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A New Siderophore Isolated from *Streptomyces* sp. TM-34 with Potent Inhibitory Activity Against Angiotensin-Converting Enzyme

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A new siderophore named tsukubachelin was isolated from an iron-deficient culture medium of newly isolated strain Streptomyces sp. TM-34. The chemical structure of tsukubachelin was established by the interpretation of 2D NMR and TOF-Mass spectroscopic data. The structure of tsukubachelin consists of six amino acid residues, including three serine, and one each of *N*-α-methyl-*N*-δ-hydroxy-*N*-δ-formylornithine, *N*-α-methyl-*N*-δ-hydroxyornithine, and cyclic *N*-hydroxyornithine. Because the structurally related siderophore, desferri-foroximithine, was reported to have potent angiotensin-converting enzyme inhibition activity, the inhibitory activity of desferri-tsukubachelin and desferri-foroximithine were tested for structure–activity comparison. Desferri-tsukubachelin showed 14 times more potent inhibitory activity than desferri-foroximithine. This result indicates that desferri-tsukubachelin may become a promising agent for angiotensin-converting enzyme inhibition.

Introduction

Siderophore is defined as a low molecular weight compound that is secreted by microorganisms to uptake ferric ions efficiently under low-iron stress. Ferric iron has very low solubility at neutral pH and, therefore, cannot be utilized by microorganisms in this form. Siderophore chelates the ferric ion with high affinity and establishes a soluble complex that can be taken up by a specific membrane transporter.[1] It has been reported that actinomycetes produce a wide variety of siderophores.[2] Desferroxiamine was isolated from *Streptomyces pilous* and has been used as an effective medical agent to cure hemochromatosis.[3] It has been found that streptomycetes produce a group of structurally related siderophores, including coelichelin,[4] and foroxymithine (**1**; Figure 1).[5] Among them, it is of interest that foroxymithine was originally isolated as an angiotensin-converting enzyme (ACE) inhibitor in 1985.

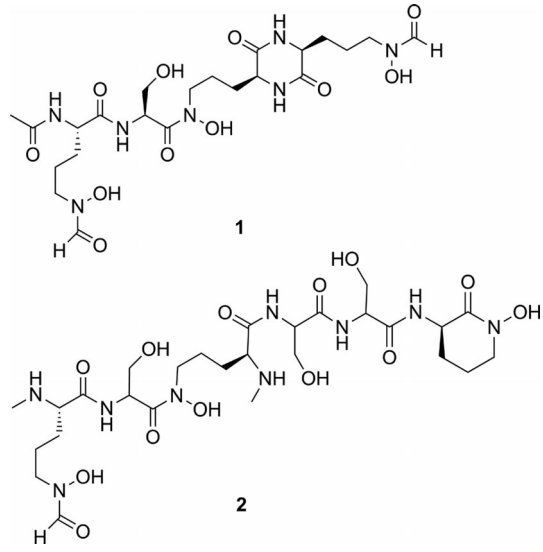

Figure 1. Chemical structures of foroxymithine (**1**) and tsukubachelin (**2**).

ACE, which catalyses the conversion of angiotensin I into angiotensin II by cleaving the C-terminal histidyl–leucine dipeptide, is a key molecule in the rennin–angiotensin system.[6] Angiotensin II works as a potent vasoconstrictor throughout the human body,[7] and it also has other important biological functions, such as a constrictor of glomerular arterioles in the kidney.[8] Therefore, ACE has been an attractive target for screening trials to find suitable inhibitors.

Several promising ACE inhibitors, including ancovenin,[9] muraceins,[10] L-681,[11] L-176,[11] I5B2,[12] and phenacein[13] have been isolated from actinomycetes to date.

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The potential of actinomycetes as a biological source prompted us to search for a new siderophore and to explore the possibility that the siderophore may be a candidate for an enzyme inhibitor. Here, we describe the exploration and structure determination of a new siderophore, tsukubachelin (**2**; Figure 1), and detail its inhibitory activity against ACE.

