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Osteogenic activity of diphenyl ether-type cyclic diarylheptanoids derived from *Acer nikoense*

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ABSTRACT

Osteogenic activity of six diarylheptanoids, acerogenin A (**1**), (*R*)-acerogenin B (**2**), aceroside I (**3**), aceroside B_1 (**4**), aceroside III (**5**) and (−)-centrolobol (**6**) and two phenolic compounds; (+)-rhododendrol (**7**) and (+)-cathechin (**8**), isolated from the stem bark of *Acer nikoense* (Nikko maple) was evaluated using alkaline phosphatase (ALP) activity as a marker for early osteoblast differentiation. We found that the diphenyl ether-type cyclic diarylheptanoids **1**–**5** promoted ALP activity in mouse preosteoblastic MC3T3-E1 cells without affecting cell proliferation, but linear-type diarylheptanoid **6** and phenolic compounds **7** and **8** did not. Diphenyl ether-type cyclic diarylheptanoids **1**–**4** also increased protein production of osteocalcin, a late stage maker for osteoblast differentiation, and induced osteoblastic mineralization. Structure–activity relationships of these compounds demonstrated that the stimulative efficacy of aglycones was higher than that of its glycosides. Taken together, diphenyl ether-type cyclic diarylheptanoids promote early- and late-stage osteoblastogenesis, which may open the possibility for the development of novel osteogenic agents.

Bone mass is maintained by continuous remodeling of osteoblastic bone formation and osteoclastic bone resorption. Excessive bone resorption is caused by imbalances in these processes, which cause several bone-decreasing diseases, such as osteoporosis, rheumatoid arthritis and periodontitis.[1] Some drugs based on the suppression of bone resorption, including bisphosphonates and selective estrogen receptor modulators, are available, but anabolic drugs that promote osteoblastogenesis and bone formation are insufficient for effective treatment of these diseases. Osteoblasts are derived from mesenchymal stem cells that can also differentiate into chondrocytes, adipocytes and myoblasts.[2] Osteoblasts produce alkaline phosphatase (ALP) and various bone matrix proteins such as osteocalcin and osteopontin, leading to mineralization. Osteoblast differentiation is regulated by many cytokines, including bone morphogenetic proteins (BMPs), which are potent inducers of osteoblastogenesis.[3] Therefore, compounds mimicking the action of BMPs may be useful as bone anabolic drugs.

The stem bark of *Acer nikoense* Maxim (Aceraceae; commonly: Nikko maple) has been used as a folk medicine for the treatment of hepatic disorders and eye diseases. Various diarylheptanoids and phenolic compounds have been identified from the stem bark of *Acer nikoense* and many of their biologic activities reported, such as the inhibition of nitric oxide production in macrophages, degranulation in basophilic cells, Na^+-glucose co-transporter, and so on.[4–9] We also reported previously that the constituents from *Acer nikoense* inhibit 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice, and melanogenesis in B16 melanoma cells.[10,11]

In the present study, we investigated the osteogenic activity of the six diarylheptanoids; acerogenin A (**1**), (*R*)-acerogenin B (**2**), aceroside I (**3**), aceroside B_1 (**4**), aceroside III (**5**), (−)-centrolobol (**6**) and two phenolic compounds, (+)-rhododendrol (**7**) and (+)-cathechin (**8**), which were isolated from *Acer nikoense* (Fig. 1).[10,11] To evaluate the effects of these compounds on osteoblast differentiation, mouse preosteoblastic MC3T3-E1 cells were cultured in the presence of these compounds at 30 μM, or BMP-2, a positive control,

Abbreviations: ALP, alkaline phosphatase; αMEM, alpha minimum essential medium; BMPs, bone morphogenetic proteins; FBS, fetal bovine serum; MAPK, mitogen-activated protein kinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; TRAP, tartrate-resistant acid phosphatase.

