JOURNAL OF NATURAL PRODUCTS

Article

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Secondary Metabolites from the Roots of *Neolitsea daibuensis* and Their Anti-inflammatory Activity

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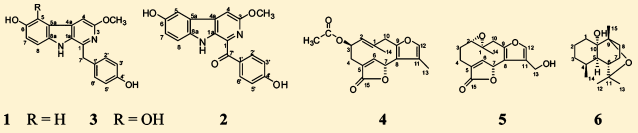
Supporting Information

ABSTRACT: Bioassay-guided fractionation of the roots of *Neolitsea daibuensis* afforded three new β-carboline alkaloids, daibucarbolines A–C (**1**–**3**), three new sesquiterpenoids, daibulactones A and B (**4** and **5**) and daibuoxide (**6**), and 20 known compounds. The structures of **1**–**6** were determined by spectroscopic analysis and single-crystal X-ray diffraction. Daibucarboline A (**1**), isolinderalactone (**7**), 7-*O*-methylnaringenin (**8**), and prunetin (**9**) exhibited moderate iNOS inhibitory activity, with IC_{50} values of 18.41, 0.30, 19.55, and 10.50 μM, respectively.

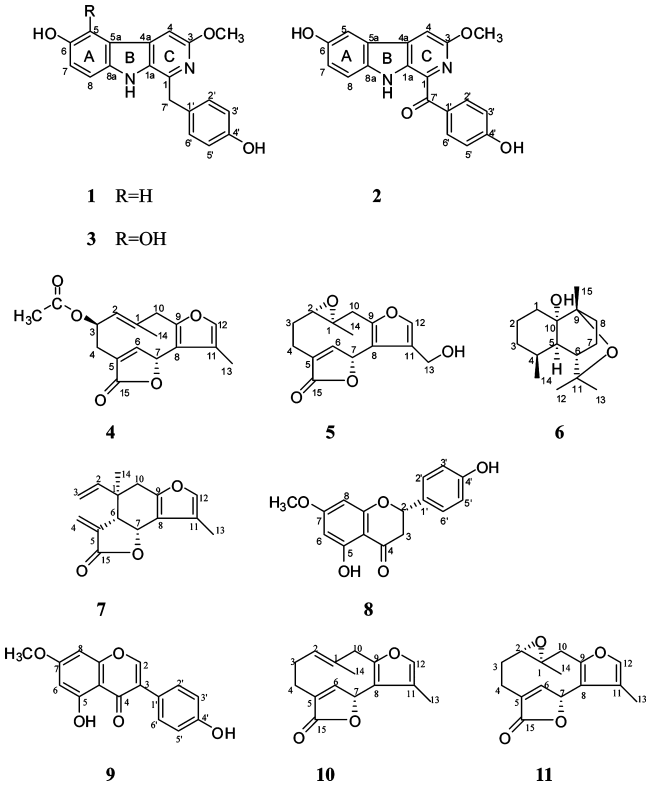


Neolitsea daibuensis Kamikoti (Lauraceae) is a small, semi-deciduous tree endemic to forests at altitudes of 800–1000 m in southern Taiwan.[1] Its chemical constituents and their bioactivity have not been analyzed; however, one alkaloid, *S*-(+)-reticuline, was isolated from the root wood and root bark of this species.[2] More than 40 species of Formosan Lauraceous plants were recently screened for anti-inflammatory activity using an inducible nitric oxide synthase (iNOS) assay, and the methanolic extract of *N. daibuensis* roots inhibits nitric oxide (NO) production in RAW 264.7 cells with no cytotoxicity. In this study, bioassay-guided fractionation led to the isolation of six new compounds (**1**–**6**) and 20 known compounds. Their structures were established from spectroscopic data and single-crystal X-ray diffraction. Isolation and elucidation of the structures of **1**–**6** together with an assessment of their anti-inflammatory activity are described herein.

RESULTS AND DISCUSSION

Compound **1** was isolated as a yellowish powder, and its molecular formula was found to be $C_{19}H_{16}N_2O_3$ using ESIMS and HR-ESIMS (321.1241 $[M + H]^+$). The observed UV absorption peaks at 229, 243, 307, and 390 nm in conjunction with a bathochromic shift observed after the addition of aqueous KOH suggest the presence of a phenolic carboline moiety.[3] The IR spectrum showed an absorption band at 3327 cm^{-1}, indicating the presence of a hydroxy group. The ^1H NMR spectrum (Table 1) showed an ABX system [δ 7.06 (1H, dd, J = 8.4, 2.4 Hz, H-7), 7.32 (1H, d, J = 8.4 Hz, H-8), 7.50 (1H, d, J = 2.4 Hz, H-5)] and confirmed the presence of a hydroxy group [δ 8.04 (1H, br s, OH-6)] on ring A, an NH proton [δ 9.89 (1H, br s)] on ring B, and a proton [δ 7.16 (1H, s, H-4)], a methoxy group [δ 3.93 (3H, s, OCH_3-3)], and a *p*-hydroxybenzyl group [δ 4.26 (2H, s, H-7′), 7.24 (2H, d, J = 9.0 Hz, H-2′, H-6′), 6.71 (2H, d, J = 9.0 Hz, H-3′, H-5′), 8.10 (1H, br s, OH-4′)] on ring C. The HMBC spectrum (Figure 1) showed correlations between H-7′ (δ_H 4.26) and C-1 (δ_C 142.1) and C-1a (δ_C 133.3), supporting the conclusion that a *p*-hydroxybenzyl group is connected to C-1. H-5 (δ_H 7.50) showed HMBC correlations with C-4a (δ_C 134.7), C-8a (δ_C 138.5), C-6 (δ_C 152.2), and C-7 (δ_C 119.7). The NOESY spectrum (Figure 2) showed a correlation between H-8 (δ 7.32) and its neighboring NH (δ 9.89) and H-7 (δ 7.06), thus confirming the location of H-8. H-4 (δ_H 7.16) showed HMBC correlations with C-5a (δ_C 123.8), C-1a (δ_C 133.3), and C-3 (δ_C 158.5) and a NOESY correlation with H-5 (δ 7.50). The OCH_3 group (δ_H 3.93) showed an HMBC correlation with C-3 (δ_C 158.5), hence assigning the locations of H-4 and OCH_3-3. On the basis of these data, the structure of **1**, named

Received: December 4, 2010
Published: December 7, 2011



daibucarboline A, was elucidated as 1-(4-hydroxybenzyl)-3-methoxy-9*H*-pyrido[3,4-*b*]indol-6-ol.

