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Neoorthosiphonone A; a nitric oxide (NO) inhibitory diterpene with new carbon skeleton from *Orthosiphon stamineus*

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Abstract—From the aerial part of *Orthosiphon stamineus* from Hainan island of China, a diterpene named neoorthosiphonone A (**1**), having a novel carbon framework, has been isolated. Neoorthosiphonone A (**1**) possessed a unique unprecedented structural feature of eight-membered ring C in its structure, which may be biogenetically derived from its isopimarane precursor, orthosiphonone A, through the insertion of vinyl group into the C_{13}–C_{14} bond. Neoorthosiphonone A (**1**) displayed potent inhibitory activity on the nitric oxide production in LPS-activated macrophage-like J774.1 cells with an IC_{50} value of 7.08 μM, more potent than the positive control L-NMMA.
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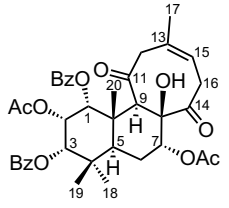
Nitric oxide (NO) is an important signaling molecule that acts in many tissues to regulate a diverse range of physiological processes. When certain cells are activated by specific proinflammatory agents such as endotoxin, tumor necrosis factor (TNF), interferon-gamma (IFN-γ), and interleukin-1 (IL-1), NO is produced by inducible nitric oxide synthase (iNOS) and acts as a host defense by damaging pathogenic DNA and as a regulatory molecule with homeostatic activities.[1] However, excessive production has detrimental effects on many organ systems of the body leading to tissue damage, even leading to a fetal development (septic shock).[2] Therefore, effective inhibition of NO accumulation by inflammatory stimuli presents a beneficial therapeutic strategy.

Orthosiphon stamineus BENTH. [syn.: *O. aristatus* (BL.) MIQ., *O. grandiflorus* BOLD., *O. spicatus* (THUMB.) BAK.; Lamiaceae] is one of the popular traditional folk medicine extensively used in Southeast Asia for the treatment of a wide range of diseases: in Indonesia for rheumatism, diabetes, hypertension, tonsillitis, epilepsy, menstrual disorder, gonorrhea, syphilis, renal calculus, gallstone etc.;[3] in Vietnam for urinary lithiasis, edema, eruptive fever, influenza, hepatitis, jaundice, and biliary lithiasis;[4] and in Myanmar to alleviate diabetes, urinary tract, and renal diseases.[5] While in Okinawa prefecture of Japan, it is consumed as a healthy Java tea to facilitate body detoxification. In our search of biologically active compounds from *O. stamineus*[6–12] recently we found that the methanolic extract of an aerial part of *O. stamineus* collected from Hainan island of China showed significant inhibitory activity on the NO production in lipopolysaccharide (LPS)-activated macrophage-like J774.1 cells (IC_{50}, 35.1 μg/mL). Further separation of the methanolic extract led to the isolation of a highly oxygenated diterpene, neoorthosiphonone A (**1**), having a novel carbon skeleton. In this paper, we wish to report the structure of the novel diterpene as a potent NO inhibitor.



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Neoorthosiphonone A (**1**)[13] was obtained as a colorless amorphous solid and showed $[\alpha]_D^{25}$ −145.5° (*c* 0.5, $CHCl_3$). Its molecular formula was determined by HR-FABMS to be $C_{38}H_{42}O_{11}$ [*m*/*z* 675.2806 $(M+H)^+$]. The IR spectrum of **1** showed the absorptions due to hydroxyl (3450 cm^{-1}), ester carbonyl (1710 cm^{-1}), and

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phenyl (1600, 1450 cm^{-1}) groups. The ^1H NMR spectrum of **1** revealed signals due to four tertiary methyls (δ_H 0.98, 1.23, 1.25, 1.60), four oxygen-substituted methines (δ_H 6.05, 5.71, 5.37, 4.70), and three aliphatic methylenes (δ_H 3.68, 3.25; 2.99, 2.56; 2.18, 1.92), together with those of two acetyl and two benzoyl groups. The ^{13}C NMR spectrum (Table 1) showed the signals of 20 carbons including those of two ketone carbonyls and two sp^2 and five oxygenated sp^3 carbons, together with those of two benzoyl and two acetyl groups. The ^1H–^1H COSY and HMQC spectra revealed the partial connectivities (bold line) of C_1–C_2–C_3, C_5–C_6–C_7, and C_{15}–C_{16}. These partial structures were connected based on the long-range correlations observed in the HMBC spectrum (Fig. 1).

