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Isolation and structure determination of new siderophore tsukubachelin B from *Streptomyces* sp. TM-74

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The new siderophore tsukubachelin B (**1**) was isolated from the iron-deficient culture medium of the newly isolated strain *Streptomyces* sp. TM-74. The chemical structure of tsukubachelin B (**1**) was established via interpretation of 2D nuclear magnetic resonance and electrospray ionization-mass spectroscopic data. The structure of tsukubachelin B (**1**) comprises 6 mol of amino acids, including 2 mol of serine and 1 mol each of ornithine, *N*-α-methyl-*N*-δ-hydroxy-*N*-δ-formylornithine, *N*-α-methyl-*N*-δ-hydroxyornithine, and cyclic *N*-hydroxyornithine.

Keywords: siderophore; *Streptomyces*; peptide; NMR spectrum

1. Introduction

Iron is essential for critical processes, such as respiration and DNA synthesis, in almost all life forms. Despite being one of the most abundant elements in the Earth's crust, the bioavailability of iron in many environments, such as the soil, is limited due to the very low aqueous solubility of the Fe^{3+} ion. Siderophore is defined as a low-molecular weight compound secreted by microorganisms to uptake ferric ion efficiently under poor iron environmental conditions. Siderophore chelates ferric ion with high affinity and establishes a soluble complex that can be effectively taken up via a specific membrane transporter (V. Braun & M. Braun, 2002). Iron uptake systems of pathogenic bacteria using siderophores have been well studied for the sake of establishing efficient clinical treatments, because iron acquisition is a critical process for pathogens to survive in host cells. In fact, the secretion of some siderophores was reported to enhance the virulence of the pathogenic bacteria (Miethke & Marahiel, 2007). Because of their biological significance, many siderophores, including pyochelin (Youard, Mislin, Majcherczyk, Schalk, & Reimmann, 2007), yersiniabactin (Lawlor, O'Connor, & Miller, 2007), petrobactin (Abergel, Zawadzka, & Raymond, 2008) and mycobactin (Luo, Fadeev, & Groves, 2005) have been isolated from pathogenic bacteria.

It has also been reported that actinomycetes produce a wide variety of siderophores (Oliveira, Batagov, Ward, Baganz, & Krabben, 2006; Schneider et al., 2007; Sontag et al., 2006). Desferroxiamine was isolated from *Streptomyces pilosus* and has been the effective medical reagent for curing hemochromatosis (Porter, 2001; Roosenberg, Lin, Lu, &

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Miller, 2000). Especially streptomycetes were reported to produce one group of structure-related siderophores that have three hydroxamate residues, including coelichelin (Challis & Ravel, 2000; Lautru, Deeth, Bailey, & Challis, 2005) and foroxymithine (Umezawa et al., 1985). These siderophores have 3 mol of hydroxamate moieties as the common functional structure to chelate ferric ion. Among them, it is of interest that foroxymithine was originally isolated as an angiotensin-converting enzyme (ACE) inhibitor in 1985. We previously found tsukubachelin, which also has ACE inhibitory activity, from *Streptomyces* sp. TM-34 (Kodani, Kameyama, Yoshida, & Ochi, 2011). As a result of further screening, we found tsukubachelin B, a related siderophore of tsukubachelin, from *Streptomyces* sp. TM-74. Herein, we describe the isolation and structure determination of tsukubachelin B (**1**, Figure 1a).

2. Results and discussion

The new bacterial strain TM-74 was isolated from the soil of the Tsukuba Mountain in Japan using ISP2 agar medium, as described in a previous report (Kodani et al., 2011). In the course of a screening search for new siderophores, the strain TM-74 was found to produce a potent amount of siderophores. To identify the genetic position of the strain TM-74, a sequencing analysis on the 16S rRNA coding gene was performed. The results of the sequencing analysis indicated that the genetic position of TM-74 is located in the genus *Streptomyces* (supplemental data, Figure S1), based on the data that were retrieved from the web database NCBI BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990). The strain TM-74 is closely related to *Streptomyces thermosacchari,* with a high identity of 99%. The 16S rDNA sequence of *Streptomyces* sp. TM74 was deposited in the DDBJ database under the accession number AB686269.

To isolate the new siderophore, cultivation of the TM-74 strain was performed with 1 L of iron-deficient media. The concentrated culture medium was subjected to HPLC purification to yield 6.0 mg of ferri-tsukubachelin B. Because the presence of ferric ion hampers the nuclear magnetic resonance (NMR) spectroscopic analysis, conversion of the ferric siderophore to a gallium ion complex via desferri-tsukubachelin was accomplished following a previously reported method (Kodani et al., 2011). As a result, desferri-tsukubachelin B (**1,** Figure 1a) and the gallium-complex of tsukubachelin B (Ga-tsukubachelin B) were obtained in yields of 2.0 and 1.6 mg, respectively.

