JOURNAL OF NATURAL PRODUCTS

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ent-Kaurane Diterpenoids from *Croton tonkinensis* Stimulate Osteoblast Differentiation

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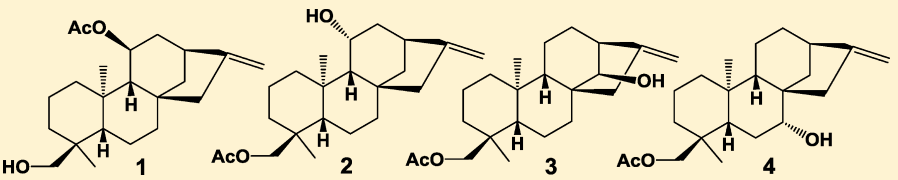
Supporting Information



ABSTRACT: Four new *ent*-kaurane diterpenoids (**1**–**4**) were isolated from the leaves of *Croton tonkinensis* by bioactivity-guided fractionation using an in vitro osteoblast differentiation assay. Their structures were identified as *ent*-11β-acetoxykaur-16-en-18-ol (**1**), *ent*-11α-hydroxy-18-acetoxykaur-16-ene (**2**), *ent*-14β-hydroxy-18-acetoxykaur-16-ene (**3**), and *ent*-7α-hydroxy-18-acetoxykaur-16-ene (**4**). Compounds **1**–**4** significantly increased alkaline phosphatase activity and osteoblastic gene promoter activity. Compounds **1**–**3** also increased the levels of ALP and collagen type I alpha mRNA in C2C12 cells in a dose-dependent manner. These results suggest that *ent*-kaurane diterpenoids from *C. tonkinensis* have a direct stimulatory effect on osteoblast differentiation and may be potential therapeutic molecules against bone diseases such as osteoporosis.

Osteoporosis, a disease caused by reduction in skeleton mass, occurs due to a decrease in bone formation by osteoblasts and an increase in bone resorption by osteoclasts.[1,2] Treatment methods for osteoporosis include inhibition of osteoclast activities or stimulation of the osteoblastic lineage proliferation and induction of osteoblast differentiation. Because many osteoporotic patients have already lost a substantial amount of bone at the time of diagnosis, bone mass must be increased by stimulating the osteoblastic lineage proliferation and inducing the differentiation of osteoblasts.[3] However, as commercially available drugs used to treat osteoporosis are mostly osteoclast inhibitors that contain drugs such as estrogen, estrogen receptor derivatives, calcitonin, and bisphosphates, their effects on increasing or recovering bone mass are relatively small.[4,5] As potential complications such as breast cancer, uterine bleeding, and cardiovascular disease have also been reported in the use of these drugs, there is major interest in finding new agents that can enhance osteoblast differentiation activity and increase skeletal bone formation.[6]

During a screening program of natural products with stimulatory activity on osteoblast differentiation, the CH_2Cl_2-soluble part of the MeOH extract of the leaves of *Cronton tonkinensis* Gagnep. (Euphorbiaceae) was found to significantly increase osteoblast differentiation in vitro. This prompted us to phytochemically examine the constituents responsible for this activity using bioassay-guided fractionation.

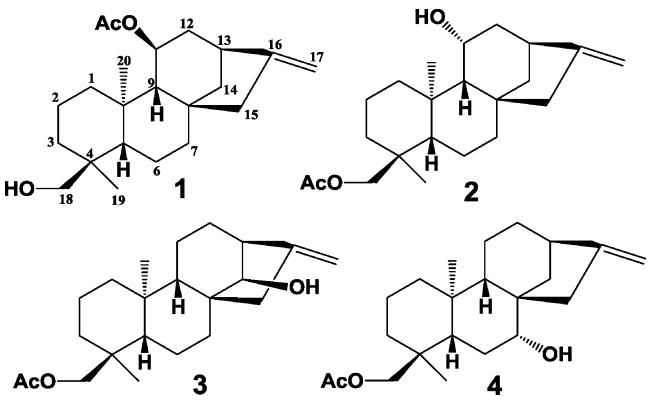
C. tonkinensis is a Vietnamese medicinal plant, and steroids, alkaloids, and diterpenoids have been reported as its main constituents.[7−11] Recently, the isolated diterpenoids from this plant were found to have anti-inflammatory[12] and inhibitory effects on silent information regulator two homologue 1 (SIRT1).[13] However, there have been no previous studies on the effects of *C. tonkinenesis* on osteoblast differentiation. Thus, here we report the isolation and structural elucidation of four *ent*-kaurane diterpenoids (**1**–**4**) and their effects on alkaline phosphatase (ALP) activity, osteoblastic gene promoter activities, and mRNA expression levels in C2C12 cells.

RESULTS AND DISCUSSION

Four new *ent*-kaurane diterpenoids (**1**–**4**) were isolated from the methanol extract of *C. tonkinensis* by successive chromatographic procedures (silica gel, Sephadex LH-20, RP-18, and HPLC).

