Four New Cembrane Diterpenes Isolated from an Okinawan Soft Coral *Lobophytum crassum* with Inhibitory Effects on Nitric Oxide Production

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Four new cembrane diterpenes (1—4) and fifteen known cembranoids (5—19) were isolated from an Okinawan soft coral *Lobophytum crassum*. The structures of these four new cembranoids were determined on the basis of spectroscopic evidence. In particular, the absolute stereochemistry of 1, 2, 5 and 6 were elucidated by the application of the modified Mosher's method and circular dichroism (CD) spectral data. The inhibitory effects of some isolates were evaluated on nitric oxide (NO) production against a murine macrophage-like cell line (Raw 264.7). Cembranoids consisting of α-methylene-γ-lactone, exhibited the significant effect on NO production.

Key words cembrane diterpene; soft coral; *Lobophytum crassum*; absolute stereochemistry; nitric oxide production

Soft corals are well known to be a rich source of terpene metabolites, especially diterpenes of the cembranoid series,[1,2] which are naturally occurring diterpenens containing a 14-carbon ring. Many of them are of great interest as they exhibit a wide range of biological activities including ichthyotoxic, anti-cancer, anti-inflammatory, anti-human immunodeficiency virus (HIV), and anti-microbial activities.[3—9] In the continuation of our ongoing research for bioactive marine natural products, we have investigated the *n*-hexane extract of the *Lobophytum crassum* (von Marenzeller, 1886) which has exhibited a significantly inhibitory effect on nitric oxide (NO) production against lipopolysaccharide (LPS)-induced Raw 264.7. Chemical investigation revealed that the *n*-hexane-soluble material in this soft coral included four new (**1**—**4**) and fifteen known (**5**—**19**) cembranoids (Fig. 1). In this paper, we report on the isolation and structure elucidation of four new cembranoids **1**—**4** and also the determination of absolute stereochemistry of the new **1** and **2** and the known cembranoids **5** and **6**. Furthermore, the inhibitory effect of some isolates was evaluated on NO production against Raw264.7.

The frozen *L. crassum* collected in Okinawa was completely extracted in *n*-hexane. The extract was partitioned between *n*-hexane and aqueous methanol. The *n*-hexane soluble-material was subjected to silica gel flash column chromatography and recycling HPLC, followed by the reversed phase (RP) HPLC to afford four new cembranoids **1**—**4** and fifteen known cembranoids **5**—**19**.

The molecular formula of **1** was determined to be $C_{22}H_{30}O_4$ by the combination of electrospray ionization (ESI)-time-of-flight (TOF)-MS [*m*/*z* 381.2039, Calcd 381.2036 $(M+Na)^+$] and ^{13}C-NMR spectroscopic data revealing eight degrees of unsaturation. The IR absorptions at 1759 and 1666 cm^{-1} and UV absorption at 229 nm were indicative of the presence of an α-methylene-λ-lactone moiety. The ^{1}H- and ^{13}C-NMR spectra of **1** showed signals assignable to three olefinic methyls, one acetylmethyl, three trisubstituted double bonds, one exomethylene, two oxymethines, one aliphatic methine, five methylenes, and two ester carbonyls. Six of the eight degrees of unsaturation in **1** were attributed to four double bonds and two ester carbonyls (Tables 1, 2). The remaining two degrees of unsaturation were attributed to the bicyclic ring system of **1**. Interpretation of correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) spectra, in combination with heteronuclear multiple bond correlation (HMBC) experiments, led to the planar structure of **1** (Fig. 2). The COSY and TOCSY spectra of **1** revealed three spin systems from H-3 [δ_H 5.13 (d, 9.6)] to H-13 [δ_H 5.18 (t, 6.0)], H-5 [δ_H 2.22] to H-7 [δ_H 4.98 (t, 6.1)], and H-9 [δ_H 2.00, 2.02] to H-11 [δ_H 5.53 (t, 6.7)]. The HMBC correlations from three olefinic methyls [H-18 (δ_H 1.66)/C-3 (δ_C 121.2), C-5 (δ_C 39.2)], [H-19 (δ_H 1.58)/C-7 (δ_C 123.9), C-9 (δ_C 34.6)], and [H-20 (δ_H 1.63)/C-11 (δ_C 72.2), C-13 (δ_C 124.6)], an oxymethine [H-2 (δ_H 5.28)/C-16 (δ_C 170.5) and an exomethylene [H-17 (δ_H 5.66, 6.22)/C-16 (δ_C 170.5), C-17 (δ_C 121.6), C-1 (δ_C 42.8)] easily merged these three spin systems and the presence of an α-methylene-λ-lactone adjacent to the 14-membered ring. Thus, the planar structure of **1** was depicted in Fig. 2. The *E*-geometry of the Δ^3 and Δ^7 was determined by the nuclear Overhauser effect (NOE) correlations between H-3/H-5 and H-7/H-9 (δ_H 2.00), and the *Z*-geometry of the Δ^{12} was determined by the strong NOE between H-13 and H-20. The *cis*-fused α-methylene-λ-lactone and the 14-membered ring was deduced from the NOE correlation between H-1 and H-2. In addition, NOE correlations were observed between H-3 and H-2/H-5/H-7, H-7 and H-9/H11, H-13 and H-1/H-17/H-20. These data revealed the conformation of the 14-membered ring and the relative configuration of C-11. Thus, the relative stereochemistry of **1** was determined to be 1*S**, 2*S** and 11*S** as shown in Fig. 3. The absolute stereochemistry of **1** was determined using modified Mosher's method.[10] Cembranoid **1** was first converted to the 11-hydroxy derivative **1a** and then prepared (*R*)- or (*S*)-α-methoxy-α-(trifluoromethyl)phenylacetic acid (MTPA) esters (**1b**, **1c**), respectively. The proton chemical shifts of each MTPA ester were assigned by 1D- and 2D-NMR experiments. The distribution of $\Delta\delta$ ($\delta_S-\delta_R$) values indicates that the absolute configuration of C-11 is *S* configuration. Thus, the absolute configuration at C-1 and C-2 was then deduced to be both *S* configurations (Chart 1).

