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Chromatographic Determination of the Absolute Configuration in Sanjoinine A That Increases Nitric Oxide Production

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Abstract

A chiral derivatization strategy with phenylglycine methyl ester (PGME) was employed to develop a straightforward method to determine the absolute configurations of *N*,*N*-dimethyl amino acids. The PGME derivatives were analyzed using liquid chromatography-mass spectrometry to identify the absolute configurations of various *N*,*N*-dimethyl amino acids based on their elution time and order. The established method was applied to assign the absolute configuration of the *N*,*N*-dimethyl phenylalanine in sanjoinine A (**4**), a cyclopeptide alkaloid isolated from Zizyphi Spinosi Semen widely used as herbal medicine for insomnia. Sanjoinine A displayed production of nitric oxide (NO) in LPS-activated RAW 264.7 cells.

Key Words: *N*,*N*-dimethyl amino acids, Phenylglycine methyl ester, Chemical derivatization, LC-MS analysis, Nitric oxide production

INTRODUCTION

The herbal medicine contains a large number of pharmacologically active substances, such as flavones, alkaloids, and triterpenes (Jeon *et al*., 2019), which have been shown to improve cognitive performance by suppressing cholinesterase activity, protect against NMDA-induced neuronal cell damage, and prolong pentobarbital-induced sleep (Habtemariam, 2019). For thousands of years, *Zizyphus jujuba* Mill *var. spinosa* (Rhamnaceae) (Zizyphi Spinosi Semen, ZSS) has been used as an analgesic, sedative, and anticonvulsant for treating sleeplessness and anxiety in Asian countries such as China and Korea (Peng and Zhu, 2001). In modern scientific analysis, extracts of ZSS in ethanol and methanol have been shown to reduce anxiety (Han *et al*., 2007). In rats, aqueous extracts of Sanjoin-Tang have exhibited anxiolytic-like effects (Ahn *et al*., 2004). Nutritional components of ZSS have been explored and employed in functional foods and biomedicine as a result of modern scientific and technical advances (Gao *et al*., 2013). Chemical substances of ZSS have been reported as terpenoids, flavonoids, alkaloids, phenolics, and fatty acids (Kim, 2016). Among them, sanjoinine A, isolated by Han *et al*. (1985), has been continuously studied for its GABA-related activity (Han *et al*., 1985; Ma *et al*., 2007). In order to further explore the activity related to sanjoinine A, we investigated the structure of this substance; however, the three-dimensional structure of sanjoinine A was not assigned by chemical derivatization. To determine the chemical structures of biologically active compounds, it is necessary to identify both planar and three-dimensional structures in chemical biology and medicine (Brown and Wobst, 2021). The three-dimensional structure analysis of a drug is especially relevant because the changes in physiological activities and side effects of pharmacological actions are reportedly related to the three-dimensional structures of the chiral compounds (De camp, 1993; Liu and Liu, 2002). Thalidomide is the most prominent example of an enantiopure drug, which is defined as a compound with a single structure. This medicine was created as a racemic structure to treat morning sickness in pregnant women; however, it was shown to cause birth abnormalities (Burley and Lenz, 1962; Lenz, 1988; Tokunaga *et al*., 2018). As a result of subsequent research, it was found that only the (*R*)-structure exhibited medicinal effects from the outset, and the (*S*)-structure was the cause of the side effects, highlighting the importance of the arrangement of a single three-dimensional structure of the drug (Blaschke *et al*., 1979). In addition, only

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the (*S*,*S*)-enantiomer of the tuberculosis medicine Ethambutol proved useful for therapy, whereas the (*R*,*R*)-enantiomer was discovered to induce blindness (Fraunfelder *et al*., 2006). Consequently, in the present pharmaceutical industry, the preparation and separation analysis of enantiopure pharmaceuticals, which vary based on the three-dimensional structural arrangement, are gaining relevance.

In the process of determining the three-dimensional structure of sanjoinine A, we intended to provide a method for evaluating enantiopure peptide-based natural substances for laboratory knowledge that fulfils the requirement of the current pharmaceutical industry. This study was conducted with the aim to perform liquid chromatography (LC) analysis through chemical asymmetric derivatization of *N*-substituted amino acids. To determine the three-dimensional structure of a general amino acid, Marfey's method was applied (Marfey, 1984); in this method, the amine group of an amino acid is subjected to an asymmetric functionalization, and amide coupling is induced between the carboxyl group of an amino acid and an asymmetric functional group (e.g., 1-fluoro-2,4-dinitrophenyl-5)-L-alanine amide (L-FDAA). It was predicated on the idea that the elution time would vary based on the arrangement of the hydrophilic and hydrophobic functional groups in the LC column of the asymmetric derivatives.

