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Osteoblast differentiation stimulating activity of biflavonoids from *Cephalotaxus koreana*

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Abstract—Six amentoflavone-type biflavonoids, bilobetin (**1**), ginkgetin (**2**), 4′,7″-di-*O*-methyl-amentoflavone (**3**), 7-*O*-methyl-isoginkgetin (**4**), sciadopitysin (**5**), and 7,4′,7″,4‴-*O*-methyl-amentoflavone (**6**), were isolated from the EtOAc fraction of *Cephalotaxus koreana* Nakai (Cephalotaxaceae) by bioactivity-guided fractionation technique using primary cultures of mouse osteoblasts as an in vitro assay system. Among the six biflavonoids isolated, bilobetin (**1**), sciadopitysin (**5**), and 7,4′,7″,4‴-*O*-methyl-amentoflavone (**6**) significantly increased osteoblast differentiation as assessed by alkaline phosphatase activity, collagen synthesis, and mineralization. Considering structure–activity relationship, methoxyl groups at 4′ and 4‴ in the B rings in amentoflavone-type biflavonoid might be important in osteoblast differentiation. Taken together, our present study suggests therapeutic potential of biflavonoids against bone diseases such as osteoporosis.

As the lifespan has increased, osteoporosis has become one of the major health problems. Osteoporosis is characterized by lower bone density and a decrease in bone mass which result in increased bone fragility and fracture risk.[1] Osteoporosis is known to occur due to a decrease in bone formation by osteoblasts and an increase in bone resorption by osteoclasts. Therefore, stimulation of bone formation and inhibition of bone resorption have been suggested to be an important therapeutic approach for prevention and/or treatment of osteoporosis.[2]

Osteoblasts play a crucial role in bone formation through the proliferation and differentiation.[3] Osteoblast differentiation, an important process for its function, confers marked rigidity and strength to the bone while still maintaining some degree of elasticity. During differentiation, osteoblast produces an extracellular matrix mainly type I collagen, which becomes mineralized by deposition of calcium. Other characteristics include upregulation of alkaline phosphatase (ALP) which is an important trigger for calcium deposition in vivo and in vitro.[4] For in vitro studies, osteoblasts isolated from calvaria of newborn animals were widely used for osteoblast differentiation because of its relatively pure population. The differentiation of osteoblast can be easily induced by ascorbic acid and also can be regulated by diverse factors such as transcriptional factors and exogenous chemicals. Therefore, we employed primary cultures of mouse calvarial osteoblast isolated from newborns as an in vitro assay system for osteoblast differentiation.[5]

In the course of screening of natural products having the stimulatory activity on osteoblast differentiation, the methanolic extract of the leaves and twigs of *Cephalotaxus koreana* Nakai (Cephalotaxaceae) was found to significantly increase osteoblast differentiation in vitro. Alkaloids, flavonoids, biflavonoids, and diterpenes were reported as constituents of *Cephalotaxus* species.[6] To date, however, there were no previous studies on osteoblast differentiation of *C. koreana*. Thus, we have attempted to isolate compounds having stimulating activity on osteoblast differentiation from *C. koreana*.

Keywords: Amentoflavone-type biflavonoid; *Cephalotaxus koreana*; Osteoblast differentiation; Alkaline phosphatase; Collagen; Mineralization; Osteoporosis.
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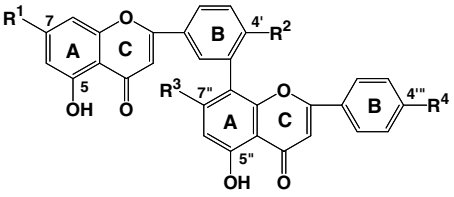
Bioassay-guided fractionation of the methanolic extract of the leaves and twigs of *C. koreana* revealed that the EtOAc fraction was the most active one (34.5% increase of ALP activity at 10 μg/ml, $p < 0.05$). Further fractionations and separation of the EtOAc fraction by several chromatographic methods yielded six biflavonoids, compounds **1**–**6**.[7] Compounds **1**–**6** were identified as bilobetin, ginkgetin, 4′,7″-di-*O*-methyl-amentoflavone, 7-*O*-methyl-isoginkgetin, sciadopitysin, 7,4′,7″,4‴-*O*-methyl-amentoflavone, respectively (Fig. 1), by the direct comparison of their physicochemical and spectroscopic data with those of previously reported.[8] Among these, compounds **3** and **4** are first reported from this plant.

