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Simultaneous detection and determination of the absolute configuration of thiazole-containing amino acids in a peptide

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Abstract—For the simultaneous detection and determination of the absolute configuration of a thiazole-containing (Tzl-) amino acid in a peptide, we have developed a reliable method using the 'advanced Marfey's method', which includes HPLC with a rational guideline, a sensitive derivatizing reagent, 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA), and a racemization procedure using DL-FDLA for determination of the absolute configuration of constituent amino acids in a peptide. Tzl-amino acids could be directly detected in the hydrolysate by this method, although they were racemized under ordinary hydrolysis conditions. In order to depress the racemization, the flash hydrolysis was introduced. As a result, the flash hydrolysis for 1 h was sufficient to detect each constituent amino acid, and it was possible to identify the original peak. Consequently, the absolute configuration of microcyclamide (**1**) possessing Tzl-amino acids was determined by the advanced Marfey's method combined with flash hydrolysis. Additionally, this method was successfully applied to the simultaneous detection and determination of the absolute configuration of two other naturally occurring peptides, waiakeamide (**2**) and goadsporin (**3**). The established method with the flash hydrolysis had an additional advantage in that labile amino acids, such as tryptophan and methionine sulfoxide, during acid hydrolysis can be detected in the intact form.

1. Introduction

A large number of naturally occurring peptides containing thiazole, oxazole and methyloxazole rings have been isolated from various origins, and almost all including antibiotics show characteristic biological activities such as cytotoxic, immunosuppressive, antifungal and enzyme inhibitory activities. Thiazole-containing (Tzl-) amino acids in such a peptide arise biosynthetically from the cyclization of cysteine and an adjacent amino acid, and oxazole- (Ozl-) and methyloxazole-containing (mOzl-) amino acids are derived from serine and threonine and their adjacent amino acids, respectively.[1,2] Recently, several cyclic peptides composed of three such cyclically modified amino acids have been isolated as a group of natural products from cyanobacteria and marine origins, and they have cytotoxic activities in particular.[3–11]

In general, the absolute configuration of such a modified amino acid in a peptide is determined on the basis of that of an amino acid adjacent to the heterocycle. Amino acids adjacent to Ozl- and mOzl-rings can be released from Ozl- and mOzl-amino acids in a peptide by acid hydrolysis.[3–11] However, no adjacent amino acid was obtained from Tzl-amino acid under the usual hydrolysis conditions, and it is difficult to isolate the intact Tzl-amino acid due to its instability. Additionally, acid hydrolysis of a peptide containing Tzl-amino acids causes a serious racemization of liberated Tzl-amino acid.[3,12] In order to obtain amino acids adjacent to Tzl-rings in Tzl-amino acids, Ireland et al. proposed a practical method including ozonolysis followed by acid hydrolysis in 1983.[12] Since then, although this method has been applied to many peptides containing Tzl-amino acids, no method has been established for the direct detection and identification of Tzl-amino acids including the absolute configuration so far.

We have carried out the isolation and the characterization of cyanobacterial peptides including toxic peptides for the elucidation of their biosynthesis.[13–18] During this study, we have developed the 'advanced Marfey's method' for the rapid and reliable structural determination of isolated cyanobacterial peptides. The advanced Marfey's method is a nonempirical method for the determination of the absolute configuration of constituent amino acids in a peptide using liquid chromatography/mass spectrometry (LC/MS).[19,20] This method consists of Marfey's method as a chromatographic technique for the separation of amino acids into their enantiomers, detection using mass spectrometry, and a

Keywords: microcyclamide; absolute configuration; advanced Marfey's method; thiazole-containing (Tzl-) amino acid.

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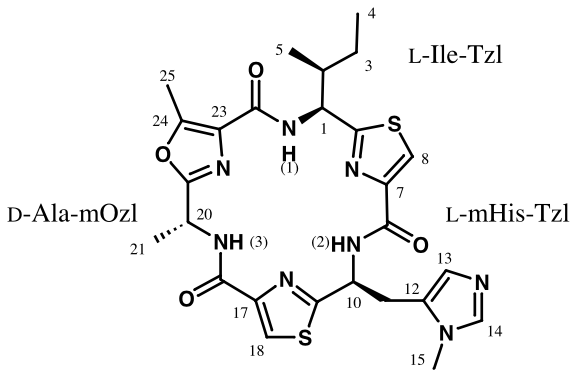
procedure for obtaining the corresponding enantiomer from either the L- or D-amino acids. For the establishment of this method, we have developed 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA) as a new derivatizing reagent instead of the original reagent to combine Marfey's method effectively with mass spectrometry and elucidated the limitation of Marfey's method and its separation mechanism.[19,21] Furthermore, we introduced a racemization procedure using 1-fluoro-2,4-dinitrophenyl-5-DL-leucinamide (DL-FDLA) to obtain the corresponding enantiomer from the original samples on the chromatogram. Thus, the advanced Marfey's method has enabled us to undertake the chromatographic determination of the absolute configuration of amino acids including unusual amino acids without a standard sample on the basis of the proposed separation mechanism and has been applied not only to amino acids but also to primary amines.[19–22] As a result, this method was successfully applied to the determination of the absolute configuration of the isolated cyanobacterial peptides possessing unusual amino acids, and the wide utility of this method has been confirmed in the study of several naturally occurring peptides.[13–18,23] In addition, this methodology is being extended to the determination of the absolute configuration of secondary alcohols.[24]

