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Pseudoalterobactin A and B, New Siderophores Excreted by Marine Bacterium *Pseudoalteromonas* sp. KP20-4

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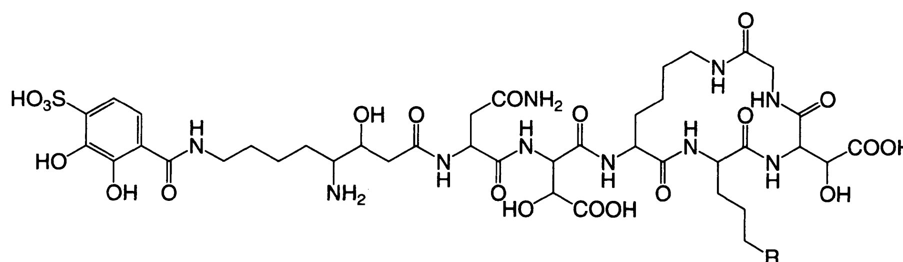
Siderophores are relatively low-molecular-weight compounds that typically have a very high affinity constant (10^{25}~10^{50})[1] for iron (Fe^{3+}), which is an essential element for most microorganisms owing to its importance in a variety of biochemical reactions. The role of siderophores is to scavenge extracellular iron from the environment and transport it into microbial cells. Hundreds of siderophores have been isolated from terrestrial, especially pathogenic, microorganisms, and their biosyntheses and iron-sequestering systems have been enthusiastically studied.[2~4] In spite of the high abundance of iron in the earth's crust, the dissolved iron concentration is particularly low (20 pM~1 nM)[5] in the surface water of the open ocean. In such an iron-deficient environment, marine bacteria are thought to get iron by siderophore-based iron-sequestering systems.[6] However, only a few studies concerning siderophores of marine bacteria have been done.[7~9,12] In the course of screening for new siderophores from marine bacteria, we found that *Pseudoalteromonas* sp. KP20-4 produced new siderophores. In this note, the isolation and structural determination of pseudoalterobactin A (**1**) and B (**2**), excreted by marine bacterium *Pseudoalteromonas* sp. KP20-4, are reported.

Pseudoalteromonas sp. KP20-4 was isolated from a marine sponge (*Cinachyrella australiensis*) obtained in the Republic of Palau. The bacterium was identified from the results of taxonomic studies and 16S rDNA sequence. All glassware used for the culture and for isolating the siderophore was washed with 6 N HCl and then rinsed with milli Q water to avoid iron contamination. *Pseudoalteromonas* sp. KP20-4 was cultured in an ASG medium (iron free) containing casamino acid (5 g/liter), glycerol (3 g/liter), glycerophosphate (0.1 g/liter), NaCl (15.5 g/liter), KCl (0.8 g/liter), $MgSO_4 \cdot 7H_2O$ (12.4 g/liter), $CaCl_2 \cdot 2H_2O$ (2.9 g/liter), NH_4Cl (1.0 g/liter), HEPES (10 mM), and $NaHCO_3$ (2 mM) at pH 6.8 (before being autoclaved) for 72 hours at 30°C with rotary shaking at 100 rpm. The culture broth was centrifuged at 8,000×*g* for 15 minutes at 4°C. The collected supernatant was batch loaded onto a Diaion HP20 (Mitsubishi Chemical Co.) column. After washing the resin with acidic water (pH 2, adjusted with conc. HCl), the Fe-binding fraction was eluted with methanol, the chrome azurol S (CAS) assay[10] being used as the index. This methanol fraction was chromatographed on a reverse-phase open column (Wakogel® 100C18, Wako Pure Chemical Industries, Ltd.), using stepwise-elution with acidified water, 20% methanol, and 50% methanol. The 20% methanol fraction including CAS-positive substances was further chromatographed on an LH20 column, using 50% methanol - water (pH 2.0) as the mobile phase. The CAS-positive fractions were collected and evaporated *in vacuo*. The resulting fraction was loaded into a reverse-phase HPLC (column: TSK gel ODS 80Ts, i.d. 7.8×300 mm, Tosoh Co.), eluting with 15%



