N-Desmethylmajusculamide B, a lipopeptide isolated from the Okinawan cyanobacterium Okeania hirsuta

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N-Desmethylmajusculamide B, a lipopeptide isolated from the Okinawan cyanobacterium *Okeania hirsuta*

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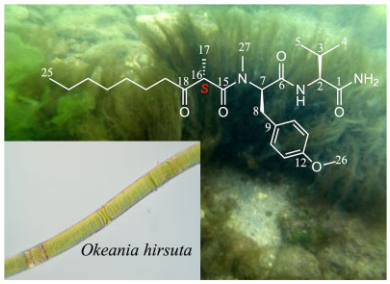
Abstract

A new lipopeptide, *N*-desmethylmajusculamide B (**1**), was isolated from the Okinawan cyanobacterium *Okeania hirsuta* along with 2 known compounds majusculamide A (**2**) and majusculamide B (**3**). The planar structure of (**1**) was elucidated by a detailed analysis of mass spectrometry and nuclear magnetic resonance spectra. The absolute configurations of the amino acid residues were determined using Marfey's analysis. The configuration of C-16 in the α-methyl-β-keto-decanoyl moiety was determined unambiguously to be *S* by conducting a semisynthesis of *N*-desmethylmajusculamide B from **3**. The cytotoxicity against mouse L1210 leukemia cells was evaluated for majusculamides (**1-3**).

Keywords: lipopeptide, cyanobacteria, *Okeania hirsuta*, Marfey's method, semisynthesis

Graphical abstract



N-Desmethylmajusculamide B, a novel lipopeptide was isolated from the marine cyanobacterium *Okeania hirsuta*.

In July 2010, a massive outbreak of toxic filamentous cyanobacteria occurred on Kuba Beach, Nakagusuku, Okinawa, Japan. Consequently, the beach was set off-limits to the public to prevent health hazards. This cyanobacterial bloom lasted for approximately a month, allowing us to collect a substantial amount of samples for investigation. The sample was initially identified as *Moorea producens* (formerly *Lyngbya majuscula*) based on morphological observations. However, a recent analysis of its 16S rRNA sequence led to its reidentification as *Okeania hirsuta* (Kanda *et al.* 2023). More than 40 compounds, including various secondary metabolites such as terpenoids, cyclic peptides, and polyketides have been characterized from this *O. hirsuta* sample, over 20 of which were novel compounds (Jiang *et al.* 2017; Nagai *et al.* 2019a, 2019b; Kawaguchi *et al.* 2020; Murakami *et al.* 2020; Iguchi *et al.* 2021; Satake *et al.* 2021). These findings have revealed that this *O. hirsuta* sample is an important source of biochemicals. In our ongoing investigation, a new lipopeptide, *N*-desmethylmajusculamide B (**1**), along with 2 known lipopeptides majusculamides A (**2**) and B (**3**) (Marner *et al.* 1977), was first isolated from this sample. The isolation, structure elucidation, and bioactivities of these compounds are reported in the present study.

Results and discussion

Structure elucidation

A frozen sample of *O. hirsuta* was extracted with methanol (MeOH). After filtration, the extract was concentrated and partitioned between 80% aqueous MeOH and hexane. The 80% aqueous MeOH layer was successively partitioned between ethyl acetate (EtOAc) and H_2O. The EtOAc-soluble material was subjected to fractionation using reversed-phase column chromatography and repeated reversed-phase high performance liquid chromatography (HPLC) to yield **1** (1.3 mg), **2** (3.8 mg), and **3** (52.1 mg) (Figure 1).

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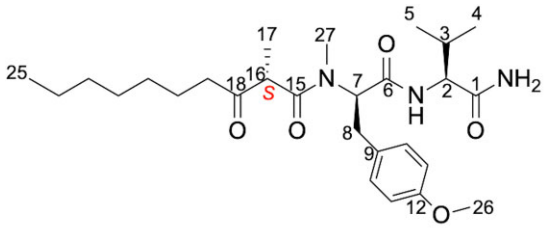


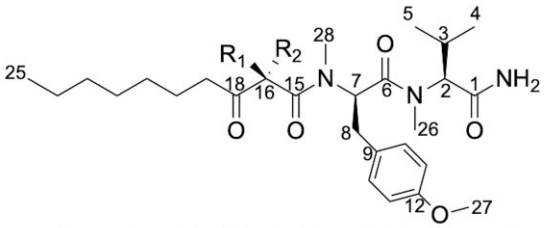


Figure 1. Structures of majusculamides isolated from *Okeania hirsuta*.

