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ORIGINAL ARTICLE

Decalpenic acid, a novel small molecule from *Penicillium verruculosum* CR37010, induces early osteoblastic markers in pluripotent mesenchymal cells

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Osteoblasts are the cells responsible for bone formation during embryonic development and adult life. Small compounds that could induce osteoblast differentiation might be promising sources of therapies for bone diseases such as osteoporosis. During screening for inducers of osteoblast differentiation of mouse pluripotent mesenchymal C3H10T1/2 cells, we isolated a small compound from the fermentation broth of *Penicillium verruculosum* CR37010. This compound, named decalpenic acid, bears a decalin moiety with a tetraenoic acid side chain. Treatment of C3H10T1/2 cells with decalpenic acid alone induced the expression of early osteoblast markers, such as alkaline phosphatase activity and osteopontin mRNA, but did not induce the late osteoblast marker osteocalcin mRNA or adipocyte markers under our experimental conditions.
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Keywords: adipocyte; alkaline phosphatase; differentiation; mesenchymal stem cell; osteoblast

INTRODUCTION

Osteoblasts produce most of the proteins present in the bone extracellular matrix, and are responsible for bone formation during both embryonic development and adult life.[1] The adult skeleton undergoes continuous coordinated remodeling involving bone formation by osteoblasts and bone resorption by osteoclasts. Dysregulation of this coupled remodeling is known to lead to bone diseases such as osteoporosis.[2] Therefore, understanding of the molecular mechanisms for the regulation of osteoblast differentiation is of great interest for developing therapies for bone disease and in bone regenerative medicine.

Osteoblasts arise from mesenchymal stem cells (MSCs) having a potential for differentiating into various cell lineages, including osteoblasts, chondrocytes, myoblasts and adipocytes.[3] Several signaling pathways, such as bone morphogenetic proteins (BMPs), hedgehog and canonical Wnt, are shown to stimulate osteoblast differentiation.[4–6] Moreover, two transcription factors, Runx2 and Osterix/Sp7, are essential for osteoblastogenesis.[7–9] During osteoblast differentiation, osteoblastic markers such as alkaline phosphatase (ALP), osteopontin (OPN), type I collagen (ColI) and osteocalcin (OCN) are induced strongly.[5,10] However, the molecular mechanisms for the regulation of osteoblast differentiation are still not completely elucidated. Small molecules that could induce osteoblastogenesis might be useful tools to investigate the mechanism of osteoblast differentiation; moreover, they might also be promising candidate therapies for bone diseases.

The mouse mesenchymal cell line C3H10T1/2 exhibits MSC-like pluripotency, and therefore provides a useful model system for the study of osteoblast differentiation.[11,12] Using ALP expression as the osteoblast marker, we screened libraries of microbial fermentation broths for natural compounds that might induce osteoblast differentiation in C3H10T1/2 cells. During screening, we isolated a small compound named decalpenic acid (Figure 1) from the fermentation broth of *Penicillium verruculosum* CR37010. In this report, we describe the isolation, structure determination and biological activity of this compound.

RESULTS AND DISCUSSION

Isolation procedure for decalpenic acid

A 10-kg culture broth of *P. verruculosum* CR37010 was extracted with 20 l of 67% aqueous acetone and filtered. The filtrate of the extracts was concentrated *in vacuo* to remove acetone. The aqueous solution (6.6 l, pH 4.0) was extracted with butanol (15 l). The extract was concentrated *in vacuo* to produce 32.1 g of dried material. This was extracted with ethyl acetate, and the extract was concentrated *in vacuo* to give 11.8 g of dried materials. These were subjected to Centrifugal Partition Chromatography (CPC) (Model LLB-M, Sanki, Kyoto, Japan) with hexane–methanol–acetonitrile (7:2:3 by volume), and active fractions were concentrated *in vacuo* to produce dried material. We repeated the CPC purification four times to give 393.6 mg of dried material. This was further applied to a reversed-phase HPLC column (ODS, 30×250 mm, 15 ml min^{-1}) and eluted with 55% acetonitrile to

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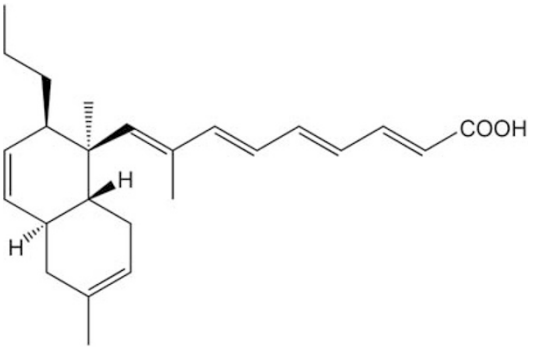

Figure 1 Structure of decalpenic acid.

give decalpenic acid. We repeated the HPLC purification three times to obtain 8.4 mg of pure decalpenic acid.