ESIMS and HR-ESIMS established the molecular formula of compound **2** as $C_{19}H_{14}N_2O_4$. The data analysis indicated that it contains one more oxygen atom and two fewer hydrogen atoms compared to compound **1**, indicating that a carbonyl group in **2** replaced a methylene group in **1**. The IR spectrum showed absorption bands corresponding to a hydroxy group at 3158 cm^{-1} and a carbonyl group at 1606 cm^{-1}. The ^1H and ^{13}C NMR spectra (Tables 1 and 2) were similar to those of **1**, except that a carbonyl group (δ_C 192.5, C-7′) in **2** replaced a methylene [δ_H 4.26 (2H, s, H-7′), δ_C 40.4 (C-7′)] in **1**. On the basis of the above-mentioned data, the structure of **2**, named daibucarboline B, was elucidated as (6-hydroxy-3-methoxy-9*H*-pyrido[3,4-*b*]indol-1-yl)(4-hydroxyphenyl)methanone. This assignment was further supported by HMBC (Figure 1) and NOESY (Figure 2) experiments.

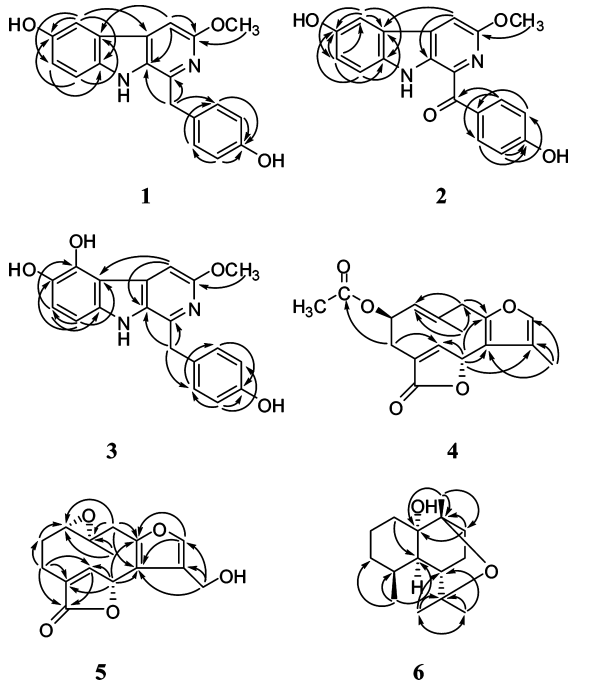


Figure 1. Key HMBC (H→C) correlations of **1**–**6**.

Compound **3** was isolated as a yellowish powder. Its UV and IR spectra were similar to those of **1**, suggesting the presence of

Table 1. ^1H NMR and ^{13}C NMR Data for Compounds 1, 2, and 3[a]

	1		2		3	
position	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)	δ_C
1		142.1 (s)		137.6 (s)		142.3 (s)
3		158.5 (s)		156.5 (s)		158.2 (s)
4	7.16, s	97.8 (d)	7.71, s	106.7 (d)	5.70, s	96.2 (d)
5	7.50, d (2.4)	107.6 (d)	7.64, br s	108.1 (d)		139.0 (s)
6		152.2 (s)		153.0 (s)		149.1 (s)
7	7.06, dd (8.4, 2.4)	119.7 (d)	7.18, dd (9.0, 2.4)	120.6 (d)	7.33, d (9.0)	119.9 (d)
8	7.32 d (8.4)	113.7 (d)	7.62, d (9.0)	114.6 (d)	7.54, d (9.0)	113.3 (d)
1a		133.3 (s)		136.7 (s)		133.1 (s)
4a		134.7 (s)		132.9 (s)		134.2 (s)
5a		123.8 (s)		122.7 (s)		122.2 (s)
8a		138.5 (s)		139.2 (s)		117.7 (s)
1′		131.7 (s)		131.4 (s)		131.1 (s)
2′, 6′	7.24, d, (9.0)	131.3 (d)	8.50, d (8.7)	135.2 (d)	7.06, d (8.7)	130.6 (d)
3′, 5′	6.71, d, (9.0)	116.6 (d)	7.00, d (8.7)	116.1 (d)	6.64, d (8.7)	116.4 (d)
4′		157.3 (s)		162.8 (s)		156.8 (s)
7′	4.26, s	40.4 (d)		192.5 (s)	4.19, d (3.0)	39.5 (d)
OMe-3	3.93, s	54.5 (q)	4.01, s	54.9 (q)	3.32, s	54.7 (q)
OH[b]	8.04, br s		8.20, brs			
OH[b]	8.10, br s		9.18, br s			
NH[b]	9.89, br s		10.8, br s			

[a]NMR data were measured in acetone-d_6 at 600 MHz for **1** and **2** and in methanol-d_4 at 600 MHz for **3**. [b]D_2O exchangeable.

