Tenacibactins A−D, Hydroxamate Siderophores from a Marine-Derived Bacterium, *Tenacibaculum* sp. A4K-17

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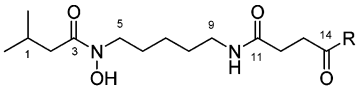
Received October 12, 2006

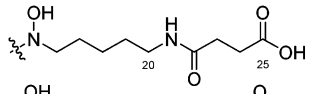
Four new hydroxamate siderophores, tenacibactins A−D (**1**−**4**), were isolated from a culture broth of the marine-derived bacterium *Tenacibaculum* sp. A4K-17. The structures of these tenacibactins were determined by NMR analyses and ESIMS/MS experiments. The iron-binding (chelating) activity of **1**−**4** was evaluated by the chrome azurol sulfonate (CAS) assay.

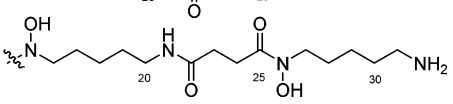
Iron is required by most living cells because of its diverse roles in important biological processes, such as DNA synthesis and repair, respiration, photosynthetic transport, nitrate reduction, nitrogen fixation, and detoxification of free radicals.[1] In spite of its high abundance in the earth's crust, the dissolved iron concentration is particularly low (<0.4 μM) in the surface waters of the open ocean.[2] Under such iron-limited conditions, marine bacteria utilize siderophores, high-affinity iron-chelating compounds, for their vital activity.[3] A wide range of structures have been reported for the siderophores produced by terrestrial and enteric bacteria.[4] Several siderophores have recently been isolated from marine-derived bacteria.[5] We have also been collecting bacteria from various marine environments, particularly those producing siderophores detectable by the CAS assay,[6] and have reported new catechol-type siderophores, named pseudoalterobactins A and B, from the marine bacterium *Pseudoalteromonas* sp. KP20-4.[7] During our subsequent screening for siderophores from marine bacteria, we found that the marine bacterium *Tenacibaculum* sp. A4K-17 produced four new hydroxamate siderophores. We describe in this paper the taxonomy, fermentation of *Tenacibaculum* sp. A4K-17, and isolation, structural determination, and iron-binding activity of tenacibactins A (**1**), B (**2**), C (**3**), and D (**4**).



Tenacibactin A (**1**) R= OCH_3

Tenacibactin B (**2**) R= OH





Results and Discussion

Strain A4K-17, producing siderophores **1**−**4**, was isolated from algae collected from Awajishima Island in Japan. Morphological, physiological, and phylogenetic properties enabled A4K-17 to be identified as the genus *Tenacibaculum*. The siderophores produced in cultures of *Tenacibaculum* sp. A4K-17 were isolated by chromatography on a porous-polymer resin Diaion HP20 column with gradient elution by aqueous MeOH, after removing the bacterial cells by centrifugation and acidifying the culture broth to pH 3. Using the CAS assay, the active fractions were further purified by preparative reversed-phase HPLC to afford four new siderophores, tenacibactins A−D (**1**−**4**).

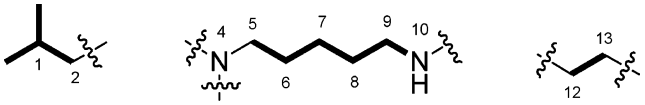


Figure 1. Partial structures ***a***−***c*** of tenacibactins A−D (**1**−**4**). Bold lines show proton spin networks.

Tenacibactin A (**1**) was obtained as a white powder, the molecular formula being established as $C_{15}H_{28}N_2O_5$ on the basis of high-resolution FABMS data [found *m*/*z* 317.2049 $(M + H)^+$] and NMR spectra. The ^{1}H NMR spectrum of **1** indicated the presence of one amide proton (δ 7.79 t), two methyl protons (δ 0.89 s and 0.88 s), one methoxy proton (δ 3.57 s), one methine proton (δ 2.00 m), and eight methylene protons (δ 3.46 t, 3.00 dt, 2.48 t, 2.34 t, 2.23 d, 1.50 m, 1.38 m, and 1.22 m). The ^{13}C NMR and HSQC spectra revealed the presence of 15 carbon signals comprising three carbonyl carbons (δ 173.4, 172.6, and 171.0), one methoxy carbon (δ 51.1), two methyl carbons (δ 22.5), six methylene carbons (δ 40.6, 29.9, 29.2, 28.7, 25.9, and 23.4), and two nitrogen-bearing methylene carbons (δ 46.9 and 38.4). These physicochemical properties and NMR data suggested that **1** was related to a ferrioxamine siderophore.[5,8]

