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Structure of vulnibactin, a new polyamine-containing siderophore from *Vibrio vulnificus*

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A new siderophore named vulnibactin has been isolated from low iron cultures of *Vibrio vulnificus*, a human pathogen. The structure was established as *N*-[3-(2,3-dihydroxybenzamido)propyl]-1,3-bis[2-(2-hydroxyphenyl)-*trans*-5-methyl-2-oxazoline-4-carboxamido]propane by a combination of acid hydrolysis, nuclear magnetic resonance spectroscopy and positive fast atom bombardment mass spectrometry. Vulnibactin is characterized as containing one residue of 2,3-dihydroxybenzoic acid as well as two residues of salicylic acid, both of which are involved in the formation of oxazoline rings with L-threonine bound to a norspermidine backbone. In addition, two other compounds with siderophore activity were purified and their structures were also determined. These two compounds provided further support for the structure of vulnibactin.

Keywords: iron transport, siderophore, *Vibrio vulnificus*, vulnibactin

Introduction

Bacteria, like most other organisms, require iron for cellular functions (Griffiths 1987). However, in mammals, most of the iron is either stored intracellularly or bound to proteins like transferrin and lactoferrin (Weinberg 1978, Griffiths 1987, Payne 1988). In addition, simple ferric salts are hydrolyzed at physiological pH in aerobic environments to form extremely insoluble oxyhydroxide polymers, thus limiting the availability of free iron to levels below that necessary to support bacterial growth (Neilands 1981). To surmount this nutritional restriction, many bacteria have elaborated high-affinity iron acquisition mechanisms. One such method comprises of low molecular weight, highly specific iron chelators termed siderophores and the cognate membrane receptors (Neilands 1982, Bagg & Neilands 1987, Braun & Winkelmann 1987, Silver & Walderhaug 1992). A correlation between siderophore production and bacterial virulence has been found in some pathogens (Griffiths 1988, Payne 1988, Martínez *et al.* 1990).

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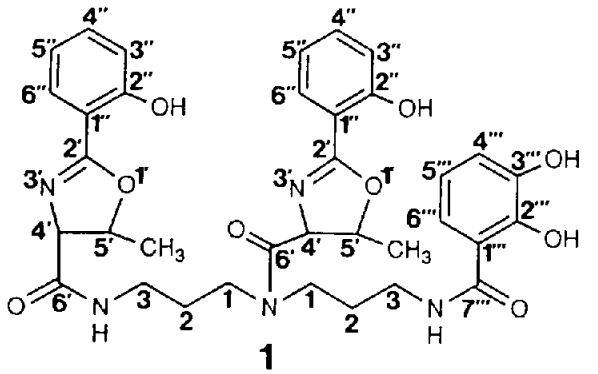
Vibrio vulnificus is an opportunistic marine pathogen capable of causing lethal septicemia or wound infections in humans (Blake *et al.* 1979, Tacket *et al.* 1984, Janda *et al.* 1988). It has been shown that ingestion of *V. vulnificus* resulting in septicemia is highly lethal, but the mortality rate after wound infections is not great (Blake *et al.* 1979). Many cases of these infections have been correlated with a pre-existing factor such as hepatic dysfunctions or hemochromatosis leading to elevated serum iron levels (Chart & Griffiths 1985, Brennt *et al.* 1991, Bullen *et al.* 1991). An *in vivo* study by Wright *et al.* (1981) directly correlated the pathogenicity of *V. vulnificus* with the availability of iron. Despite the long-standing recognition of putative siderophores of both catecholate (phenolate) and hydroxamate types in this bacterium (Andrus *et al.* 1983, Simpson & Oliver 1983, Wright *et al.* 1986), their structures have not been fully characterized.

In the present study, the structures of a catecholate-type siderophore, named vulnibactin (**1**), and related compounds (**2** and **3**) isolated from culture supernatants of *V. vulnificus* M-2799 grown in a low-iron medium were determined, as shown in Figure 1, by a combination of acid hydrolysis, nuclear magnetic resonance (NMR) and fast atom



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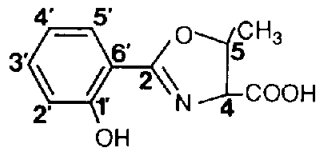

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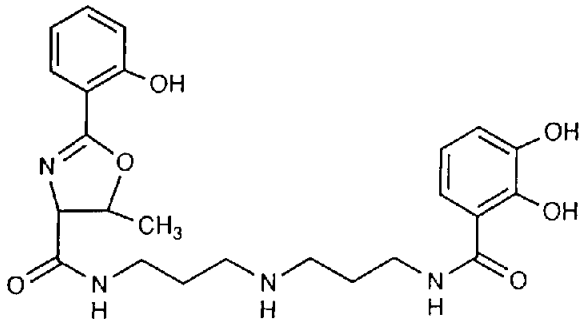

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Figure 1. Proposed structures of vulnibactin (**1**), **2** and **3** isolated from *V. vunificus* M-2799. The order of the numbering is adopted according to Griffiths *et al.* (1984).

ment mass spectrometry (FAB-MS). Vulnibactin is distinct from known siderophores in that it contains two salicyloyl residues, each of which forms an oxazoline ring with L-threonine.