dx.doi.org/10.1021/np100874f | *J. Nat. Prod.* 2011, 74, 2489–2496

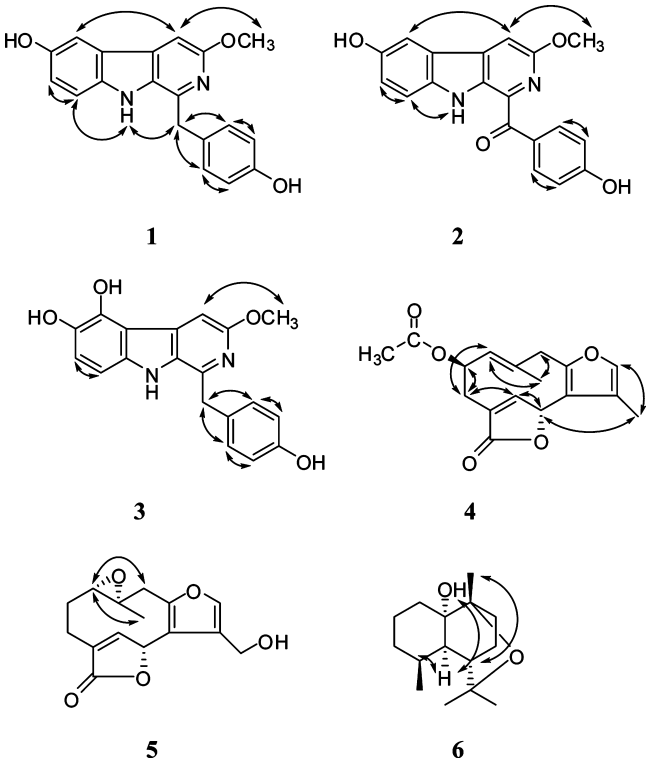


Figure 2. Key NOESY (H↔H) correlations of **1**–**6**.

a phenolic *β*-carboline moiety. The ESIMS and HR-ESIMS data determined the molecular formula of **3** to be $C_{19}H_{16}N_2O_4$. Compound **3** has one more oxygen atom than **1**, indicating the addition of one hydroxy group. The ^{1}H NMR spectrum (Table 1) was similar to that of **1**, except that an AB coupling pattern involving H-7 [*δ* 7.33 (1H, d, *J* = 9.0 Hz)] and H-8 [*δ* 7.54 (1H, d, *J* = 9.0 Hz)] on ring A of **3** replaced the ABX spin system observed for ring A of **1**. The chemical shift of H-4 [δ_H 5.70 (1H, s)] suggested the presence of a neighboring hydroxy group at C-5 (δ_C 139.0), which in turn showed an HMBC (Figure 1) correlation with H-7 (δ_H 7.33). Thus, the structure of **3** was elucidated as 1-(4-hydroxybenzyl)-3-methoxy-9*H*-pyrido[3,4-*b*]indole-5,6-diol, and it was termed daibucarboline C, the structure of which was further confirmed by ^{13}C NMR, HSQC, DEPT, and NOESY (Figure 2) experiments.

Compound **4** was isolated as optically active colorless needles with $[\alpha]_D^{25}$ +39.4 (*c* 0.04, $CHCl_3$). The molecular formula was established as $C_{17}H_{18}O_5$ by ESIMS and HR-ESIMS. The UV spectrum showed absorptions at 208 and 269 nm, and the IR spectrum showed a lactone carbonyl group at 1734 cm^{-1}. The ^{1}H and ^{13}C NMR spectra (Table 2) suggested the presence of a germacrane-type sesquiterpene moiety.[4] The ^{1}H NMR spectrum (Table 2) showed one methyl group [*δ* 2.09 (3H, s, H-13)], an olefinic proton in a furan ring [*δ* 7.14 (1H, s, H-12)], an oxymethine proton [*δ* 5.86 (1H, br s, H-7)], and an olefinic proton in a lactone ring [*δ* 7.06 (1H, d, *J* = 1.2 Hz, H-6)]. One vinylic methyl group [*δ* 1.44 (3H, s, H-14)] and one olefinic proton [*δ* 5.02 (1H, d, *J* = 10.2 Hz, H-2)], together with the methylene protons [*δ* 3.38 (1H, d, *J* = 15.6 Hz, H-10b) and 3.62 (1H, d, *J* = 15.6 Hz, H-10a)], were similar to those of linderalactone,[4] which was also isolated in this study. In compound **4** an acetoxy group [δ_H 2.10 (3H, s, OAc-3), δ_C 173.1] and an oxymethine proton [δ_H 5.58 (1H, ddd, *J* = 10.2, 9.6, 6.6 Hz, H-3)] replaced the methylene protons [δ_H 2.28 (1H, m, H-3b) and 2.37 (1H, m, H-3a)] of C-3 in linderalactone.[4] Thus, the planar structure of **4** was determined to be 3-acetoxylinderalactone. The ^{1}H NMR spectrum of **4** was also similar to the sesquiterpene pseudoneolinderane (**11**), except for the C-3 acetoxy group of **1** instead of the 1,2-epoxy group in **11**. The ^{1}H NMR chemical shifts and coupling patterns of H-6 [*δ* 7.06 (1H, d, *J* = 1.2 Hz, H-6)] ($CDCl_3$, 400 MHz) in **4** and H-6 [*δ* 7.05 (1H, s, H-6)] ($CDCl_3$, 200 MHz) in **11**[5] suggested the same relative configuration at C-7. The relative configuration of C-7 in pseudoneolinderane has been determined as *R**.[5] The single-crystal X-ray diffraction pattern of **4** (Figure 3) indicated that the relative configuration is (3*R**, 7*R**). Compound **4** was designated as daibulactone A.

Compound **5** was obtained as yellowish needles with $[\alpha]_D^{25}$ −2.4 (*c* 0.04, $CHCl_3$). The UV, IR, and ^{1}H NMR spectra (Table 2) of **5** were similar to those of pseudoneolinderane (**11**),[5] which was also isolated in this study, suggesting that **5** is also a germacrane-type sesquiterpene. The ^{1}H and ^{13}C NMR spectra of **5** (Table 2) indicated that a C-11 hydroxymethyl group [δ_H 5.00 (1H, d, *J* = 13.0 Hz, H-13b) and 5.18 (1H, d, *J* = 13.0 Hz, H-13a); δ_C 56.0 (C-13); δ_C 122.3 (C-11)] replaced a C-11 methyl group [δ_H 2.11 (3H, s, H-13) and δ_C 8.3 (C-13)] in pseudoneolinderane.[5] Referring to the X-ray diffraction data, NOESY associations, and ^{1}H NMR coupling patterns of pseudoneolinderane,[5] the relative configuration of **5** was suggested as (1*R**, 2*R**, 7*R**). The structure of **5**, designated as daibulactone B, was further confirmed by HMBC (Figure 1) and NOESY (Figure 2) experiments.

