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Anti-inflammatory Amino Acid Derivatives from the Ascidian *Herdmania momus*

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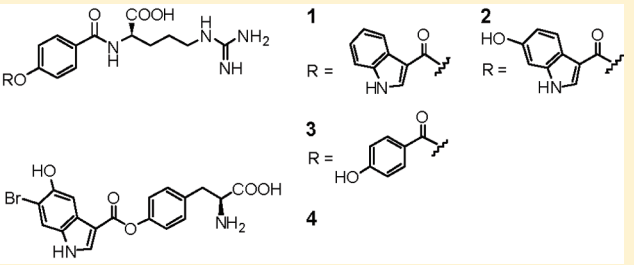
Supporting Information

ABSTRACT:

Herdmania momus



Four new amino acid derivatives, herdmanines A−D (**1**−**4**), were isolated from the marine ascidian *Herdmania momus*. Herdmanines A−C contain the unusual D-form of arginine. Compounds **3** and **4** had a moderate suppressive effect on the production of NO, with IC_{50} values of 96 and 9 μM, respectively. These compounds were found to inhibit the mRNA expression of *iNOS*. The inhibitory activities on the production and mRNA expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 were evaluated.

Marine ascidians are a very rich source of chemically unique and biologically active secondary metabolites that have attracted the interest of both chemists and pharmacologists.[1] Ascidians are particularly rich in amino-acid-derived metabolites such as cyclic peptides and alkaloids. The cyclic depsipeptide didemnin B, isolated from the ascidian *Tridemnum solidum*, is the first marine natural product to enter phase I and II clinical trials as an anticancer agent, but discontinued for its high cardiac and neuromuscular toxicity.[2]

The solitary tunicate *Herdmania momus* (order Pleurogona, family Pyuridae) is a frequently encountered species distributed primarily in the Indo-Pacific Ocean. However, only one chemical study of this organism has been reported.[3] Over the past few years, there has been an unusual bloom of this ascidian around the Korean peninsula, and this has caused severe economic and ecological problems. In an approach to utilize *H. momus* in a productive manner, we performed a chemical investigation of the bioactive metabolites of this ascidian, which we collected off the coast of Jeju Island, Korea. By solvent partition and chromatography, we isolated four new amino acid derivatives, herdmanines A−D (compounds **1**−**4**), and evaluated their anti-inflammatory activity. In this paper, we describe the structure elucidation and biological evaluation of the amino-acid-derived metabolites of the ascidian *H. momus*.

RESULTS AND DISCUSSION

Combined MeOH and CH_2Cl_2 extracts of *H. momus* were subjected to solvent partitioning between CH_2Cl_2 and H_2O. The H_2O layer was partitioned with *n*-BuOH, and the *n*-BuOH-soluble fraction was subjected to chromatographic separation and purification to afford a series of amino-acid-derived metabolites (**1**−**4**).

Herdmanine A (**1**) was obtained as a yellow gum. The (+)-FABMS spectrum showed a pseudomolecular ion peak at *m*/*z* 438 $[M + H]^+$, and the molecular formula of this compound was established as $C_{22}H_{23}N_5O_5$ on the basis of the (+)-HRFABMS and NMR data, indicating 14 degrees of unsaturation.

The ^1H NMR spectrum of **1** (Table 1) revealed resonances of an AA′XX′ spin system at δ_H 7.15 (H-10/14) and 7.81 (H-11/13), attributable to a *p*-disubstituted phenyl moiety. This was further corroborated by COSY data (Figure 1). Furthermore, an

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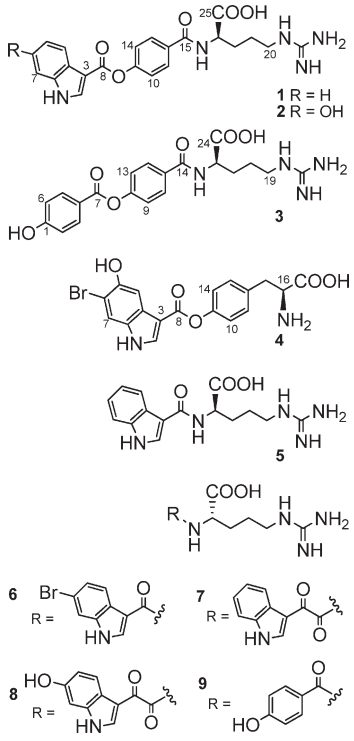