Table 1. NMR data for *N*-desmethylmajusculamide B (**1**) in $CDCl_3$

No.	δ H[a] (*J* in Hz)	δ C[b], type
1		173.6, qC
2	4.25, dd (8.6, 5.5)	58.9, CH
3	2.29, m	29.8, CH
4	0.88, d (6.9)	19.5[c], CH_3
5	0.88, d (6.9)	17.6[c], CH_3
6		170.4, qC
7	5.43, dd (9.3, 7.1)	57.7, CH
8a	2.90, dd (14.7, 9.3)	32.8, CH_2
8b	3.36, dd (14.8, 7.1)	32.8, CH_2
9		128.8, qC
10	7.11, d (8.6)	129.9, CH
11	6.80, d (8.6)	114.1, CH
12		158.5, qC
13	6.80, d (8.6)	114.1, CH
14	7.11, d (8.6)	129.9, CH
15		172.3, qC
16	3.61, q (7.1, 7.1, 7.1)	51.0, CH
17	1.32, d (7.1)	13.7, CH_3
18		209.2, qC
19a	2.47, dd (7.4, 7.4)	40.5, CH_2
19b	2.50, dd (7.4, 7.4)	40.5, CH_2
20	1.54, m	23.7, CH_2
21	1.25, m	29.2, CH_2
22	1.25, m	29.2, CH_2
23	1.24, m	31.8, CH_2
24	1.28, m	22.7, CH_2
25	0.87, dd (7.3, 7.3)	14.2, CH_2
26-OCH_3	3.77, s	55.4, CH_3
27-NCH_3	2.80, s	31.3, CH_3
NH	6.76, d (8.6)	
NH_2	6.42, brs	
	5.35, brs	

[a]Measured at 800 MHz.
[b]Measured at 200 MHz.
[c]These carbon signals are interchangeable.

Majusculamide A (**2**) and majusculamide B (**3**) were obtained as white amorphous solids. Both compounds have the molecular formula $C_{28}H_{45}N_3O_5$, as confirmed by high-resolution (HR)-electrospray ionization (ESI)-mass spectrometry (MS). The structural elucidation of **2** and **3** was carried out by comparing the nuclear magnetic resonance (NMR) data and specific rotations with their synthetic counterparts (Nakajima *et al.* 2019). The NMR spectra of **2** and **3** measured in $CDCl_3$ exhibited 2 interconverting conformers with the NMR signal ratio of 5:2 (Figures S1-S4), which can be attributed to the presence of tertiary amide moieties (Fischer 2000). A comprehensive analysis of the 2-dimensional NMR spectra, particularly the heteronuclear multiple bond coherence (HMBC) and heteronuclear single quantum coherence-total correlation spectroscopy (HSQC-TOCSY) spectra, accomplished the complete assignment of **2** and **3**, including their conformers (Tables S1 and S2).

N-Desmethylmajusculamide B (**1**) was obtained as a white amorphous solid ($[\alpha]_D^{23} = +19.8$ (c 0.61, EtOH)). Its molecular formula was determined to be $C_{27}H_{43}N_3O_5$ based on a prominent $[M + H]^+$ ion peak at *m/z* 490.3254 (calculated for $C_{27}H_{44}N_3O_5$: 490.3281), which is 14 mass units (CH_2) less than that of the major ions in **2** and **3**. Moreover, the ^1H NMR spectra of **1** (Table 1) and **3** were similar, and both included signals for a terminal NH_2, a para-substituted anisole ring, and a linear alkyl group. However, **1** presented only 1 *N*-methyl proton signal at δ_H 2.80, whereas **3** had 2 *N*-methyl proton signals at δ_H 2.91 and δ_H 3.02. Combined with the lower molecular weight of **1** than that of **3**, it can be readily inferred that **1** is an *N*-demethylation analog of **3**. Interestingly, the NMR spectra of **1** measured in $CDCl_3$ presented only a very small proportion of the conformer (Figures S7 and S8), which was different from those of **2** and **3**. The *N*-methyl proton signal in **1** was assigned to an *N*,*O*-dimethyltyrosine moiety based on the HMBC correlations from H_3-27 (δ_H 2.80) to C-7 and C-15 amide. The correlation spectroscopy (COSY) correlations from H_3-4, H_3-5 to H-3, H-3 to H-2, and H-2 to an NH proton at δ_H 6.76, along with the HMBC correlations of an NH proton to C-1, C-2, and C-3, clearly indicated that **1** contained a valine residue instead of an *N*-methylvaline. Furthermore, the HMBC correlations from H-16 to C-15, C-18, and H_3-17 to C-15, C-18, combined with 6 aliphatic methylene groups observed in ^{13}C and HSQC NMR spectra, suggested the presence of an α-methyl-β-keto-decanoyl moiety in **1**. The planar structure of **1** and the key COSY and HMBC correlations were established as Figure 2.

