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 focused 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 7α,8β,9α,9β-trihydroxy-3,3′-dimethoxy-8-O-4′-neolignan-7-α-β-d-glucopyranoside (2), 7R,8R-dihydrodihydrodiconiferyl alcohol-9′-O-β-d-glucopyranoside (3), urolignoside (4), junipetrioside A (5), isolarctiresinol (6), corchoicoside C (7), and blumeon C glycoside (8).5) 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−H2O+Na]⁺ peak at m/z 531.1824 (Caled for C25H32O11Na, 531.1842), corresponding to the molecular formula C25H34O12Na lost of a water molecule. The 1H- and 13C-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-glucopyranosyl-type glycoside. The 1H-NMR (CD3OD) 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 methylene protons [δ 1.87 (m, H-8), 2.60 (t, J = 6.4 Hz, H-7′)], two methine protons [δ 4.84 (over-
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 CD3OD, the proton signal H-7 of 1 was buried in the solvent signal. Thereby, 1H-NMR spectrum of 1 was checked in the other NMR solvent, DMSO-d6, in which, the proton signal H-7 was appeared as a doublet at δ 4.96 (J=4.8 Hz), correspondingly. The 13C-NMR (CD3OD) 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). In addition, it was possible to assign the carbon signals corresponding to each proton signal using the obtained HMBC 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 observed. The HMBC spectrum revealed correlations of H-7/C-1,2; H-8/C-4; H-5′/C-1′,2′. Moreover, further analysis of the HMBC spectrum found the correlations from anomic 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 anomic 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 1H-NMR spectrum of 1a in CDCl3, 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′-neolignan diastereoisomers correspond to the threo and erythro forms, in terms of the possible staggered conformers with intramolecular hydrogen bonding of the benzylid hydroxyl and arylxyl groups. 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. The CD spectrum of 1 showed a strong negative Cotton effect at 233 nm (Δε = -14.45), opposite of that of 7S,8S,4,7,9,9′-tetrahydroxy-3′,5′-dimethoxy-8-O′-neolignan-9′-O-β-D-glucopyranoside which reported recently from Iodes cirrhosa. Based on these evidence, compound 1 has been identified as 7R,8R-3′,4,7,9-tetrahydroxy-3-methoxy-8-O′-neolignan-9′-O-β-D-glucopyranoside, which has been named “camellioside A”.

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 and stronger than the positive control, 17β-estradiol.

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 vitro and in vivo studies. For instance, lignans from Machilus thunbergii Sieb. et Zucc. (Lauraceae) were found to increase osteoblast differentiation in primary cultures of mouse osteoblasts. Another study reported that secoisolariciresinol diglycoside from flaxseed showed anti-

![Fig. 2. Selected HMBC and COSY Correlations of 1](image-url)
osteoporotic activity in postmenopausal women.\(^{(19)}\) Also, iso-
taxiresinol from *Taxus yunnanensis* has been found to pre-
vent 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 pro-
moting 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 a Bruker DRX 400 and 500 NMR spectrometers. The **HR-ESI-MS** spectra were recorded 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 (30–60 

**Extraction and Isolation** The air-dried sample of *C. amplexicaulis* (5 kg) was extracted with methanol (101×3 times). Evaporation of the com-

**Plant Material** The leaves and branches of *C. amplexicaulis* were collected in Tamda National Park, Vinhphuc province, Vietnam, in April 2006. They were identified by Dr. Tran Huy Thai, Institute of Ecology and

**Chemical Methods**

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

<table>
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<tr>
<th>Compounds</th>
<th>Mineralization (% of control)</th>
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<tbody>
<tr>
<td></td>
<td>0.01 mm</td>
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<tr>
<td>1</td>
<td>104.7±5.3</td>
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<tr>
<td>2</td>
<td>100.3±3.9</td>
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<tr>
<td>3</td>
<td>100.5±4.6</td>
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<tr>
<td>4</td>
<td>107.8±2.1*</td>
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<tr>
<td>5</td>
<td>100.5±1.7</td>
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<tr>
<td>6</td>
<td>101.1±1.5</td>
</tr>
<tr>
<td>7</td>
<td>101.4±1.2</td>
</tr>
<tr>
<td>8</td>
<td>105.9±3.9</td>
</tr>
<tr>
<td>17β-Estradiol(^{22})</td>
<td>126.9±6.0*</td>
</tr>
</tbody>
</table>

Data are the mean±S.D. (n=5), expressed as a percentage of the control. The control mineralization value was 302±0.011 O.D. a) Positive control. p<0.05 vs. control.

### Results and Discussion

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

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Calcium Deposition Assay  The cells were treated, at 90% confluence, with a culture medium containing 10 mM \( \beta \)-glycerophosphate and 50 \( \mu \)g/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% cetylpyridinium 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 \pm S.D. (n=5). Statistical analysis was performed using a one-way ANOVA \((p<0.05)\) with SAS statistical software.

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References