indole spin system was suggested, which was further supported by the UV absorption at 283 nm. In the HMBC spectrum (Figure 1), the correlations from H-4 to C-3, C-6, and C-7a, from H-5 to C-3a and C-7, from H-6 to C-4 and C-7a, and from H-7 to C-5 and C-3a were assigned to an indole moiety. Correlations from the indole proton H-2 to C-3, C-3a, and C-7a were also observed in the HMBC spectrum. The H-4 and H-2/C-2 signals were significantly shifted downfield owing to the carbonyl substitution at C-3 of the indole group, and a correlation from H-2 to the carbonyl carbon C-8 (δ_C 163.8) was observed in the HMBC spectrum. The carbon resonance at δ_C 55.1 and the resonance due to the associated proton at δ_H 4.19 indicated the presence of an amino acid moiety, and the carbon signal at δ_C 158.0 (C-22) was in good agreement with the expected value for a terminal guanidyl group. The presence of the guanidyl group was further corroborated by the formation of a pyrimidine derivative in the reaction between **1** and acetylacetone (Figure 2). Peaks due to six methylene protons, a methine proton, and two exchangeable protons appeared at δ_H 8.78 (16-NH) and 9.29 (21-NH), and these protons were thought to be associated with a 2,5-diaminopentanoic acid moiety. Successive COSY connections from 16-NH to 21-NH suggested the presence of an arginine moiety. The amide proton (16-NH) and α-proton (H-17) of arginine showed HMBC correlations to the carbonyl carbon C-15, which in turn showed correlations with the aromatic protons H-11/13, indicating that **1** contained a *p*-oxybenzoyl group directly attached to an arginine moiety. On the basis of the molecular formula of **1**, it was assumed that the *N*-(*p*-oxybenzoyl)arginine and 3-carboxyindole units were joined via an ester linkage. This was further supported by the chemical shifts of C-8 (δ_C 163.8) and C-9 (δ_C 153.1) and the results of FAB-CID-MS/MS analysis (Figure 3). The arginine moiety was suggested to be in the D-form by comparison of the specific rotation of **1** ($[\alpha]^{23}_D$ −18) with that of the analogous D-arginine (**5**: $[\alpha]^{20}_D$ −133) and L-arginine model compounds (**6**: $[\alpha]^{20}_D$ +3.6; **7**: $[\alpha]^{23}_D$ +14; **8**: $[\alpha]^{23}_D$ +26; **9**: $[\alpha]^{23}_D$ +19.5).[4,5] This

Table 1. ^{1}H (500 MHz) and ^{13}C (100 MHz) NMR Data for 1[a] and 2[b]

	herdmanine A (**1**)		herdmanine B (**2**)	
position	δ_C	δ_H (*J* in Hz)	δ_C[c]	δ_H (*J* in Hz)
1-NH		12.17, brs		
2	134.7	8.33, s	133.4	8.01, s
3	105.9		106.0	
3a	126.5		120.9	
4	121.0	8.02, dd (6.0, 1.0)	122.3	7.87, d (8.5)
5	122.4	7.24, ddd (7.5, 6.0, 1.5)	113.1	6.78, dd (8.5, 2.0)
6	123.4	7.26, ddd (7.5, 6.0, 1.0)	155.2	
7	113.3	7.42, dd (6.0, 1.5)	98.4	6.88, d (2.0)
7a	137.2		139.4	
8	163.8		164.8	
9	153.1		155.7	
10	122.2	7.15, d (8.5)	123.3	7.33, dd (8.5, 2.0)
11	129.0	7.81, d (8.5)	129.7	7.95, dd (8.5, 2.0)
12	132.3		132.7	
13	129.0	7.81, d (8.5)	129.7	7.95, dd (8.5, 2.0)
14	122.2	7.15, d (8.5)	123.3	7.33, dd (8.5, 2.0)
15	165.1		168.5	
16-NH		8.78, d (9.0)		
17	55.1	4.19, m	55.8	4.50, dd (7.0, 5.0)
18a	29.9	1.87, m	31.3	2.00, m
18b		1.81, m		1.87, m
19	25.3	1.58, m	26.1	1.68, quint (7.5)
20	41.2	3.15, m	42.1	3.21, m
21-NH		9.29, s		
22	158.0		157.6	
23-NH		7.20, brs		
24-NH_2		7.20, brs		
25-COOH[d]	177.5		178.3	

[a] Spectra recorded in d_6-DMSO. [b] Spectra recorded in CD_3OD. [c] Chemical shifts obtained from gHSQC and gHMBC spectra. [d] Proton signals were not observed.

was confirmed by Marfey's method;[6] the pyrimidine derivative of **1** (**1a**) was hydrolyzed and allowed to react with Marfey's reagent to give **1c**. TLC analysis showed that **1c** was identical to the compound derived from authentic D-arginine.

