussis* and*B. bronchiseptica*, both colonizing the upper respiratory tract of human and animals (Moore *et al.* 1995; Brickman *et al.* 1996) A reduced virulence has been reported for a *B. bronchiseptica* mutant defective in alcaligin biosynthesis after insertion of a kanamycin resistance gene into *alcA* (Register *et al.* 2001). Alcaligin, containing additional hydroxy groups located at the diamine backbone has been shown to be recognized by the FauA receptor in *Bordetella* species (Brickman & Armstrong 1999). Bisucaberin and alcaligin are highly pre-organized for metal ion chelation, leading to different binding modes depending on the prevailing pH (Hou *et al.* 1998; Spasojevic *et al.* 2001; Boukhalfa & Crumbliss 2002). Most other natural hydroxamate siderophores form simple 1:1 ligand to metal complexes, whereas bisucaberin and alcaligin show monobridged $Fe_2L_3$ complexes as derived from the crystal structure of alcaligin (Hou *et al.* 1998). The conformationally pre-organized orientation of the bidentate ligands of bisucaberin results in a higher (32-fold) complexation constant of K = 32.2 (1/2 log $\beta_{230}$), compared to the more flexible Fe-rhodotorulate ($Fe_2L_3$) complex. The high stability of the ferric bisucaberin complex ($Fe_2L_3$) even exceeds the commonly found values (log ß $\sim$ 30) of most other hydroxamate siderophores (ferrichrome, coprogen, ferrioxamines) which makes bisucaberin well suited for scavenging iron from iron-binding host proteins in salmon tissue and serum.

## Acknowledgements

We thank Thomas Neu and Sandra Frick for technical assistance.

## References

Audi G, Wapstra AH. 1995 Update to the atomic mass evaluation. *Nucl Phys A*, **595**, 409–480.

Boukhalfa H, Crumbliss AL. 2002 Chemical aspects of siderophore mediated iron transport. BioMetals (in press).

Brickman TJ, Hansel J-G, Miller MJ, Armstrong SK. 1996 Purification, spectroscopic analysis and biological activity of the macrocyclic dihydroxamate siderophore alcaligin produced by *Bordetella pertussis* and *Bordetella bronchiseptica*. *BioMetals* **9**, 191–203.

Brickman TJ, Armstrong SK. 1999 Essential role of the iron-regulated outer membrane receptor FauA in alcaligin siderophore-mediated iron uptake in Bordetella species. *J Bacteriol* **181**, 5958–5966.

Caravatti P, Allemann M. 1991 The infinity cell: a new trapped-ion cell with radiofrequency covered trapping electrodes for Fourier transform ion cyclotron resonance mass spectrometry. *Org Mass Spectrom* **26**, 514–518.

Colquhoun DJ, Sørum H. 2001 Temperature dependent siderophore production in *Vibrio salmonicida*. *Microbial pathogen* **31**, 213–219.

Colquhoun DJ, Alvheim K, Dommarsnes K, Sørum H. 2002. Relevance of incubation temperature for *Vibrio salmonicida* vaccine production. *J. Appl. Microbiol.* (in press).

Crosa JH. 1989 Genetics and molecular biology of siderophore-mediated iron transport in bacteria. *Microbiol Rev* **53**, 517–530.

Drechsel H, Winkelmann G. 1997 Iron chelation and siderophores. In: Winkelmann G and Carrano CJ, eds. *Transition Metals in Microbial Metabolism*, Harwood Academic Publishers, Amsterdam, pp. 1–49.

Deiss K, Hantke K, Winkelmann G. 1998 Molecular recognition of siderophores: A study with cloned ferrioxamine receptors (FoxA) from *Erwinia herbicola* and *Yersinia enterocolitica*. *BioMetals* **11**, 131–137.

Dhungana S, White PS, Crumbliss AL. 2001 Crystal structure of ferrioxamine B: comparative analysis and implications for molecular recognition. *J Biol Inorg Chem* **6**, 810–818.

Egidius E, Wilk R, Andersen K, Hoff KA, Hjeltnes B. 1986 *Vibrio salmonicida* sp. nov., a new fish pathogen. *Int J Syst Bacteriol* **36**, 518–520.

Fouz B, Toranzo AE, Biosca EC, Mazoy R, Amaro C. 1994 Role of iron in the pathogenicity of *Vibrio damsela* for fish and mammals. *FEMS Microbiol Lett* **21**, 181–188.

Gauthier JW, Trautman TR, Jacobson DB. 1991 Sustained off-resonance irradiation for CAD involving FTMS. *Anal Chim Acta* **246**, 211–225.

Griffith GL, Sigel S P, Payne SM, Neilands JB. 1984 Vibriobactin, a siderophore from *Vibrio cholerae*. *J Biol Chem* **259**, 383–385.

Haygood MG, Holt PD, Butler A. 1993 Aerobactin production by planktonic marine *Vibrio* sp. *Limol Oceanogr* **38**, 1091–1097.

Hou Z, Raymond KN, O'Sullivan B, Esker TW. 1998 A pre-organized siderophore: Thermodynamic and structural characterization of alcaligin and bisucaberin, microbial macrocyclic dihydroxamate chelating agents. *Inorg Chem* **37**, 6630–6637.