Partial structures (***a***−***c***) shown in Figure 1 were elucidated by the interpretation of data from ^{1}H−^{1}H COSY and TOCSY experiments. Partial structure ***a*** was determined by the spin network from terminal dimethyl protons (δ 0.89 s and 0.88 s) to H-2 methylene protons (δ 2.23, 2H, d). The COSY and TOCSY signals revealed five contiguous methylenes in partial structure ***b*** and an isolated $-CH_2-CH_2-$ (δ 2.48, 2H, t and 2.34, 2H, t) in partial structure ***c***. The H-9 methylene protons were coupled to an amide proton (δ 7.79, NH-10), while the H-5 methylene protons appeared to be adjacent to a nitrogen atom on the basis of the ^{1}H and ^{13}C chemical shifts (δ_H/δ_C 3.46/46.9) in partial structure ***b*** (a cadaverine moiety). Partial structure ***c*** contained a succinyl moiety by HMBC correlations from H-12 and H-13 to two carbonyl carbons (δ 173.4 and 171.0). The methoxy group (δ 3.57, s) attached at C-14 was assigned on the basis of the HMBC correlations observed from these protons and C-14. The connectivity among these partial structures was elucidated by an HMBC experiment (Figure 2). The HMBC correlation from H-5 and H-2 to C-3 established the linkage between partial structures ***a*** and ***b***. The connectivity between partial structures ***b*** and ***c*** was confirmed by the HMBC correlation from an amide proton (δ 7.79, t, NH-10) and H-9 to C-11 through an amide bond. Taking the molecular formula of **1** into consideration, the remaining hydroxyl group (δ 9.48 brs) was assigned to a

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10.1021/np060502b CCC: $37.00
Published on Web 02/24/2007

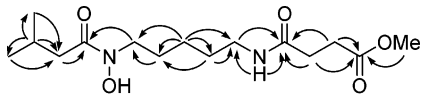

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Figure 2. HMBC correlations for tenacibactin A (**1**).

substituent at the N-4 position of the nitrogen atom, resulting in the hydroxamate moiety [−CO−N(OH)−].

Tenacibactin B (**2**) was also obtained as a white powder, the molecular formula being established as $C_{14}H_{26}N_2O_5$ on the basis of the HRFABMS data [found *m*/*z* 303.1882 $(M + H)^+$] and ^{13}C NMR spectra. This compound had similar ^1H and ^{13}C NMR spectra to those of **1**, except for the lack of the methoxyl group signal. Furthermore, compound **2** was 14 mass units less than that of **1** (HRFABMS *m*/*z* 317.2049). These data suggested the formation of a terminal hydroxyl group instead of a methoxyl group. Interpretation of the 2D NMR data allowed the assignment of partial structures ***a***−***c***, and further interpretation of the NMR data led to the structure of tenacibactin B (**2**).

Tenacibactin C (**3**) was obtained as a white powder. The molecular formula of **3** was established as $C_{23}H_{42}N_4O_8$ from the HRFABMS data (*m*/*z* 503.3079 $[M + H]^+$) and NMR data. The ^1H NMR spectrum was similar to that of **2**, except that integration of the 1D spectrum indicated the presence of 42 protons including four exchangeable ^1H signals (δ 9.58 NOH, 9.50 NOH, 7.78 NH, and 7.76 NH) observed in DMSO-d_6. Although the limited quantity of the sample precluded direct acquisition of a ^{13}C NMR spectrum, the ^{13}C chemical shifts were obtained by an analysis of the HSQC and HMBC data. Interpretation of the ^1H NMR, COSY, TOCSY, HSQC, and HMBC spectra revealed the presence of two succinyl (δ_H 2.25, 2H, t and 2.58, 2H, t; 2.25, 2H, t and 2.41, 2H, t) and two cadaverine moieties in the molecule. The HMBC correlation from H-23 to C-22 (δ 170.4) and from H-24 to C-25 (δ 173.5) established a newly appearing succinyl moiety (C-22−C-25). Thus, the structure of compound **3** was proposed as that shown in Figure 3.