Herdmanine B (**2**) was isolated as a white powder. The pseudomolecular ion $[M + H]^+$ peak at *m/z* 454.1755 in the (+)-HRFABMS spectrum matched well with the expected formula $C_{22}H_{24}N_5O_6$. The spectroscopic properties of **2** were similar to those of **1**. The only difference between the ^{1}H NMR spectra of **1** and **2** was in the signals associated with the indole moiety. COSY and ^{1}H NMR analysis indicated that three of the aromatic protons were part of an AMX system. Correlations were observed from H-4 to C-3, C-6, and C-7a, from H-5 to C-3a and C-7, and from H-7 to C-5 and C-3a. The chemical shift of C-6 (δ_C 155.2) was indicative of hydroxylation. The negative specific rotation of **2** ($[\alpha]^{23}_D$ −33) suggested that it contained D-arginine, and this was confirmed by the analogy of the specific rotation with that observed for (−)-herdmanine A ($[\alpha]^{23}_D$ −18).

Herdmanine C (**3**) was isolated as a white powder, and the (+)-HRFABMS data of this compound supported the molecular

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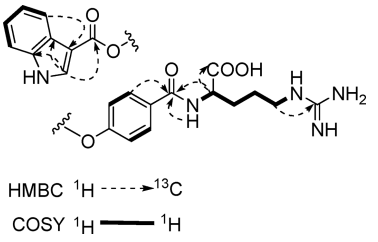

Figure 1. Selected HMBC and COSY correlations of compound **1**.

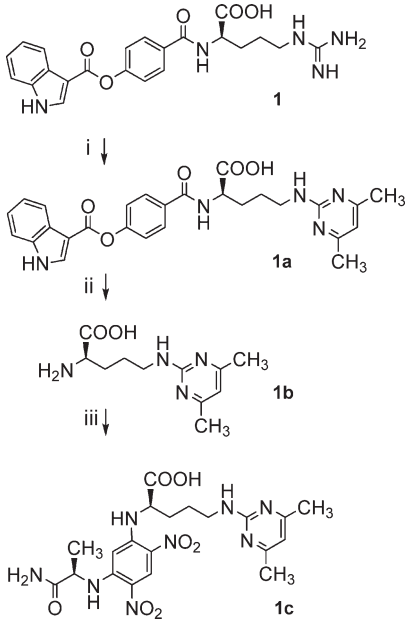

Figure 2. Derivatization of compound **1** for Marfey analysis: (i) H_2O (25 μL), EtOH (50 μL), triethylamine (25 μL), and acetylacetone (50 μL), 110 °C, 4 h; (ii) 2 N HCl, 110 °C, 6 h; (iii) FDAA (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide), 40 °C, 2 h.

formula $C_{20}H_{22}N_4O_6$. The ^{1}H NMR and ^{13}C NMR data of **3** showed two sets of AA′XX′ spin systems, suggesting the presence of two *p*-disubstituted phenyl moieties: δ_H 6.92 (H-2/6) and 7.79 (H-3/5); δ_H 7.10 (H-9/13) and 7.98 (H-10/12). The HMBC and COSY correlations for the remaining proton signals suggested the presence of an arginine moiety. Comparison of the NMR data of **3** and **1** suggested that the 3-carboxyindole ring was replaced with a *p*-hydroxybenzoyl moiety in the former. The planar structure of **3**, derived from the observations mentioned above, was further corroborated by FAB-CID-MS/MS analysis (Figure 3). The negative specific rotation of **3** ($[\alpha]^{23}_D$ −22) suggested that it contained D-arginine.

Herdmanine D (**4**) was obtained as a white powder. The (+)-FABMS spectrum showed isotopic $[M + H]^+$ ion peaks at *m/z* 421/419 (the peak intensities were in a 1:1 ratio), which is characteristic of a monobrominated compound. The molecular formula of **4** was deduced as $C_{18}H_{15}BrN_2O_5$ on the basis of the (+)-HRFABMS and NMR data. Peaks due to all 18 carbons were visible in the ^{13}C NMR spectrum, and HSQC analysis allowed the appropriate assignment of 10 carbon-bound protons (seven aromatic, one methine, and two methylene protons). The five remaining protons were exchangeable. ^{1}H NMR analyses and the correlations observed in the COSY and HMBC spectrum indicated

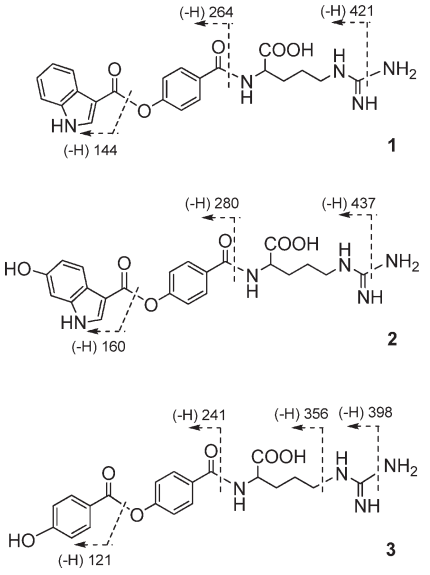

Figure 3. Prominent fragmentations of the $[M + H]^+$ ions of compounds **1**−**3** in FAB-CID tandem mass spectrometry.

