New Pyrano-Pyrone from *Goniothalamus tamirensis* Enhances the Proliferation and Differentiation of Osteoblastic MC3T3-E1 Cells

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The new pyrano-pyrone, (+)-8-epi-9-deoxygoniopypyrone (1) and (+)-9-deoxygoniopypyrone (2) were isolated from a chloroform extract of *Goniothalamus tamirensis* leaves. Their absolute stereostructures were discussed and confirmed by using infrared (IR), Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), one (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) spectra, Mosher's method, and comparison with the known compounds leiocapin A (3), deoxygoniopypyrone A (4), and (−)-8-epi-9-deoxygoniopypyrone (5). At concentrations of 2.67 μM, compounds 1 and 2 significantly increased the growth of osteoblastic MC3T3-E1 cells and caused a significant elevation of collagen content, alkaline phosphatase activity, and nodule mineralization in the cells ($p<0.05$). Our data suggest that the enhancement of osteoblast function by 1 and 2 may result in the prevention of osteoporosis.

Key words (+)-8-epi-9-deoxygoniopypyrone; pyrano-pyrone; *Goniothalamus tamirensis*; MC3T3-E1 cell; osteoporosis

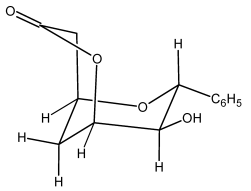
The genus *Goniothalamus* comprises about 160 species of shrubs and trees growing in tropical and subtropical Asia.[1] Many of these species are used for timber, as fiber sources, as mosquito repellant, and most interestingly in folk medicine in several countries.[2,3] Phytochemical studies of *Goniothalamus* species have led to the isolation and characterization of a large number of styryl-lactones. These can be classified into several main subtypes: styryl-pyrones, furano-pyrones, furano-furones, and pyrano-pyrones.[4] Styryl-lactones possess significant cytotoxic activity against several human tumor cell lines such as A-549 (lung carcinoma), HT-29 (colon adenocarcinoma), MCF-7 (breast carcinoma), RPMI (melanoma), U251 (brain carcinoma),[3,5] and to induce apoptosis in HL-60 leukemic cell.[6] Some of styryl-lactones have been synthesized *via* total synthesis.[3,7—10] Goniopypyrone, the first pyrano-pyrone compound, was first isolated in 1990 from *G. giganteus*.[11,12] It was high bioactive, showing ED_{50} values of *ca.* 0.67 μg/ml in the cytotoxicity of human tumor cell lines (A-549, MCF-7, HT-29), high toxicity to the brine shrimp (BS), and significant inhibition of the formation of crown gall tumors on potato discs (PD). Typically, up to date, pyrano-pyrone compounds are found in some *Goniothalamus* species.

The formation of bone involves a complex series of events, including the proliferation and differentiation of osteoprogenitor cells. Eventually, this results in the formation of a mineralized extracellular matrix. Most effective osteoporosis therapies reduce bone loss but do not restore lost bone mass and strength. Therefore, it is desirable to discover bone-building (anabolic) agents that stimulate new bone formation and correct the imbalance of microarchitecture characteristic of established osteoporosis.[13] As new bone formation is primarily a function of the osteoblasts, agents that regulate bone formation act either by increasing the proliferation of cells in the osteoblastic lineage or inducing osteoblast differentiation.[13,14] Early intervention is now possible with the help of some effective medications, which may reduce the risk of first and recurrent fractures.

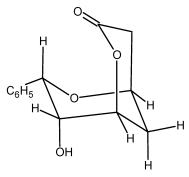
In our investigation of phytochemistry of the leaves of *G. tamirensis*, 9-deoxygoniopypyrone (**2**) and a new pyrano-pyrone, (+)-8-epi-9-deoxygoniopypyrone (**1**), were isolated and identified using spectral methods. To investigate whether these compounds could stimulate the function of osteoblasts, their effects on cell growth, collagen content, alkaline phosphatase (ALP) activity, and calcium deposition were assessed in the pre-osteoblastic target cell line, MC3T3-E1, which is a well-characterized *in vitro* model of osteoblast differentiation.[15]

Results and Discussion

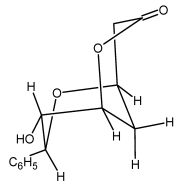
Compound **1** was obtained as white needle-like crystal, $[\alpha]_D^{20}$ +93.3°, mp 131—132 °C. The molecular formula of **1** was deduced to be $C_{13}H_{14}O_4$ from the Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) ion



