

# Anachelin, the Siderophore of the Cyanobacterium *Anabaena cylindrica* CCAP 1403/2A<sup>#</sup>

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*Anabaena cylindrica*, Anachelin, Siderophore

A catecholate siderophore – anachelin – has been isolated from the cyanobacterium *Anabaena cylindrica* CCAP 1403/2A. The central part of the siderophore is a tripeptide consisting of L-Thr, D-Ser and L-Ser. Its C-terminus is linked amidically to a 1,1-dimethyl-3-amino-1,2,3,4-tetrahydro-7,8-dihydroxyquinolinium system and its N-terminus to 6-amino-3,5,7-trihydroxyheptanoic acid. The 7-hydroxyl group of the latter is esterified with salicylic acid whose carboxyl group is condensed with the 6-amino group to an oxazoline ring. Anachelin is the first genuine siderophore of a cyanobacterium whose structure has been elucidated.

## Introduction

Cyanobacteria were the first organisms to perform oxygenic photosynthesis and the oxygen they released was probably responsible for changing the Earth's originally reductive atmosphere into an oxidative one. This had a detrimental side effect: iron, necessary for many enzymatic processes and originally readily available in the form of soluble Fe<sup>2+</sup> salts, became transformed in scarcely soluble Fe<sup>3+</sup> oxide hydrates. To cope with this problem many organisms release iron chelating

compounds, called siderophores, which make iron available more or less specifically to the producing species. Cyanobacteria produce catecholate as well as hydroxamate siderophores as was shown by colour reactions (Wilhelm and Trick, 1994; Wilhelm *et al.*, 1996; Wilhelm *et al.*, 1998), but nothing is known about their structures with the exception of the citrate-hydroxamate, schizokinen (Simpson and Neilands, 1976). Interestingly, schizokinen is also produced by other types of bacteria (Mullis *et al.*, 1971; Budzikiewicz *et al.*, 1997), which suggests that the ability to produce this substance is either inherited from a common ancestor or has been acquired, by convergent evolution or gene transfer.

Fogg (1942) demonstrated that the nitrogen-fixing cyanobacterium *Anabaena cylindrica* Lemm. released nitrogenous substances into its culture medium. Much of the extracellular nitrogen was in the form of peptidic substances, more of which were produced when iron was deficient in the culture medium (Fogg, 1952). Whitton (1965) obtained an extracellular fraction of high molecular mass containing peptides and a brown pigment. Walsby (1974a) isolated the major extracellular pigment-peptide complex in which Ser and Thr formed >90% of the amino acid residues and which bound >10% of the iron supplied to the culture. This complex appeared to be formed by poly-

**Abbreviations:** Common amino acids, 3-letter code; TAP, N/O-trifluoroacetyl (amino acid) isopropyl ester; RP-HPLC, reversed phase high performance liquid chromatography; GC, gas chromatography; MS, mass spectrometry; ESI, electrospray ionization; MALDI, matrix assisted laser desorption ionization; TOF, time of flight; PSD, post source decay; NMR, nuclear magnetic resonance; COSY, correlated spectroscopy; DEPT, distortionless enhancement by polarization transfer; HMBC, heteronuclear multiple bond correlation; HMQC, heteronuclear multiple quantum coherence; NOE, nuclear Overhauser effect; ROESY, rotating frame nuclear Overhauser and exchange spectroscopy; WATERGATE, water suppression by gradient-tailored excitation; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate; TMS, tetramethylsilane; CCAP, Culture Collection of Algae and Protozoa.

<sup>#</sup> Part XCI of the series "Bacterial constituents". For part XC see Sultana *et al.* (2000); for part LXXXIX Münzinger *et al.* (2000).

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merisation in the light of a smaller molecule that contained 2 Ser, 1 Thr and a colourless fluorescent moiety (Walsby, 1974b). We report here the elucidation of the structure of a siderophore produced by *Anabaena cylindrica* CCAP 1403/2A, which contains these structural elements (Beiderbeck, 2000).