Results and Discussion

The new bacterial strain TM-34 was isolated from soil of the Tsukuba Mountain in Japan using ISP2 agar medium[14] along with 50 other bacterial strains. With the aim of screening for siderophore, these strains were cultured in iron-deficient liquid media for four days. After removal of the cells, each culture medium was added to a $FeCl_3$ solution to generate the ferric complex of siderophore. Each solution was screened for siderophore by HPLC analysis with UV/Vis detection at 435 nm. As a result, strain TM-34 was found to produce a significant amount of siderophore. To identify the genetic position of strain TM-34, sequencing analysis on the 16S rRNA coding gene was performed. Based on the standard PCR method with universal primers for bacterial 16S rRNA gene,[15] the complete length of 16S rRNA gene was amplified. The sequence analyses were accomplished using an automated DNA sequencer with eight universal primers. The obtained sequence was used to construct a phylogenetic tree with the multiple-alignment program ClustalX.[16] As shown in Figure 2, the genetic position of TM-34 is located in the genus of Streptomyces, and is closely related to Streptomyces prunicolor with a high similarity of 99%.

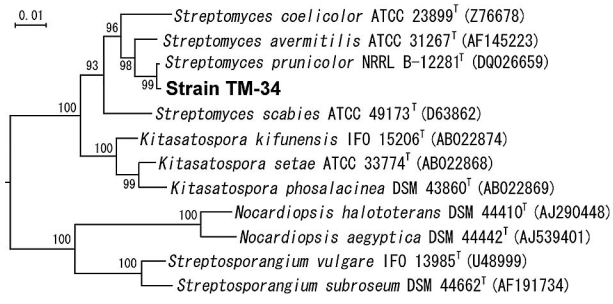

Figure 2. Phylogenetic position of strain TM-34.

To purify the new siderophore, large-scale cultivation of the TM-34 strain was performed with 1 L of iron-deficient media. To avoid contamination of ferric ion, flasks and funnels made of polystyrene were used to culture and harvest the cells. After cultivation for six days, the bacterial cells were removed from the culture media by filtration. A solution of $FeCl_3$ (1 M, 0.1 mL) was added to the culture media to generate the complex of siderophore with ferric ion. The culture media was concentrated to a volume of 50 mL by rotary evaporation. The concentrated solution was centrifuged at 3000 rpm for 10 min and filtered using a membrane filter to remove insoluble compounds. The solution was subjected to HPLC purification to yield 2.5 mg of the ferri-tsukubachelin (Figure 3). Because the presence of ferric ion hampers NMR spectroscopic analysis, the conversion of the ferric siderophore into the corresponding gallium ion complex via desferri-tsukubachelin was accomplished by a previously described method.[17] As a result, 1.5 mg of gallium-chelating tsukubachelin was obtained after HPLC purification.

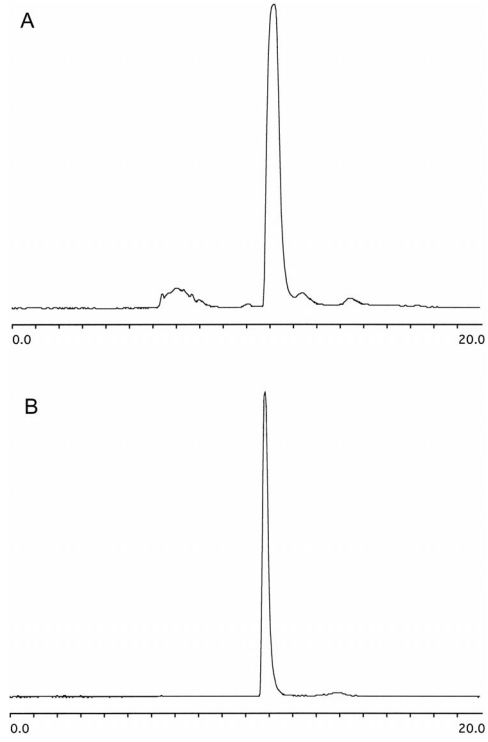

Figure 3. HPLC separation of ferri-tsukubachelin. (A) Crude ferri-tsukubachelin; (B) purified ferri-tsukubachelin.