Tenacibactin D (**4**) was obtained as a white powder. Its molecular formula was determined to be $C_{28}H_{54}N_6O_8$ by HRFABMS (*m*/*z* 603.4080 $[M + H]^+$) and NMR data. The ^1H NMR spectrum was very similar to that of **3** except for the presence of one more cadaverine moiety and one NH_2 (δ 7.62 brs) proton signal in the molecule. The ^{13}C NMR chemical shift assignments were conducted by an analysis of the HSQC and HMBC data. Careful interpretation of the 1D and 2D NMR spectra revealed the presence of a newly appearing terminal cadaverine moiety as $-NOH(CH_2)_5NH_2$. The spin network of five contiguous methylenes (H-27−H-31) was revealed by COSY and TOCSY correlations, in particular, by the COSY correlation from the terminal methylene protons (δ 2.75, H-31) to the NH_2 protons (δ 7.62 brs). The H-27 methylene protons appeared to be connected to a nitrogen atom on the basis of the ^1H and ^{13}C chemical shifts (δ_H/δ_C 3.47/46.9).

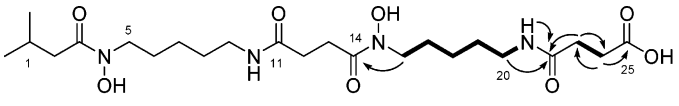
The proposed structures of **3** and **4** were supported by the ESIMS/MS data (see Supporting Information), which revealed the characteristic fragment peaks shown in Figure 4. The prominent fragment ions (**3**, *m*/*z* 201, 219, 285, and 301; **4**, *m*/*z* 201, 285, 319, 401, and 485) were strongly characteristic of these compounds, as has been reported for the prominent fragment ions in feroxamine-related compounds.[9]

The tenacibactins possessed Fe-chelating activity comparable to that of deferoxamine mesylate by a CAS assay. The ED_{50} values for tenacibactins C (**3**) and D (**4**) were 110 and 115 μM, similar to that of deferoxamine mesylate (ED_{50} of 120 μM), while tenacibactins A (**1**) and B (**2**) showed a weak color change, but both were inactive (ED_{50} >1 mM) under our assay conditions. These findings suggest that the number of hydroxamate moieties plays an importance role in the interaction of these compounds with iron. Although the chemical structures are closely related to those of feroxamine siderophores, tenacibactins A−D (**1**−**4**) are the first siderophores from a marine-derived bacterium of the genus *Tenacibaculum* to be structurally determined, to our knowledge. The filamentous organism *Tenacibaculum* sp. (especially *Tenacibaculum maritimum*) is the causative agent of marine flexibacterious or tenacibaculousis, which affects a large number of farmed marine fish throughout the world and thus has considerable economic significance to aquaculture producers.[10] Many bacterial pathogens, owing to their limited ability to take up iron during infection, secrete siderophores as an essential factor in their pathogenicity.[11] Tenacibactins A−D (**1**−**4**) are therefore likely to be involved in the pathogenicity of the genus *Tenacibaculum*.

Experimental Section

General Experimental Procedures. UV spectra were recorded with a Beckman DU 640 spectrometer and IR spectra with a JASCO FT/IR-430 instrument. The ^1H and all 2D NMR spectra were recorded with a Varian Unity INOVA 750 instrument at 750 MHz. The ^{13}C NMR spectrum was recorded on a Varian Unity INOVA 500 instrument at 125 MHz. Chemical shifts are referenced to the solvent peaks of δ_H 2.49 and δ_C 39.5 for DMSO-d_6. Low- and high-resolution mass spectrometric data were obtained on a JEOL JMS700 spectrometer. ESIMS/MS spectra were measured with a ThermoFinigan LCQ Advantage instrument.

