Highly-Oxygenated Isopimarane-Type Diterpenes from *Orthosiphon stamineus* of Indonesia and Their Nitric Oxide Inhibitory Activity

Suresh AWALE, Yasuhiro TEZUKA, Arjun H. BANSKOTA, I Ketut ADNYANA, and Shigetoshi KADOTA*

Institute of Natural Medicine, Toyama Medical and Pharmaceutical University; 2630-Sugitani, Toyama 930–0194, Japan.
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From the methanolic extract of Indonesian *Orthosiphon stamineus*, nine new highly-oxygenated isopimarane-type diterpenes [7-*O*-deacetylorthosiphol B (1), 6-hydroxyorthosiphol B (2), 3-*O*-deacetylorthosiphol I (3), 2-*O*-deacetylorthosiphol J (4), siphonols A—E (5—9)] have been isolated together with nine known diterpenes [orthosiphols H (10), K (11), M (12) and N (13); staminols A (14) and B (15); neoorthosiphols A (16) and B (17); norstaminol A (18)]. Their structures were determined based on the spectroscopic data. The isolated diterpenes inhibited nitric oxide (NO) production in lipopolysaccharide (LPS)-activated macrophage-like J774.1 cells. Compounds 4—7, 9, 10, 14, and 17 showed inhibitory activities more potent (IC_{50}, 10.8—25.5 μM) than a positive control N^G-monomethyl-L-arginine (L-NMMA; IC_{50}, 26.0 μM).

Key words *Orthosiphon stamineus*; isopimarane-type diterpene; staminane-type diterpene; nitric oxide inhibitory activity; Indonesia

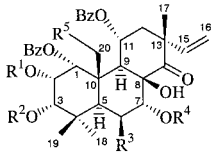
Orthosiphon stamineus BENTH. [syn.: *O. aristatus* (BL.) MIQ., *O. grandiflorus* BOLD., *O. spicatus* (THUMB.) BAK.; Lamiaceae] is one of the popular traditional folk medicines used extensively in Southeast Asia for the treatment of a wide range of diseases. This plant is known as "kumis kucing" in Indonesia and regarded as an effective folk medicine for the treatment of diabetes, hypertension, rheumatism, tonsillitis, menstrual disorder, *etc.*[1] In Vietnam, the aerial part is known by the name of "Rau meo" and used for the treatment of urinary lithiasis, edema, eruptive fever, influenza, hepatitis, jaundice, *etc.*[2] In Myanmar, this plant is known as "Secho" or "Myit-shwe", the leaves are reputed to be an antidiabetic drug and decoctions of the air-dried leaves are used to cure urinary tract and renal diseases.[3] In Okinawa prefecture of Japan, the plant is known as "Neko no hige" and consumed as a healthy Java tea to facilitate body detoxification. Because of its popularity and demonstrated effectiveness, phytochemical and pharmacological studies have been conducted since the 1930's, and highly-oxygenated isopimarane-type and migrated pimarane-type diterpenes were reported together with monoterpenes, triterpenes, saponins, flavonoids, hexoses, organic acids, rosmarinic acid, chromene, and myo-inositol.[4—12] On the other hand, as a part of our study on the biologically active constituents from this plant species, we also reported two diterpenes with a novel carbon-framework named "staminane" (staminols A and B), three secostaminanes (staminolactones A and B, norstaminolactone A), four norstaminanes (norstaminols A—C, norstaminone A), three secoisopimaranes (secoorthosiphols A—C), a norisopimarane (nororthosiphonolide A), and fifteen isopimaranes (orthosiphols F—T) from *O. stamineus* of Vietnam,[13—15] Myanmar,[16,17] and Okinawa[18,19] of Japan. In our continued search for biologically active compounds from *O. stamineus*, we found that a methanolic extract of an aerial part collected from Indonesia showed significant inhibitory activity on nitric oxide (NO) production[20] in lipopolysaccharide (LPS)-activated J774.1 macrophage-like cells (IC_{50}, 42 μg/ml). Thus, further separation of the methanolic extract was carried out to isolate the active constituent and six new diterpenes, orthosiphols U—Z, were reported from less polar fractions of the $CHCl_3$-soluble extract.[21] We recently isolated, from more polar fractions having strong nitric oxide inhibitory activity, nine new highly-oxygenated isopimarane-type diterpenes, namely 7-*O*-deacetylorthosiphol B (**1**), 6-hydroxyorthosiphol B (**2**), 3-*O*-deacetylorthosiphol I (**3**), 2-*O*-deacetylorthosiphol J (**4**), and siphonols A—E (**5**—**9**), together with nine known diterpenes.[22] In this paper, we report the isolation and structure elucidation of the new diterpenes together with their NO inhibitory activity.

Results and Discussion

Air-dried aerial parts of *O. stamineus* from Indonesia were extracted by refluxing with MeOH and the water suspension of the MeOH extract was successively partitioned into hexane, $CHCl_3$, EtOAc, BuOH, and H_2O fractions. The $CHCl_3$ fraction was subjected to a series of chromatographic separation and preparative TLC to afford nine new highly-oxygenated isopimarane-type diterpenes (**1**—**9**), together with nine previously-reported compounds (**10**—**18**). The known compounds were identified by analysis of their spectroscopic data and comparison with literature data to be orthosiphols H[13] (**10**), K[16] (**11**), M[16] (**12**), and N[16] (**13**); staminols A[13] (**14**) and B[13] (**15**); neoorthosiphols A[6,16] (**16**) and B[6,19] (**17**); and norstaminol A[13,19] (**18**).

Compound **1** was obtained as a colorless amorphous solid with an $[\alpha]_D^{25}$ −94.4° ($CHCl_3$). It showed the quasimolecular ion at *m/z* 635.2836 $(M+H)^+$ in high-resolution FAB-MS (HR-FAB-MS), which corresponds to the molecular formula $C_{36}H_{42}O_{10}$. The IR spectrum of **1** showed absorptions of hydroxyl (3450 cm^{-1}), ester carbonyl (1720 cm^{-1}), and phenyl (1605, 1455 cm^{-1}) groups. The ^{1}H-NMR spectrum of **1** exhibited signals due to four tertiary methyls (δ_H 1.49, 1.21, 1.09, 0.97), a vinyl (δ_H 5.78, 4.95, 4.82), five oxygen-substituted methines (δ_H 5.88, 5.03, 5.01, 4.42, 4.29), and two methylenes (δ_H 2.01, 1.92; δ_H 2.70, 2.00), together with those of an acetyl and two benzoyl groups (Table 1), while its ^{13}C-NMR spectrum revealed the signals of a ketone, three ester carbonyls, five oxygen-substituted carbons, and three oxygen-nonsubstituted quaternary carbons (Table 2). These ^{1}H- and ^{13}C-NMR data were similar to those of orthosiphol B (**19**) isolated previously from *O. stamineus* of Vietnam,[13] Myanmar,[16] and Okinawa,[19] but they were characterized by