Compound **1** was obtained as a white, amorphous powder. A molecular formula of $C_{22}H_{34}O_3$ was determined from the molecular ion peak at m/z 346.2509 (calcd for $C_{22}H_{34}O_3$, 346.2508) in the HR-EIMS spectrum. The IR spectrum revealed the presence of hydroxy and carbonyl groups at 3498 and 1710 cm^{-1}, respectively. The ^1H and ^{13}C NMR spectroscopic data (Table 1) showed an *ent*-kaurane diterpenoid with 22 carbons, which were assignable to two

Received: August 9, 2011
Published: November 15, 2011



tertiary methyl groups [δ_H 1.01 (3H, s, H-20), 0.76 (3H, s, H-19); δ_C 17.9 (C-20), 17.5 (C-19)], an *O*-acetyl group [δ_H 1.93 (3H, s, 11-OAc); δ_C 170.1, 21.7], nine methylene groups including one oxymethylene [δ_H 3.42 (1H, d, J = 10.5 Hz, H-18), 3.11 (1H, d, J = 10.5 Hz, H-18); δ_C 71.9 (C-18)], four methine groups including an oxymethine [δ_H 5.07 (1H, t, J = 3.0 Hz, H-11); δ_C 69.1 (C-11)], three quaternary carbons, and an exocyclic double bond in ring D [C-17: δ_H 4.82 (1H, s, H-17ax), 4.68 (1H, s, H-17eq); δ_C 155.2 (C-16), 103.1 (C-17)]. These signals were similar to those of *ent*-11α-acetoxykaur-16-en-18-oic acid,[11] except for the presence of oxymethylene signals at C-18 instead of the carboxylic carbon in *ent*-11α-acetoxykaur-16-en-18-oic acid. These data indicated that one of the three tertiary methyl groups was oxidized to a hydroxymethylene group in compound **1**. Analysis of the HMQC spectrum revealed signals for methyl groups at C-19 and C-20. The hydroxy group was assigned to C-18 by HMBC correlations between H-18/C-3, C-4, C-5, and C-19 (Figure 1).[11,14] The attachment of the acetoxy group to C-11 was also determined by HMBC correlations from both the methyl protons of the acetoxy group and H-11 (δ_H 5.07) to the ester carbonyl at δ_C 170.1 (Figure 1). A strong NOE correlation between H-11 and the C-20 methyl protons revealed that the acetoxy group at C-11 had a β-orientation (Figure 1).[11,15] In addition, the CD data and the negative value of the optical rotation of **1** indicated the *ent*-configuration.[11,16] Therefore, the structure of compound **1** was characterized as *ent*-11β-acetoxykaur-16-en-18-ol.

Compound **2** was isolated as a brown, amorphous powder with absorption bands at 3482 (OH) and 1725 cm^{-1} (CO) in

Table 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data[a] of 1−4

	1		2		3		4	
position	δ_H mult. (J in Hz)	δ_C	δ_H mult. (J in Hz)	δ_C	δ_H mult. (J in Hz)	δ_C	δ_H mult. (J in Hz)	δ_C
1	0.92 ddd (13.0, 13.0, 3.0)	39.4	1.06 m	39.2	0.78 m	39.8	0.75 ddd (13.5, 13.5, 3.5)	39.6
	1.96 m		1.92 dd (11.0, 2.5)		1.80 m		1.82 dt (13.5, 2.0)	
2	1.52 m	17.7	1.42 m	17.6	1.59 m	17.6	1.46 m	17.7
	1.62 m		1.55 m		1.61 m		1.51 m	
3	1.28 m	35.1	1.38 m	35.6	1.38 m	35.6	1.37 m	35.6
	1.43 m		1.52 m		1.40 m		1.48 m	
4		37.6		36.5		36.4		36.3
5	1.20 dd (12.5, 1.5)	49.0	1.18 dd (12.0, 1.5)	49.8	1.12 d (12.0)	50.3	1.18 d (12.5)	46.8
6	1.33 dd (12.5, 2.5)	19.7	1.35 m	19.9	1.37 m	19.7	1.41 m	29.4
	1.49 m		1.50 m		1.47 m		1.69 m	
7	1.55 m	40.6	1.47 m	43.2	1.14 m	31.9	3.45 dd (11.0, 4.0)	74.8
	1.67 m		1.93 m		2.18 m			
8		42.9		42.8		49.3		49.7
9	1.37 m	61.8	1.44 m	64.9	1.35 m	58.7	1.09 m	55.3
10		37.9		37.8		39.2		39.2
11	5.07 t (3.0)	69.1	3.86 dd (11.0, 5.5)	66.9	1.32 m	17.9	1.67 m	17.9
					1.56 m		1.70 m	
12	1.87 m	39.7	1.97 m	40.5	1.64 m	33.0	1.53 m	33.6
	1.94 m		2.22 m		1.83 m		1.69 m	
13	2.63 br, s	42.4	2.72 br, s	42.1	2.62 br, s	51.8	2.69 br, s	43.3
14	1.17 dd (11.5, 5.0)	39.3	1.13 m	39.4	4.15 br, s	76.2	1.43 m	30.6
	1.95 m		1.89 m				1.65 m	
15	2.03 dt (16.5, 3.0)	47.9	2.11 dt (17.0, 3.0)	48.8	2.02 dt (16.5, 1.5)	44.6	1.95 d (16.0)	43.2
	2.48 d (16.5)		2.50 d (17.0)		2.30 d (16.5)		2.66 dt (16.0, 2.0)	
16		155.2		156.0		152.7		154.9
17	4.68 s	103.1	4.87 s	105.3	4.93 s	106.7	4.78 s	103.6
	4.82 s		5.04 s		4.95 s		4.85 s	
18	3.11 d (10.5)	71.9	3.64 d (11.0)	72.8	3.66 d (10.5)	73.1	3.66 d (11.0)	72.7
	3.42 d (10.5)		3.89 d (11.0)		3.86 d (10.5)		3.86 d (11.0)	
19	0.76 s	17.5	0.84 s	17.5	0.84 s	17.4	0.85 s	17.5
20	1.01 s	17.9	0.95 s	17.7	1.09 s	18.5	1.08 s	18.2
11-OAc	1.93 s	21.7						
		170.1						
18-OAc			2.08 s	20.9	2.07 s	21.0	2.07 s	21.0
				171.3		171.3		171.2