Compound **6** was isolated as optically active colorless needles with $[\alpha]_D^{25}$ −5.3 (*c* 0.05, $CHCl_3$). The molecular formula of **6**, $C_{15}H_{26}O_2$, was determined by ESIMS and HR-ESIMS to have three indices of hydrogen deficiency (IHD), which indicated the presence of a sesquiterpenoid framework. The ^{1}H NMR spectrum (Table 2) showed signals for three methines at *δ* 1.68 (1H, m, H-6), 1.81 (1H, m, H-5), and 2.60 (1H, m, H-4), four methyl groups at *δ* 0.95 (3H, d, *J* = 7.2 Hz, H-14), 1.10 (3H, s, H-15), 1.19 (3H, s, H-12), and 1.29 (3H, s, H-13), and five methylenes (Table 2). The ^{13}C (Table 2) and DEPT spectra also indicated that **6** was a sesquiterpene, showing signals for 15 carbons, which were classified as three quaternary O-bearing carbons with signals at *δ* 75.6 (C-9), 90.7 (C-10), and 73.6 (C-11), three sp^3 carbons with signals at *δ* 35.4 (C-4), 47.9 (C-5), and 36.4 (C-6), and five aliphatic methylene carbons. The COSY spectrum (Figure 4, Supporting Information) showed correlations indicating a C-1−C-2−C-3−C-4−C-5−C-6−C-7−C-8 linkage. The HMBC spectrum (Figure 1) showed several correlations, including H-1 (δ_H 1.66) to C-5 (δ_C 47.9), C-9 (δ_C 75.6), and C-10 (δ_C 90.7); H-8 (δ_H 1.64) to C-7, C-9, and C-10; H-15 (δ_H 1.10) to C-8 (δ_C 26.2), C-9, and C-10; H-14 (δ_H 0.95) to C-3 (δ_C 29.5), C-4 (δ_C 35.4), and C-5 (δ_C 47.9); both H-12 (δ_H 1.19) and H-13 (δ_H 1.29) to C-6 (δ_C 36.4) and C-11 (δ_C 73.6); and H-14 to C-3, C-4, and C-5. Furthermore, we propose that the two O-bearing carbons, C-9 and C-11, are connected through an oxygen to form the third ring, as indicated by the three IHDs in **6**. The NOESY spectrum (Figure 2) showed correlations between H-4, H-5, and OH-10 and between H-6 and CH_3-9, but it showed no correlation between H-5 and H-6 or between CH_3-9 and OH-10. These data indicated that H-4, H-5, and OH-10 occupied *α*-equatorial positions and H-6 and CH_3-9 *β*-axial positions. The structure of **6**, designated daibuoxide, was further confirmed by DEPT, HSQC, HMBC (Figure 1), and NOESY (Figure 2) experiments.

dx.doi.org/10.1021/np100874f | *J. Nat. Prod.* 2011, 74, 2489–2496

Table 2. ^{1}H NMR and ^{13}C NMR Data for Compounds 4, 5, and 6[a]

	4		5		6	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
1		131.0 (s)		59.3 (s)	1.66, m	19.2 (t)
					1.93, m	
2	5.02, d (10.2)	129.6 (d)	2.77, dd (11.8, 2.0)	69.4 (d)	1.31, m	32.3 (t)
					2.47, m	
3	5.58, ddd (10.2, 9.6, 6.6)	70.9 (d)	1.48, br ddd,	22.2 (t)	1.36, m	29.5 (t)
			(13.0, 11.8, 5.6)		1.88, m	
			2.15, m			
4	2.41, dd (12.0, 9.6)	32.6 (d)	2.58, ddd (13.1, 12.8, 5.6)	21.6 (t)	2.60, m	35.4 (d)
5			2.83, dd (13.1, 5.6)			
		137.8 (s)		134.0 (s)	1.81, m	47.9 (d)
6	7.06, d (1.2)	153.8 (d)	7.35, s	148.1 (d)	1.68, m	36.4 (d)
7	5.86, br s	74.6 (d)	6.01, s	73.9 (d)	1.41, t (3.2)	24.4 (t)
					1.49, m	
8		122.4 (s)		116.1 (s)	1.64, m	26.2 (t)
					2.07, t (3.2)	
9		151.9 (s)		150.8 (s)		75.6 (s)
10	3.38, d (15.6)	40.7 (t)	2.46, d (16.2)	38.2 (t)		90.7 (s)
	3.62, d (15.6)		3.44, d (16.2)			
11		116.0 (s)		122.3 (s)		73.6 (s)
12	7.14, s	137.7 (d)	7.40, s	140.6 (d)	1.19, s	29.1 (q)
13	2.09, s	8.4 (q)	5.00, d (13.0)	56.0 (t)	1.29, s	29.3 (q)
			5.18, d (13.0)			
14	1.44, s	16.6 (d)	0.94, s	16.3 (q)	0.95, d (7.2)	15.1 (q)
15		170.2 (s)		172.7 (s)	1.10, s	26.7 (q)
OAc-3	2.10, s	21.1 (q)				
		173.1 (s)				
OH[b]					1.65	

[a]NMR data were measured in $CDCl_3$ at 600 MHz for **4** and at 400 MHz for **5** and **6**. [b]D_2O exchangeable.

dx.doi.org/10.1021/np100874f | J. Nat. Prod. 2011, 74, 2489–2496

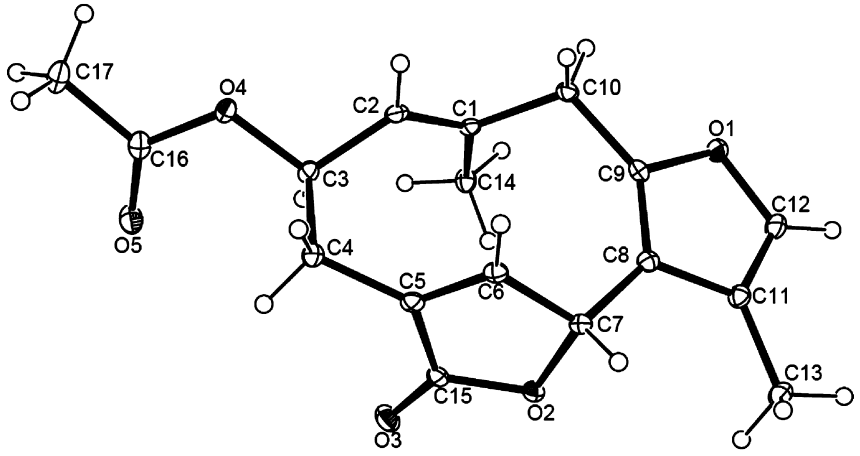


Figure 3. ORTEP[32] drawing of **4** with ellipsoids at the 30% probability level.