To obtain information on the chemical structure of tsukubachelin (**2**; Figure 1), NMR spectroscopic analyses, including 2D NMR measurements such as DQF-COSY, TOCSY, ROESY, HMBC, and HSQC were performed on the gallium complex of tsukubachelin dissolved in 0.5 mL of [D_6]DMSO. The ^{1}H NMR spectroscopic data revealed a peptidic nature, with peaks of several amide residues over the region δ = 8–9 ppm and α-protons over the range δ = 4–5 ppm (Figure 4). By interpretation of the DQF-COSY and TOCSY proton spin systems, the connectivity of each amino acid was constructed as shown by the bold line in Figure 5. The assignments of the C–H spin system were performed on the basis of the HSQC data (Table 1), and revealed that the molecular structure of tsukubachelin consisted of six amino acid residues, including three Serine and one each of *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). As shown in Figure 5, the HMBC correlations from the α-proton or amide proton to the carboxyl carbon (one head arrow) were used to establish the connections between *N*-Me hfOrn1/Ser2, Ser4/Ser5, and Ser5/chOrn6. The presence of a formyl residue was confirmed by the HMBC correlation from the

δ-protons (δ = 3.38 and 3.47 ppm) to the carbonyl carbon with a characteristic chemical shift value of δ = 152.7 ppm. The moiety of chOrn6 was determined from the HMBC correlations between the α- and γ-protons to the carbonyl carbon. The connection between *N*-Me hOrn3 and Ser4 was elucidated by the NOESY correlation between the α-proton of *N*-Me hOrn3 and the amide proton of Ser4.

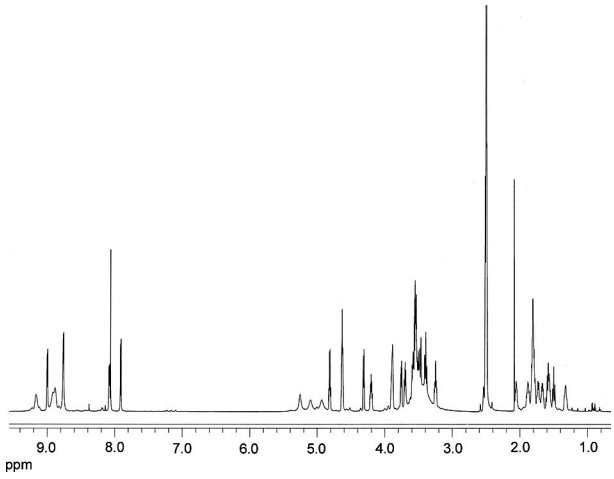

Figure 4. ^{1}H NMR spectrum of Ga-tsukubachelin in [D_6]DMSO.

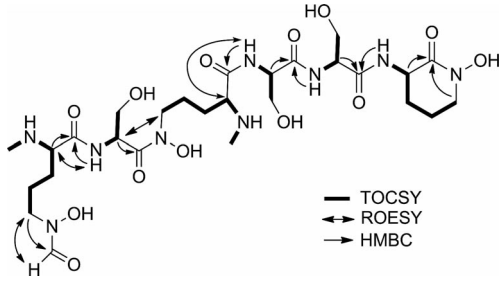

Figure 5. Selected correlations in 2D NMR spectra of tsukubachelin.

The hydroxyl residues in *N*-Me hfOrn1, *N*-Me hOrn3, and chOrn6 were not detected by ^{1}H NMR analysis, however the result of an ESI-MS/MS experiment supported the indicated positions of the hydroxyl residues (Figure 6). The major fragmentation ion peaks at *m*/*z* 536.25, 449.23, and 305.14 were observed, which corresponded to the fragmentations of *N*-Me hfOrn (172 Da), Ser (87 Da), and *N*-Me hOrn (144 Da). The molecular formula of tsukubachelin was determined as $C_{27}H_{49}N_9O_{13}$ by HRMS (ESI) analysis of desferri-tsukubachelin, with $[M + H]^+$ at *m*/*z* 708.3526 corresponding to the molecular formula of $C_{27}H_{50}N_9O_{13}$ (protonated MW, *m*/*z* calcd. 708.3522). By combining the above data, the structure of tsukubachelin **2** was determined to be as depicted in Figure 1.