Pseudoalterobactin A (**1**): R= CH_2NH_2

Pseudoalterobactin B (**2**): R= $NHC(NH)NH_2$

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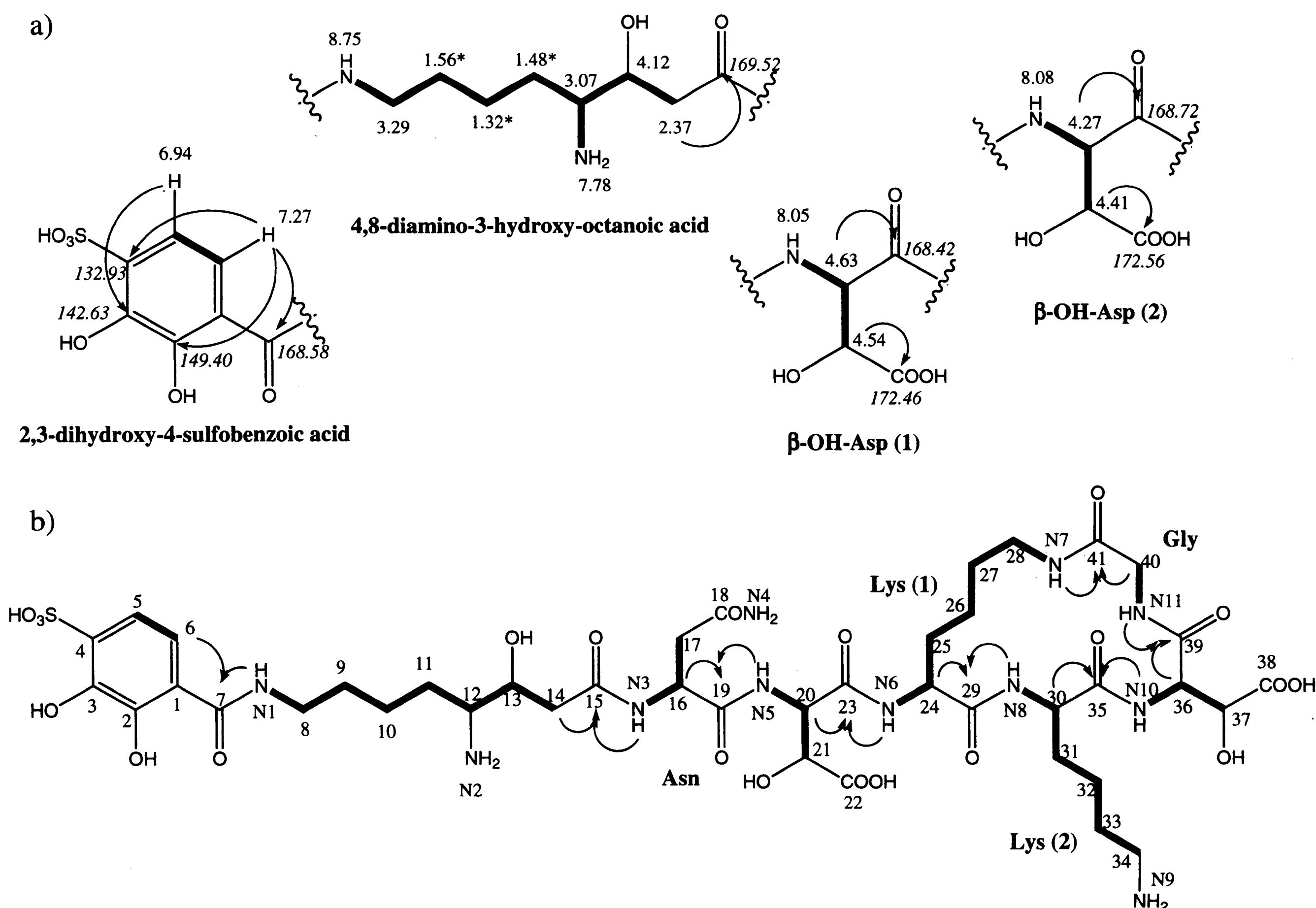
Fig. 1. Structural determination of pseudoalterobactin A (**1**) by NMR data.



