New Neolignan Component from *Camellia amplexicaulis* and Effects on Osteoblast Differentiation

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A new neolignan named "camellioside A" (1) has been isolated from the leaves and branches of *Camellia amplexicaulis*, along with seven known glycosides (2—8). Their structures were determined by a variety of spectroscopic analyses. Among them, compounds 2, 3, 4, and 6 significantly ($p<0.05$) increased the alkaline phosphatase activity and the mineralization of the nodules of the MC3T3-E1 osteoblastic cells compared to those of the control.

Key words *Camellia amplexicaulis*; Theaceae; neolignan; camellioside A; osteoblast differentiation; MC3T3-E1 cell

Osteoporosis is a major health concern for aging communities. The associated progressive decrease in bone mass leads to an increased susceptibility to fractures, which results in substantial morbidity and mortality.[1)] Bone tissue is constantly being resorbed and rebuilt in a coupled process known as remodeling. The bone remodeling process is controlled by the rates of bone formation and bone resorption.[2)] A rapid rate of bone resorption that exceeds the rate of bone formation results in bone abnormalities, such as osteoporosis. Since many osteoporotic patients have already lost a substantial amount of bone, a method of increasing bone mass by stimulating new bone formation is required.[3)]

In our ongoing study to find active natural compounds to differentiate MC3T3-E1 osteoblastic cells, potent activity was found in the MeOH extract of the aerial parts of *Camellia amplexicaulis* (Theaceae). This plant is only really known for its flowers, which are a favorite choice for New Year decorations. To date, there appears to have been no study that focussed on the phytochemistry and bioactivity of this plant.[4)] Based on a bioactivity-guided fractionation and isolation method, one new neolignan glycoside named "camellioside A" (**1**) has been isolated from the leaves and branches of *C. amplexicaulis*, along with seven known compounds including 7*S*,8*S*-4,9,9′-trihydroxy-3,3′-dimethoxy-8-*O*-4′-neolignan-7-*O*-β-D-glucopyranoside (**2**),[5)] 7*R*,8*R*-dihydrodehidrodiconiferyl alcohol-9′-*O*-β-D-glucopyranoside (**3**),[6)] urolignoside (**4**),[7)] junipetrioloside A (**5**),[8)] isolarciresinol (**6**),[9)] corchoionoside C (**7**),[10)] and blumeon C glycoside (**8**).[11)] This paper describes the isolation method, the structure elucidation of camellioside A, and the evaluation of the effects of compounds **1**—**8** on osteoblast differentiation in MC3T3-E1 cells.

Results and Discussion

Compound **1** was obtained as a white amorphous powder. Its HR-ESI-MS showed a $[M-H_2O+Na]^+$ peak at *m/z* 531.1824 (Calcd for $C_{25}H_{32}O_{11}Na$, 531.1842), corresponding to the molecular formula $C_{25}H_{34}O_{12}Na$ lost of a water molecule. The ^{1}H- and ^{13}C-NMR spectra of **1** displayed typical features that were similar to those of **2**, which was first isolated from *Lonicera gracilipes* var. *glandulosa* Maxim.[5)] This suggests that **1** was a 8-*O*-4′ neolignan-type glycoside. The ^{1}H-NMR (CD_3OD) spectrum of **1** showed six aromatic protons [δ 6.68 (dd, J=8.0, 2.0 Hz), 6.75 (d, J=2.0 Hz), 6.81 (d, J=8.0 Hz), 6.83 (d, J=8.0 Hz), 6.87 (dd, J=8.0, 2.0 Hz), 6.97 (d, J=2.0 Hz)] revealing two ABX proton systems of two 1,3,4-trisubstituted aromatic rings, one methoxy proton at δ 3.84 (s), two methylen protons [δ 1.87 (m, H-8′), 2.60 (t, J=6.4 Hz, H-7′)], two methine protons [δ 4.84 (over-