Recently, Ishida et al. have isolated a cyclic peptide, microcyclamide, composed of two Tzl-amino acids and a mOzl-amino acid from the toxic cyanobacterium, *Microcystis aeruginosa* NIES-298. Their constituent amino acids were determined to have all L-configurations using Marfey's method after ozonolysis.[25] We also isolated a cyclic peptide (**1**) with the same planar structure as microcyclamide as a cytotoxic peptide not only from the cultured cells of the same strain but also from Philippine bloom samples; we also detected **1** in the nontoxic *M. aeruginosa* NIES-101. However, it was found that the absolute configurations of the constituent amino acids in **1** were not consistent with those of microcyclamide from our preliminary experiments, which prompted us to establish an effective method for the determination of the absolute configuration of constituent amino acids including Tzl-amino acid. We considered that Tzl-amino acids in the hydrolysate can be directly detected and that their absolute configuration can be determined by the advanced Marfey's method without any chemical degradation, if it was possible to control the racemization of the Tzl-amino acids during the acid hydrolysis. In this study, we developed a reliable method composed of the advanced Marfey's method and flash (shorter reaction time) hydrolysis for the simultaneous detection and determination of the absolute configuration for Tzl-amino acids in a peptide, and the established method was successfully applied to **1** and two other naturally occurring peptides.

2. Results and discussion

2.1. Isolation and characterization of microcyclamide

Microcyclamide (**1**) was isolated together with microcystins from a 5% AcOH aq. extract of the cultured cells of *M. aeruginosa* NIES-298 and the field samples collected from Lake Laguna de Bay in the Philippines and was also detected in the extract of the cultured cells of nontoxic *M. aeruginosa* NIES-101. Compound **1** was purified by repeated silica gel and TOYOPEARL HW-40F chromatographies and has been characterized using 2D NMR techniques and HRFABMS experiments as follows. The molecular formula of **1** was established to be $C_{26}H_{31}N_8O_4S_2$ based on the HRFABMS and NMR spectral data (see Section 4). In the ^{13}C NMR spectrum, six sp^2 carbon signals in the amide region (159.0–169.4 ppm in DMSO-d_6) were observed, and three broadened doublet signals due to amide NH protons were found in the ^1H NMR spectrum (8.36, 8.41 and 8.52 ppm in DMSO-d_6). The 2D NMR spectral analysis of **1** confirmed that the gross structure of microcyclamide was unambiguously determined as shown in **1**. The absolute configurations of the constituent amino acids in **1** were investigated by subsequent experiments using the advanced Marfey's method.



microcyclamide (**1**)

2.2. Direct detection of Tzl-amino acids in microcyclamide

In order to detect directly Tzl-amino acids by the advanced Marfey's method, we carried out the following experiment using **1**. Microcyclamide (**1**) from the NIES-298 was hydrolyzed in 6 M HCl at 110°C for 17 h, and the hydrolysate was derivatized with L-FDLA as a derivatizing reagent. The resulting derivative was applied to ESI-LC/MS analysis under the negative ion mode (Fig. 1(a)). Although the 2,4-dinitrophenyl-5-L-leucinamide (DLA) derivative of the intact Ala-mOzl was not detected, the peak of Ala derived from Ala-mOzl was observed as its DLA derivative on the mass chromatogram monitored at *m/z* 382 $[M-H]^-$, showing that Ala has the D-configuration based on its retention time. The same analysis of **1** from the Philippine bloom samples gave the same results (data not shown). These results indicated that the absolute configuration of Ala-mOzl in **1** was D.

On the other hand, the peaks of the Tzl-amino acids, mHis-Tzl and Ile-Tzl, were surely detected as their DLA derivatives on the mass chromatograms monitored at *m/z* 545 $[M-H]^-$ and *m/z* 507 $[M-H]^-$, respectively. However, the two desired peaks were observed with almost the same intensity on each mass chromatogram, showing that the racemization of Tzl-amino acids occurred during the hydrolysis as expected. Although it was possible to detect intact Tzl-amino acids in peptides by the advanced Marfey's