Physicochemical properties and structure of decalpenic acid

The physicochemical properties of decalpenic acid are summarized in Table 1. It was isolated as a yellow powder and was soluble in $CHCl_3$, methanol and dimethylsulfoxide (DMSO). It had a characteristic UV maximum at 334 nm (ε=18 000) in the UV spectrum, suggesting the presence of conjugated double bonds. The molecular formula of decalpenic acid was determined to be $C_{25}H_{34}O_2$ by HRESI-MS (*m/z* 365.2480 Δ+0.5 mDa $(M\text{-}H)^-$), indicating nine degrees of unsaturation.

The ^1H and ^{13}C NMR spectra including DEPT135 and HMQC in $CDCl_3$ revealed that decalpenic acid had 4 methyl, 4 methylene, 3 sp3 methine, 10 sp2 methine and 4 quaternary carbons, including 1 sp3, 2 sp2 and 1 carboxyl carbon atoms, as summarized in Table 2. The molecular formula ($C_{25}H_{34}O_2$) and the NMR data suggested the presence of an exchangeable proton (OH). Based on the molecular formula, ^{13}C NMR signal at δ 171.1, UV spectra and characteristic absorption at 1684 cm^{-1} in IR spectrum, decalpenic acid appears to have a conjugated carboxylic acid moiety. Results of 2D NMR experiments are summarized in Figure 2a. According to ^1H–^1H COSY experiments, decalpenic acid had three ^1H spin–spin networks from the olefinic proton at 2-H (δ 5.85) to 3-H (δ 7.39), 4-H (δ 6.34), 5-H (δ 6.65), 6-H (δ 6.24) and 7-H (δ 6.46), from the methyl proton at 22-H (δ 0.85) to 21-H (δ 1.17 and 1.45), 20-H (δ 1.44), 19-H (δ 2.19), 18-H (δ 5.75) and 17-H (δ 5.50), and from the olefinic proton at 13-H (δ 5.42) to 12-H (δ 1.81 and 2.05), 11-H (δ 1.63), 16-H (δ 2.07) and 15-H (δ 1.81 and 2.05). These three partial structures and the remaining signals were connected by the heteronuclear multiple bond connectivity analysis. The connectivities from the first singlet methyl protons at 23-H (δ 1.89) to C-7 (δ 145.3), C-8 (δ 134.0) and C-9 (δ 145.7), and from the olefinic protons at 2-H and 3-H to C-1 (δ 171.1) suggested the presence of a tetraenoic acid moiety. In addition, long-range coupling from the second singlet methyl proton at 25-H (δ 1.67) to C-13 (δ 121.6), C-14 (δ 133.8) and C-15 (δ 37.7), the third one at 24-H (δ 1.17) to C-9 (δ 145.7), C-10 (δ 40.8), C-11 (δ 40.5) and C-19 (δ 46.1), the olefinic proton at 17-H to C-11, C-15 and C-16 (δ 35.3), and the olefinic proton at 18-H to C-10 (δ 40.8) established a bicyclic ring structure attached to the propyl and tetraenoic acid side chains of decalpenic acid. The remaining part was an exchangeable proton, which should sit adjacent to a carbonyl carbon as mentioned above. Taken together, the planar structure of decalpenic acid was determined as shown in Figure 2a.