Producing Microorganism and Taxonomy. The producing microorganism, strain A4K-17, was isolated from an alga (*Chondrus ocellatus*) collected at Awajishima Island, Japan. A pure culture of strain A4K-17 was preserved in 10% glycerol at −80 °C. Strain A4K-17 was characterized by its morphological, physiological, and biochemical properties, as well as by an analysis of the 16S rDNA sequence. The strain was grown at 30 °C for 2 days on MB2216 agar (Becton Dickinson, MD) for taxonomic characterization. Its morphological characteristics were observed with a BX50F4 optical microscope (Olympus, Tokyo, Japan). The physiological and biochemical properties were tested according to the method of Barrow et al.[12] with an API20NE kit (bioMériux, Lyon, France). Strain A4K-17 was a Gram-negative bacillus. The cell size was 0.3−0.4 × 3.0−5.0 μm. The colonies were smooth, circular, and yellow in color. Furthermore, a BLAST search of 16S rDNA sequences available in the DDBJ/EMBL/GenBank database showed the highest similarity of 95% to *Tenacibaculum marilutum* strain TF-53, *Tenacibaculum cellulophagum* strain D30, and *Tenacibaculum mesophilum*. Therefore, the strain was identified from the results of the taxonomic studies and 16S rDNA sequence (Supporting Information) as being of the genus *Tenacibaculum*. This strain has been deposited as NITE P-166 at the National Institute of Bioscience



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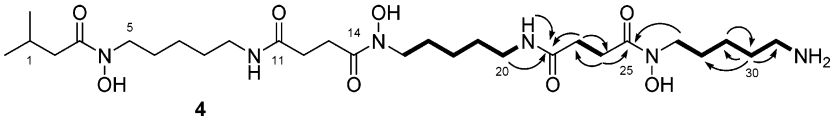

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Figure 3. Selected COSY (bold) and HMBC (arrows) correlations for tenacibactins C (**3**) and D (**4**).

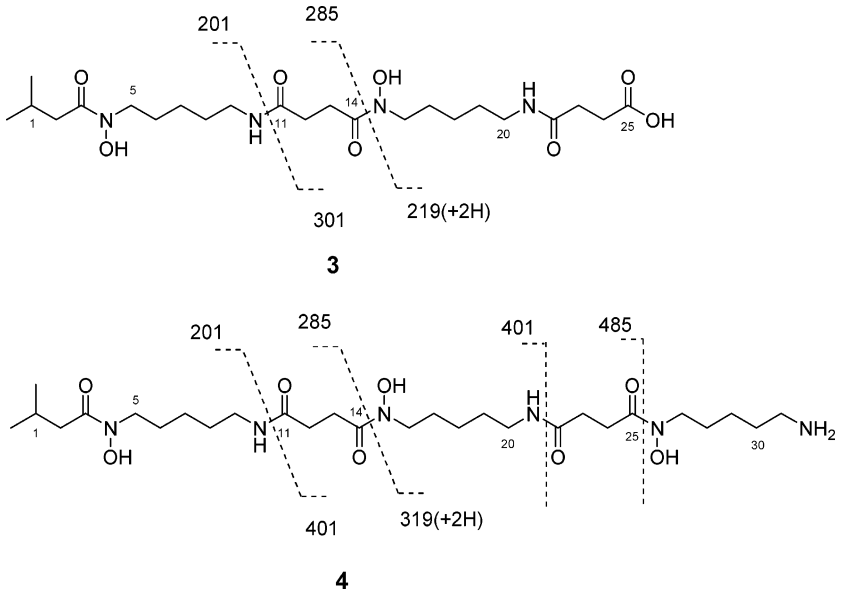

Figure 4. Fragmentation of tenacibactins C (**3**) and D (**4**) by ESIMS/MS.