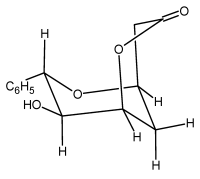
(+) 8-epi-9-deoxygoniopypyrone (**1**)



9-deoxygoniopypyrone (**2**)



deoxygoniopypyrone A (**4**)



(−) 8-epi-9-deoxygoniopypyrone (**5**)

Fig. 1. (+)-8-Epi-9-deoxygoniopypyrone and Known Goniopypyrone Compounds from *Goniothalamus* Species

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Table 1. ^{1}H-NMR Spectral Data and Physicochemical Data for Compounds **1** and **2**, Deoxygoniopypyrone, and (−)-8-Epi-9-deoxygoniopypyrone[a)]

H	**1** 500 MHz, $CDCl_3$	**2** 500 MHz, $CDCl_3$	**4**[18)] 400 MHz $CDCl_3$	**5**[3)] 200 MHz $CDCl_3$
1	4.93 (1H, br s)	4.86 (1H, quin, 2.0)	4.76 (1H, dd, 1.0, 4.2)	4.87 (1H, m)
4	2.99 (1H, d, 19.5)	2.96 (1H, d, 19.0)	2.96 (1H, dt, 2.6, 18.2)	2.95 (1H, d, 19.5)
	2.88 (1H, dd, 5.5, 19.5)	2.86 (1H, dd, 5.5, 19.0)	2.59 (1H, dd, 2.8, 18.2)	2.84 (1H, dd, 4.8, 19.5)
5	4.47 (1H, br s)	4.52 (1H, br s)	4.61 (1H, dd, 2.8, 5.4)	4.42 (1H, s)
7	4.46 (1H, d, 10.0)	4.94 (1H, s)	4.35 (1H, d, 8.8)	4.44 (1H, d, 9.2)
8	3.59 (1H, ddd, 2.0, 8.5, 10.0)	3.94 (1H, br s)	4.02 (1H, dd, 1.0, 8.8)	3.50 (1H, td, 2.2, 9.2)
9	2.23 (2H, m)	1.84 (1H, dquin, 2.0, 14.0)	2.02 (1H, dd, 4.2, 14.6)	2.08 (2H, m)
		2.59 (1H, dd, 3.5, 14.0)	2.68 (1H, dddd, 1.0, 2.6, 5.4, 14.6)	
OH	2.43 (1H, d, 8.5)	1.67 (1H, s)	—	3.07 (d, 9.2)
Ph	7.35—7.42 (5H, m)	7.31—7.48 (5H, m)	7.31—7.43 (5H, m)	7.20—7.50 (5H, m)
$[\alpha]_D^{20}$	+93.3	+10.2	−98.4	−90.0
mp	131—132	201—203	132—134	130—131

a) Chemical shift values are given in ppm, and *J* values in parenthesis are given in Hz. Assignment were confirmed by COSY, HMBC, and HSQC experiments.

Table 2. ^{13}C-NMR Data (Measured in $CDCl_3$) for Compounds **1** and **2**, Deoxygoniopypyrone, and (−)-8-Epi-9-deoxygoniopypyrone[a)]

C	**1** 125 MHz	**2** 125 MHz	**4**[18)] 100 MHz	**5**[3)] 50 MHz
C-1	76.8	74.8	79.1	76.8
C-3	169.1	169.3	168.9	169.2
C-4	36.6	36.3	40.8	36.9
C-5	65.8	66.1	65.2	65.6
C-7	74.3	70.5	76.0	74.0
C-8	72.6	68.2	75.8	72.3
C-9	29.9	24.0	23.8	29.6
C-1′	137.9	136.8	139.3	138.0
C-2′	127.4	126.2	126.9	127.3
C-3′	128.6	128.8	128.4	128.4
C-4′	128.7	128.3	128.6	128.5
C-5′	128.6	128.8	128.4	128.4
C-6′	127.4	126.2	126.9	127.3

a) Chemical shift values are given in ppm. Assignments were confirmed by HMBC, and HSQC experiments.