The geometry of the double bond of the side chain was determined to be 2*E*, 4*E*, 6*E* and 8*E* by the coupling constants and NOEs shown in Figure 2b. The coupling constants of 2-H to 7-H were all large (14.8–15.4 Hz) and NOEs were observed between 6-H and 23-H, and between 7-H and 9-H. The relative stereochemistry of decalpenic acid was mainly established from the NOEs. The bicyclic ring junction was determined as *trans* from the coupling constant between 11-H and 16-H (11.0 Hz). NOEs observed from 9-H (δ 5.71) to 11-H and 12-Heq (δ 2.05), between 19-H and 23-H, 24-H and from 24-H to 16-H and 12-Hax (δ 1.81) revealed that the tetraenoic acid and propyl side chains were present in the same surface on the decalin ring. From these results, the relative stereochemistry of decalpenic acid was elucidated to be 10*R**, 11*R**, 16*S**, 19*R** in Figure 2b.

Table 1 Physicochemical properties of decalpenic acid

Appearance	Yellow powder
Molecular formula	$C_{25}H_{34}O_2$
Molecular weight	366.54
HRESI-MS (negative, *m/z*)	
Found	365.2480 $(M\text{-}H)^-$
Calcd.	365.2475 (for $C_{25}H_{33}O_2$)
UV λ_{max} (nm, ε) in MeOH	334 (18,000)
$[\alpha]_D^{22}$ (*c* 0.39, $CHCl_3$)	−130.0°
IR ν_{max} (cm^{-1}) (KBr)	3438, 2958, 2871, 1684, 1621, 1591, 1269, 1144, 1005

Table 2 ^{13}C- and ^1H-NMR data of decalpenic acid in $CDCl_3$

Position	^{13}C		^1H	*Multiplicity*
1	171.1	>C=O		
2	118.5	=CH–	5.85	d 15.4
3	147.0	=CH–	7.39	dd 11.5, 15.4
4	128.4	=CH–	6.34	dd 11.5, 14.8
5	142.8	=CH–	6.65	dd 11.0, 14.8
6	124.7	=CH–	6.24	dd 11.0, 15.1
7	145.3	=CH–	6.46	d 15.4
8	134.0	=C<		
9	145.7	=CH–	5.71	brs
10	40.8	>C<		
11	40.5	>CH–	1.63	ddd 5.0, 11.0, 11.0
12	27.0	$-CH_2-$	1.81	m
			2.05	m
13	121.6	=CH–	5.43	brd 4.1
14	133.8	=C<		
15	37.7	$-CH_2-$	1.81	m
			2.05	m
16	35.3	>CH–	2.07	m
17	129.2	=CH–	5.50	d 10.4
18	129.3	=CH–	5.75	m
19	46.1	>CH–	2.19	m
20	20.9	$-CH_2-$	1.44	m
21	36.0	$-CH_2-$	1.16	m
			1.45	m
22	14.4	$-CH_3$	0.85	t 6.9
23	14.2	$-CH_3$	1.89	brs
24	19.5	$-CH_3$	1.17	s
25	23.3	$-CH_3$	1.67	brs

Chemical shifts in p.p.m. from TMS were used as an internal standard.

Biological activities

The effect of decalpenic acid on osteoblast differentiation of mouse pluripotent mesenchymal C3H10T1/2 cells was examined using an early osteoblast marker, ALP activity. As reported previously, a 4-day treatment of the cells with 1 μM PM, a small-molecule agonist of hedgehog signaling,[12,13] induced ALP activity determined by ALP staining (Figure 3a). Four days of treatment with 1 μM decalpenic acid also induced ALP staining (Figure 3a). The same results were obtained when ALP activity was determined using the fluorescent substrate (Figure 3b). Treatment with decalpenic acid at concentrations ranging from 0.37 to 10 μM also induced ALP activity in a dose-dependent manner. The half maximal effective concentration (EC_{50}) based on ALP expression for decalpenic acid was estimated at 2 μM. Thus, decalpenic acid alone was able to induce ALP activity in C3H10T1/2 cells.

