Pergamon

Tetrahedron 57 (2001) 9093–9099

TETRAHEDRON

Two structural isomeric siderophores from the freshwater cyanobacterium *Anabaena cylindrica* (NIES-19)

Yusai Itou, Shigeru Okada and Masahiro Murakami*

Laboratory of Marine Biochemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

Received 2 August 2001; accepted 19 September 2001

Abstract—Siderophore is the iron-chelating agent excreted by microorganisms for acquisition of trace of iron. It has been reported that cyanobacteria also could utilize siderophores, but few structural properties of cyanobacterial siderophores were reported. We screened the siderophore producing activity for freshwater cyanobacterial species and found that *Anabaena cylindrica* (NIES-19) secreted two structural isomeric siderophores. Those structures were elucidated by FABMS analysis and $^{1}H-^{1}H$, $^{1}H-^{13}C$, and $^{1}H-^{15}N$ 2D NMR analyses.

1. Introduction

Iron is the most important transition metal ion for all organisms. It forms versatile electron transfer agents in a variety of important proteins for basic physiological processes. However, under aerobic and the neutral pH condition iron forms highly insoluble ferric hydroxide complex to limit the concentration of biologically available iron. It is well known that under iron deficient condition a large number of terrestrial microorganisms biosynthesize and secrete the low-molecular weight chelating compounds, siderophores, and acquire the iron–siderophore complex into cells with their outer membrane specific receptors.[1] The knowledge of siderophore had been intensively collected about these terrestrial microorganisms, especially pathogenic species, because they use siderophores to sequester iron from host cells. In the last decade, however, it has been elucidated that low levels of iron in ocean limit marine microorganisms including phytoplankton[2] and it has been vigorously reported new siderophores from heterotrophic marine bacteria and some of which had the noble structural features that had never reported from terrestrial species,[3] suggesting the possibility that aquatic microorganisms independently evolved their siderophores to survive in habitual low-iron condition.

Cyanobacteria are the prokaryotic phytoplankton and the representative primary producer in aquatic environment to perform oxygenic photosynthesis using the iron-containing catalysis, cytochrome and ferredoxin, and many species of them can also fix nitrogen by using the iron-containing enzyme, nitrogenase. Therefore, the iron-acquisition mechanism of cyanobacteria would have seemed of special interest. Murphy et al. suggested that cyanobacteria could produce siderophores as well as other prokaryote and may become dominant by using them to monopolize iron in freshwater system.[4] In fact, Trick and co-worker demonstrated that many cyanobacterial species are capable of producing and/or utilizing siderophores.[5] However little is known about the structural property of cyanobacterial siderophores. Schizokinen from *Anabaena* sp. (PCC-7120),[6] which was first reported from *Bacillus megaterium* (ATCC 19213),[7] was the only characterized cyanobacterial siderophore until quite recently.

Then, we screened the ability of siderophore production of cyanobacteria, especially bloom-forming freshwater species, by using Chrome azurol S (CAS) assay which estimated the iron-chelating activity,[8] and found that some *Anabaena* species produced siderophores. In this paper, we report the isolation and the structural elucidation of two structural isomeric siderophores from *A. cylindrica* (NIES-19) by using FABMS, 2D NMR (^{1}H, ^{13}C, and ^{15}N) analyses, and chemical degradation.

2. Result

Eleven species of freshwater cyanobacteria were cultured in iron-deficient medium ($FeCl_3=1\times10^{-9}$ M) for two weeks and tested for the siderophore production. Siderophores in the extraceller supernatants were determined by liquid CAS assay.[8] *Microcystis aeruginosa* (NIES-44, 87, 90, 98, and 298), *M. viridis* (NIES-102), *M. wesenbergii* (NIES-111 and 604), and *Oscillatoria agardhii* (NIES-596), which are the major bloom forming species, were negative for this test.

Keywords: iron; siderophore; cyanobacteria; *Anabaena cylindrica*; CAS.

* Corresponding author. Tel.: +81-3-5841-5302 ext. 5298; fax: +81-3-5841-8166; e-mail: amura@mail.ecc.u-tokyo.ac.jp

