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Aphanamenes A and B, Two New Acyclic Diterpene [4 + 2]-Cycloaddition Adducts from *Aphanamixis grandifolia*

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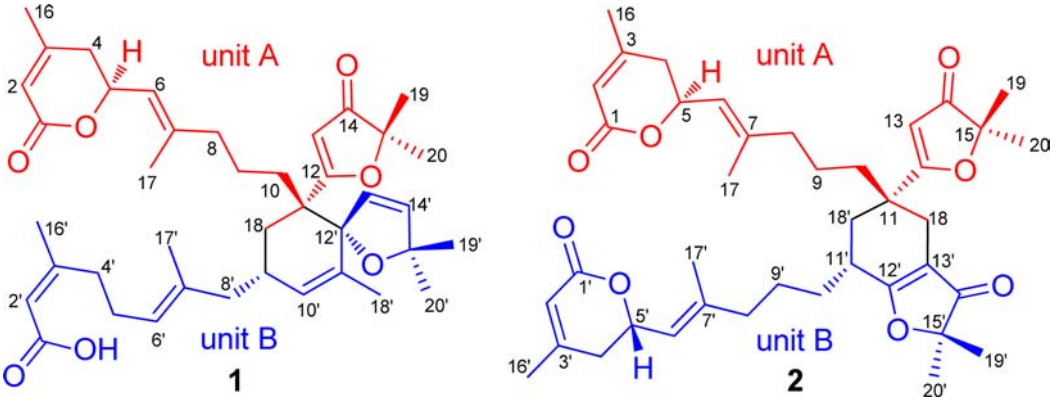
ABSTRACT



Aphanamenes A (1) and B (2), two unprecedented acyclic diterpene dimers formed via a [4 + 2]-cycloaddition, were isolated from the root bark of *Aphanamixis grandifolia*. Their structures were elucidated by spectroscopic analyses, and the absolute configuration of 1 was determined by ECD calculations. Both 1 and 2 showed significant inhibition on NO production on lipopolysaccharide-induced RAW264.7 macrophages.

Aphanamixis grandifolia Bl., a wild timber tree from the Meliaceae family, is distributed mainly in the tropical and subtropical areas of South and Southeast Asia.[1] The roots and leaves are utilized to relieve rheumatoid joint pain and numbness of limbs in some regions of China.[2] Previous phytochemical studies on the genus *Aphanamixis* indicated that they are a rich resource of structurally diverse and highly oxygenated triterpenoids and limonoids,[3] which has been a research focus of natural products from the Meliaceae family in recent decades.[4]

In our continuing study on *A. grandifolia*, two acyclic diterpene dimers with an unprecedented carbon skeleton, namely aphanamenes A (**1**) and B (**2**) (Figure 1), were isolated from the EtOH extract of the root barks of the plant collected from the Yunnan province of China. This unique dimeric carbon framwork was likely formed via a [4 + 2]-cycloaddition between $\Delta^{11(18)}$ and $\Delta^{9'(10'),11'(12')}$ in the respective acyclic diterpene unit of **1** and formed between $\Delta^{11(18)}$ and $\Delta^{11'(18'),12'(13')}$ of **2**. Both **1** and **2** showed significant inhibition on nitric oxide (NO) production

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on lipopolysaccharide-induced RAW264.7 macrophages with IC_{50} values of 9.72 and 7.98 μM, respectively. Herein, we report their isolation, structural elucidation, plausible biosynthetic pathway as well as inhibitory activities on NO production.

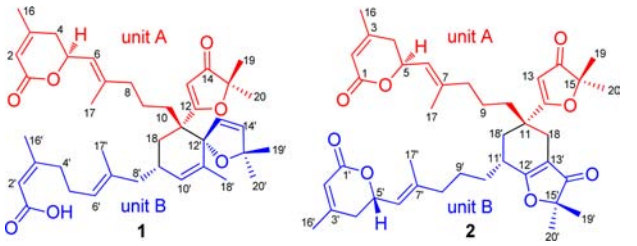

Figure 1. Structures of aphanamenes A (**1**) and B (**2**).