N,*N*-Dimethyl amino acids are frequently found in natural compounds derived from peptides such as IB-01212, symplocin A, ochraceopetalin, sanjoinine A, citrinadin, kinenzoline, and OA-7653 (Han *et al*., 1985; Jeffs *et al*., 1988; Tsuda *et al*., 2004; Cruz *et al*., 2006; Molinski *et al*., 2012; Kurisawa *et al*., 2021; Park *et al*., 2021) (Fig. 1). However, chiral derivatization of the *N*,*N*-dimethyl amino acids using L-FDAA is inapplicable in the case of *N*,*N*-dimethyl amino acids, which include the fully substituted dimethyl at the amine functional group. In previous studies, the following approaches have been used to determine the absolute configurations of the *N*,*N*-dimethyl amino acids:

(1) The absolute configuration of the *N*,*N*-dimethyl leucine in cyclodepsipeptide (IB-01212) from a fungal strain *Clonostachys* sp. was determined by derivatization with menthol. Acid hydrolysis of cyclodepsipeptide was treated with menthol in toluene and a catalytic *p*-toluene sulfonic acid and refluxed. The standards and reaction mixture were subjected to reversed-phased high-performance liquid chromatography (HPLC) and compared the retention time (Cruz *et al*., 2006). However, this procedure requires harsh reaction conditions and the absence of ultraviolet (UV) chromophores in menthol makes it difficult to analyze chiral derivatives using reverse-phased HPLC.

(2) The absolute configuration of the *N*,*N*-dimethyl isoleucine and valic acid in linear peptide symplocin A from cyanobacterium *Symploca* sp. were determined by chiral HPLC analysis of the naphthacyl esters derivatives (Molinski *et al*., 2012). Disadvantage of this method is the requirement of chiral HPLC column and all kinds of diastereomer standards.

(3) Recently, the basic PGME analysis (Yabuuchi and Kusumi, 2000) was employed to determine the *N*,*N*-dimethyl valine and prolinol in ochraceopetalin from marine fungus *Aspergillus ochraceopetaliformis*. Hydrolysates were treated with *S*- and *R*-PGME to produce the corresponding PGME amides and $\Delta\delta_H$ values ($\Delta\delta=\delta_S-\delta_R$) were compared (Park *et al*., 2021). This approach necessitates a large amount of the substance, and it is cumbersome to purify the reactants by HPLC and perform nuclear magnetic resonance (NMR) spectroscopy analysis.

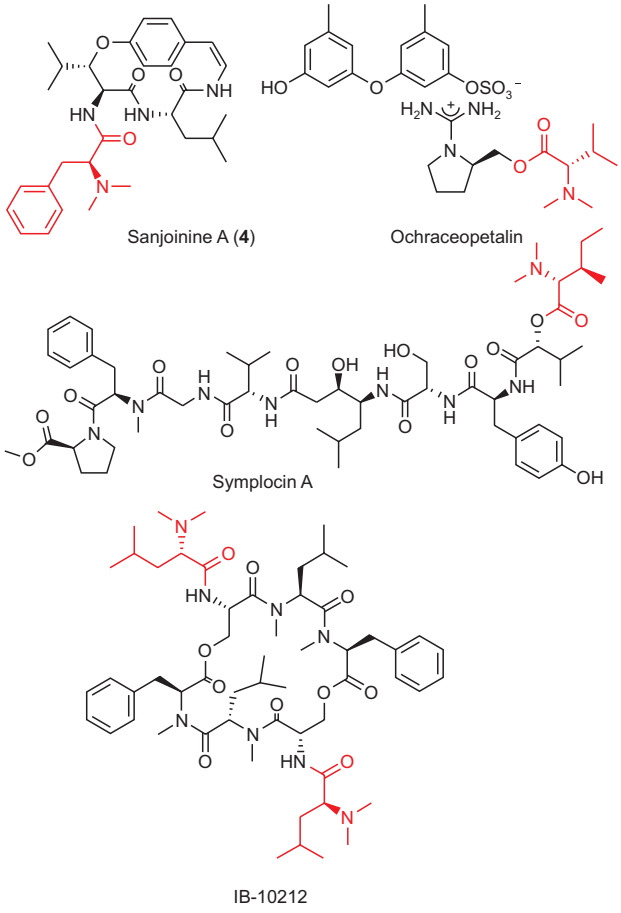

Fig. 1. Representative bioactive natural substances bearing *N*,*N*-dimethyl amino acid.