that the molecule contained a 3,5,6-trisubstituted indole moiety and a tyrosine moiety. Comparison of the spectroscopic data with those of 6-bromo-5-hydroxyindole[6] and the chemical shift of the quaternary carbons of the indole ring, δ_C 150.0 (C-5) and 107.0 (C-6), indicated that those positions were substituted by a hydroxy group and a bromine atom, respectively. A correlation was observed between H-2 and C-8 (δ_C 163.2), indicating that the C-3 position of indole is substituted by a carboxy group. The molecular formula of **4** suggested that these two moieties must be linked between C-8 and C-9 via an ester linkage. The negative specific rotation of **4** ($[\alpha]^{23}_D$ −4.7) suggested that the tyrosine moiety had the L-configuration. This was corroborated by the hydrolysis of **4** and derivatization with Marfey's reagent, followed by TLC analysis. Herdmanine D contains a rare 6-bromo-5-hydroxyindole moiety. This moiety has been found only in the metabolites of the muricid gastropod *Drupella fragum*,[7] the sponge *Oceanapia bartschi*,[8] and the ascidians *Syncarpa oviformis*[9] and *Eudistoma olivaceum*.[10]

Arginine derivatives (**5**−**9**) isolated from the ascidian *Leptoclinides* sp.[4,5] are analogous to compounds **1**−**3**. However, it is noteworthy that compounds **1**−**3** include the less common D-arginine unit. The occurrence of D-amino acids in vivo has been shown by several authors.[11,12] Specifically, microorganisms transform L-amino acids to D-isomers with amino acid oxidase, transaminases, and epimerases (racemases).[13,14] From the presence of the unusual D-arginine moiety, we can speculate that compounds **1**−**3** of this tunicate are the metabolites of symbiotic microorganisms with an arginine epimerase. On the other hand, it has been reported that the total tissue concentration of the D-amino acids in 18 species of marine invertebrates ranges from 0.04 to 0.44 mM.[15] This may support another suggestion that the uncommon D-arginine originates from seawater, which contains significant amounts of D-amino acids.[15]

The effects of compounds **1**, **3**, and **4** on LPS-induced nitric oxide (NO) production in RAW 264.7 cells were screened by measuring the accumulated nitrite in the culture medium. Compounds **3** and **4** had moderate suppressive effects on the production of NO, with IC_{50} values of 96 and 9 μM, respectively, and compound **4** had minimal cytotoxicity, with a IC_{50} value of

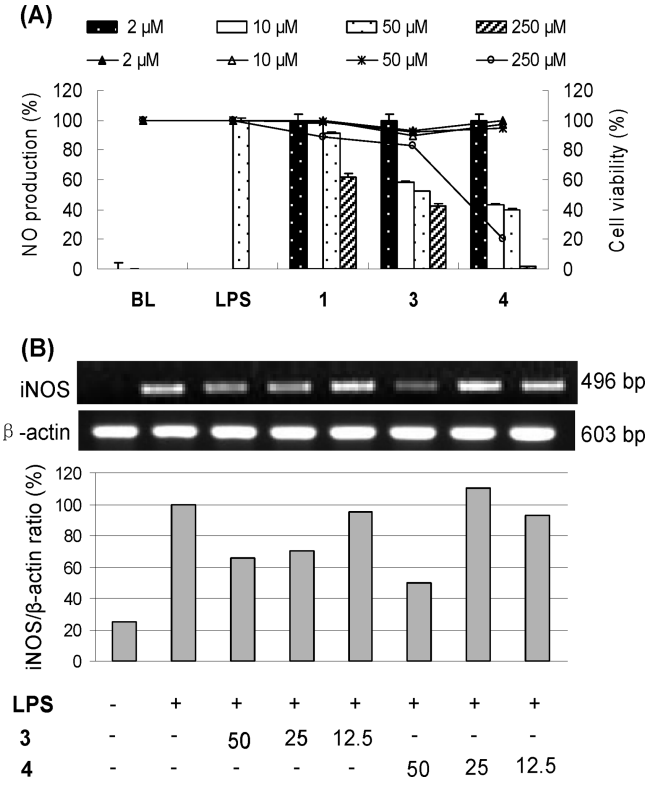

Figure 4. Inhibitory effect of compounds **1**, **3**, and **4** on NO production (A) and mRNA expression of *iNOS* of compounds **3** and **4** (B) in the LPS-stimulated RAW264.7 cells. (A) Cells were stimulated for 24 h with pure LPS (1 μg/mL) or with LPS containing the test samples at different concentrations (250, 50, 10, and 2 μM). NO production (columns) was determined using the Griess reagent method. Cell viability (lines) was determined using the WST method. The data represent the mean ± SD of triplicate experiments. (B) Total RNA was isolated in an RNase-free environment, and *iNOS* mRNA expression was determined by Western blotting. β-Actin was used as an internal control.