[a]Recorded in $CDCl_3$.

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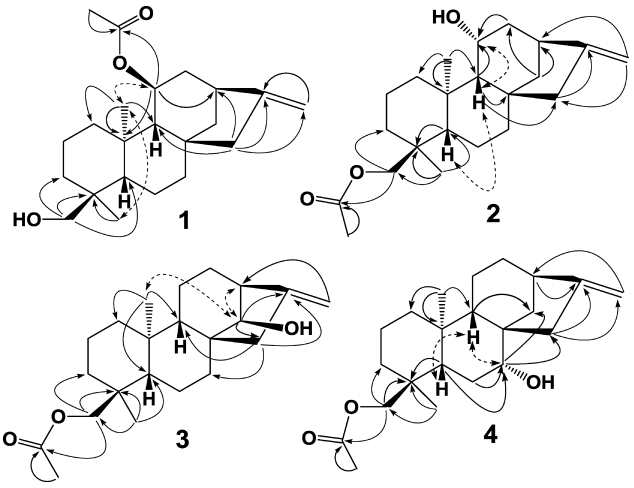


Figure 1. Selected HMBC [H (solid arrow) C] and NOESY [H (dotted arrow) H] correlations for compounds **1**–**4**.

its IR spectrum. The molecular formula of **2** was determined as $C_{22}H_{34}O_3$ from the HR-EIMS molecular ion peak at m/z 346.2507 $[M]^+$ (calcd for $C_{22}H_{34}O_3$, 346.2508). Comparison of the ^1H and ^{13}C NMR data of **2** with those of **1** (Table 1) showed the presence of a 20-carbon skeleton of an *ent*-kaurane diterpenoid having a hydroxy and an acetoxy group. However, the ^1H and ^{13}C NMR signals of the oxygenated methylene group at C-18 of **2** (δ_H 3.89, 3.64; δ_C 72.8) were shifted downfield compared to those of **1**. HMBC correlations between H-18/C-3, C-5, C-19, and the ester carbonyl at δ_C 171.3 suggested the presence of an acetoxy group at C-18 (Figure 1).[11] The oxygenated methine at δ_H 3.86 (1H, dd, J = 11.0, 5.5 Hz) and δ_C 66.9 was located at C-11 on the basis of HMBC correlations between H-9/C-1, C-8, C-10, C-11, C-14, and C-15. The hydroxy group at C-11 was in the α-orientation on the basis of the coupling pattern of H-11, a doublet of doublets (δ_H 3.86, dd, J = 11.0, 5.5 Hz),[11,15] and the NOE correlation between H-11 and H-9 (Figure 1). Thus, compound **2** was *ent*-11α-hydroxy-18-acetoxykaur-16-ene.