* To whom correspondence should be addressed. e-mail: kadota@ms.toyama-mpu.ac.jp



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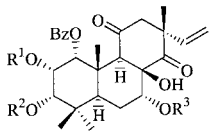
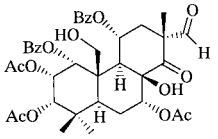
5 $R^1 = R^2 = R^4 = Ac$, $R^3 = H$, $R^5 = OH$
6 $R^1 = R^2 = Ac$, $R^3 = R^4 = H$, $R^5 = OH$
7 $R^1 = R^3 = H$, $R^2 = R^4 = Ac$, $R^5 = OH$
8 $R^1 = R^2 = Ac$, $R^3 = R^4 = H$, $R^5 = OAc$
10 $R^1 = R^2 = R^4 = Ac$, $R^3 = R^5 = H$
11 $R^1 = R^2 = R^3 = R^5 = H$, $R^4 = Ac$
19 $R^1 = R^3 = R^5 = H$, $R^2 = R^4 = Ac$



3 $R^1 = Ac$, $R^2 = R^3 = H$
4 $R^1 = H$, $R^2 = R^3 = Ac$
12 $R^1 = R^3 = Ac$, $R^2 = H$
13 $R^1 = Ac$, $R^2 = Bz$, $R^3 = H$
20 $R^1 = R^2 = Ac$, $R^3 = H$
21 $R^1 = R^2 = R^3 = Ac$
22 $R^1 = R^3 = Ac$, $R^2 = Bz$



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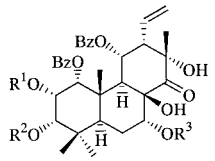

14 $R^1 = R^2 = R^3 = Ac$
15 $R^1 = R^2 = Ac$, $R^3 = H$
16 $R^1 = R^3 = Ac$, $R^2 = H$
17 $R^1 = H$, $R^2 = R^3 = Ac$

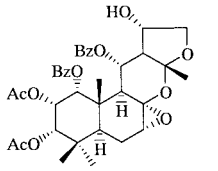

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Chart 1

the lack of one of the two acetyl groups in **19**. Analysis of the ^1H–^1H shift correlation spectroscopy (COSY) and heteronuclear multiple-quantum coherence (HMQC) spectra indicated a high-field shift of H-7 (**1**, δ_H 4.29; **19**, δ_H 5.44). Thus, **1** was assumed to be 7-*O*-deacetylorthosiphol B.

The planar structure of **1** was determined on the basis of the significant heteronuclear multiple-bond correlation (HMBC) spectrum depicted in Fig. 1a. The location of an acetyl substituent at C-3 and two benzoyl substituents at C-1 and C-11 were elucidated from the HMBC correlations between the ester carbonyl carbon at δ_C 170.9 (3-OCO) and the protons at δ_H 1.41 (3-$OCOCH_3$) and δ_H 5.01 (H-3), between the ester carbonyl carbon at δ_C 167.9 (C-7′) and the protons at δ_H 7.73 (H-2′,6′) and δ_H 5.03 (H-1), and between the ester carbonyl carbon at δ_C 166.2 (C-7″) and the protons at δ_H 7.53 (H-2″,6″) and δ_H 5.88 (H-11), respectively.

The relative stereochemistry of **1** was assigned on the basis of the rotating-frame Overhauser enhancement spectroscopy (ROESY) correlations and the coupling constant data. The ROESY correlations H-1/H-2, H-2/H-3, H-2/H_3-19, H-2/H_3-20, H-3/H_3-19, H_3-19/H_3-20, H-5/H-6α, H-5/H-9, H-5/H_3-18, H-6β/H_3-20, and H-6β/H-7 indicated rings A and B to have a chair conformation (Fig. 1b) with *trans*-fused ring junctions and β-axial orientation of H-2. On the other hand, small coupling constants for H-1 (J=3.4 Hz), H-2 (br s), and H-3 (br s) indicated the benzoyloxy, hydroxy, and acetoxy substituents at C-1, C-2, and C-3 to be α-equatorially oriented. Similarly, a small coupling constant observed for H-7 (br s) indicated it to be in β-equatorial orientation. As for ring C, the ROESY correlations H-1/H-11, H-9/H-11, H_3-20/H-11, H-11/H-12α, H-11/H-12β, and H-12β/H_3-17 indicated the boat conformation of ring C and a small coupling constant between H-9 and H-11 (J=5.1 Hz) indicated the β-equatorial orientation of H-11. This is also supported by the absence of *trans*-diaxial coupling between H-11 and H-12α. The significant ROESY correlations between H-12β and H_3-17 indicated that the vinyl group at C-13 should be α-oriented.

The absolute configuration of **1** was established by application of the exciton chirality method.[23,24] In the circular dichroism (CD) spectrum (Fig. 2) of **1**, a positive maximum ($[\theta]_{238}$ +44590) and a negative maximum ($[\theta]_{213}$ −11390) due to two chromophoric benzoates at C-1 and C-11 were observed indicating that the two benzoyloxy groups have a clockwise relationship.[4,6] Thus, compound **1** was concluded to be 7-*O*-deacetylorthosiphol B.

The HR-FAB-MS of **2** showed the quasimolecular ion at *m/z* 693.2941 $(M+H)^+$, consistent with the molecular formula $C_{38}H_{44}O_{12}$. The IR spectrum of **2** showed absorptions of hydroxyl, ester carbonyl, and phenyl groups. The ^1H- and ^{13}C-NMR spectra of **2** (Tables 1, 2) also resembled those of orthosiphol B (**19**), but they showed the presence of one more oxymethine (δ_H 4.60, δ_C 69.4) and the lack of methylene signals assigned to H_2-6 in **19** (δ_H 2.01, 2.15; δ_C 21.5), indicating the presence of a hydroxyl group at C-6 in **2**. This and the planar structure of **2** were confirmed by the COSY, HMQC, and HMBC data. The significant correlations observed in the ROESY spectrum were similar to **1**, which indicated that rings A and B have a chair conformation and that ring C has a boat conformation, as in **1** and **19**. The broad singlet nature of H-6 indicated the C-6 hydroxyl group was β-oriented. The low-field resonances of H_3-19 (δ 1.47) and H_3-20 (δ 1.78) may be due to the anisotropic effect of 6β-OH. A positive cotton effect ($[\theta]_{238}$ +62925, $[\theta]_{221}$ −10543) observed in the CD spectrum (Fig. 2) revealed the absolute stereostructure of **2** to be the same as **1**. From these data, compound **2** was concluded to be 6-hydroxyorthosiphol B.