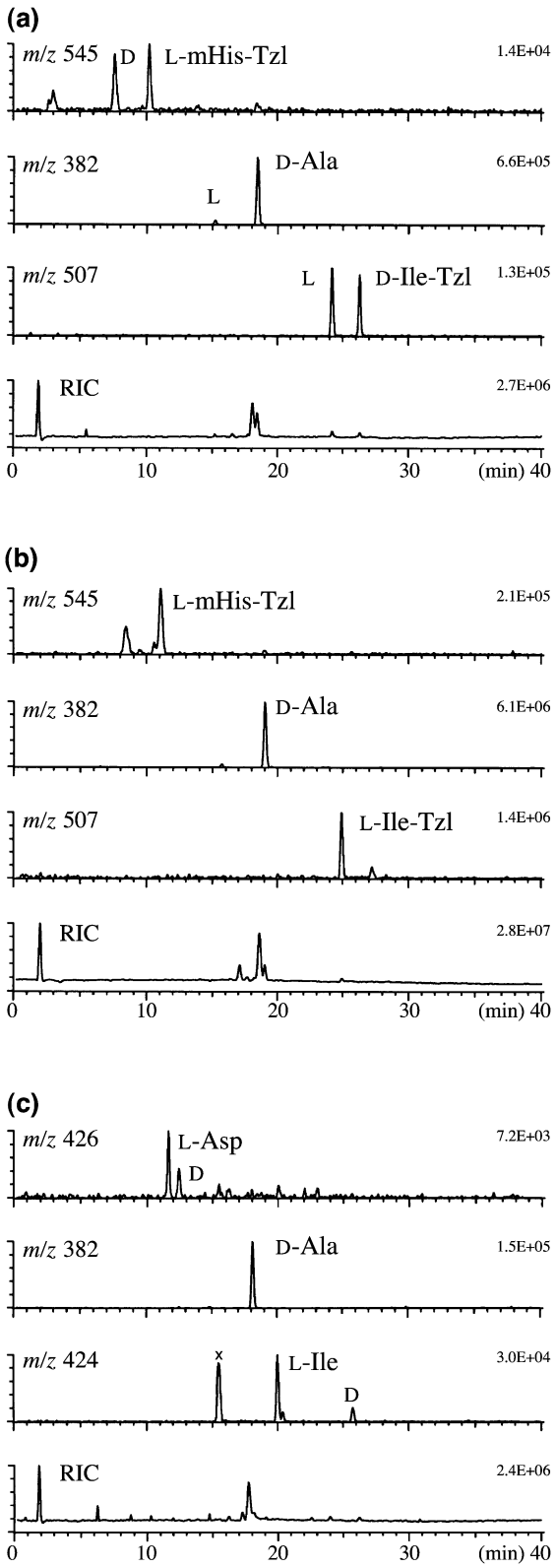


Figure 1. Mass chromatograms monitored at the *m/z* values of the deprotonated molecules of the L-DLA derivatives of each constituent amino acid in the hydrolysate for 17 h (a), for 1 h (b), and the hydrolysate after ozonolysis (c) of microcyclamide (**1**) using ESILC/MS in the negative ion mode.

method, the results also suggested that reaction conditions should be carefully considered in order to avoid the racemization.

2.3. Determination of the absolute configuration of Tzl-amino acids in microcyclamide using the flash hydrolysis

Among the reaction conditions for acid hydrolysis of a peptide, we tried to shorten the reaction time for depression of the racemization of Tzl-amino acids. The ratio of both enantiomers for **1** was examined at different reaction times (1, 2, 4, 7 and 11 h) using HPLC analysis after the derivatization with L-FDLA. On the basis of the resulting peak heights of the two Tzl-amino acids, the ratio of peak height for the resulting enantiomeric isomer to the original isomer is shown in Fig. 2. Although the racemization of Tzl-amino acid depended on its amino acid moiety, their racemization during the acid hydrolysis was minimally depressed at 1 h. Fig. 1(b) shows the results of ESI-LC/MS analysis of the flash hydrolysate (1 h). Each original peak for Tzl-amino acids was clearly detected on their mass chromatograms, indicating that both Tzl-amino acids have L-configurations as will be discussed later. In addition, these results indicated strongly that the flash hydrolysis for a 1 h reaction time was sufficient to hydrolyze constituent amino acids from a peptide.

As shown in Fig. 1(b), the original peak for Ile-Tzl was eluted prior to the corresponding enantiomer, whereas with mHis-Tzl the opposite situation occurred. According to the proposed separation mechanism for Marfey's method, the elution order of an amino acid derivatized with FDLA can be elucidated from the comparison of hydrophobicity between the α-carboxyl group and the side chain.[19,21] Generally, the L-amino acid derivative is eluted faster than the D-amino acid derivative, but the elution order becomes opposite if the hydrophobicity of the side chain is lower than that of the α-carboxyl group.[19] Ile-Tzl showed the normal elution order (L⟶D) based on the separation mechanism. However, mHis-Tzl was oppositely eluted (D⟶L) under acidic conditions, because the side chain containing a methylimidazole moiety was ionized, so that its hydrophobicity was lower than that of the corresponding free base.[†] Therefore, the absolute configurations of each of the two peaks from mHis-Tzl and Ile-Tzl were assigned as shown in Fig. 1(a). Thus, the absolute configuration of **1** possessing Tzl-amino acids was determined by the advanced Marfey's method combined with the flash hydrolysis. These results were confirmed as shown in Fig. 1(c) by the analysis of the hydrolysate after ozonolysis, indicating that Asp and Ile derived from mHis-Tzl and Ile-Tzl both had L-configurations.[25] Consequently, the original structure of microcyclamide proposed by Murakami et al. should be revised to be **1**.