## Materials and Methods

### Instruments and chemicals

Mass spectrometry: ESI Finnigan-MAT 900 ST; GC/MS Incos 500 (both Finnigan-MAT, Bremen) with Varian (Sunnyvale CA, USA) GC 3400; MALDI Reflex III with SCOUT source (Bruker, Bremen). For MALDI using an N<sub>2</sub>-laser the sample was added in a matrix: sample ratio of ca. 500:1 to the matrix solution (saturated solution of 4-hydroxy- $\alpha$ -cyanocinnamic acid in 0.1% aqueous CF<sub>3</sub>COOH in H<sub>2</sub>O/CH<sub>3</sub>CN 1:2). 2  $\mu$ l of the solution were applied to the target and allowed to crystallize. The accelerating voltage was 20 kV for normal spectra and 26.3 kV for PSD measurements.

NMR: DRX 500 (<sup>1</sup>H 500, <sup>13</sup>C 125 MHz) (Bruker, Karlsruhe). Chemical shifts relative to TMS with the internal standard DSS;  $\delta(\text{TMS}) = \delta(\text{DSS})$  for <sup>1</sup>H,  $\delta(\text{DSS}) = -1.61$  ppm for <sup>13</sup>C. For suppression of the H<sub>2</sub>O signal by the WATERGATE pulse sequence, 10 mg of the sample was dissolved in 0.6 ml H<sub>2</sub>O/D<sub>2</sub>O 9:1.

UV/Vis: Lambda 7 (Perkin-Elmer, Überlingen) 1 mg of the sample was dissolved in 10 ml buffer solution (100 mM, phosphate buffer, pH 4.2 and NH<sub>4</sub>HCO<sub>3</sub> buffer, pH 9.0) with the buffer solution used as reference.

Chromatography: Low pressure chromatography columns used included XAD-4 (Serva, Heidelberg), Biogel P-2 (Bio-Rad, Richmond CA, USA), CM-Sephadex C-25 and DEAE Sephadex A-25 (Pharmacia, Uppsala, S), MN-Polyamid SC6 (Machery & Nagel, Düren); Sep-Pak RP<sub>18</sub> cartridges (Waters Millipore, Milford MA, USA); GC/MS: Chirasil-L-Val (Chrompack, Frankfurt); paper chromatography paper type No. 2668 (Schleicher & Schüll, Dassel).

Chemicals: Water was desalted and distilled twice in a quartz apparatus. Organic solvents were distilled over a column. Reagents were of p. a. quality.

### Production and isolation of anachelin (1)

The strain of *Anabaena cylindrica* CCAP 1403/2A, originally isolated from a pond in Surrey, England (Fogg, 1942), was obtained from the Culture Collection of Algae and Protozoa at Ambleside, UK. It was grown in a Jaworski medium: 1 ml each of the following solutions to be combined and diluted with bidistilled water to a final volume of 1 litre: a) 4.0 g Ca(NO<sub>3</sub>)<sub>2</sub> · 4 H<sub>2</sub>O, b) 2.48 g KH<sub>2</sub>PO<sub>4</sub>, c) 10.0 g MgSO<sub>4</sub> · 7 H<sub>2</sub>O, d) 3.18 g NaHCO<sub>3</sub>, e) 0.45 g EDTAFeNa and 0.45 g EDTAH<sub>2</sub>Na<sub>2</sub>, f) 0.496 g H<sub>3</sub>BO<sub>3</sub>, 0.278 g MnCl<sub>2</sub> · 4 H<sub>2</sub>O and 0.20 g (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub> · 4 H<sub>2</sub>O, g) 0.008 g cyanocobalamin, 0.008 g thiamine hydrochloride and 0.008 g biotin, h) 16.0 g NaNO<sub>3</sub>, i) 7.2 g Na<sub>2</sub>HPO<sub>4</sub> · 12 H<sub>2</sub>O, each dissolved in 200 ml H<sub>2</sub>O. This medium was modified for maximum siderophore production by decreasing the amount of iron-chelate solution e) from 1 ml to 0.025 ml. 300 ml of the culture solution in a 500 ml Erlenmeyer flask with indentations was inoculated, the flask was closed with a sterile stopper and shaken for two weeks at 100 rpm. Four 5-l Erlenmeyer flasks containing 3.5 l of the culture medium were inoculated with a stock culture (ratio 1:50). The flasks were aerated continuously with sterile air, illuminated with 10,000 lux in a 12 h:12 h light:dark cycle, and kept at 21 °C until the end of the exponential growth was reached (ca. 3–4 weeks). To the culture freed from cell material by centrifugation were added 0.1 g NaN<sub>3</sub> (to prevent further cell growth) and 20 ml of a 5% Fe(III) citrate solution per litre. The removal of gelatinous polysaccharides was effected by filtration through cotton wool, a paper filter and a glass fibre filter.