The ^1H- and ^{13}C-NMR spectra of compound **3** also closely resembled those of orthosiphol I (**20**) obtained from Vietnamese *O. stamineus*,[13] but they were characterized by the disappearance of signals of one of two acetyl groups in **20**. The location of deacetylation was determined to be at C-3 based on the high-field shift of H-3 (**3**: δ_H 3.56; **20**: δ_H 5.08), as indicated by the COSY and HMQC spectra. Thus, com-

Table 1. ^{1}H-NMR Data for Compounds **1**—**9** in $CDCl_3$, 400 MHz (*J* Values (Hz) in Parentheses)

Position	**1**	**2**	**3**	**4**
1	5.03 d (3.4)	5.01 d (2.7)	6.44 d (3.0)	6.37 d (2.9)
2	4.42 br s	4.60 t (2.7)	5.55 t (3.0)	4.53 t (2.9)
3	5.01 br s	4.95 d (2.7)	3.56 br s	5.08 d (2.9)
5	2.63 dd (15.0, 2.2)	2.30 d (3.9)	2.46 dd (13.5, 2)	2.22 dd (11.3, 4.3)
6	2.01 m	4.60 br s	2.08 m	1.98 m
	1.92 dd (6.6, 2.2)		1.83 m	
7	4.29 br s	5.49 d (3.9)	4.26 br s	5.38 t (4.3)
9	3.27 d (5.1)	3.27 d (5.1)	3.45 s	3.65 s
11	5.88 t (5.1)	6.00 t (5.1)		
12	2.70 dd (15.4, 5.1)	2.66 dd (15.9, 5.1)	2.72 d (18.1)	2.69 d (4.7)
	2.00 m	1.96 dd (15.9, 5.1)	2.61 d (18.1)	2.69 d (4.7)
15	5.78 dd (17.5, 10.9)	5.76 dd (17.4, 11.0)	5.31 dd (17.3, 10.5)	5.30 dd (17.5, 10.5)
16	4.95 d (17.5)	4.87 d (17.4)	4.63 d (17.3)	4.74 d (17.5)
	4.82 (10.9)	4.77 d (11.0)	4.10 d (10.5)	4.21 d (10.5)
17	1.21 s	1.19 s	1.13 s	1.13 s
18	0.97 s	0.98 s	1.03 s	0.89 s
19	1.09 s	1.47 s	1.13 s	1.07 s
20	1.49 s	1.78 s	1.40 s	1.40 s
1-OBz				
2′,6′	7.73 d (7.5)	7.77 dd (7.6, 1.2)	8.01 d (7.6)	8.09 d (7.1)
3′,5′	7.35 t (7.5)	7.36 t (7.6)	7.44 t (7.6)	7.43 t (7.1)
4′	7.56 t (7.5)	7.61 tt (7.6, 1.2)	7.56 t (7.6)	7.59 t (7.1)
11-OBz				
2″,6″	7.53 d (7.5)	7.58 dd (7.4, 1.2)		
3″,5″	7.09 t (7.5)	7.15 t (7.4)		
4″	7.42 t (7.5)	7.47 tt (7.4, 1.2)		
2-OAc				
$COCH_3$			2.05 s	
3-OAc				
$COCH_3$	1.41 s	1.43 s		1.71 s
7-OAc				
$COCH_3$		2.33 s		2.08 s

Position	**5**	**6**	**7**	**8**	**9**
1	5.47 d (2.9)	5.76 d (2.9)	5.49 d (3.2)	5.66 br s	5.70 br s
2	5.49 t (2.9)	5.49 t (2.9)	4.49 t (3.2)	5.41 t (3.0)	5.51 t (3.4)
3	5.03 d (2.9)	5.04 d (2.9)	5.03 d (3.2)	4.94 d (3.0)	5.03 d (3.4)
5	2.60 dd (13.5, 2.2)	2.82 dd (15.2, 2.8)	2.53 d (12.4)	2.76 dd	2.63 d (12.4)
6	2.34 d (13.5)	2.22 m	2.22 m	1.91 m	2.23 dd (12.4, 4.5)
	2.01 m	1.90 m	2.01 d (12.4)	1.80 m	2.00 m
7	5.51 br s	4.31 br s	5.51 br s	3.56 br s	5.51 br s
9	3.35 d (5.6)	3.26 d (2.7)	3.44 d (5.6)	3.10 d (3.9)	3.14 d (2.6)
11	6.04 t (5.6)	5.82 t (2.7)	6.13 t (5.6)	5.86 t (3.9)	5.83 t (2.6)
12	2.72 dd (15.6, 5.6)	2.86 dd (16.2, 2.7)	2.73 dd (15.6, 5.6)	2.73 dd (11.5, 3.9)	2.74 dd (12.6, 2.6)
	2.05 d (15.6)	2.40 dd (16.2, 2.7)	2.06 d (15.6)	2.14 d (11.5)	2.65 dd (12.6, 2.6)
15	5.66 dd (17.5, 10.9)	5.86 dd (16.6, 10.8)	5.70 dd (17.5, 10.6)	5.71 dd (17.6, 10.7)	9.34 s
16	4.83 (17.5)	4.86 d (16.6)	4.90 d (17.5)	4.80 d (17.6)	
	4.60 (10.9)	4.58 d (10.8)	4.66 d (10.6)	4.55 d (10.7)	
17	1.22 s	1.39 s	1.24 s	1.88 s	1.33 s
18	0.94 s	1.03 s	0.92s	0.95 s	0.94 s
19	1.20 s	1.09 s	1.13 s	1.08 s	1.09 s
20	4.45 d (12.2)	4.31 d (12.1)	4.48 d (12.0)	5.01 d (12.7)	4.32 d (12.0)
	4.22 d (12.2)	4.19 d (12.1)	4.25 d (12.0)	4.58 d (12.7)	4.21 d (12.0)
1-OBz					
2′,6′	7.71 d (7.1)	7.63 d (7.3)	7.25 d (7.4)	7.59 d (7.8)	7.59 d (7.5)
3′,5′	7.28 t (7.1)	7.11 t (7.3)	7.26 t (7.4)	7.13 t (7.8)	7.11 t (7.5)
4′	7.51 t (7.1)	7.36 t (7.3)	7.52 t (7.4)	7.35 dt (7.8, 1.2)	7.4 t (7.5)
11-OBz					
2″,6″	7.63 d (7.5)	7.41 d (7.3)	7.53 d (7.6)	7.40 dd (7.6, 1.0)	7.33 d (7.5)
3″,5″	7.12 t (7.5)	6.96 t (7.3)	7.13 t (7.6)	6.95 t (7.6)	6.97 t (7.5)
4″	7.40 t (7.5)	7.25 t (7.3)	7.43 t (7.6)	7.25 dt (7.6, 1.0)	7.27 t (7.5)
2-OAc					
$COCH_3$	1.84 s	1.85 s		1.75 s	1.87 s
3-OAc					
$COCH_3$	1.51 s	1.57 s	1.52 s	1.45 s	1.54 s
7-OAc					
$COCH_3$	2.22 s		2.22 s		2.27 s
20-OAc					
$COCH_3$				2.20 s	