The absolute configurations of the 2 amino acid residues in **1** were determined using Marfey's method (Marfey 1984). Following acid hydrolysis, the hydrolysate of **1** was derivatized using 1-fluoro-2,4-dinitrophenyl-5-L-leucinamide (L-FDLA, Marfey's reagent) and compared with valine and *N*,*O*-dimethyltyrosine standards that were similarly derivatized using L-FDLA. The *N*,*O*-dimethyltyrosine standard was synthesized by the methylation of commercially available Boc-protected tyrosine following a literature procedure (Boger and Yohannes 1988). These analyses revealed that **1** contained L-valine and *N*,*O*-dimethyl-D-tyrosine (Figures S13-S15).

With regard to the fatty acid moiety, the absolute configuration of stereocenter at C-16 had to be determined. Therefore, we conducted a semisynthesis of *N*-desmethylmajusculamide B from **3** (Scheme 1). Acid hydrolysis of **3** under mild conditions (9 M HCl, 25 °C) followed by HPLC purification afforded a partial hydrolysate **4** (Figures S16-S18). Compound **4** was then condensed with a commercially available L-valinamide

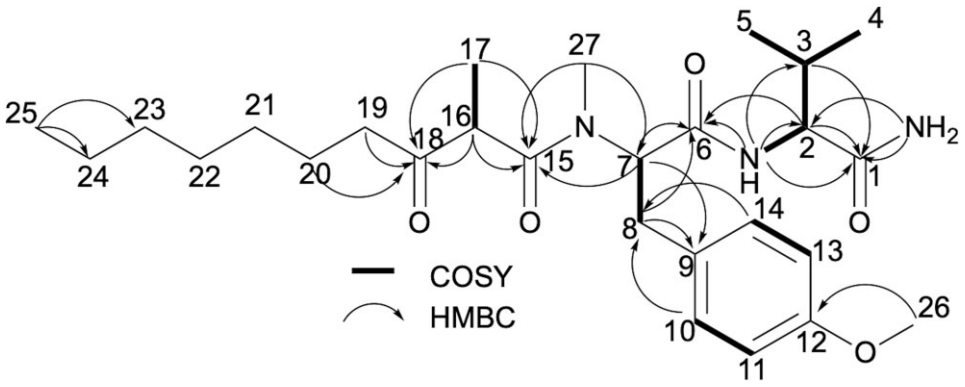
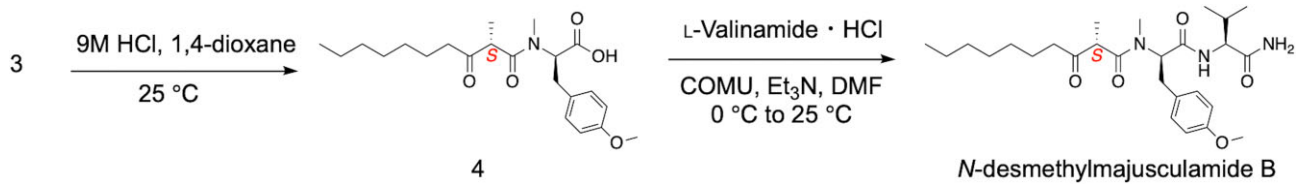


Figure 2. Key COSY and HMBC correlations of *N*-desmethylmajusculamide B (**1**).