The methods available for determining the structures of *N*,*N*-dimethyl amino acids involve complex procedures. Thus, a straightforward method for determining the absolute configurations of *N*,*N*-dimethyl amino acids in the analyte is desired. We hypothesize that a PGME reaction of the *N*,*N*-dimethyl amino acids would provide derivatives appropriate for chromatographic mass spectrometry without requiring an extra chemical derivatization or the NMR analysis step. Herein, we report a "PGME method" that facilitates a simple and easy determination of the absolute configurations of *N*,*N*-dimethyl amino acids without requiring any purification process.

Additionally, we investigated bioactivity screening of the isolated sanjoinine A from Zizyphi Spinosi Semen extract.

MATERIALS AND METHODS

General experimental procedure

An Agilent G6125B MSD system was used to record the UV spectra and liquid chromatography-mass spectrometry (LC-MS) data. The system was paired with an Agilent Technologies 1260 series Infinity II LC system that employed a reverse-phase C18 column (Phenomenex Luna, 100 mm×4.6

mm, 5 μm). The ionization source of the mass spectrometer was operated in both positive and negative ionization modes. The LC-MSD ChemStation software (Agilent Technologies, Santa Clara, CA, USA) was used to control the whole system. An Agilent Technologies 1290 series HPLC instrument paired with an Agilent 6530 iFunnel Q-TOF LC/MS system was utilized in order to acquire high-resolution electrospray ionization mass spectra. ^{1}H and ^{13}C NMR spectra were recorded using a Bruker Avance II 800 MHz NMR spectrometer at the Korea Basic Science Institute, Ochang, Korea. The HPLC purification was performed on a Waters system (1525 binary HPLC pump and 996 photodiode array detector) with a YMC Pack-ODS-A-C18 column (250×10 mm, 5 μm). All the L-amino acids, 37% formaldehyde, and sodium cyanoborohydrate were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the solvents were of a quality suitable for HPLC, and they were acquired from Merck (Mumbai, India). The three-dimensional structure of the asymmetric derivatized product was formed using the database obtained from the virtual molecular structure energy minimization program Avogadro (https://avogadro.cc).

Extraction and HPLC purification for sanjoinine A (4)

The plant material, Zizyphi Spinosi Semen (ZSS), was purchased at Malbawoo market in Gwangju (250 g, Oherb, Seoul, Korea). Maceration of the powdered dry seeds of *Z. jujuba* (ZSS) with MeOH (2 times×2 L, overnight) was used to extract the desired compound from the plant, After removing the solvent in vacuo from the extraction process, 132 mg of the crude extract was obtained. In order to purify sanjoinine A from the crude extract, HPLC was performed (an isocratic system, 35% MeCN and 0.1% formic acid, 3 mL/min, column), and the extracted sanjoinine A (9.3 mg) was eluted at 25 min.

Molecular modeling of (*S*)-PGME derivatives of *N*,*N*-dimethyl phenylalanine

In order to establish the energy minimized conformations of (*S*)-PGME derivatives of L- and D-*N*,*N*-dimethyl phenylalanine, computational density function theory (DFT) calculations were performed as we described previously (Shin *et al*., 2021). By utilizing Avogadro 1.2.0 software under an MMFF94s force field, the ground-state geometries of (*S*)-PGME derivatives of L- and D-*N*,*N*-dimethyl phenylalanine were obtained. The geometries were further optimized using TmoleX 4.3.2 software with DFT settings (B3-LYP functional/M3 grid size, energy 10-6 Hartree, gradient norm |dE/dxyz|=10-3 Hartree/Bohr, and 6-31G basis set.

N,*N*-dimethyl amino acid synthesis and PGME derivatization

All the dimethyl amino acids including dimethyl phenylalanine, dimethyl alanine, dimethyl valine, dimethyl leucine, dimethyl threonine, dimethyl methionine, dimethyl tryptophan, dimethyl aminobutyric acid, and dimethyl allothreonine (Fig. 2) were synthesized by methylation of free amino acids according to the established protocol (Dearborn and Jentoft, 1983). In a nutshell, one equivalent of amino acid was dissolved in a buffer solution of 50 mM sodium acetate with a pH of 5. Afterward, 10 equivalents of 37% formaldehyde and 10 equivalents of sodium cyanoborohydride were added simultaneously. The mixture was swirled at room temperature for 15-20 min, while the temperature was held constant. After stirring, the reaction mixture was placed in a refrigerator. Before being derivatized with *S*-, *R*-PGME and the dimethyl amino acids were first acidified with diluted hydrochloric acid and then dissolved in methanol.