Aphanamene A (**1**)[5] was obtained as colorless gum. Its molecular formula of $C_{40}H_{54}O_7$ was determined by sodiated molecular ion $[M + Na]^+$ at m/z 669.3763 (calcd for 669.3762) in the HR-ESI-MS, corresponding to 14 degrees of unsaturation. The UV absorptions at 203 and 266 nm and bands at 1695 and 1567 cm^{-1} in the IR spectrum indicated the presence of carbonyl and α,β-unsaturated ketone moieties. Analysis of the NMR data (Table 1) of **1** revealed resonances for 40 carbons including 9 methyls, 8 methylenes, 10 methines, and 13 quaternary carbons. In the ^1H NMR spectra, 8 characteristic olefinic protons (δ_H 5.23 to 5.81) and 9 singlet methyls (δ_H 1.13 to 1.98) were observed (Table 1). The above information indicated that compound **1** was not a characteristic triterpenoid or limonoid previously reported from *Aphanamixis*.[3] Considering the 40 carbon atoms and 14 degrees of unsaturation in the structure of **1**, together with the number of methyls, it could be speculated that compound **1** may be a diterpene dimer.

More detailed information about the structure of **1** came from interpretation of its HSQC, HMBC, and $^1H-^1H$ COSY spectra. A furan-3(2*H*)-one unit was established by HMBC correlations (Figure 2) from H-13 to C-12 and C-14 and from Me-19 to C-15 and C-14. An α,β-unsaturated δ-lactone moiety was confirmed by HMBC correlations from H-2 to C-1 and C-3, from Me-16 to C-2 and C-4, and from H-4 to C-3 and C-5. The structural fragments of C-4 to C-6 and C-8 to C-10 were readily established from the $^1H-^1H$ COSY spectrum (Figure 2). Finally, the connection between the furanone and the α,β-unsaturated δ-lactone units through an aliphatic chain was established by key HMBC correlations (Figure 2) from H-6/C-8 and C-17, H-13/C-11, and H-10/C-12 which enabled us to establish an acyclic diterpene moiety, similar to nemoralisin C which was previously isolated.[3d]

By deducting the acyclic diterpene moiety (unit A), the remaining signals of NMR data allowed us to construct another acyclic diterpene moiety (unit B). In particular, a dihydrofuran unit (δ_H 5.71, 5.75; δ_C 94.3, 126.7, 136.5 and 86.8) and a carboxyl group (δ_C 168.6) were easily distinguished from key HMBC correlations (Figure 2). The connection of these two characteristic parts was also established by key $^1H-^1H$ COSY correlations (H-4′/H-5′/H-6′ and H-8′/H-9′/H-10′) and HMBC correlations (H-2′ to C-4′, H-6′ to C-8′ and H-10′ to C-12′) showed in Figure 2. The above information suggested that unit B of **1** was characterized as melidianolic acid A derivative.[6]

Finally, unit A was fused with unit B via two C−C bonds (between C-11 and C-12′, C-18, and C-9′) as part of an additional cyclohexene unit due to the key $^1H-^1H$ COSY correlation of H-18 with H-9′ and key HMBC correlations of H-18 with C-9′, H-10′ with C-12′, and H-13′ with C-11. Thus, the planar structure of **1** was determined as depicted in Figure 1.

The relative configuration of **1** was determined by ROESY experiment (Figure 2). Correlations from H-13′ (δ_H 5.71) to H-10a (δ_H 1.67) and H-10b (δ_H 1.56) to H-9′ (δ_H 2.17) indicated that the above protons were cofacial and the cyclohexene ring adopted a half-chair conformation. Therefore, a quasi axial relationship for the H-10a of

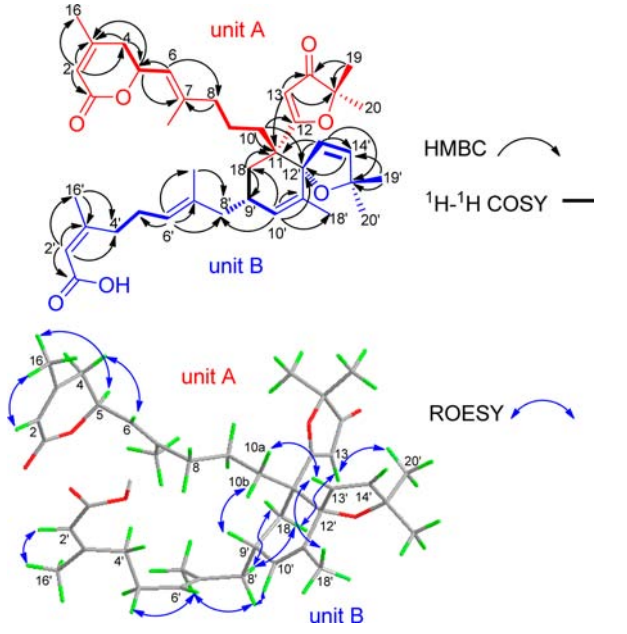

Figure 2. Key HMBC(→), $^1H-^1H$COSY(—), and ROESY (↔) correlations for **1**.