Materials and methods

Chemicals and reagents

2,3-Dihydroxybenzoic acid (DHBA) and ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (EDDA) were obtained from Sigma (St Louis, MO); norspermidine (NSPD) was from Aldrich (Milwaukee, WI); Amberlite XAD-7 resin was from Nacalai Tesque (Kyoto, Japan) and purified as described by Actis *et al.* (1986). All other reagents were of commercially available analytical grade.

Bacterial strain and culture conditions

V. vulnificus M-2799, a clinical isolate, was used for siderophore production which showed a strongly positive result in the Chrome Azurol S test of Schwyn & Neilands (1987). Cultures were grown in a modified Tris-buffered medium (T medium) (Yamamoto *et al.* 1993) containing $FeCl_3$ at a final concentration of 0.15 μM. Routinely, each 1 l of the medium was inoculated with 20 ml of the preculture which had been grown to the mid-log phase and shaken vigorously on a rotary shaker at 37 °C for 28 h in a dark room, after which the cells were removed by centrifugation to obtain the culture supernatant. To minimize iron contamination, all glassware was washed with 6 M HCl, and reagents and media were prepared with glass-distilled water. In experiments to find an optimal iron concentration for siderophore production, strain M-2799 was cultured in T medium containing varying amounts of added $FeCl_3$ ranging from 0 to 10 μM.

Thin layer chromatography (TLC)

Analytical and preparative TLC was carried out in a dark room on precoated plates of silica gel 60 (thickness 0.2 mm) or cellulose (thickness 0.1 mm) (Merck, Darmstadt, Germany) in one of the following solvent systems (v/v): (A) chloroform:methanol, 4:1; (B) benzene:acetic acid:water, 125:72:3; (C) toluene:benzene:acetic acid, 2:2:1; (D) *iso*-propyl alcohol:acetic acid:water, 16:8:1. After development, the plates were examined under long wave UV light followed by spraying with 1% $FeCl_3$ in 0.05 M HCl or 0.25% ninhydrin in acetone. DHBA and salicylic acid (SA) were used as reference standards.

Plate bioassay for siderophore activity

The procedure was described in the previous paper (Yamamoto *et al.* 1993). Agar plates containing EDDA at 100 μg ml^{-1} were used for all strains tested.

Purification of vulnibactin and related compounds

During purification, siderophore activity in TLC band fractions was checked by the plate bioassay. The culture supernatants were subjected to batchwise adsorption onto XAD-7 resin followed by elution with methanol as described earlier for fluvibactin, a catecholate-type siderophore of *V. fluvialis* (Yamamoto *et al.* 1993). The combined methanolic eluate from 4 l of the supernatant was taken to dryness at 30 °C. The residue was dissolved in 100 ml of water, adjusted to pH 2 with 60% (w/v) citric acid and immediately extracted twice with 200 ml of ethyl acetate. The dried ethyl acetate layer was evaporated to dryness at 30 °C *in vacuo* in an atmosphere of N_2 to give a crude vulnibactin fraction (fraction A). The aqueous layer after extraction with ethyl acetate was again treated with XAD-7 and the adsorbed materials (not extracted with ethyl acetate) were eluted with methanol to obtain another extract (fraction B). Both fractions A and B were active in the plate bioassay for siderophores. These fractions were stored under N_2 at −20 °C until further purification.

To each of pooled fractions A and B prepared from 40 l of culture supernatant was added 5 ml of methanol and the insoluble materials were removed by centrifugation. The

resulting solutions were subjected to preparative TLC to purify active compounds. The fluorescent blue bands visible under UV light were scraped from the plates and eluted with appropriate solvents described below.