Compound **2** displayed a molecular ion peak at *m*/*z* 381.2037 $(M+Na)^+$ and gave the same molecular formula $C_{22}H_{30}NaO_4$ as for **1**. From ^{1}H- and ^{13}C-NMR data, compound **2** was found to be an isomer of **1**. The IR spectrum of

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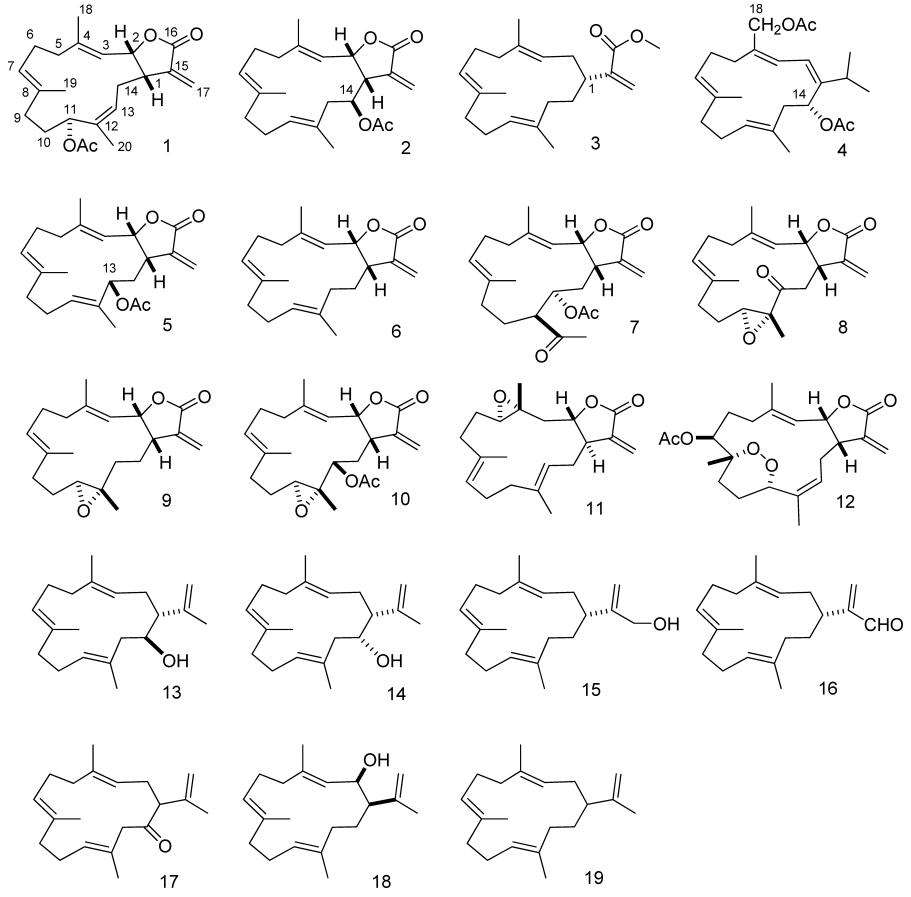

Fig. 1. Structures of Compounds **1**—**19**

Table 1. ^{1}H-NMR (600 MHz) Data of Compounds **1**—**4** in $CDCl_3$

Proton	**1**	**2**	**3**	**4**
1	3.03 (m)	3.02 (m)	2.56 (m)	—
2	5.28 (dd, 7.7, 9.6)	4.87[a)]	1.97β (m) 2.10α[a)]	6.24 (d, 11.6)
3	5.13 (d, 9.6)	5.08 (d, 9.4)	5.15 (t, 7.0)	6.30 (d, 11.6)
5	2.22 (2H)[a)]	2.17 (2H)[a)]	2.11α[a)] 2.17β[a)]	2.04 (2H)[a)]
6	2.22 (2H)[a)]	2.18β[a)] 2.29α[a)]	2.15α[a)] 2.23β (m)	2.16 (2H)[a)]
7	4.98 (t, 6.1)	4.87[a)]	4.96 (br t, 6.9)	4.96 (br t, 6.9)
9	2.00β (m) 2.02α (m)	2.02β[a)] 2.17α[a)]	2.04β (m) 2.08α[a)]	2.02β[a)] 2.11α[a)]
10	1.61β[a)] 1.85α (m)	2.18β[a)] 2.29α[a)]	2.10 (2H)[a)]	2.09 (2H)[a)]
11	5.53 (t, 6.7)	5.02 (t, 6.1)	5.05 (dd, 5.4, 6.5)	5.05 (br s)
13	5.18 (t, 6.0)	2.29β (br) 2.42α (m)	1.70β[a)] 1.95α[a)]	2.37 (2H, m)
14	2.30β (m) 2.41α (m)	4.97 (m)	1.54β[a)] 1.76α (m)	5.97 (dd, 3.9, 10.0)
15	—	—	—	2.51 (m)
16	—	—	—	1.02 (3H, d, 6.9)
17	5.66α (d, 2.4) 6.22β (d, 2.7)	5.70 (d, 1.9) 6.34 (d, 2.5)	5.49 (s) 6.17 (d, 1.2)	1.06 (3H, d, 6.9)
18	1.66 (3H, s)	1.67 (3H, s)	1.56 (3H, s)	4.65 (d, 12.4) 4.73 (d, 12.4)
19	1.58 (3H, s)	1.57 (3H, s)	1.57 (3H, s)	1.46 (3H, s)
20	1.63 (3H, s)	1.64 (3H, s)	1.52 (3H, s)	1.54 (3H, s)
OAc	2.00 (3H, s)	2.02 (3H, s)		2.00 (3H, s) 2.05 (3H, s)
OMe	—	—	3.73 (3H, s)	

a) Submerged by other signals.