To elucidate the absolute stereochemistries of the amino acids, the hydrolysate of desferri-tsukubachelin was derivatized with *N*-α-(5-fluoro-2,4-dinitrophenyl)-L-valinamide (L-FDVA),[18] and the derivative was subjected to HPLC analysis to compare with standard amino acid derivatives

Table 1. NMR chemical shift values of Ga-tsukubachelin in [D_6]-DMSO.

Residue	Position	^{1}H NMR [δ] (*J* in Hz)	^{13}C NMR [δ]
N-Me hfOrn1	N-CH_3	2.50 (m)	31.5
	NH	8.74 (br.), 8.91 (br.)	
	CO		167.5
	α	3.89 (m)	59.5
	β	1.49 (m)	25.3
		1.87 (m)	
	γ	1.32 (m)	17.5
		1.58 (m)	
	δ	3.38 (m)	49.8
		3.47 (m)	
	formyl	8.05(s)	152.7
Ser2	NH	8.99 (d, 8.21 Hz)	
	CO		160.4
	α	4.80 (q, 7.39 Hz)	49.7
	β	3.52 (m)	60.3
		3.69 (dd, 10.59, 7.51 Hz)	
	OH	5.24 (br.)	
N-Me hOrn3	N-CH_3	2.48 (m)	31.2
	NH	8.87 (br.), 9.15 (br.)	
	CO		167.1
	α	3.90 (m)	60.2
	β	1.72 (m)	27.3
		1.78 (m)	
	γ	1.55 (m)	24.3
		1.66 (m)	
	δ	3.24 (ddd, 14.03, 8.78, 5.44 Hz)	50.4
		4.19 (td, 14.03,6.98 Hz)	
Ser4	NH	8.75 (d, 7.37 Hz)	
	CO		169.2
	α	4.62 (m)	55.6
	β	3.52 (m)	62.0
	OH	5.11 (br.)	
Ser5	NH	8.07 (d, 9.24 Hz)	
	CO		169.8
	α	4.30 (m)	55.3
	β	3.55 (m)	62.0
		3.75 (dd, 10.71,4.65 Hz)	
	OH	4.91 (br.)	
chOrn6	NH	7.90 (d, 8.76 Hz)	
	CO		159.2
	α	4.61 (m)	46.0
	β	1.80 (m)	26.7
	γ	1.80 (m)	18.5
		2.05 (m)	
	δ	3.49 (m)	49.9
		3.58 (m)	

with L-FDVA or D-FDVA. To obtain Orn and *N*-Me Orn, hydrogen iodide (HI) was used for complete hydrolysis. As a result, 1 mol each of D-Orn and L-Ser, 2 mol of D-Ser, and 0.5 mol of *N*-Me-Orn were detected. Considering that chOrn was converted into Orn by hydrolysis, the stereochemistry of chOrn was reasoned to be in the D-form. Regarding L-Me-Orn, although the yield of L-FDVA derivatization was low, only the L-form was detected, which determined the stereochemistries of the 2 mol of *N*-Me-Orn to be in the L-form. Although 2 mol of L-Ser and 1 mol of D-

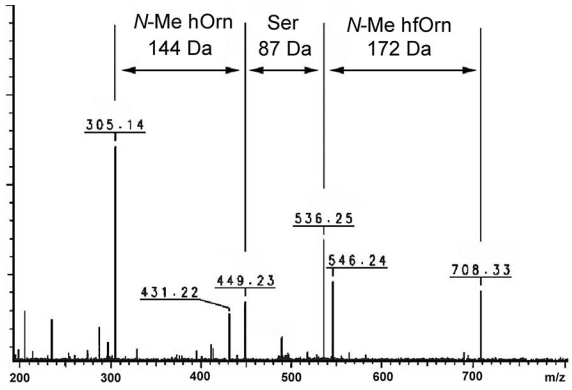

Figure 6. ESI MS/MS spectrum of desferri-tsukabachelin.