Desferri-tsukubachelin B (**1**) was obtained as a colourless powder and had a molecular formula of $C_{29}H_{54}N_{10}O_{12}$ based on the high resolution electrospray ionization mass spectrometry (HR-ESIMS) analysis ($[M + H]^+$ at m/z 735.3975, calcd 735.4000). To obtain further information on the chemical structure of tsukubachelin B, analysis of NMR spectra, including 2D NMR measurements: correlation spectroscopy (COSY), total COSY (TOCSY), rotating-frame Overhauser effect spectroscopy (ROESY), heteronuclear multiple bond correlation (HMBC), and heteronuclear multiple quantum coherence (HMQC) were performed on Ga-tsukubachelin B dissolved in 0.5 mL of DMSO-d_6. The ^{1}H-NMR spectral data showed a peptidic nature, with peaks of several amide residues in the range of 8–9 ppm, and α-protons in the range of 4–5 ppm. From the interpretation of the COSY and TOCSY spectra, the proton spin system of each amino acid was constructed, as shown by the bold line in Figure 1(b). The assignments for the C-H spin system were performed by interpreting the HMQC data, and revealed that tsukubachelin B consists 6 mol of amino acids, including 2 mol of Serine (Ser) and 1 mol each of ornithine (Orn), *N*-α-methyl-*N*-δ-hydroxy-*N*-δ-formylornithine (*N*-Me hfOrn), *N*-α-methyl-*N*-δ-hydroxyornithine (*N*-Me hOrn) and cyclic *N*-hydroxyornithine (chOrn). The presence of a formyl residue was confirmed by the HMQC correlation of the protons (8.03 ppm) to the

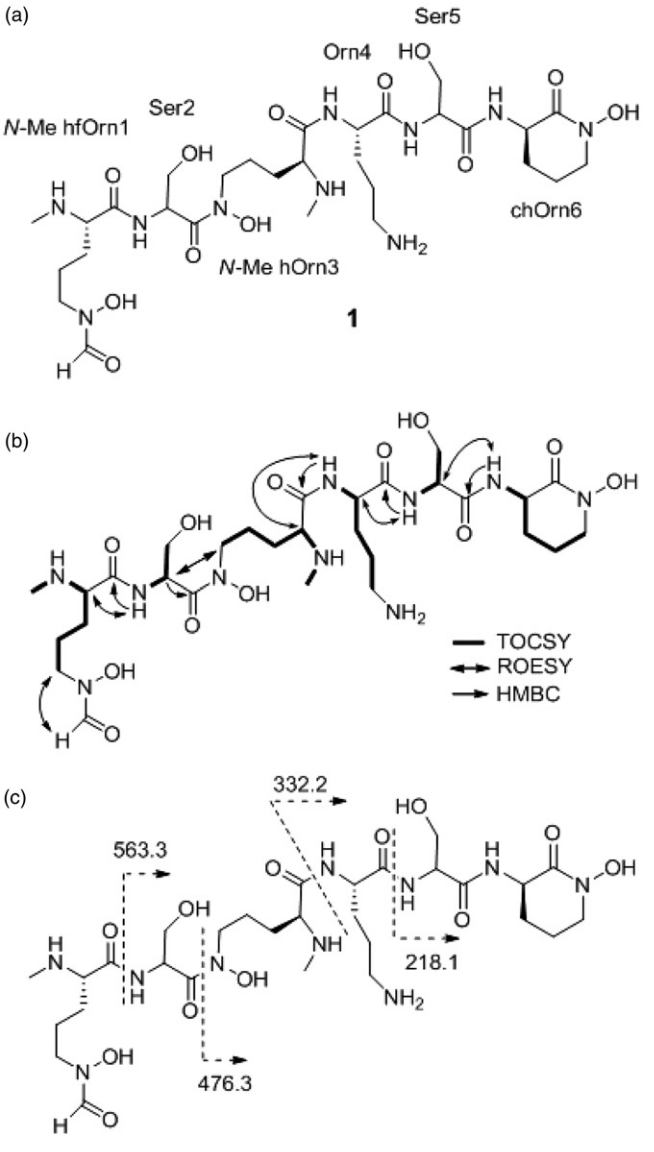

Figure 1. (a) Chemical structure of Tsukubachelin B (**1**). (b) Selected correlations of 2D NMR of **1.** (c) ESI-MS fragmentats of **1** by in-source CID experiment.