Scheme 1. Semisynthesis of *N*-desmethylmajusculamide B from **3**.

hydrochloride using (1-cyano-2-ethoxy-2-oxoethylidenaminooxy) dimethylamino-morpholino-carbenium hexafluorophosphate (COMU), resulting in *N*-desmethylmajusculamide B with a 26% yield (El-Faham *et al.* 2009). The ^{1}H NMR spectrum of the synthesized *N*-desmethylmajusculamide B was consistent with that of the natural product (**1**) (Figures S19-S21). Consequently, the configuration of C-16 was unambiguously determined to be S for **1**. These results led us to establish the absolute configurations of **1** as shown in Figure 1.

The presence of *N*-desmethylmajusculamide A in the original sample was also considered. Liquid chromatography-mass spectrometry (LC-MS) was performed in both positive and negative modes for the EtOAc extract of *O. hirsuta*. However, only one signal corresponding to $C_{27}H_{43}N_3O_5$, *N*-desmethylmajusculamide B (**1**), could be detected. Additionally, 3.8 mg of majusculamide A (**2**) and 52.1 mg of majusculamide B (**3**) were obtained simultaneously, which revealed that **3** was more abundant than **2** in this sample. Consequently, even if *N*-desmethylmajusculamide A was present in the *O. hirsuta* sample, the amount might be too small to be detected.

Biological activities of majusculamides

The cytotoxicity of isolated compounds against mouse L1210 leukemia cells was evaluated using WST-8 assay. The IC_{50} value of majusculamide A (**2**) was determined to be 34 μM. On the other hand, *N*-desmethylmajusculamide B (**1**) and majusculamide B (**3**) showed only 41% and 43% inhibition of cell growth at up to 100 μM, respectively. In addition, the growth-inhibitory activity toward the marine diatom *Nitzschia amabilis* was also conducted for the majusculamides (**1**-**3**). As a result, neither **1**, **2**, nor **3** showed growth-inhibitory activity at 10 μM.

In the cytotoxicity test, *N*-desmethylmajusculamide B (**1**) and majusculamide B (**3**) showed similar activities, which were clearly weaker than majusculamide A (**2**). From the perspective of the structure-activity relationship, the presence of a methyl group on *N*-methylvaline in majusculamides appears to be less important for their bioactivities. Furthermore, it was reported that **2** had a more significant effect in promoting osteoblast differentiation compared to **3** in MC3T3-E1 cells (Natsume *et al.* 2020). However, **3** exhibited more toxic to larvae of the barnacle, *Amphibalanus amphitrite* than **2** in the cyprid settlement assay (Tan *et al.* 2010). It is intriguing to note that **2** and **3**, 2 epimers that differ only in their configuration at C-16, display remarkably distinct activities in various biological experiments, including the present study.

Conclusion

A new lipopeptide, *N*-desmethylmajusculamide B (**1**), together with 2 known compounds majusculamide A (**2**) and majusculamide B (**3**), was isolated from the cyanobacterium *O. hirsuta* from Okinawa, Japan. The absolute configurations of the amino acid residues were determined using Marfey's method. Furthermore, a semisynthesis of *N*-desmethylmajusculamide B from **3** was conducted to establish the absolute configuration of C-16 as S. In the cytotoxicity test against mouse L1210 leukemia cells, the activities of **1** and **3** were almost identical. In terms of structure-activity relationship, the presence of a methyl group on *N*-methylvaline in majusculamides appears to be less important for their bioactivities. In addition, the cytotoxicity of **2** was more potent than those of **1** and **3**. In this regard, the configuration of the C-16 position was again shown to have a significant impact on biological activity, as reported by Natsume *et al.* (2020).

Experimental

General experimental procedures

The specific rotations were measured using a JASCO P-2100 (JASCO, Tokyo, Japan) polarimeter with a 10-mm-long cell. All NMR spectral data were recorded on a Bruker AVANCE III 800 MHz (Bruker, Billerica, MA, USA) or a Bruker AVANCE III 600 MHz (Bruker, Billerica, MA, USA) spectrometer at 298 K and referenced to residual solvent signals (δ_H 7.26, δ_C 77.2 for $CDCl_3$). The HR-ESI-MS data were acquired using a Bruker micrOTOF QII mass spectrometer (Bruker, Billerica, MA, USA). HPLC purification was performed using a Hitachi Chromaster HPLC system equipped with a 5110 pump and a 5430 diode array detector (Hitachi High-Tech Science Co., Tokyo, Japan). Bioassay results were recorded using a Model 550 microplate reader (Bio-Rad, Hercules, CA, USA).