Ser were determined to exist in the molecule, the specific positions of L- and D-Ser in the molecule were not elucidated.

With respect to the inhibitory activity against ACE,[19] desferri-tsukubachelin showed the most potent inhibitory activity, with an IC50 value of 1.4 μg/mL. For structure–activity comparison, the IC50 value of authentic desferrioroxymithine was also determined to be 20.1 μg/mL in the same experiment. Because the inhibitory activity of desferri-tsukubachelin was 14 times more potent, tsukubachelin may be promising reagent as an ACE inhibitor. The X-ray crystallographic analysis of human ACE revealed that it has a structural similarity to zinc metalloproteases,[20,21] so there was the possibility that the inhibition was caused by deprival of zinc from ACE. Umezawa et al. reported that the ACE inhibition activity of foroxymithine was completely abolished upon addition of $FeCl_3$ and partially abolished with $ZnCl_2$.[5] In this experiment, ferri-tsukubachlin did not show inhibitory activity at a concentration of 100 μg/mL.

As shown in Figure 1, tsukubachelin (**2**) and foroximithine (**1**) both have three hydroxyamate moieties, which have the ability to chelate ferric iron in the molecule. The N-terminus part (hfOrn1-Ser2-hOrn3), which contains two hydroxyamate moieties, was very similar in both tsukubachelin and foroxymithine, however, the third hydroxyamate group, in the cyclic Orn of tsukubachelin was slightly further away from the other two hydroxamate moieties than in foroxymithine. Because the three hydroxamate moieties are critical for ferric ion chelation, the distances between these groups are very important. Moreover, the diketopiperazine structure in foroxymithine was thought to be less flexible than a normal peptide bond. These structural features may influence the affinity of these peptides towards zinc ions and may affect the inhibitory activities.

Conclusions

ACE is a very promising target for screening agents that have the potential to be used against kidney disease caused by high blood pressure or diabetes. In the present study, a new siderophore named tsukubachelin was discovered and found to exert potent ACE inhibitory activity. The mechanism of inhibition was thought to arise through deprivation of zinc ions from ACE, however, further study is needed to clarify the inhibitory mechanism.

So far, a series of similar siderophores: foroximithine, coelichelin, and tsukubachelin, have been isolated from streptomycetes. Considering the variety of secondary metabolite biosynthetic genes in streptomycetes, this class of siderophore may be broadly distributed in streptomycetes. Further extensive screening for siderophores may lead to the discovery of more potent ACE inhibitors.

Experimental Section

NMR and Mass Spectroscopic Measurements: The gallium complex of tsukubachelin (1 mg) was dissolved in [D_6]DMSO (0.6 mL), and NMR spectra including ^{1}H NMR at 800.23 MHz, ^{13}C NMR at 201.24 MHz, ^{1}H-^{1}H correlation 2D NMR (DQF-COSY, TOCSY, ROESY, and NOESY), and ^{1}H-^{13}C correlation 2D NMR (HSQC and HMBC) were measured with an AVANCE 800 spectrometer with a CryoProbe (Bruker Biospin, Karlsruhe, Germany) at 303 K. An electrospray ionization (ESI) Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer (Apex II, Bruker Daltonics, Billerica, MA, USA) was used for accurate mass analyses.