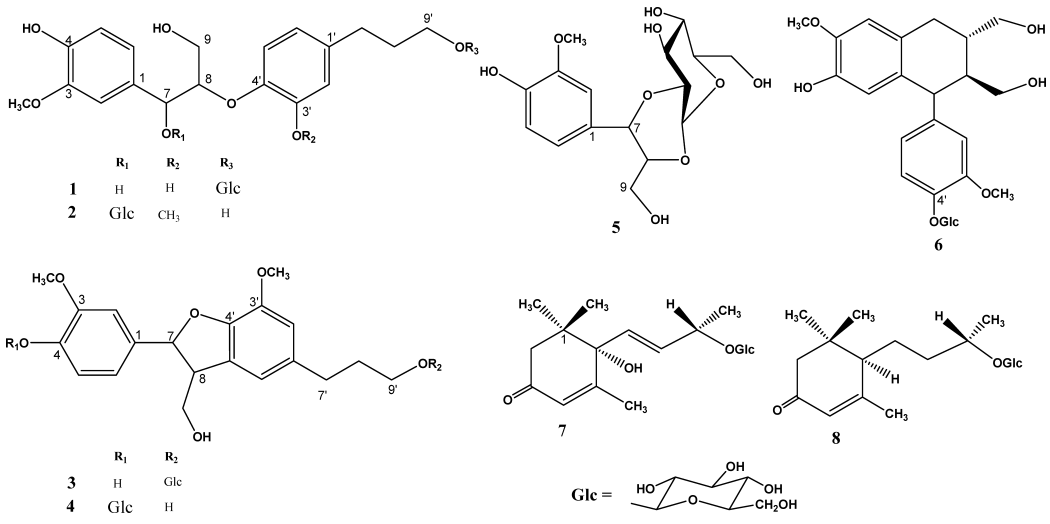

Fig. 1. Structures of **1**—**8**

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lapped, H-7), 3.98 (1H, m, H-8)], and a glucose anomeric proton at δ 4.22 (d, J=7.6 Hz, H-1″). However, in CD_3OD, the proton signal H-7 of **1** was buried in the solvent signal. Thereby, ^{1}H-NMR spectrum of **1** was checked in the other NMR solvent, DMSO-d_6; in which, the proton signal H-7 was appeared as a doublet at δ 4.96 (J=4.8 Hz), considerately. The ^{13}C-NMR (CD_3OD) spectrum of **1** showed 25 carbon signals, including one methoxy carbon at δ 55.3, 18 carbons of two phenyl propanoid units forming neolignan, and six carbons of an *O*-glucose unit (δ 103.2, 74.0, 76.9, 70.5, 76.7, 61.6).[12] In addition, it was possible to assign the carbon signals corresponding to each proton signal using the obtained HMQC spectrum. Furthermore, the partial structures of **1** were confirmed from COSY and HMBC NMR spectra; key correlations for these experiments are illustrated in Fig. 2. In the COSY spectrum, correlations of H-5/H-6, H-7/H-8, H-8/H-9, H-5′/H-6′, H-7′/H-8′, and H-8′/H-9′ were obsevered. The HMBC spectrum revealed correlations of H-7/C-1,2; H-8/C-4′; H-7′/C-1′,2′. Moreover, further analysis of the HMBC spectrum found the correlations from anomeric proton H-1″ to C-9′ at δ 68.7 and H-9′ to C-1″ at δ 103.2, indicating the linkage position with glucose unit at C-9′. The mode of glycosidic linkage were determined to be β form due to the large coupling constant of the anomeric proton doublet at δ 4.22 (J=7.6 Hz).

Enzymatic hydrolysis of **1** gave D-glucose (determined by a GC experiment) and an aglycone (**1a**). In the ^{1}H-NMR spectrum of **1a** in $CDCl_3$, the proton H-7 was a doublet with a large coupling constant (J=8.0 Hz) at δ 4.92. Braga *et al.* reported that large and small J values for H-7 and H-8 of 8-*O*-4′ neolignan diastereoisomers correspond to the *threo* and *erythro* forms, respectively, in terms of the possible staggered conformers with intramolecular hydrogen bonding of the benzylic hydroxyl and aryloxyl groups.[13] Accordingly, **1** was determined to have relative *threo* configuration. Furthermore, based on the CD spectrum the stereochemistry of C-7 and C-8 were both assigned to be *R* according to the study of related structures.[5,14—16] The CD spectrum of **1** showed a strong negative Cotton effect at 233 nm ($\Delta\varepsilon$ −14.45), opposite of that of 7*S*,8*S*-4,7,9,9′-tetrahydroxy-3,3′-dimethoxy-8-*O*-4′-neolignan-9′-*O*-β-D-glucopyranoside which reported recently from *Iodes cirrhosa*.[14] Based on these evidence, compound **1** has been identified as 7*R*,8*R*-3′,4,7,9-tetrahydroxy-3-methoxy-8-*O*-4′-neolignan-9′-*O*-β-D-glucopyranoside, which has been named "camellioside A".

