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Note

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Resolution of the Confusion in the Assignments of Configuration for the Ciliatamides, Acylated Dipeptides from Marine Sponges

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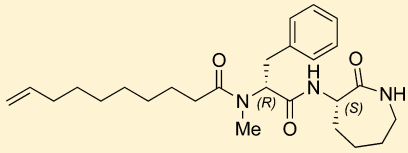
Supporting Information

ABSTRACT: Direct comparison of authentic ciliatamide A with four synthetic isomers (**1**−**4**) by means of NMR and chiral-phase HPLC revealed that ciliatamide A possesses the 12*R* (D-*N*-MePhe residue) and 22*S* (L-Lys residue) configurations, which were not identical with either our previous assignment or those proposed by others through total synthesis. The absolute configuration of the methionine sulfoxide residue in ciliatamide D was also revised to be D.



ciliatamide A

Ciliatamide A was originally reported as an antileishmanial compound from the deep-sea sponge *Aaptos ciliata*. Its structure was proposed as **1** (12*S*,22*S*), being composed of *N*-methyl-L-phenylalanyl-L-lysine with the N-terminus 9-decenoylated and the C-terminus cyclized to form an ε-caprolactam.[1] Later, Lindsley and co-workers reported that the synthetic compound with the proposed (12*S*,22*S*)-configuration exhibited NMR data identical with the natural product, but the sign of the specific rotation was opposite.[2] They synthesized all four possible stereoisomers (**1**−**4**), measured their NMR spectra and optical rotations, and concluded that ciliatamide A was **4** with the (12*R*,22*R*)-configuration.[2] Later, we isolated ciliatamide A together with a new analogue, ciliatamide D (**5**, assigned as 12*S*, 18*S*), from a *Stelletta* sp. marine sponge.[3] At this time we reinvestigated the configuration of ciliatamide A and again proposed the (12*S*,22*S*)-configuration for ciliatamide A. Mohapatra et al. synthesized the (12*S*,22*S*)-isomer that had a specific rotation of −33, the same as observed by Lindsley et al. for the (12*S*,22*S*)-isomer.[4,5] However, the true configuration of ciliatamide A has not been fully resolved due to the lack of direct comparison between the natural product and the synthetic derivatives.[3] Therefore, we synthesized the four possible stereoisomers of ciliatamide A and compared each of them with the natural product by ^{1}H NMR spectroscopy and HPLC using a chiral stationary phase.

We employed a synthetic route essentially identical with the one reported by Lindsley et al. (Scheme 1).[2] Either commercially available L-α-amino-ε-caprolactam hydrochloride or its D-isomer prepared from *N*-α-Boc-D-lysine was condensed with D- or L-*N*-methyl-phenylalanine (*N*-MePhe) and 9-decenoic acid to afford **1** with the (12*S*,22*S*)-configuration, **2** with the (12*R*,22*S*)-configuration, **3** with the (12*S*,22*R*)-configuration, and **4** with the (12*R*,22*R*)-configuration. The signs and magnitudes of the specific rotations of the four compounds prepared in this study were in agreement with those reported by Lindsley et al. (Table 1), and the sign and magnitude of the specific rotation value of the natural product was closest to those of the (12*R*,22*R*)-isomer as described by Lindsley et al..[3]

Next, we rigorously assigned the ^{1}H and ^{13}C NMR signals of the (12*S*,22*S*)- and (12*R*,22*S*)-isomers (**1** and **2**, respectively) including those of the minor conformers which arose from the *cis*−*trans* isomerism of the amide bond between the 9-decenoyl residue and the *N*-MePhe residue.[6] As expected, the NMR spectra of the enantiomeric pairs [(12*S*,22*S*)- and (12*R*,22*R*)-isomers as well as (12*R*,22*S*)- and (12*S*,22*R*)-isomers] were superimposable. The ^{1}H NMR spectra of the diastereomeric pairs were different, as briefly noted by Lindsley et al.[2] In the major isomer, chemical shifts of H-12 (**1**: δ 5.38 vs **2**: δ 5.43), H-13a (**1**: δ 3.04 vs **2**: δ 2.96), and H-23b (**1**: δ 1.93 vs **2**: δ 1.89) signals were slightly different between the diastereomers, whereas noticeable differences were observed for the H-2a (**1**: δ 1.67 vs **2**: δ 1.77), H-4a (**1**: δ 1.06 vs **2**: δ 1.10), H_3-20 (**1**: δ 2.89 vs **2**: δ 2.92), H-23a, (**1**: δ 1.59 vs **2**: δ 1.54), and H-23b (**1**: δ 1.92 vs **2**: δ 1.89) signals in the minor isomers (Table S1). ^{1}H NMR signals of natural ciliatamide A for H-12 and H-13a in the major isomer and H-4a and H_3-20 in the minor isomer were identical with those of the (12*R*,22*S*)-isomer but different from those of the (12*S*,22*S*)-isomer (Figure 1 and Tables S1−S3).[7] Marfey's analysis of the natural ciliatamide A clearly showed the presence of D-*N*-MePhe and L-Lys, demonstrating that the structure of the natural product was **2** (12*R*,22*S*) (Figure S17). The configuration of the *N*-MePhe residue was erroneously assigned in our previous publications.[1,3,8]