unit A and the olefinic proton (H-13′) of dihydrofuran in unit B. Additionally, H-9′ and H-10b were axially located and randomly assigned as β-configuration. Moreover, the furan-3(2*H*)-one in unit A and the C-8′ of unit B were located at opposite side and α-configured. Thus, the relative configuration of compound **1** was established as shown in Figure 1. The absolute configuration of **1** was established as 5*S*,11*S*,9′*R*,12′*R* by its well matched

(5) Aphanamene A (**1**): $C_{40}H_{54}O_7$; colorless gum; $[\alpha]^{25}_D$ +2.6 (c 0.18, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.30), 266 (3.97) nm; CD (c 0.41 mmol/L, MeOH, 25 °C) λ_{max} ($\Delta\varepsilon$) 215 (−1.37), 252 (−1.61);IR (KBr) ν_{max} 3450, 1695, 1567, 1384, 1249 cm^{-1}; for ^1H and ^{13}C NMR data, see Table 1; ESIMS (positive) m/z 647.4 $[M + H]^+$; HR-ESI-MS m/z 669.3763 $[M + Na]^+$ (calcd for $C_{40}H_{54}O_7Na$, 669.3762).

calculated and experimental ECD spectrum (Figure 3), and comparison of ^{1}H and ^{13}C NMR data of C-5 between **1** and those of nemoralisins.[3d]

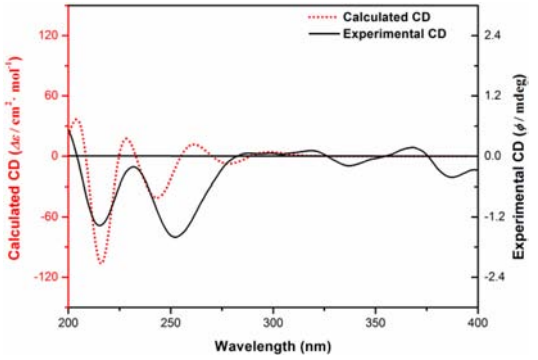

Figure 3. Calculated and experimental ECD spectra of **1**.

Aphanamene B (**2**),[7] obtained as colorless gum, was determined to have the molecular formula of $C_{40}H_{52}O_8$ by HR-ESI-MS data (*m*/*z* 661.3737 $[M + H]^+$, calcd for 661.3735) with 15 degrees of unsaturation. The ^{1}H and ^{13}C NMR spectral data of **2** were similar to those of **1** (Table 1). The notable difference is that **2** possessed an additional α,β-unsaturated δ-lactone instead of the acyclic carboxylic acid moiety in **1**, suggesting that **2** was a diterpene dimer derived from two nemoralisin C derivative units.[3d] By detailed analysis of the 1D and 2D NMR spectra of **2**, two furanone, two α,β-unsaturated δ-lactone and two aliphatic chain moieties were confirmed from two nemoralisin C derivative units (Figure S1, Supporting Information).

The key HMBC correlations (Figure S1, Supporting Information) from H-18 to C-13′ and from H-18′ to C-10 combined with the key ^{1}H−^{1}H COSY correlation of H-11′ with H-18′ indicated that the connection of the two nemoralisin C derivative units via two C−C bonds (between C-11 and C-18′, C-18 and C-13′) which were part of cyclohexene unit. Finally, the planar structure of **2** was established as depicted in Figure 1.

The key ROESY correlations (Figure S1, Supporting Information) of H- 13 (δ_H 5.19) with H-18′ (δ_H 1.43), H-10′ (δ_H 2.07), and Me-20′ (δ_H 1.34) established that the cyclohexene moiety adopted a half-chair conformation and the furan-3(2*H*)-one moiety in unit A and C-10′ of unit B were cofacial. Furthermore, observation of correlations of H-11′ (δ_H 2.55) with H-18′ (δ_H 1.94) and H-18′ (δ_H 1.94) with H-10b (δ_H 1.65) indicated that a quasi axial relationship for H-10b and H-11′, and they were arbitrarily assigned as β-configuration. Consequently, the relative configuration of compound **2** was established as depicted in Figure 1.