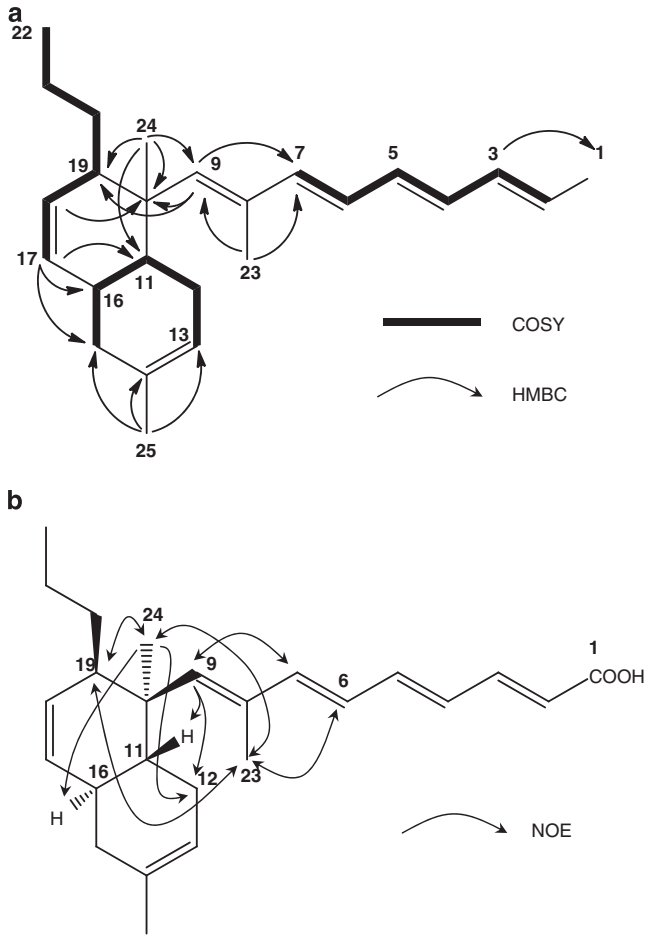

Figure 2 (**a**) ^{1}H–^{1}H COSY and selected HMBC correlations of decalpenic acid in $CDCl_3$. (**b**) NOEs of decalpenic acid in $CDCl_3$.

Next, to examine whether decalpenic acid could induce the expression of other osteoblast differentiation markers, real-time PCR analysis of a panel of osteoblast marker genes was performed (Figure 4). Treatment of the cells with 1 μM of decalpenic acid for 8 days markedly increased the expression of *Alpl* mRNA, the gene encoding ALP. It also increased the expression of mRNAs for the early osteoblast markers *CollA1*, a gene that encodes the major component of ColI and *Opn* (Figures 4a–c). However, decalpenic acid treatment could not increase *Osterix*, which encodes an essential transcription factor for osteoblast differentiation,[9] or *Ocn*, a late osteoblast marker gene (Figures 4d and e). On the other hand, treatment for 8 days with 1 μM of PM increased the expression levels of *Alpl*, *Ocn* and *Osterix*. These results suggest that decalpenic acid could promote early, but not late steps of osteoblast differentiation.

C3H10T1/2 cells, like MSCs, can differentiate into various cells, including adipocytes as well as osteoblasts, and treatment of C3H10T1/2 cells with BMP-2 induced osteoblastogenesis and adipocytogenesis.[14,15] Therefore, we next examined whether decalpenic acid could induce C3H10T1/2 cells to differentiate into adipocytes (Figure 5). As reported previously, BMP-2 treatment of C3H10T1/2 cells induced adipocyte differentiation as indicated by Oil Red O staining (Figure 5c) and *Fabp4* mRNA expression (Figure 5e), both markers of adipocyte differentiation. BMP-2 treatment also induced markers of osteoblast differentiation such as *Opn* and *Ocn* mRNA expression (Figures 4c and d). In contrast, treatment with 1 μM of decalpenic acid or 1 μM of PM did not induce adipocyte differentiation markers (Figures 5d and e). The same results were obtained when the cells were treated with 100 nM or 10 μM of decalpenic acid (data not