Due to the absence of the detailed experimental data for our first report,[1] we speculated the cause of the misassignment during the Marfey's analysis of ciliatamide A was as follows. The retention times of the *N*-(2,4-dinitrophenyl)-L-alanine amide (DAA) derivatives of *N*-MePhe and Lys are as follows: L-*N*-MePhe (13.1 min), D-*N*-MePhe (13.6 min), L-Lys (13.7 min), D-Lys (17.8 min), among which D-*N*-MePhe and L-Lys are overlapped (Figure S17). Our hydrolysis experiments

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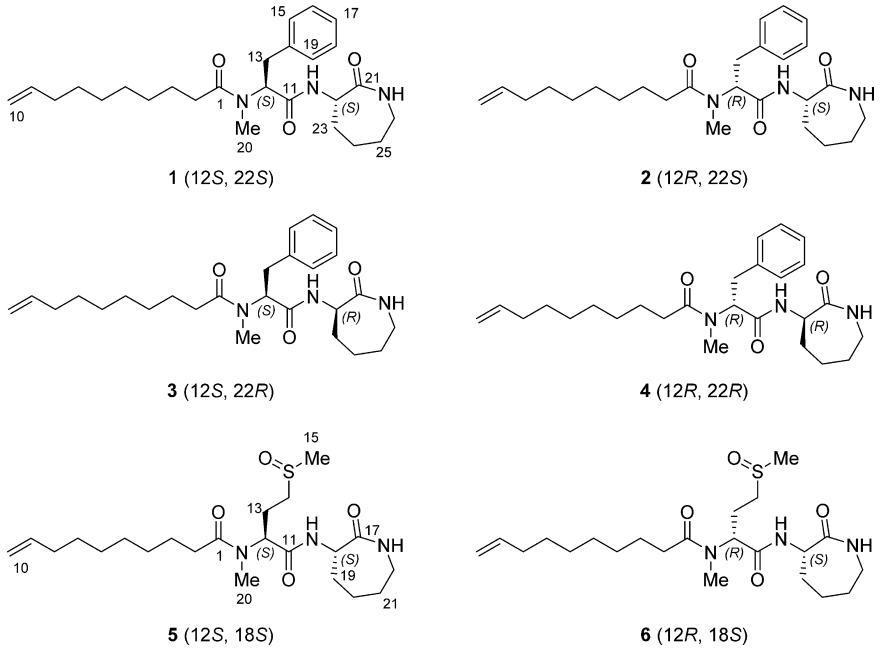
Chart 1



Scheme 1. Synthesis of 1

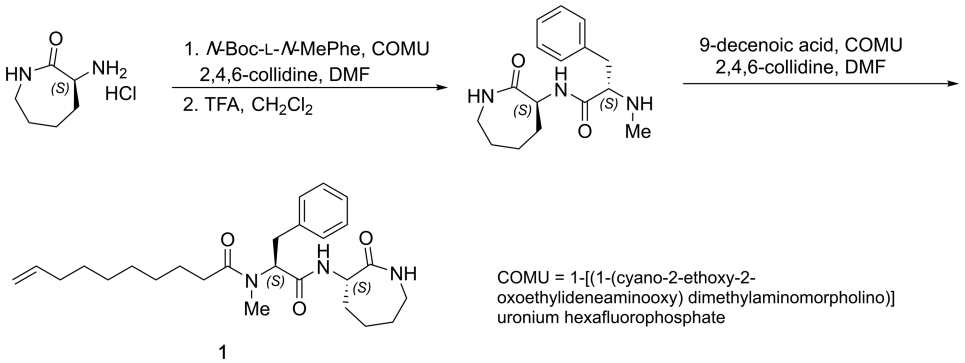


Table 1. Specific Rotation Values (MeOH) for Natural Ciliatamide A and Synthetic Diastereomers