Flavonoids are polyphenolic compounds which show many biological and pharmacological activities, including antioxidative, anti-inflammatory, antitumor, and neuroprotective effects.[9] Related to bone, soybean isoflavonoids such as daidzein and genistein have received considerable attention for their potential role in preventing postmenopausal bone loss.[10] Recently, it has been suggested that flavonoids may have beneficial effect on bone health.[11] Biflavonoids consist of a dimer of flavonoids that linked to each other. Although biflavonoids are known to exert diverse activities, such as anti-inflammatory, anti-viral activity, and anti-tuberculosis,[12] there has been no report on the effects of biflavonoids on osteoblast differentiation. Therefore, we further assessed the effects of six biflavonoids isolated from *C. koreana* on osteoblast differentiation in primary cultures of mouse calvarial osteoblasts as an in vitro assay system.

The effect of biflavonoids on osteoblast differentiation was first assessed by measuring the ALP activity in primary cultures of mouse osteoblasts.[13] To test the effects of biflavonoids on osteoblast differentiation, osteoblast differentiation was induced by changing the medium containing 50 μg/ml ascorbic acid and cells were then treated with vehicle or compounds to be tested for 7 days.[14] As expected, treatment of primary cultured osteoblasts with ascorbic acid for 7 days increased ALP activity almost 10 times, whereas weak increase in ALP activity was observed in nontreated control cells (data not shown). As shown in Table 1, among the six biflavonoids tested, compounds **1**, **4**, **5**, and **6** significantly increased the ALP activity at concentrations ranging from 1.0 to 20.0 μM. However, compounds **2** and **3** showed cytotoxicity at a concentration of 20.0 μM as measured by MTT assay (data not shown), which resulted in the decrease in ALP activity. The effect of compounds **1**, **4**, **5**, and **6** on ALP activity was also visualized by ALP staining.[15] As shown in Figure 2, ALP staining was consistent with ALP activity.

Since biflavonoids **1**, **4**, **5**, and **6** significantly increased ALP activity in primary cultures of mouse osteoblasts, we further investigated their effects on collagen synthesis.[16] Collagen content was quantified by Sirius red-based colorimetric assay.[17] As shown in Figure 3, compounds **1**,



Compounds	R^1	R^2	R^3	R^4
1	OH	OCH_3	OH	OH
2	OCH_3	OCH_3	OH	OH
3	OH	OCH_3	OCH_3	OH
4	OCH_3	OCH_3	OCH_3	OH
5	OCH_3	OCH_3	OH	OCH_3
6	OCH_3	OCH_3	OCH_3	OCH_3

Figure 1. Biflavonoids isolated from the leaves and twigs of *Cephalotaxus koreana*.

Table 1. Effects of biflavonoids isolated from *Cephalotaxus koreana* on the ALP activity in primary cultures of mouse osteoblasts

Compounds	ALP activity[b] (% of control)		
	1.0 (μM)	10.0 (μM)	20.0 (μM)
Control[a]		100.0 ± 1.4	
Bilobetin (**1**)	100.9 ± 8.3	131.9 ± 18.7**	130.6 ± 14.7**
Ginkgetin (**2**)	116.8 ± 18.3	69.3 ± 13.1	39.9 ± 15.7
4′,7″-Di-*O*-methyl-amentoflavone (**3**)	101.1 ± 13.3	113.0 ± 13.4	73.5 ± 16.2
7-*O*-Methyl-isoginkgetin (**4**)	108.4 ± 9.6	129.7 ± 19.9*	122.0 ± 15.8*
Sciadopitysin (**5**)	121.1 ± 16.6*	149.0 ± 14.1***	153.0 ± 18.5***
7,4′,7″,4‴-*O*-Methyl-amentoflavone (**6**)	105.1 ± 14.4	147.3 ± 5.0***	149.5 ± 9.2***
Daidzein[c]	107.8 ± 9.2	132.8 ± 8.2***	128.1 ± 5.7**

The values are expressed as means ± SD of triplicate experiments. Mean value is significantly different. (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) from the control.

[a] ALP activity of control was 19.3 ± 0.3 U/mg protein.

[b] ALP activity (%) was calculated as 100 × (ALP activity of compound-treated/ALP activity of control).

[c] Daidzein was used as a positive control.

* $p < 0.05$.

** $p < 0.01$.

*** $p < 0.001$.

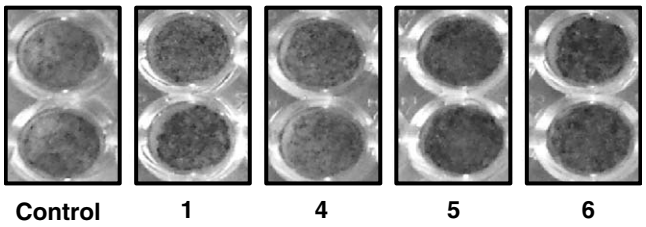


Figure 2. Effect of biflavonoids on ALP staining in primary cultures of mouse osteoblasts. Cultures were treated with biflavonoids in the presence of 50 μg/ml ascorbic acid for 1 week and subjected to ALP staining. The figures are representative of three independent experiments with similar results.