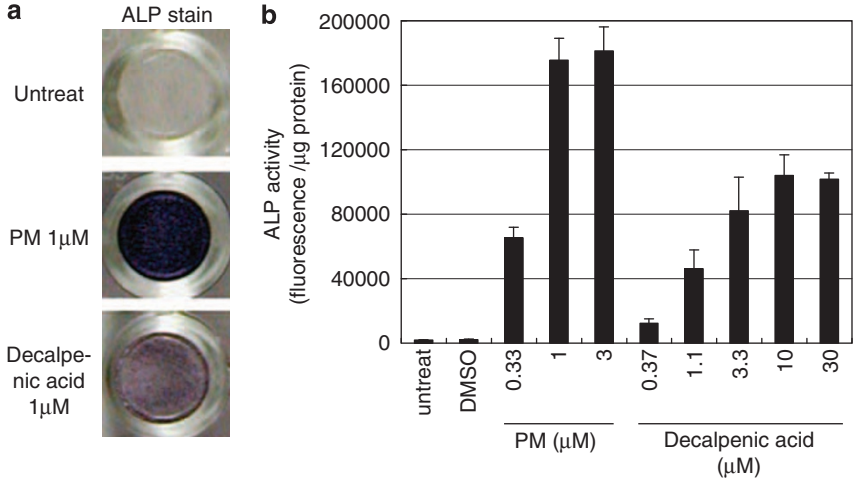

Figure 3 Decalpenic acid treatment induces expression of the early osteoblastic marker alkaline phosphatase (ALP) in C3H10T1/2 cells. (**a**) C3H10T1/2 cells were treated with 1 μM PM or 1 μM decalpenic acid. After 4 days of treatment, cells were fixed and stained for ALP. (**b**) C3H10T1/2 cells were treated with 0.1% dimethylsulfoxide (DMSO), purmorphamine (PM) or decalpenic acid. After 4 days of treatment, ALP activities were measured as described in Methods. Data are shown as the mean ± s.d. of three independent experiments performed in duplicate.

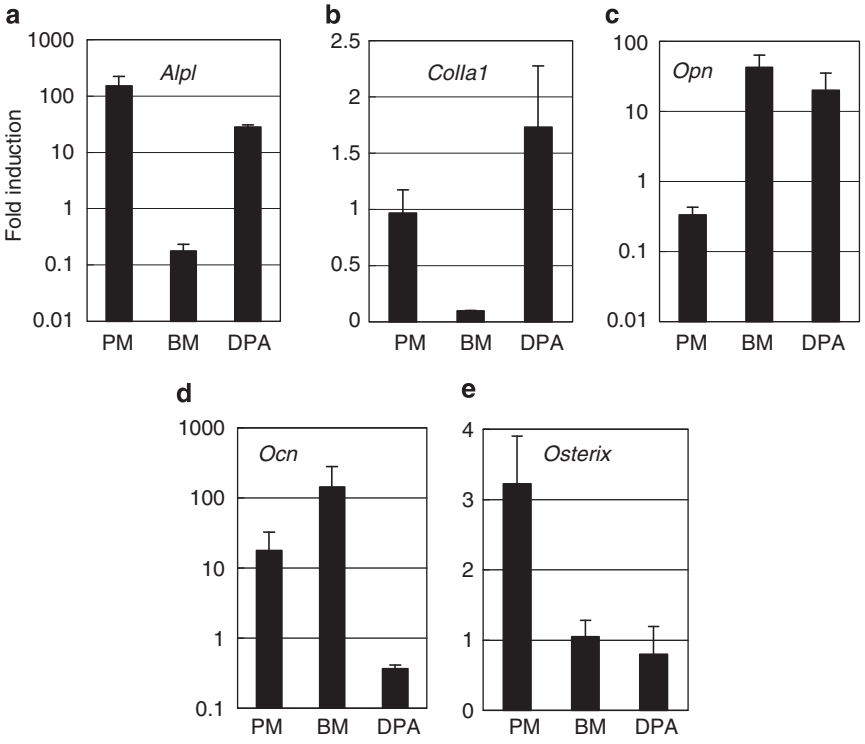

Figure 4 Real-time PCR analysis of osteoblastic marker genes in C3H10T1/2 cells treated with decalpenic acid. C3H10T1/2 cells were treated with 1 μM PM, 400 ng/ml BMP-2 (BM) or 1 μM decalpenic acid (DPA). After 8 days of treatment, total RNA was isolated from the cells and mRNA expression was determined by real-time PCR analysis for *Alpl* (**a**), *Colla1* (**b**), *Osteopontin* (**c**), *Osteocalcin* (**d**) and *Osterix* (**e**). Data were first normalized to the level of *Gapdh* and are presented as fold induction versus untreated controls. Data represent the mean ± s.d. of three independent experiments.