LC-MS analysis for PGME derivatives

We then investigated the optimum PGME reaction conditions and methodologies that are suited for chromatography-mass spectrometry and were able to establish the proper procedure for LC-MS analysis as follows (Fig. 2):

1) To a stirred solution of the synthesized *N*,*N*-dimethyl amino acid (<1 mg, ca. 5.0 mmol) in tetrahydrofuran (1.0 mL), EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, 1.6 mg, 10 mmol) was added at room temperature. 2) After 5 min, PGME (1.2 mg, 7.5 mmol) was added, and the reaction mixture was stirred under an argon or nitrogen gas for 30 min. 3) A 30 μL aliquot of the reaction mixture was quenched by adding 30 μL of MeOH. 4) Data analysis based on the UV spectra and mass values were performed after injecting 10 μL of the reaction mixture onto an LC-MS. The mobile phase was controlled under the linear gradient condition: 10% MeCN to 50% MeCN in H_2O with 0.1% formic acid over 30 min, 100% MeCN after 30 min at flow rate of 0.4 mL/min. The products were detected by UV analysis at 210 nm (200-600 nm by photodiode array detection).

Antimicrobial assay

The bacteria and fungi were first cultured on Luria-Bertani agar (LB) and Yeast extract Peptone Dextrose (YPD) agar at 30°C overnight, respectively, and then transferred into LB broth and YPD broth for further incubation. After incubation at 30°C for 24 h, the harvested microbial cells were inoculated into Mueller Hilton broth and YPD broth, respectively, with an initial optical density value (OD_{600}) of 0.0008. The compound was prepared in DMSO and diluted in a 96 well plate to a range of 200 to 0.8 μg/mL using two-fold dilutions. The OD values of the plate were measured at 30°C after 24 h. Gentamicin and cycloheximide were used as positive control compounds.

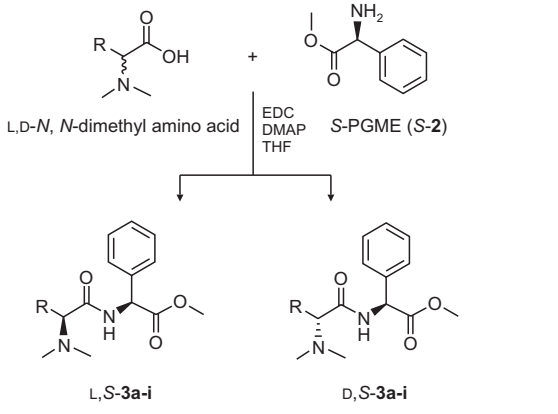

Fig. 2. Chemical derivatization for PGME application.