The first TLC of fraction A on silica gel with Solvent A revealed two active bands, which were colored by spraying with 1% (w/v) $FeCl_3$ in 0.01 M HCl. The band of R_f 0.71 (dark red purple) was further chromatographed on the same matrix with Solvent B (R_f 0.48) followed by Solvent A. In each case, the band was extracted with chloroform: benzene:methanol (16:4:1, v/v). The residue from the final TLC was dissolved in acetone, from which a white precipitate, vulnibactin (**1**), was obtained by addition of *n*-hexane. The yield was 21 mg. The second active band with an R_f of 0.18 (red purple with 1% $FeCl_3$ and fading with time) was separated by subsequent TLC on silica gel with Solvent C into two bands, which were extracted with ethanol. One compound (R_f 0.48) with a yield of less than 1 mg was identified as SA by gas chromatography (GC)-MS after derivatization to its methyl ester and the other (R_f 0.13) (**2**) was obtained with a yield of 11 mg. The latter compound showed siderophore activity. Cellulose TLC of fraction B with Solvent D gave an active band (R_f 0.65, dark red purple with 1% $FeCl_3$), and this was extracted with methanol and further purified with Solvent C on the same matrix. Finally, an oily material (R_f 0.37) (**3**) was obtained with a yield of about 1 mg.

Analysis of hydrolytic products

Three kinds of purified compounds with siderophore activity were hydrolyzed in 6 M HCl under N_2 in sealed tubes at 110 °C for 72 h. The hydrolyzates were processed and analyzed by the gas liquid chromatography (GLC) and GC-MS methods as described previously (Yamamoto *et al.* 1993). SA was analyzed as its methyl ester after derivatization with ethereal diazomethane. The amounts of NSPD, threonine, SA and DHBA determined by GLC were corrected for each recovery rate of the corresponding references which were heated under the same conditions as described above. SA and DHBA were separated by TLC on a cellulose plate with Solvent C (R_f for SA, 0.72; R_f for DHBA, 0.30). The configuration of threonine was determined by HPLC using a chiral column of Crownpack CR (+) (Daicel Chemical, Osaka, Japan) according to a supplier's manual.

Spectroscopy

Absorption spectra in the visible and UV ranges were obtained with a Shimadzu UV-260 spectrophotometer. NMR spectra were recorded in CD_3OD at ambient temperature on a Varian VXR 500 spectrometer (500 MHz for ^{1}H-NMR and 125.7 MHz for ^{13}C-NMR). The chemical shifts are given in δ values (p.p.m.) relative to tetramethyl silane and the coupling constants are expressed as J values in Hz. Varian's standard programs were used for J-resolution 2D spectrum, ^{1}H–^{1}H shift correlation spectroscopy (COSY), ^{1}H–C COSY and heteronuclear multiple bond coherence (HMBC). The ^{1}H–^{13}C COSY and HMBC spectra were obtained with average J_{CH} values of 140 and 7 Hz for overnight runs, respectively. GC-MS and FAB-MS (nominal and accurate mass) were performed with a VG Analytical 70SE mass spectrometer. Positive FAB-MS spectra were measured with a glycerol matrix at 8 kV (accelerating voltage). For GC-MS (electron impact ionization mode, 70 eV), a Quadrex bonded-fused silica capillary column coated with OV-1 (25 m × 0.25 mm i.d.) was used.

Results

Optimal iron concentration in the medium for the production of vulnibactin was examined by measuring NSPD in the acid hydrolyzates of crude siderophore fractions (corresponding to fraction A) obtained at various added iron concentrations. The maximum quantity of vulnibactin (about 1 μmol l^{-1}) was obtained at 0.15 μM added iron. Complete repression of vulnibactin synthesis was observed at 10 μM added iron and the yield was greatly reduced at 1 μM added iron.

Vulnibactin, as well as **2** and **3**, did not react with ninhydrin, suggesting the absence of a primary amine group. Purified vulnibactin decomposed at 93–97 °C without melting to a clear liquid and the specific rotation was $[\alpha]^{20}_D$ + 101.6° (*c*. 1.28, methanol). The absorption spectrum in methanol showed a broad peak at 307 nm with ε_{mM} = 9.8, a minimum at 274.5 nm and a sharper peak at 248 with ε_{mM} 21.3 (Figure 2A). The complex of vulnibactin with ferric ion (ferric vulnibactin) prepared by mixing vulnibactin in methanol with equimolar $FeCl_3$ displayed a broad absorption band at 523.5 nm (Figure 2B). The complex was more soluble in water than the iron-free form and could be stored dry for months with no deterioration. Mild acid hydrolysis (0.1 M HCl in 90% ethanol for 3 h at room temperature) completely converted **2** to a compound which was not extractable with ethyl acetate, implying hydrolysis of the oxazoline ring to the open form.