The filtrate was adsorbed on a 5 cm × 50 cm XAD-column. The dark brown adsorbate was washed with 5 l NaN<sub>3</sub> solution (0.1 g/l) and with 10 l H<sub>2</sub>O to elute salts and retained polysaccharides, and then desorbed with 1 l 80% aqueous CH<sub>3</sub>OH and 2 l pure CH<sub>3</sub>OH. If the resin remained dark the siderophores were decomplexed with 100 ml of a 4% oxalate buffer (pH 4.0). After washing out salts with H<sub>2</sub>O organic material was desorbed with 1 l 80% and 1 l pure CH<sub>3</sub>OH. The combined methanol phases were brought to dryness i. v. at 35 °C. The residue was dissolved in a minimal amount of 0.2 M pyridinium acetate buffer (pH 5.0) and chromatographed on Biogel P-2 (6 × 20 cm, detection at 405

and 280 nm). The brownish-violet main fraction was brought to dryness i. v. at 35 °C and chromatographed again on DEAE Sephadex (2.6 cm × 20 cm, detection at 280 nm). In this way a negatively charged brown pigment could be removed. The remainder was again brought to dryness as described above and rechromatographed on CM Sephadex (2.6 × 30 cm, detection at 280 nm). The brownish-violet main fraction was brought to dryness again and 40 mg fractions were further purified by descending preparative paper chromatography (15 × 40 cm) with *n*-C<sub>4</sub>H<sub>9</sub>OH/CH<sub>3</sub>COOH/H<sub>2</sub>O 90:10:29 (v/v). The dark violet main fraction (*R<sub>f</sub>* ca. 0.5) was cut out and eluted with H<sub>2</sub>O and 0.1 M NH<sub>4</sub>HCO<sub>3</sub> solution. The eluates were concentrated i. v. and chromatographed on CM Sephadex (2.6 × 20 cm, detection at 280 nm) to remove contaminants contained in the paper. The siderophore fraction was brought to dryness i. v. and kept at -25 °C. 10 l of culture yielded ca. 100 mg of the iron complex.

For decomplexation all glass vessels were rinsed with 6 M HCl. 15 mg of the ferri siderophore were adsorbed on a SepPak cartridge (rinsed with 5 ml CH<sub>3</sub>OH and activated 4 times with 5 ml H<sub>2</sub>O) and treated with 5 ml of a 4% oxalate buffer (pH 4.0). After washing with 5 ml of 1 M NaCl and 5 ml H<sub>2</sub>O the free siderophore was desorbed with 5 ml 80% and 5 ml pure CH<sub>3</sub>OH. The eluate was concentrated i. v. at 30 °C and lyophilized. In cases where the substance still had a yellowish tinge it was chromatographed on MN-Polyamid and treated as before.