In the HMBC spectrum of **1**, the methyl protons at δ_H 1.60 (H_3-20) showed the long-range correlations with three methine carbons at δ_C 74.5 (C-1), 36.7 (C-5), and 50.9 (C-9) and the quaternary carbon at δ_C 43.4 (C-10), indicating the carbons C-1, C-5, and C-9 and the methyl group (C-20) to be connected with the quaternary carbon C-10. Similarly, the methyl protons at δ_H 0.98 (H_3-18) and 1.23 (H_3-19) both revealed the long-range correlations with the carbons at δ_C 36.7 (C-5), 37.9 (C-4), and 76.3 (C-3). Thus, the carbons C-3, C-5, C-18, and C-19 should be connected with the quaternary carbon C-4. Likewise, long-range correlations of the hydroxyl proton (δ_H 4.12) with the oxygen-substituted quaternary carbon at δ_C 80.0 (C-8), C-9, and a ketone carbonyl at δ_C 203.3 (C-14) and the correlations of the methine proton at δ_H 4.02 (H-9) with C-8, C-14 and the ketone carbonyl at δ_C 214.5 (C-11) and the oxymethine at δ_C 76.7 (C-7) indicated that C-8 should be flanked by C-7, C-14, and C-9. In addition, the methyl protons at δ_H 1.25 (H_3-17) showed correlations with the methylene carbon at δ_C 48.6 (C-12), olefinic quaternary carbon at δ_C 131.6 (C-13) and olefinic methine at δ_C 119.8 (C-15), indicating C-13 to be connected with C-17, C-12, and C-15. Furthermore, long-range correlations were observed between the methylene protons at δ_H 2.99 and 2.56 (H_2-12) and C-11 and between the protons at δ_H 4.60 (H-15), 3.68 and 3.25 (H_2-16) and C-14. Analysis of these data led to the novel framework (Fig. 1a). The locations of two acetoxyl groups were determined to be at C-2 and C-7 based on the correlations between the ester carbonyl carbon at δ_C 170.1 (2-OCO) and the protons at δ_H 1.93 (2-OCOCH_3) and 5.71 (H-2) and between the ester carbonyl carbon at δ_C 167.8 (7-OCO) and the protons at δ_H 2.19 (7-OCOCH_3) and 4.70 (H-7), while the location of two benzoyloxy groups were determined to be at C-1 and C-3 based on the correlations between the ester carbonyl carbon at δ_C 164.7 (1-OCO) and the protons at δ_H 8.12 (H-2′,6′) and 6.05 (H-1) and between the ester carbonyl carbon at δ_C 166.4 (3-OCO) and the protons at δ_H 7.91 (H-2″,6″) and 5.37 (H-3).

Table 1. ^1H NMR and ^{13}C NMR data for neoorthosiphonone A (**1**) in $CDCl_3$ (*J* values in parenthesis)

Position	^1H	^{13}C
1	6.05 d (3.0)	74.5
2	5.71 t (3.0)	65.8
3	5.37 d (3.0)	76.3
4		37.9
5	2.45 dd (13.2, 1.7)	36.7
6	2.18 td (13.2, 2.9) 1.92 m	22.5
7	4.70 t (2.9)	76.7
8		80.0
9	4.02 d (1.5)	50.9
10		43.4
11		214.5
12	2.99 d (13.9) 2.56 d (13.9)	48.6
13		131.6
14		203.3
15	4.60 t (5.1)	119.8
16	3.68 dd (15.8, 5.1)	
	3.25 ddd (15.8, 5.1, 1.7)	43.6
17	1.25 d (1.7)	24.7
18	0.98 s	28.3
19	1.23 s	22.8
20	1.60 s	15.7
8-OH	4.12 d (1.5)	
1-OBz		
1′		130.2
2′, 6′	8.12 dd (8.3, 1.2)	129.8
3′, 5′	7.37 dd (8.3, 1.2)	128.1
4′	7.61 tt (8.3, 1.2)	133.1
*C*O		164.7
3-OBz		
1″		130.1
2″, 6″	7.91 dd (8.3, 1.2)	129.6
3″, 5″	7.09 dd (8.3, 1.2)	128.0
4″	7.40 tt (8.3, 1.2)	132.7
*C*O		166.4
2-CO*CH*$_3$	1.93 s	20.8
*C*O		170.1
7-CO*CH*$_3$	2.19 s	21.1
*C*O		167.8

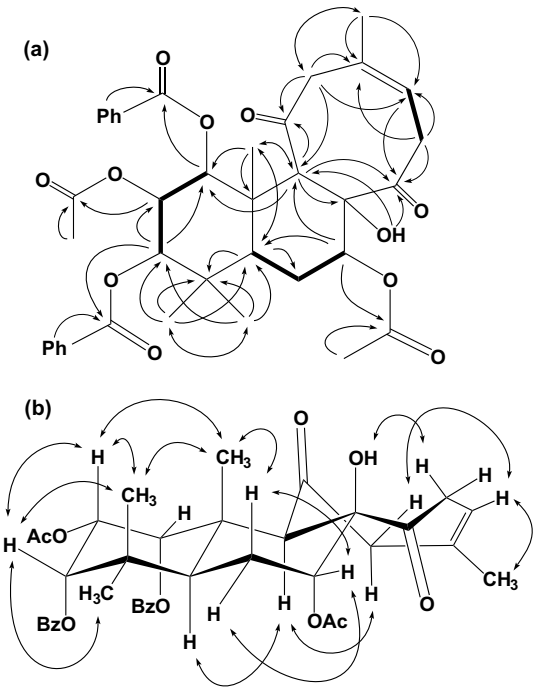
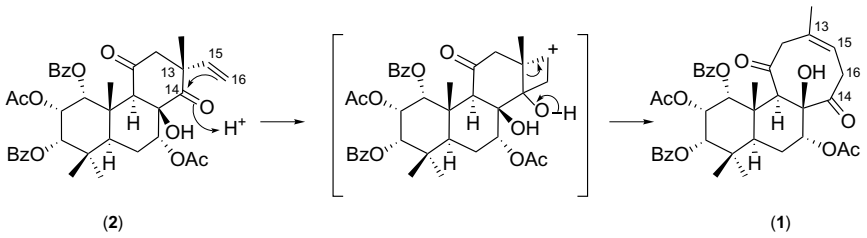