The components released from vulnibactin, **2** and **3** upon hydrolysis were identified by GC-MS (not shown) and their molar ratios were determined by GLC performed with appropriate controls. Vulnibactin yielded NSPD, DHBA, threonine and SA, and the molar ratio was calculated to be approximately 1:1:2:2. Threonine was determined to have an L-configuration. On the other hand, **2** was hydrolyzed to release threonine and SA at a molar ratio of 1:1. When **2** was analyzed by GC-MS after treatment with ethereal diazomethane, prominent ion peaks at m/z 235 $[M]^+$ and 176 $[M-COOCH_3]^+$ were detected for a main product peak, indicative of

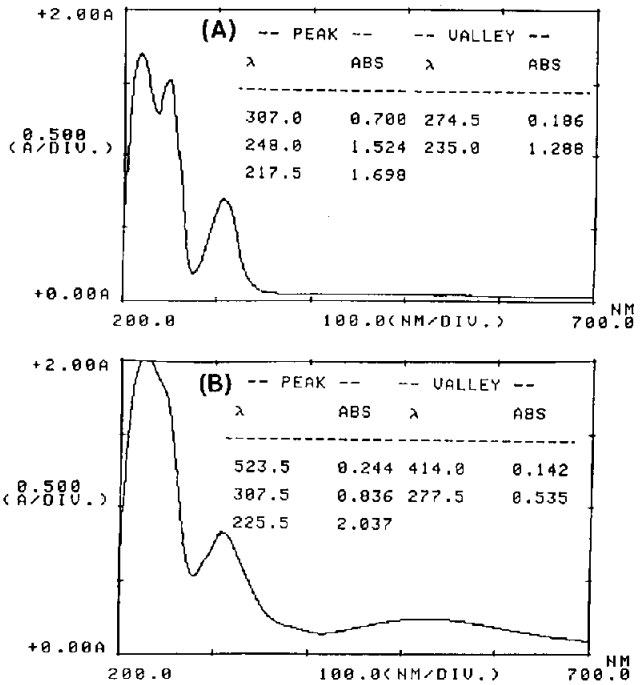

Figure 2. Absorption spectra of vulnibactin (**A**) and its ferric complex (**B**) in methanol (72 μM).

the formation of a monomethyl ester derivative with an oxazoline ring. Compound **3** provided NSPD, DHBA, threonine and SA at a molar ratio of 1:0.8:1.1:0.9.

The NMR spectra of vulnibactin and **2** were measured to determine the connectivity of the components identified in their hydrolyzates. The ^{1}H-NMR spectrum of vulnibactin is shown in Figure 3. It is notable that all signals in the spectrum exist 'in duplicate'. This might be attributed to the siderophore existing as a mixture of conformational isomers, as reported for vibriobactin from *V. cholerae*, which contains two oxazoline rings on the NSPD backbone (Griffiths *et al.* 1984). Two multiple peaks at δ 1.87 and 2.05 integrated to four protons were assigned to the two internal methylene groups of the NSPD backbone, while the four external methylene groups adjacent to the amides were responsible for the eight proton signals observed from δ 3.17 to 3.92. The duplicated peaks centered at δ 1.45 (3H, 2d, $J = 6.5$), 4.81 (1H, 2d, $J = 6.5$) and 5.29 (1H, 2 quintet, $J = 6.5$) were correlated with each other in the ^{1}H–^{1}H COSY spectrum, and assigned to C-5′-methyl, H-4′ and H-5′ protons, respectively, on the oxazoline ring attached to the central nitrogen (N-5) of NSPD. Three similar peaks centered at δ 1.55 (3H, 2d, $J = 6.5$), 4.47 (1H, 2d, $J = 7.0$) and 4.91 (2 quintet, $J = 7.0$, the integration was disturbed with the DOH signal) were assigned to the resonances due to the presence of the second oxazoline ring attached to the external nitrogen (N-1) of NSPD. In the HMBC spectrum, the peaks at δ 4.47 and 4.81 were correlated with the peaks at δ 20.40 and 19.22, respectively, due to 5′-methyl carbons by a long-range coupling. The chemical shifts of two sets of these three peaks described above showed a close similarity to those of the protons on each of the oxazoline rings (N-1 and N-5) in vibriobactin. The coupling constants between H-4′ and H-5′ in vulnibactin were 7.0 Hz for the central and 6.5 Hz for the external oxazoline rings. Since these values were similar to those reported for vibriobactin ($J = 6.9$ and 7.2), we have concluded that both oxazoline rings of vulnibactin are also *trans*. The aromatic proton signals were observed between δ 6.66 and 7.70 and integrate to approximately 11 protons, suggesting that there may be two SA and one DHBA moiety in the molecule. This was confirmed by the *J*-resolution 2D spectrum (not shown). Eight doublets and eight triplets due to two SA moieties, and four doublets and two triplets due to one DHBA moiety were definitely detected. Their assignments were further supported by the HMBC spectrum (*vide infra*).