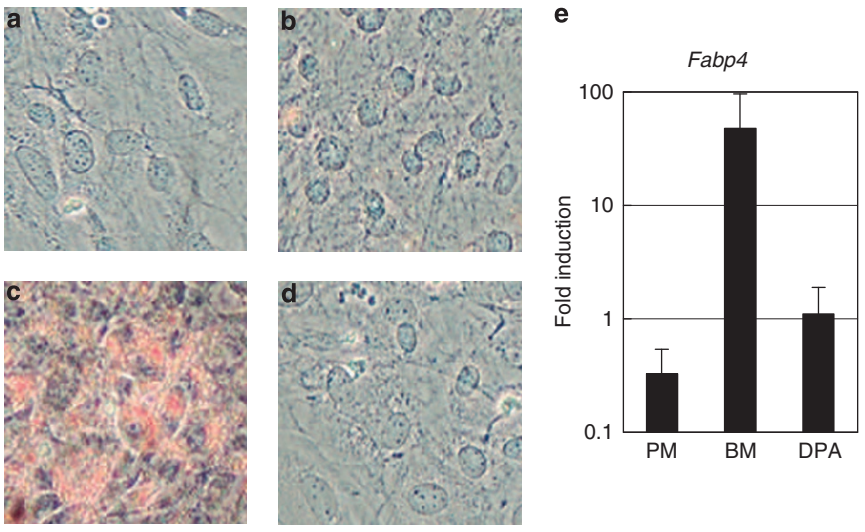

Figure 5 Decalpenic acid treatment does not induce adipocyte differentiation in C3H10T1/2 cells. C3H10T1/2 cells were treated with decalpenic acid for 8 days and stained with Oil Red O (**a**–**d**). Total RNA was isolated to determine the level of *Fabp4* mRNA expression by real-time PCR (**e**). (**a**) Untreated control, (**b**) PM 1 μM, (**c**) BMP-2 400 ng/ml, (**d**) decalpenic acid 1 μM. The images were taken at ×400 magnification. (**e**) Data were first normalized to the level of *Gapdh* and are presented as fold induction versus untreated controls. Data represent the mean ± s.d. of three independent experiments.

shown). These results show that treatment of decalpenic acid alone could not induce adipocyte differentiation in pluripotent mesenchymal C3H10T1/2 cells under our experimental conditions.

In conclusion, we report the isolation of a novel small compound from the fermentation broth of *P. verruculosum* CR37010. This compound, named decalpenic acid, consists of a decalin moiety and a tetraenoic acid side chain (Figure 1). Compounds structurally related to decalpenic acid include the antifungal polyketide hamigerone, dihydrohamigerone[16] and the cytotoxic polyketides cladobotric acids A–F.[17] These compounds also bear a decalin moiety, but differ

from decalpenic acid in the nature of the side chain, such as the presence of an epoxide. Moreover, decalpenic acid has an unusual connection of tetraenoic acid to a quaternary carbon atom of the decalin moiety. Thus, decalpenic acid is a fungal polyketide with a unique structural feature.

Decalpenic acid can induce the expression of early osteoblast markers such as ALP activity and *Opn* mRNA in C3H10T1/2 cells. It is well known that osteoblast differentiation is influenced by multiple signaling pathways including hedgehog and BMPs.[4,5] In this study, we compared the effects of decalpenic acid on C3H10T1/2 cells with the effects of a hedgehog signaling agonist and BMP-2, one of the BMP family of proteins. As shown in Figures 4 and 5, treatment with PM could induce both early and late markers of osteoblast differentiation, but did not induce adipocyte markers. Treatment with BMP-2 can induce the expression of markers of both osteoblasts and adipocytes. On the other hand, treatment with decalpenic acid induced neither late osteoblast markers nor adipocyte markers under the same experimental conditions (Figures 4 and 5). These differences in the effects of these reagents suggest that decalpenic acid affects signaling pathways other than those involving hedgehog and BMPs. We are currently investigating the molecular mechanism of the biological activity of decalpenic acid.

METHODS

Materials

The C3H10T1/2 cell line was obtained from American Type Culture Collection (Manassas, VA, USA). Purmorphamine (PM) was purchased from Calbiochem (La Jolla, CA, USA). BMP-2 was purchased from Wako Pure Chemical Industry (Tokyo, Japan). All other chemicals and solvents used for purifications were purchased from Wako Pure Chemical Industry.