Table 2. ^{13}C-NMR Data for Compounds **1**—**9** in $CDCl_3$

Position	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	78.4	79.3	75.3	77.1	69.8	69.3	74.4	68.5	69.1
2	66.2	65.8	67.4	65.5	67.6	67.3	66.6	67.0	67.0
3	78.1	79.2	77.6	78.0	75.8	75.7	78.1	75.7	75.6
4	37.3	37.9	38.2	36.9	37.1	37.5	37.0	35.8	37.4
5	35.3	39.9	33.5	36.1	36.6	36.3	36.7	37.0	37.5
6	23.6	69.4	21.0	21.2	21.7	23.5	21.6	23.3	22.0
7	69.2	70.3	69.1	71.2	70.7	68.6	70.7	68.4	69.4
8	77.5	77.2	78.0	76.3	74.0	76.6	74.1	77.7	74.8
9	40.9	42.0	51.0	51.9	43.2	44.6	43.0	42.5	45.1
10	44.1	43.0	42.7	43.0	48.4	49.3	48.7	47.1	49.0
11	69.0	68.9	205.8	205.6	69.5	70.3	70.1	69.6	69.0
12	40.0	39.5	47.1	47.3	38.6	37.3	38.5	38.2	32.8
13	48.0	47.8	49.5	49.4	47.3	47.3	47.3	47.8	56.3
14	212.6	205.7	211.0	208.0	207.2	215.3	207.3	214.0	204.9
15	141.6	142.0	38.8	138.4	142.3	142.5	142.2	141.9	199.0
16	114.0	113.5	116.2	116.6	112.9	112.7	113.2	113.2	
17	26.5	26.3	25.1	25.1	27.4	29.2	27.4	28.1	23.9
18	27.7	28.0	28.7	28.0	28.0	27.5	27.9	27.9	27.6
19	22.6	24.9	22.0	22.1	21.7	21.9	22.0	21.9	21.8
20	16.6	19.1	16.3	16.3	61.9	62.6	61.9	63.6	62.7
1-OBz									
1′	130.3	130.2	129.9	130.0	130.1	129.5	129.8	130.0	129.5
2′,6′	130.1	129.9	129.8	129.9	129.6	129.6	129.7	129.6	129.3
3′,5′	128.2	28.1	128.6	12.2	127.9	128.0	128.0	127.9	127.9
4′	133.2	133.5	133.1	133.4	132.8	132.7	133.2	132.5	132.9
7′	167.9	167.4	164.2	166.5	164.1	165.2	166.9	164.3	165.3
11-OBz									
1″	129.8	129.9			130.0	129.5	129.6	129.9	129.2
2″,6″	129.6	129.6			129.5	129.4	129.5	129.4	129.1
3″,5″	128.0	128.1			127.7	127.5	127.9	127.5	127.8
4″	132.6	132.7			132.4	132.1	132.6	132.0	132.4
7″	166.2	166.0			166.4	165.9	166.0	165.7	164.6
2-OAc									
CO$\underline{C}$H$_3$			23.7		20.7	20.7		20.6	20.7
$\underline{C}$OCH$_3$			170.0		170.6	170.8		169.9	170.5
3-OAc									
CO$\underline{C}$H$_3$	20.4	20.4		20.8	20.3	20.6	21.1	20.4	20.5
$\underline{C}$OCH$_3$	170.9	170.5		171.0	170.4	170.6	171.0	170.7	170.3
7-OAc									
CO$\underline{C}$H$_3$		20.9		20.8	21.1		20.4		21.3
$\underline{C}$OCH$_3$		168.5		168.3	169.0		169.0		169.3
20-OAc									
CO$\underline{C}$H$_3$								21.1	
$\underline{C}$OCH$_3$								171.4	

pound **3** was assumed to be 3-*O*-deacetylorthosiphol I, which was confirmed by the COSY, HMQC, HMBC, and ROESY spectra. The absolute stereochemistry of **3** was determined by comparing its CD spectrum (Fig. 2) with that of orthosiphonone A (**22**), of which absolute stereochemistry was determined by Shibuya *et al.*[7]

Compound **4** showed the quasimolecular ion at *m/z* 571.2507 $(M+H)^+$ in HR-FAB-MS, which corresponded to the molecular formula $C_{31}H_{38}O_{10}$. The IR spectrum of **4** showed absorptions of hydroxyl (3450 cm^{-1}), ester carbonyl (1725 cm^{-1}), and phenyl (1605, 1455 cm^{-1}) groups. The ^{1}H-NMR spectrum of **4** displayed signals due to four tertiary methyls, a vinyl, and five oxygen-substituted and two aliphatic methines, together with those of an acetyl and two benzoyl groups (Table 1). Its ^{13}C-NMR spectrum, on the other hand, revealed the signals of two ketone and two ester carbonyl carbons, five oxygen-substituted carbons, and three oxygen-nonsubstituted quaternary carbons (Table 2). These ^{1}H- and ^{13}C-NMR data were similar to those of orthosiphol J (**21**) isolated from *O. stamineus* of Vietnam.[13] However, they were characterized by the disappearance of signals due to one of three acetyl groups in **21**. Analysis of the COSY and HMQC spectra showed a high-field shift of H-2 (**4**, δ_H 4.53; **21**, δ_H 5.60), indicating the location of deacetylation to be at C-2. The coupling constants of each proton and the ROESY correlations H-1/H-2, H-1/H$_3$-20, H-1/H-9, H-2/H-3, H-2/H$_3$-19, H-2/H$_3$-20, H-5/H-9, H$_3$-19/H-6β, H$_3$-19/H$_3$-20, H$_3$-20/H-6β, H-12α/H$_2$-16, and H-12β/H$_3$-17 indicated the rings A—C all to have the chair conformation (Fig. 3b). The absolute stereostructure of **4** was determined by comparing the CD data with **3** and **22** (Fig. 2). From these data, compound **4** was concluded to be 2-*O*-deacetylorthosiphol J.