Table 2. ^{13}C-NMR (150 MHz) Data of Compounds **1**—**4** in $CDCl_3$

Carbon	1	2	3	4
1	42.8 (d)	46.9 (q)	9.6 (d)	146.0 (s)
2	77.7 (d)	76.8 (d)	32.8 (t)	120.0 (d)
3	121.2 (d)	123.2 (d)	123.5 (d)	126.4 (d)
4	141.2 (s)	141.7 (s)	135.4 (s)	133.0 (s)
5	39.2 (t)	38.5 (t)	38.9 (t)	35.4 (t)
6	24.9 (t)	24.3 (t)	24.9 (t)	25.8 (t)
7	123.9 (d)	124.8 (d)	125.8 (d)	123.9 (d)
8	134.3 (s)	133.9 (s)	133.4 (s)	134.8 (s)
9	34.6 (t)	38.6 (t)	39.5 (t)	38.6 (t)
10	29.2 (t)	24.3 (t)	23.8 (t)	24.6 (t)
11	72.2 (d)	127.9 (d)	122.3 (d)	126.9 (d)
12	135.8 (s)	129.4 (s)	133.9 (s)	130.0 (s)
13	124.6 (d)	39.9 (t)	34.2 (t)	41.9 (t)
14	27.3 (t)	71.7 (d)	29.2 (t)	72.5 (d)
15	138.8 (s)	134.8 (s)	144.7 (s)	28.1 (d)
16	170.5 (s)	170.0 (s)	168.0 (s)	24.8 (q)
17	121.6 (t)	124.2 (t)	123.6 (t)	23.8 (q)
18	15.9 (q)	16.2 (q)	15.4 (q)	61.7 (t)
19	16.5 (q)	15.4 (q)	15.3 (q)	15.7 (q)
20	17.7 (q)	18.3 (q)	17.7 (q)	18.5 (q)
OAc	21.1 (q)	21.0 (q)	—	21.0 (q)
OAc	170.3 (s)	170.1 (s)	—	21.3 (q)
OAc	—	—	—	170.0 (s)
OAc	—	—	—	170.5 (s)
OMe	—	—	51.7 (q)	—

2, similar to that of **1**, also revealed the presence of an α-methylene-λ-lactone (1759, 1663 cm^{-1}) and an ester group (1737 cm^{-1}) moieties. The ^{1}H- and ^{13}C-NMR spectra of **2** also exhibited some similarities to those of **1**. The exomethylene in **2** was confirmed by the presence of two characteristic proton signals at δ_H 5.70 (d, 1.9, 1H) and δ_H 6.34 (d, 2.5, 1H), both connected to C-17 at δ_C 124.2. The presence of two carbonyl esters in **2** was supported by ^{13}C-NMR signals at δ_C 170.0 and δ_C 170.1. ^{1}H- and ^{13}C-NMR signals at δ_H 4.87 (m), δ_C 124.8; δ_H 5.02 (d, 6.1), δ_C 127.9; δ_H 5.08 (d, 9.4), δ_C 123.2 suggested the presence of three trisubstituted double bonds in **2** (Tables 1, 2). By the analysis of HSQC, COSY, TOCSY and HMBC spectral data, the acetoxy group was located at C-14 [δ_H 4.97 (m), δ_C 71.7]. Additionally, the double bond at C-12/C-13 in **1** shifted to C-11/C-12 in **2**. Thus, the planar structure was established as shown in Fig. 2. The *E*-geometry of the three trisubstituted double bonds in **2** was determined by the NOE correlations between H-3 and H-5, H-7 and H-9 and H-11 and H-14. Furthermore, a *cis*-fused ring and the a oriented acetoxy at C-14 were confirmed by the intense NOE correlations, demonstrating 1*R**, 2*S**, and 14*S** configurations of **2**. The absolute configuration in **2** was determined by following the same procedure subsequent to **1**. Then, the absolute configuration of **2** was determined to be 1*R*, 2*S*, and 14*S* (Chart 1).

The molecular formula of $C_{21}H_{32}O_2$ for **3** was determined

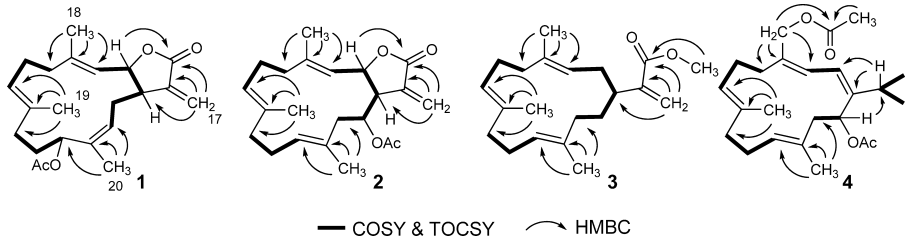

Fig. 2. ^{1}H–^{1}H COSY, TOCSY, and Selected HMBC Correlations of Compounds **1**—**4**

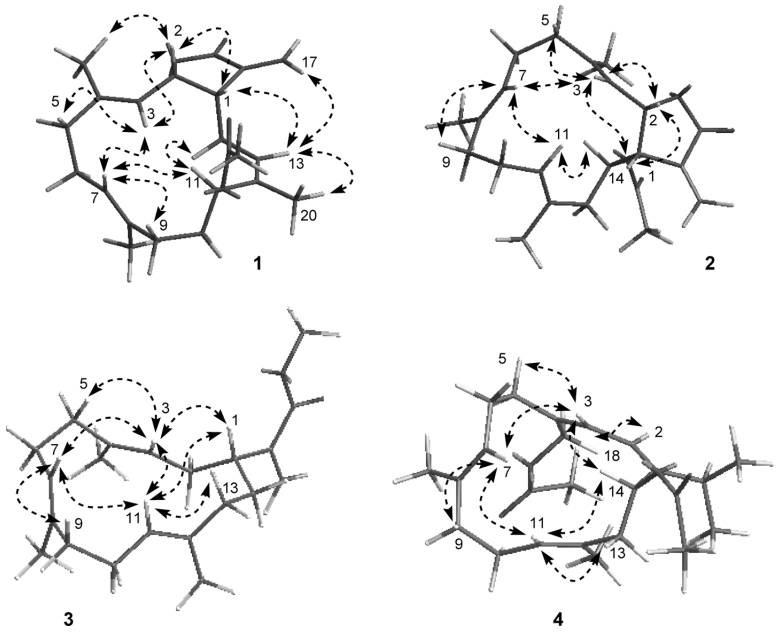

Fig. 3. Stereochemistry of Compounds **1**—**4**, and Selected NOE Correlations

Structures were refined by performing an optimized using MM2.