0040–4020/01/$ - see front matter
PII: S0040-4020(01)00934-6

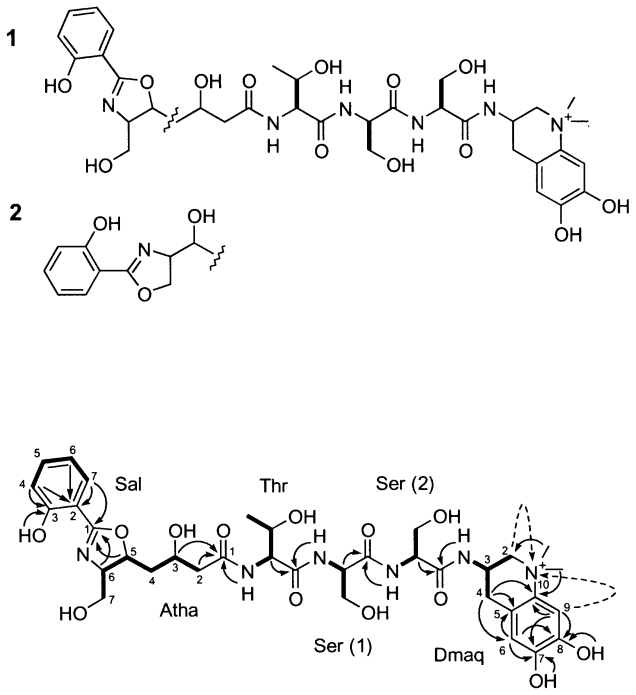


Figure 1. Selected $^{1}H-^{1}H$ COSY (bold lines), $^{1}H-^{13}C$ HMBC (half arrows) and $^{1}H-^{15}N$ HMBC (dashed half arrows) correlations for anachelin-2(**1**).

The culture fluid of *Anabaena cylindrica* (NIES-19) and *A. variabilis* (NIES-23) showed the strong iron chelating activities. The brownish supernatant of iron-starved *A. cylindrica* culture was collected and lyophilized, followed to subject to ODS open column chromatography and eluted with 0, 20, 50 and 100% MeOH. The 50% MeOH fraction which showed the activities in CAS assay was subjected to HPLC on ODS column with H_2O/MeCN/0.05%TFA as mobile phase to yield two active principals, anachelin-2 (**1**, 50.5 mg) and anachelin (**2**, 52.5 mg), as white solids.

2.1. Anachelin-2 (1)

The positive FABMS spectra of **1** gave an intense ion peak at *m/z* 761. Amino acid analysis revealed the presence of one residue of Thr and two of Ser in **1**. The ^{1}H and ^{13}C NMR spectra of **1** in DMSO-d_6 suggested the peptidic nature of **1** containing four amide protons. The 2D NMR analyses including $^{1}H-^{1}H$ COSY, $^{1}H-^{13}C$ HMBC and HMQC experiments easily assigned three usual amino acids residues, and HMBC correlations between amide protons and amide carbons constructed their successive connection (NH-Thr-Ser(1)-Ser(2)-CO). In addition, HMBC spectrum showed that unusual units attached to both terminus of this sequence through the amide linkage (Fig. 1). Each moiety attached to C and N terminus was designated right moiety and left moiety, respectively.

Right moiety. $^{1}H-^{1}H$ COSY experiment gave the straightforward connectivities from the amide proton at δ_H 8.00 to H-2 and H-4 methylene protons (Fig. 1). HMBC correlations from two singlet signal of H-6 at δ_H 6.62 and H-9 at δ_H 7.28 to the aromatic carbon signals, and from two catecholic proton signals at δ_H 9.40 and 9.90 to their respective carbon signals at δ_C 145.0 and 148.0 indicated the presence of a 4,5-disubstituted catechol moiety. H-4 methylene signals showed HMBC correlations to C-5, 6, and 10, and H-6 also showed to C-4 (Fig. 1). This data indicated that C-4 coupled to C-5. The strong HMBC correlations from two methyl proton signals at δ_H 3.48 and 3.55 to C-2 at δ_C 64.4 and C-10 at δ_C 133.0 were observed (Fig. 1). These correlations and the fact that these chemical resonance unusually shifted to downfield suggested that C-2 and C-10 linked through a *N*,*N*-dimethyl quaternary nitrogen, which was confirmed by $^{1}H-^{15}N$ HMBC correlations from H-2, H-9 and *N*,*N*-dimethyl proton signals to the quaternary nitrogen signal at δ_N 58.2 (Fig. 1).[9] Thus the 1,1-dimethyl-3-amino-1,2,3,4-tetrahydro-7,8-dihydroxyquinolin unit (Dmaq) was ambiguously assigned as right moiety.

Left moiety. $^{1}H-^{1}H$ COSY, HMQC, and HMBC experiments constructed the 3,5,6,7-tetrasubstituted heptanoic acid moiety and also assigned the salicylic acid moiety (Sal). Both H-5 and H-6 methine proton signals in the 3,5,6,7-tetrasubstituted heptanoic acid moiety showed the HMBC correlations to the C-1 carbon signal at δ_C 164.5 in Sal moiety (Fig. 1). This suggested the presence of the 2-hydroxyphenyl-oxazoline system in **1**, which was established well in mycobactins, the lipophilic siderophores produced by mycobacteria.[10] Interestingly, when **1** was left in H_2O containing 0.05% TFA for several days, it was observed that **1** converted to **3** which gave an ion peak at *m/z* 779 in positive FABMS analysis. $^{1}H-^{1}H$ COSY spectrum of **3** showed the correlation between the H-6 signal and a new proton signal at δ_H 8.11 (Fig. 2), which was considered to be an amide or a primary ammonium proton signal. Since the chemical resonances of H-6 and C-6 shifted to upfield (Table 1), it was firstly expected that the hydrolysis of the oxazoline ring was occurred and the ring was opened as **5** (Fig. 2), which was the spontaneous hydrolysis product of anachelin (**2**) reported by Beiderbeck et al. as described below. However, the chemical resonance of H-5 in **3** shifted to downfield from δ_H 4.73