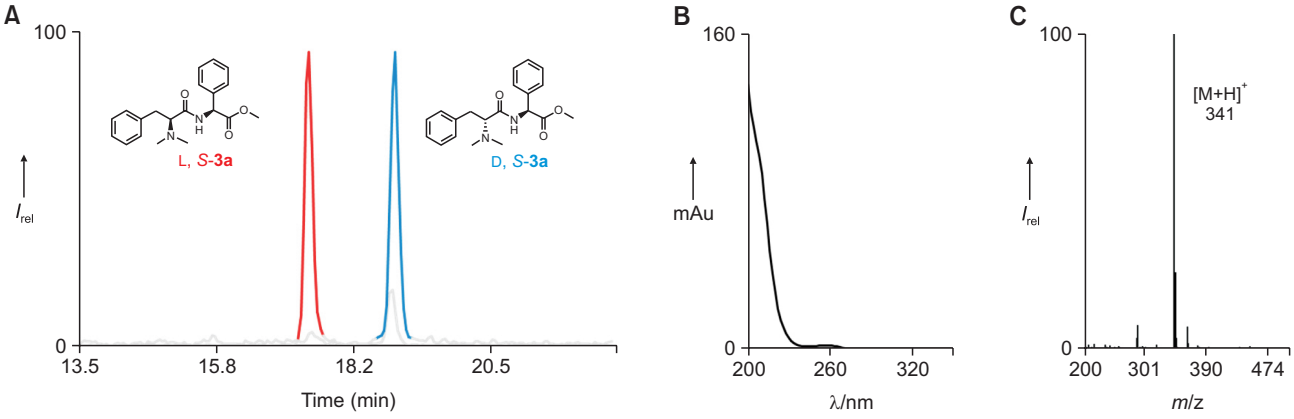

Fig. 3. LC-MS analysis of the PGME derivatives of L- and D-*N*,*N*-dimethyl phenylalanine (L,*S*- and D,*S*-**3a**). (A) The overlaid extracted ion mass chromatogram of L,*S*-**3a** and D,*S*-**3a** (B) Characteristic UV spectrum of **3a**. (C) Mass spectrum of **3a** in the positive ionization mode, *m/z* 341 $[M+H]^+$.

Table 1. LC-MS analysis of *S*-PGME derivatives of *N*,*N*-dimethyl amino acids

N,*N*-dimethyl amino acid	*S*-PGME derivative	t_{RL}[a] (min)	t_{RD}[b] (min)	Δt[c] (min)	Elution order
Phenylalanine (**1a**)	3a	17.4	18.7	1.3	L→D
Alanine (**1b**)	3b	10.3	11.3	1.0	L→D
Valine (**1c**)	3c	13.6	45.0	1.4	L→D
Leucine (**1d**)	3d	16.6	18.1	1.5	L→D
Threonine (**1e**)	3e	12.2	12.9	0.7	L→D
Methionine (**1f**)	3f	15.3	16.1	0.8	L→D
Tryptophan (**1g**)	3g	18.9	19.6	0.7	L→D
Aminobutyric acid (**1h**)	3h	12.0	13.1	1.1	L→D
Allothreonine (**1i**)	3i	10.2	10.5	0.3	L→D

[a]Retention times of L,*S*-3, [b]Retention times of D,*S*-3, [c]$\Delta t = t_{RD} - t_{RL}$.

Griess assay

RAW 264.7 murine macrophage cells were purchased from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco Modified Eagle's medium (DMEM) (Mumbai, India) containing 1 % penicillin-streptomycin and 10 % fetal bovine serum (FBS) (Gibco BRL, Rockville, MD, USA) under a 5 % CO_2 atmosphere at 37°C. RAW 264.7 cells were seeded in 96-well plates (SPL Life sciences Co., Pocheon, Korea). The cells were treated with sanjoinine A (30, 10, and 3 μg/mL) for 2 h and then stimulated with 500 ng/mL of LPS (Sigma-Aldrich). The NO production in the cell culture supernatant was measured using Griess reagent (1% sulfanilamide in 2.5% H_3PO_4, 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride). The cell supernatant was mixed with Griess Reagent for 30 min at room temperature. Absorbance was measured at 570 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader (ELx808; BioTek Instrumnents, Inc., Winooski, VT, USA) (Robbins and Grisham, 1997; Albina and Reichner, 1998; Zhang *et al*., 2007, 2020).

RESULTS

PGME derivatization and LC-MS analysis

Once the PGME-*N*,*N*-dimethyl amino acid derivative is produced, it is essential to ascertain that it exhibits an unique retention time relative to its diastereomer under specified LC-MS analytical conditions. Initially, L-dimethyl phenylalanine and D-dimethyl phenylalanine were derivatized with *S*-PGME, and then the resultant diastereomers, L,*S*-**3a** and D,*S*-**3a** were examined by LC-MS (Fig. 3). Using the previously described reverse-phase LC-MS analysis technique, we observed a distinct separation between the two diastereomers. The L,*S*-**3a** diastereomer eluted at 17.4 min, whereas the D,*S*-**3a** diastereomer eluted at 18.7 min (Table 1, Fig. 3). Similarly, six more amino acids found in natural products were selected and derivatized to *N*,*N*-dimethyl amino acids, including dimethyl alanine, dimethyl valine, dimethyl leucine, dimethyl methionine, dimethyl threonine, and dimethyl tryptophan. Under the specified conditions (Fig. 2), both the L and D forms of such *N*,*N*-dimethyl amino acids coupled with *S*-PGME to yield L,*S*-**3** and D,*S*-**3**. The elution times of the obtained products determined after the LC-MS analysis are listed in Table 1 (Fig. 4). All the *N*,*N*-dimethyl amino acid derivatives exhibited identical elution orders (L→D) as dimethyl phenylalanine derivatives (L,*S*-**3a** and D,*S*-**3a**). We also investigated the retention times of the PGME derivatives with unusual amino acids, such as aminobutyric acid (**3h**) and allothreonine (**3i**), which eluted in an identical elution order (L→D). These results suggest that the absolute configurations of the *N*,*N*-dimethyl amino acids discovered in natural products can be determined by *S*-PGME derivatization. We examined the three-dimensional structures