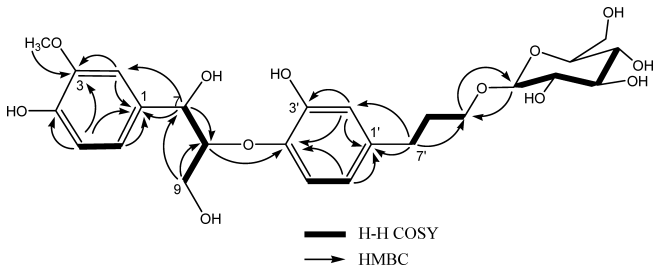

Fig. 2. Selected HMBC and COSY Correlations of **1**

To evaluate the effect of the isolated compounds on MC3T3-E1 differentiation, the alkaline phosphatase (ALP) activity and calcium deposition were examined. Osteoblastic MC3T3-E1 cells, at confluence, were cultured with a differentiation-inducing medium, and then incubated in a medium containing the individual isolated compounds. Among the eight compounds tested, compounds **2**, **3**, **4**, and **6** significantly increased the ALP activity. Compounds **3** and **4** showed the most potent activity. At concentrations of 0.01 μM, compounds **3** and **4** increased the ALP activity up to 152.2 and 142.3%, respectively, compared to that of the control and stronger than the positive control, 17β-estradiol. Compounds **1**, **5**, **7**, and **8** showed comparatively weaker effect (Table 1). The effect of the isolated compounds on mineralization was then examined by measuring the calcium deposition by Alizarin Red staining. As was found for the ALP activity study, compounds **2**, **3**, **4**, and **6** showed significant stimulatory effects on mineralization (Table 2). They increased the mineralization to 115, 118, 124, and 113%, respectively, compared to that of the control at a concentration of 10 μM. When the cell viability was measured using the MTT assay, no toxic effects were observed after incubation of the cells with 0.01 to 10 μM of the eight compounds (data not shown). This result indicated that the compounds were not toxic at the concentrations used in this study.

Of the eight isolated compounds, compounds **1**—**4**, and **6** belong to the lignan group. As the results, lignans **2**, **3**, **4**, and **6** showed more significant stimulatory effects in ALP activity and calcium deposition than did the rest of the compounds. Recently, lignans have received considerable attention due to their noteworthy role in stimulating osteoblast differentiation both in *in vitro* and *in vivo* studies.[17] For instance, lignans from *Machilus thunbergii* SIEB. *et* ZUCC. (Lauraceae) were found to increase osteoblast differentiation in primary cultures of mouse osteoblasts.[18] Another study reported that secoisolariciresinol diglycoside from flaxseed showed anti-

Table 1. Effects of Compounds **1**—**8** on the ALP Activity of MC3T3-E1 Cells

Compounds	ALP activity (% of control)			
	0.01 μM	0.1 μM	1.0 μM	10 μM
1	113.5±5.5*	112.0±13.6	100.9±4.3	102.6±1.9
2	110.7±7.3	117.9±5.1*	126.2±18.3*	114.8±13.2
3	152.2±8.8*	121.0±8.1*	120.8±3.1*	101.6±4.0
4	142.3±3.7*	136.9±12.9*	103.9±5.8	112.5±2.4*
5	129.6±15.1	102.9±2.9	121.0±12.1*	134.8±12.9
6	137.4±14.3*	137.9±12.4*	109.9±9.9	121.0±15.4
7	119.7±2.6	111.4±6.2	108.3±1.8	129.6±20.2*
8	103.6±1.8	110.1±8.8	114.0±3.3*	113.3±5.1*
17β-Estradiol[a)]	123.0±4.7*	114.0±6.5*	104.7±3.1	98.0±3.1

Data are the mean±S.D. (n=5), expressed as a percentage of the control. The control ALP activity was 0.193±0.001 Unit/10^6 cells. *a*) Positive control. * p<0.05 *vs.* control.