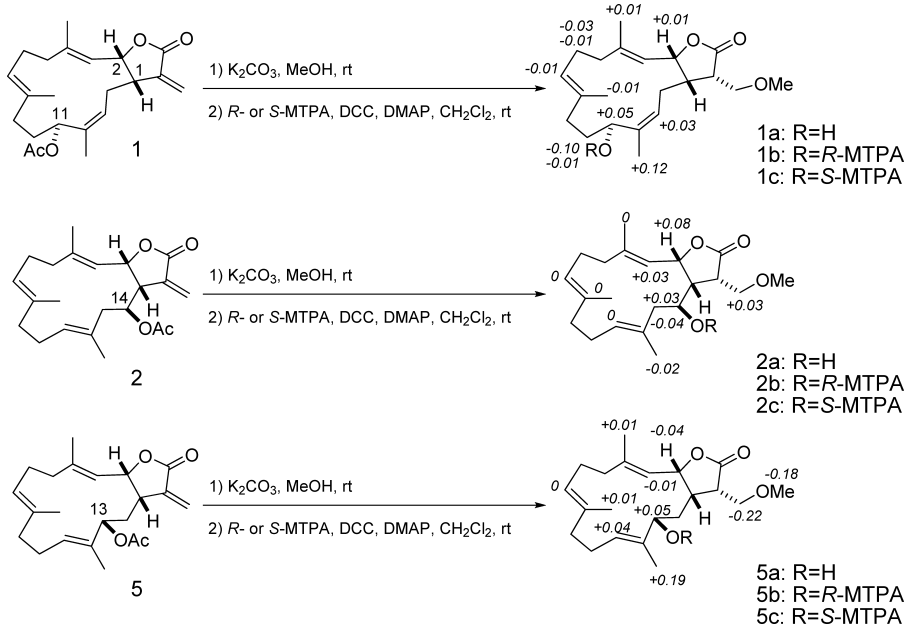

Chart 1. Preparation of MTPA Esters of Compounds **1**, **2**, and **5**, and Their $\Delta\delta$ Values

by the ESI-TOF-MS, and therefore, possessed six degrees of unsaturation. A signal of a carbonyl ester at 1714 cm^{-1} appeared in the IR spectrum of **3**. The ^{1}H-NMR spectra of **3** displayed characteristic proton signals for the exomethylene at δ_H 5.49 (s, 1H) and δ_H 6.17 (d, 1.2, 1H). In addition, ^{1}H- and ^{13}C-NMR data of **3** revealed the presence of three olefinic protons corresponding to trisubstituted double bonds at δ_H 5.15 (t, 7.0), δ_H 4.96 (br s) and δ_H 5.05 (dd, 5.4, 6.5) with olefinic carbon signals at δ_C 123.5 and 135.4, δ_C 125.8 and 133.4, and δ_C 122.3 and 133.9, respectively. Moreover, the proton resonance appearing at δ_H 3.73 and carbon resonances at δ_C 51.7 and 168.0 unequivocally revealed the presence of a methyl ester moiety. It was then concluded that **3** is a monocyclic compound. The cembranoid backbone of **3** was assembled through the interpretation of COSY, TOCSY and HMBC correlations (Fig. 2). The geometry of all three double bonds in **3** was characterized to be of the *E*-geometry, in accordance with the NOE correlations, because the NOE correlations between H-3 and H-5, H-7 and H-9, H-11 and H-13 were observed. Furthermore, the NOE correlations between H-1 and H-3/H-11, H-7 and H-3/H-11 were observed, the methine proton at C-1 was oriented to the same side of the three olefinic protons in **3**. Finally, the relative configuration of the stereogenic center at C-1 in **3** was determined to be 1*R** from a perspective of biosynthetic pathway (Fig. 3).

The molecular ion peak at *m/z* 411.2505 $(M+Na)^+$ in the ESI-TOF-MS spectrum of **4**, in combination with ^{13}C-NMR, allowed us to establish the molecular formula of **4** as $C_{24}H_{36}O_4$. The IR spectrum of **4** showed characteristic peaks at 1732 cm^{-1} attributable to the ester group. The presence of two doublet secondary methyl groups [δ_H 1.02 (d, 6.9) and δ_H 1.06 (d, 6.9)] suggested an isopropyl group (C-15/C-16/C-17). In fact, the presence of this isopropyl unit was confirmed by the COSY correlations between the methine proton H-15 [δ_H 2.51 (m)] to both secondary methyl groups (H_3-16 and H_3-17). Proton signals at δ_H 1.46 and δ_H 1.54 were assigned to olefinic methyl groups (H_3-19 and H_3-20). The HMBC spectrum showed that the two olefinic methyls, H_3-19 and H_3-20, were coupled to C-7, C-8, C-9 and C-11, C-12, C-13, respectively. In the same way, the oxymethylene protons at C-18 [(δ_H 4.65 (d, 12.4) and δ_H 4.73 (d, 12.4)] exhibited the HMBC correlations to C-3, C-4, C-5 and also to one of the carbonyl carbons of the ester group found in **4**. The oxymethine proton [δ_H 5.97 (dd, 3.9, 10.0)] at C-14 showed the HMBC correlations to the second ester group and also to the methine C-15. Finally, the COSY and TOCSY correlations between H-2 and H-3, H-6 and H-5/H-7, H-10 and H-9/H-11, H-13 and H-14 achieved the connection of all partial structures, and which led to the proposed structure of **4** (Fig. 2).

The stereochemistry of the four double bonds and that of the acetate group in **4** were established by analyzing the NOESY spectrum of **4**. The NOE correlations from H-2 to H_2-18 and from H-3 to H-5 allowed us to confer both Δ^1 and Δ^3 *Z*-geometry. The *Z*, *Z* nature of the conjugated diene in **4** was confirmed by the coupling constant of H-2/H-3 (J=11.6 Hz). On the other hand, the NOE correlations from H-7 to H-9, H-11 to H_2-13 and those from H-3 to H-7 and H-14, H-7 to H-11, H-11 to H-14 revealed that the geometry of Δ^7 and Δ^{11} were both *E*, and that the acetate group at C-14 was on the opposite side of the olefinic protons H-3, H-7 and H-11 (Fig. 3).

Compound **5** was a known compound isolated previously from *L. denticulatum*[11] and the Okinawan soft coral *Sinularia mayi*.[12] The structure of compound **5** was established by comparison of ^{1}H-NMR data reported in the literature. The relative and absolute stereochemistry of **5** has not been determined yet. Then again, from the NOE correlations of **5** and by applying Modified Mosher's method as for **1** and **2**, we have determined the absolute configuration of **5** to be 1*S*, 2*S*, and 13*S* (Chart 1).

Compounds **1**, **2**, and **5** were confirmed to be isomeric cembranoids, which differ from each other in the location of the acetoxy group and the position of one double bond at Δ^{11}

or Δ^{12}. Furthermore, compound **6**, which is the deacetoxy cembranoid of **1**, **2**, and **5**, was isolated from *L. michaelae*,[13] and *S. mayi*,[14] and the spectral data and specific rotation were identical with those reported. However, the absolute stereochemistry was reported to be 1*R* and 2*R*[15] which indicated the enantiomer of our isolates.