Table 1. ^{1}H and ^{13}C NMR Data for **1** and **2** in DMSO-d_6

	1		**2**	
position	δ_C	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)
1	24.5	2.00, m	24.5	2.00, m
2	40.6	2.23, d, 6.75	40.6	2.23, d, 7.06
3	172.6		172.6	
4-NOH		9.48, brs		9.49, brs
5	46.9	3.46, t, 7.07	46.9	3.46, t, 7.07
6	25.9	1.50, m	25.9	1.50, m
7	23.4	1.22, m	23.4	1.22, m
8	29.2	1.38, m	28.8	1.38, m
9	38.4	3.00, dt, 6.7, 6.1	38.4	3.00, dt, 6.7, 5.7
10-NH		7.79, t, 6.1		7.77, t, 5.7
11	171.0		171.2	
12	29.9	2.34, t, 7.1	29.8	2.30, t, 7.4
13	28.7	2.48, t, 7.1	28.7	2.40, t, 7.4
14	173.4		174.2	
$-OCH_3$	51.1	3.57, s		
CH_3	22.5	0.89, s	22.5	0.89, s
CH_3	22.5	0.88, s	22.5	0.88, s

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Fermentation of *Tenacibaculum* sp. A4K-17. All glassware used for the culture and isolation of the siderophore was washed with 6 N HCl and then rinsed with Milli Q water to avoid any iron contamination. Strain *Tenacibaculum* sp. A4K-17 was cultured in 5 L of an ASG medium containing casamino acid (5 g/L), glycerol (3 g/L), glycerophosphate (0.1 g/L), NaCl (15.5 g/L), KCl (0.8 g/L), $MgSO_4 \cdot 7H_2O$ (12.4 g/L), $CaCl_2 \cdot 2H_2O$ (2.9 g/L), NH_3Cl (1.0 g/L), HEPES (2.6 g/L), $NaHCO_3$ (0.17 g/L), and 10 mM $FeCl_3$ (100 μL/L, final concentration of 1 μM) at pH 6.8 (before autoclaving) for 72 h at 30 °C with rotary shaking at 100 rpm.

Extraction and Isolation of Siderophores. The culture broth (5 L) was centrifuged at 8000*g* for 15 min at 4 °C. The resulting supernatant was acidified to pH 3 with concentrated 6 N HCl and applied to a column of Diaion HP20 (Mitsubishi Chemical Co.). The column was eluted with a stepwise gradient of aqueous MeOH (H_2O, 50% MeOH, and 100% MeOH, pH 3). The Fe-binding fraction was eluted with 50% MeOH using the CAS assay as the index. The 50% MeOH fraction was purified by HPLC (TSK gel ODS 80Ts, 2.0 × 25 cm, 10 mL/min, UV detection at 210 nm) with 5% MeCN in water for 5 min and then a linear gradient up to 50% over 50 min containing 0.1% TFA. The final purification was performed by RP-HPLC (TSK gel ODS 80Ts, 1.0 × 25 cm, 3 mL/min, UV detection at 210 nm) eluting with 20% MeCN containing 0.1% TFA to yield tenacibactins A (**1**, 0.6 mg), B (**2**, 0.5 mg), C (**3**, 0.2 mg), and D (**4**, 0.1 mg).

Table 2. ^{1}H and ^{13}C NMR Spectral Data for **3** and **4** in DMSO-d_6

	3		**4**	
position	δ_C[a]	δ_H (mult., *J* in Hz)	δ_C[a]	δ_H (mult., *J* in Hz)
1	24.1	2.00, m	24.2	2.00, m
2	40.2	2.22, d, 6.75	40.6	2.22, d, 6.75
3	171.6		171.9	
4-NOH		9.58[b]		9.58[d]
5	46.6	3.45, t, 7.07	46.9	3.45, t, 7.07
6	25.6	1.49, m	25.9	1.50, m
7	23.0	1.21, m	23.4	1.20, m
8	28.4	1.38, m	28.6	1.38, m
9	38.0	2.98, dt, 6.7, 6.1	38.2	3.00, dt, 6.7, 6.1
10-NH		7.78[c]		7.75
11	171.0		171.4	
12	29.8	2.25, t, 7.1	29.7	2.28, t, 7.1
13	27.4	2.58, t, 7.1	27.4	2.58, t, 7.1
14	171.6		171.8	
15-NOH		9.50[b]		9.50[d]
16	46.6	3.45, t, 7.07	46.9	3.45, t, 7.07
17	25.6	1.49, m	25.9	1.50, m
18	23.0	1.21, m	23.4	1.20, m
19	28.4	1.38, m	28.6	1.38, m
20	38.0	2.98, dt, 6.7, 6.1	38.2	3.00, dt, 6.7, 6.1
21-NH		7.76[c]		7.75
22	170.4		171.3	
23	29.8	2.25, t, 7.1	29.7	2.28, t, 7.1
24	28.8	2.41, t, 7.1	27.4	2.58, t, 7.1
25	173.5		171.9	
26-NOH				9.62[d]
27			46.9	3.47, t, 7.07
28			25.9	1.52, m
29			22.8	1.26, m
30			28.8	1.55, m
31			38.6	2.75, m
32-NH2				7.62, brs
CH_3	22.1	0.89, s	22.4	0.89, s
CH_3	22.1	0.88, s	22.4	0.88, s