Bacterial Strain and Culture Media: Soil samples were collected from the ground of Tsukuba Mountain, Ibaraki Prefecture, Japan. The samples were suspended in sterile water (10 mL), and spread over ISP2 agar medium. After 4–5 d of incubation at 30 °C, developed colonies were isolated and stored at –80 °C. The iron-deficient medium consisted of K_2SO_4 (2 g), K_2HPO_4 (3 g), NaCl (1 g), and NH_4Cl (5 g) in deionized water (1 L). To remove ferric ions, the solution was stirred with 50 g of chelex-100 Na form (Bio-Rad) for 16 h. The solution was filtered through filter paper (Whatman No.1) and combined with the stock solutions: 100 μL of thiamine (20 mg/mL), 100 μL of $ZnSO_4{\cdot}7H_2O$ (20 mg/mL), 20 μL of $CuSO_4{\cdot}7H_2O$ (0.5 mg/mL), 20 μL of $MnSO_4{\cdot}4H_2O$ (3.5 mg/mL), followed by autoclaving. The separately sterilized solutions (10 mL each) of $CaCl_2{\cdot}H_2O$ (10 mg/mL), glucose (250 mg/mL), and 0.5% yeast extract (Difco) were added to the medium.

Screening for Siderophore Using HPLC: Each bacterial strain was cultured using a shaker (180 rpm) set at 30 °C for 4 d in a conical flask containing 10 mL of the iron-deficient culture medium. Each cultured medium was centrifuged at 3000 rpm, and 5 mL of the supernatant was collected in a new conical flask. $FeCl_3$ (1 M, 0.05 mL) was added to the supernatant to generate the ferric siderophore. After centrifugation at 10000 rpm for 5 min, 50 μL of the solution was subjected to HPLC analysis. The HPLC analysis was performed using an analytical C18 column (Nacalai tesque, Cosmosil 5C18MS-II), eluted with solvent A (distilled water containing 0.05% TFA) and solvent B (MeCN containing 0.05%TFA) using time program: 0 to 10 min with 100% solvent A, then 10 min to 60 min increasing solvent B with the ratio of 1%/min. The UV/Vis detector was set at the wavelength of 435 nm.

Polymerase Chain Reaction (PCR) Amplification, Sequencing, and Phylogenetic Analysis of 16S rRNA Genes: The extraction of total DNA from the cells of TM-34 was performed according to the previous paper.[19] The 16S rRNA-encoding sequence was amplified from the total DNA by PCR using two universal primer pairs: 9F (5′-GAGTTTGATCCTGGCTCAG-3′) and 1541R (5′-CTCCTACGGGTGAGTAACAC-3′). The reaction mixture for PCR was prepared by adding 0.5 μL of total DNA of TM-34 (100

ng), 0.5 μL of LA taq DNA polymerase (Takara, Japan), 25 μL of X2 GC buffer (Takara, Japan), 4 μL of 2.5 mM dNTPmix solution (Takara, Japan), and 20 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 2 min at 95 °C, followed by 30 cycles consisting of denaturation for 10 s at 95 °C, annealing for 10 s at 55 °C, and DNA synthesis for 3 min at 72 °C. A final extension of 3 min at 72 °C was added at the end of the 30 cycles. The PCR product was purified with QIAGEN PCR PURE kit following the manufacturer's instruction. The reactions for sequencing were performed in a solution containing 2 μL of purified PCR product, 1 μL of ABI Prism BigDye (Applied Biosystems), 7.4 μL of sterile distilled water, and 1.6 μL of each primer with the following PCR protocol: 96 °C for 3 min and 25 cycles of 95 °C for 40 s, 55 °C for 40 s, and 60° for 4 min. The eight primers used for the reaction: 9F (5′-GAGTTTGATCCTGGCTCAG-3′), 339F (5′-CTCCTACGGGTGAGTAACAC-3′), 686F (5′-TAGCGGTGAAATGCGTAGA-3′), 1099F (5′-GCAACGAGCGCAACCC-3′), 1541R (5′-AAGGAGGTGATCCAGCC-3′), 1510R (5′-GGCTACCTTGTTACGA-3′), 926R (5′-CCGTCAATTCCTTTGAGTTT-3′), 536R (5′-GTATTACCGCGGCTGCTG-3′).