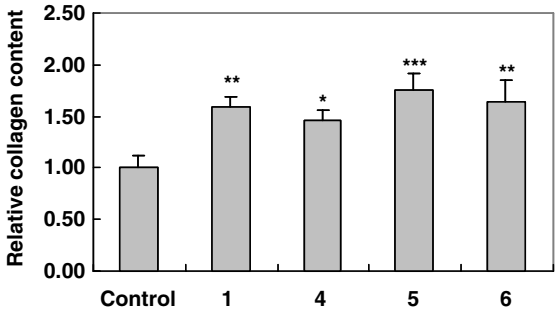


Figure 3. Effect of biflavonoids on collagen synthesis in primary cultures of mouse osteoblasts. Cultures were treated with biflavonoids in the presence of 50 μg/ml ascorbic acid for 2 weeks and collagen content was quantified by Sirius red-based colorimetric assay. The values are expressed as means ± SD of triplicate experiments. Mean value is significantly different (*$p < 0.05$, **$p < 0.01$, and *** $p < 0.001$) from the control.

5, and **6** significantly increased collagen synthesis. At a concentration of 10.0 μM, compound **5** increased collagen synthesis up to 180% compared to that of control cells.

Next, we examined the effects of biflavonoids on mineralization by measuring the calcium deposition, another important process in differentiation.[18] The degree of mineralization was determined by Alizarin red staining.[19] Consistent with the effects on ALP activity and collagen synthesis, compounds **1**, **5**, and **6** showed significant stimulatory effect on mineralization (Fig. 4).

All the six biflavonoids isolated from *C. koreana* are classified as amentoflavone-type and only differ in the number of methoxyl groups at 7 and 7″ in A rings and at 4′ and 4‴ in B rings. Regarding the structure–activity relationship, compound **1** with a methoxyl group at 4′ in the B ring significantly increased osteoblast differentiation. Compounds **5** and **6** with two methoxyl groups at 4′ and 4‴ in the B rings increased osteoblast differentiation higher than compound **1**. On the other hand, addition of a methoxyl group on 7 and 7″ in A rings decreased osteoblast differentiation, as observed in compounds **2** and **3**. Although more derivatives should be assessed for relevant relationship between the structure and activity, our study suggested that methoxyl groups at 4′ and 4‴ in the B rings might be important in osteoblast differentiation.

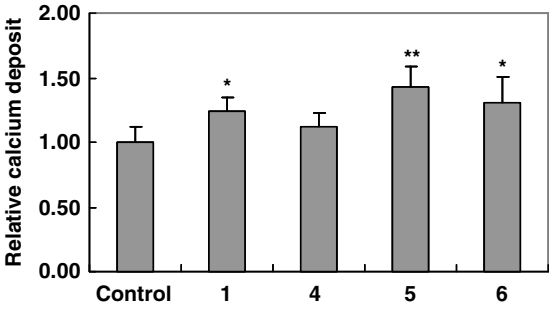


Figure 4. Effect of biflavonoids on mineralization in primary cultures of mouse osteoblasts. Cultures were treated with biflavonoids in the presence of 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate for 2 weeks and mineralization was quantified by Alizarin red staining. The values are expressed as means ± SD of triplicate experiments. Mean value is significantly different (*$p < 0.05$ and **$p < 0.01$) from the control.

Osteoblast differentiation includes various characteristics in time-dependent manner: increase in ALP activity, followed by ECM synthesis, resulting in mineralization. ALP, which hydrolyzes the ester bond of organic phosphate compounds under alkaline conditions, plays an important role in the calcification of bond. ALP produces phosphate required for mineralization and, in addition, hydrolyzes substances that inhibit calcification.[4] Differentiated osteoblasts also produce collagen, a major constituent of extracellular matrix in bone, which is further mineralized by calcium deposition. In our present study, compounds **1**, **5**, and **6** increased ALP activity as well as collagen synthesis and calcium deposition. Therefore, we suggest that high ALP activity by compounds **1**, **5**, and **6** might further promote mineralization. In addition, mineralization was also achieved via increased collagen synthesis.

In conclusion, our data suggest that biflavonoids isolated from *C. koreana* increased osteoblast differentiation in vitro. To our knowledge, this is the first report to suggest that naturally occurring biflavonoids increased osteoblast differentiation. Thus, it will be of interest to test further whether these biflavonoids increase osteoblast differentiation in vivo, for example, in animal models of osteoporosis, to explore their therapeutic potentials for osteoporosis. This will provide further insight into the design of new approaches to osteoporosis.

Acknowledgment

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.03.018.