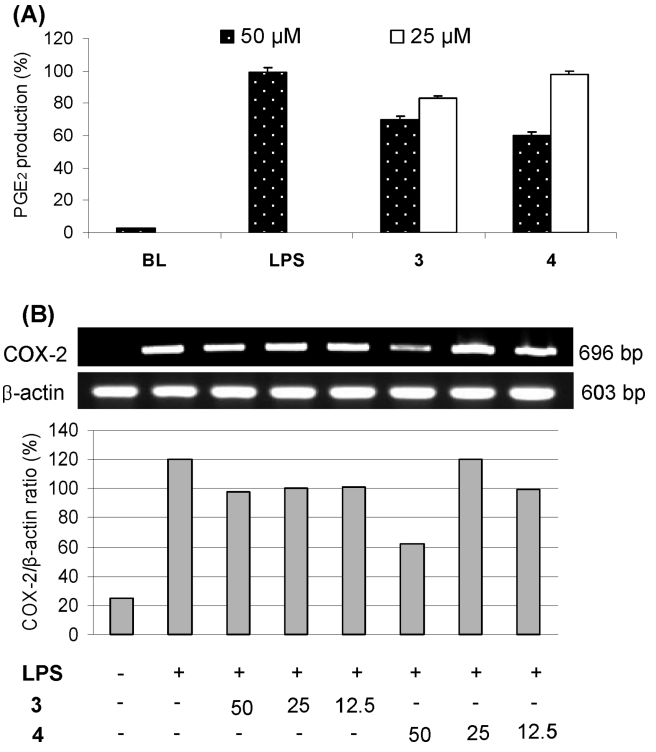

Figure 5. Inhibitory effect of compounds **3** and **4** on PGE_2 production (A) and mRNA expression of COX-2 (B) in the LPS-stimulated RAW264.7 cells. (A) Cells were stimulated for 24 h with LPS (1 μg/mL) only or with LPS containing the test samples at different concentrations (50 and 25 μM). PEG_2 amounts in the culture supernatant were determined by ELISA. The data represent the mean ± SD of triplicate experiments. (B) mRNA expression was determined by RT-PCR. The graph represents the changes in the mRNA levels of COX-2 normalized by β-actin. The data represent the mean ± SD.

170 μM (Figure 4A). To examine whether this suppression of NO production was due to the reduced *iNOS* expression, RT-PCR analysis of LPS-stimulated cells was performed. Both compounds inhibited the mRNA expression of *iNOS* (Figure 4B). The results indicated that these compounds inhibit NO production via modulation of *iNOS* gene expression. Compounds **3** and **4** were also applied to RAW 264.7 cells to investigate the inhibitory effect on the production of cyclooxygenase-2 (COX-2)-derived prostaglandin E_2 (PGE_2). As depicted in Figure 5A, compounds **3** and **4** showed moderate inhibition of PGE_2 production. The correlation of PGE_2 production with the mRNA expression of COX-2 was confirmed (Figure 5B).

In a subsequent investigation on the ability of the compounds to inhibit the production of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6), RAW 264.7 cells were cultured with **3** or **4** in the presence of LPS, and the respective cytokines were measured using ELISA. Compounds **3** and **4** showed moderate suppressive effects on the production of IL-6 (Figure 6A). Compound **4** also showed clear inhibition of the mRNA expression of IL-6 (Figure 6B).

Compounds **1**, **3**, and **4** were also evaluated for antibacterial activity against human pathogenic bacteria (*Staphylococcus aureus*, *Escherichia coli*, *Klebsiella aerogenes*, *Salmonella typhimurium*, and *Enterobacter cloacae*) because **3** and **4** were isolated from antibacterial fractions. However, the aforementioned three compounds did not show significant activity up to a concentration of 30 μg/disk (agar diffusion assay).

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured using a Jasco DIP-370 digital polarimeter. UV spectra were recorded using a Shimadzu UV-1601 UV/visible spectrophotometer. 1D and 2D NMR spectra were recorded using Varian UNITY 400 and Varian INOVA 500 spectrometers, respectively. Chemical shifts were reported with reference to the respective residual solvent or deuterated solvent peaks (δ_H 2.50 and δ_C 39.7 for DMSO). FABMS data were obtained using a JEOL JMS SX-102A. HRFABMS data were obtained using a JEOL JMS-700 Mstation mass spectrometer. FAB accurate mass measurement was carried out at a resolution of 10 000. HPLC was performed using a Gilson 370 pump with a YMC-packed J'sphere ODS-H80 column (250 × 10 mm, 4 μm, 80 Å) and a Shodex packed NH-5E column (250 × 10 mm, 5 μm) using Shodex RI-71 and Jasco UV-975 detectors.