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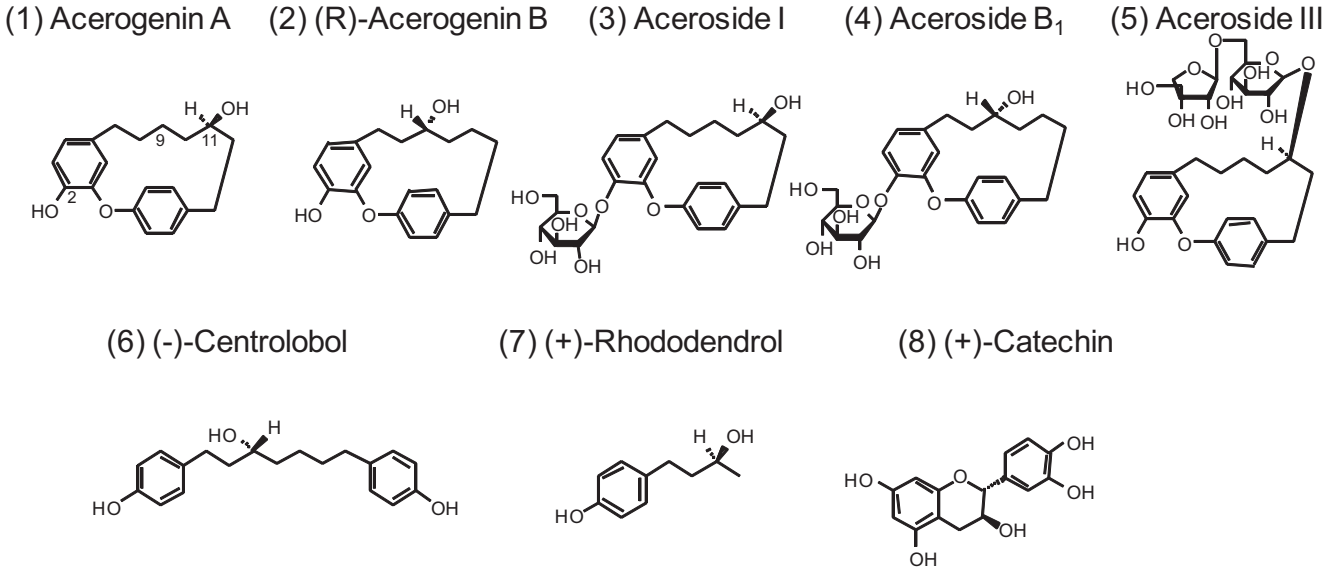

Figure 1. Chemical structures of the compounds isolated from *Acer nikoense* (Nikko maple).

at 50 ng/ml, for 6 days. Alkaline phosphatase activity was measured as a marker of early-stage osteoblastogenesis.[12]

We found that diphenyl ether-type cyclic diarylheptanoids **1**–**5** significantly increased ALP activity; treated cells were strongly stained for ALP to a similar extent as the BMP-2-treated cells without affecting total cell number (Fig. 2). The ALP activity was dose-dependently increased by compounds **1**–**5** (Supplementary Fig. 1). However, linear diarylheptanoid **6** exhibited cytotoxicity, while the phenolic compounds, **7** and **8,** had no effect on either ALP activity or cell number (Fig. 2). Even at lower concentrations where cytotoxicity was not shown, compound **6** did not promote ALP activity (data not shown).

The effect of compound **1** was confirmed in primary calvarial osteoblasts. Compound **1** dose-dependently promoted the ALP activity, and also enhanced staining for ALP in mouse primary osteoblasts (Supplementary Fig. 2). Recently, we reported on the action