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7. The air-dried leaves and twigs of *C. koreana* (5 kg) were extracted three times with 80% MeOH and yielded the methanolic extract (460 g). The methanolic extract was suspended in H_2O and partitioned successively with *n*-hexane, $CHCl_3$, EtOAc, and *n*-BuOH. The EtOAc fraction (40 g) which significantly increased ALP activity was chromatographed on silica gel (Kiesgel 60, 40–60 μm, 230–400 mesh, Merck) column with a $CHCl_3$–MeOH step gradient to give nine fractions (Fr. 1 to Fr. 9). Fr. 3 was rechromatographed on an ODS RP (Lichroprep ODS RP-18, 40–63 μm, EM science) with a H_2O–MeOH to give six fractions (Fr. 3–1 to Fr. 3–6). Compounds **1** (4 mg), **5** (5 mg), and **6** (5 mg) were isolated from Fr. 3–1 by repeated semi-preparative HPLC (Microsorb C_{18} 80–299, 10×250 mm, AcCN/MeOH/H_2O 50:20:30, 2 ml/min, rt: 9.46, 26.28, and 27.40 min, respectively). Fr. 4 was applied on an ODS RP column using H_2O–MeOH and repeated semi-preparative HPLC (Microsorb C_{18} 80–299, 10×250 mm, AcCN/MeOH/H_2O 50:20:30, 2 ml/min, rt: 14.30 and 13.04 min, respectively) to yield compounds **2** (15 mg) and **3** (4 mg). Fr. 5 was applied on Sephadex LH-20 (25–100 μm, Pharmacia) using MeOH to yield compound **6** (4 mg). The purity of each compound was higher than 95.0%, respectively, as measured by HPLC.
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13. For the assessment of ALP activity, cells were rinsed with phosphate-buffered saline and lysed in 0.01% sodium dodecyl sulfate in PBS followed by sonication. After clarification by centrifugation, cell lysates were assayed for ALP activity using the Alkaline Phosphate Assay Kit (Youngdong Pharmaceutical Co., Korea). Each value was normalized with the protein content of cell lysate, measured using bicinchoninic acid with bovine serum albumin as a standard. The evaluation of statistical significance was determined by the one-way ANOVA with a value of $p < 0.05$ or less considered to be statistically significant.
14. Biflavonoids to be tested were dissolved in dimethylsulfoxide (DMSO). Our preliminary study showed that DMSO at a final concentration of 0.1% in media did not affect the cell viability or differentiation. Osteoblast differentiation was induced by changing the medium containing 50 μg/ml ascorbic acid and cells were then treated with vehicle or compounds to be tested. After 3 days, medium was changed with fresh medium containing each compound and maintained for further 4 days.
15. (a) For ALP staining, cells were rinsed with PBS and fixed in 100% methanol for 1 h at room temperature. Cells were rinsed with PBS and stained with 0.15 mg/ml 5-bromo-4-chloro-3-indolylphosphate plus 0.3 mg/ml nitroblue tetrazolium chloride in 0.1 M Tris–HCl, 0.01 N NaOH, and 0.05 M $MgCl_2$ for 2 h in the dark; (b) Sowa, H.; Kaji, H.; Yamaguchi, T.; Sugimoto, T.; Kazuo, C. *J. Biol. Chem.* **2002**, *39*, 36204.
16. To evaluate the effects of biflavonoids on collagen synthesis, osteoblasts were treated with vehicle or biflavonoids to be tested in the presence of ascorbic acid and grown for 2 weeks by changing the medium containing compounds every 3 days.
17. (a) Cultured osteoblasts were washed with PBS, followed by fixation with Bouin's fluid for 1 h. After fixation, the fixation fluid was removed and the culture dishes were washed by immersion in running tap water for 15 min. The culture dishes were air-dried and stained by Sirius red dye reagent for 1 h under mild shaking on a shaker. Thereafter, the solution was removed and the cultures were washed with 0.01 N HCl to remove nonbound dye. The stained material was dissolved in 0.1 N NaOH and absorbance was measured at 550 nm against 0.1 N NaOH as a blank; (b) Tullberg-Reinert, H.; Jundt, G. *Histochem. Cell Biol.* **1999**, *112*, 271.
18. To evaluate the effects of biflavonoids on calcium deposition, osteoblasts were treated with vehicle or biflavonoids to

be tested in the presence of 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate, and grown for 2 weeks by changing the medium containing compounds every 3 days.
19. For Alizarin red staining, the cells were rinsed with PBS and fixed with ice-cold 70% ethanol for 1 h. The ethanol was removed and rinsed with deionized water. The cells were then stained with 40 mM Alizarin red S in deionize d water (adjusted to pH 4.2) for 10 min at room temperature. The Alizarin red S solution was removed and rinsed with deionized water and PBS. The stained material was extracted in DMSO and the absorbance was measured at 562 nm.