[†] Basic amino acids such as ornthine, lysine and histidine yield mono- and di-DLA derivatives in the derivatization with FDLA. All α-mono-DLA derivatives of these basic amino acids showed short retention time and opposite elution order (D⟶L) under the same LC conditions.[19,21] These occur for the reason that the hydrophobicity of the side chain of the α-mono-DLA derivatives of basic amino acids is lower than that of the α-carboxyl group, because the side chain moiety is ionized under the conditions used. In the case of methylhistidine (mHis), only the α-mono-DLA derivatives with short retention time were obtained and also showed opposite elution order (D⟶L).

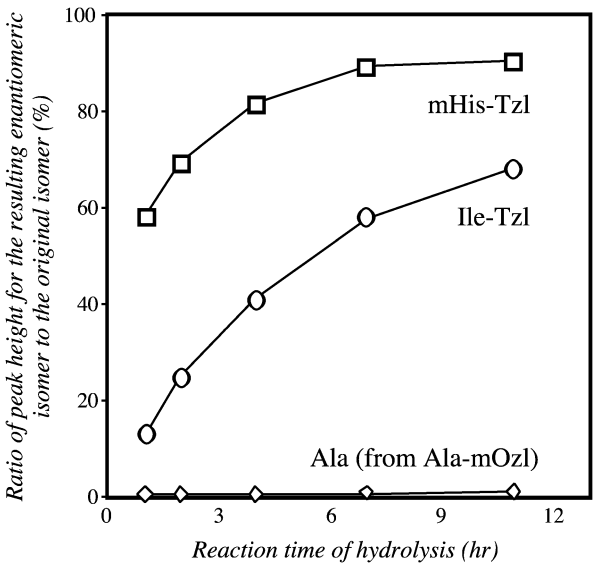


Figure 2. Time course for racemization of Tzl-amino acids in microcyclamide by hydrolysis.

2.4. Application of the advanced Marfey's method with flash hydrolysis

To confirm the utility of the advanced Marfey's method with flash hydrolysis, the combination method was applied to two naturally occurring peptides possessing Tzl-amino acids. One is a cyclic hexapeptide containing phenlyalanine-Tzl (Phe-Tzl), waiakeamide (**2**), which was originally isolated from the sponge *Ircinia dendroides*.[26] Another is a linear peptide composed of 13 amino acid moieties containing glycine- and leucine-Tzl (Gly- and Leu-Tzl), goadsporin (**3**), which was isolated from *Streptomyces* sp. as an inducer of sporulation and secondary metabolism in streptomycetes.[27] Each peptide (500 μg) was subjected to the flash hydrolysis (6 M HCl, 110°C, 1 h), and the hydrolysate was divided into two portions, and each portion was derivatized with L- or D-FDLA. The L-DLA derivatives alone and an equal mixture of D- and L-DLA derivatives, the DL-DLA derivatives, were analyzed using ESI-LC/MS in the positive ion mode. The L-DLA derivative of Phe-Tzl from waiakeamide (**2**) were detected as two peaks, large and small peaks (retention time: 15.2 and 16.3 min), on the mass chromatograms monitored at *m/z* 545 $[M+H]^+$ (Fig. 3).

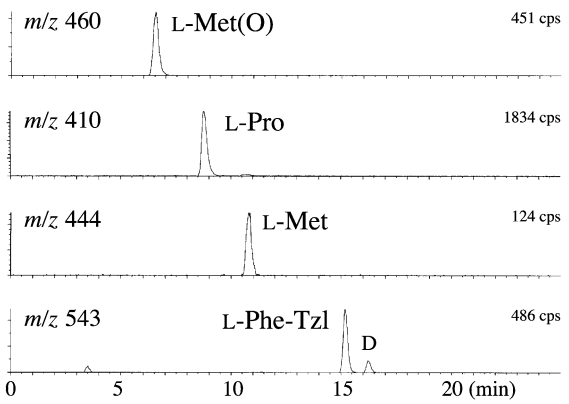
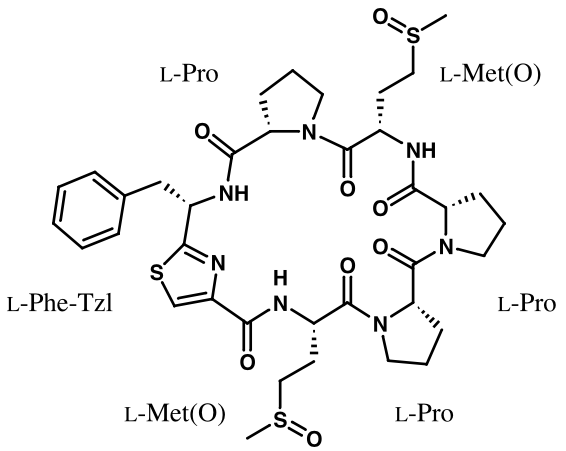


Figure 3. Mass chromatograms monitored at the *m/z* values of the protonated molecules of the L- (a) and DL-DLA derivatives (b) of each constituent amino acid in waiakeamide (**2**) using ESILC/MS in the positive ion mode.