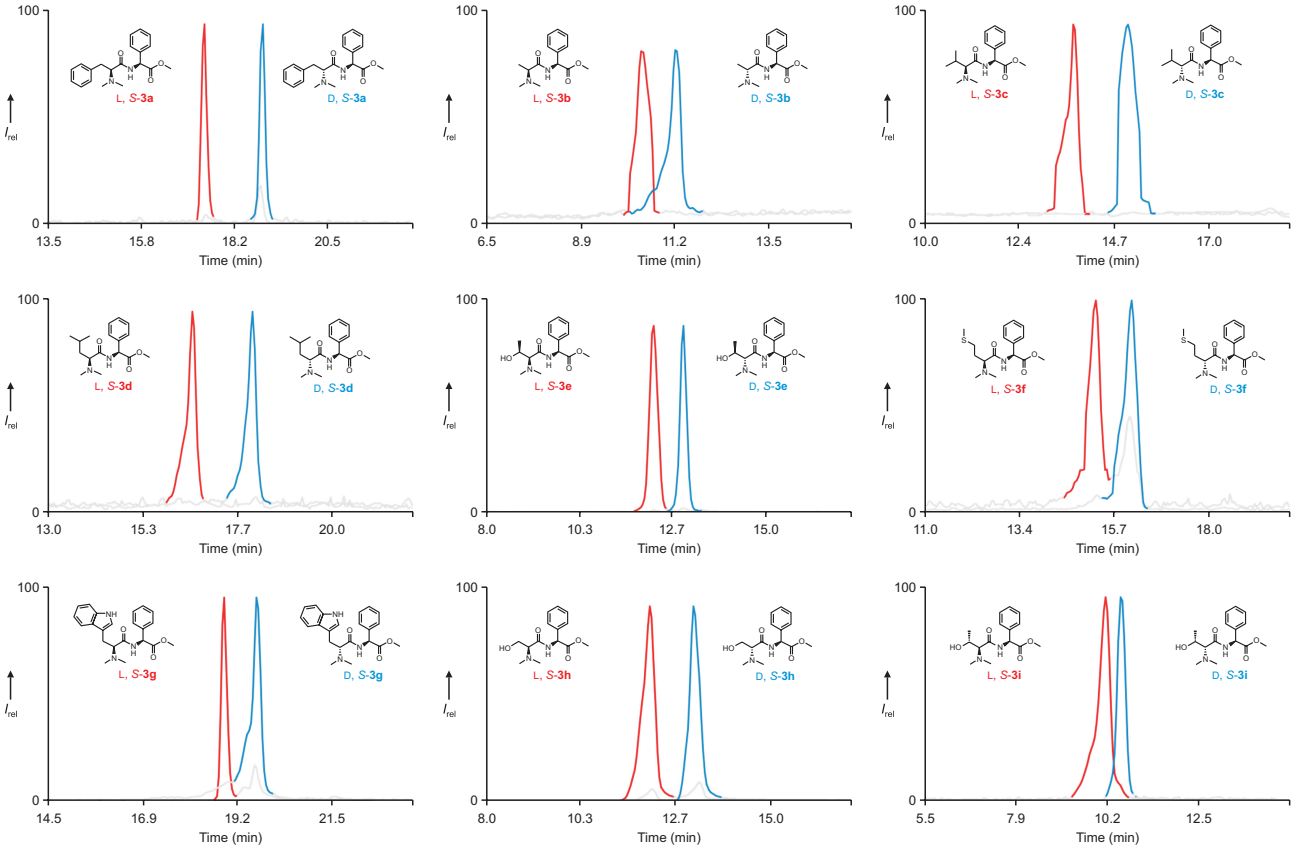

Fig. 4. Elution order of *S*-PGME derivatives of *N*,*N*-dimethyl amino acids in LC-MS analysis.