peak at *m/z* 234.0852 $[M]^+$, which is in agreement with thirteen carbon signals observed on ^{13}C-NMR (two quaternary, nine methine and two methylene carbons). The presence of the hydroxyl group was suggested by successive loss of a water molecule from the $[M+H]^+$ ion giving the $[M+H-H_2O]^+$ ion peak at *m/z* 217 in the APCI-MS, and a broad infrared (IR) absorption band at 3397 cm^{-1}. The similarity of ^{1}H- and ^{13}C-NMR spectra of **1** to those of known pyrano-pyrones leiocapin A (**3**), deoxygoniopypyrone A (**4**), and (−)-8-epi-9-deoxygoniopypyrone (**5**) (Tables 1, 2) showed that **1** had a pyrano-pyrone type skeleton.[12,16—18)] The presence of a saturated pyrone ring in **1** was suggested by an IR carbonyl absorption band at 1737 cm^{-1} and a slight upfield-shifted carbonyl signal at δ_C 169.1 ppm (C-3) in the ^{13}C-NMR spectrum. The heteronuclear single quantum coherence (HSQC) spectrum of **1** showed the direct ^{1}H–^{13}C correlations between the doublet [δ_H 2.99 (H-4a), 2.88 (H-4b)], and multiplet methylene proton signals at δ_H 2.23 (H-9, 2H) to the methylene carbon signals at δ_C 36.6 (C-4) and 29.9 (C-9), respectively, confirming the existence of two methylene groups. The long range ^{1}H–^{13}C heteronuclear multiple bond correlation (HMBC) and ^{1}H–^{1}H correlation spectroscopy (COSY) spectra of **1** also confirmed the assignment of the pyrone ring. Four methine proton signals at δ_H 4.93, 4.47, 4.44 and 3.59 ppm were correlated, as showed in the ^{1}H–^{13}C HSQC spectrum, respectively, to four methine carbon signals at δ_C 76.8, 65.8, 74.3 and 72.6, indicating the presence of four oxygen bearing carbons of the pyrano ring in **1**. The presence of a mono-substituted phenyl group was also confirmed by five proton signals appeared around δ_H 7.35—7.42 and six carbon signals at δ_C 127.4, 128.6, 128.7, and 137.9 ppm.

Tables 1 and 2 showed that compound **1** has ^{1}H- and ^{13}C-NMR parameters similar to those of compound **5**. However, a careful examination of 2D-NMR spectral data of **1** including COSY, HMBC, HSQC, especially nuclear Overhauser effect spectroscopy (NOESY), and further examination using Mosher's method suggested that **1** is a chiral isomer of **5**, a new goniopypyrone-type isomer, named (+)-8-epi-9-deoxygoniopypyrone. The presence of the cross peaks between protons H-1 and H-5 in the COSY spectra specifically illustrate the "W-type" interaction in the bicyclic structural skeleton of pyrano-pyrones, as described by Lan *et al.*[18)] (Fig. 3). The coupling constant between protons H-7 and H-8 (J=10.0 Hz), was evidence of the *axial-trans* configuration between H-7 and H-8 protons. As a result, the two substituted groups at C-7 and C-8 were in the *equatorial* configuration. The *axial* methine proton signal at δ_H 3.59 (H-8) had NOESY correlation to the *axial*-oriented methylene proton signal at δ_H 2.23 (H-9), while the methylene proton signals (H-4a, H-4b) did not have correlation to methylene proton signals (H-9). Furthermore, the NOESY spectrum of **1** showed cross peaks between the *axial* methine proton signal at δ_H 4.46 (H-7) to the *axial* methylene proton signal at δ_H 2.88 (H-4a). These above evidences showed that in **1** the pyrano ring had the chair form and the lactone ring had the boat form. The chair-boat form of **1** was also confirmed by interactions of protons, *i.e.*, H-7 and H-4a, H-8 and H-9b on the NOESY spectrum of both **1** and acetylated product (**1a**). Finally, the absolute stereostructure of **1** was investigated through configuration of C-8. Following Mosher's method, the differences in the proton chemical shifts between (*S*)-methoxytrifluoromethylphenylacetic acid (MTPA)-ester and (*R*)-MTPA-ester of **1** (Fig. 2, Table 3) indicated the "*S*" configuration for this carbon atom.[18,19)] As a result, we concluded that **1** is a new pyrano-pyrone and its absolute configuration is described as 1*S*, 5*S*, 7*R*, 8*S*, 3*exo*, 7*endo*. This was also confirmed by its physical properties, such as melting point and optical rotation of **1** in comparison with **5** (Table 1).