	Lindsley (*c* 0.05)	Mohapatra (*c* 0.05)	this work (*c* 0.2)	natural product (*c* 0.1)
12*S*,22*S*	−35	−33	−42	+40[a] (+52[b])
12*S*,22*R*	+70		+65	
12*R*,22*S*	−48		−67	
12*R*,22*R*	+42		+46	

[a]The value reported in ref 1. [b]The value reported in ref 3.

showed that *N*-MePhe was partially racemized, but Lys was not racemized (Figure S17). Because DAA_2-L-Lys and DAA-D-*N*-MePhe overlap, the hydrolysate of **2** (composed of D-*N*-MePhe and L-Lys) will give a small peak of DAA-L-*N*-MePhe, generated by partial racemization, and a large overlapped peak of DAA_2-L-Lys and DAA-D-*N*-MePhe. By detecting the HPLC with UV absorption, it was not possible to distinguish between DAA_2-L-Lys and DAA-D-*N*-MePhe. After detection of L-*N*-MePhe, it was reasonable to consider another peak as derived only from L-Lys (Figure S17).

In our second report[3] Marfey's analysis of *N*-MePhe was conducted by inadvertently using the mass chromatogram with detection at m/z 418, which corresponded to the $[M + H]^+$ ion of DAA-Phe, not of DAA-*N*-MePhe (Figure S10 in ref 2). The D-*N*-MePhe and L-*N*-MePhe used as the standards were prepared from D-*N*-Boc-Phe and L-*N*-Boc-Phe, respectively, and used without rigorous purification. In the Marfey's analysis of the natural ciliatamide A, a peak coeluting with DAA-L-Phe derived from the unreacted L-Phe during the preparation of L-*N*-MePhe was detected. The misleading peak turned out to derive from an unidentified impurity.

With the four isomers as well as the natural product in hand, we proceeded to analyze them by HPLC with a chiral stationary phase. By using a CHIRALPAK IG-3 column, the (12*R*,22*R*)-, (12*S*,22*S*)-, and (12*R*,22*S*)-isomers each gave a sharp peak with different retention times. However, the (12*S*,22*R*)-isomer (**3**) did not give a peak under the same HPLC condition. We examined a variety of chiral stationary phases,[9] among which CHIRALPAK ID-3 afforded a peak for **3** and baseline separated the four isomers (Figure 2). Under this condition natural ciliatamide A coeluted with the (12*R*,22*S*)-isomer, in accordance with the assignment described above.

The revision of the configuration of ciliatamide A prompted us to reanalyze the configuration of ciliatamide D (**5**), a 1:1 epimeric mixture of diastereomers due to asymmetry at the sulfur atom of the methionine sulfoxide residue.[3] We conducted

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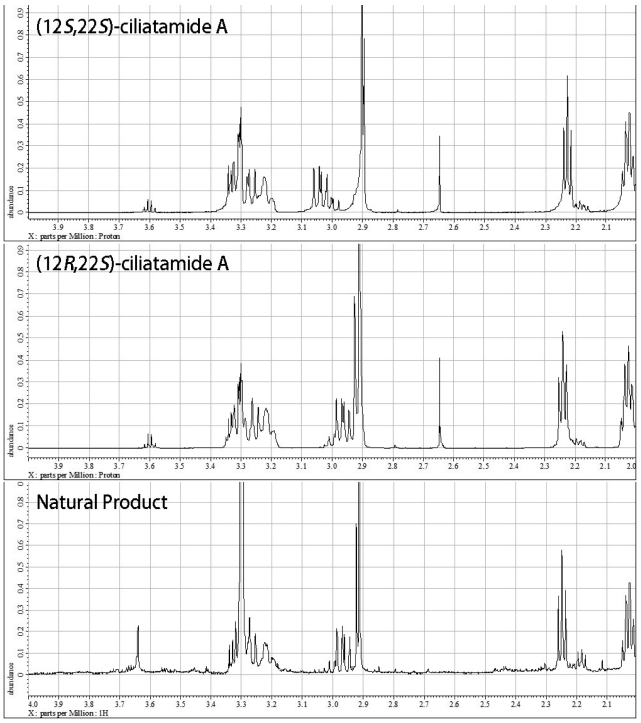

Figure 1. ^{1}H NMR (600 MHz) spectra of **1**, **2**, and natural ciliatamide A.