Marine cyanobacterium sample

The marine cyanobacterium *O. hirsuta* was collected from Kuba Beach, Nakagusuku, Okinawa, Japan, in July 2010. The sample was immediately frozen at −30 °C in Okinawa. The frozen sample was brought to the laboratory and stored at −30 °C until the experiments were performed. The reidentification of this sample has been reported in our previous article (Kanda *et al.* 2023). A voucher specimen of this sample (20100713-a) was deposited at the Tokyo University of Marine Science and Technology.

Extraction and isolation

The frozen *O. hirsuta* sample (860 g, wet weight) was soaked in MeOH for several days at room temperature. After filtration, the sample was extracted 5 times with methanol and once with acetone. These extracts were then combined and concentrated *in vacuo* to obtain a residue (37.8 g), which was subsequently partitioned between 80% aqueous MeOH and hexane. The 80% aqueous MeOH layer was then evaporated and partitioned between EtOAc and water to obtain an EtOAc layer. Next, the EtOAc extract (1.65 g) was separated on an open glass column (Cosmosil 75C18-OPN resin, 40 × 400 mm) through stepwise elution with 50%, 70%, 90%, and 100% MeOH. The 70% MeOH fraction (450 mg) was then subjected to a semipreparative HPLC [column, SHISEIDO CAPCELL PAK-ODS (20 × 250 mm); flow rate, 4.0 mL/min; detection at 210 nm; solvent MeOH/H_2O gradient condition, 40-min linear gradient elution from 70% to 100% MeOH, after which the 100% MeOH extract was held for an additional 20 min] to produce 9 subfractions (Fr. 1-9). Subfraction 7 (183 mg) was further separated by HPLC [column, Cosmosil 5C_{18}-AR-II (10 × 250 mm); flow rate, 2.0 mL/min; detection at 210 nm; solvent 75% MeOH] to obtain *N*-desmethylmajusculamide B (**1**, 1.3 mg), majusculamide A (**2**, 3.8 mg), and majusculamide B (**3**, 52.1 mg).

Acid hydrolysis and Marfey's analysis

N-Desmethylmajusculamide B (**1**) (0.225 mg, 0.46 µmol) in 6 M HCl (100 µL) was heated at 110 °C for 24 h under reduced pressure in a sealed mini vacuum tube RT-1 (Osaka chemical, Osaka, Japan). The resulting hydrolysate was then concentrated to dryness under a stream of dry N_2 and redissolved in H_2O (20 µL). A 1% L-FDLA (Marfey's reagent) solution in acetone (40 µL) and 10 µL of 1 M aqueous $NaHCO_3$ were added. The mixture was stirred at 40 °C for 1 h and subsequently cooled to room temperature. The reaction mixture was neutralized with 10 µL of 1 M aqueous HCl and evaporated to dryness. The residue was resuspended in 50 µL of acetonitrile (MeCN)-H_2O (1:1), and the solution was analyzed by reversed-phase HPLC. [Conditions for HPLC separation: column, TSKgel ODS-120H (5 µm, 4.6 × 150 mm); flow rate, 1.0 mL/min; temperature 40 °C; detection at 340 nm; solvent system (a) MeCN/H_2O acidified with 0.1% formic acid (HCOOH), 35-min linear gradient elution from 20% to 55% MeCN; solvent system (b) MeCN/H_2O acidified with 0.1% HCOOH, 30-min isocratic elution with 35% MeCN.] The retention times (t_R min) of the authentic standards in the solvent system (a) were as follows: *N,O*-dimethyl-L-Tyr-L-DLA (26.9), *N,O*-dimethyl-D-Tyr-L-DLA (28.1), L-Val-L-DLA, (23.2); and D-Val-L-DLA (29.3). However, the peaks of L-Val-L-DLA overlapped with unreacted L-FDLA under the conditions of solvent system (a). Thus, a solvent system (b) was performed specifically for L-Val-L-DLA. The recorded retention times were 25.2 min for unreacted L-FDLA and 26.2 min for the L-Val-L-DLA standard. Regarding the DLA derivatives of amino acids derived from **1**, the hydrolysate gave peaks at 23.2 min and 28.1 min in the solvent system (a). The peak eluted at 23.2 min was isolated and further analyzed with the solvent system (b), yielding 2 peaks at 25.3 min (L-FDLA) and 26.2 min (L-Val-L-DLA). These analyses revealed that *N*-desmethylmajusculamide B (**1**) comprised L-valine and *N,O*-dimethyl-D-tyrosine (Figures S13-S15).