Siphonol A (**5**) was obtained as a colorless amorphous solid and showed the quasimolecular ion at *m/z* 735.3030 $(M+H)^+$ in HR-FAB-MS, which corresponds to the molecular formula $C_{40}H_{46}O_{13}$. IR spectrum of **5** was similar to that of orthosiphol H (**10**), a known compound isolated from the same extract, and showed absorptions of hydroxyl, ester car-

bonyl, and phenyl groups. The ^{1}H-NMR spectrum of **5** displayed a unique feature consisting of only three tertiary methyl signals, a vinylic and five oxygen-substituted methines, an oxygen-substituted methylene, and two methylenes, together with those of three acetyl and two benzoyl groups (Table 1). Except for the difference in the disappearance of one tertiary methyl signal with the presence of one more oxymethylene signal, the remaining data were similar to those of orthosiphol H (**10**). Thus, one of the four tertiary methyls in **10** was assumed to be oxygenated, and the position of oxygenation was determined to be C-20 based on the significant correlations H-1/C-20, H_2-20/C-1, H-5/C-20, H_2-20/C-5 and H-9/C-20 observed in the HMBC spectrum (Fig. 4a). The locations of two benzoyl and three acetyl substituents were determined to be the same as those in **10** based on the HMBC correlations between ester carbonyl carbons of the substituents and the respective oxymethine protons (Fig. 4a).

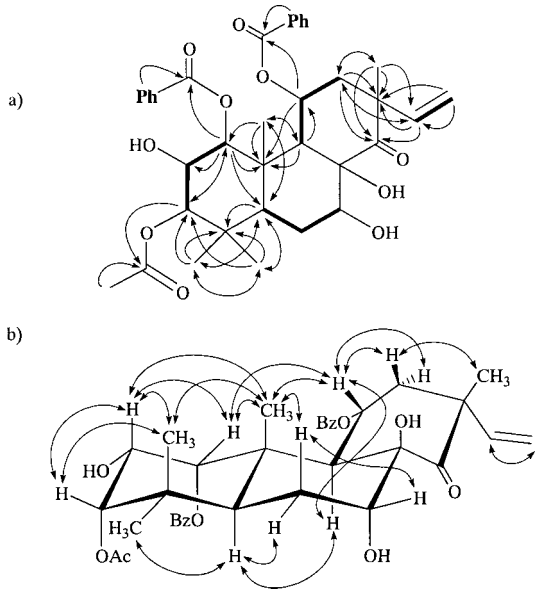

Fig. 1. (a) Connectivities (Bold Line) Deduced by the COSY and HMQC Spectra and Significant HMBC Correlations (Arrow) and (b) ROESY Correlations Observed for **1**

The relative stereochemistry of **5** was assigned on the basis of the ROESY correlations and the coupling constant data. The ROESY correlations H-2/H-3, H-2/H_3-19, H-2/H_2-20, H-3/H_3-19, H_3-19/H_2-20, and H-5/H-9 indicated rings A and B to have a chair conformation (Fig. 4b), while the ROESY correlations H-1/H-11, H_2-20/H-11, H-11/H-12β, and H-12β/H_3-17 and the small coupling constant between H-9 and H-11 (J=5.6 Hz) indicated a boat conformation of ring C and a gauche relationship between H-11 and H-9. In the CD spectrum, a positive maximum ($[\theta]_{241}$ +29815) due to two chromophoric benzoates at C-1 and C-11 revealed its absolute stereostructure. Thus, orthosiphonol A was concluded to be 20-hydroxyorthosiphol H (**5**).

Siphonols B (**6**) and C (**7**) were obtained as colorless amorphous solids and their molecular formulas were determined to be the same ($C_{38}H_{44}O_{12}$, 692) by HR-FAB-MS spectra. The ^{1}H- and ^{13}C-NMR spectra of **6** and **7** were similar to each other and showed the signals of a vinyl, five oxymethines, three methylene, and three methyls, together with those of two benzoyl and two acetyl groups. These data closely resembled those of siphonol A (**5**), but they were characterized by the disappearance of signals due to one of three acetyl groups in **5**. The location of the deacetylation was determined to be at C-7 for **6** and C-2 for **7** based on the upfield shift of H-7 (**6**: δ_H 4.31; **5**: δ_H 5.51) and H-2 (**7**: δ_H 4.49; **5**: δ_H 5.49), respectively. In both **6** and **7**, the ROESY correlations of H-2 with H-1, H-3, H_3-19, and H_2-20, of H-11 with H_2-20, and of H-5 with H-9 and the small coupling constant value of H-9/H-11 (**6**, J=2.7 Hz; **7**, J=5.6 Hz) indicated the stereochemistry of **6** and **7** to be the same as **5**, *i.e.* chair conformation in rings A and B and boat conformation in ring C. The CD data for **6** and **7** indicated that they have the same absolute stereostructures as **1**. Thus, siphonols B and C were concluded to be 7-*O*-deacetylsiphonol A (**6**) and 2-*O*-deacetylsiphonol A (**7**), respectively.