the Marfey's analysis of ciliatamide D after converting MetO to $MetO_2$, which permitted us to detect D-$MetO_2$ (Figure S18) and L-Lys, indicating that the absolute configuration of ciliatamide D has to be revised as **6** (12*R*,18*S*).[9]

In this study, we showed that ciliatamide A possesses the (12*R*,22*S*)-configurations, which were not identical with either our previous assignment or those proposed by others through total synthesis.[1−3] In spite of possessing all isomers for comparison with the reported data of ciliatamide A, Lindsley et al. drew an incorrect conclusion.[2] Because the differences of the NMR data between the diastereomers were small and the value of the specific rotation of the synthetic (12*R*,22*R*)-isomer matched well with that of the natural product, more serious consideration was apparently given to the latter evidence. It is worthy to note that the ^{1}H NMR spectra of synthetic ciliatamide A derivatives are highly reproducible, but the magnitude of specific rotation is prone to fluctuate due to the inconsistent purity and errors in weighing of the sample. It is not possible for us to study the configuration of ciliatamides B and C due to the lack of authentic samples. The synthetic studies showed that the sign and magnitude of specific rotations of ciliatamide A and its isomers depend largely on the configuration of the *N*-MePhe residue; the D-*N*-MePhe residue gives a positive value, whereas L-*N*-MePhe gives a negative value. Because the Lys residue was correctly assigned by Marfey's analysis in the two isolation studies,[1,3] we anticipate that the configurational assignment of the Orn residue, a minor homologue of Lys, was also correct. From these analyses and the reported positive specific rotation values of both ciliatamides B and C, we speculate that the *N*-MePhe residues in these compounds are in the D-form and the Lys and Orn residues in the L-form. This study provides a cautionary note for those who conduct structure elucidation of natural products that the direct comparison of the synthetic compounds with the natural product is crucial.[11]

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on a Jasco DIP-1000 polarimeter. UV spectra were measured on a Shimadzu Biospec 1600. NMR spectra were recorded on a JEOL alpha 600 NMR spectrometer. Chemical shifts were referenced to a solvent peak: δ_H 3.30 and δ_C 49.0 (CD_3OD). HRESI mass spectra were measured on a JEOL JMS-T100LC. LC-MS experiments were performed on a Shimadzu LC-20AD solvent delivery system and interfaced to a Bruker amaZon SL mass spectrometer. Ciliatamides A and D were isolated as described in ref 3.

Syntheses of (12*S*,22*S*)- and (12*R*,22*S*)-Ciliatamide A. To a solution of L-α-amino-ε-caprolactam hydrochloride (24.5 mg, 0.15 mmol) and *N*-Boc-*N*-methyl-L-phenylalanine (41.6 mg, 0.15 mmol) and 1-[(1-(cyano-2-ethoxy-2-oxoethylideneaminooxy)-dimethylaminomorpholino)]uronium hexafluorophosphate (COMU) (64.2 mg, 0.15 mmol) in DMF (1 mL) was added 2,4,6-collidine (0.060 mL, 0.45 mmol) at 0 °C. The reaction mixture was allowed to warm to room temperature (rt) and stirred for 21 h. The reaction mixture was then cooled to 0 °C, quenched with saturated aqueous

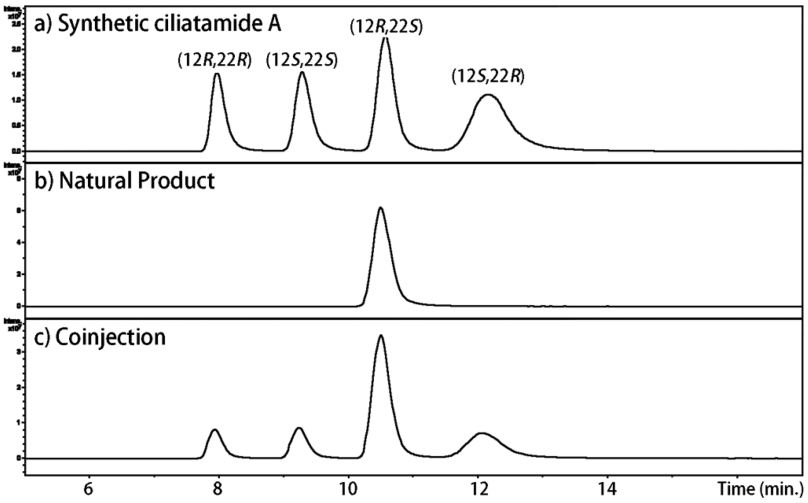

Figure 2. LC-MS analyses of the synthetic ciliatamide A isomers and natural product. (a) Chromatogram of a mixture of the four synthetic isomers. (b) Chromatogram of the natural ciliatamide A. (c) Co-injection of the mixture of the four synthetic isomers and natural ciliatamide A. LC-MS conditions are as follows: CHIRALPAK ID-3 (ϕ 4.6 × 250 mm); flow rate, 0.8 mL/min.; solvent, MeCN/H_2O containing 20 mM NH_4HCO_3 (7:3).