carbonyl carbon with a characteristic chemical shift value of 153.8 ppm. No correlation peaks to the carbonyl carbon in chOrn6 were observed in the HMBC experiment, but the similar chemical shift values indicated the presence of chOrn compared with the previously reported data for tsukubachelin (Kodani et al., 2011). The sequence of peptides was determined by ROESY correlations between the α-protons and amide protons, as shown by the two-ended arrow in Figure 1(b). Although the hydroxyl residues in *N*-Me hfOrn1, *N*-Me hOrn3, and chOrn6 were not detected by ^{1}H-NMR, the result of an in-source collision-induced dissociation (CID) experiment using ESI-time-of-flight (TOF) MS supported the positions of the hydroxyl residues. The fragmentation ion peak at m/z 563.3 indicated the removal of *N*-Me hfOrn1 (Figure 1c). The other fragmentation ion peaks at

m/*z* 476.3, 332.2 and 218.1 correspond to the truncated peptides of *N*-Me hOrn3-Orn4-Ser5-chOrn6, Orn4-Ser5-chOrn6 and Ser5-chOrn6, respectively.

To elucidate the absolute stereochemistries of the amino acids, the hydrolysate of ferri-tsukubachelin B was derivatised with *Nα*-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide (L-FDLA), and the derivative was subjected to HPLC analysis for comparison with the standard amino acid derivatives with L-FDLA or D-FDLA (Harada et al., 1996; Marfey, 1984). When HCl was used for the hydrolysis, 1 mol each of L-Ser, D-Ser, L-Orn, and a trace amount of D-Orn were detected. To obtain Orn derived from chOrn, and *N*-Me Orn derived from *N*-Me hfOrn and *N*-Me hOrn, hydrogen iodide (HI) was used for hydrolysation with reduction of the hydroxylamine (Okujo & Yamamoto, 1994). As a result, 1 mol each of L-Ser, D-Ser, L-Orn, D-Orn, and 0.5 mol of L-*N*-Me-Orn were detected. Considering that chOrn was converted to Orn by hydrolysis using HI, the stereochemistry of chOrn was elucidated to be the D-form, therefore the original Orn was determined to be the L-form. Regarding *N*-Me-Orn, the yield of L-FDLA derivatisation was low; however, only the L-form was detected, which determined the stereochemistry of the 2 mol of *N*-Me-Orn as the L-form. Although 1 mol each of L-Ser and D-Ser was determined to exist in the molecule, their specific positions in the molecule were not determined.

Because the structurally related compound desferri-tsukubachelin A was reported to be a moderate ACE inhibitor (Kodani et al., 2011), the inhibitory activity of desferri- and ferri-tsukubachelin B was tested. Ferri- and desferri-tsukubachelin B did not show inhibition at a concentration of 50 $\mu g\,mL^{-1}$.

3. Experimental

3.1. *General methods*

NMR spectra were obtained with a JEOL ECA-600 in DMSO-d_6 at 27.0°C. The resonances of the residual DMSO-d_6 at δ_H2.49 and δ_C39.5 were used as internal references for the ^{1}H- and ^{13}C-NMR spectra, respectively. ESI-TOF MS spectra were recorded using a JEOL JMS-T100LP mass spectrometer. For the CID experiment using the ESI-TOF MS spectrometer, orifice 1, orifice 2 and the ring lens voltage were set at 100, 10 and 12 V, respectively.

3.2. *Culture media*

The iron-deficient medium consisted of 2 g of K_2SO_4, 3 g of K_2HPO_4, 1 g of NaCl, and 5 g of NH_4Cl in 1 L of deionised water. To remove ferric ions, the solution was stirred with 50 g of the Na form of chelex-100 (Bio-Rad) for 16 h. The solution was filtered through filter paper (Whatman No.1) and stock solutions were then added: 100 μL of thiamine (20 $mg\,mL^{-1}$), 100 μL of $ZnSO_4 \cdot 7H_2O$ (20 $mg\,mL^{-1}$), 20 μL of $CuSO_4 \cdot 7H_2O$ (0.5 $mg\,mL^{-1}$), and 20 μL of $MnSO_4 \cdot 4H_2O$ (3.5 $mg\,mL^{-1}$), followed by autoclaving. Next, separately sterilised solutions (10 mL each) of $CaCl_2 \cdot H_2O$ (10 $mg\,mL^{-1}$), glucose (250 $mg\,mL^{-1}$) and 0.5% yeast extract (Difco) were added to the medium.