## Results

**1** has an absorption maximum at 287 nm and shoulders at 247 and 305 nm. These values make a

2,3-dihydroxybenzoic acid system unlikely (cf. e.g., Ehlert *et al.*, 1994). The Fe<sup>3+</sup> complex shows pH dependent broad charge transfer bands between 500 and 600 nm. The hypsochromic shift with increasing pH is reflected in a change of colour from dark violet to pink due to the change of the protonation of phenolic OH groups. The molecular mass of **1** could be determined as 761.3355 ± 0.0026 u. This is in excellent agreement with the gross formula C<sub>35</sub>H<sub>49</sub>N<sub>6</sub>O<sub>13</sub> (761.3357 u) corresponding to the structure deduced below. The mass of the Fe<sup>3+</sup> complex is 814.4 u corresponding to M + <sup>56</sup>Fe - 3H. Amino acid analysis after total hydrolysis and TAP derivatization on a chiral column (see Briskot *et al.*, 1986 and Mohn *et al.*, 1990) demonstrated that **1** contained D- and L-Ser and L-Thr. **1** is very sensitive to hydrolysis: an aqueous solution of **1** kept at 4 °C for several days is transformed completely to a product 18 u heavier (**2**).

Further information regarding the structural elements contained in **1** can be gained from NMR data: the basis of this is the unambiguous identification of all <sup>1</sup>H- and <sup>13</sup>C-signals by a combination of homo- and heteronuclear one- and two-dimensional experiments: COSY allows detection of the <sup>3</sup>J-, TOCSY higher H,H-couplings. HMQC identifies <sup>1</sup>J-C,H, HMBC <sup>2</sup>J- and <sup>3</sup>J-coupling and thus allows the identification of quaternary C-atoms. HMBC indicates the number of H-atoms bound to a carbon. Sequence information is obtained by ROESY which correlates spatially close protons and by HMBC correlating carbonyl and amide signals of neighbouring amino acids. To avoid complications arising from the facile hydrolysis of **1**, structural studies were performed with **2** (see Fig. 1), whose NMR-signals are compiled in Table I.

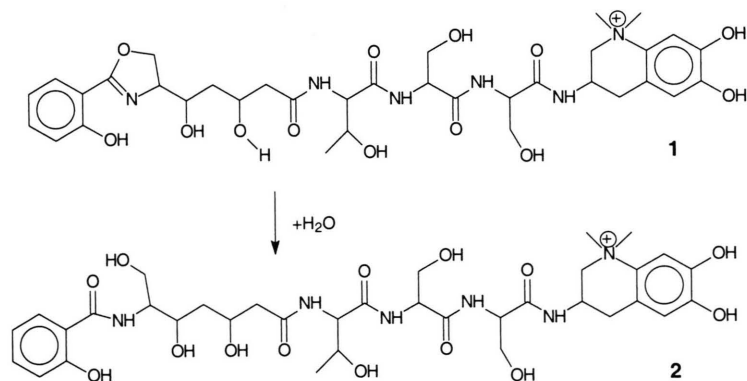


Fig. 1. Structure of anachelin (**1**) and of its hydrolysis product (**2**).

Table I.  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of **2** ( $\text{H}_2\text{O}/\text{D}_2\text{O}$  9:1, 298 °C)<sup>a</sup>.

Position		$^1\text{H}$	$^{13}\text{C}$	Position		$^1\text{H}$	$^{13}\text{C}$
Sal	1	–	117.2	Ser-1	1	–	173.1
	2	–	157.5		2	4.40	56.4
	3	6.97	117.5		3	3.86	61.1
	4	7.45	134.6	Ser-2	NH	8.56	–
	5	7.00	120.5		1	–	172.1
	6	7.77	129.2		2	4.37	56.7
	CO	–	170.3	Quin	3	3.94	61.2
Hep	1	–	174.4		NH	8.62	–
	2	2.02/2.29	43.4		1-CH <sub>3</sub>	3.50	57.9/58.7
	3	4.09	66.6		2	3.38/3.80	65.1
	4	1.65/1.55	40.3		3	4.56	41.6
	5	4.06	68.1		4	2.92/3.05	30.9
	6	4.21	55.0		4a	–	120.2
	7	3.72	61.9		5	6.72	116.8
Thr	6-NH	8.48	–		6	–	144.9
	1	–	173.1		7	–	146.6
	2	4.36	59.3		8	7.05	107.9
	3	4.21	67.9		8a	–	133.4
	4	1.15	19.2		3-NH	8.09	–
	NH	8.15	–				

<sup>a</sup> Sal – salicylic acid unit, Hep – 6-amino-3,5,7-trihydroxyheptanoic acid unit (cf. Fig. 4), Quin – 1,1-dimethyl-1,2,3,4-tetrahydro-7,8-dihydroxyquinolinium unit (cf. Fig. 3).