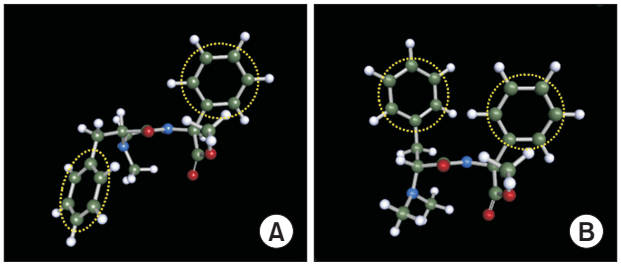

Fig. 5. Energy-minimized conformations of (A) L-*N*,*N*-dimethyl phenylalanine-S-PGME (L,S-3a, left panel) and (B) D-*N*,*N*-dimethyl phenylalanine-S-PGME (D,S-3a, right panel) in the gas phase. Hydrophobic groups are presented by yellow circles.

of (*S*)-PGME adducts of L,*S*-**1a** and D,*S*-**1a** using computational density functional theory (DFT) calculations (Fig. 5). That according our DFT calculations, the two hydrophobic benzyl groups of L,*S*-**3a** are arranged on opposing faces, although in D,*S*-**3a** they are located on the identical face. Reversed-phase HPLC predicts a shorter retention time for L,*S*-**3a** than D,*S*-**3a** because the latter interacts stronger with the hydrophobic stationary phase of the column (Bhushan and Brückner, 2004) (Fig. 2).

LC-MS retention time comparison for achiral PGME derivatives of *N*,*N*-dimethylamino acids

We performed a second procedure that does not require authentic standards to compare the LC-MS retention time order. In an achiral chromatography system, the L-*R* and D-*S* combination were eluted at the same retention times as those deduced from the advanced Marfey's method (Fujii *et al*., 1998) for the α-amino acids and the *O*-Marfey's methods (Moon *et al*., 2013) for α-hydroxy acids. In addition, the derivative containing the L-*S* combination of the dimethyl amino acid PGME is expected to display a retention time that is identical to that of its D-*R* combination, which is the enantiomeric configuration. We derivatized the L and D forms of the *N*,*N*-dimethyl amino acids with both S-and R-PGME. As expected, the tR values of the L-*S* and D-*R* derivatives were identical and the L-*R* and D-*S* derivatives as well as the D-*R* and L-*S* derivatives were also the same. Based on these elution times and orders, we could determine the absolute configuration of the *N*,*N*-dimethyl amino acids without using the l or D-dimethyl amino acid authentic standards by comparing the elution order of the diastereomer derivatives.

Application of the PGME method to a natural product, sanjoinine A (4)

In an effort to broaden the scope of our work into natural substances, we attempted to apply the PGME method with *S*, *R*-PGME derivatization to characterize the *N*,*N*-dimethyl phenylalanine in sanjoinine A (**4**) (Han *et al*., 1985) (Fig. 1). Sanjo-

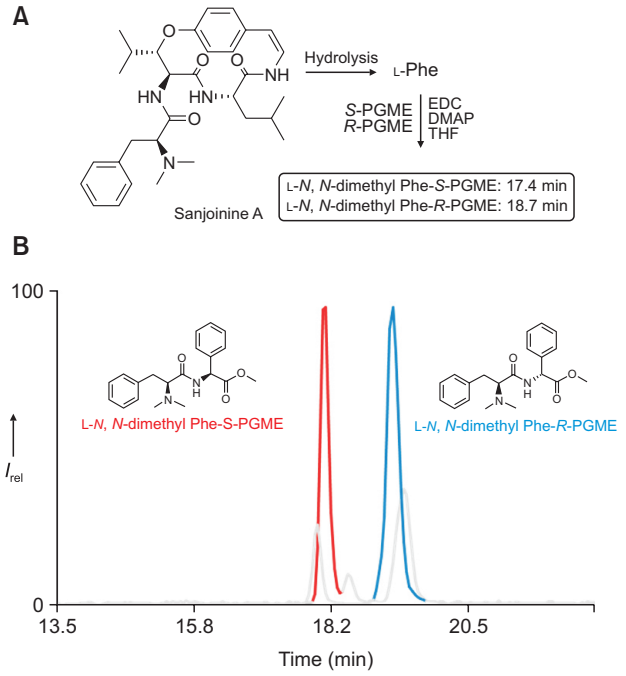

Fig. 6. Applicaiton of the PGME method to sanjoinine A. (A) Determination of the absolute configuration of *N*,*N*-dimethyl phenylalanine in sanjoinine A. (B) LC-MS analysis of the PGME derivatives of L-*N*,*N*-dimethyl phenylalanine (L,*S*- and L,*S*-*N*,*N*-dimethyl Phe) from sanjoinine A hydrolysis.