The HR-FAB-MS of siphonol D (**8**) showed a quasimolec-

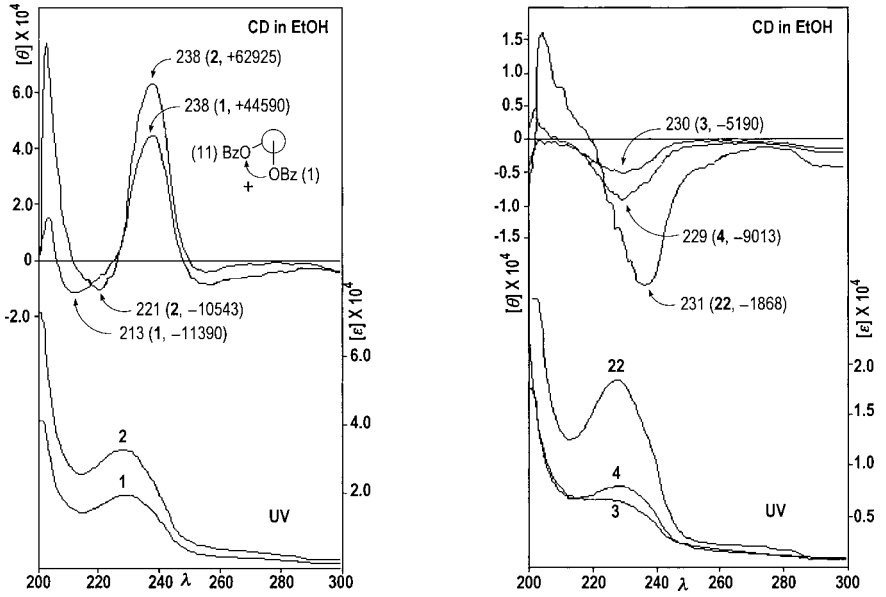

Fig. 2. CD and UV Spectra of **1**—**4** and **22** in EtOH

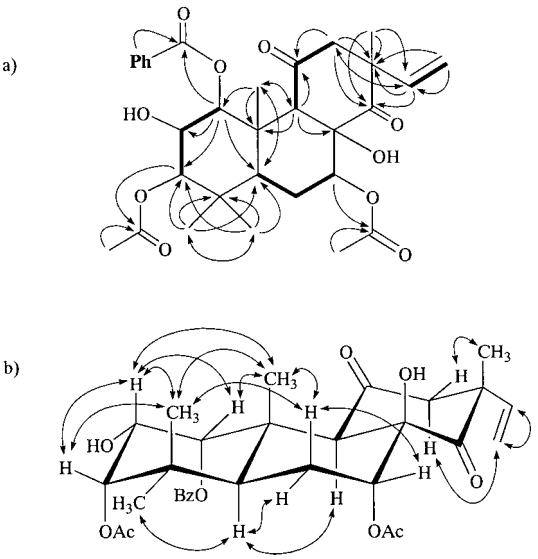

Fig. 3. (a) Connectivities (Bold Line) Deduced by the COSY and HMQC Spectra and Significant HMBC Correlations (Arrow) and (b) ROESY Correlations Observed for **4**

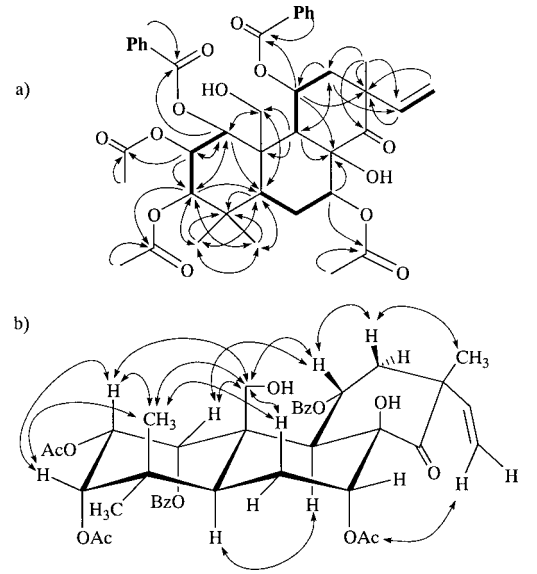

Fig. 4. (a) Connectivities (Bold Line) Deduced by the COSY and HMQC Spectra and Significant HMBC Correlations (Arrow) and (b) ROESY Correlations Observed for **5**

ular ion at *m*/*z* 735.3030 $(M+H)^+$ indicating its molecular formula ($C_{40}H_{46}O_{13}$) to be the same as siphonol A (**5**). The ^{1}H-NMR data of **8** were also similar to **5** and displayed signals due to three tertiary methyls, five oxygen-substituted methines, an oxygen-substituted methylene, and two oxygen-nonsubstituted methylenes, together with those of three acetyl and two benzoyl groups (Table 1). However, its ^{1}H-NMR spectrum showed an upfield shift of H-7 (δ_H 3.56) and a downfield shift of H_2-20 (δ_H 5.01, 4.58). These data suggested that the acetoxyl group at C-7 in **5** should be replaced by a hydroxyl group in **8**, and the hydroxyl group at C-20 in **5** should be replaced by an acetoxyl group in **8**. This was confirmed by the HMBC correlations between the ester carbonyl carbon at δ_C 171.4 (20-OCO) and the protons at δ_H 2.20 (20-OCOC$\underline{H}_3$) and δ_H 5.01 and 4.58 (H_2-20). The relative stereochemistry was assigned by the ROESY experiment to be the same as that of **1** and the absolute stereostructure was confirmed by the CD data.

Siphonol E (**9**) was obtained as a colorless amorphous solid. The HR-FAB-MS showed a quasimolecular ion at *m*/*z* 737.2818 $(M+H)^+$, consistent with the molecular formula $C_{39}H_{44}O_{14}$. The IR spectrum of **9** showed absorptions due to hydroxyl (3400 cm^{-1}), aldehyde (2850, 2750 cm^{-1}), ester carbonyl (1725 cm^{-1}), and phenyl (1605, 1455 cm^{-1}) groups. The ^{1}H-NMR spectrum of **9** displayed signals due to an aldehyde proton (δ_H 9.34), three tertiary methyls, five oxygen-substituted and two aliphatic methines, and one oxygen-substituted methylene, together with those of three acetyl and two benzoyl groups (Table 1), while its ^{13}C-NMR spectrum revealed the signals of a ketone carbonyl, an aldehyde carbonyl, five ester carbonyls, seven oxygen-substituted carbons, and two quaternary carbons (Table 2). Excluding the ^{13}C-NMR signals for two benzoyl and three acetyl groups, **9** possessed only 19 carbon signals in its main carbon framework, suggesting it to be a norditerpene.

The partial connectivities between C_1–C_2–C_3, C_5–C_6–C_7 and C_9–C_{11}–C_{12} were obtained by the analysis of the COSY and HMQC spectra, and these were connected from the long-range correlations observed in the HMBC spectrum. Significant long-range correlations between the aldehyde carbon (δ_C 199.0) with H_3-17 and H_2-12 confirmed the aldehyde group to be C-15. On the other hand, the locations of the two benzoyl and three acetyl groups were determined to be at C-1 and C-11 and at C-2, C-3, and C-7, respectively, based on the HMBC correlations between the ester carbonyl carbon at δ_C 165.3 (1-OCO) and the protons at δ_H 5.70 (H-1) and 7.59 (H-2′,6′), between the ester carbonyl carbon at δ_C 170.5 (2-OCO) and the protons at δ_H 5.51 (H-2) and 1.87 (2-OCOCH_3), between the ester carbonyl carbon at δ_C 170.3 (3-OCO) and the protons at δ_H 5.03 (H-3) and 1.54 (3-OCOCH_3), between the ester carbonyl carbon at δ_C 169.3 (7-OCO) and the protons at δ_H 5.51 (H-7) and 2.27 (7-OCOCH_3) and between the ester carbonyl carbon at δ_C 164.6 (11-OCO) and the protons at δ_H 5.83 (H-11) and 7.33 (H-2″,6″). The relative stereochemistry of **9** was determined to be the same as **5**, on the basis of the ROESY correlations and the coupling constant data and the absolute stereochemistry was established by the CD spectrum.