The biosynthetic origin of **1** and **2** was proposed to be acyclic diterpenes reported from this plant,[3d,6] and a [4 + 2]-cycloaddition[9] probably played a key role in the dimerization of the carbon skeleton of **1** and **2** (Scheme 1). In the plausible biosynthetic pathway of **1**, the precursor (unit A)

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data of **1** and **2**

	1[b]		**2**[b]	
no.	δ_C	δ_H (mult, *J*, Hz)	δ_C	δ_H (mult, *J*, Hz)
1	165.1		165.2	
2	116.6	5.81 (s)	116.8	5.79 (s)
3	156.7		157.0	
4	35.0	2.34 (m)	35.2	2.33 (m)[a]
		2.20 (m)[a]		2.20 (m)[a]
5	74.0	5.09 (m)	74.2	5.08 (m)[a]
6	122.1	5.28 (d, 8.5)	122.5	5.29 (d, 8.5)
7	141.9		142.2	
8	39.8	1.99 (m)[a]	39.4	2.00 (m)[a]
9	22.6	1.20 (m)	25.1	1.94, 1.36 (m)[a]
10a	31.3	1.67 (m)[a]	30.5	1.72 (m)[a]
10b		1.56 (m)[a]		1.65 (m)[a]
11	50.2		42.2	
12	195.8		194.8	
13	105.2	5.54 (s)	102.0	5.19 (5.18, 5.20) (s)[c]
14	207.8		207.2	
15	88.1		89.1	
16	22.8	1.98 (s)	23.0	1.97 (s)
17	16.7	1.66 (s)	16.9	1.68 (s)
18α	30.3	1.87 (m)[a]	34.5[c]	2.09 (2.13)(m)[a,c]
18β		1.63 (m)[a]	(34.1)[c]	1.87 (1.79) (m)[a,c]
19	23.3	1.33 (s)	23.0	1.34 (s)
20	23.4	1.34 (s)	22.9	1.32 (s)
1′	168.6		165.2	
2′	115.8	5.70 (s)	116.7	5.78 (s)
3′	161.9		157.0	
4′	33.2	2.78 (m)	35.2	2.33 (m)[a]
		2.66 (m)		2.20 (m)[a]
5′	26.4	2.22 (m)[a]	74.2	5.08 (m)[a]
6′	125.9	5.23 (t, 7.0)	122.1	5.25 (d, 9.0)
7′	133.0		142.0	
8′	45.7	2.13, 1.99 (m)[a]	39.3	2.00 (m)[a]
9′	30.5	2.17 (m)[a]	22.5	1.94, 1.21 (m)[a]
10′	129.5	5.43 (s)	30.4	2.07, 1.43 (m)[a]
11′	134.6		36.6	2.55(m)[a]
12′	94.3		189.8	
13′	126.7	5.71 (d, 6.0)	111.4	
14′	136.5	5.75 (d, 6.0)	204.3	
15′	86.8		87.9	
16′	25.1	1.92 (s)	22.9	1.96 (s)
17′	15.7	1.62 (s)	16.7	1.65 (s)
18′α	21.1	1.65 (s)	24.2 (24.1)[c]	1.43 (m)[a]
18′β				1.94 (m)[a]
19′	28.9	1.30 (s)	23.3	1.35 (s)
20′	29.2	1.13 (s)	23.0	1.34 (s)

[a] Singal pattern unclear due to overlapping. [b] Measured in $CDCl_3$. [c] Peak splitting due to cyclohexene conformation isomerization.[8]

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(7) Aphanamene B (**2**): $C_{40}H_{52}O_8$; colorless gum; $[\alpha]^{25}_D$ +18.5 (*c* 0.33, MeOH); UV (MeOH) λ_{max} (log ε) 204 (4.54), 265 (4.46) nm; IR (KBr) ν_{max} 3450, 1693, 1573, 1384, 1247 cm^{-1}; for ^{1}H and ^{13}C NMR data, see Table 1; ESIMS(positive) *m*/*z* 661.6 $[M + H]^+$; HR-ESI-MS *m*/*z* 661.3737 $[M + H]^+$ (calcd for $C_{40}H_{53}O_8$, 661.3735).