Table 2. Effects of Compounds **1**—**8** on the Mineralization of MC3T3-E1 Cells

Compounds	Mineralization (% of control)			
	0.01 μM	0.1 μM	1.0 μM	10 μM
1	104.7±5.3	103.5±5.4	102.7±4.0	109.7±6.3
2	100.3±3.9	105.2±1.5	106.3±1.8	115.0±3.5*
3	100.5±4.6	105.9±2.1	104.2±2.0	117.7±1.6*
4	107.8±2.1*	107.1±2.2*	113.4±3.4*	124.0±5.8*
5	100.5±1.7	101.3±1.9	105.2±3.5*	100.8±2.1
6	101.1±1.5	100.9±1.2	106.2±2.5	113.1±3.6*
7	101.4±1.2	102.7±1.3	104.7±2.2	103.4±1.7
8	105.9±3.9	101.6±2.3	101.9±3.4	105.4±4.2
17β-Estradiol[a)]	126.9±6.0*	124.3±7.7*	120.5±8.7*	114.6±12.8

Data are the mean±S.D. (n=5), expressed as a percentage of the control. The control mineralization value was 0.302±0.011 O.D. *a*) Positive control. * $p<0.05$ *vs.* control.

osteoporotic activity in postmenopausal women.[19] Also, isotaxiresinol from *Taxus yunnanensis* has been found to prevent bone loss in an ovariectomized model.[20] This current study suggests that, among the compounds obtainable from *C. amplexicaulis*, the lignans play an important role in promoting MC3T3-E1 osteoblast cell differentiation *in vitro*.

Experimental

General Experimental Procedures Optical rotations were determined using a JASCO DIP-360 digital polarimeter. UV spectra were recorded using a Beckman Du-650 UV–VIS recording spectrometer. CD spectra were recorded using a JASCO J-700 spectropolarimeter. IR spectra were measured by a Perkin-Elmer 577 spectrometer. NMR spectra were recorded on Bruker DRX 400 and 500 NMR spectrometers. The HR-ESI-MS spectra were obtained using a JMS-T100TD spectrometer with an electrospray ion source (Tokyo, Japan). Column chromatography (CC) was performed on silica gel (70—230 and 230—400 mesh, Merck), YMC RP-18 resins (30—50 μm, Fuji Silysia Chemical Ltd.), and HP-20 dianion (Mitshubishi Chemical Corporation). Thin layer chromatography (TLC) was performed on Kieselgel 60 F_{254} (Merck 1.05715) or RP-18 F_{254s} (Merck) plates. Spots were visualized by spraying the plates with 10% aqueous H_2SO_4 solution and heating them for 5 min.

Plant Material The leaves and branches of *C. amplexicaulis* were collected in Tamdao National Park, Vinhphuc province, Vietnam, in April 2006. They were identified by Dr. Tran Huy Thai, Institute of Ecology and Biological Resources, VAST. A voucher specimen (No. 20060408) was deposited at the Institute of Natural Products Chemistry, VAST.