3.3. *Polymerase chain reaction amplification, sequencing and phylogenetic analysis of the 16 S rRNA genes*

Extraction of the total DNA from the cells of TM-74 was performed according to the previously reported procedure (Kodani, Imoto, Mitsutani, & Murakami, 2002). The 16S rRNA-encoding sequence was amplified from the total DNA via the Polymerase chain reaction (PCR) method using two sets of universal primer pairs (Baker, Smith, & Cowan, 2003): 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 926R (5′-CCGTCAATTCCTTT GAGTTT-3′), and 686F (5′-TAGCGGTGAAATGCGTAGA-3′) and 1510R (5′-GGC

TACCTTGTTACGA-3′). The reaction mixture for PCR was prepared by adding 1.0 μL of the total DNA of TM-74 (100 ng), 1.0 μL of Pfu DNA polymerase (Bioneer, Korea), 5.0 μL of X10 reaction buffer (Bioneer, Korea), 4 μL of 2.5 mM dNTPmix solution (Bioneer, Korea) and 31 μL of distilled water into the PCR reaction tube. PCR amplification was carried out with a thermal cycler using the following program: initial denaturation for 10 min at 94°C, followed by 34 cycles consisting denaturation for 40 s at 94°C, annealing for 60 s at 55°C, and DNA synthesis for 1 min at 72°C. A final extension of 5 min at 72°C was added at the end of the 34 cycles. The PCR product was purified with a QIAGEN PCR PURE kit following the manufacturer's instructions. The reactions for sequencing were performed using a Beckman DTCS-Quick Start Kit, also following manufacture's instructions. The four primers used for the reacton included 339F (5′-CTCCTACGGGTGAGTAACAC-3′), 536R (5′-GTATTACCGCGG CTGCTG-3′), 686F (5′-TAGCGGTGAAATGCGTAGA-3′) and 1099F (5′-GCAACGAGCGCAACC C-3′). The sequencing was performed with the CEQ8000XL capillary DNA sequencer (Beckman Coulter). The obtained sequence was used with the multiple-alignment program ClustalX (Larkin et al., 2007) for the construction of the phylogenetic tree.

3.4. *Isolation of tsukubachelin B*

Streptomyces sp. TM-74 was cultured in 1 L of iron-deficient media for six days. The culture medium was harvested by filtering through filter paper (Whatman No. 1). Next, 0.5 mL of 1 M $FeCl_3$ was added to the medium and the solution was evaporated using a rotary evaporator to concentrate it to a final volume of 20 mL. The concentrated solution was centrifuged at 3000 rpm for 10 min and then filtered through a membrane filter (Millipore, 0.45 um pore size) to remove insoluble materials. HPLC purification was then performed on a C18 Semi-Prep column (10 mm × 250 mm, Capcell Pak C18 UG80, Shiseido) eluted with 2% MeCN/98% water containing 0.05% TFA and monitored at a UV-Vis absorbance 435 nm to yield 6.0 mg of ferri-tsukubachelin.

3.5. *Conversion of ferri-tsukubachelin B into Ga-tsukubachelin B via desferri-tsukubachelin B*

Ferri-tsukubachelin B (5.0 mg) was dissolved in 3 mL of water. The solution was mixed with 3 mL of 1 M 8-quinolinol and stirred at room temperature for 30 min. Partitioning of the two layers was performed a total of three times using 6 mL of $CHCl_3$ each time to remove ferri-8 quinolinol. The water layer was immediately collected and lyophilised in a freeze-dryer. After dissolving the dry material in 2 mL of water, HPLC purification was performed on a C18 Semi-Prep column (10 mm × 250 mm, Capcell Pak C18 UG80, Shiseido) eluted with 3% MeCN/97% water containing 0.05% TFA and monitored at a UV-Vis absorbance of 215 nm to yield 4.0 mg of desferri-tsukubachelin B. Desferri-tsukubachelin B (2.0 mg) was dissolved in 2 mL of distilled water, and 10 mg of gallium chloride was added to form Ga-tsukubachelin. After HPLC purification in the same manner as described above, 1.6 mg of Ga-tsukubachelin B was obtained.