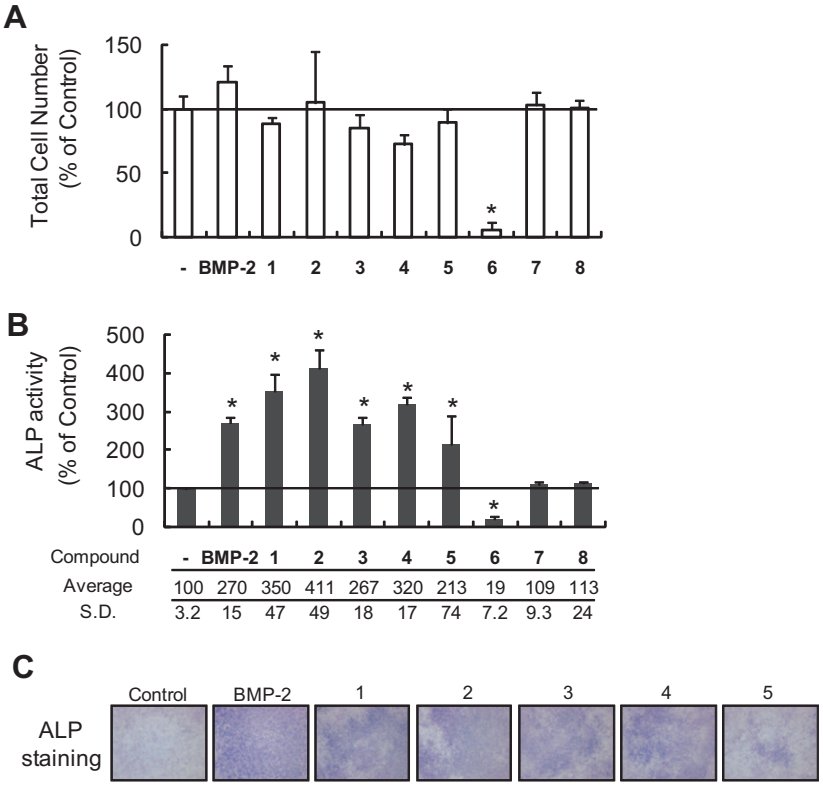

Figure 2. Effects of the compounds **1**–**8** on cell number and ALP activity in MC3T3-E1 cells. The MC3T3-E1 cells were cultured with compounds **1**–**8** (30 μM) or with BMP-2 (50 ng/ml) for 6 day. Total cell number (A) and ALP activity (B) were then measured and the cells were stained for ALP (C). Data represent means ± SD of more than three cultures. *P <0.05 versus the control. The photographs show representative views of three or more independent experiments.

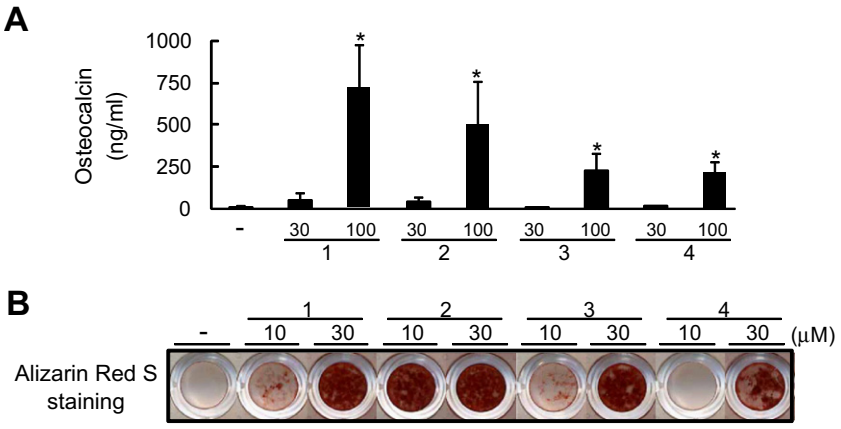

Figure 3. Effects of compounds **1**–**4** on the production of osteocalcin and mineralization in MC3T3-E1 cells. The MC3T3-E1 cells were cultured with compounds **1–4** in the presence of ascorbic acid (50 μg/ml) and β-glycerophosphate (10 mM) for 14 days. The concentration of osteocalcin in cultured media was measured using a mouse osteocalcin EIA kit (A). The cells were fixed and the mineralized matrix was stained with 1% Alizarin red S solution (B). The data represent means ± SD of three or more cultures. $^{*}P$ <0.05 versus the control. The photographs show representative views of three or more independent experiments.

of compound **1** on ALP activity and marker gene expression only.[13] Here, the effects of its derivatives and glycosides on early- and late-stage osteoblastogenesis and osteoblastic mineralization were further investigated.

The protein production of osteocalcin, a late-stage maker for osteoblast differentiation, was also evaluated using mouse osteocalcin EIA kit (Biomedical Technologies, Inc., Stoughton, MA). Compounds **1**–**4** significantly increased concentration of osteocalcin in the cultured media (Fig. 3A). Next, mineralization capacity was evaluated using Alizarin red S staining for calcium to assess the osteoblast function.[14] As a result, compounds **1**–**4** dose-dependently and dramatically stimulated mineralization in MC3T3-E1 cells (Fig. 3B). These results demonstrate that diphenyl ether-type cyclic diarylheptanoids promote not only early-stage but also late-stage osteoblastogenesis, and induce osteoblastic mineralization.