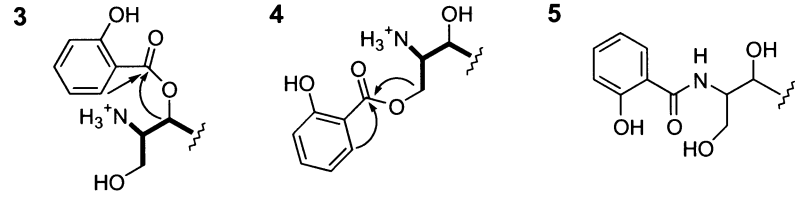


Figure 2. Structures of ester intermediate of anachelin-2 (**3**) and that of anachelin (**4**): $^{1}H-^{1}H$ COSY (bold lines) and $^{1}H-^{13}C$ HMBC (half arrows) correlations. **5** is the expected final hydrolysis compound of the oxazoline ring.

Table 1. ^{1}H and ^{13}C NMR Data for anachelin-2 (**1**) and ester intermediate of anachelin-2 (**3**) in DMSO-d_6

		Anachelin-2 (**1**)			Ester intermediate of anachelin-2 (**3**)	
		^{1}H *J* (Hz)	^{13}C		^{1}H *J* (Hz)	^{13}C
Sal	1		164.5 (s)	1		167.9 (s)
	2		110.1 (s)	2		112.8 (s)
	3		159.2 (s)	3		160.2 (s)
	4	6.96 (d, 7.7)	116.3 (d)	4	6.98 (d, 7.7)	117.2 (d)
	5	7.40 (t, 7.7)	133.8 (d)	5	7.40 (t, 7.7)	133.8 (d)
	6	6.90 (t, 7.7)	118.8 (d)	6	6.90 (t, 7.7)	118.8 (d)
	7	7.60 (d, 7.7)	128.0 (d)	7	8.01 (d, 7.7)	131.0 (d)
	OH	10.3 (s)		OH	10.4 (s)	
Atha	1		171.4 (s)	1		170.8 (s)
	2a	2.18 (brd, 14.1)	43.1 (t)	2a	2.20 (dd, 14.1, 4.3)	43.2 (t)
	2b	2.38 (dd, 14.1, 8.2)		2b		2.35 (dd, 14.1, 8.1)
	3	4.00 (m)	64.1 (d)	3	3.96 (m)	64.2 (d)
	4a	1.73 (m)	41.5 (t)	4a	1.83 (m)	36.9 (t)
	4b	1.86 (m)		4b		1.95 (m)
	5	4.73 (m)	78.9 (d)	5	5.38 (m)	70.2 (d)
	6	4.06 (m)	71.4 (d)	6	3.61 (m)	53.6 (d)
	7a	3.55 (m)	62.3 (t)	7a	3.61 (m)	58.7 (t)
	7b	3.59 (m)		7b	3.69 (m)	
				3-OH	5.06 (brs)	
				7-OH	5.43 (brs)	
				6-NH	8.11 (br)	
Thr	1		170.8 (s)	1		170.8 (s)
	2	4.23 (m)	58.4 (d)	2	4.22 (m)	58.2 (d)
	3	3.94 (m)	66.5 (d)	3	3.93 (m)	66.6 (d)
	4	1.05 (d, 6.0)	19.8 (q)	4	1.00 (d, 6.0)	19.5 (q)
				OH	4.93 (d, 5.1)	
	NH	7.92 (d, 7.7)		NH	7.86 (d, 7.7)	
Ser (1)	1		170.4 (s)	1		170.3 (s)
	2	4.24 (m)	55.5 (d)	2	4.26 (m)	55.4 (d)
	3a	3.58 (m)	61.3 (t)	3a	3.59 (m)	61.3 (t)
	3b	3.64 (m)		3b		3.63 (m)
				OH	5.07 (br)	
	NH	8.04 (m)		NH	8.04 (m)	
Ser (2)	1		170.0 (s)	1		169.8 (s)
	2	4.20 (m)	55.6 (d)	2	4.21 (m)	55.5 (d)
	3a	3.60 (m)	61.1 (t)	3a	3.58 (m)	61.3 (t)
	3b	3.67 (m)		3b		3.65 (m)
				OH	4.99 (br)	
	NH	8.09 (m)		NH	8.06 (m)	
Dmaq	2a	3.38 (m)	64.3 (t)	2a	3.41 (t, 11.7)	64.3 (t)
	2b	3.73 (m)		2b		3.75 (m)
	3	4.46 (m)	39.2 (d)	3	4.46 (m)	39.2 (d)
	4a	2.83 (dd, 15.4, 11.1)	30.9 (t)	4a	2.82 (dd, 15.8, 11.1)	30.9 (t)
	4b	2.88 (dd, 15.4, 5.1)		4b	2.90 (dd, 15.8, 5.7)	
	5		119.0 (s)	5		119.0 (s)
	6	6.62 (s)	115.5 (d)	6	6.92 (s)	115.5 (d)
	7		145.0 (s)	7		145.0 (s)
	8		148.0 (s)	8		148.0 (s)
	9	7.28 (s)	107.0 (d)	9	7.28 (s)	108.0 (d)
	10		133.0 (s)	10		133.0 (s)
	N-Me	3.55 (s)	56.9 (q)	*N*-Me	3.55 (s)	56.9 (q)
	N-Me	3.48 (s)	57.8 (q)	*N*-Me	3.48 (s)	57.8 (q)
	7-OH	9.40 (s)		7-OH	9.40 (s)	
	8-OH	9.90 (s)		8-OH	9.90 (s)	
	NH	8.00 (m)		NH	8.05 (m)	