Animal Material. *Herdmania momus* was collected by hand with scuba (15 to 25 m depth) in 2008, off the coast of Jeju Island, Korea. The fresh specimens were immediately frozen and maintained at −20 °C until the chemical investigation. A voucher specimen (J08J-1) was taxonomically identified by one of the authors (S.S.) and deposited at the Laboratory of Phylogenetic Systematics, Sahmyook University.

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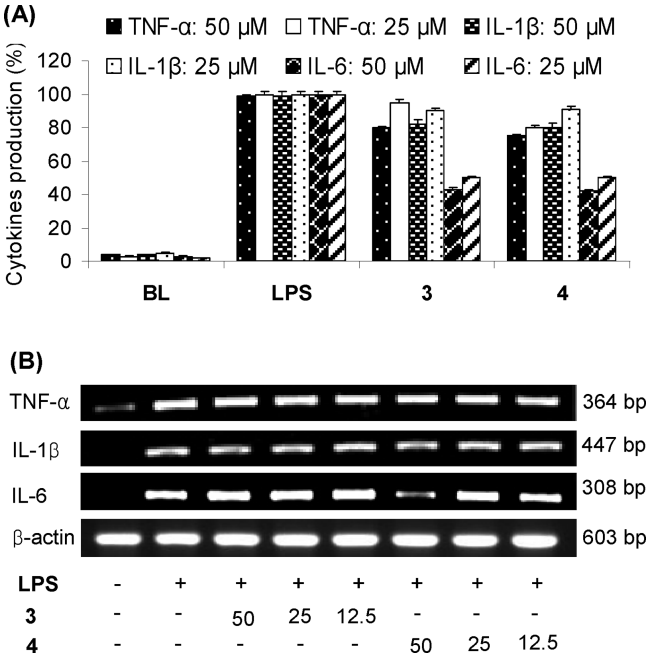

Figure 6. Inhibitory effect of compounds **3** and **4** on production (A) and mRNA expression (B) of cytokines (IL-6, IL-1β, and TNF-α) in the LPS-stimulated RAW264.7 cells. (A) The RAW264.7 cells (1.5×10^5 cells/mL) were stimulated for 24 h with pure LPS (1 μg/mL) or with LPS containing test samples at different concentrations (50 and 25 μM). The production of cytokines was determined using the Griess ELISA method. The data represent the mean ± SD of triplicate experiments. (B) Effects of compounds **3** and **4** on the mRNA expression of cytokines in the LPS-stimulated RAW 264.7 cells. Total RNA was isolated in an RNase-free environment, and the cytokine and β-actin mRNA levels were measured by RT-PCR.

Extraction and Isolation. The frozen ascidians (0.4 kg) were chopped into small pieces and extracted with MeOH and CH_2Cl_2 at room temperature. The combined extract was partitioned between CH_2Cl_2 and H_2O. The H_2O layer was further extracted by *n*-BuOH. The *n*-BuOH fraction (6 g) was subjected to reversed-phase MPLC column chromatography (YMC gel ODS-A, 60 Å, 230 mesh) with a step gradient solvent system, 30% to 100% MeOH/H_2O, to afford 21 fractions. Fraction 11 (37.3 mg), which gave interesting signals in the ^{1}H NMR spectrum, was subjected to reversed-phase HPLC (eluent: 50% MeOH) to afford **1** (3.8 mg). Fraction 8 (136.9 mg), which gave ^{1}H NMR signals similar to those given by fraction 11, was first purified on a Shodex packed NH-5E column using 85% MeOH as the eluent and further purified on a YMC-packed J'sphere ODS-H80 column using 35% MeOH as the eluent to afford **2** (1.0 mg). Fraction 9 (120.3 mg), one of the antibacterial fractions, was subjected to reversed-phase HPLC (eluent: 50% MeOH) to obtain **3** (2.6 mg) and **4** (5.8 mg).

Herdmanine A (***1***): yellow gum; $[\alpha]^{23}_D$ −18 (*c* 0.10, MeOH); ^{1}H and ^{13}C NMR (see Table 1); (+)-FABMS *m*/*z* 438 $[M + H]^+$; (+)-HRFABMS *m*/*z* 438.1774 (calcd for $C_{22}H_{24}N_5O_5$, 438.1771); FAB-CID-MS/MS *m*/*z* 438 (100), 421 (0.4), 294 (2.5), 264 (1.8), 144 (3.4), 121 (0.7).