Extraction and Isolation The air-dried sample of *C. amplexicaulis* (5 kg) were extracted with methanol (10 l×3 times). Evaporation of the combined MeOH extracts *in vacuo* gave a residue (300 g), which was then partitioned between CH_2Cl_2 and H_2O. The H_2O layer was fractionated over a Dianion HP-20 column eluted with H_2O and MeOH–H_2O (20%, 50%, 70%, and 100% MeOH) to give four fractions: 1A (9 g), 1B (18 g), 1C (40 g), and 1D (60 g). Fractions 1A and 1B were combined due to their similar TLC behaviour. They were then chromatographed over a silica gel column with CH_2Cl_2–MeOH (10 : 1—0 : 1), gradiently, to give five fractions (2A—E). Fraction 2C (8 g) was rechromatographed on a silica gel column with $CHCl_3$–MeOH–H_2O (70 : 30 : 4) to give six subfractions (3A—F). Compound **7** (12 mg) was obtained from subfraction 3A (30 mg) by silica gel column chromatography with $CHCl_3$–MeOH–H_2O (5 : 1 : 0.1). Fraction 2D (9 g) was chromatographed on a YMC RP-18 column (MeOH–H_2O, 4 : 1) to afford **5** (100 mg). Fraction 1D was subjected to silica gel column chromatography ($CHCl_3$–MeOH, 10 : 1—0 : 1) to give ten fractions (4A—J). Fraction 4A was purified using a YMC RP-18 column (MeOH–H_2O, 1 : 1.5) to give **8** (8.3 mg). Fraction 4D (3 g) was rechromatographed on a YMC RP-18 column (MeOH–H_2O, 1 : 4) to give **1** (30 mg) and **3** (10 mg). Fraction 4H (300 mg) was rechromatographed on a YMC RP-18 column (MeOH–H_2O, 1 : 2) to give **4** (5.2 mg). Fraction 4I was rechromatographed on a YMC RP-18 column (MeOH–H_2O, 1 : 1) to afford **2** (3.3 mg). Fraction 4J was rechromatographed on a YMC RP-18 column (MeOH–H_2O, 1 : 2) to give **6** (5.5 mg).

Camellioside A White amorphous powder; $[\alpha]_D^{25}$ +14.8 (c=0.3, MeOH); IR ν_{max} cm^{-1} (KBr): 3415, 1605, 1514, 1276, 1032; UV λ_{max} nm (log ε) (MeOH): 227 (4.2), 277 (3.8); CD nm ($\Delta\varepsilon$) (MeOH): 233 (−14.5); HR-ESI-MS m/z: 531.1824 $[M-H_2O+Na]^+$ (Calcd for $C_{25}H_{32}O_{11}Na$, 531.1842); ^{1}H-NMR (CD_3OD, 400 MHz) δ: 1.87 (2H, m, H-8′), 2.60 (2H, t, J=6.4 Hz, H-7′), 3.16 (1H, dd, J=7.6, 8.4 Hz, glc H-2″), 3.25—3.38 (3H, overlapped, glc H-3″,4″,5″), 3.44 (1H, dd, J=4.8, 12.4 Hz, H-9), 3.52 (1H, m, H-9′), 3.63 (1H, dd, J=6.4, 12.4 Hz, H-9), 3.65 (1H, dd, J=4.0, 12.4 Hz, glc H-6″), 3.82 (1H, m, glc H-6″), 3.84 (3H, s, 3-OCH_3), 3.88 (1H, m, H-9′), 3.98 (1H, m, H-8), 4.22 (1H, d, J=7.6 Hz, glc H-1″), 4.84 (1H, overlapped, H-7), 6.68 (1H, dd, J=2.0, 8.0 Hz, H-6′), 6.75 (1H, d, J=2.0 Hz, H-2′), 6.81 (1H, d, J=8.0 Hz, H-5), 6.83 (1H, d, J=8.0 Hz, H-5′), 6.87 (1H, dd, J=2.0, 8.0 Hz, H-6), 6.97 (1H, d, J=2.0 Hz, H-2); ^{1}H-NMR (DMSO-d_6, 400 MHz) δ: 1.76 (2H, m, H-8′), 2.32 (2H, t, J=6.4 Hz, H-7′), 3.76 (3H, s, 3-OCH_3), 4.04 (1H, m, H-8), 4.12 (1H, d, J=7.6 Hz, glc H-1″), 4.96 (1H, d, J=4.8 Hz, H-7), 6.68—6.98 (6H, aromatic protons); and ^{13}C-NMR (CD_3OD, 100 MHz) δ: 128.5 (C-1), 110.8 (C-2), 147.1 (C-3), 55.26 (3-O<u>C</u>H_3) 141.8 (C-4), 115.1 (C-5), 120.5 (C-6), 76.5 (C-7), 78.6 (C-8), 61.00 (C-9), 135.3 (C-1′), 116.6 (C-2′), 143.8 (C-3′), 148.0 (C-4′), 116.4 (C-5′), 121.3 (C-6′), 31.2 (C-7′), 31.5 (C-8′), 68.7 (C-9′), 103.2 (glc C-1″), 74.0 (glc C-2″), 76.9 (glc C-3″), 70.5 (glc C-4″), 76.7 (glc C-5″), 61.6 (glc C-6″).