inine A is a cyclopeptide alkaloid isolated from Zizyphi Spinosi Semen, which is widely used as a sedative herbal medicine for the treatment of insomnia (Ma *et al*., 2007). The absolute configuration of the alpha position of *N*,*N*-dimethyl phenylalanine in sanjoinine A had to be proven through a complex total synthesis (Xiao *et al*., 1998); hence, we were interested in verifying the three-dimensional structure through the simple chemical derivatization. In order to employ the PGME method, purified sanjoinine A was first hydrolyzed, and then the analyte was derivatized using *S*- and *R*-PGME under the previously established conditions. After confirming the UV spectra as well as the mass values of their peaks using electrospray ionization (ESI) LC-MS, the PGME derivatives as well as an equal combination of the *S*- and *R*-PGME derivatives from standard *N*,*N*-dimethyl phenylalanine were analyzed in the positive ion mode. We could identify the absolute configuration of *N*,*N*-dimethyl phenylalanine in **4** as L form by comparing the retention times of the product components in the LC-MS analysis (Fig. 3, 6). This result is in good agreement with that of an earlier study, which was based on the total synthesis, proving that the PGME approach is reliable.

Antimicrobial assay for sanjoinine A

Contrary to what was previously reported about sanjoinine A's bioactivity regarding GABA-related activities, our extensive antimicrobial experiment did not reveal any inhibitory activity against *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, or *Erwinia rhapontici*.

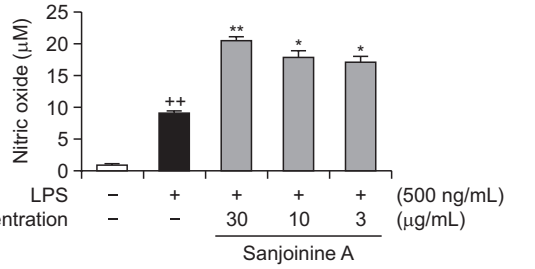

Fig. 7. Effect of sanjoinine A on the production of nitric oxide (NO) in LPS-activated RAW 264.7 cells. $^{++}p<0.01$ compared with the non-treated group. $^{*}p<0.05$ and $^{**}p<0.01$ compared with the LPS treated group.

Effect of sanjoinine A on the production of NO in LPS-activated RAW 264.7 cells

To investigate the effect of sanjoinine A on NO production, RAW 264.7 cells were pretreated with sanjoinine A for 2 h and then the cells were activated with LPS (500 ng/mL). NO production was detected by Griess assay. Sanjoinine A induced NO production in LPS-activated RAW 264.7 cells in a dose-dependent manner (Fig. 7).

DISCUSSION

In summary, we devised a facile LC-MS-based approach (PGME method) for the determination of the configurations of *N*,*N*-dimethyl amino acids by chiral derivatization using a commercially available PGME reagent, and validated the method using a variety of *N*,*N*-dimethyl amino acids. The method is practical and straightforward because the reaction is rapid (30 min), all stages are carried out at room temperature, and the analytical conditions do not need further purification of the reaction mixture. PGME derivatives of the *N*,*N*-dimethyl amino acids can be determined simply and precisely with their distinct UV and mass spectra through the LC-MS analysis, and the reaction conditions are benign and uncomplicated. This analytical PGME method, which can be successfully applied to the three-dimensional structural analysis of peptide-based natural substances, can be used to analyze pure drug contents and to further refine the structure of natural products. The PGME method allowed us to establish the absolute configuration of a natural cyclopeptide-alkaloid that bears an *N*,*N*-dimethyl phenylalanine. It is anticipated that this work will be widely employed in configurational analysis because it requires the analytical conditions utilizing single quad LC/MS commonly utilized in analytical laboratories. In addition to the development of analysis methods for three-dimensional structure analysis of sanjoinine A, we evaluated the effect of sanjoinine A on NO production from macrophage cells. Sanjoinine A showed significant activity in NO production (Fig. 7). This result suggests that NO produced by sanjoinine A treatment to macrophage cells may show anti-tumor activity (Xu *et al*., 2002) and blood vessel dilation to lowering blood pressure (Chen *et al*., 2008). In addition, an increase in NO production by sanjoinine A may be evidence for the fact that *Zizyphus jujuba* Mill *var. spinosa* (Rhamnaceae) has been used for treating sleeplessness and anxiety in Asian countries (Gautier-Sauvigné *et al*., 2005). Further studies on the effect of gam-

ma-aminobutyric acid (GABA) according to the increased NO production of sanjoinine A are needed.

CONFLICT OF INTEREST

The authors declare no competing financial interest.

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