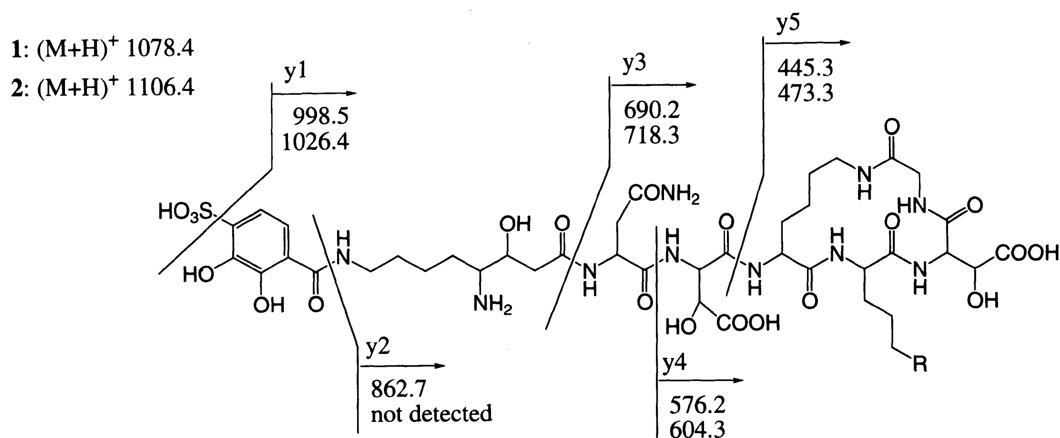
a) Partial structures determined from COSY, TOCSY and HMBC data. Numbers reveal ^{1}H chemical shifts, italic numbers reveal ^{13}C chemical shifts. Bold lines reveal the connections obtained from COSY or TOCSY data. Arrows indicate HMBC signals. * Chemical shifts were obtained from the HSQC spectrum.

b) Connection of the partial structures and amino acids. Bold lines reveal the connections obtained from COSY or TOCSY data. Arrows indicate HMBC signals.

acetonitrile - 0.1% TFA - water at a flow rate of 2 ml/minute with detection at UV λ 250 nm. The CAS-positive fractions were purified twice by HPLC, finally yielding **1** (8 mg) and **2** (6 mg) from 40 liters of the culture broth.

The ESI-MS spectrum of **1** displayed an $[M+H]^+$ ion at *m/z* 1078.4. The molecular formula of **1** ($C_{41}H_{63}N_{11}O_{21}S$) was established from ^{13}C-NMR and HR-FAB-MS data: $(M+H)^+$ peak at $m/z=1078.3986$ [calcd. for $C_{41}H_{64}N_{11}O_{21}S$, 1078.3999]. The ^{1}H- and ^{13}C-NMR spectra suggested that **1** had a peptide moiety. The results of an amino acid analysis indicated that **1** contained Asp (or Asn; HR-FAB-MS and MS/MS analyses described later indicate the presence of Asn) as well as Gly, Lys and an unusual amino acid. This unusual amino acid was clarified to be *threo*-β-hydroxy-Asp by an analysis of the NMR data (Figure 1a), and confirmed by its comparison with an authentic sample. Two partial structures, 4,8-diamino-3-hydroxyoctanoic acid and one-substituted (at the C-4 position) 2,3-dihydroxy benzoic acid, were constructed from 2D NMR spectra including COSY, TOCSY and HMBC (Figure 2a). The sequences of these two parts and the amino acids were determined by the correlation signals from the amide proton and the α-proton of amino acid to the neighboring carbonyl carbon in the HMBC spectra (Figure 1b). The HMBC spectra also indicated the ring structure by amide bond formation between ε-NH of Lys

Fig. 2. Characteristic fragmentation of pseudoalterobactin A (**1**) and B (**2**) in the ESI-MS/MS data.



The upper numbers correspond to **1**, and the lower to **2**.

(1) and the C-end of Gly. The ESI-MS/MS data for the protonated molecule exhibited the formation of Y-type ions (nomenclature according to ROEPSTORFF).[11] The characteristic fragmentation of **1** is shown in Figure 2. The fragmentation of **1** reveals that the substitution group at the C-4 position was sulfonic acid, and the fragment ion [y3: 690.2−y4: 576.2=114.0] indicates that the amino acid residue at this position was Asn. The fragmentation in the ESI-MS/MS data also exhibits the complete sequence of the 2,3-dihydroxy-4-sulfobenzoic acid, 4,8-diamino-3-hydroxyoctanoic acid, Asn, *threo*-β-hydroxy-Asp (1) and a cyclic peptide moiety composed of four amino acids (Lys (1), Lys (2), *threo*-β-hydroxy-Asp (2) and Gly), in agreement with the structure determined from the NMR data. These results established the planar structure of pseudoalterobactin A as **1**, the assignment of ^{1}H and ^{13}C chemical shifts being summarized in the Table 1.