Compound **2** was also isolated as white powder, and its

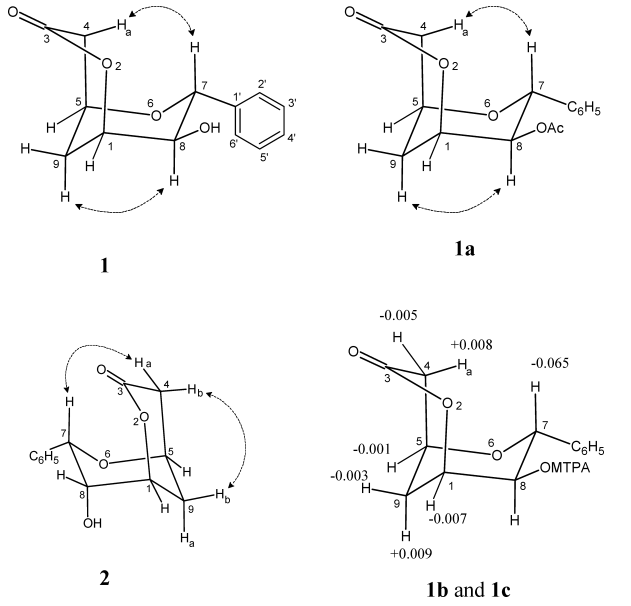

Fig. 2. ^{1}H–^{1}H Interaction in NOESY Spectra and Absolute Stereostructure of **1**—**2**

Table 3. ^{1}H-NMR Data of (*R*)-MTPA Ester (**1b**), (*S*)-MTPA Ester (**1c**) of **1**

Position	**1b** (δ_H)	**1c** (δ_H)	$\Delta\delta=\delta_S-\delta_R$
1	5.149	5.142	−0.007
4a	3.007	3.002	−0.005
4b	2.893	2.901	+0.008
5	4.511	4.51	−0.001
7	4.698	4.633	−0.065
8	5.058	5.037	−0.021
9a	2.371	2.368	−0.003
9b	2.236	2.245	+0.009

molecular weight was indicated by a peak in the APCI-MS at *m/z* 235 $[M+H]^+$, as in the case of **1**, corresponding to a molecular formula of $C_{13}H_{14}O_4$. Like **1**, compound **2** also is a pyrano-pyrone compound. Comparison of the ^{1}H- and ^{13}C-NMR spectral data of **2** and **1** and other known pyrano-pyrones (Tables 1, 2) showed that **2** was 9-deoxygoniopypyrone, a known compound isolated from the leaves of *G. giganteus*.[12] In addition, the absolute stereostructure of **2** was confirmed by 2D-NMR spectral data and its optical rotation in comparison with 9-deoxygoniopypyrone. The presence of the long range correlations through four bonds in the ^{1}H–^{1}H COSY spectrum between H-1 and H-5, H-8 and H-9b, H-4a and H-9a of **2** are due to "W-type" interaction of those protons in pyrano-pyrone structure (Fig. 3). In addition, the NOESY spectrum of **2** showed the cross peaks between H-4b and H-9b, and between the *axial* proton H-7 and H-4a. This evidence confirmed the chair–chair form of **2** (Fig. 2), and this is the first report about chair–chair conformation of this compound.

In this study, we investigated the effects of **1** and **2** on the osteoblast function using pre-osteoblastic target cell line, MC3T3-E1, as an *in vitro* model of osteoblast differentiation. At the range of concentration of 2.67—10.68 μM, the compounds significantly increased MC3T3-E1 cell growth (Fig. 4). Given that they significantly increased osteoblast growth,