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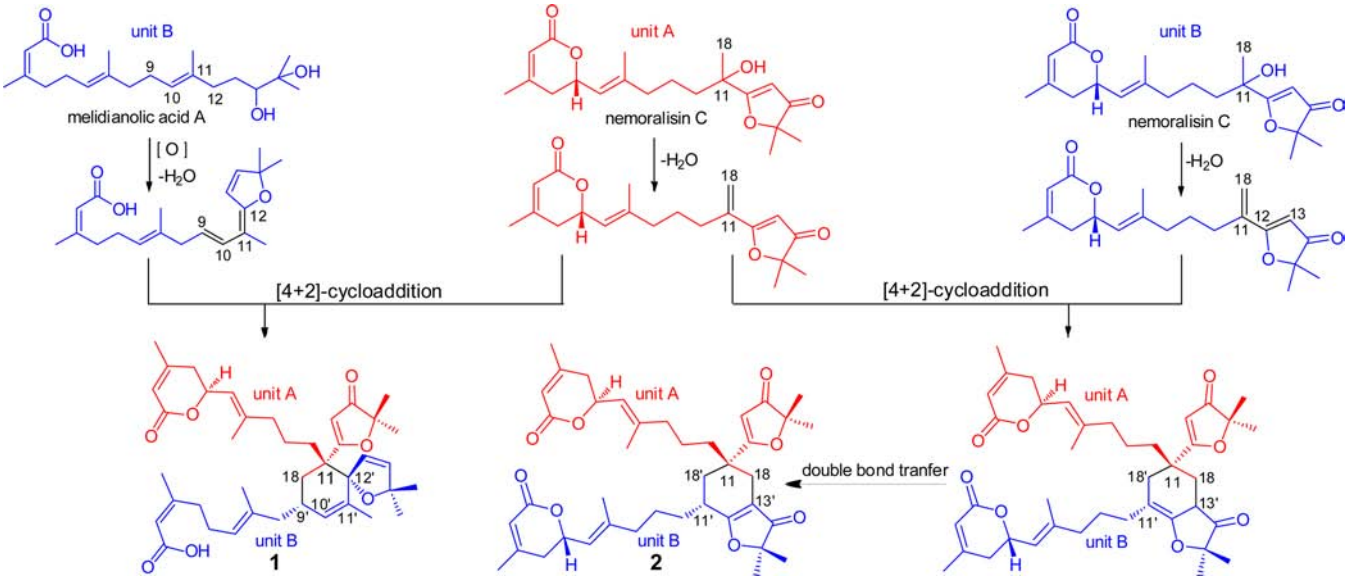
Scheme 1. Plausible Biogenetic Pathway of **1** and **2**



with $\Delta^{11(18)}$ was formed through dehydration between OH-11 and Me-18 of nemoralisin C,[3d] and another precursor (unit B) with $\Delta^{9(10),11(12)}$ was produced by dehydration and double bond formation between C-9 and C-10, C-11 and C-12 of melidianolic acid A.[6] On the catalysis of enzyme and UV, a new cyclohexene ring was formed between the $\Delta^{11(18)}$ of unit A and $\Delta^{9'(10'),11'(12')}$ of unit B. In the same manner as **1**, compound **2** was also generated via a [4 + 2]-cycloaddition. Different from **1**, compound **2** had the same precursor with $\Delta^{11(18)}$ formed through dehydration between OH-11 and Me-18 of nemoralisin C.[3d] The basic skeleton of **2** was first formed between the $\Delta^{11(18)}$ and $\Delta^{11'(18'),12'(13')}$, then the transfer of $\Delta^{11'(12')}$ to $\Delta^{12'(13')}$ produced the final compound **2**.

Compounds **1** and **2** were tested for their inhibitory effects on NO production induced by LPS in a macrophage cell line RAW264.7. Cell viability was first determined by the MTT method to find whether inhibition of NO production was due to the cytotoxicity of the tested compounds. There was no obvious cytotoxic effect (over 90% cell survival) on RAW264.7 cells treated with compounds **1** and **2** at concentrations of up to 25 μM. Compounds **1** and **2**, respectively, showed significant inhibition on NO production on LPS-activated RAW264.7 cells with IC_{50} values of 9.72 and 7.98 μM, comparrable to that of the positive control *N*-monomethyl-L-arginine at 40.45 μM.[10] The result suggests that this unique acyclic diterpene dimeric skeleton may be a potential candidate as an anti-inflammatory agent.

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Supporting Information Available. Experimental procedures, quantum chemical ECD calculations, and CD, UV, IR, ESIMS, HRESIMS, 1D and 2D NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.

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