In this paper, we have reported nine new diterpenes, 7-*O*-deacetylorthosiphol B (**1**), 6-hydroxyorthosiphol B (**2**), 3-*O*-deacetylorthosiphol I (**3**), 2-*O*-deacetylorthosiphol J (**4**), and siphonols A—E (**5**—**9**), together with nine known diterpenes, orthosiphols H (**10**), K (**11**), M (**12**), and N (**13**), staminols A (**14**) and B (**15**), neoorthosiphols A (**16**) and B (**17**), and norstaminol A (**18**). Except for **3**, all the new diterpenes (**1**, **2**, **4**—**6**) possessed a benzoyl substituent at C-1 and an acetyl substituent at C-3, respectively. Interestingly, the ^{1}H chemical shifts for acetyl methyl at C-3 appeared at higher field than the usual methyl protons, which may be due to orientation of the 3-OCOC$\underline{H}_3$ group to lie in the shielding zone of the benzene ring of 1-OBz. Among the new diterpenes, siphonols A—E (**6**—**9**) represent the first examples of isopimarane-type diterpenes oxygenated at C-20. Siphonol E (**9**) is a biogenetically interesting norisopimarane-type diterpene having an aldehyde functional group, which might have been produced by the oxidative cleavage of the vinylic group in **1**,

Table 3. Inhibitory Effects of Diterpenenes on NO Production in LPS-Activated Macrophage-Like J774.1 Cells

Compound	IC_{50} (μM)[a]
1	102
3	66.3
4	24.1
5	10.8
6	17.3
7	22.9
8	46.5
9	23.0
10	24.1
11	27.3
12	>200
13	35.9
14	25.5
15	67.9
16	40.7
17	14.0
18	44.4
L-NMMA	26.0
Polymixin B (μg/ml)	27.8
Dexamethasone	170

a) IC_{50} values were calculated from the mean of data of four determinations.

and thus represent a new carbon skeleton.

All the isolated compounds, except for **2**, were tested for their inhibitory activities against NO production in LPS-activated macrophage-like J774.1 cells. All of them displayed significant dose-dependent inhibition, and the activities of **1**, **3**—**7**, **9**, **10**, **14**, and **17** were more potent than the positive controls N^G-monomethyl-L-arginine (L-NMMA), polymixin B, and dexamethasone. Among the isolated compounds, siphonol A (**5**) displayed the most potent activity with an IC_{50} value of 10.8 μM (Table 4). The diterpenes isolated from this plant species have been shown to exhibit a suppressive effect on contractile responses in rat thoracic aorta[6,8] and inhibitory activity against the inflammation induced by a tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) on mouse ears.[4] The NO inhibitory activity in endotoxin-activated macrophages by the diterpenes further verifies the anti-inflammatory utility of *O. stamineus*.

Experimental

General Methods Optical rotations were recorded on a JASCO DIP-140 digital polarimeter. IR spectra were measured with a Shimadzu IR-408 spectrophotometer in $CHCl_3$ solutions. NMR spectra were taken on a JEOL JNM-LA400 spectrometer with tetramethylsilane (TMS) as an internal standard, and chemical shifts are expressed in δ values. HR-FAB-MS measurements were carried out on a JEOL JMS-700T spectrometer and glycerol was used as matrix. CD spectra were measured in a JASCO J-805 spectropolarimeter. Column chromatography was performed with BW-820MH silica gel (Fuji Silysia, Aichi, Japan). Analytical and preparative TLC were carried out on precoated silica gel plates (Merck, 0.25 or 0.50 mm thickness).

Plant Material The aerial parts of cultivated *O. stamineus* BENTH. were collected at Bangdung, Indonesia in August, 2000. A voucher sample (TMPW 20628) is preserved in the Museum for Materia Medica, Analytical Research Center for Ethnomedicines, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan.

Extraction and Isolation Air-dried aerial parts of *O. stamineus* (2.8 kg) were extracted with MeOH (6 l, reflux, 3 h×3). The MeOH extract (200 g) was suspended in H_2O (1 l) and partitioned successively with hexane, $CHCl_3$, and EtOAc (each 1 l×3) to yield hexane (25 g), $CHCl_3$ (90 g), EtOAc (15 g), and H_2O (68 g) fractions, respectively. The $CHCl_3$ fraction (87 g) was chromatographed (8×46 cm) with an EtOAc–hexane solvent system to give six fractions [Fr. 1: EtOAc–hexane (1 : 4) eluate, 18.8 g; Fr. 2: EtOAc–hexane (1 : 3) eluate, 9.1 g; Fr. 3: EtOAc–hexane (1 : 2) eluate, 12.3 g; Fr. 4: EtOAc–hexane (1 : 1) eluate, 7.0 g; Fr. 5: EtOAc–hexane (3 : 2) eluate, 10.7 g; Fr. 6: EtOAc–hexane (3 : 1) eluate, 21.2 g].

Fraction 4 (6 g) was rechromatographed (5×45 cm) with MeOH–$CHCl_3$ (1%→5%) to afford four subfractions (fr. 4-1, 2.1 g; fr. 4-2, 0.5 g; fr. 4-3, 1.8 g; fr. 4-4, 0.5 g). Subfraction 4-1 was rechromatographed with 2.5% MeOH–$CHCl_3$, followed by preparative TLC with 15% acetone–benzene, to give 7-*O*-deacetylorthosiphol B (**1**, 8.0 mg), siphonol D (**8**, 15 mg), orthosiphols K[16] (**11**, 10 mg) and N[16] (**13**, 24 mg), staminol A[13] (**14**, 7.0 mg), and neoorthosiphol A[6] (**16**, 5.0 mg). Subfraction 4-2 was rechromatographed with 2.5% MeOH–$CHCl_3$, followed by preparative TLC with hexane–EtOAc (2 : 1), to give **16** (10 mg). Subfraction 4-3 was rechromatographed with 2.5% MeOH–$CHCl_3$, followed by preparative TLC with hexane–EtOAc (2 : 1), to give 6-hydroxyorthosiphol B (**2**, 0.5 mg), 3-*O*-deacetylorthosiphol I (**3**, 4.0 mg), 2-*O*-deacetylorthosiphol J (**4**, 3.0 mg), siphonol B (**6**, 10 mg), siphonol C (**7**, 10 mg), siphonol D (**8**, 14 mg), siphonol E (**9**, 1.4 mg), and neoorthosiphol B[6] (**17**, 5.0 mg). Subfraction 4-4 was rechromatographed with 2.5% MeOH–$CHCl_3$, followed by preparative TLC with 15% acetone–benzene, to give siphonol A (**5**, 8.0 mg), **7** (11 mg), **8** (4.0 mg), **11** (7.0 mg), **17** (4.0 mg), and staminol B[13] (**15**, 8.0 mg).