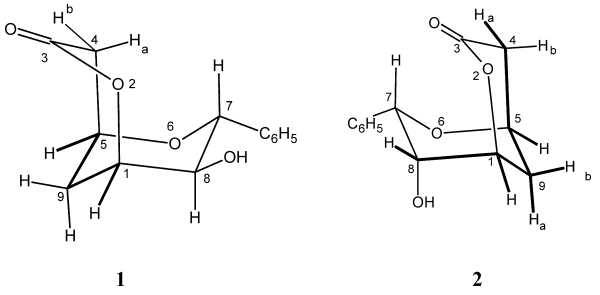

Fig. 3. "W-Type" Interactions in the COSY Spectra of Compounds **1** and **2**

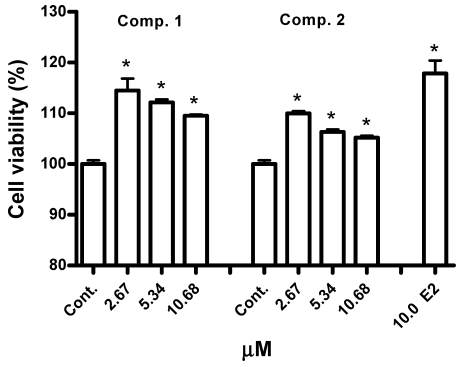

Fig. 4. Effects of **1** and **2** on the Viability of MC3T3-E1 Cells

E2: 17β-estradiol. Data are expressed as a percentage of the control. The control value was 0.437±0.006 OD. $*p<0.05$ *vs.* control.

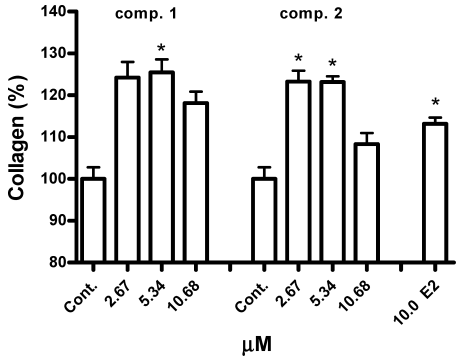

Fig. 5. Effects of **1** and **2** on the Alkaline Phosphatase Activity of MC3T3-E1 Cells

E2: 17β-estradiol. Data are expressed as a percentage of the control. The control value was 0.984±0.031 Unit/10^6 cells. $*p<0.05$ *vs.* control.

we then investigated the effect of these compounds on collagen synthesis using Sirius Red-based colorimetric assay. Both **1** and **2** increased collagen synthesis at a concentration of 2.67 μM (Fig. 5). Next, the effects of **1** and **2** on the osteoblast differentiation were assessed by measuring the ALP activity, a major osteoblast differentiation marker. Both **1** and **2** increased the ALP activity up to 125% at concentration of 2.67 μM (Fig. 6). Finally, we examined the effects of **1** and **2** on mineralization, another important process in differentiation of MC3T3-E1 cells, by measuring the calcium deposition by Alizarin Red staining. Consistent with the effects on ALP activity and collagen synthesis, **1** and **2** (2.67 μM) significantly stimulated mineralization (Fig. 7). At this concentration, both **1** and **2** did not show any cytotoxicity. Further-

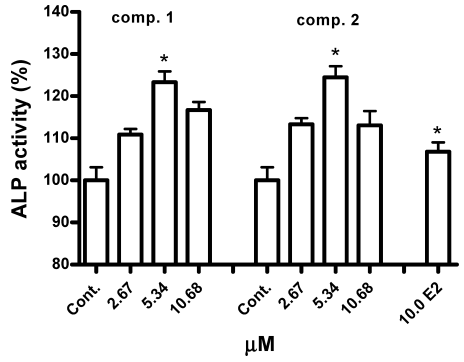

Fig. 6. Effects of **1** and **2** on the Collagen Content of MC3T3-E1 Cells

E2: 17β-estradiol. Data are expressed as a percentage of the control. The control value was 11.8±0.328 mg. $*p<0.05$ *vs.* control.

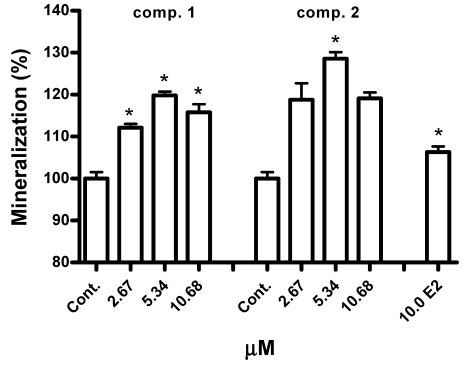

Fig. 7. Effects of **1** and **2** on the Mineralization of MC3T3-E1 Cells

E2: 17β-estradiol. Data are expressed as a percentage of the control. The control value was 0.191±0.003 OD. $*p<0.05$ *vs.* control.

more, 17β-estradiol (E2) used as a positive control significantly increased cell growth, collagen content, ALP activity, and mineralization at 10.0 μM. The greater activity of **1** and **2** compared with the positive control demonstrated that **1** and **2** increased the proliferation and differentiation of osteoblastic MC3T3-E1 cells.