Isolation of Tsukubachelin: Streptomyces sp. TM-34 was cultured in 1 L of iron-deficient media for 5 d. The culture medium was harvested by filtering through filter paper (No. 1 paper filter, Whatmann). The medium was added with 0.5 mL of 1 M $FeCl_3$ and evaporated using a rotary evaporator to concentrate to 20 mL final volume. The concentrated solution was centrifuged at 3000 rpm for 10 min and filtered through a membrane filter (Millipore, 0.45 μm pore size) to remove insoluble materials. HPLC purification was performed to obtain 2.5 mg of ferri-tsukubachelin using C18 Semi Prep column (10 × 250 mm, Capcell Pak C18 UG80, Shiseido), eluted with MeCN/water (2:98) containing 0.05% TFA and monitored at a UV/Vis absorbance 435 nm.

Conversion of Ferri-Tsukubachelin into Ga-tsukubachelin via Desferri-Tsukubachelin: Ferri-tsukubachelin (2.0 mg) was dissolved in water (3 mL) and the solution was mixed with 8-quinolinol (1 M, 3 mL) and stirred at room temperature for 30 min. The reaction mixture was extracted with CH_2Cl_2 (4 × 6 mL) to remove ferri-8-quinolinol and the aqueous layer was immediately collected and lyophilized by freeze-drying. After dissolving the dry material in water (2 mL), HPLC purification was performed using a C18 Semi-Prep column (10 × 250 mm; Capcell Pak C18 UG80, Shiseido), eluted with MeCN/water (3:97) containing 0.05% TFA, and monitored at a UV/Vis absorbance of 215 nm, to yield desferri-tsukubachelin (1.9 mg). Desferri-tsukubachelin (1.5 mg) was dissolved in distilled water (2 mL), and gallium chloride (10 mg) was added to generate Ga-tsukubachlin. After HPLC purification in the same manner as described above, Ga-tsukubachelin (1.1 mg) was obtained.

Modified Marfey Method: Desferri-tsukubachelin (0.2 mg) was subjected to acid hydrolysis at 110 °C for 16 h with HI (6 N, 0.5 mL), and then the hydrolysates were dried under a stream of N_2 gas and resuspended in H_2O (200 μL). To the hydrolysate, 10 μL of a solution of either *N*-α-(5-fluoro-2,4-dinitrophenyl)-L-valinamide (L-FDVA, Sigma–Aldrich) or D-FDVA (Sigma–Aldrich) in acetone was added at a concentration of 10 mg/mL, followed by $NaHCO_3$ (1 M, 100 μL). The mixture was heated at 80 °C for 3 min, then cooled, neutralized with HCl (2 N, 50 μL), and diluted with MeCN (200 μL). About 10 μL of each solution of FDVA derivative was subjected to HPLC analysis with a C18 column (4.6 × 50 mm; Cosmosil MSII); the elution was performed at a flow rate of 1 mL/min using solvent A (distilled water containing 0.05% TFA) and solvent B (MeCN containing 0.05%TFA) with a linear gradient mode from 0 min to 60 min increasing solvent B at 1%/min. The UV/Vis detector was set at the wavelength of 340 nm. Retention times for L- and D-FDVA derivatized amino acids were as follows: L-Ser-L-FDVA (35.3 min), D-Ser-D-FDVA (36.2 min), L-Orn-L-FDVA (30.2 min), L-Orn-D-FDVA (31.8 min), N-Me Orn-L-FDVA (32.3 min), *N*-Me Orn-D-FDVA (31.30 min).

Inhibition Test with Angiotensin-Converting Enzyme (ACE): ACE inhibitory activity was determined by the modified method of Cushman and Cheung.[22] The enzyme ACE was dissolved in distilled water at 8 mU/mL. 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 the addition of 100 μL enzyme solution. The mixture was incubated at 37 °C for 30 min, then 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 redissolved in 3 mL of 1 M NaCl and the UV absorbance at 228 nm was measured to calculate the inhibitory concentration.

Acknowledgments

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