Compound **3** was obtained as a brown, amorphous powder. The IR spectrum indicated the presence of hydroxy (3471 cm^{-1}) and carbonyl (1710 cm^{-1}) groups. A molecular formula of $C_{22}H_{34}O_3$ was identified from the molecular ion peak at m/z 346.2503 $[M]^+$ (calcd for $C_{22}H_{34}O_3$, 346.2508). The ^1H and ^{13}C NMR data of **3** (Table 1) were similar to those of **2** except that the chemical shift of the hydroxymethine group (δ_H 4.15, δ_C 76.2) was shifted downfield compared with that of C-11 in **2** (δ_H 3.86; δ_C 66.9). The location of the hydroxy group at C-14 was identified by HMBC correlations of H-14/C-15 and C-16 and of H-9/C14. Finally, the β-orientation of the hydroxy group was determined from the NOE correlations between H-14/H-13 and H-20.[10] On the basis of the above evidence, the structure of **3** was characterized as *ent*-14β-hydroxy-18-acetoxykaur-16-ene.

Compound **4** was obtained as a white, amorphous powder. The IR spectrum indicated the presence of hydroxy (3437 cm^{-1}) and carbonyl (1726 cm^{-1}) groups. The molecular formula of compound **4** was $C_{22}H_{34}O_3$ from its molecular ion peak at m/z 346.2509 $[M]^+$ (calcd for $C_{22}H_{34}O_3$, 346.2508) in the HR-EIMS spectrum. Signals of an oxymethylene group at δ_H 3.86 (1H, d, J = 11.0 Hz) and 3.66 (1H, d, J = 11.0 Hz), corresponding to δ_C 72.7 in the ^1H and ^{13}C NMR spectra, indicated that an acetoxy group was located at C-18. This was further confirmed by HMBC correlations of H-18/C-3, C-4, C-5, and C-19, and the ester carbonyl at δ_C 171.2 (Figure 1). The location of the hydroxy group at C-7 was assigned by comparison of 1D NMR data with reported data[15] and by HMBC correlations of H-7/C-14 and C-15 and of H-5, H-6, and H-15/C-7 (Figure 1). The α-orientation of the 7-OH was derived from the coupling constants of the H-7 axial signal at δ_H 3.45 (1H, dd, J = 11.0 and 4.0 Hz)[15,17] and confirmed by the NOE correlations between H-5/H-7 and H-9. Therefore, compound **4** was identified as *ent*-7α-hydroxy-18-acetoxykaur-16-ene.

Osteoblasts are the most important cells in bone tissues and are critical for bone formation through proliferation and differentiation. During osteoblast differentiation, bone morphogenetic protein (BMP) induces the expression of osteoblastic markers such as alkaline phosphatase (ALP), osteopontin (OPN), and osteocalcin (OCN). BMP also produces extracellular matrix type I collagen and finally induces mineralization by deposition of calcium.[18] To evaluate the effects of compounds **1**–**4** on osteoblast function, ALP activity, which is related to the osteoid and initiates the deposition of minerals, was determined. When ALP was stained in C2C12 cells, treatment with compounds **1**–**4** (10 μM) and BMP2 (5 ng/mL) dramatically increased ALP activity compared to treatment with BMP2 alone (Figure 2).

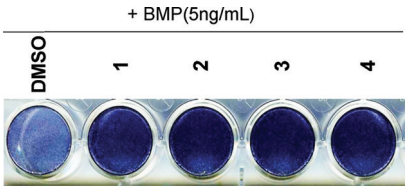


Figure 2. Effect of compounds **1**–**4** on alkaline phosphatase (ALP) activity in C2C12 cells. Cells were incubated until subconfluent and then cultured in the presence of compounds **1**–**4** (10 μM) with BMP2 (5 ng/mL). On day 2, the cells were subjected to ALP staining.

Since compounds **1**–**4** induced a strong increase in ALP activity, we further assessed their effects on osteoblast differentiation by determining the promoter activity of ALP, osteocalcin, OSE, and bone sialoprotein. Treatment of C2C12 cells with the compounds (10 μM) for 24 h significantly increased the promoter activity of early signals of genes related to bone formation (Figure 3). While ALP activity is a phenotypic marker for early and mature differentiations of osteoblasts, osteocalcin secretion and collagen type I alpha are reported biologic markers for terminal differentiation. ALP and osteocalcin promoter activities were increased in a dose-dependent manner in C2C12 cells after treatment with compounds **1**–**4** (Figure 4A and B). Moreover, compounds **1**–**3** also increased the levels of ALP and collagen type I alpha mRNA in a dose-dependent manner in C2C12 cells (Figure 4C).