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$NaHCO_3$, extracted with EtOAc, washed with brine, and dried over $MgSO_4$. The solution was concentrated and dissolved in CH_2Cl_2 (5 mL), to which was added trifluoroacetic acid (TFA) (1 mL) at 0 °C. The reaction mixture was stirred at rt for 4 h and concentrated to afford a crude dipeptide, which was used in the next reaction without further purification. To a solution of the crude dipeptide, COMU (64.2 mg, 0.15 mmol), and 9-decenoic acid (0.028 mL, 0.15 mmol) in dimethylformamide (DMF) (1 mL) was added 2,4,6-collidine (0.040 mL, 0.30 mmol) at 0 °C. The reaction mixture was allowed to warm to rt and stirred for 16 h. The reaction mixture was then cooled to 0 °C, quenched with saturated aqueous $NaHCO_3$, extracted with EtOAc, washed with brine, and dried over $MgSO_4$. The solution was concentrated, and the residue was purified by RP-HPLC (50−65% MeCN containing 0.5% AcOH) to afford (12*S*,22*S*)-ciliatamide A (**1**, 29 mg) as a colorless oil. (12*R*,22*S*)-Ciliatamide A (**2**) was synthesized by using *N*-Boc-*N*-methyl-D-phenylalanine instead of *N*-Boc-*N*-methyl-L-phenylalanine.

(12S,22S)-Ciliatamide A (1): ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz), Table S21; HRESIMS *m/z* 464.2867 $[M + Na]^+$ (calcd for $C_{26}H_{40}N_3O_3Na$, 464.2884).

(12R,22S)-Ciliatamide A (2): ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz), see Table S32; HRESIMS *m/z* 464.2866 $[M + Na]^+$ (calcd for $C_{26}H_{40}N_3O_3Na$, 464.2884).

Syntheses of (12*S*,22*R*)- and (12*R*,22*R*)-Ciliatamide A. To a solution of *N*-α-Boc-D-lysine (150.0 mg, 0.60 mmol) and COMU (771.1 mg, 1.80 mmol) in CH_2Cl_2/DMF (9:1, 120 mL) was added 2,4,6-collidine (0.480 mL, 3.60 mmol) at 0 °C. The reaction mixture was allowed to warm to rt and stirred for 13 h. The reaction mixture was then cooled to 0 °C, quenched with saturated aqueous $NaHCO_3$, extracted with EtOAc, washed with brine, and dried over $MgSO_4$. The solution was concentrated, the residue was extracted with Et_2O and filtered, and the filtrate was concentrated. The residue was dissolved in CH_2Cl_2 (5 mL), to which was added TFA (1 mL) at 0 °C. The reaction mixture was stirred at rt for 4 h and concentrated to afford a crude D-α-amino-ε-caprolactam (78.5 mg), which was used in the next reaction without further purification. (12*S*,22*R*)- and (12*R*,22*R*)-Ciliatamide A were synthesized as described above by using D-α-amino-ε-caprolactam.

(12S,22R)-Ciliatamide A (3): ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data were identical with those of **2** (Table S3); HRESIMS *m/z* 464.2868 $[M + Na]^+$ (calcd for $C_{26}H_{40}N_3O_3Na$, 464.2884).

(12R,22R)-Ciliatamide A (4): ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data were identical with those of **1** (Table S2); HRESIMS *m/z* 464.2868 $[M + Na]^+$ (calcd for $C_{26}H_{40}N_3O_3Na$, 464.2884).

LC-MS Analysis of the Four Isomers with a Chiral Stationary Phase. The mode of separation of **1**−**4** was screened by a combination of a chiral stationary phases (CHIRALPAK IA, IB, IC, ID, IE, IF, IG, AS-RH, AY-RH, OD-RH, OJ-RH, and OZ-RH) and various solvent systems (mixtures of H_2O, MeOH, and MeCN). The four isomers were separated by LC-MS [CHIRALPAK ID-3 (ϕ 4.6 × 250 mm); flow rate, 0.8 mL/min; solvent, MeCN/H_2O containing 20 mM NH_4HCO_3 (7:3)].