The ^{13}C NMR spectrum of vulnibactin is shown in

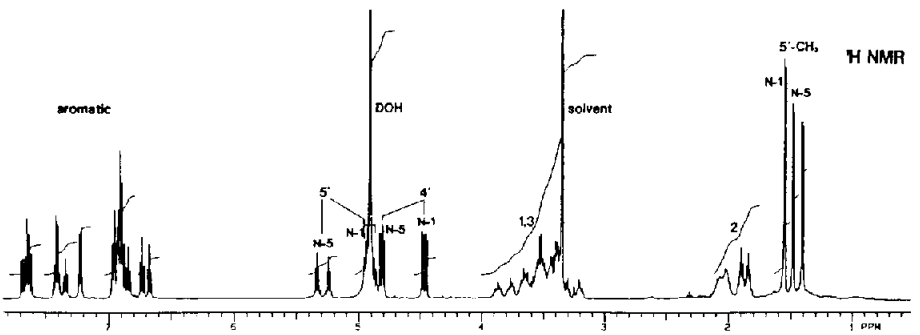

Figure 3. ^{1}H-NMR spectrum of vulnibactin in CD_3OD (20 mg/ml). N-1 and N-5 represent the external and central nitrogens of NSPD, respectively.

Figure 4. Most of the signals are split by the presence of two conformers. The peaks responsible for the carbons on the NSPD backbone and the oxazoline rings except for C-2′ were readily assigned as shown in Figure 4. In the $^{1}H-^{13}C$ COSY spectrum, the peaks at δ 72.09 and 74.86 (C-4′) were correlated with H-4′ (N-5) and H-4′ (N-1), respectively, while the peaks at δ 78.62 and 79.57 (C-5′) were correlated with H-5′ (N-5) and H-5′ (N-1), respectively. The signals at δ 166.35 and 166.92 were assigned to C-2′ based on the lack of correlation with any proton. The downfield portion containing the aromatic and carbonyl carbons was further examined with the $^{1}H-^{13}C$ COSY and HMBC spectra. The HMBC spectrum (Figure 5) allowed us to assign the aromatic carbons as well as the aromatic protons. Formation of two oxazoline rings between threonine and SA was supported by the following: (i) the long-range couplings of C-2′ with H-6″ (Figure 5) and H-4′, (ii) the long-range coupling of C-6′ with H-4′ and H-5′, and (iii) the presence of three carbonyl carbons, which were verified by the lack of correlation with any proton in the $^{1}H-^{13}C$ COSY spectrum. The long-range coupling was also observed between C-7‴ and H-6‴, indicating a direct attachment of the DHBA moiety to one of the NSPD nitrogens. However, we were unable to definitely determine which of the nitrogens in NSPD was linked with DHBA until **3** was isolated.

In the ^{1}H-NMR spectrum of **2** (Figure 6), the

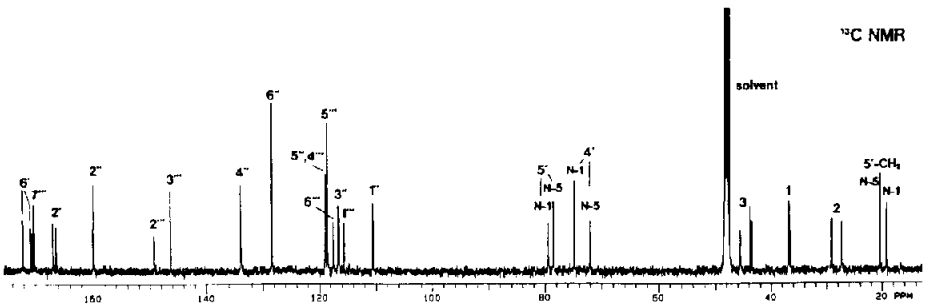

Figure 4. ^{13}C-NMR spectrum of vulnibactin in CD_3OD (20 mg/ml). N-1 and N-5 represent the external and central nitrogens of NSPD, respectively.