Table 3. Mean E_{max}, IC_{50}, and CC_{50} of Isolates from Roots of *N. daibuensis* for Nitrite Production Induced by LPS in RAW 264.7 Cells

compound	E_{max} (%)[a]	IC_{50} (μM)	CC_{50} (μM)[e]
daibucarboline A (**1**)	96.14 ± 2.64	18.41 ± 0.47	94.80 ± 2.55
isolinderalactone (**7**)[b]	100.00 ± 0.00	0.30 ± 0.01	66.41 ± 1.34
7-*O*-methylnaringenin (**8**)	85.17 ± 0.43	19.55 ± 0.89	>100.00
prunetin (**9**)	94.83 ± 0.56	10.50 ± 0.33	>100.00
aminoguanidine[c]	83.50 ± 0.39	28.47 ± 0.36	>100.00
N^{ω}-nitro-L-arginine[d]	41.21 ± 0.47	143.55 ± 10.66	>100.00

[a] E_{max} indicates mean maximum inhibitory effect, at a concentration of 100 μM, expressed as a percentage inhibition of nitrite production induced by LPS (200 ng/mL) in the presence of vehicle; IC_{50} means concentration producing 50% E_{max} (n = 4–6 in each group). [b] The E_{max} of isolinderalactone (**7**) was evaluated at a concentration of 25 μM because this compound was cytotoxic at a concentration of 50 μM. [c] Positive control (a selective iNOS inhibitor). [d] Positive control (a nonselective iNOS inhibitor). [e] CC_{50} indicates the compound concentration required to reduce cell viability by 50%.

The known compounds linderalactone (**10**),[4] pseudoneolinderane (**11**),[5] linderadine,[4] epicubebol methyl ether,[6] eudesm-4(15)-ene-1β,6α-diol,[7] hiiranlactone C,[8] sericealactone,[9] isolinderalactone (**7**),[10] 8α,11-elemdiol,[11] 7-*O*-methylnaringenin (**8**),[12,13] prunetin (**9**),[14] β-sitosterol,[15] methyl linoleate,[16] 2,3-dihydroxypropyl palmitate,[17] a mixture of methyl palmitate[16] and methyl stearate,[18] a mixture of stearic acid[19] and docosanoic acid,[20] oleic acid,[21] and α-tocopheryl quinoine[22] were identified by comparing their physical and spectroscopic data ($[\alpha]_D$, UV, IR, ^{1}H NMR, and MS) with their literature values. *S*-(+)-Reticuline was isolated from the phenolic layer of the roots of this plant in a previous study,[2] but none was isolated in this study. Most likely, *S*-(+)-reticuline may be present in the unsolved fractions, including the water layer of this plant.

Twelve of the isolates were evaluated for their ability to inhibit nitrite production stimulated by bacterial endotoxin lipopolysaccharide (LPS). Compounds **1**, **7**, **8**, and **9** were moderate inhibitors, with IC_{50} values of 18.41, 0.30, 19.55, and 10.50 μM, respectively (Table 3).

Bioassay-guided fractionation of the EtOAc-soluble layer led to the isolation of 26 compounds, including six new compounds. Sesquiterpenoids, including germacrane-, elemane-, cadinane-, and eudesmane-type sesquiterpenes, were the most common compounds isolated from the roots of *N. daibuensis* and correlate with the results of previous investigations of the *Neolitsea* genus. Previously, 1-benzyl-β-carboline-type alkaloids were isolated only from the Lauraceous plant *Aniba santalodora*[3] and the Hernandiaceous plant *Illigera luzonensis*,[23] and ours is the first report of the isolation of **1**–**3** from a plant in the *Neolitsea* genus. Among the tested compounds, daibucarboline A (**1**) concentration-dependently suppressed LPS-induced nitrite accumulation with an IC_{50} value of 18.41 μM and a CC_{50} value (compound concentration required to reduce cell viability by 50%) of 94.80 μM. The 1-benzyl-β-carboline-type alkaloid luzongerine, which was isolated from *I. luzonensis*,[23] and its synthetic 3-carboxy analogue[23] also showed anti-inflammatory effects by suppressing LPS/IFN-γ-induced NO production (IC_{50} = 12.67 and 18.39 μM, respectively). These results are similar to our result for daibucarboline A, which suggests that 1-benzyl-β-carboline-type alkaloids dose-dependently inhibit iNOS. The elemane-type sesquiterpene isolinderalactone (**7**) significantly inhibited NO production (IC_{50} = 0.30 μM, CC_{50} = 66.41 μM). This result suggests that the lactone ring connected to C-5, C-6, and C-7 of sesquiterpenoids plays an important role in anti-iNOS activity. The two flavonoids 7-*O*-methylnaringenin (**8**) and prunetin (**9**) showed moderate and concentration-dependent suppression of NO production (IC_{50} = 19.55 and 10.50 μM, respectively, and the CC_{50} values for both compounds were >100 μM). These results agree with the results of a study that revealed 7-*O*-methylnaringenin (**8**) and prunetin (**9**) are moderate and stable iNOS inhibitors.[24] This finding also agrees with the results of a previous study that demonstrated the ability of flavonoids such as daidzein, genistein, naringenin, and taxifolin to inhibit LPS-induced NO production.[25] 7-*O*-Methylnaringenin (**8**) inhibited NO production by 85.17%, showing stronger activity than naringenin (59.6% inhibition) and taxifolin (23.9% inhibition).[25] Furthermore, the isoflavone prunetin (**9**) showed 94.83% inhibition of NO production and

was more active than daidzein (70.3% inhibition) and genistein (97.4% inhibition).[25] These findings indicate that the presence of OCH_3-7 in these two flavonoids (**8**, **9**) plays an important role in iNOS inhibition.