### The tripeptide Thr–Ser–Ser

The spin systems of the two Ser and of Thr were established by COSY and TOCSY crosspeaks between the respective side-chain H atoms and that of the proper NH group; the corresponding C atoms were identified by HMQC, the CO groups by HMBC. The shift values indicate that the three amino acids are connected by peptide bonds and especially that the OH groups are free (ester bonds would result in a downfield shift of ca. 0.5 ppm of the  $\beta$ -protons; Budzikiewicz, 1997). The sequence Thr–Ser–Ser follows from ROESY experiments (see Fig. 2) and from mass spectral data (see below). The chirality of the three amino acids had been determined (see above) as L-Thr, D-Ser and L-Ser.

### The 1,1-dimethyl-3-amino-1,2,3,4-tetrahydro-7,8-dihydroxyquinolinium system

The connectivities of the aliphatic part of the molecule were established by COSY cross peaks of H-3 with the neighbouring  $\text{CH}_2$ -groups and with the NH at 8.09 ppm. In the TOCSY experiment the coupling of the NH proton with the two  $\text{CH}_2$  groups can also be seen. The corresponding C-resonances were identified by the  $^1J$  coupling in a HMQC experiment. The proton signals of the two N-methyl groups coincide, but they show  $^1J$  coupling to two C-atoms at 57.9 and 58.7 ppm and  $^2J$  coupling to the other ones, respectively; hence, they are both bound to the same atom. The low shift value suggests that it is a strongly electron withdrawing centre. In addition, long-range cou-

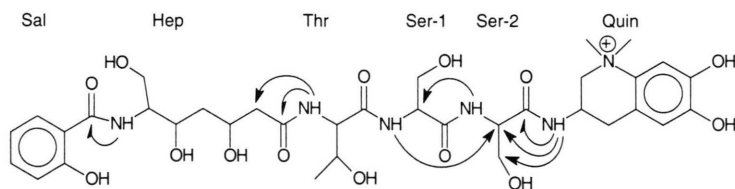


Fig. 2. Sequence relevant cross peaks for **1** derived from ROESY (full arrows) and HMBC (half arrows) experiments.

pling is observed with the CH<sub>2</sub> group at 65.1 ppm (C-2) and an aromatic quaternary C at 133.4 ppm. In the ROESY spectrum cross signals between the CH<sub>3</sub> protons and those of the neighbouring CH<sub>2</sub> group (C-2) as well as with an aromatic proton at 7.05 ppm can be observed.

The connection of the aliphatic with the aromatic part of the molecule can be deduced from the protons of the CH<sub>2</sub> group C-4. They show HMBC connectivities with a CH-carbon (116.8 ppm) and 2 quaternary C-atoms (according to the HMQC and DEPT experiments) at 120.2 and 133.4 ppm, the last one in the neighbourhood of the CH<sub>3</sub> groups mentioned above, as well as with the H at 6.72 ppm (C-5). The connectivities within the aromatic ring can be seen in Fig. 3. The assignment of the respective shift values to the two phenolic carbon atoms could not be deduced from the HMBC experiments as both showed equally strong coupling to the CH protons at C-5 and C-8. A comparison with the structurally related dihydropyoverdin chromophore (Gwose and Taraz, 1992) suggests the assignment given in Table I, which is in agreement with the more pronounced deshielding of the quaternary N<sup>+</sup> to the *meta* as compared with the *para* position. HMBC and ROESY correlates the NH signal with the C-terminal Ser (see Fig. 2).