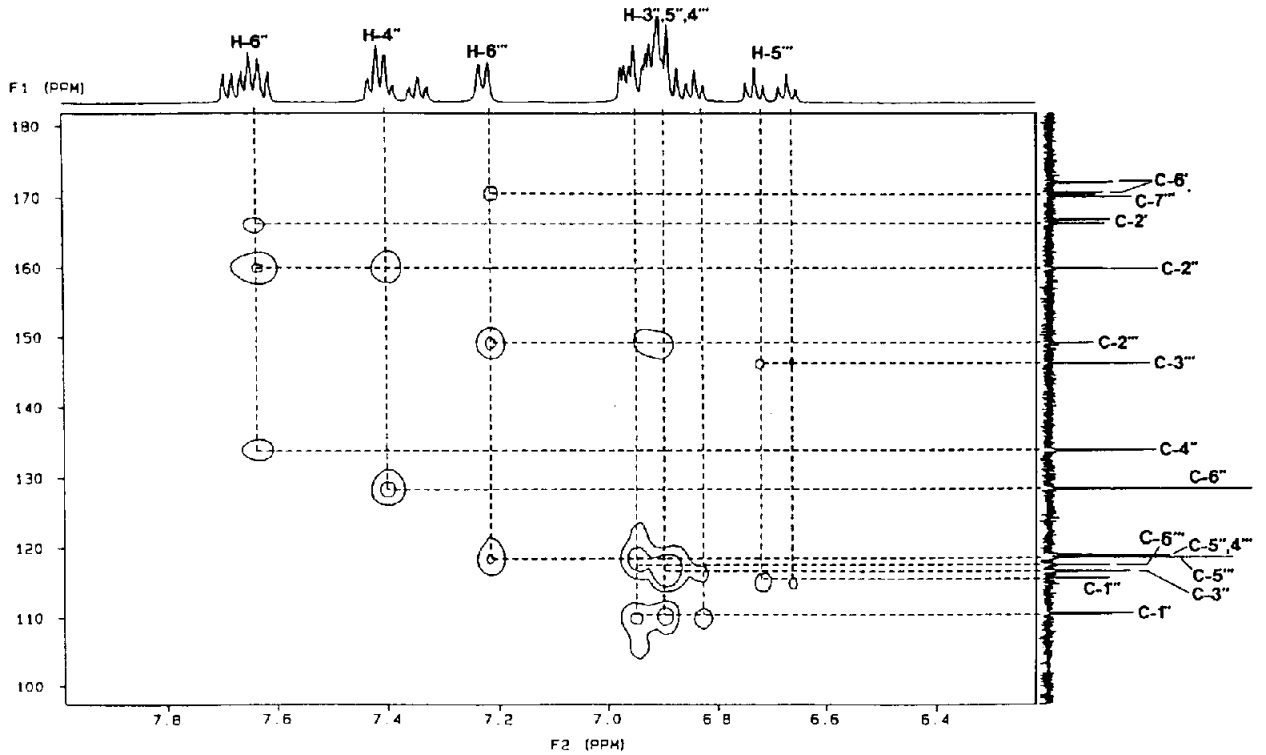

Figure 5. HMBC spectrum of vulnibactin (δ_H 6.4–7.8).

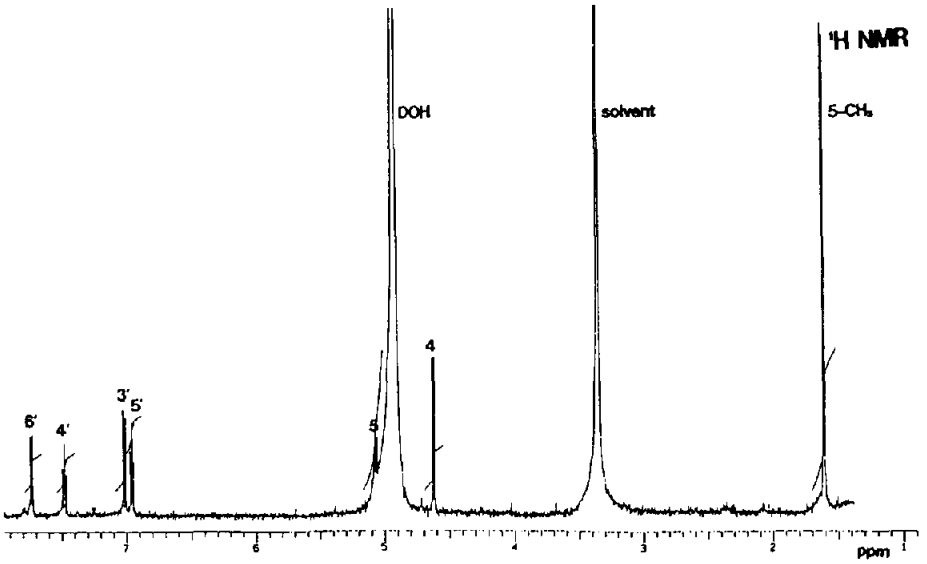

Figure 6. ^{1}H-NMR spectrum of **2** in CD_3OD (5 mg/ml).