^{1}H-NMR chemical shift values of Ga-tsukubachelin B in DMSO-d_6: *N*-Me hfOrn1, *N*-CH_3; 2.47 (m), NH; 8.75 (br), 8.98 (br), H-*α*; 3.85 (m), H-*β*; 1.30 (m), 1.87 (m), H-*γ*; 1.60 (m), H-*δ*; 3.40 (m), H-formyl; 8.03 (s), Ser2, NH; 8.97 (d, 8.3 Hz), H-*α*; 4.77 (m), H-*β*; 3.50 (m), 3.68 (m), OH; 5.20 (br), *N*-Me hOrn3, *N*-CH_3; 2.45 (m), NH; 8.90 (br), 9.32 (br), H-*α*; 3.84 (m), H-*β*; 1.76 (m), H-*γ*; 1.58 (m), H-*δ*; 3.20 (m), 4.18 (m), Orn4, NH; 8.84 (d, 7.5 Hz), H-*α*; 4.55 (m), H-*β*; 1.68 (m), H-*γ*; 1.54 (m), H-*δ*; 2.79 (m), NH_2; 7.85 (br), Ser5, NH; 8.28 (d, 8.9 Hz), H-*α*; 4.28 (m), H-*β*; 3.55 (m), H-*γ*; 3.70 (m), OH; 4.99 (br), chOrn6, NH;

7.90 (d, 8.9 Hz), H-α; 4.54 (m), H-β; 1.78 (m), H-γ; 1.80 (m), 2.00 (m), H-δ; 3.45 (m), 3.53 (m).

^{13}C-NMR chemical shift values of Ga-tsukubachelin B in DMSO-d_6: *N*-Me hfOrn1, *N*-CH_3; 31.4, CO; 168.3, C-α; 60.0, C-β; 26.1, C-γ; 18.0, C-δ; 50.8, formyl; 153.8, Ser2, CO; 175.0, C-α; 50.6, C-β; 61.2, *N*-Me hOrn3, *N*-CH_3;31.4, CO; 168.0, C-α; 60.0, C-β; 27.6, C-γ; 24.3, H-δ; 51.2, Orn4, CO; 171.1, C-α; 53.6, C-β; 30.9, C-γ; 23.2, C-δ; 39.3, Ser5, CO; 170.2, C-α; 56.0, C-β; 62.7, chOrn6, C-α; 46.8, C-β; 20.0, C-γ; 19.8, C-δ; 50.8.

3.6. *Modified Marfey method*

Ferri-tsukubachelin B (0.2 mg) was subjected to acid hydrolysis at 110°C for 16 h with 6 N HCl or 50% HI solution (0.5 mL), and then the hydrolysates were dried using a rotary evaporator and resuspended in 1 M $NaHCO_3$ (200 µL). To the hydrolysate, 10 µL of a solution of *N*α-(5-fluoro-2,4-dinitrophenyl)-L-leucinamide (L-FDLA, Sigma-Aldrich) or D-FDLA (Sigma-Aldrich) in acetone was added at a concentration of 10 mg mL^{-1} along with 100 µL of 1 M $NaHCO_3$, after which the mixtures were heated to 80°C for 3 min. The reaction mixtures were cooled, neutralised with 2 N HCl (50 µL), and diluted with MeCN (200 µL). About 10 µL of each solution of FDLA derivatives was subjected to HPLC analysis using a C18 column (Cosmosil MSII, 4.6 mm × 50 mm). The elution was performed at a flow rate of 1 mL min^{-1} using solvent A (distilled water containing 0.05% TFA) and solvent B (MeCN containing 0.05% TFA) with a linear gradient mode from 0 to 60 min and increasing solvent B at a ratio of 1% per min. The UV/VIS detector was set at a wavelength of 340 nm. The retention times (min) of the L- and D-FDLA derivatised amino acids were as follows; L-Ser-L-FDLA (44.8 min), L-Ser-D-FDLA (45.5 min), L-Orn-L-FDLA (39.4 min), L-Orn-D-FDLA (36.6 min), L-*N*-Me Orn-L-FDLA (37.1 min), and L-*N*-Me Orn-D-FDLA (36.8 min).

3.7. *Inhibitory test of ACE*

ACE inhibitory activity was determined using the modified method of Cushman and Cheung (1971). The enzyme ACE was dissolved in distilled water at 8 mU mL^{-1}. The substrate hippuryl-L-histydyl-L-leucine was dissolved in water at a concentration of 5 mM. The test solution (30 µL) was added to 70 µL of the substrate solution, followed by addition of 100 µL of the enzyme solution. The mixture was incubated at 37°C for 30 min. The enzyme reaction was terminated by adding 300 µL of 0.5 N HCl. The hippuric acid was extracted with 1.5 mL of ethyl acetate. The ethyl acetate layer (0.5 mL) was evaporated by heating at 100°C for 30 min. The hippuric acid was then redissolved in 3 mL of 1 M NaCl, and the UV absorbance at 228 nm was measured to calculate the inhibitory concentration.

Supplementary material

Figures S1–S10 relating to this article are available online.

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