To clear up any doubts, we examined the absolute stereochemistry of **1**, **2**, **5**, and **6** using the circular dichroism (CD) spectra. The Cotton effect in the 260 nm region corresponds to an n–π^* transition in the α-methylene-γ-lactone chromophore. Because the positive Cotton effect at 260 nm was observed in the CD spectra of **1**, **5**, and **6**, the absolute stereochemistry of **6** was identical to those of **1** and **5**. On the other hand, the CD spectrum of **2** exhibited a negative Cotton effect in the same region contrary to compounds **1**, **5** and **6**. At first glance, the inversion in the sign of the Cotton effect between 240 nm and 260 nm observed for **2** indicated the opposite absolute configuration. However, previous studies on correlations between the sign of the CD spectrum and the absolute configuration in γ-lactones provided evidence of the dependence of the sign of the n–π^* Cotton effect upon the location of C_β relative to the planar lactone system.[16,17] The inversion in the sign of the Cotton effect in **2** can be explained from the conformational change in the lactone ring as likewise reported for some sesquiterpenes, such as germacranolides.[18] From the above results, the absolute configuration of **1**, **2**, **5**, and **6** as obtained by Modified Mosher's method and the CD analysis, is still the same and can be easily established as shown in Fig. 4.

The inhibitory effects of compounds **1**—**3**, **5**—**16** on LPS-induced NO production against Raw 264.7 cells were evaluated. Compounds **1**, **2**, and **5**—**12** showed significant inhibitory effect of NO production, and their IC_{50} values were less than 10 μM without any cytotoxic effect (Table 3). These data indicated that the α-methylene-γ-lactone moiety obviously plays a role for an inhibitory effect on NO production.[19] The inhibitory mechanism of these cembranoids was confirmed by the inhibition of inducible NO synthase (iNOS) expression *via* suppression of a transcription factor nuclear factor κB (NF-κB).[20] These biological properties will be reported in a specialized journal. It is expected these cembranoids will contribute to the development of therapies for anti-inflammatory diseases.

Experimental

General Experimental Procedures Optical rotations were measured with a Jasco Dip-370 digital polarimeter at 23 °C. IR spectra were measured with a JASCO FT/IR-410 spectrophotometer. CD spectra were measured with a Jasco J-720W spectropolarimeter at 22 °C. The NMR spectra were recorded on a Varian INOVA 600 operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C in $CDCl_3$. ^{1}H- and ^{13}C-NMR chemical shifts are reported in ppm (δ)

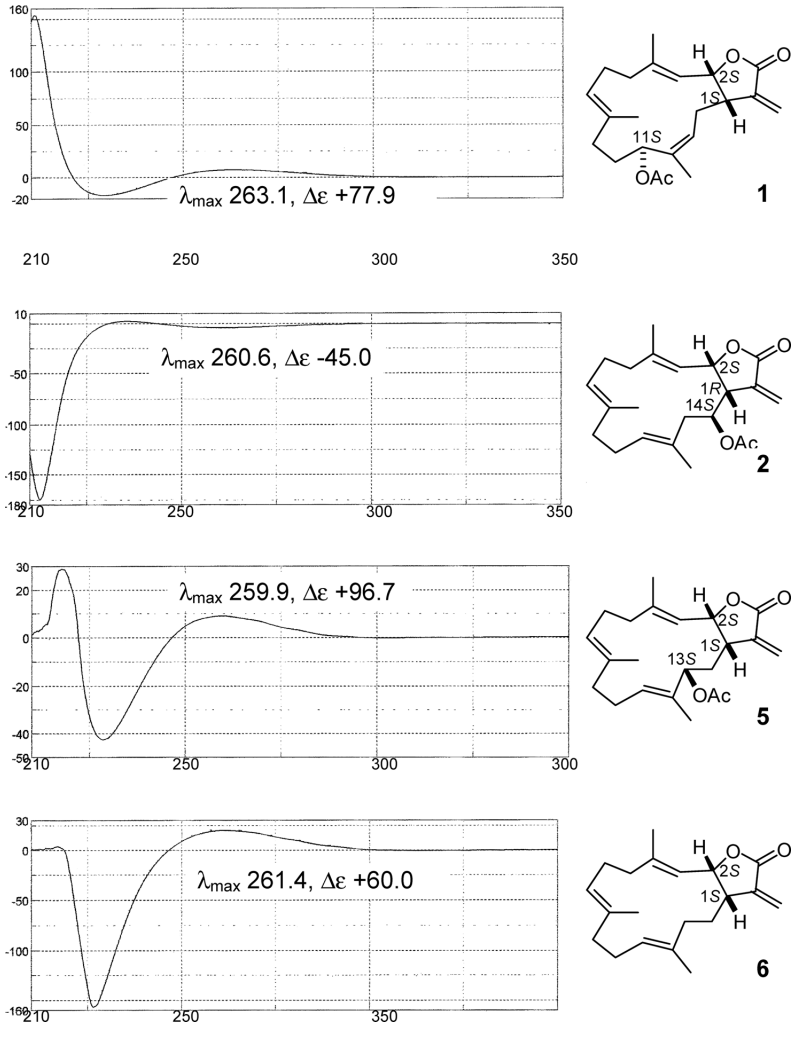

Fig. 4. CD Spectra of Compounds **1**, **2**, **5**, and **6**, and Their Absolute Stereochemistry

Table 3. Inhibitory Effect of Compounds **1**—**3**, and **5**—**16** on LPS-Induced NO Production (IC_{50}, μM), and Cell Viability at the Concentration of 10 and 50 μM (% of Control)

Compounds	IC_{50} [a]	10 μM [a]	50 μM [a]
1	3.8±0.97	107.3±5.5	26.1±6.7
2	4.0±0.91	100.0±6.6	50.5±9.1
3	>50	106.0±6.3	108.1±3.1
4	NT		
5	4.8±0.75	105.9±7.2	28.5±12.0
6	2.6±0.12	103.5±10.1	31.3±10.0
7	3.8±1.97	99.9±1.4	78.3±6.1
8	5.7±1.77	97.9±2.4	92.0±9.2
9	7.9±1.19	102.2±0.53	101.5±5.8
10	2.4±0.21	101.2±1.7	98.6±0.19
11	4.9±1.33	104.3±2.1	74.9±15.2
12	6.4±2.13	103.2±1.9	104.3±2.8
13	>50	100.8±0.20	106.5±1.9
14	>50	103.3±0.70	102.4±1.5
15	>50	100.2±2.4	101.9±1.3
16	16.6±1.70	97.2±0.05	45.8±5.5
17	NT		
18	NT		
19	NT		
L-NMAA	32.9±5.90	96.7±1.0	97.5±1.4

a) Each value represents the mean±S.D. (n=5) of three experiments.