Because the large peak has the original absolute configuration of Phe-Tzl, it was determined to be the L-configuration on the basis of the proposed application guideline.[19] Indeed, this result was consistent with the absolute configuration confirmed previously. Furthermore, the peak of methionine sulfoxide [Met(O)], which is usually decomposed into Met by the acid hydrolysis,[28] was detected without decomposition on the mass chromatogram monitored at *m/z* 460 $[M+H]^+$ (Fig. 3).

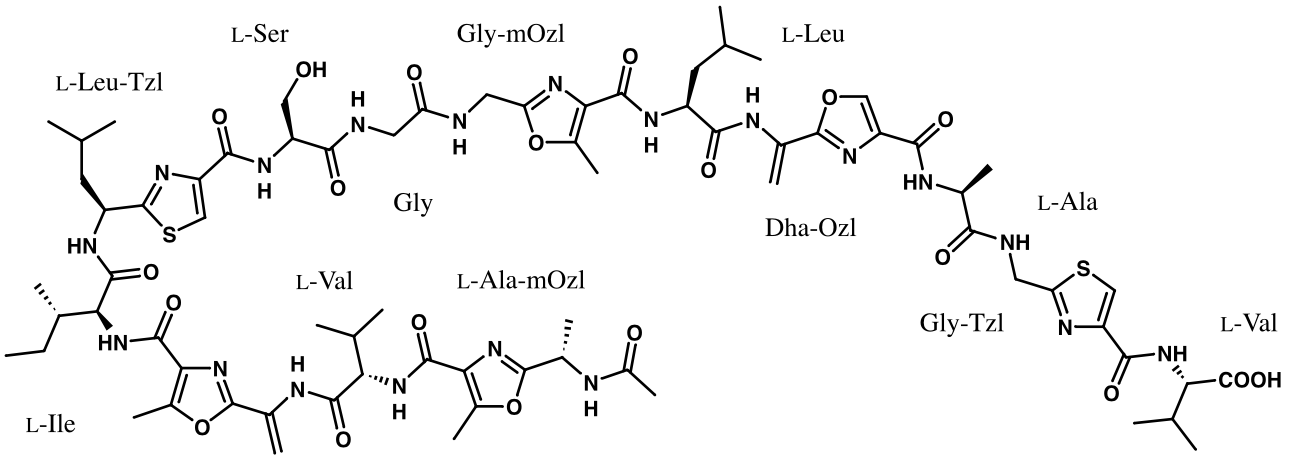


waiakeamide (**2**)

The DL-DLA derivatives of each constituent amino acid from goadsporin (**3**) except for Gly and Gly-Tzl were detected as two peaks corresponding to each enantiomer, and the L-DLA derivatives was found to be clearly detected as the original peak. As a result, it was concluded that all constituent amino acid moieties had L-configurations.[27] Additionally, the presence of Gly-Tzl was confirmed on the mass chromatogram, and the absolute configuration of Leu-Tzl can be determined to be L-configuration according to the proposed application guideline.[19] Thus, the advanced Marfey's method was successfully applied to the detection and determination of the absolute configuration of Tzl-amino acids in a peptide by the combination with flash hydrolysis. Further, it was found that the established method has an additional advantage in that labile amino acids such as tryptophan and Met(O) during acid hydrolysis can be detected in the intact form.

3. Conclusion

For the detection and determination of the absolute configuration of Tzl-amino acids in a peptide, we developed a reliable method using the advanced Marfey's method. Tzl-amino acids could be directly detected in the hydrolysate by this method, although they were racemized under ordinary hydrolysis conditions. In order to depress the racemization, flash hydrolysis was introduced. As a result, the flash hydrolysis for 1 h was sufficient to detect each constituent amino acid and made it possible to identify the original peak. Consequently, the absolute configuration of microcyclamide (**1**) possessing Tzl-amino acids was determined by the advanced Marfey's method combined with the flash hydrolysis. Additionally, this method was successfully



Goadsporin (**3**)

applied to the simultaneous detection and determination of the absolute configuration of two other naturally occurring peptides possessing Tzl-amino acids. The established method is being further extended for the structural determination of various naturally occurring peptides possessing modified amino acids and D-amino acids.