The position of hydroxyl groups at C9 and C11 may be changeable for osteogenic action, because the activities of compounds **1** and **2** on ALP activity, production of osteocalcin and mineralization were almost the same. Effects of 2- or 11-*O*-glycoside compounds **3**–**5** were less than those of their aglycone compounds **1** and **2**. This indicates that hydroxyl groups at C2, and C9 or C11 are vital for osteogenic action of diphenyl ether-type cyclic diarylheptanoids. We speculate that lower osteogenic activities of glycosylated compounds are caused by the differences in cell permeability between glycosylated compounds and its aglycone compounds, since substitution of hydroxyl groups into glycosyl groups often decrease hydrophobicity and membrane permeability. To elucidate this feasibility, further studies such as metabolite analysis and structure–activity relationships analysis using more wide-substituted derivatives will be also required.

Reportedly, some osteogenic agents such as statins and flavonoids induce osteoblast differentiation via BMP signaling pathways.[15] The BMP signaling cascades depend on interaction with type I and type II serine/threonine kinase receptors, which subsequently activate distinct pathways, transcription factor Smad and p38 mitogen-activated protein kinase (MAPK), independently.[16] To show participation of these BMP signaling pathways in the promotive effect of diphenyl ether-type cyclic diarylheptanoids on osteoblast differentiation, pathway specific inhibitors were treated with compound **1** in MC3T3-E1 cells and ALP activity was measured. The BMP antagonist noggin, type I BMP receptor kinase inhibitor (LDN-193289) and p38 MAPK inhibitor (SB203580) significantly reduced compound **1**-promoted ALP activity (Supplementary Fig. 3). These results suggest that osteogenic actions of diphenyl ether-type cyclic diarylheptanoids are mediated by BMP-Smad/p38 MAPK signaling pathways, and support our study showing possible involvement of BMP production in compound **1**-induced osteoblastic gene expression.[14]

Osteoclasts are dedicated bone-resorbing multinuclear cells derived from hematopoietic stem cells of the monocyte–macrophage lineage. The effects of compounds **1**–**8** on osteoclast differentiation in mouse monocytic RAW264 cells were determined by measuring the activity of tartrate-resistant acid phosphatase (TRAP), which is an osteoclast-specific marker enzyme. The methods are described in our previous paper.[17] However, these compounds did not specifically affect TRAP activity (data not shown), which implies that these compounds might not affect osteoclast differentiation.

In conclusion, we have found a novel osteogenic action of the diphenyl ether-type cyclic diarylheptanoids **1**–**5** isolated from *Acer nikoense*. We showed that these diphenyl ether-type cyclic diarylheptanoids promote production of osteocalcin and mineralization in vitro for the first time. These compounds may activate BMP-Smad/p38 MAPK signaling pathways and promote osteoblast differentiation, accompanied by increased ALP and osteocalcin expression and mineralization. Although further studies are required to clarify their in vivo action and mechanisms, diphenyl ether-type cyclic diarylheptanoids offer the possibility of new bone anabolic drugs that support bone formation and regeneration.

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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.2011.04.041.

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12. Mouse preosteoblastic MC3T3-E1 cells were provided from the Riken Cell Bank (Tsukuba, Japan) and maintained in α-minimum essential medium (αMEM) containing 10% fetal bovine serum (FBS). To assess the effects of compounds on osteoblast differentiation, the cells were seeded in 96-well plates (5×103 cells/well) and cultured for 2 days. After the cells reached confluence, the medium was changed into 10% FBS-αMEM each with one of the compounds, and further cultured for 6 days. Cells were then fixed with ice-cold 100% methanol. To measure alkaline phosphatase (ALP) activity, fixed cells were incubated in ALP substrate buffer (100 mM Tris–HCl pH 8.5, 2 mM $MgCl_2$, 6.6 mM 4-nitrophenyl phosphate) for 30–120 min. Absorbance at 410 nm was measured as ALP activity and values (% of control) were shown. Total cell number was determined by a cell-counting kit 8 (Dojindo, Kumamoto, Japan), used according to the manufacturer's protocol. For the ALP staining, fixed cells were incubated in ALP-staining buffer (100 mM Tris–HCl pH 8.5, 2 mM MgCl2, 1% *N*,*N*-dimethyl formamide, 0.01% napthol AS-MX phosphate (Sigma) and 0.06% fast blue BB salt (Sigma)).
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