Figure 1. (a) Partial structures (bold line) and key HMBC correlations (arrows) and (b) Key ROESY correlations observed for **1**.



Scheme 1. Possible biogenesis of neorothosiphonone A (**1**) from orthosiphonone A (**2**).

The relative stereochemistry of **1** 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_3-20, H-3/H_3-19, H_3-19/H_3-20, and H_3-20/H-6β 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, a small axial–equitorial coupling constant (3.0 Hz each) for H-1/H-2 and H-2/H-3 indicated two benzoyloxy groups at C-1 and C-3 and an acetoxyl substituent at C-2 to be all α-orientated. The large coupling constant for H-5 ($J_{5,6ax} = 13.2$ Hz) and ROESY correlation H-5/H-9 indicated them to have α-axial orientation, while the small coupling constant for H-7 ($J = 2.9$ Hz) indicated it to be in β-equitorial orientation. The stereochemical correlation between the H-9 and OH-8 was also suggested to be *trans* from the observation of long-range w-type correlations between H-9 and the hydroxyl group at C-8 ($J = 1.5$ Hz). The low-field shift of 20-H_3, which was assumed to be due to an anisotropic effect of OH-8,[14] also supported the 8β-axial-OH orientation. As for ring C, the ROESY correlations H_2-12/H-15, H_2-12/H-9, H_2-16/8β-OH, and H-15/H_3-17 (Figure 1b) indicated the geometry of an olefin at C_{13}–C_{14} to be *Z*. Analysis of these ROESY signals as well as MM2 energy minimized structure indicated that rings A and B have chair conformation and ring C has the conformation shown in Figure 1b.

To the best of our knowledge, neoorthosiphonone A (**1**) represents a biogenetically interesting diterpene with a novel carbon framework, which may be produced by insertion of vinylic group to the C_{13}–C_{14} bond in an isopimarane-type diterpene, orthosiphonone A (**2**),[8,10,15] isolated from the same extract (Scheme 1). The new diterpene **1** was tested for the inhibitory activity on NO production by LPS-activated macrophage-like J774.1 cells.[16] N^G-monomethyl-L-arginine (L-NMMA, a non-selective NOS inhibitor)[17] and caffeic acid phenethyl ester (CAPE, an inhibitor of nuclear factor κB activation)[18] were used as positive control substances. Neoorthosiphonone A (**1**) displayed significant dose-dependent inhibition (Fig. 2) with an IC_{50} value of 7.08 μM, which was more potent than the positive control L-NMMA (IC_{50}, 38.1 μM) and was identical with the positive control CAPE (IC_{50}, 2.24 μM). The significant increase in the activity of neoorthosiphonone A (**1**) compared to orthosiphonone A (**2**) (IC_{50}, 32.1 μM) further indicated the importance of the novel carbon framework for its antiinflammatory actions.

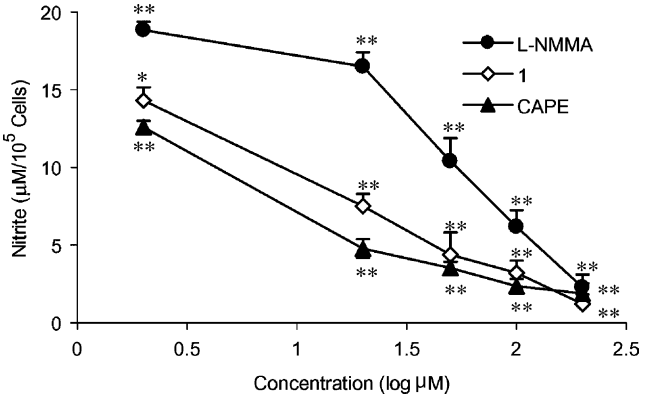


Figure 2. Dose-dependent inhibition of NO production in macrophage-like J774.1 cells by 1. Each value represents the mean ± SD of four determinations. Significantly different from control: $*p < 0.05$, $**p < 0.01$.

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13. Neoorthosiphonone A (**1**). Colorless amorphous solid, $[\alpha]_D^{25}$ −145.5° (*c* 0.5, $CHCl_3$). IR ν_{max} ($CHCl_3$) 3450, 1710, 1600, 1450, 1450, 1370, 1280, 1230–1200, 1160, 1110, 1040 cm^{-1}. HRFABMS 675.2806 [calcd for $C_{38}H_{43}O_{11}$ $(M+H)^+$, 675.2805]. ^1H and ^{13}C NMR, see Table 1.
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