4. Experimental

4.1. Materials

The cyanobacteria *M. aeruginosa* NIES-298 and -101 were obtained from the NIES collection (Microbial Culture Collection, the National Institute for Environmental Studies, Japan) and cultivated in the defined inorganic nutrient culture medium, called MA.[25] Cells were harvested after 4 weeks of cultivation and freeze-dried. Field samples were collected from the east curve of Lake Laguna de Bay in the Republic of the Philippines on 23 June 1999. Waiakeamide and goadsporin were kindly provided by Dr Kyoko Adachi of Marine Biotechnology Institute Co., Ltd, and Dr Yasuhiro Igarashi of Biotechnology Center, Toyama Prefectural University, respectively. Derivatizing reagents, L-FDLA and D-FDLA, were synthesized according to the procedure shown in a previous paper.[19] All chemicals and solvents were of analytical grade.

4.2. Isolation of microcyclamide (1)

Dried cells (32 g) of *M. aeruginosa* NIES-298 were extracted four times with 5% AcOH aq. (800 mL) for 30 min while stirring. The combined extracts were centrifuged at 9000 rpm for 30 min, and the supernatant was applied to a preconditioned ODS silica gel cartridge (100 g, Chromatorex ODS) after filtration on a glass microfiber filter (GF/C). The cartridge was rinsed with water (1 L) and 20% MeOH aq. (1 L) and then eluted with 90 and 100% MeOH (each 600 mL) to give a fraction containing **1**. The fraction was separated to give **1** (13.4 mg) using the following chromatography: silica gel (Silica gel 60 (230–400 mesh)) using AcOEt/*i*-PrOH/H_2O (8:1:2, upper layer), $CHCl_3$/MeOH/H_2O (65:35:10 and 65:10:5, lower phase) and TOYOPEARL HW-40F (890×11 mm i.d.; flow rate, 0.3 mL/min; detection, UV230 nm) using MeOH. The peptide **1** (1.0 mg) from the Philippine bloom samples (159 g) were isolated in the same manner as mentioned above.

4.2.1. Characterization of microcyclamide (1)

The peptide **1** isolated from *M. aeruginosa* NIES-298 was an amorphous powder; $[\alpha]_D^{27}=-35.6°$ (*c* 0.100, MeOH); positive FABMS (glycerol) *m/z* 583 $[M+H]^+$; negative FABMS (glycerol) *m/z* 581 $[M-H]^-$; HRFABMS *m/z* 583.1909 $[M+H]^+$, calcd for $C_{26}H_{31}N_8O_4S_2$, Δ −0.1 mmu (JMS-HX110, JEOL, Tokyo, Japan). NMR spectra were measured at 150 MHz for the ^{13}C and at 600 MHz for the ^1H in DMSO-d_6 (JNM-A600, JEOL). The ^{13}C and ^1H chemical shifts [position: ^{13}C (ppm), ^1H (ppm) (mult; *J*)] are referenced to the solvent peaks (^{13}C: 39.5 and ^1H: 2.49 ppm in DMSO-d_6): NH(1): 8.41 (br d; *J*=7.3 Hz), 1: 54.3, 5.33 (dd; *J*=7.7, 5.5 Hz), 2: 40.1, 2.01 (m), 3: 25.3, 1.57 (m), 1.17 (m), 4: 11.5, 0.94 (t; *J*=7.3 Hz), 5: 14.4, 0.79 (d; *J*=7.0 Hz), 6: 167.7, 7: 147.9, 8: 125.1, 8.36 (s), 9: 159.0, NH(2): 8.73 (br d; *J*=8.4 Hz), 10: 50.5, 5.87 (dt; *J*=8.4, 6.2 Hz), 11: 30.8, 3.30 (m), 12: 126.3, 13: 127.8, 6.58 (s), 14: 138.2, 7.47 (s), 15: 30.8, 3.46 (s), 16: 169.4, 17: 149.4, 18: 125.7, 8.39 (s), 19: 159.3, NH(3): 8.52 (br d; *J*=5.9 Hz), 20: 44.0, 5.16 (dq; *J*=6.0, 6.6 Hz), 21: 19.0, 1.53 (d; *J*=6.6 Hz), 22: 161.2, 23: 127.9, 24: 153.4, 25: 11.2, 2.59 (s), 26: 159.5. The peptide **1** from the Philippine bloom samples was identified using FABMS, HRFABMS, ^1H NMR spectra and amino acid analysis, and the obtained data were completely consistent with the above data. The 5% AcOH extract from *M. aeruginosa* NIES-101 also contained **1**, which was confirmed by the HPLC analysis. At 13.7 and 26.6 μg/mL (IC_{50}), **1** showed a cytotoxicity against the lymphocytic mouse leukemia L1210 and L929 cells, respectively, and showed an anticyanobacterial activity against *Anabaena* sp.

4.3. Acid hydrolysis and ozonolysis of microcyclamide (1)

A 200 μg sample of **1** was hydrolyzed at 110°C with 400 μL of 6 M HCl for 1, 2, 4, 7, 11 and 17 h and subjected to the FDLA derivatization after each solution was evaporated to dryness. Alternatively, **1** (500 μg) in MeOH (1 mL) was ozonized at −80°C for 30 min. After the solvent was

evaporated to dryness, the reaction mixture was dissolved in 6 M HCl (400 μL) and heated at 110°C for 17 h. The hydrolysate was subjected to the FDLA derivatization after evaporation.