Fraction 5 (8.5 g) was rechromatographed (5×45 cm) with hexane–EtOAc (2 : 1) to afford three subfractions (fr. 5-1, 1.5 g; fr. 5-2, 3.3 g; fr. 5-3, 2.7 g). Subfraction 5-1 was rechromatographed with 2.5% MeOH–$CHCl_3$, followed by preparative TLC with hexane–EtOAc (2 : 1), to give **5** (4.0 mg), **10** (7.0 mg), **12** (3.0 mg), **14** (7.0 mg), and **15** (2.0 mg). Subfraction 5-2 was also rechromatographed with 2.5% MeOH–$CHCl_3$, followed by preparative TLC with hexane–EtOAc (2 : 1), to give **5** (8.0 mg), **6** (3.0 mg), **8** (7.0 mg), **16** (13 mg), and **17** (6.0 mg). Subfraction 5-3 was rechromatographed with 2.5% MeOH–$CHCl_3$ followed by preparative TLC with 15% acetone–benzene, to give **5** (9.0 mg), **6** (7.0 mg), **7** (4.0 mg), **8** (11 mg), **14** (2.0 mg), **15** (3.5 mg), **16** (13 mg), **17** (10 mg), and norstaminol A[13] (**18**, 5.0 mg).

7-*O*-Deacetylorthosiphol B (**1**): Colorless amorphous solid, $[\alpha]_D^{25}$ −94.4° (c=0.033, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3450, 1720, 1605, 1590, 1495, 1455, 1370, 1280, 1180, 1120, 1045. CD λ_{max} (EtOH) nm: 238 ([θ] +44590), 213 ([θ] −11390). HR-FAB-MS: 635.2836 [Calcd for $C_{36}H_{43}O_{10}$ $(M+H)^+$, 635.2856]. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

6-Hydroxyorthosiphol B (**2**): Colorless amorphous solid, $[\alpha]_D^{25}$ −53.4° (c=0.027, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3450, 1720, 1605, 1590, 1495, 1455, 1370, 1280, 1180, 1110, 1040. CD λ_{max} (EtOH) nm: 238 ([θ] +62925), 221 ([θ] −10543). HR-FAB-MS: 693.2941 [Calcd for $C_{38}H_{45}O_{12}$ $(M+H)^+$, 693.2911]. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

3-*O*-Deacetylorthosiphol I (**3**): Colorless amorphous solid, $[\alpha]_D^{25}$ −47.8° (c=0.04, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3450, 1720, 1605, 1585, 1510, 1455, 1370, 1315, 1270, 1175, 1120. CD λ_{max} (EtOH) nm: 230 ([θ] −5190). HR-FAB-MS: 529.2437 [Calcd for $C_{29}H_{37}O_9$ $(M+H)^+$, 529.2438]. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

2-*O*-Deacetylorthosiphol J (**4**): Colorless amorphous solid, $[\alpha]_D^{25}$ −48.6° (c=0.044, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3450, 1725, 1605, 1590, 1495, 1455, 1370, 1270, 1180, 1110. CD λ_{max} (EtOH) nm: 229 ([θ] −9013). HR-FAB-MS: 571.2507 [Calcd for $C_{31}H_{39}O_{10}$ $(M+H)^+$, 571.2543]. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Siphonol A (**5**): Colorless amorphous solid, $[\alpha]_D^{25}$ −146.5° (c=0.07, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3450, 1720, 1605, 1590, 1495, 1455, 1370, 1320—1200, 1180, 1110, 1040. CD λ_{max} (EtOH) nm: 230 ([θ] +29815). HR-FAB-MS: 735.3030 [Calcd for $C_{40}H_{47}O_{13}$ $(M+H)^+$, 735.3017]. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Siphonol B (**6**): Colorless amorphous solid, $[\alpha]_D^{25}$ −103.4° (c=0.08, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3450, 1720, 1605, 1590, 1495, 1455, 1370, 1320—1200, 1180, 1110, 1040. CD λ_{max} (EtOH) nm: 230 ([θ] +34704). HR-FAB-MS: 693.2943 [Calcd for $C_{38}H_{45}O_{12}$ $(M+H)^+$, 693.2911]. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Siphonol C (**7**): Colorless amorphous solid, $[\alpha]_D^{25}$ −49.9° (c=0.06, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3400, 1725, 1605, 1590, 1495, 1455, 1370, 1320—1200, 1180, 1110. CD λ_{max} (EtOH) nm: 230 ([θ] +31485). HR-FAB-MS: 693.2875 [Calcd for $C_{38}H_{45}O_{12}$ $(M+H)^+$, 693.2911]. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Siphonol D (**8**): Colorless amorphous solid, $[\alpha]_D^{25}$ −92.8° (c=0.09, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3450, 1720, 1605, 1585, 1510, 1455, 1370, 1315, 1285—1200, 1175, 1120. CD λ_{max} (EtOH) nm: 230 ([θ] +18414). HR-FAB-MS: 735.3030 [Calcd for $C_{40}H_{47}O_{13}$ $(M+H)^+$, 735.3017]. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Siphonol E (**9**): Colorless amorphous solid, $[\alpha]_D^{25}$ −135.7° (c=0.06, $CHCl_3$). IR ($CHCl_3$) cm^{-1}: 3400, 2850, 2750, 1725, 1605, 1590, 1495, 1455, 1370, 1320, 1280, 1240—1210, 1115, 1040, 980. CD λ_{max} (EtOH) nm: 230

([θ] +14249). HR-FAB-MS: 737.2818 [Calcd for $C_{39}H_{45}O_{14}$ $(M+H)^+$, 737.2809]. ^{1}H- and ^{13}C-NMR: Tables 1 and 2.

Nitric Oxide Inhibitory Assay Macrophage-like J774.1 cell line was purchased from Riken Cell Bank (Tsukuba, Japan) and propagated in 75-cm^2 plastic culture flasks (Falcone, Becton Dickinson, NJ, U.S.A.), containing RPMI-1640 medium supplemented with penicillin G (100 units/ml), streptomycin (100 μg/ml), and 10% fetal calf serum. The cells were harvested with trypsin and diluted to a suspension in fresh medium, then seeded in 96-well plastic plates with 1×10^5 cells/well and allowed to adhere for 2 h at 37 °C in a humidified atmosphere containing 5% CO_2. Then the medium was replaced with fresh medium, containing LPS (10 μg/ml) and test compounds at indicated concentrations, and the cells were incubated for 24 h. NO production was determined by measuring the accumulation of nitrite in the culture supernatant with Griess reagent.[25] Briefly, 50 μl of the supernatant from each well of 96-well plates was incubated with an equal volume of Griess reagent (0.5% sulfanilamide and 0.05% naphthylene-diamide dihydrochloride in 2.5% H_3PO_4) and then allowed to stand for 10 min at room temperature. Absorbance at 550 nm was measured using a HTS 7000 microplate reader. The nitrite concentration in the medium was determined from the calibration curve ($Y=0.0038X-0.0043$, $r=0.9998$) obtained by using different concentrations of sodium nitrite ($NaNO_2$) in the culture medium as standard. The blank correction was carried out by subtracting the absorbance due to medium only from the absorbance reading of each well.

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