and referenced to the solvent signal ($CDCl_3$: δ_H=7.24, δ_C=77.2). EI-MS were performed on Gas Chromatography Mass Spetrometer-QP5050 (Direct injection). ESI-TOF-MS were measured with a Bruker microTOF mass spectrometer. Chromatographic separations were carried out using silica gel (Merck Silica gel 60 F254), Recycling Preparative HPLC (JAIGEL 1H×2, 600×20 mm i.d., $CHCl_3$, JAI) with a JAI Model LC-9201 system, and RP HPLC (Cosmosil cholester, 250×4.6 mm i.d., Nakalai Tesque, YMC Pack Pro C18, 250×10 mm i.d., YMC) with a Jasco PU2089 gradient pump and PU2075 UV/VIS detector. Thin layer chromatography (TLC) analysis were carried out using Merck precoated TLC plates Silica gel 60 F_{254} and RP-18F_{254s}.

Animal Material The soft coral *L. crassum* (No. OCE 2007-01) was collected on a coral reef off Sesoko-cho (Okinawa, Japan) by hand in January 2008 at a depth of 2—3 m. A voucher specimen was deposited at the Graduate School of Pharmaceutical Sciences, Kyushu University.

Extraction and Isolation The fresh material of *L. crassum* was chopped and exhaustively extracted with *n*-hexane (10 l×3). After removal of solvent *in vacuo*, 90.38 g of the *n*-hexane extract was obtained. Part of this extract (5.5 g) was chromatographed over silica gel with *n*-hexane/Et_2O (9/1—0/1, stepwise) to yield 10 fractions, and Fr. 7 was identified as lobophytol acetate (**7**, 422.9 mg).[21] Fr. 3 (235.0 mg) was subjected to recycling preparative HPLC to yield denticulatolide (**12**, 28.8 mg),[22] and **8** (112.6 mg).[23] Fr. 4 (70.0 mg) was also subjected to recycling HPLC to yield **10** (15.2 mg).[24] Part of the *n*-hexane extract (21.1 g) was dissolved in *n*-hexane and was further partitioned against 90% of aqueous methanol to yield a *n*-hexane soluble part (7.2 g). Flash chromatography of this part (7.0 g) on silica gel with *n*-hexane/EtOAc yielded 13 fractions (Fr. 1′—Fr. 13′). Part of Fr. 1′ (149.0 mg) was applied to RP-HPLC [MeOH–H_2O (85/15)] to yield **19** (14.1 mg).[25] Fr. 2′ (714.0 mg) was chromatographed over silica gel with *n*-hexane/EtOAc (30/1) to afford Fr. 2′-1 (15.8 mg) and Fr. 2′-2 (490.8 mg). Part of Fr. 2′-2 (250.0 mg) was subjected to recycling HPLC to give eight fractions (Fr. 2′-2-1—Fr. 2′-2-8). Purification of Fr. 2′-2-4 (8.5 mg) by RP-HPLC [MeOH/H_2O (85/15)] resulted in the isolation of **3** (1.4 mg) and **17** (1.6 mg).[26] Fr. 2′-2-5 (12.3 mg) was purified RP-HPLC [MeOH/H_2O (85/15 and 80/20) to give **16** (1.8 mg),[26] **17** (1.0 mg), and **18** (0.7 mg).[27] Fr. 3′ (101.0 mg) was flash chromatographed over silica gel using *n*-hexane/EtOAc (20 : 1) to give five fractions. Fr. 3′-5 (47.6 mg) was subjected to recycling HPLC to afford four fractions (Fr. 3′-5-1—Fr. 3′-5-4). Fr. 3′-5-2 (6.6 mg) was purified by RP-HPLC [CH_3CN/H_2O (70/30)] to afford **4** (1.0 mg). Fr. 4′ (0.5 g) was subjected to silica gel column with *n*-hexane/EtOAc (14/1) to afford six fractions (Fr. 4′-1—Fr. 4′-6). Fr. 4′-5 (277.9 mg) was chromatographed over silica gel using *n*-hexane/EtOAc (14/1) to give **6** (217.1 mg).[14] Fr. 4′-6 was purified by RP-HPLC [MeOH–H_2O (85/15)] to give **13** (16.3 mg)[26] and **14** (4.9 mg).[27] Fr. 5′ (50.1 mg) was subjected to silica gel column with *n*-hexane/EtOAc (25/1), then to the RP-HPLC [MeOH/H_2O (80/20)] to yield **15** (2.0 mg).[26] Fr. 7′ (0.4 g) was chromatographed on reverse phase column (Cosmosil 140 C_{18} Prep, Nakalai Tesque) using MeOH/H_2O (9/1) to give four fractions (Fr. 7′-1—Fr. 7′-4). Fr. 7′-1 (114.3 mg) was subjected to Recycling HPLC, and RP-HPLC [MeOH/H_2O (85/15)] to afford **1** (6.4 mg), **2** (10.4 mg) and **5** (65.7 mg).[11,12] Finally, Fr. 8′ (401.0 mg) was subjected to Recycling HPLC to afford seven fractions (Fr. 8′-1—Fr. 8′-7). Fr. 8′-4 was chromatographed on a reversed phase column (Cosmosil) using MeOH/H_2O (85/15) to give two fractions (Fr. 8′-4-1—Fr. 8′-4-2). Fr. 8′-4-1 (27.2 mg) was then purified by a reverse phase HPLC on a YMC Pack Pro C_{18} [250×10 mm, MeOH/H_2O (80/20)] to yield **11** (20.0 mg).[28]

Compound **1**: Coloress oil; $[\alpha]_D^{23}$ +40.0 (c=0.26, $CHCl_3$); IR ($CHCl_3$) cm^{-1}: 2933, 1759, 1727, 1666, 1438, 1372, 1249, 1112, 1018, 982; UV λ_{max} (n-hexane) nm (log ε): 229 (3.74); EI-MS m/z: 358 $[M]^+$; ESI-TOF-MS m/z: 381.2039 $[M+Na]^+$ (Calcd for $C_{22}H_{30}O_4Na$, 381.2036). ^{1}H- and ^{13}C-NMR see Tables 1 and 2.