#### 6-Amino-3,5,7-trihydroxyheptanoic acid

The structural studies of this part of the molecule are complicated by the overlap of the proton CHOH signals (C-3 and C-5) and the coincidence of that of C-6 with the one of the β-CH of Thr (see Table I). The connectivities of the <sup>1</sup>H NMR signals were determined starting from the CH<sub>2</sub> protons at C-4 (1.55/1.65 ppm). A TOCSY experiment gave cross peaks with the second CH<sub>2</sub> group

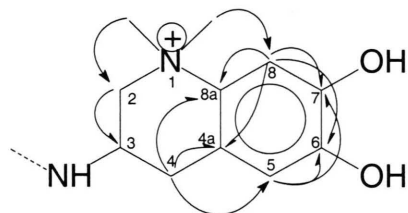


Fig. 3. Selected cross peaks for the dihydroxytetrahydroquinolinium system (ROESY full arrows, HMBC half arrows).

(2.02/2.29 ppm) as well as with the two CHOH groups (4.06 and 4.09 ppm). The system can be extended by the observation of cross peaks from H-5 to H-6 (4.21 ppm) and from H-6 to H-7 (3.72 ppm), and from the NH-signal at 8.48 ppm to H-7 and H-6. COSY <sup>3</sup>J couplings confirm the relationships NH/H-6, H-7/H-6, and show *inter alia* that the two CH<sub>2</sub> groups are not connected with each other.

The H-bearing C atoms were identified by DEPT and HMQC experiments. C,H-correlations confirmed the structure deduced from the proton spectrum (see Fig. 4). Of special importance is the correlation of the CO group at 174.4 ppm with the CH<sub>2</sub> group C-2 only; this excludes an α-hydroxy acid, which might have been expected as a complexing site. HMBC and ROESY data connect the CO group with the NH group of Thr.

#### Salicylic acid part

The proton spin system corresponds to literature data (Okujo *et al.*, 1994) and could be confirmed by TOCSY, HMQC and HMBC experiments. A HMBC cross peak connects its CO group with the NH of the heptanoic acid system.

#### The additional ring system of 1

The molecular mass of **2** is 18 u higher than that of **1**. This indicates that during hydrolysis a ring was opened. NMR data were obtained only from mixtures of **1** and **2**. Changes in the spectra occur only in the salicylic acid/amino-trihydroxyheptanoic acid part of the structure; this can be explained by the formation of an oxazoline ring. Especially evident are the signals at 5.08 (H-6, now H-4 of the ring) and 5.33 ppm (H-7, now H-5 of the ring). In a HMBC experiment both couple to C-2 and C-4 of the chain. In the <sup>13</sup>C spectrum signals occur at 80.2 ppm (C-7, now C-5 of the ring) showing geminal coupling with the proton signal

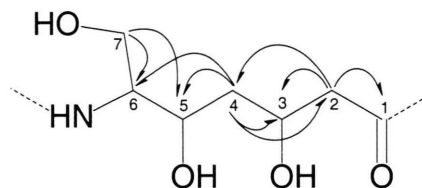


Fig. 4. Selected heteronuclear long-range couplings in the amino-trihydroxy-heptanoic subunit.

at 5.08 ppm, and at 72.3 ppm (C-6, now C-4 of the ring). In the salicylic acid part, the signal of the phenolic carbon (C-2) is shifted to 160.1 ppm and that of the former carboxyl group is now found at 166.8 ppm. These values agree well with those obtained from other siderophores containing the 2-*o*-hydroxyphenyl-oxazoline substructure (Okujo *et al.*, 1994). The signals of Thr as the next unit show only minute shifts.

#### Sequence of the subunits

Cross peaks relevant to the connections between the structural subunits were mentioned in the preceding sections and are combined in Fig. 2. The sequence is confirmed by characteristic fragments observed in a MALDI-TOF-PSD mass spectrum (Tang *et al.*, 1988; Spengler *et al.*, 1992). The B- ( $R-CO^+$ ) and Y'-ions ( $H_2N-R'$ ) (Roepstorff and Fohlman, 1984) formed by cleavage of the amide bonds are depicted in Fig. 5. They are accompanied by ions formed by the loss of  $H_2O$ . Of interest are two ions ( $m/z$  236 and 256) which are formed by a McLafferty rearrangement of the  $\beta$ -hydroxycarbonyl unit. The stereochemistry of most of the chiral centres remains to be determined.