Preparation of *N*-Methyl-D- and *N*-Methyl-L-methionine Sulfone. To a solution of *N*-Boc-L-methionine (50 mg) in MeOH (2 mL) was added Oxone (246 mg), and the solution was stirred at rt for 1 h. The mixture was diluted with H_2O and extracted with CH_2Cl_2 to give *N*-Boc-L-methionine sulfone (*m/z* 282 $[M + H]^+$). To a solution of the above *N*-Boc-L-methionine sulfone in THF (2 mL) was added NaH (15 mg) and MeI (165 μL), and the mixture was stirred at rt overnight. The reaction mixture was diluted with H_2O and extracted with EtOAc. The organic layer was concentrated to afford *N*-Boc-*N*-methyl-L-methionine sulfone (*m/z* 296 $[M + H]^+$), which was dissolved in 6 N HCl (100 μL) and heated at 110 °C for 2 h. The solution was concentrated to provide *N*-methyl-L-methionine sulfone (*m/z* 196 $[M + H]^+$). *N*-Methyl-D-methionine sulfone was synthesized from *N*-Boc-D-methionine in the same manner.

Oxidation of Ciliatamide D. To a solution of ciliatamide D (50 μg) in MeOH (0.5 mL) was added Oxone (1 mg in 100 μL of H_2O), and the mixture was stirred at rt for 2 h. The solution was diluted with H_2O, applied on InertSep PLS-2 (GL Science), washed with 10% MeOH, and eluted with MeOH to afford ciliatamide D sulfone (*m/z* 458 $[M + H]^+$).

Marfey's Analysis. Ciliatamide D sulfone was dissolved in 6 N HCl (100 μL) and heated at 110 °C for 2 h. The solution was concentrated and redissolved in 100 μL of H_2O. L-FDAA (1%) in acetone (100 μL) and 1 M $NaHCO_3$ (10 μL) were added to the solution. The mixture was heated at 55 °C for 30 min. After cooling to rt, the reaction mixture was quenched with 2 N HCl (5 μL), concentrated, and redissolved in MeOH. D- and L-Methionine sulfone were treated with L-DAA in the same manner. The L-FDAA derivatives were analyzed by LC-MS [Cosmosil 2.5C_{18}-MS-II (ϕ 2.0 × 100 mm); flow rate, 0.5 mL/min; solvent, MeOH/MeCN containing 1% formic acid/H_2O (27:5:68)].[11] Retention times (t_R) of the amino acids were as follows: *N*-methyl-D-methionine sulfone derivative (t_R = 22.2 min) and *N*-methyl-L-methionine sulfone derivative (t_R = 23.7 min). The t_R of the derivatized methionine sulfone from ciliatamide D was 22.2 min.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.7b00684.

NMR and MS data of **1** and **2** (PDF)

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Notes

The authors declare no competing financial interest.

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(5) In a symposium paper, it was mentioned that the synthetic **1** exhibited the specific rotation value as reported by Lindsley et al. Akiyama, S.; Nakao, Y.; Matsumoto, Y.; Goto, Y.; Sanjoba, C.; Osada, Y.; Umehara, M.; Kimura, J. *Symposium Paper of 55th Symposium on The Chemistry of Natural Products* **2013**, *55*, 591−596.

(6) ^{1}H NMR signal assignments for the ε-caprolactam residue reported in refs 1 and 3 have been corrected.

(7) This analysis contradicts with the one reported by Lindsley et al.,[2] who stated that the NMR spectra of the synthetic **1** were identical with those of the natural product.

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(9) In our previous study of ciliatamide D, the Marfey's analysis was conducted by using an ODS column (Cosmosil MS-II) as the stationary phase and a gradient elution of 10−50% MeCN containing 0.5% AcOH as the mobile phase. Under this condition DAA-D-$MetO_2$ and FDAA-L-$MetO_2$ were barely separable. The assignment of the configuration of $MetO_2$ was conducted by considering the difference of the retention times observed in experiments carried out on separate days as the true difference, leading to the misassignment. In the current study HPLC of the DAA derivatives was conducted with the same stationary phase but with a different mobile phase [an isocratic elution with a mixture of MeOH/MeCN/H_2O/HCOOH (27:5:68:1)],[10] which gave two distinct peaks for the two DAA derivatives and permitted us to unambiguously assign the chirality of $MetO_2$ as D.

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