The structure of pseudoalterobactin B (**2**) was determined by the same procedure as that used for **1**. The ESI-MS data for **2** displayed an $[M+H]^+$ ion at *m/z* 1106.5, and the molecular formula of **2** was determined to be $C_{41}H_{63}N_{13}O_{21}S$ (HR-FAB-MS data: $(M+H)^+$ peak at *m/z*=1106.4089; calcd. for $C_{41}H_{64}N_{13}O_{21}S$, 1106.4060). The amino acid analysis and NMR data for **2** indicate that **2** contained Asn (or Asp), *threo*-β-hydroxy-Asp, Lys, Arg and Gly. Two partial structures, 4,8-diamino-3-hydroxyoctanoic acid and 2,3-dihydroxy-4-sulfobenzoic acid, were constructed from the 2D NMR spectra. The connection of these two components and the amino acids was determined by the HMBC spectra and confirmed by ESI-MS/MS data; the ESI-MS/MS data also indicated the presence of Asn [y3: 718.3−y4: 604.3=114.0], as in the case of **1** (Figure 2). These data determined the planar structure of pseudoalterobactin B to be **2**, the assignment of ^{1}H and ^{13}C chemical shifts being summarized in the Table 1. The structural difference between **1** and **2** is only in the replacement of Lys(2) in **1** by Arg in **2**. An investigation of the absolute stereochemistry of **1** and **2** is now being undertaken.

REID *et al.* have reported the marine siderophores, alterobactins A and B, which had exceptionally high affinity for the ferric ion (affinity constant of $10^{49}\sim10^{53}$).[12] They speculated that this high affinity was derived from the coordination by a catechol and two β-hydroxy-Asp residues with the ferric ion. The structural resemblance between these alterobactins and pseudoalterobactins **1** and **2**, having a catechol and two β-hydroxy-Asp residues, led us to presume that the pseudoalterobactins would also have extraordinary affinity to the ferric ion. In fact, the psedoalterobactins exhibited strong activity comparable to that of enterobactin by a CAS assay, the affinity constant of enterobactin to the ferric ion being 10^{49}.[13] Both **1** and **2** exhibited an ED_{50} value, the concentration that reduced the absorbance at 630 nm of the CAS solution by 50% in 2 hours, of 20 μM under our assay conditions. Enterobactin and desferrioxamine B (affinity constant of 10^{31})[14] exhibited ED_{50} values of 60 μM and 500 μM, respectively, under the same conditions. Detailed analysis of the binding

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) chemical shift of pseudoalterobactin A and B (DMSO-d_6).

Pseudoalterobactin A (1)

position	^{13}C (δ ppm)	^{1}H (δ ppm) (multiplicity J (Hz))	HMBC(H→C)
2,3-dihydroxy-4-sulfobenzoic acid			
C1	115.41	—	
C2	149.40	12.51(-OH) (br s)	
C3	142.63	10.55(-OH) (br s)	
C4	132.93	—	
C5	115.37	6.94 (d 8.5)	C3
C6	115.77	7.27 (d 8.5)	C2, C4, C7
C7	168.58	—	
4,8-diamino-3-hydroxyoctanoic acid			
N1	—	8.75 (bt 5.5)	C7
C8	38.73	3.29 (m)	C9, C10
C9	28.58	1.55# (m)	
C10	22.36	1.31# (m)	
		1.40# (m)	
C11	26.31**	1.49# (m)	
C12	54.32	3.07 (m)	
N2	—	7.78 (bs)	
C13	67.19	4.12*** (m)	
C14	38.56	2.37 (d 6.0)	C12, C13, C15
C15	169.52	—	
Asparagine			
N3	—	8.32 (bd 8.0)	C15
C16	49.38	4.66 (dd 14.0, 7.0)	C15, C17, C18, C19
C17	36.93	2.5*	C16, C18, C19
		2.65 (dd 15.5, 7.0)	C16, C18, C19
C18	171.80	—	
N4	—	ND	
C19	170.83	—	
β-Hydroxyaspartic acid (1)			
N5	—	8.05 (bd 8.5)	C19
C20	55.72	4.63 (dd 8.5, 3.0)	C23
C21	69.97	4.54 (d 3.0)	C22
C22	172.46	—	
C23	168.42	—	
Lysine (1)			
N6	—	7.99 (bd 7.5)	C23
C24	54.51	4.07 (bdd 12.5, 7.5)	C25, C29
C25	30.58	1.51# (m)	
		1.64# (m)	
C26	21.20	1.20# (m)	
		1.29# (m)	
C27	27.40	1.35# (m)	
		1.38# (m)	
C28	37.27	2.88 (m)	
		3.26 (m)	
N7	—	7.17 (bt 5.5)	C41
C29	171.21	—	
Lysine (2)			
N8	—	7.65 (bd 10.5)	C29
C30	53.22	4.16*** (m)	C31, C35
C31	30.58	1.61# (m)	
		1.68# (m)	
C32	21.96	1.30# (m)	
C33	26.38**	1.50# (m)	
C34	38.58	2.77 (bd 6.0)	
N9	—	ND	
C35	172.60	—	
β-Hydroxyaspartic acid (2)			
N10	—	8.08 (bd 7.5)	C35
C36	55.56	4.27 (bt 7.5)	C37, C38, C39
C37	68.47	4.41 (d 6.0)	C36, C38, C39
C38	172.56	—	
C39	168.72	—	
Glycine			
N11	—	8.18 (bt 6.5)	C39
C40	42.95	3.32 (dd 16.0, 6.5)	C41
		3.91 (dd 16.0, 6.5)	C41
C41	168.11	—	