4.4. Preparation of DLA derivatives of microcyclamide (1)

The hydrolysate of **1** under various conditions and the hydrolysate after ozonation of **1** were dissolved in 100 μL of water. To each solution, 40 μL of 1 M sodium bicarbonate and then 100 μL of 1% L-FDLA in acetone were added. The solution was vortexed and incubated at 40°C for 60 min. The reaction was quenched by the addition of 40 μL of 1 M HCl. After dilution with 220 μL of acetonitrile, 0.1 and 1 μL of the L-DLA derivatives of all samples were analyzed by HPLC and ESILC/MS, respectively.

4.5. HPLC conditions

HPLC was performed using a TOSOH (Tokyo, Japan) dual-pump delivery system composed of two Model CCPS pumps, a Model SD-8022 degasser, a Model MX-8010 mixer, a Model CO-8020 column oven, a Model UV-802O UV–Vis detector, a Model PX-8020 system controller, and a Model C-R6A integrator from Simadzu (Kyoto, Japan). Separations were carried out on a TSK gel 80Ts (150×4.6 mm i.d., TOSOH) column heated at 40°C. Acetonitrile–0.01 M trifluoroacetic acid (TFA) was used as the mobile phase under a linear gradient elution mode (acetonitrile, 30→70%, 40 min). The system was allowed to equilibrate for 10 min at 30% B prior to the next analysis. The flow rate was 1 mL/min with UV detection at 340 nm. Retention times of the constituent amino acids of **1** (min): D-mHis-Tzl (6.8), L-mHis-Tzl (8.5), L-Asp (10.2), D-Asp (10.9), L-Ala (13.2), D-Ala (16.5), L-Ile (19.0), D-Ile (26.3), L-Ile-Tzl (24.3), D-Ile-Tzl (27.2).

4.6. ESILC/MS conditions for microcyclamide (1)

The separation of the L- and DL-FDLA derivatives of **1** was performed on a Develosil ODS-HG-5 (150×2.0 mm i.d., Nomura Chemical, Seto, Japan) column maintained at 40°C using a HP1050 (Hewlett–Packard, Novi, MI). Acetonitrile–water containing 0.01 M TFA was used as the mobile phase under a linear gradient elution mode (acetonitrile, 30–70%, 40 min) at a flow rate of 0.2 mL/min. The mass spectrometer used was a Finnigan TSQ7000 (Finnigan-Mat, San Jose, CA). All mass spectra were acquired using Q1 as the scanning quadrupole. The ESI voltage was 4.5 kV with the auxiliary and sheath gas nitrogen pressure set at 5 units and 60 psi, respectively, and the capillary was heated to 250°C. A mass range of *m/z* 300–1000 was covered with a scan time of 1.5 s, and data were collected in the negative ion mode using an electron multiplier voltage of 1200 V.

4.7. Preparation of DLA derivatives of waiakeamide (2) and goadsporin (3)

Each 500 μg of **2** and **3** was hydrolyzed at 110°C for 1 h with 500 μL of 6 M HCl. This solution was divided into two portions, and each portion was derivatized with L- or D-FDLA. Each solution was then evaporated to dryness, and the residue was dissolved in 100 μL of water. To each amino acid solution, 20 μL of 1 M sodium bicarbonate and then 100 μL of 1% L- or D-FDLA in acetone were added. These solutions were vortexed and incubated at 40°C for 60 min. These reactions were quenched by the addition of 20 μL of 1 M HCl. After dilution with 260 μL of acetonitrile, 1 μL of the L-DLA derivative and an equal mixture of the L- and D-DLA derivatives were analyzed by ESILC/MS.

4.8. ESI-LC/MS conditions for waiakeamide (2) and goadsporin (3)

The separation of the L- and DL-DLA derivatives of **2** and **3** was performed on an TSK gel super-ODS (100×2.0 mm i.d., TOHSO) column maintained at 40°C using a HP1100 (Hewlett–Packard). Acetonitrile–water containing 1% formic acid was used as the mobile phase under a linear gradient elution mode (acetonitrile, 30–80%, 25 min) at a flow rate of 0.2 mL/min. The mass spectrometer used was an API Qstar Pulser-*i* (MDS Sciex, Toronto, Canada). All mass spectra were acquired using TOFMS. A mass range of *m/z* 350–1500 was covered with a scan time of 1 s, and data were collected in the positive ion mode. The HPLC and mass spectrometer were interfaced with laboratory-made flow splitter and an IonSpray ion source (MDS Sciex). The effluent from the HPLC was split at a ratio of 1:40, and a smaller portion of the effluent was introduced into the ion source at a flow rate of 5 μL/min. The IonSpray voltage was 5.5 kV with the nebulizer gas air pressure and curtain gas nitrogen pressure set at 20 and 25 psi, respectively.