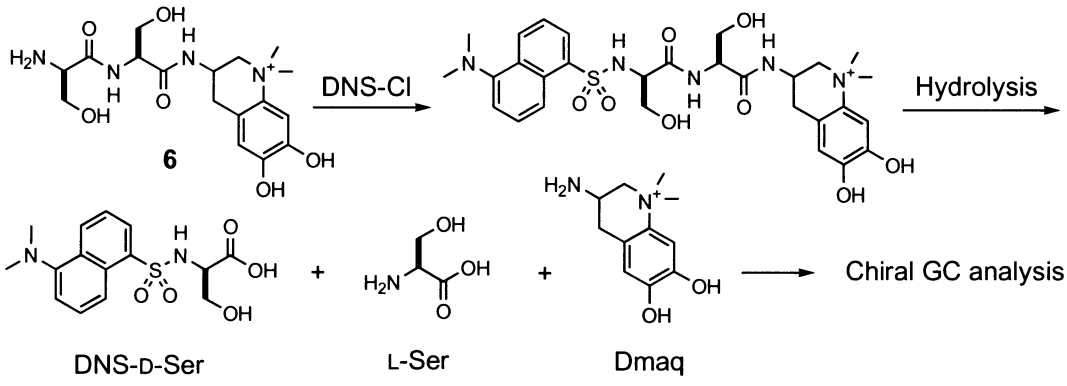
to 5.39 indicating the presence of ester moiety, and the 3-OH and 7-OH proton signals were clearly observed in ^{1}H NMR spectrum of **3** but the 5-OH proton signal was not (Table 1). Furthermore, although intense HMBC correlation from the H-5 proton signal to the C-1 carbon signal of Sal observed, the correlations from the new proton signal at δ_H 8.11 and the H-6 proton signal to that were not (Fig. 2). Therefore it was presumed that the proton signal at δ_H 8.11 was a primary ammonium proton signal and the oxazoline ring opened to afford the ester (Fig. 2).[11] Consequently, these data demonstrated the presence of the 2-hydroxyphenyl-oxazoline system in **1**, which was formed from 6-NH_2 and 5-OH of the 6-amino-3,5,7-trihydroxyheptanoic acid moiety (Atha) and Sal. Thus the gross planar structure of **1** was determined.

Table 2. ^{1}H and ^{13}C NMR data for anachelin (**2**) and ester intermediate of anachelin (**4**) in DMSO-d_6