Preparation of 4 from 3

Majusculamide B (**3**) (10.0 mg, 19.9 µmol) was dissolved in 1,4-dioxane (0.5 mL) and 9 M HCl (1 mL). The mixture was then stirred at 25 °C for 8 h, after which the solvent was evaporated to dryness. The residue was purified using reversed-phase HPLC [Column, GL-Sciences InertSustain C18 (5 µm, 10 × 250 mm); flow rate, 3.0 mL/min; detection, UV210 nm; solvent 70% MeOH with 0.1% HCOOH; t_R = 34.3 min] to give partial hydrolysate **4** (5.5 mg, 14.1 µmol) as a white amorphous solid. ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.12 (d, J = 8.2 Hz, 3H), 6.82 (d, J = 8.5 Hz, 2H), 5.25 – 5.18 (m, 1H), 3.77 (s, 3H), 3.51 (q, J = 6.9 Hz, 1H), 3.36 (dd, J = 15.0, 4.4 Hz, 1H), 3.06 (dd, J = 14.2, 11.8 Hz, 1H), 2.85 (s, 3H), 2.20 (dt, J = 17.7, 7.4 Hz, 1H), 1.82 (dt, J = 17.6, 7.2 Hz, 1H), 1.46 – 1.32 (m, 2H), 1.29 (d, J = 7.0 Hz, 3H), 1.26 – 1.20 (m, 6H), 1.17 – 1.09 (m, 2H), 0.87 (t, J = 7.0 Hz, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 206.74, 173.95, 171.90, 158.73, 129.94, 128.60, 114.18, 59.93, 55.30, 51.70, 39.25, 34.23, 33.64, 31.83, 29.32, 29.11, 23.55, 22.77, 14.24, 13.47. HR-ESI-MS *m/z* 390.2292 $[M-H]^-$ (calculated for $C_{22}H_{32}NO_5$: 390.2286).

Semisynthesis of *N*-desmethylmajusculamide B from 4

To a solution of **4** (2.75 mg, 7.0 µmol) and triethylamine (Et_3N, 1 µL) in N,N-dimethylformamide (DMF) (100 µL) COMU (3.4 mg, 7.9 µmol) was added at 0 °C. After stirring for 30 min, a solution of L-valinamide hydrochloride (1.2 mg, 7.9 µmol) and Et_3N (1 µL) in DMF (100 µL) was added dropwise at 0 °C. The resulting mixture was stirred at 0 °C for 1 h and at 25 °C for 5 h. The reaction mixture was then quenched with 50% aqueous MeOH (2 mL) and evaporated to dryness. The residue was purified by reversed-phase HPLC [Column, GL-Sciences InertSustain C18 (5 µm, 10 × 250 mm); flow rate, 3.0 mL/min; detection, UV210 nm; solvent 70% MeOH with 0.1% HCOOH; t_R = 38.4 min] to give *N*-desmethylmajusculamide B (0.9 mg, 1.8 µmol).

Biological assays

Cytotoxicity test against mouse L1210 leukemia cells and growth inhibition assay against the marine diatom *N. amabilis* (Suzuki, Nagumo and Tanaka 2010) were performed for the isolated compounds (**1**-**3**). Details of these 2 bioactivity assays have been previously reported (Kawabata *et al.* 2013; Jiang *et al.* 2016). However, we used the WST-8 colorimetric reagent (Dojindo Lab. Kumamoto, Japan) instead of the XTT in this study.

Supplementary material

Supplementary material is available at *Bioscience, Biotechnology, and Biochemistry* online.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Author contribution

H. Nagai and M.S. designed the research; B.Z., H. Nishino, R.K., R.W., and H.U. performed the experiments; and B.Z., M.K., M.S., and H. Nagai wrote the manuscript.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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