The known compounds linderalactone (**10**) and pseudoneolinderane (**11**) weakly inhibited iNOS activity. However, **10** and **11** showed significant anti-inflammatory properties by suppressing the generation of superoxide anion by human neutrophils in response to formylmethionyl-leucyl-phenylalanine (fMLP) and dihydrocytochalasin B stimulation (IC_{50} = 8.48 and 3.21 μg/mL, respectively).[26] The discrepancy is most likely because we used a different anti-inflammatory pathway (iNOS suppression) as compared with the previous study (fMLP suppression). Superoxide formation caused by stimulation of the fMLP receptor involves various signal transduction pathways. The major pathway is the synergistic activation of p110β/p85-type phosphatidylinositol (PI) 3-kinase, p110/p101-type PI 3-kinase, and Gab2-associated PI 3-kinase in neutrophil-type cells.[27] In contrast, Toll-like receptor-4 (TLR4) is necessary for signal transduction induced by LPS. The interaction between TLR4 and myeloid differentiation factor 88 (MyD88) initiates a complex signaling pathway, including activation of mitogen-activated protein kinases (MAPKs), Akt, and inhibitory κB (IκB). These changes eventually lead to the activation of nuclear factor-kappa B (NF-κB), which is responsible for the induction of various inflammatory molecules.[28]

The isolation of anti-inflammatory compounds, especially 1-benzyl-β-carboline-type alkaloids, from the *Neolitsea* genus is significant. The dose-dependent inhibition of iNOS by these compounds requires further investigation. In addition, taxonomic identification of the *Neolitsea* species may be important.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined with a Yanaco micromelting point apparatus and were uncorrected. Optical rotations were measured on a JASCO P-1020 digital polarimeter. UV spectra were obtained with a Jasco V-530 UV/vis spectrophotometer, and IR spectra (KBr or neat) were acquired with a Genesis II FTIR spectrophotometer. 1D (^{1}H, ^{13}C, DEPT) and 2D (COSY, NOESY, HSQC, HMBC) NMR spectra using $CDCl_3$, acetone-d_6, or methanol-d_4 as solvents were recorded on a Varian Unity Plus 400 spectrometer (400 MHz for ^{1}H NMR; 100 MHz for ^{13}C NMR) and a Varian VNMRS-600 spectrometer (600 MHz for ^{1}H NMR; 150 MHz for ^{13}C NMR). Chemical shifts are given as δ (ppm) using TMS as the internal standard. Low-resolution ESIMS were obtained with a Micromass Trio-2000 GC/MS, a VG Biotech Quattro 5022 mass spectrometer, and a JEOL-JMS-HX 100 mass spectrometer. High-resolution ESIMS spectra were recorded on JEOL JMS-SX102A GC/LC/MS and Finnigan MAT-95XL high-resolution mass spectrometers. Silica gel (70−230 and 230−400 mesh; Merck) and spherical C_{18} 100 Å reversed-phase silica gel (RP-18; particle size 20−40 μm; Silicycle) were used for column chromatography, and silica gel 60 F254 (Merck) and RP-18 F254S (Merck) were used for TLC and preparative TLC. Further purification was performed by HPLC (Shimadzu; pump, LCC-6AD; UV/vis detector, SPD-10A; integrator, C-R7A Plus). Relative configuration was determined by X-ray diffraction patterns measured with a Bruker SMART diffractometer.

Plant Material. Roots of *N. daibuensis* were collected at Mt. Damumu, Pingtung County, Taiwan, in July 2008, and identified by one of the authors (I.-S.C.). A voucher specimen (Chen 6252) was deposited in the Herbarium of the School of Pharmacy, College of Pharmacy, Kaohsiung Medical University, Kaohsiung, Taiwan, Republic of China.

Extraction and Isolation. The dried roots (10.3 kg) of *N. daibuensis* were sliced and extracted three times with cold MeOH (30 L) at room temperature, and the solution was concentrated under vacuum. The MeOH extract (950 g) inhibited nitrite production below 25%, with no observed cytotoxicity at 100 μg/mL. The afforded MeOH extract was partitioned between EtOAc and H_2O to obtain EtOAc-soluble (400 g) and H_2O-soluble (400 g) fractions. The EtOAc-soluble fraction exhibited iNOS inhibitory activity, whereas the H_2O-soluble fraction was inactive in the bioassay system. The EtOAc-soluble fraction (120 g) was subjected to silica gel chromatography (70−230 mesh, 3.5 kg) with a gradient of *n*-hexane−EtOAc to obtain fractions 1−18. Fractions 2−4, 8, and 10−16 showed anti-inflammatory activity.

Fraction 7 (2.4 g) was subjected to silica gel column chromatography (70−230 mesh, 65 g), eluting with *n*-hexane−acetone (10:1), to yield fractions 7-1 to 7-10. Fraction 7-1 (116 mg) was chromatographed on a silica gel column (230−400 mesh, 3 g), eluting with *n*-hexane−CH_2Cl_2 (4:1), to afford epicubebol methyl ether (4.2 mg). Fraction 7-3 (804 mg) was chromatographed on a silica gel column (230−400 mesh, 22 g), eluting with CH_2Cl_2−acetone (100:1), to provide fractions 7-3-1 to 7-3-11. Fraction 7-3-7 was comprised of α-tocopheryl quinone (3.5 mg). Fraction 7-5 (301 mg) was recrystallized from MeOH to yield **7** (7.1 mg). The mother liquor (290 mg) was chromatographed on silica gel (230−400 mesh, 8 g), eluting with *n*-hexane−CH_2Cl_2−acetone (15:1:1), to afford fractions 7-5-1 to 7-5-9. Fraction 7-5-8 was comprised of oleic acid (16.2 mg). Fraction 7-8 (128 mg) was recrystallized from MeOH to afford a mixture of stearic acid and docosanoic acid (3.6 mg).