Pseudoalterobactin B (2)

position	^{13}C (δ ppm)	^{1}H (δ ppm) (multiplicity J (Hz))	HMBC(H→C)
2,3-dihydroxy-4-sulfobenzoic acid			
C1	115.45	—	
C2	149.35	12.52(-OH) (br s)	
C3	142.63	10.56(-OH) (br s)	
C4	132.93	—	
C5	115.38	6.95 (d 8.5)	C3
C6	115.75	7.25 (d 8.5)	C2, C4, C7
C7	168.54**	—	
4,8-diamino-3-hydroxyoctanoic acid			
N1	—	8.73 (bt 5.5)	C7, C8
C8	38.72	3.31 (m)	C7, C9, C10
C9	28.60	1.55# (m)	
C10	22.35	1.31# (m)	
		1.43# (m)	
C11	24.62	1.49# (m)	
C12	54.27	3.08*** (br d)	C11
N2	—	7.74 (bs)	
C13	67.18	4.12 (m)	
C14	38.50	2.36 (d 7.0)	C12, C13, C15
C15	169.51	—	
Asparagine			
N3	—	8.33 (bd 7.5)	C15, C16
C16	49.32	4.66 (dd 7.0, 6.5)	C15. C17, C19
C17	36.87	2.5*	C16, C18
		2.65 (dd 16.0, 6.5)	C16, C18
C18	171.73	—	
N4	—	ND	
C19	170.84	—	
β-Hydroxyaspartic acid (1)			
N5	—	8.03 (bd 7.5)	C19
C20	55.66	4.62 (dd 8.0, 2.5)	C23
C21	69.98	4.53 (d 2.5)	C22
C22	172.55	—	
C23	168.54**	—	
Lysine			
N6	—	7.98 (bd 7.0)	C23
C24	54.48	4.08 (bdd 13.0 7.5)	C25, C29
C25	30.44	1.53# (m)	
		1.64# (m)	
C26	21.18	1.18# (m)	
		1.28# (m)	
C27	27.43	1.34# (m)	
		1.36# (m)	
C28	37.31	2.91 (m)	
		3.22 (m)	
N7	—	7.16 (bt 6.0)	C28, C41
C29	171.23	—	
Arginine			
N8	—	7.69 (bd 7.5)	C29
C30	53.14	4.17 (m)	C35
C31	26.32	1.48# (m)	
		1.55# (m)	
C32	28.25	1.73# (m)	
C33	40.19	3.07*** (br d)	C34
N9	—	7.35 (bt 5.0)	
C34	156.32	—	
N10	—	ND	
N11	—	ND	
C35	172.28	—	
β-Hydroxyaspartic acid (2)			
N12	—	8.01 (bd 7.0)	C35
C36	55.56	4.29 (dd 7.0, 6.0)	C37, C38, C39
C37	68.50	4.41 (d 6.0)	C36, C38, C39
C38	172.49	—	
C39	168.68	—	
Glycine			
N13	—	8.21 (bt 6.0)	C39
C40	42.99	3.34 (dd 16.5, 5.5)	C39, C41
		3.91 (dd 16.5, 6.0)	C41
C41	168.10	—	

ND: not determined.

*: overlapping with DMSO. **, ***: overlapping with each other, interchangable.

#: Overapping. Chemical shifts were determined from HSQC and TOCSY spectra.

affinity between the ferric ion and pseudoalterobactins should be done.

The only current application of a siderophore to pharmaceuticals is · desferrioxamine B (desferal^{TM}). However, the recent application of a siderophore as the carrier for drug derivery systems is being studied,[15] and the immunosuppressive activity of the siderophores, IC202A, B and C, has been reported.[16~18] The detailed biological activities of pseudoalterobactins and their application to pharmaceutical uses should be examined.

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