Herdmanine B (***2***): white powder; $[\alpha]^{23}_D$ −33 (*c* 0.09, MeOH); ^{1}H and ^{13}C NMR (see Table 1); (+)-FABMS *m*/*z* 454 $[M + H]^+$; (+)-HRFABMS *m*/*z* 454.1755 (calcd for $C_{22}H_{24}N_5O_6$, 454.1727); FAB-CID-MS/MS *m*/*z* 454 (100), 437 (0.5), 294 (1.6), 280 (1.0), 160 (2.6).

Herdmanine C (***3***): white powder; $[\alpha]^{23}_D$ −22 (*c* 0.10, MeOH); ^{1}H NMR (DMSO, 500 MHz) δ 9.17 (1H, brs, 20-NH), 8.85 (1H, d, *J* = 7.5 Hz, 15-NH), 7.89 (2H, d, *J* = 9.0 Hz, H-10, H-12), 7.79 (2H, d, *J* = 8.5 Hz, H-3, H-5), 7.26 (3H, brs, 22-NH, 23-NH_2), 7.10 (2H, d, *J* = 9.0 Hz, H-9, H-13), 6.92 (2H, d, *J* = 8.5 Hz, H-2, H-4), 4.20 (1H, m, H-16), 3.15 (2H, m, H-19), 1.89 (1H, m, H-17a), 1.81 (1H, m, H-17b), 1.57 (2H, quintet, *J* = 6.5 Hz, H-18); ^{13}C NMR (DMSO, 100 MHz) δ 177.6 (C-24), 165.0 (C-14), 164.7 (C-7), 163.5 (C-1), 157.9 (C-21), 153.1 (C-8), 132.9 (C-3, C-5), 132.5 (C-11), 129.0 (C-10, C-12), 122.0 (C-9, C-13), 119.8 (C-4), 116.3 (C-2, C-6), 55.0 (C-16), 41.2 (C-19), 29.9 (C-15), 25.3 (C-18); (+)-FABMS *m*/*z* 415 $[M + H]^+$; (+)-HRFABMS *m*/*z* 415.1595 (calcd for $C_{20}H_{23}N_4O_6$, 415.1618); FAB-CID-MS/MS *m*/*z* 417 (100), 398 (1.2), 356 (0.7), 294 (2.0), 241 (3.4), 121 (1.8).

Herdmanine D (***4***): white powder; $[\alpha]^{23}_D$ −4.7 (*c* 0.30, MeOH); ^{1}H NMR (DMSO, 500 MHz) δ 12.07 (1H, brs, 1-NH), 10.14 (1H, brs, 18-NH_2), 9.95 (1H, brs, 18-NH_2), 7.80 (1H, s, H-7), 7.61 (1H, s, H-4), 7.33 (2H, d, *J* = 8.5 Hz, H-11, H-13), 7.16 (2H, d, *J* = 8.5 Hz, H-10, H-14), 3.45 (1H, dd, *J* = 8.5, 4.5 Hz H-16), 3.16 (1H, dd, *J* = 14.5, 4.5 Hz H-15a), 2.91 (1H, dd, *J* = 14.5, 8.0 Hz H-15b); ^{13}C NMR (DMSO, 100 MHz) δ 170.4 (C-17), 163.2 (C-8), 150.1 (C-9), 150.0 (C-5), 135.2 (C-12), 135.1 (C-2), 131.7 (C-7a), 130.9 (C-11, C-13), 127.0 (C-3a), 122.5 (C-10, C-14), 116.8 (C-7), 107.0 (C-6), 106.3 (C-4), 105.3 (C-3), 56.1 (C-16), 37.0 (C-15). (+)-FABMS *m*/*z* 421/419 $[M + H]^+$, 375/373 $[M - COOH]^+$; (+)-HRFABMS *m*/*z* 421.0224/419.0223 (calcd for $C_{18}H_{16}BrN_2O_5$, 421.0222/419.0242).