		Anachelin (**2**)			Ester intermediate of anachelin (**4**)	
		^{1}H *J* (Hz)	^{13}C		^{1}H *J* (Hz)	^{13}C
Sal	1		165.2 (s)	1		168.2 (s)
	2		110.2 (s)	2		112.8 (s)
	3		159.2 (s)	3		160.2 (s)
	4	6.97 (d, 7.7)	116.5 (d)	4	6.98 (d, 7.7)	117.2 (d)
	5	7.43 (t, 7.7)	133.8 (d)	5	7.54 (t, 7.7)	136.0 (d)
	6	6.91 (t, 7.7)	118.8 (d)	6	6.96 (t, 7.7)	119.2 (d)
	7	7.99 (d, 7.7)	127.8 (d)	7	7.99 (d, 7.7)	131.0 (d)
	OH	10.4 (s)		OH	10.4 (s)	
Atha	1		171.5 (s)	1		171.2 (s)
	2a	2.12 (dd, 13.7, 3.4)	42.5 (t)	2a	2.21 (dd, 14.1, 4.3)	42.8 (t)
	2b	2.18 (dd, 13.7, 8.1)		2b	2.28 (dd, 14.1, 8.1)	
	3	4.00 (m)	65.5 (d)	3	4.04 (m)	65.0 (d)
	4a	1.54 (m)	40.5 (t)	4	1.67 (m, 2H)	40.2 (t)
	4b	1.68 (m)				
	5	3.62 (m)	68.6 (d)	5	3.91 (m)	65.5 (d)
	6	4.38 (m)	69.0 (d)	6	3.61 (m)	53.2 (d)
	7a	4.34 (m)	68.6 (t)	7a	4.44 (dd, 11.5, 6.5)	63.3 (t)
	7b	4.47 (m)		7b	4.49 (dd, 11.5, 3.8)	
				3-OH	5.06 (brs)	
				5-OH	5.59 (brs)	
				6-NH	8.08 (m)	
Thr	1		170.8 (s)	1		170.8 (s)
	2	4.23 (m)	58.4 (d)	2	4.24 (m)	58.4 (d)
	3	3.96 (m)	66.5 (d)	3	3.95 (m)	66.7 (d)
	4	1.04 (d, 6.4)	19.8 (q)	4	1.03 (d, 6.4)	19.8 (q)
				OH	4.95 (brs)	
	NH	7.85 (d, 7.7)		NH	7.86 (d, 7.7)	
Ser (1)	1		170.5 (s)	1		170.3 (s)
	2	4.23 (m)	55.6	2	4.26 (m)	55.4 (d)
	3a	3.60				
	(m)	61.3 (t)				
	(m)	61.3 (t)				
	3b	3.62 (m)		3b	3.62 (m)	
				OH	5.07 (brs)	
	NH	8.10 (5.8)		NH	8.07 (m)	
Ser (2)	1		170.0	1		170.0 (s)
	2	4.19 (m)	55.7	2	4.22 (m)	55.8 (d)
	3a	3.61 (m)	61.3	3a	3.60 (m)	61.3 (t)
	3b	3.68 (dd, 10.9, 5.3)		3b	3.66 (m)	
				OH	5.00 (brs)	
	NH	8.13 (7.7)		NH	8.06 (m)	
Dmaq	2a	3.36 (t, 11.8)	64.3	2a	3.41 (m)	64.4 (t)
	2b	3.74 (brd, 10.7)		2b	3.76 (brd, 9.8)	
	3	4.47 (m)	39.3	3	4.47 (m)	39.3 (d)
	4a	2.86 (m)	30.6	4a	2.83 (dd, 15.4, 10.9)	30.6 (t)
	4b	2.90 (m)		4b	2.90 (dd, 15.4, 5.1)	
	5		119.0	5		119.0 (s)
	6	6.64 (s)	116.0	6	6.64 (s)	115.5 (d)
	7		145.0	7		145.0 (s)
	8		148.0	8		148.0 (s)
	9	7.29 (s)	108.0	9	7.28 (s)	108.0 (d)
	10		133.0	10		133.0 (s)
	N-Me	3.55 (s)	56.9	*N*-Me	3.55 (s)	57.2 (q)
	N-Me	3.48 (s)	57.8	*N*-Me	3.48 (s)	58.0 (q)
	7-OH	9.40 (s)		7-OH	9.40 (s)	
	8-OH	9.90 (s)		8-OH	9.90 (s)	
	NH	7.97 (d,7.3)		NH	8.05 (m)	

2.2. Anachelin (2)

The positive FABMS spectra of **2** also gave an ion peak at *m/z* 761 as well as **1**, suggesting that **2** was a structural isomer of **1**. The planer structure of **2** was elucidated in a similar way and it was found that an oxazoline ring in **2** was formed from 7-OH of Atha, instead of 5-OH in **1**. As a consequence, **2** was identical to anachelin recently reported from *A. cylindrica* (CCAP1403/2A) by Beiderbeck et al.[12] Therefore, **1** was named anachelin-2. The oxazoline ring of **2** was more sensitive to hydrolysis than that of **1** and it was observed that **2** entirely converted to **4** in the NMR tube in



Scheme 1. DNS-Cl: 5-[Dimethylamino]naphthalene-1-sulfonyl chloride.

several days. NMR spectral data of **4** (Table 2) revealed that the oxazoline ring in **4** was also opened to afford the ester as well as **3** (Fig. 2).

2.3. The stereochemistry of Thr and Sers

The stereochemistry of Thr in **1** was determined to be L form by HPLC analysis of the acid hydrolysate derivatized with Marfey's reagent.[13] Since racemic Ser was not separated with this method, chiral GC analysis was performed, which revealed that D and L-Ser existed in **1**. The mild hydrolysis of **1** (6N HCl at 110°C for 30 min) gave the fragment of Ser(1)-Ser(2)-Dmaq (**6**), which was confirmed FABMS analysis, amino acid analysis and chiral GC analysis. After the derivatization of **6** with the dansyl-chrolide,[14] the chiral GC analysis of the acid hydrolysate of the derivative detected L-Ser, but not D-Ser (Scheme 1). Therefore the sequence of usual amino acids was determined to be L-Thr-D-Ser-L-Ser. But the stereochemistry of both Dmaq and Atha remained to be determined.

3. Discussion

In our screening, the major bloom forming species as *Microcystis* did not show the positive activity against CAS assay, but this result could not lead the conclusion that these species did not have the faculty of producing siderophores because their siderophores might be very weak complex to iron. But in consideration of their lower growth in iron-deficient condition as compared with *Anabaena* species (data not shown), axenic strains of *Microcystis* species seemed not to have the effective system for acquisition the trace of iron from iron-starved environment. It is known that in natural environment most of *Microcystis* species form multicellular colonies in their slime polysaccharide layer, and in which many microorganisms exist. It might be that *Microcystis* species did not have their own siderophores but the specific receptors for siderophores produced by symbiotic microorganisms in slime layer.