While *ent*-kaurane diterpenoids containing a carbonyl group at C-15 are the main compounds in *C. tonkinensis*,[9−13] compounds **1**–**4** without the C-15 carbonyl group are rare.[11] Diterpenoids with an *ent*-kaur-16-en-15-one functionality exhibited no or very weak osteoblast differentiation activity in an in vitro assay (data not shown). Although the structure–activity relationships of *ent*-kaurane diterpenoids have not been investigated thoroughly, our results suggest that the skeleton of *ent*-kaur-16-ene is important for the in vitro osteoblast

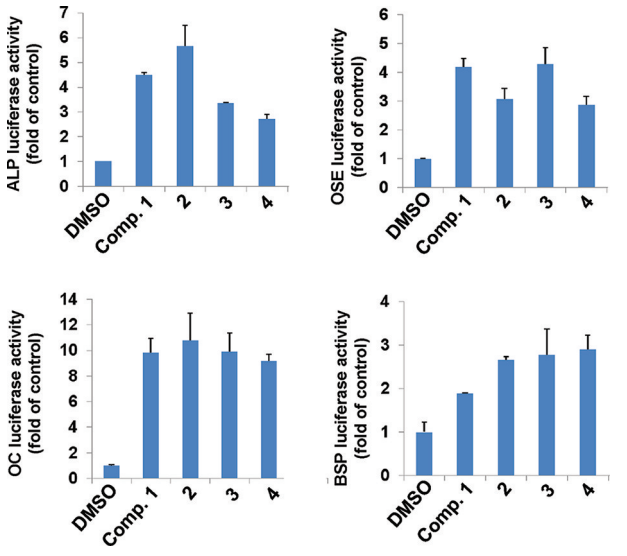


Figure 3. Compounds **1**–**4** increased the osteoblastic gene promoter activity in C2C12 cells. The compounds (10 μM) increased ALP, osteocalcin (OC), osteoblast specific element of OC (OSE, Runx2 binding site), and bone sialoprotein (BSP) promoter activity. C2C12 cells were transfected with the OC-, ALP-, BSP-, or OSE-Luc plasmid, and a luciferase reporter assay was performed 48 h later. Each bar shows three independent experiments.

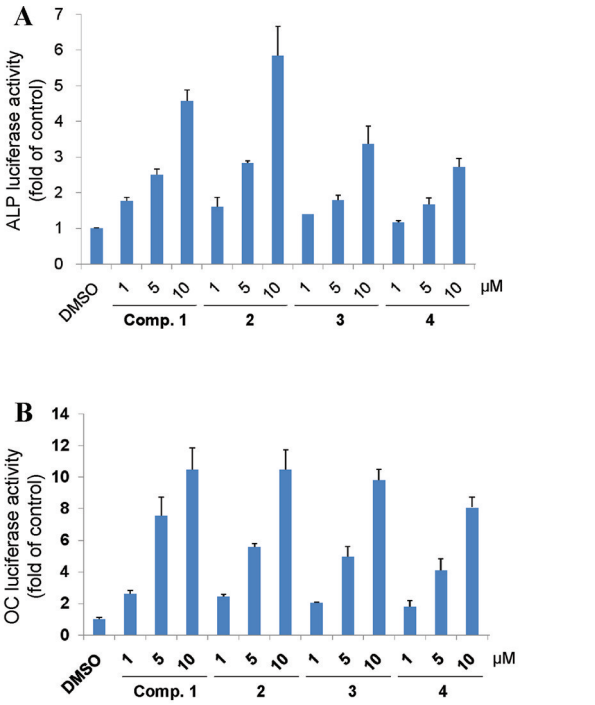
differentiation activity, while the presence of a C-15 carbonyl group may decrease this activity.

Osteoporosis is the most common cause of age-related bone loss. All of the current drugs used to treat osteoporosis are bone resorption inhibitors but have associated risks.[2,3] While many previous researchers have reported that natural phenolics have inhibitory activity against osteoclast differentiation,[19−24] our study shows that *ent*-kaurane diterpenoids **1**–**4** stimulate differentiation in osteoblasts, accompanied by increases in ALP activity and collagen content in vitro. This is the first report to suggest that *ent*-kaurane diterpenoids increase osteoblast differentiation. Thus, further investigation and optimization of these derivatives may enable the design of new approaches to osteoporosis.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were determined on a Rudolph Autopol IV polarimeter using a 100 mm glass microcell. UV spectra in MeOH were recorded on a JASCO V-550 UV/vis spectrometer with 0.5 nm resolution, and IR spectra (KBr) on a Nicolet 6700 FT-IR (Thermo Electron Corp.). NMR spectra were recorded on a Varian Unity Inova 500 MHz spectrometer with TMS as the internal standard at the Korea Basic Science Institute (KBSI, Gwangju Center, Korea). EIMS and HR-EIMS data were recorded on a Micromass QTOF2 (Micromass, Wythenshawe, UK) mass spectrometer. Silica gel (Merck, 63−200 μm particle size), RP-18 (Merck, 40−63 μm particle size), and Sephadex LH-20 were used for column chromatography. TLC was carried out with silica gel 60 F_{254} and RP-18 F_{254} plates. HPLC was carried out using a Gilson system with a UV detector and an Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size, RS Tech, Korea). All solvents used for extraction and isolation were of analytical grade.