In conclusion, the new pyrano-pyrone, (+)-8-epi-9-deoxygoniopypyrone (**1**) and 9-deoxygoniopypyrone (**2**) were isolated from a chloroform extract of *G. tamirensis* leaves. Their absolute stereostructures are discussed and confirmed using IR, MS, and NMR spectra. The effects of the isolated compounds on the proliferation and differentiation of MC3T3-E1 cells were also investigated. We showed that both **1** and **2** at concentration of 2.67 μM stimulated the growth and differentiation of osteoblastic MC3T3-E1 cells. Therefore, **1** and **2** may be beneficial in stimulating the osteoblastic activity, resulting in bone formation.

Experimental

General Experimental Procedures Melting points were taken on a Boetius melting point apparatus and the optical rotations were determined on an ATAGO POLAR-2X polarimeter. The FT-IR spectra were measured on an IMPAC-410 (Nicollet America) spectrometer. APCI-MS was performed on a LC-MSD-Trap-SL Agilent 1100 series spectrometer and NMR spectra were recorded in $CDCl_3$ with a Bruker Avance 500 (Germany) spectrometer using tetramethylsilane (TMS) as the internal standard. The high resolution mass spectra were obtained using a Variant 910 FT-ICR mass spectrometer. Column chromatography (CC) was performed on silica gel (70—230, 230—400 mesh, Merck). Thin layer chromatography (TLC) was carried out on pre-coated DC Alufolien 60 F_{254} plates (Merck). Spots were detected under UV radiation (254, 365 nm) and by spraying the plates with 10% H_2SO_4 followed by heating with a heat gun.

Plant Material Leaves of *G. tamirensis* were collected in Huong Son District, Ha Tinh Province, Vietnam, and identified by an experienced botanist at the Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology (VAST). A voucher of specimen (No. VN-1087) was deposited at the Institute of Ecology and Biological Resources, VAST.

Isolation and Purification Dried powdered leaves of *G. tamirensis* (760 g) were extracted with methanol at room temperature three times. After removing the solvent under reduced pressure, the crude extract (90 g) was dissolved in 1 l of water to form a suspension. This was partitioned successively with *n*-hexane, chloroform, and *n*-butanol to give *n*-hexane (7.3 g), chloroform (37.5 g) and *n*-butanol (39.0 g) residues, respectively. Then, the chloroform extract was subjected to a flash silica gel column chromatography and eluted with a gradient of dichloromethane–methanol (0, 2, 4, 8, 16, 32, 64, 100% MeOH by volume) to give eight fractions (C1—8). Fraction C5 was chromatographed repeatedly on a silica gel column and eluted with an isocratic solvent system of *n*-hexane/ethyl acetate (EtOAc) (7/3, v/v). Based on thin-layer chromatography (TLC) monitoring, it was then recrystallized from the mixture of *n*-hexane/ethyl acetate (7/3, v/v) to yield compound **1** (1.1650 g, 0.1533% from dried leaves). Next, the fraction C4 was also subjected to a silica gel column chromatography and eluted with *n*-hexane/EtOAc (4/1, v/v) to obtain 54 mg (7.1053×10^{-3} %) of compound **2**. The purity of **1** and **2** exceeded 97% by high-performance liquid chromatography (HPLC) analysis.

Compound **1** was obtained as white needle-like crystals; ^{1}H- and ^{13}C-NMR data are provided in Tables 1 and 2. IR (KBr) cm^{-1}: 3397, 3290, 3039, 1737, 1082. FT-ICR-MS *m/z*: 234.0852 (Calcd for $C_{13}H_{14}O_4$: 234.0886). APCI-MS *m/z*: 235 $[M+H]^+$. $[\alpha]_D^{20}$ +93.3° (*c*=0.4, $CHCl_3$). mp 131—132 °C.

Acetylation A mixture of 3 ml anhydride acetic acid and 30 mg of **1** was placed in a 10 ml round-bottomed flask. The reaction mixture was stirred well with a magnetic stirrer and refluxed for 5 h in an oil bath. Then, the solvent was removed under low pressure. The residues were washed several times with saturated $NaHCO_3$ and distilled water and dried to obtain a crude product. Finally, an acetylated (**1a**) product of **1** (25 mg) was obtained by column chromatography with *n*-hexane/ethyl acetate (3/1, v/v) as eluent.