aromatic protons appeared at δ 6.96 (td, $J = 8.0$, H-5'), 7.01 (dd, $J = 8.0$, H-3'), 7.48 (td, $J = 8.0$, H-4') and 7.73 (dd, $J = 8.0$, H-6'). The splitting pattern of these protons supported the presence of a salicyloyl moiety. The doublet at δ 4.62 ($J = 7.5$) and the quintet at δ 5.07 ($J = 7.5$) were assigned to the H-4 and H-5, respectively. The methyl protons at C-5 showed a doublet at δ 1.61 ($J = 7.5$). The coupling constant between H-4 and H-5 indicated the presence of a *trans* oxazoline ring.

These NMR data were consistent with the structures **1** and **2** proposed for vulnibactin and compound **2** (see Figure 1).

Positive FAB mass spectrum of vulnibactin gave a molecular ion peak $[M + H]^+$ at m/z 674, indicative of a molecular mass of 673 Da (Figure 7A). The accurate mass of the $[M + H]^+$ ion was determined by high-resolution MS to be 674.2852 (calculated for $C_{35}H_{40}O_9N_5$, 674.2826). FAB-MS of **2** indicated its molecular mass to be 221 Da [m/z 222 for $M + H]^+$. High-resolution MS gave the molecular formula of **2**: m/z 222.0724 (calculated for $C_{11}H_{12}O_4N$, 222.0766). These data were in agreement with the molecular formulas of vulnibactin and **2** deduced from the NMR analyses. FAB-MS of **3** revealed a prominent ion peak at m/z 471 (Figure 7B) corresponding to the $[M + H]^+$ ion, which was substantiated by a remarkable increase in intensity of the peak at m/z 493 $[M + Na]^+$ upon addition of NaCl to the glycerol matrix. The molecular mass was thus 18 mass units lower than that calculated based on its components, indicative of the presence of an oxazoline ring. The exact mass number (m/z 471.2200) determined by high resolution MS supported the molecular formula, $C_{22}H_{31}O_6N_4$; 471.2244. These data along with no reaction with ninhydrin indicate that **3** lacks a substituent on the central nitrogen of NSPD.

When ferric vulnibactin was subjected to FAB-MS, new intense peaks at m/z 727 $[M + H + Fe-3H]^+$ and 728 $[M + H + Fe-2H]^+$ in addition to the previously observed ion at m/z 674 were obtained. No other peaks containing chloride atoms or with higher mass numbers were detected. These results suggested that vulnibactin would make a 1:1 siderophore–iron complex.

The abilities of vulnibactin, **2** and **3** to reverse growth inhibition of the producer strain imposed by EDDA were compared. When applied on disks ranging from 50 to 200 nmol, vulnibactin was, unexpectedly, less potent than **2** and **3**, e.g. growth halo diameter at 100 nmol measured after 12 h incubation at 37 °C was 15 mm for vulnibactin and 22 mm for **2** and **3**. This may be accounted for by the fact that vulnibactin is less soluble in water than the others. Moreover, vulnibactin was equally effective for *V. cholerae* Non O1 NCTC 8042 in alleviating iron deficiency, but not at all for *V. fluvialis* AQ 0012.

Discussion

In this study, we have purified and structurally characterized vulnibactin and two related compounds from the culture supernatants of *V. vulnificus*. Batchwise adsorption by means of XAD-7 and subsequent TLC purification according to the biological activity facilitated the isolation of these compounds present in relatively small amounts. Although measurement of the NMR spectrum of **3**