Plant Material. The dried leaves of *C. tonkinensis* were collected from a medicinal garden in December 2009 in Hanoi, Vietnam, and were identified botanically by one of the authors (B.-T.N.). A voucher specimen (NIMM2009-15) was deposited at the herbarium of the National Institute of Medicinal Materials, Hanoi, Vietnam.



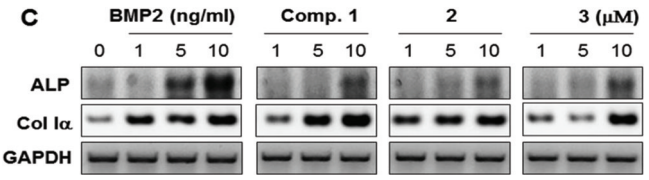


Figure 4. Compounds **1**–**4** increased ALP, OC promoter, ALP, and collagen type I alpha mRNA in a dose-dependent manner. (A, B) Compounds **1**–**4** stimulate ALP and OC promoter-driven transcription in a dose-dependent manner. C2C12 cells were transfected with the ALP- or OC-Luc plasmid and then treated with compounds **1**–**4** (1, 5, 10 μM). A luciferase reporter assay was performed 48 h later. Each bar shows three independent experiments. (C) C2C12 cells were treated with compounds **1**–**4** (1, 5, 10 μM) or BMP2. Total RNA was extracted from the cell lysates, and cDNA was prepared from 1 μg of the total RNA. RT-PCR was performed using the cDNA and primers for ALP, collagen type 1α, and GAPDH.

Extraction and Isolation. The dried leaves of *C. tonkinensis* (2 kg) were extracted with MeOH (3 × 4 L) at room temperature for a week. The combined methanol extracts were concentrated to yield a dry residue (120.0 g). This crude extract was suspended in H_2O (2.0 L) and partitioned successively with CH_2Cl_2 (3 × 1.5 L), EtOAc (3 × 1.5 L), and *n*-BuOH (3 × 2 L). The CH_2Cl_2 fraction (32.0 g), which significantly increased ALP activity, was chromatographed over a silica gel column (10 × 25 cm; 63−200 μm particle size) and eluted with a gradient solvent of *n*-hexane/EtOAc (9:1, 8:2, ..., 1:9, each 2.5 L) to yield six fractions (F1: 10.6 g; F2: 3.5 g; F3: 2.5 g; F4: 4.2 g; F5: 5.6 g; F6: 3.6 g) based on the TLC profile. The active fractions, F3 and F4, were subjected to additional chromatography. Fraction F3 was applied to an RP-18 column (4 × 30 cm; 40−63 μm particle size) with a stepwise gradient of MeOH/H_2O (3:1 to 10:1) to afford four subfractions (F3.1−F3.4). Fraction F3.2 (110 mg) was further separated by HPLC [Optima Pak C_{18} column (10 × 250 mm, 10 μm particle size, RS Tech, Korea); mobile phase MeOH in H_2O containing 0.1% HCO_2H (0−65 min: 63% MeOH, 65−70 min: 63−100% MeOH, 70−80 min: 100% MeOH); flow rate 2 mL/min; UV detection at 205 and 254 nm] to give compound **1** (t_R = 37.5 min, 8.5 mg) and compound **2** (t_R = 46.0 min, 5.0 mg). Fraction F4 was

dx.doi.org/10.1021/np200667f|*J. Nat. Prod.* 2011, 74, 2526−2531

chromatographed over a Sephadex LH-20 column (5 × 30 cm) using MeOH as the eluting solvent to afford three subfractions (F4.1−F4.3). Fraction F4.3 (450 mg) was applied to an RP-18 column (4 × 25 cm; 40−63 μm particle size) using a stepwise gradient of MeOH/H_2O (2:1 to 10:1) to yielded three subfractions (F4.3.1−F4.3.3). From fraction F4.3.2 (148 mg), compound **3** (t_R = 36.0 min, 7.0 mg) and a mixture (t_R = 40.0−43.5 min, 42.0 mg) were afforded by HPLC (0−45 min: 58% MeOH, 45−50 min: 58−100% MeOH, 50−60 min: 100% MeOH). This mixture was finally purified by HPLC (0−45 min: 65% MeCN; 45−50 min: 65−100% MeCN; 50−60 min: 100% MeCN) to yield compound **4** (t_R = 24.0 min, 6.0 mg).

ent-11β-Acetoxykaur-16-en-18-ol (**1**): white, amorphous powder; $[\alpha]_D^{25}$ −20 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (log ε) 210 (3.82) nm; IR (film) ν_{max} 3498 (OH), 2927, 1710 (C=O), 1631, 1444, 1378, 1265, 1033 cm^{-1}; CD (MeOH, $\Delta\varepsilon$) λ_{max} 288 (+0.32), 218 (−12.19), 209 (+0.83); ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; EIMS *m*/*z* (rel int) 284 (29), 269 (23), 255 (100), 239 (35), 213 (14), 199 (19); HR-EIMS *m*/*z* 346.2509 $[M]^+$ (calcd for $C_{22}H_{34}O_3$, 346.2508).