[a] Chemical shifts were determined from the HSQC and HMBC spectra. [b] Interchangeable. [c] Interchangeable. [d] Interchangeable.

Tenacibactin A (1): white powder; UV λ_{max} (MeOH) (log ϵ) 205 (2.69) nm; IR (KBr) ν_{max} 3434, 1736, 1685, 1655, 1638, 1560, 1543, 1459, 1205 cm^{-1}; ^{1}H and ^{13}C NMR data (DMSO-d_6), see Table 1; FABMS (positive mode) *m*/*z* 317 $[M + H]^+$; HRFABMS *m*/*z* 317.2049 (calcd for $[C_{15}H_{28}N_2O_5 + H]^+$ 317.2076).

Tenacibactin B (2): white powder; UV λ_{max} (MeOH) (log ϵ) 205 (2.72) nm; IR (KBr) ν_{max} 3433, 1735, 1685, 1655, 1637, 1560, 1543, 1458, 1204 cm^{-1}; ^{1}H and ^{13}C NMR data (DMSO-d_6), see Table 1;

FABMS (positive mode) *m*/*z* 303 $[M + H]^+$; HRFABMS *m*/*z* 303.1882 (calcd for $[C_{14}H_{26}N_2O_5 + H]^+$ 303.1920).

Tenacibactin C (3): white powder; UV λ_{max} (MeOH) (log ϵ) 214 (3.14) nm; IR (KBr) ν_{max} 3448, 1735, 1685, 1638, 1560, 1543, 1509, 1459, 1204 cm^{-1}; ^1H and ^{13}C NMR data (DMSO-d_6), see Table 2; FABMS (positive mode) *m*/*z* 503 $[M + H]^+$; HRFABMS *m*/*z* 503.3079 (calcd for $[C_{23}H_{42}N_4O_8 + H]^+$ 503.3081).

Tenacibactin D (4): white powder; UV λ_{max} (MeOH) (log ϵ) 212 (3.12) nm; IR (KBr) ν_{max} 3448, 1735, 1685, 1655, 1637, 1560, 1543, 1509, 1458, 1206 cm^{-1}; ^1H and ^{13}C NMR data (DMSO-d_6), see Table 2; FABMS (positive mode) *m*/*z* 603 $[M + H]^+$; HRFABMS *m*/*z* 603.4080 (calcd for $[C_{28}H_{54}N_6O_8 + H]^+$ 603.4081).

CAS Assay. Siderophore activity was evaluated by the chrome azurol sulfonate (CAS) assay.[13] The principle of the assay is based on the color change of CAS from blue to orange, resulting from the removal of iron from CAS. Standard curves relating the CAS reactivity to iron-binding ligands were determined by using deferoxamine mesylate. The ED_{50} value is defined as the concentration of a compound that reduced the absorbance at 630 nm of the CAS solution by 50% in 4 h under our assay conditions.

Acknowledgment. We thank Assistant Professor R. Sakai of Kitasato University for the HRFABMS measurements. This work was performed as part of the project entitled "Constructing the Genetic Resource Library of Unidentified Microbes Based on Genome Information" supported by New Energy and Industrial Technology Development Organization of Japan (NEDO).

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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NP060502B