Enzymatic Hydrolysis β-Glucosidase (10 mg, from almond) was added to a suspension of **1** (2 mg) in 50 mM phosphate buffer (5 ml) adjusted to pH 7.0 by NaOH. The resulting mixture was stirred at 37 °C for 48 h. The reaction mixture was extracted with EtOAc (5 ml×3), and the organic layer was evaporated *in vacuo*. The residue was subjected to preparative TLC with $CHCl_3$–MeOH (10 : 1) to afford aglycone **1a** (1 mg). The sugar residue, obtained by concentration of the water layer, was dissolved in dry pyridine (0.1 ml). Then L-cysteine methyl ester hydrochloride in pyridine (0.06 M, 0.1 ml) was added to the solution. After heating the reaction mixture at 60 °C for 2 h, 0.1 ml of trimethylsilylimidazole was added. Heating at 60 °C was continued for a further 2 h. The dried product was partitioned between hexane and H_2O. The hexane layer was analyzed by GC (Shimadzu-2010) using a DB-05 capillary column (0.5 mm ID×30 m) (column temperature: 210 °C; detector temperature: 300 °C; injector temperature: 270 °C; He gas flow rate: 30 ml/min).[21] The peak of the hydrolysate of **1** was detected at t_R 14.12 min for D-glucose. The retention times for the authentic samples (Sigma), after being treated in the similar manner, were 14.12 min (D-glucose) and 14.25 min (L-glucose). Co-injection of the hydrolysates of **1** with standard D-glucose gave single peaks. **1a**: ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 1.90 (2H, m, H-8′), 2.64 (2H, t, J=7.2 Hz, H-7′), 3.86 (3H, s, OCH_3), 4.02 (1H, m, H-8), 4.92 (1H, d, J=8.0 Hz, H-7), 6.60—7.00 (6H, aromatic protons).

Cell Culture MC3T3-E1 cells (RCB1126, an osteoblast-like cell line from C57BL/6 mouse calvaria) were obtained from the RIKEN Cell Bank (Tsukuba, Japan), and cultured at 37 °C in a 5% CO_2 atmosphere, in an α-modified minimal essential medium (α-MEM; GIBCO). Unless otherwise specified, the medium contained 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Alkaline Phosphatase Activity The cells were treated, at 90% confluence, with a culture medium containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid to initiate *in vitro* mineralization. The medium was changed every 2—3 d. After 6 d, the cells were cultured with a medium containing 0.3% bovine serum albumin (BSA) and the isolated compounds for 3 d. On harvesting, the medium was removed and the cell monolayer was gently washed twice with phosphate buffered saline (PBS). The cells were lysed with 0.2% Triton X-100, with the lysate centrifuged at 14000×g for 5 min. The clear supernatant was used to measure the ALP activity, which was determined using an ALP activity assay kit (Asan Co., Korea).

Calcium Deposition Assay The cells were treated, at 90% confluence, with a culture medium containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid to initiate *in vitro* mineralization. After 10 d, the cells were cultured with a medium containing 0.3% BSA and the isolated compounds individually for 3 d. On harvesting, the cells were fixed with 70% ethanol for 1 h, and then stained with 40 mM Alizarin Red S for 10 min with gentle shaking. To quantify the bound dye, the stain was solubilized with 10% cetylpyridinum chloride by shaking for 15 min, while shielded from light. The absorbance of the solubilized stain was measured at 561 nm.

Statistics The results are expressed as the mean±S.D. ($n=5$). Statistical analysis was performed using a one-way ANOVA ($p<0.05$) with SAS statistical software.

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