ent-11α-Hydroxy-18-acetoxykaur-16-ene (**2**): brown, amorphous powder; $[\alpha]_D^{25}$ −26 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 206 (3.63) nm; IR (film) ν_{max} 3482 (OH), 2936, 1725 (C=O), 1644, 1458, 1368, 1249, 1043 cm^{-1}; CD (MeOH, $\Delta\varepsilon$) λ_{max} 285 (+4.24), 227 (−5.87), 209 (+3.37); ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; EIMS *m*/*z* (rel int) 346 $[M]^+$ (83), 328 (15), 303 (100), 286 (13), 273 (20), 255 (61); HR-EIMS *m*/*z* 346.2507 $[M]^+$ (calcd for $C_{22}H_{34}O_3$, 346.2508).

ent-14β-Hydroxy-18-acetoxykaur-16-ene (**3**): brown, amorphous powder; $[\alpha]_D^{25}$ −17 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 206 (3.69) nm; IR (film) ν_{max} 3471 (OH), 2929, 1710 (C=O), 1632, 1456, 1381, 1271, 1033 cm^{-1}; CD (MeOH, $\Delta\varepsilon$) λ_{max} 286 (+1.35), 223 (−4.42), 203 (+0.36); ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; EIMS *m*/*z* (rel int) 346 $[M]^+$ (47), 328 (15), 273 (24), 255 (35), 123 (30), 109 (100), 107 (30); HR-EIMS *m*/*z* 346.2503 $[M]^+$ (calcd for $C_{22}H_{34}O_3$, 346.2508).

ent-7α-Hydroxy-18-acetoxykaur-16-ene (**4**): white, amorphous powder; $[\alpha]_D^{25}$ −22 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 208 (3.78) nm; IR (film) ν_{max} 3437 (OH), 2932, 1726 (C=O), 1646, 1446, 1382, 1240, 1037 cm^{-1}; CD (MeOH, $\Delta\varepsilon$) λ_{max} 268 (+0.73), 225 (−10.63), 207 (+1.72); ^1H (500 MHz) and ^{13}C (125 MHz) NMR data, see Table 1; EIMS *m*/*z* (rel int) 346 $[M]^+$ (100), 328 (17), 286 (10), 268 (51), 255 (32); HR-EIMS *m*/*z* 346.2509 $[M]^+$ (calcd for $C_{22}H_{34}O_3$, 346.2508).

Cell Culture. All tissue culture media and antibiotics were from Gibco-BRL. C2C12 cells were maintained in DMEM with 20% fetal bovine serum, antibiotics, and antimycotics at 37 °C in an atmosphere of 5% CO_2.

Plasmids. The plasmid pGL3-12-OSE-luc was constructed by inserting 12 tandem repeats of the RUNX binding site (AACCACA) into the multiple cloning site of pGL3-luciferase (Promega, Madison, WI, USA). OC promoter-Luc (1.3 kb of the osteocalcin promoter), ALP-Luc, and BSP-Luc were generously provided by Dr. N. Kim (Chonnam National University).

DNA Transfections and Reporter Assay. Transient transfections were performed using the Ca_3PO_4 or Lipofectamine Plus method, with pCMV β-gal as an internal control for transfection efficiency. For the luciferase assay, C2C12 cells were seeded on 24-well plates and incubated for 24 h before transfection with the reporter constructs. At 36 h post-transfection, the cell lysates were analyzed for luciferase activity using a luciferase reporter assay kit (Promega).

ALP Staining. Differentiated osteoblast cells were stained for ALP activity using CIP/NBT color development substrate (Promega).

RNA Preparation and Semiquantitative RT-PCR. Total cellular RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Random primer and reverse transcriptase (Invitrogen) were used to synthesize cDNA from 1 μg of total RNA. The following conditions were used for PCR amplification: initial denaturation at 94 °C for 1 min, followed by 28−30 cycles of denaturation at 94 °C for 30 s, annealing at an optimal temperature for each primer pair for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min. The PCR primer sequences were as follows: ALP, forward 5′-GAT CAT TCC CAC GTT TTC AC-3′ and reverse 5′-TGC GGG CTT GTG GGA CCT GC-3′; OC, forward 5′-CTC CTG AGT CTG ACA AAG CCT T-3′ and reverse 5′-GCT GTG ACA TCC ATT ACT TGC-3′; CollA, forward 5′-TCT CCA CTC TTC TAG GTT CCT-3′ and reverse 5′-TTG GGT CAT TTC CAC ATG C-3′; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), forward 5′-ACC ACA GTC CAT GCC ATC AC-3′ and reverse 5′-TCC ACC ACC CTG TTG CTG TA-3′. GAPDH was used as an internal control.