Compound **2**: Coloress oil; $[\alpha]_D^{23}$ −79.1 (c=0.34, $CHCl_3$); IR ($CHCl_3$) cm^{-1}: 2925, 1759, 1737, 1663, 1437, 1374, 1217, 1130, 1027, 978, 942; UV λ_{max} (n-hexane) nm (log ε): 213 (4.21); EI-MS m/z: 358 $[M]^+$; ESI-TOF-MS m/z: 381.2036 $[M+Na]^+$ (Calcd for $C_{22}H_{30}O_4Na$, 381.2036); ^{1}H- and ^{13}C-NMR see Tables 1 and 2.

Compound **3**: Coloress oil; $[\alpha]_D^{23}$ +22.3 (c=0.13, $CHCl_3$); IR ($CHCl_3$) cm^{-1}: 2927, 1714, 1456, 1438, 1141, 948; EI-MS m/z: 316 $[M]^+$; ESI-TOF-MS m/z: 339.2284 $[M+Na]^+$ (Calcd for $C_{21}H_{32}O_2Na$, 339.2295); ^{1}H- and ^{13}C-NMR see Tables 1 and 2.

Compound **4**: Coloress oil; $[\alpha]_D^{23}$ +93.3 (c=0.09, $CHCl_3$); IR ($CHCl_3$) cm^{-1}: 2928 1732, 1244, 1020, 960; ESI-TOF-MS m/z: 411.2505 $[M+Na]^+$ (Calcd for $C_{24}H_{36}O_4Na$, 411.2506); ^{1}H- and ^{13}C-NMR see Tables 1 and 2.

Compound **5**—**19**: The known compounds were identified by comparison of their ^{1}H- and ^{13}C-NMR data and specific rotations with previously published data.

Preparation of Secondary Alcohol Derivative (1a) To a solution of compound **1** (9.9 mg, 27.6 mmol) in dry MeOH (2.5 ml) at room temperature were added potassium carbonate (20.1 mg, 145.4 μmol) and the resulting mixture was stirred for 22 h at room temperature. The reaction mixture was quenched by adding some amount of ion-exchange resine (Dowex 50WX8-200) and then filtrated. The filtrate was dried with N_2, and the resulting residue was subjected to silica gel column chromatography with *n*-hexane/EtOAc (8/1—4/1) to give 2.2 mg of **1a**. ^{1}H-NMR ($CDCl_3$) δ: 2.61 (1H, m, H-1), 5.17 (1H, dd, J=7.6, 9.5 Hz, H-2), 5.05 (1H, d, J=9.7 Hz, H-3), 4.93 (1H, br t, H-7), 4.45 (1H, t, J=6.4 Hz, H-11), 2.20 (1H, m, H-15), 3.54 (1H, dd, J=3.7, 9.5 Hz, H-17), 3.63 (1H, m, H-17′), 1.60 (3H, s, H-18), 1.55 (3H, s, H-19), 1.63 (3H, s, H-20), 3.28 (3H, s, OMe-17).

Preparation of (*R*)- or (*S*)-MTPA Esters (1b, 1c) 1.1 mg (3.2 μmol) of **1a** was dissolved in 1.5 ml of dry methylene chloride. The solution was treated with (*R*)-α-methoxy(trifluoromethyl)phenylacetic acid (MTPA) (45.7 mg, 195.1 μmol), *N*,*N*′-dicyclohexyl carbodiimide DCC (45.1 mg, 218.6 μmol) and a catalytic amount of 4-dimethylaminopyridine (DMAP). The solution was stirred at room temperature for 91 h and filtered. The filtrate was dried with N_2 and subjected to silica gel column with *n*-hexane/EtOAc (12/1) to afford (*R*)-MTPA ester (**1b**, 0.8 mg, 1.5 μmol). With (*S*)-MTPA acid, **1a** (1.1 mg) was esterified in the same manner to yield (*S*)-MTPA ester (**1c**, 0.8 mg). **1b**: ^{1}H-NMR ($CDCl_3$) δ: 5.16 (2H, overlap, H-2, H-3), 2.17 (1H, m, H-6), 2.24 (1H, m, H-6′), 5.01 (1H, br t, H-7), 1.63 (1H, m, H-10), 1.91 (overlap, H-10′), 5.83 (1H, br t, H-11), 5.22 (1H, br t, H-13), 1.613 (3H, s, H-18), 1.52 (3H, s, H-19), 1.44 (3H, s, H-20). **1c**: ^{1}H-NMR ($CDCl_3$) δ: 5.17 (2H, overlap, H-2, H-3), 2.14 (1H, m, H-6), 2.23 (1H, m, H-6′), 5.00 (1H, br t, H-7), 1.53 (overlap, H-10), 1.86 (1H, br ?, H-10′), 5.88 (1H, br t, H-11), 5.25 (1H, br t, H-13), 1.615 (3H, s, H-18), 1.51 (3H, s, H-19), 1.56 (3H, s, H-20).

Preparation of Secondary Alcohol Derivative (2a), and (*R*)- , (*S*)-MTPA Esters (2b, 2c) from 2 Secondary alcohol derivative (**2a**), and (*R*)- , (*S*)-MTPA esters (**2b**, **2c**) were prepared as described for **1** using **2** (4.7 mg, 13.1 μmol). **2a** (2.2 mg): ^{1}H-NMR ($CDCl_3$) δ: 2.39 (1H, m, H-1), 4.88 (1H, dd, J=9.1 Hz, H-2), 5.04 (1H, d, J=9.2 Hz, H-3), 4.84 (1H, br t, H-7), 4.93 (1H, br t, H-11), 3.68 (1H, br d, H-14), 3.00 (1H, m, H-15), 3.60 (1H, d, J=3.2 Hz, H-17), 3.63 (1H, d, J=5.0 Hz, H-17′), 1.64 (3H, s, H-18), 1.51 (3H, s, H-19), 1.56 (3H, s, H-20), 3.29 (3H, s, OMe-17). ^{13}C-NMR ($CDCl_3$) δ: 49.9 (CH, C-1), 79.2 (CH, C-2), 124.7 (CH, C-3), 143.0 (qC, C-4), 39.9 (CH_2, C-5), 25.9 (CH_2, C-6), 126.4 (CH, C-7), 135.6 (qC, C-8), 40.0 (CH_2, C-9), 25.9 (CH_2, C-10), 127.7 (CH, C-11), 132.0 (qC, C-12), 46.3 (CH, C-13), 72.8 (CH_2, C-14), 43.1 (CH, C-15), 181.1 (qC, C-16), 60.6 (CH_2, C-17), 18.7 (CH_3, C-18), 17.6 (CH_3, C-19), 20.1 (CH_3, C-20). **2b**