Anachelin (**2**) was isolated from the iron-starved culture of *A. cylindrica* (CCAP1403/2A) by Beiderbeck et al. and this was the first genuine cyanobacterial siderophore whose structure had been elucidated.[12] They reported that **2** was isolated as the iron-complex based on its brownish violet color, and after decomplexation the structural studies were performed with the spontaneous hydrolysis product (**5**), which could not be obtained in our work, and the oxazoline ring structure was assigned from NMR data of mixtures of **2** and **5**.

In this report, we could success to isolate two iron-free siderophores (**1** and **2**) from the iron-deficient culture supernatant of *A. cylindrica* (NIES-19) by the combination of liquid CAS assay and HPLC purification system, and assigned their structure directly by NMR experiment. Since only **2** was isolated from *A. cylindrica* (CCAP1403/2A), the question that **1** might be an artifact forming under the isolation procedure arose. In order to clarify that **1** was not artifact, each of **1** and **2** was left in H_2O containing 0.05% TFA and monitored for a week. In H_2O containing 0.05% TFA **1** and **2** were converted to **3** and **4**, respectively, but the conversion between **1** and **2** was not observed. Furthermore, the direct HPLC analysis of the supernatant of *A. cylindrica* (NIES-19) in fourth day under iron-starved condition revealed the presence of both **1** and **2** in culture supernatant, which was in the identical ratio to the isolates. Therefore, it was confirmed that both compounds were produced by *A. cylindrica* (NIES-19). As well as **2**,[12] the FABMS spectrum of the iron complex of **1** gave an ion peak at *m/z* 815 for a 1:1 complex as $[M^+ + Fe^{3+} - 3H]^+$ when this complex was synthesized by mixing a solution of $FeCl_3$ and **1** with a ratio 5:1, which suggested that the functional groups for binding Fe^{3+} was the catecholate in Dmaq and the 2-hydroxyphenyl-oxazoline system. Catecholate was a typical functional group of siderophore for binding Fe^{3+} as well as hydroxamate and a 2-hydroxyphenyl-oxazoline system was also established well in mycobactins. But it remains unknown whether the iron complexes of two structural isomeric anachelins were transported equally into *A. cylindrica* (NIES-19) cells, and whether other cyanobacterial species or microorganisms can use these complexes.

4. Experimental section

4.1. Instrumentation

NMR spectra were recorded on a JEOL JNM-A600 spectrometer using DMSO-d_6 as solvent at 27°C. ^1H and ^{13}C NMR chemical shifts were referenced to residual solvent peaks of DMSO-d_6 at δ_H 2.49 and δ_C 39.5. ^{15}N chemical shifts were

referenced to δ_N 112.0 for NH_2CHO. FABMS spectra were measured by using glycerol as matrix on a JEOL JMS SX-102 mass spectrometer. Amino acid analysis was carried out with a Hitachi L-8500A amino acid analyzer. Chiral GC analysis was carried out on a Shimadzu GC-9A gas chromatograph. Optical rotation was determined on a JASCO DIP-1000 digital polarimeter in CH_3OH. UV spectra were measured on a Hitachi 330 spectrometer.

4.2. Strains and growth condition

All axenic cyanobacterial species: *A. cylindrica* (NIES-19), *A. variabilis* (NIES-23), *M. aeruginosa* (NIES-44, 87, 90, 98, and 298), *M. viridis* (NIES-102), *M. wesenbergii* (NIES-111 and 604), and *O. agardhii* (NIES-596); were obtained from the NIES-collection (Microbial Culture Collection, the National Institute Environmental Studies, Japan) and was maintained axenically at 25°C in modified CB medium containing (per liter): 150 mg of $Ca(NO_3)\cdot 4H_2O$, 100 mg of KNO_3, 100 mg of K_2HPO_4, 40 mg of $MgSO_4\cdot 7H_2O$, 0.588 mg of $FeCl_3\cdot 6H_2O$ (2.18×10^{-6} M), 0.108 mg of $MnCl_2\cdot 4H_2O$, 0.066 mg of $ZnSO_4\cdot 7H_2O$, 0.012 mg of $CoCl_3\cdot 6H_2O$, 0.0075 mg of $Na_2MoO_4\cdot 2H_2O$, 500 mg of BICIN (*N*,*N*-bis[2-hydroxyethyl]glycine) at pH 8.5. Cultures were illuminated with fluorescent lights on a 12L/12D cycle at an intensity of 25 μE/m^2 s and aerated (filtered, 0.3 mL/min, without CO_2).