Acknowledgments

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References

1. Marahiel, M. A.; Stachelhaus, T.; Mootz, H. D. *Chem. Rev.* **1997**, *97*, 2651–2673.
2. Konz, D.; Klens, A.; Schörgendorfer, K.; Marahiel, M. A. *Chem. Biol.* **1997**, *4*, 927–937.
3. Davidson, B. S. *Chem. Rev.* **1993**, *93*, 1771–1791.
4. Ojika, M.; Nemoto, T.; Nakamura, M.; Yamada, K. *Tetrahedron Lett.* **1995**, *36*, 5057–5058.
5. Todorova, A. K.; Jüttner, F.; Linden, A.; Plüss, T.; von Philipsborn, W. *J. Org. Chem.* **1995**, *60*, 7891–7895.

6. Admi, V.; Afek, U.; Carmeli, S. *J. Nat. Prod.* **1996**, *59*, 396–399.
7. Ogino, J.; Moore, R. E.; Patterson, G. M. L.; Smith, C. D. *J. Nat. Prod.* **1996**, *59*, 581–856.
8. Sone, H.; Kigoshi, H.; Yamada, K. *Tetrahedron* **1997**, *53*, 8149–8154.
9. Banker, R.; Carmeli, S. *J. Nat. Prod.* **1998**, *61*, 1248–1251.
10. Mitchell, S. S.; Faulkner, D. J.; Rubins, K.; Bushman, F. D. *J. Nat. Prod.* **2000**, *63*, 279–282.
11. Jutter, F.; Todorova, A. K.; Walch, N.; von Philipsborn, W. *Phytochemistry* **2001**, *57*, 613–619.
12. Biskupiak, J. E.; Ireland, C. M. *J. Org. Chem.* **1983**, *48*, 2304–2306.
13. Harada, K.-I.; Mayumi, T.; Shimada, T.; Suzuki, M.; Kondo, F.; Watanabe, M. F. *Tetrahedron Lett.* **1993**, *34*, 6091–6094.
14. Harada, K.-I.; Fujii, K.; Shimada, T.; Suzuki, M.; Sano, H.; Adachi, K.; Carmichael, W. W. *Tetrahedron Lett.* **1995**, *36*, 1511–1514.
15. Fujii, K.; Harada, K.-I.; Suzuki, M.; Kondo, F.; Ikai, Y.; Oka, H.; Carmichael, W. W.; Sivonen, K. In *Harmful and Toxic Algal Blooms*; Yasumoto, T., Oshima, Y., Fukuyo, Y., Eds.; Intergovernmental Oceanographic Commission of UNESCO: Sendai, 1996; pp 559–562.
16. Fujii, K.; Sivonen, K.; Adachi, K.; Noguchi, Y.; Shimizu, Y.; Sano, H.; Hirayama, M.; Suzuki, M.; Harada, K.-I. *Tetrahedron Lett.* **1997**, *38*, 5525–5528, see also pp 5529–5532.
17. Fujii, K.; Sivonen, K.; Kashiwagi, T.; Hirayama, K.; Harada, K.-I. *J. Org. Chem.* **1999**, *64*, 5777–5782.
18. Fujii, K.; Sivonen, K.; Naganawa, E.; Harada, K.-I. *Tetrahedron* **2000**, *56*, 725–733.
19. Fujii, K.; Ikai, Y.; Mayumi, T.; Oka, H.; Suzuki, M.; Harada, K.-I. *Anal. Chem.* **1997**, *69*, 3346–3352.
20. Fujii, K.; Ikai, Y.; Oka, H.; Suzuki, M.; Harada, K.-I. *Anal. Chem.* **1997**, *69*, 5146–5151.
21. Harada, K.-I.; Matsui, A.; Shimizu, Y.; Ikemoto, R.; Fujii, K. *J. Chromatogr. A* **2001**, *921*, 187–195.
22. Fujii, K.; Shimoya, T.; Ikai, Y.; Oka, H.; Harada, K.-I. *Tetrahedron Lett.* **1998**, *39*, 2579–2582.
23. Suzuki, Y.; Ojika, M.; Sakagami, Y.; Kaida, K.; Fudou, R.; Kameyama, T. *J. Antibiot.* **2001**, *54*, 22–28.
24. Harada, K.-I.; Shimizu, Y.; Kawakami, A.; Norimoto, M.; Fujii, K. *Anal. Chem.* **2000**, *72*, 4142–4147.
25. Ishida, K.; Nakagawa, H.; Murakami, M. *J. Nat. Prod.* **2000**, *63*, 1315–1317.
26. Mau, C. M. S.; Nakao, Y.; Yoshida, W. Y.; Scheuer, P. J.; Kelly-Borges, M. *J. Org. Chem.* **1996**, *61*, 6302–6304.
27. Igarashi, Y.; Kan, Y.; Fujii, K.; Fujita, T.; Harada, K.-I.; Naoki, H.; Tabata, H.; Onaka, H.; Furumai, T. *J. Antibiotics* **2001**, *54*, 1045–1053.
28. Morihara, K. *Bull. Chem. Soc. Jpn* **1964**, *37*, 1781–1784.