Fraction 8 (5.9 g) was recrystallized from MeOH to give **10** (244 mg), and the mother liquor was subjected to a Sephadex LH-20 column, eluting with 100% MeOH, to afford fractions 8-1 to 8-9. Fraction 8-2 (24 mg) was further separated by preparative TLC developed with *n*-hexane−acetone (3:1) to give a mixture of methyl palmitate and methyl stearate (3.6 mg). Fraction 8-3 (184 mg) and fraction 8-5 (141 mg) were treated as described for fraction 7 to obtain β-sitosterol (32.4 mg) and methyl linoleate (2.8 mg), respectively. Fraction 8-4 (28 mg) was purified as described for fraction 8-2 to afford **6** (2.6 mg). Fraction 8-6 (76 mg) was separated as described for fraction 7 and further purified by preparative HPLC (RP-18 column 250 × 10 mm, 5 μm, Merck), eluting with MeOH−H_2O (3:1), to obtain **11** (1.4 mg, t_R 9.0 min, 2 mL/min).

Fraction 12 (2.32 g) was purified with a Sephadex LH-20 column, eluting with 100% MeOH, to afford fractions 12-1 to 12-11. Fraction 12-8 (500 mg) was separated by MPLC (300 × 20 mm, 15−35 μm, Silicycle) with *n*-hexane−CH_2Cl_2−EtOH (3:1:1) as the eluent to afford linderadine (8.5 mg). Fraction 12-11 (32.8 mg) was purified by preparative HPLC (RP-18 column, 250 × 10 mm, 5 μm, Merck), eluted with MeOH−H_2O (4:1), to afford **8** (8.3 mg, t_R 10.40 min, 2 mL/min) and **9** (8.2 mg, t_R 11.84 min, 2 mL/min).

Fraction 13 (1.5 g) was purified as described for fraction 7 to afford fractions 13-1 to 13-6. Fraction 13-7 (73 mg) was purified by preparative TLC and developed with CH_2Cl_2−acetone (30:1) to afford eudesm-4(15)-ene-1β,6α-diol (2.3 mg). Fraction 13-7-7 (8.1 mg) was purified as described for fraction 8-2 to yield 8α,11-elemdiol (3.5 mg). Fraction 13-12 (202 mg) was separated as described for fraction 12-8 to afford fractions 13-12-1 to 13-12-9. Fraction 13-12-5 (75 mg) was further purified by preparative RP-18 TLC with acetone−H_2O (3:1) to obtain sericealactone (3.2 mg). Fraction 13-13 (79 mg) was separated as described for fraction 13-12 to afford **5** (2.2 mg).

Fraction 14 (596 mg) was treated as described for fraction 12-8 to afford fractions 14-1 to 14-20. Fraction 14-8 (78 mg) was recrystallized from MeOH to afford 2,3-dihydroxypropyl palmitate (2.8 mg).

Fraction 16 (4.27 g) was separated as described for fraction 7 to produce 10 fractions, 16-1 to 16-10. Fraction 16-7 (989 mg) was subjected to silica gel column chromatography (28 g), eluting with $CHCl_3$−MeOH (10:1), to afford fractions 16-7-1 to 16-7-10. Fraction 16-7-5 (132 mg) was separated as described for fraction 13-7 to afford **1** (1.7 mg, t_R 7.48 min, 2 mL/min). Fraction 16-4 (254 mg) was treated as described for fraction 12-8 to yield hiiranlactone C (2.8 mg). Fraction 16-9 (448 mg) was separated as described for fraction 8 to

produce fractions 16-9-1 to 16-9-16. Fraction 16-9-6 (20.9 mg) was purified by preparative TLC with CH_2Cl_2–MeOH (8:1) to afford **3** (2.4 mg). Fraction 16-5 (473 mg) was separated as described for fraction 12-8 to afford fractions 16-5-1 to 16-5-14. Fraction 16-5-9 (43.8 mg) was recrystallized from acetone to give **4** (7.2 mg). Fraction 16-5-14 (165.7 mg) was purified as described for fraction 8 to afford **2** (0.9 mg).

*Daibucarboline A (**1**):* yellowish prisms (MeOH); mp 162–165 °C (MeOH); UV (MeOH) λ_{max} (log ε) 229 (4.27), 243 (4.07), 307 (3.99), 390 (3.43) nm; with KOH λ_{max} (MeOH) (log ε) 209 (4.6), 239 (4.3), 307 (3.9), 319 (3.8) nm; IR (KBr) ν_{max} 3327 (OH) cm^{-1}; ^1H NMR and ^{13}C NMR, see Table 1; ESIMS m/z 321 $[M + H]^+$; HR-ESIMS m/z 321.1241 (calcd for $C_{19}H_{17}N_2O_3$, 321.1239).

*Daibucarboline B (**2**):* yellowish prisms (acetone); mp 192–193 °C (MeOH); UV (MeOH) λ_{max} (log ε) 223 (3.72), 285 (3.18), 330 (3.44) nm; with KOH λ_{max} (MeOH) (log ε) 218 (4.7), 359(3.4) nm; IR (KBr) ν_{max} 3158 (OH), 1606 (C=O) cm^{-1}; ^1H NMR and ^{13}C NMR, see Table 1; ESIMS m/z 335 $[M + H]^+$; HR-ESIMS m/z 357.0849 (calcd for $C_{19}H_{14}N_2O_4Na$, 357.0851).

*Daibucarboline C (**3**):* yellowish prisms (MeOH); mp 180–182 °C (MeOH); UV (MeOH) λ_{max} (log ε) 225 (3.87), 270(3.49), 308 (3.49), 396 (3.07) nm; with KOH λ_{max} (MeOH) (log ε) 214 (4.7), 243(3.8), 314 (3.4); IR (KBr) ν_{max} 3433 (OH) cm^{-1}; ^1H NMR and ^{13}C NMR, see Table 1; ESIMS m/z 337 $[M + H]^+$; HR-EIMS m/z 336.1108 (calcd for $C_{19}H_{16}N_2O_4Na$, 336.1110).