In iron-starved cultural experiment, to avoid the contamination of iron, water and all nutrient stocks except $MgSO_4\cdot 7H_2O$ and trace metals were treated with Chelex-100 resin. And all glassware was soaked in 3N HCl for two days and rinsed with Milli-Q water, which was treated by Chelex-100 resin (Chelex Milli-Q water) to remove residual iron. For the iron-deficient culture, $FeCl_3\cdot 6H_2O$ was added to a final concentration of 1.0×10^{-9} M and for the sufficient culture 1.0×10^{-6} M. The presence of siderophore in culture supernatant was determined by the liquid phase CAS assay on a 96-well microplate (50 μL each of sample and CAS assay solution). As the positive control for CAS assay, deferoxamine mesylate (Sigma) was used.

4.3. Isolation of anachelins

The iron-deficient culture (10 L) of *A. cylindrica* (NIES-19) was continuously centrifuged at 10,000 rpm to obtain the brownish supernatant, which was concentrated in vacuo and lyophilized. The resulting material was redissolved with Chelex Milli-Q water (50 mL) and placed on a YMC-ODS column (50×150 mm), which was previously treated with one column volume of EDTA solution (10 mg/mL) and washed with three column volume of Chelex Milli-Q water, and eluted with two column volume each of Chelex Milli-Q water, 20, 50 and 100% MeOH. The presence of siderophore was detected by the liquid phase CAS assay. The CAS active 50% MeOH fraction (160 mg) was lyophilized and the resultant material was redissolved in Chelex Milli-Q water and applied to a reversed-phase HPLC column (Cosmosil C_{18}, 10×250 mm; 12–16% MeCN containing 0.05% TFA in 16 min; flow rate 2.0 mL/min; UV detection at 210 nm) to yield anachelin-2 (**1**, 50.5 mg) and anachelin (**2**, 52.5 mg) as CAS active principles. Retention times (min) on HPLC: **1** (16.00), **2** (22.00).

4.3.1. Anachelin-2 (1). Colorless amorphous powder; $[\alpha]_D -65.7°$ (*c* 0.06, H_2O); UV (H_2O) λ_{max} 286 nm (ϵ 1800); HRFABMS *m/z* 761.3328 (M^+) calculated for $C_{35}H_{49}N_6O_{13}$ (Δ −2.9 mmu); For ^1H and ^{13}C data, see Table 1.

4.3.2. Anachelin (2). Colorless amorphous powder; $[\alpha]_D -34.8°$ (*c* 0.05, H_2O); UV (H_2O) λ_{max} 286 nm (ϵ 1900); HRFABMS *m/z* 761.3403 (M^+) calculated for $C_{35}H_{49}N_6O_{13}$ (Δ 4.5 mmu); For ^1H and ^{13}C data, see Table 2.

4.3.3. Ester intermediate of anachelin-2 (3). Colorless amorphous powder; $[\alpha]_D -18.2°$ (*c* 0.1, H_2O); UV (H_2O) λ_{max} 286 nm (ϵ 1800); HRFABMS *m/z* 779.3478 $(M^{2+}-H)^+$ calculated for $C_{35}H_{51}N_6O_{14}$ (Δ 1.5 mmu); For ^1H and ^{13}C data, see Table 1.

4.3.4. Ester intermediate of anachelin (4). Colorless amorphous powder; $[\alpha]_D -8.4°$ (*c* 0.1, H_2O); UV (H_2O) λ_{max} 286 nm (ϵ 1700); HRFABMS *m/z* 779.3484 $(M^{2+}-H)^+$ calculated for $C_{35}H_{51}N_6O_{14}$ (Δ 2.1 mmu); For ^1H and ^{13}C data, see Table 2.

4.3.5. Fragment-1 (6). Colorless solid; HRFABMS *m/z* 383.1919 (M^+) calculated for $C_{17}H_{27}N_4O_6$ (Δ −1.2 mmu).

4.4. Amino acid analysis of anachelins

Each solution of **1** and **2** (100 μg) in 6N HCl (500 μL) was heated to 110°C in a sealed tube for 16 h and then cooled. The solvent was removed in a stream of dry N_2, and the residue was dissolved in 0.02N HCl (500 μL) and subjected to amino acid analysis. Retention times (min) in amino acids analysis: Thr (15.12), Ser (16.17).

4.5. HPLC analysis of the Marfey's derivatives

To each acid hydrolysate of a 100 μg portion of **1** and **2**, L-FDAA in acetone (50 μL) and 1 M $NaHCO_3$ (100 μL) were added. The mixture was kept at 80°C for 3 min followed by neutralization with 2N HCl (50 μL). The reaction mixtures were dissolved in 50% MeCN and subjected to HPLC (Cosmosil C_{18} MS column, 4.6× 250 mm; 10–20% MeCN containing 0.1% TFA in 30 min; flow rate 1 mL/min, UV detection at 340 nm). The identity of each peak was confirmed by coinjection with a solution of a standard that had been derivatized in the same manner. But racemic Ser was not separated by this method. Retention times (min): L-Thr (23.2), L-*allo*-Thr (24.0), D-*allo*-Thr (26.6), D-Thr (28.8).