(0.9 mg): ^{1}H-NMR ($CDCl_3$) δ: 4.51 (1H, dd, J=8.9 Hz, H-2), 5.04 (2H, overlap, H-3, H-14), 4.90 (1H, br t, H-7), 5.10 (1H, br t, H-11), 2.40 (1H, br d, H-13), 3.72 (1H, dd, J=3.62, 9.74 Hz, H-17), 1.64 (3H, s, H-18), 1.57 (3H, s, H-19), 1.63 (3H, s, H-20). **2c** (0.8 mg): ^{1}H-NMR ($CDCl_3$) δ: 4.59 (1H, dd, J=8.9 Hz, H-2), 5.07 (2H, overlap, H-3, H-11, H-14), 4.90 (1H, br t, H-7), 5.10 (1H, br t, H-11), 2.28 (1H, br d, H-13), 3.75 (1H, dd, J=3.62, 9.74 Hz, H-17), 1.64 (3H, s, H-18), 1.57 (3H, s, H-19), 1.61 (3H, s, H-20).

Preparation of Secondary Alcohol Derivative (5a), and (*R*)- , (*S*)-MTPA Esters (5b, 5c) from 5 Secondary alcohol derivative (**5a**), and (*R*)- , (*S*)-MTPA esters (**5b**, **5c**) were prepared as described for **1** using **5** (22.7 mg, 63.0 μmol). **5a** (11.1 mg): ^{1}H-NMR ($CDCl_3$) δ: 2.98 (1H, m, H-1), 5.36 (1H, dd, J=7.8, 10.8 Hz, H-2), 5.09 (overlap, H-3), 2.19 (overlap, H-5), 2.04 (1H, br d, J=15.9 Hz, H-6), 4.71 (1H, br d, J=8.7 Hz, H-7), 1.81 (1H, m, H-9), 2.19 (overlap, H-9′), 1.96 (1H, m, H-10), 2.19 (overlap, H-10′), 5.06 (overlap, H-11), 3.74 (1H, d, J=10.5 Hz, H-13), 1.52 (overlap, H-14), 1.90 (1H, m, H-14′), 2.47 (1H, dt, J=4.2, 12.4 Hz, H-15), 3.60 (2H, d, J=4.2 Hz, H-17), 1.66 (3H, s, H-18), 1.57 (3H, s, H-19), 1.58 (3H, s, H-20), 3.36 (3H, s, OMe-17). ^{13}C-NMR ($CDCl_3$) δ: 38.7 (CH, C-1), 77.5 (CH, C-2), 119.4 (CH, C-3), 143.2 (qC, C-4), 39.6 (CH_2, C-5), 24.4 (CH_2, C-6), 125.4 (CH, C-7), 133.3 (qC, C-8), 39.7 (CH_2, C-9), 22.9 (CH_2, C-10), 126.5 (CH, C-11), 136.9 (qC, C-12), 76.9 (CH, C-13), 35.8 (CH_2, C-14), 44.7 (CH, C-15), 176.6 (qC, C-16), 69.7 (CH_2, C-17), 15.0 (CH_3, C-18), 9.6 (CH_3, C-19), 15.1 (CH_3, C-20). **5b** (1.1 mg): ^{1}H-NMR ($CDCl_3$) δ: 5.45 (1H, dd, J=7.7, 10.8 Hz, H-2), 5.08 (1H, d, J=10.8 Hz, H-3), 4.71 (1H, br d, J=8.7 Hz, H-7), 5.28 (1H, br, H-11), 5.16 (1H, d, J=11.1 Hz, H-13), 3.60 (2H, d, J=4.0 Hz, H-17), 1.76 (3H, s, H-18), 1.58 (3H, s, H-19), 1.38 (3H, s, H-20), 3.38 (3H, s, OMe-17). **5c** (2.0 mg): ^{1}H-NMR ($CDCl_3$) δ: 5.41 (1H, dd, J=6.9, 10.8 Hz, H-2), 5.07 (1H, d, J=10.8 Hz, H-3), 4.71 (1H, br d, J=8.2 Hz, H-7), 5.32 (1H, br, H-11), 5.21 (1H, d, J=11.3 Hz, H-13), 3.55 (1H, dd, J=2.9, 9.7 Hz, H-17), 3.38 (1H, br dd, J=4.9 Hz, 9.5 Hz, H-17), 1.77 (3H, s, H-18), 1.59 (3H, s, H-19), 1.57 (3H, s, H-20), 3.20 (3H, s, OMe-17).

Cell Culture The RAW 264.7 macrophage cell line was purchased from HSRRB. RAW 264.7 macrophages were grown in Dulbecco's modification of Eagle's Medium (DMEM) medium (IWAKI) containing 10% fetal bovine serum (FBS) (IWAKI), 2% of penicillin–streptomycin (GibcoBRL, U.S.A.), 1% of NEAA (GibcoBRL, U.S.A.) and kept in an incubator at 37 °C in a humidified air containing 5% CO_2 for 24 h.

Inhibition of LPS-Induced NO Production Briefly, macrophages were plated (96-well plate) at a density of 1×10^6 cells/ml (200 μl). After pre-incubation for 24 h, cells were stimulated with LPS (1 μg/ml) for 3 h and followed by further incubation with, or without, the addition of cembranoids **1**—**3** and **5**—**12** at various concentrations for 21 h. The method is related on the measurement of the nitrite accumulated in culture medium as an indicator of NO production based on the Griess reaction. In brief, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine–HCl). Incubated at r.t. for 10 min, then the absorbance was measured at 540 nm using microplate reader (Multiskan FC). Fresh culture medium was used as the blank in all experiments. N^G-monomethyl-L-arginine monoacetate (L-NMMA, Wako), NOS inhibitor, was used for the positive control.

Cell Viability Cell viability was assessed using Cell Titer 96 AQ (MTS assay, Promega) according to the manufactureís protocol. In brief, 10 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, innersalt (MTS) was added to the 100 μl of cell culture medium after the measurement of the nitrate, and incubated for 2 h at 37 °C. The relative amount of formazan was measured by the absorbance at 492 nm using microplate reader. The cells treated with LPS were used as control.

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