Analytical measurements

Optical rotation was measured using a P-1030 spectropolarimeter (JASCO, Tokyo, Japan). UV spectra were obtained using a U-2800 spectrophotometer (Hitachi High-Tech, Tokyo, Japan). Infrared (IR) spectra were recorded on a FT-210 spectrometer (Horiba, Kyoto, Japan). ^{1}H and ^{13}C NMR spectra were measured using a JNM-ECA600 spectrometer (JEOL, Tokyo, Japan), using TMS as an internal standard. HRESI-MS spectra were measured using an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA).

Fermentation of fungal strain CR37010

The producing strain, *P. verruculosum* CR37010, was isolated from a soil sample collected in Hiroshima Prefecture, Japan. A slant culture of *P. verruculosum* CR37010 was used for inoculation in 100-ml Erlenmeyer flasks. Each flask contained 20 ml of a seed medium consisting of 2.0% soluble starch, 1.0% glucose, 0.2% soybean meal, 0.6% wheat germ, 0.5% polypeptone, 0.3% yeast extract and 0.2% $CaCO_3$ in de-ionized water adjusted to pH 7.2 with NaOH solution before sterilization. The flasks were incubated at 25 °C for 3 days on a rotary shaker at 220 r.p.m. Aliquots of 1.0 ml of this seed culture were transferred into four 500-ml Erlenmeyer flasks, each of which contained 100 ml of seed medium. The flasks were incubated at 25 °C for 2 days on a rotary shaker at 220 r.p.m. Portions of the obtained seed culture were transferred into stainless steel vats, each of which contained 2.5% oatmeal and water-absorbed rice as a solid production medium. The stainless vats were stirred thoroughly and then cultured statically at 25 °C for 14 days. After incubation, 10 kg of the obtained culture was extracted with 20 l of 67% aqueous acetone.

Cell culture and osteogenic differentiation

C3H10T1/2 cells were cultured at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industry) containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Invitrogen). To induce osteoblast differentiation for ALP staining and assay, C3H10T1/2 cells were seeded in 96-well plates (Corning, Rochester, NY, USA) at a density of 10 000 cells per well. After reaching confluence, cells were treated with PM or decalpenic acid for 4 days. For RNA isolation, cells were seeded in six-well plates (Corning) at a density of 188 000 cells per well. After reaching confluence, cells were treated with PM, BMP-2 or decalpenic acid for 8 days.

ALP staining and assay

For ALP staining, cells were washed with phosphate-buffered saline (PBS) and fixed with 10% formalin for 10 min. The fixed cells were washed three times with distilled water and stained with the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) (Sigma-Aldrich, St Louis, MO, USA) for 30 min at 37 °C. The cells were then washed twice with PBS. For ALP assay, cells were washed with PBS and lysed with passive lysis buffer (Promega, Madison, WI, USA) for 15 min. The cell lysate was mixed with an equal volume of AttoPhos ALP substrate solution (Promega) in a black 96-half-well plate (Corning). After 15 min of incubation at room temperature, fluorescence intensities of the reaction solution (excitation at 440 nm; emission at 560 nm) were measured using an ARVO plate reader (Perkin-Elmer, Waltham, MA, USA). ALP activity was normalized by protein content quantified using a BCA protein kit (Pierce, Rockford, IL, USA) and expressed as fluorescent intensity per μg of total protein.

Oil red O staining

Cells were washed twice with PBS and fixed with 10% formalin for 10 min. The cells were then washed twice with PBS and rinsed with 60% isopropanol. The cells were then stained with Oil Red O dye solution (0.3% Oil Red O from Sigma-Aldrich in 60% isopropanol) for 20 min. The cells were then rinsed with 60% isopropanol and washed twice with PBS. The red-stained lipid droplets were analyzed using a CKX30 microscope (Olympus, Tokyo, Japan).

RNA isolation and real-time PCR amplification

Total RNA was isolated from cells using an RNeasy plus kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol. cDNA was synthesized using the SuperScriptVILO first-strand synthesis system (Invitrogen). Quantitative real-time PCR was performed on a Thermal Cycler Dice Real Time System (Takara, Shiga, Japan) using SYBR Premix Ex Taq II (Takara). All the primers were purchased from Takara and the primer sequences are available upon request. All reactions were run in triplicate and relative expression levels were calculated using the ΔΔCT method. Values were normalized to *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*) level as an internal control gene.

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