Statistical Analysis. Statistical calculations were carried out using one-way ANOVA ($p < 0.05$). The results are expressed as the mean ± SD of three to five independent experiments.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

^1H and ^{13}C NMR, HSQC, and HMBC spectra of compounds **1**−**4** are available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENTS

This study was supported by research funds from Chosun University, 2011.

■ REFERENCES

(1) Lee, M. K.; Lim, S. W.; Yang, H.; Sung, S. H.; Lee, H. S.; Park, M. J.; Kim, Y. C. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 2850−2854.

(2) Riggs, B. L.; Parfitt, A. M. *J. Bone Miner. Res.* **2005**, *20*, 177−184.

(3) Lane, N. E.; Kelman, A. *Arthritis Res. Ther.* **2003**, *5*, 214−222.

(4) Rodan, G. A.; Martin, T. J. *Science* **2000**, *289*, 1508−1514.

(5) Lo, Y. C.; Chang, Y. H.; Wei, B. L.; Huang, Y. L.; Chiou, W. F. *J. Agric. Food Chem.* **2010**, *58*, 6643−6649.

(6) Han, C. Y. E.; Wang, Y.; Yu, L.; Powers, D.; Xiong, X.; Yu, V.; Nguyen, Y.; Jean, D. J. S. Jr.; Babij, P. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 1442−1445.

(7) Loi, D. T. *The Medicinal Plants and Remedies of Vietnam*; Medicinal Publishing House: Hanoi, Vietnam, 2003; pp 826−828.

(8) Ngoc, P. H.; Minh, P. T. H. *Vietnamese J. Pharm.* **1996**, *36*, 15−17.

(9) Minh, P. T. H.; Ngoc, P. H.; Quang, D. N.; Hashimoto, T.; Takaoka, S.; Asakawa, Y. *Chem. Pharm. Bull.* **2003**, *51*, 590−591.

(10) Giang, P. M.; Jin, H. Z.; Son, P. T.; Lee, J. H.; Hong, Y. S.; Lee, J. J. *J. Nat. Prod.* **2003**, *66*, 1217−1220.

(11) Giang, P. M.; Son, P. T.; Hamada, Y.; Otsuka, H. *Chem. Pharm. Bull.* **2005**, *53*, 296−300.

(12) Thuong, P. T.; Dao, T. T.; Pham, T. H. M.; Nguyen, P. H.; Le, T. V. T.; Lee, K. Y.; Oh, W. K. *J. Nat. Prod.* **2009**, *72*, 2040−2042.

(13) Dao, T. T.; Le, T. V. T.; Nguyen, P. H.; Thuong, P. T.; Minh, P. T. H.; Woo, E. R.; Lee, K. Y.; Oh, W. K. *Planta Med.* **2010**, *76*, 1011−1014.

(14) Monte, F. J. Q.; Dantas, E. M. G.; Braz-Filho, R. *Phytochemistry* **1988**, *27*, 3209−3212.

(15) Fraga, B. M.; Gonzalez, P.; Hernandez, M. G.; Suarez, S. *Tetrahedron* **2005**, *61*, 5623−5632.

(16) Kuo, P. C.; Shen, Y. C.; Yang, M. L.; Wang, S. H.; Thang, T. D.; Dung, N. X.; Chiang, P. C.; Lee, K. H.; Lee, E. J.; Wu, T. S. *J. Nat. Prod.* **2007**, *70*, 1906−1909.

(17) Fernandez, C.; Fraga, B. M.; Hernandez, M. G. *Phytochemistry* **1986**, *25*, 2573−2576.

(18) Canalis, E.; Economides, A. N.; Gazzerro, E. *Endocrinology* **2003**, *24*, 218−235.

(19) Zeng, G. Z.; Tan, N. H.; Hao, X. J.; Mu, Q. Z.; Li, R. T. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 6178−6180.
(20) Zeng, G. Z.; Pan, X. L.; Tan, N. H.; Xing, J.; Zhang, Y. M. *Eur. J. Med. Chem.* **2006**, *41*, 1247−1252.
(21) Ozaiki, K.; Kawata, Y.; Amano, S.; Hanazawa, S. *Biochem. Pharmacol.* **2000**, *59*, 1577−1581.
(22) Meng, F. H.; Li, Y. B.; Xiong, Z. L.; Jiang, Z. M.; Li, F. M. *Phytomedicine* **2005**, *12*, 189−193.
(23) Zang, G.; Qin, L.; Hung, W. Y.; Shi, Y. Y.; Leung, P. C.; Yeung, H. Y.; Leung, K. S. *Bone* **2006**, *38*, 818−825.
(24) Yoon, K. D.; Jeong, D. G.; Hwang, Y. H.; Ryu, J. M.; Kim, J. *J. Nat. Prod.* **2007**, *70*, 2029−2032.