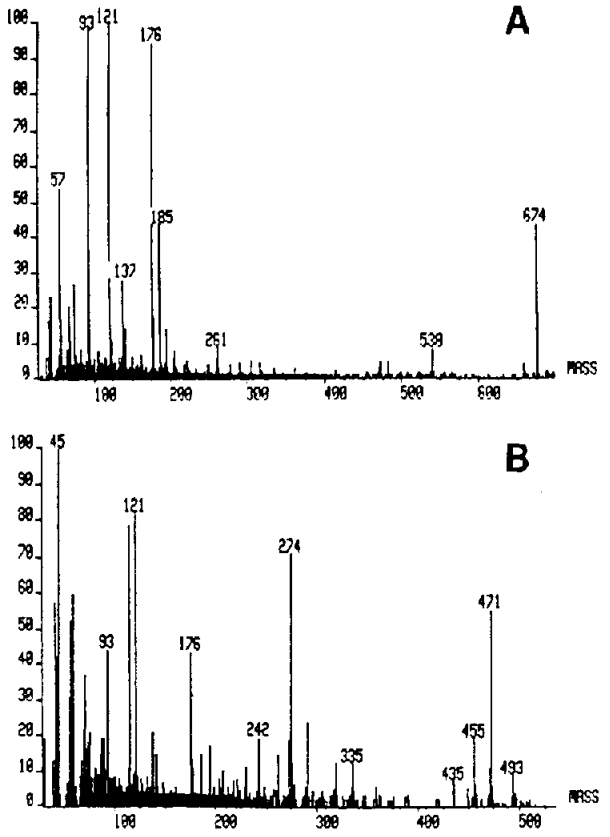

Figure 7. Positive FAB-MS spectra of vulnibactin (**A**) and **3** (**B**).

was hampered by insufficient quantity, its structure deduced from other data supported the contention that the central nitrogen of NSPD in vulnibactin is not substituted with the DHBA moiety. The isolation of **2** provided further evidence that SA and threonine in vulnibactin are also linked to form the oxazoline rings.

The FAB-MS spectrum of ferric vulnibactin suggests that vulnibactin may be a hexadentate compound which can chelate with one ferric ion. However, which of its ligand groups vulnibactin uses in chelating iron is uncertain. Moreover, the synthesis of vulnibactin was tightly regulated by the availability of iron in the growth medium, being totally repressed at 10 μM added iron. These results indicate that the biological role of vulnibactin is in fact to transport iron, i.e. to function as a siderophore.

The salicyloyl moieties have been found in siderophores such as mycobactins (Snow 1970), parabactin (Tait 1975, Person & Neilands 1979), pyochelin (Cox *et al.* 1981) and maduraferrin (Keller-Schierlein *et al.* 1988); however, these contain only one salicyloyl moiety. Vulnibactin might be the first example that contains two SA moieties. Like vibriobactin and fluvibactin isolated from *V. cholerae* and *V. fluvialis*, respectively, vulnibactin also contains NSPD as a triamine backbone. This is consistent with the finding that many *Vibrio* species contain NSPD in its free form as a major polyamine (Yamamoto *et al.* 1991). Moreover, vulnibactin can be structurally related to vibriobactin, from which it differs only in its salicyloyl moieties on two oxazoline rings, while fluvibactin contains only one oxazoline ring consisting of L-threonine and DHBA attached to the central nitrogen of NSPD. Therefore, the structural resemblance of vulnibactin to vibriobactin may explain its cross-utilization by *V. cholerae*. Interestingly, **2** and **3**, presumably the precursors of vulnibactin synthesis, were also biologically active, as shown by their abilities to restore growth inhibition of the producer strain imposed by EDDA, although their iron-binding properties and transport systems remains to be determined. DHBA derivatives substituted with an amino acid including threonine have been reported to be functioning as siderophores in certain microorganisms (reviewed in Persmark *et al.* 1989). However, an SA derivative conjugated with an amino acid, having siderophore activity, is, to our knowledge, unprecedented.

Simpson & Oliver (1987) reported that the virulent strains of *V. vulnificus* were able to compete more efficiently with transferrin, when highly saturated with iron, to acquire iron for growth than were the avirulent ones. Similarly, Brennt *et al.* (1991) showed the clinical correlation of the increase in transferrin iron saturation with susceptibility to overwhelming infection with *V. vulnificus*. In this context, it is of interest to note that the strain used for siderophore purification in this study was able to grow in the presence of human transferrin (100% iron-saturated) as a sole source of iron (Nishina *et al.* 1992). In addition, a putative hydroxamate compound(s) was detected in only a trace amount when the iron-deficient culture supernatant was assayed with an appropriate control by a modification of the Csáky method (Andrus *et al.* 1983). These observations may at least emphasize a functional role of the catecholate-type siderophores isolated in this study, although virulence of this bacterium cannot be fully attributed to transferrin utilization. Accordingly, further investigations on the ability of each of these siderophores to acquire iron from host iron sources as well as their distribution in other *V. vulnificus* strains, when correlated to the virulence, would elucidate their relevance to the pathogenesis of this invading pathogen. Moreover, it would be of interest to clarify whether two apparently iron-regulated membrane proteins reported for this species

(Chart & Griffiths 1985, Wright *et al.* 1986) are implicated as receptors for the iron complexes of these chelators.

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