#### Discussion

The structure of anachelin (**1**) from *Anabaena cylindrica* differs considerably from those obtained so far from other bacteria although it is typical in its use of the catecholate and the 2-*o*-hydroxyphenyloxazoline system for binding  $Fe^{3+}$ . It is the combination of structural elements that makes the structure unique. The discovery of this novel structure suggests that cyanobacteria independently evolved their own siderophores. Anachelin contains only the two binding sites for  $Fe^{3+}$  mentioned above. In the ESI-MS spectrum only an ion for a 1:1 complex ( $[1 - 3H^+ + Fe^{3+}]^+$ ) can be observed. In such cases the two remaining coordination sites of  $Fe^{3+}$  are usually occupied by two molecules of  $H_2O$  (Scarrow *et al.*, 1991).

Nothing is known about the mechanisms of the iron transport into the cell and the intracellular release in cyanobacteria. For *Synechococcus* a so-called taxi mechanism has been proposed:  $Fe^{3+}$  is transported by a hydroxamate siderophore to the cell surface and transferred there to a catecholate siderophore. In this way only a biosynthetically less costly hydroxamate (as, e.g., schizokinen) is lost in part by diffusion, an advantage in the aquatic realm (Wilhelm *et al.* 1994; Wilhelm *et al.*,

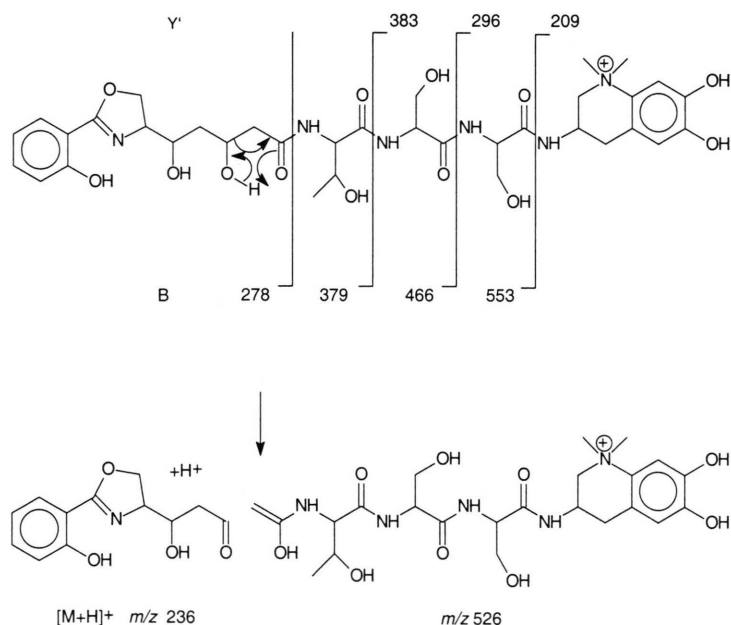


Fig. 5. Characteristic fragments in the MALDI-TOF mass spectrum of **1**.

1996). In eubacteria iron is released from its siderophore by reduction to  $\text{Fe}^{2+}$ . Since the redox potential of complexed  $\text{Fe}^{3+}$  in most siderophores is much too negative for a reduction in the equilibrium state by biological reducing agents as NADH,  $\text{Fe}^{2+}$  must be removed by complexation in, e.g., porphyrins. This renders according to the

Nernst equation the redox potential more positive (Pierre and Fontecave, 1999). The same effect is achieved by replacing one of the bidentate siderophore ligands of  $\text{Fe}^{3+}$  by 2  $\text{H}_2\text{O}$  (Crumbliss *et al.*, 2000). This would explain why **1** contains an oxazoline ring that is not very resistant to hydrolysis.

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