Jalal MAF, Hossain MB, van der Helm D, Sanders-Loehr J, Actis LA, Crosa JH. 1989 Structure of anguibactin, a unique plasmid-related bacterial siderophore from the fish pathogen *Vibrio anguillarum*. *J Am Chem Soc* **111**, 292–296.

Marshall AG, Hendrickson CL, Jackson GS. 1998 Fourier transform ion cyclotron resonance mass spectrometry: A primer. *Mass Spectrom Rev* **17**, 1–35.

Martinez JS, Haygood MG, Butler A. 2001 Identification of a natural desferrioxamine siderophore produced by a marine bacterium. *Limnol Oceanogr* **46**, 420–424.

Moore CH, Foster L-A, Gerbig DG Jr., Dyer DW, Gibson BW. 1995 Identification of alcaligin as the siderophore produced by *Bordetella pertussis* and *B. bronchiseptica*. *J Bacteriol* **177**, 1116–1118.

Murakami K, Fuse H, Takimura O, Kamimura K, Yamaoka Y. 1998 Phylogenetic analysis of marine environmental strains of Vibrio that produce aerobactin. *J Mar Biotechnol* **6**, 76–79.

Nishio T, Tanaka N, Hiratake J, Katsube Y, Ishida Y, Oda J. 1988 Isolation and structure of the novel dihydroxamate siderophore alcaligin. *J Chem Soc* **110**, 8733–8734.

Nishio T, Ishida Y. 1990 Production of dihydroxamate siderophore alcaligin by *Alcaligenes denitrificans* subsp. *xylosoxydans*. *Agric Biol Chem* **54**, 1837–1839.

Okujo N, Saito M, Yamamoto S, Yoshida T, Miyoshi S, Shinoda S. 1994 Structure of vulnibactin, a new polyamine-containing siderophore from *Vibrio vulnificus*. *BioMetals* **7**, 109–116.

Rabsch W, Winkelmann G. 1991 The specificity of bacterial siderophore receptors probed by bioassays. *BioMetals* **4**, 244–250.

Register KB, Ducey TF, Brockmeier SL, Dyer DW. 2001 Reduced virulence of a Bordetella *bronchiseptica* siderophore mutant in neonatal swine. Infect Immun **69**, 2137–2143.

Reissbrodt R, Rabsch W, Chapeaurouge A, Jung G, Winkelmann G. 1990 Isolation and identification of ferrioxamine G and E in *Hafnia alvei*. *BioMetals* **3**, 54–60.

Schwyn B & Neilands JB 1987 Universal chemical assay for the detection and determination of siderophores. *Anal Biochem* **160**, 47–56.

Sebulsky MT, Hohnstein D, Hunter MD, Heinrichs DE. 2000 Identification and characterization of membrane permease involved in iron-hydroxamate transport in *Staphylococcus aureus*. *J Bacteriol* **182**, 4394–4400.

Spasojevic I, Boukhalfa H, Stevens RD, Crumbliss AL. 2001 Aqueous solution speciation of Fe(III) complexes with dihydroxamate siderophores alcaligin and rhodotorulic acid and synthetic analogues using electropsray ionization mass spectrometry. *Inorg Chem* **40**, 49–58.

Takahashi A, Nakamura H, Kameyama T, Kurasawa S, Naganawa H, Okami Y, Takeuchi S, Umezawa H. 1987 Bisucaberin, a new siderophore, sensitizing tumor cells to macrophage mediated cytolysis. II Physico-chemical properties and structure determination. *J Antibiotics* **40**, 1671–1676.

Wertheimer AM, Verweij W, Chen Q, Crosa LM, Nagasawa M, Tolmasky ME, Actis LA, Crosa JH. 1999 Characterization of the angR gene of *Vibrio anguillarum*: Essential role in virulence. *Infect Immun* **67**, 6496–6509.

Wolf MK, Crosa JH. 1986 Evidence for a role of a siderophore in promoting *Vibrio anguillarum* infection. *J Gen Microbiol* **132**, 2949–2952.

Yamamoto S, Fujita Y, Okujo N, Matsuura S, Shinoda S. 1992 Isolation and partial characterization of a compound with siderophore activity from *V. parahaemolyticus*. *FEMS Microbiol Lett* **94**, 181–186.

Yamamoto S, Okujo N, Fujita Y, Saito M, Yoshida T, Shinoda, S. 1993a Structures of two polyamine containing catecholate siderophores from *Vibrio fluvialis*. *J Biochem* **113**, 538–544.

Yamamoto S, Okujo N, Yoshida T, Matsuura S, Shinoda, S. 1993b Structure and iron transport activity of vibrioferrin, a new siderophore of *Vibrio parahaemolyticus*. *J Biochem* **115**, 868–874.

Yamamoto S, Okuio N, Sakakibara Y. 1994 Isolation and structure elucidation of acinetobactin, a novel siderophore from *Acinetobacter baumannii*. *Arch Microbiol* **162**, 249–254.

Young IG, Gibson F. 1979 Isolation of enterochelin from *Escherichia coli*. *Methods Enzymol* **56**, 394–398.