Acetylated product of **1** was obtained as white amorphous powder; ^{1}H-NMR ($CDCl_3$) δ: 1.94 (3H, s), 2.21 (1H, dd, *J*=4.5, 14.0 Hz), 2.32 (1H, dt, *J*=2.5, 14.0 Hz), 2.90 (1H, dd, *J*=5.0, 19.0 Hz), 3.02 (1H, d, *J*=19.0 Hz), 4.51 (1H, br s), 4.67 (1H, d, *J*=10.0 Hz), 4.84 (1H, dd, *J*=2.5, 10.0 Hz), 5.02 (1H, br s), 7.34 (5H, m). ^{13}C-NMR ($CDCl_3$) δ: 20.64 (q), 29.58 (t), 36.41 (t), 66.07 (d), 71.20 (d), 73.03 (d), 73.95 (d), 127.16 (d), 128.48 (d), 128.81 (d), 136.98 (s), 168.56 (s), 169.70 (s).

Methoxytrifluoromethylphenylacetic Acid (MTPA) Reaction A solution of **1** (2.5 mg) in 300 μl of dehydrated CH_2Cl_2 was reacted with (*R*)-MTPA-Cl (4 μl) in the presence of *N*,*N*-dimethyl-4-aminopyridine (4-DMAP) (3.2 mg); the mixture was stirred occasionally at room temperature for 1 h. After adding 300 μl of CH_2Cl_2, the solution was washed with H_2O (300 μl), 5% HCl (300 μl), successively. The organic layer was evaporated and the residue was purified by preparative TLC silica gel (0.25 mm thickness), developed with *n*-hexane/ethyl acetate/methanol (2/1/0.1, v/v/v) to furnish the ester, **1b** (4.2 mg, 88%). In a similar manner, **1c** (3.5 mg, 73%) was prepared from **1** (2.5 mg) using (*S*)-MTPA-Cl (4 μl), and 4-DMAP (3.5 mg). The ^{1}H-NMR data of compounds **1b** and **1c** are provided in Table 3.

Cell Culture and Materials Murine osteoblastic MC3T3-E1 cells were cultured at 37 °C in 5% CO_2 atmosphere in α-modified minimal essential medium (α-MEM; GibcoBRL, Grand Island, NY, U.S.A.). Unless otherwise specified, the medium contained 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. When cells reached confluence, cells were subcultured using 0.02% EDTA–0.05% trypsin solution.

Cell Viability Cells were suspended in medium supplemented with 10% FBS, and cell suspension containing 5×10^3 cells was added to the individual wells of 48-well microplates. The plates were incubated at 37 °C in a CO_2 incubator for 48 h. After discarding the culture medium and washing the cells with phosphate-buffered saline (PBS), serum-free medium containing 0.3% bovine serum albumin (BSA) and compounds at appropriate concentrations was added to the cell culture and incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 48 h. Surviving cells was counted by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. A 20 μl volume of MTT in 7.2 mM phosphate buffer solution, pH 6.5 (5 mg/ml), was added to each well, and the plates were incubated for an additional 2 h. After the removal of solutions in the well, dimethyl sulfoxide was added to dissolve formazan products, and the plates were shaken for

5 min. The absorbance of each well was recorded on a microplate spectrophotometer at 570 nm.

Alkaline Phosphatase Activity The cells were treated, at 90% confluence, with culture medium containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid, to initiate differentiation. The medium was changed every 2—3 d. After 8 d, the cells were cultured with medium containing 0.3% BSA and compounds individually for 2 d. On harvesting, the medium was removed and the cell monolayer gently washed twice with PBS. The cell number was determined microspically and then calculated as number of living cells (*i.e.*, those not stained with trypan blue). The cells were lysed with 0.2% Triton X-100, and the lysate was 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).

Collagen Content The cells were treated, at 90% confluence, with culture medium containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. The medium was changed every 2—3 d. After 8 d, the cells were cultured with medium containing 0.3% BSA and compounds for 2 d. On harvesting, the medium was removed and the cell monolayer gently washed twice with PBS. Collagen content was quantified by Sirius Red-based colorimetric assay. 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 non-bound dye. The stained material was dissolved in 0.1 N NaOH and absorbance was measured at 550 nm.

Calcium Deposition Assay The cells were treated, at 90% confluence, with culture medium containing 10 mM β-glycerophosphate and 50 μg/ml ascorbic acid. After 12 d, the cells were cultured with medium containing 0.3% BSA and compounds individually for 2 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. The absorbance of the solubilized stain was measured at 561 nm.

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

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