4.6. Chiral GC analysis

A solution of 10% HCl in MeOH was added to each 100 μg portion of **1** and **2** in vials and heated to 100°C for 2 h. The solvent was removed in a stream of N_2. Trifluoroacetic anhydrate (300 μL) in CH_2Cl_2 (300 μL) was added to the each residue, followed to heat to 100°C for 5 min in sealed vials. The solvent was removed in a stream of N_2. The residues were dissolved in CH_2Cl_2 (500 μL) and

immediately analyzed by chiral GC using Chirasil-Val capillary column. Column temperature was kept at 80°C for 5 min and then increased at a rate of 4°C/min to 120°C. The identity of each peak was confirmed by coinjection with a solution of a standard that had been derivatized in the same manner. Retention times (min): L-Thr (9.873), D-Ser (13.375), L-Ser (14.233).

4.7. Mild hydrolysis of anachelin-2 (1) and isolation of the fragment (6)

A solution of **1** (1.5 mg) in 6N HCl (500 μL) was heated to 100°C in a sealed tube for 45 min and then cooled. The solvent was removed in a stream of dry N_2, and the residue was dissolved in H_2O containing 0.05% TFA and subjected to HPLC (Cosmosil C_{18} MS column, 10.0×250 mm^2; 0–10% MeCN containing 0.05% TFA in 10 min; flow rate 2 mL/min, UV detection at 274 nm) to yield the fragment (**6**) containing Dmaq unit. Retention time (min) on HPLC: **6** (13.6). Amino acid analysis and chiral GC analysis of the hydrolysate of **6** was performed with same manner mentioned above.

4.8. Dansylation of the fragment (6)

To **6** (100 μg) dissolved in 0.2 M $NaHCO_3$ (50 μL), dansyl-chrolide solution (50 μL, 2.5 mg/mL in acetone) was added and placed at room temperature for 1 h. Reaction mixture was concentrated in vacuo, and the resulting material was dissolved in 6N HCl (500 μL) and kept to 110°C for 8 h, followed to subject to chiral GC analysis with same manner mentioned above.

Acknowledgements

This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan. Y. Itou is financially supported by Research Fellowship of Japan Society for the Promotion of Science for Young Scientists.

References

1. Neilands, J. B. *J. Biol. Chem.* **1995**, *270* (45), 26723.
2. (a) Martin, J. H. et-al., *Nature* **1994**, *371*, 123. (b) Coale, K. H. et-al., *Nature* **1996**, *383*, 495.
3. (a) Reid, T. R.; Butler, A. *Limnol. Oceanogr.* **1991**, *36*, 1783. (b) Reid, R. T.; Live, D. H.; Faulkner, D. J.; Butler, A. *Nature* **1993**, *366*, 455. (c) Martinez, J. S.; Zhang, G. P.; Holt, P. D.; Jung, H.-T.; Carrano, C. J.; Haygood, M. G.; Butler, A. *Science* **2000**, *287*, 1245.
4. Murphy, T. P.; Lean, D. R. *Science* **1976**, *192*, 900.
5. (a) Kerry, A.; Laudenbach, D. E.; Trick, C. G. *J. Phycol.* **1988**, *24*, 566. (b) Trick, C. G.; Kerry, A. *Curr. Microbiol.* **1992**, *24*, 241. (c) Wilhelm, S. W.; Trick, C. G. *Limnol. Oceanogr.* **1994**, *39*, 1979. (d) Wilhelm, S. W.; Maxwell, D. P.; Trick, C. G. *Limnol. Oceanogr.* **1996**, *41*, 89.
6. Simpson, F. B.; Neilands, J. B. *J. Phycol.* **1976**, *12*, 48.
7. Byers, B. R.; Powell, M. V.; Lankford, C. E. *J. Bacteriol.* **1967**, *93*, 286.
8. Schwyn, B.; Neilands, J. B. *Anal. Biochem.* **1987**, *160*, 47.
9. ^{15}N NMR data for anachelin-2 assigned from $^{1}H–^{15}N$ HMBC experiment: Thr [δ_N 117.5], Ser (1) [δ_N 115.0], Ser (2) [δ_N 114.2], Dmaq [δ_N 58.2 (N-1), δ_N 117.0 (N-3)]. In this experiment, unfortunately, the chemical resonance of the nitrogen in the oxazoline ring was not assigned since the HMBC cross-peak between this nitrogen and H-5, 6, and 7 of Atha was not clearly observed.
10. Vergne, A. F.; Walz, A. J.; Miller, M. J. *Nat. Prod. Rep.* **2000**, *17*, 99.
11. Elliott, D. F. *J. Chem. Soc.,* **1949**, 589.
12. Beiderbeck, H.; Taraz, K.; Budzikiewicz, H.; Walsby, A. E. *Z. Naturforsch.* **2000**, *55c*, 681.
13. Marfey, P. *Carlsberg Res. Commun.* **1984**, *49*, 591.
14. Hartley, B. S.; Massey, V. *Biochim. Biophys. Acta* **1956**, *21*, 58.