*Daibulactone A (**4**):* colorless needles (EtOAc); mp 200–202 °C (MeOH); $[\alpha]_D^{25}$ +39.4 (*c* 0.04, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 208 (4.35), 269 (4.45); IR (KBr) ν_{max} 1734 (lactonic carbonyl) cm^{-1}; ^1H NMR and ^{13}C NMR, see Table 2; ESIMS m/z 325 $[M + Na]^+$; HR-ESIMS m/z 325.1049 (calcd for $C_{17}H_{18}O_5Na$, 325.1052).

*Daibulactone B (**5**):* yellowish needles (EtOAc); mp 138–140 °C (MeOH); $[\alpha]_D^{25}$ −2.4 (*c* 0.04, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 214 (4.08); IR (KBr) ν_{max} 1738 (C=O) cm^{-1}; ^1H NMR and ^{13}C NMR, see Table 2.

*Daibuoxide (**6**):* colorless needles (acetone); mp 98–99 °C (MeOH); $[\alpha]_D^{26}$ −5.3 (*c* 0.05, $CHCl_3$); IR (KBr) ν_{max} 3401(OH) cm^{-1}; ^1H NMR and ^{13}C NMR, see Table 2; ESIMS m/z 239 $[M + H]^+$; HR-ESIMS m/z 261.1831 (calcd for 261.1830, $C_{15}H_{26}O_2Na$).

Anti-iNOS Activity Assay. RAW 264.7 cells (a transformed murine macrophage cell line), obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan), were maintained by once-weekly passage in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum and penicillin–streptomycin.

The nitrite measurement was based on our published technique.[29] Cell aliquots (5×10^5 cells/mL) were grown to confluence on 24-well plates for 24 h. The medium was changed to serum-free DMEM for another 4 h to render the attached cells quiescent. To assess the effects on LPS-induced NO production, the compounds and two positive controls, N^{ω}-nitro-L-arginine (a nonselective NOS inhibitor; 100 μM) and aminoguanidine (a selective iNOS inhibitor; 100 μM), or the vehicle, DMSO (0.1%), were added in the absence or presence of LPS (200 ng/mL) to the cells for another 24 h. The culture supernatant was subsequently collected and assayed for nitrite as a reflection of NO production.[30] Briefly, an aliquot of supernatant was mixed with an equal volume of Griess reagent (prepared by adding 1 part 0.1% naphthylethylenediamine dihydrochloride to 1 part 1% sulfanilamide in 5% H_3PO_4) and incubated at room temperature for 10 min. The absorbance at 550 nm was measured with a microplate spectrophotometer (Bio-Tek Instrument, Inc., Winooski, VT, USA). Fresh medium was used as the blank. The nitrite concentration was determined by reference to a standard curve, using $NaNO_2$ diluted in the stock culture medium. The results are expressed as the percentage of inhibition calculated relative to the cells treated with vehicle and LPS.

Cell Viability Assay. A redox indicator, AlamarBlue, was used to measure cytotoxicity, as previously described.[31] After the culture supernatant was removed for NO measurement as described above, a solution of 10% AlamarBlue in DMEM was added to each well containing RAW 264.7 cells. The plates were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 3 h. Following incubation, the absorbance of the AlamarBlue was read spectrophotometrically at dual wavelengths of 570 and 600 nm against the blank prepared from cell-free wells. The absorbance for cultures treated with LPS plus vehicle was considered to indicate 100% cell viability.

Statistical Analysis. For each experimental series, data are given as the mean ± SE, with *n* representing the number of independently performed experiments. All data were analyzed by an IBM-compatible statistical software package (SPSS for Windows, ver. 10.0). The significance of the concentrations and sample treatments was determined by two-way analysis of variance (ANOVA) with repeated measurements. For all significant interactions, the simple main effect of each factor was assessed using Kruskal–Wallis nonparametric ANOVA. Post hoc comparisons were carried out between means, according to the suitability. A *p* value of less than 0.05 was considered to indicate a statistically significant difference.

X-ray Crystallographic Study of Daibulactone A (4). Crystal data: $C_{17}H_{18}O_5$, M = 302.31, monoclinic system, space group $P2_1$, a = 7.3433(3) Å, b = 11.4642(4) Å, c = 9.1189(3) Å, β = 107.807(2)°, V = 730.90(5) Å^3, Z = 2, d = 1.374 g/cm^3. A crystal of dimensions 0.32 × 0.32 × 0.20 mm was used for low-temperature (150 K) measurements on a Bruker SMART diffractometer with a graphite monochromator (ϕ and ω scans, $2\theta_{max}$ = 52.0°), Mo Kα radiation (λ = 0.71073 Å). The total number of independent reflections measured was 2404, of which 2362 were observed ($|F|^2 \geq 2\sigma|F|^2$). The crystal structure was solved using the direct method (program Sir92[32]) and then expanded using difference Fourier techniques. The structure was further refined with the SHEXTL 97[32] program (Sheldrick, G. M., University of Göttingen, Göttingen, Germany, 1997) and full-matrix least-squares calculations. Final refinement on all 2404 reflections against 202 variables with one restraint gave the following final indices: R_f = 0.0260, R_w = 0.0637 ($w = 1/[\sigma^2(F_o^2) + (0.0335P)^2 + 0.1693P]$ where $P = (F_o^2 + 2F_c^2)/3$), GOOF = 1.068. Although the relative configuration of **4** has been established, the absolute configuration of **4** could not be determined with a Flack parameter[33] of 0.5(7). Copies of the deposited crystal data can be obtained free of charge from the Cambridge Crystallographic Data Center (CCDC) (784937; 12 Union Road, Cambridge CB2 1EZ, UK; fax: +44 (0) 1223 336033; e-mail: deposit@ccdc.cam.ac.uk).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

NMR spectra for compounds **1**–**6**. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENTS

This work was supported by a grant (NSC 99-2300-B-037-009) from the National Science Council of the Republic of China.

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