Preparation of Pyrimidine Derivative and Marfey Analysis of 1. To a 1 mL vial containing pure **1** (0.5 mg) was added a mixture of H_2O (25 μL), EtOH (50 μL), triethylamine (25 μL), and acetylacetone (50 μL), and the resulting solution was maintained at 110 °C for 4 h in a screw-cap vial. The reaction mixture was evaporated to dryness under a stream of N_2 to yield the dimethylpyrimidine derivative of **1** (**1a**). Compound **1a** was dissolved in 6 N HCl (1 mL) and hydrolyzed at 110 °C for 24 h. The acid hydrolysate was dried under N_2 to yield the arginine dimethylpyrimidine derivative **1b**. To the acid hydrolysate, 0.1% FDAA (1-fluoro-2,4-dinitrophenyl-5-L-alanine amide) solution in acetone (100 μL) and 1 M $NaHCO_3$ (20 μL) were added, and the mixture was heated at 40 °C for 1 h. After cooling to room temperature, the reaction mixture containing **1c** was neutralized with 2 M HCl (10 μL) and diluted with 0.5 mL of MeOH for TLC analysis (Figure 2). (**1c** and those derived from authentic D- and L-arginine showed R_f values of 0.36 and 0.41, respectively, on a silica gel TLC plate; mobile phase, 3:1 $CHCl_3$/MeOH.) ^{1}H NMR data of **1b** in CD_3OD: δ_H 6.38 (1H, s), 4.05 (1H, t), 3.39 (2H, m), 2.21 (6H, s), 1.83 (2H, m), 1.65 (2H, m).

Acid Hydrolysis and Marfey Analysis of 4. Compound **4** (0.5 mg) was dissolved in 6 N HCl (1 mL) and hydrolyzed at 110 °C for 24 h. The acid hydrolysate was dried under N_2. The residue was treated with FDAA prior to TLC analysis, as described above. (D-Tyr-FDAA and L-Tyr-FDAA showed R_f values of 0.46 and 0.54, respectively, on an RP-18 F_{254} TLC plate; mobile phase, 1:1 MeOH/H_2O.)

Nitrite Assay. The production of NO was measured, as previously described by Ryu et al.,[16] by using Griess reagent (Sigma). Briefly, RAW 264.7 cells were stimulated with LPS (1 μg/mL), and 100 μL of the supernatant was mixed with 100 μL of the Griess reagent (0.1% naphthalene diamine dihydrochloride, 1% sulfanilamide, and 2.5% H_3PO_4). The mixture was incubated for 10 min at room temperature. The absorbance at 540 nm was measured using an ELISA reader (Amersham Pharmacia Biotech), and the results were compared against a calibration curve using sodium nitrite as the standard.

Cytotoxicity Assay. Cell viability was determined by the WST-1 (4-[3-iodophenyl]-2-(4-nitrophenyl)-2*H*-5-tetrazolio)-1,3-benzene disulfonate) assay. Briefly, the cells were stimulated for 24 h with 1 μg/mL of pure LPS or LPS containing different concentrations of compounds **1**, **3**, and **4**. Then, the cell proliferation reagent WST-1 was added, and the cells were reincubated for 2 h. Thereafter, the formazan formed was quantified by measuring the light absorbance at 450 nm using an ELISA plate reader.

Determination of PGE_2. Raw 264.7 cells were seeded in wells and incubated for 24 h. After incubation, the cells were incubated with

different concentrations of **3** and **4** in the presence of LPS (1 μg/mL) for 24 h. The PGE_2 concentration in the culture medium was determined using an ELISA kit.

Measurement of Production of Pro-inflammatory Cytokines (IL-6, IL-1β, and TNF-α). The inhibitory effects of the isolated compounds on IL-6, IL-1β, and TNF-α production were determined by the method previously described by Cho et al.[17] The samples were dissolved with EtOH and diluted with DMEM. The final concentration of chemical solvents did not exceed 0.1% in the culture medium. Under these conditions, none of the solubilized solvents altered IL-6, IL-1β, or TNF-α production in RAW 264.7 cells. Before stimulation with LPS (1 μg/mL) and testing samples, RAW 264.7 cells were incubated for 18 h in 24-well plates under the same conditions. Stimuli and the testing samples were then added to the culture cells for 24 h. Supernatants were then collected and assayed for IL-6, IL-1β, and TNF-α content using mouse ELISA kits (R & D Systems Inc.).

RNA Preparation and RT-PCR. Total RNA was extracted from cells in an RNase-free environment using the Tri-Reagent method (MRC) in accordance with the manufacturer's instructions. Reverse transcription was performed using a First-Strand cDNA Synthesis kit (Promega). Briefly, total RNA (1 μg) was incubated with oligo$(dT)_{18}$ primer at 70 °C for 5 min and cooled on ice for 5 min. After the addition of M-MuLV reverse transcriptase (Promega), dNTP (0.5 μM), and 1 U RNase inhibitor, the reactions were incubated at 25 °C for 5 min and 37 °C for 60 min. M-MuLV reverse transcriptase was inactivated by heating at 70 °C for 15 min. The PCR was performed using a DNA gene cycler (BIO-RAD), and the amplification was performed in 30 cycles: 94 °C for 45 s (denaturing), 50−58 °C for 45 s (annealing), and 72 °C for 1 min (extension). The PCR products were separated on 1.2% agarose gel.

■ ASSOCIATED CONTENT

Supporting Information. ^1H and ^{13}C NMR spectra of compounds **1**−**4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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