The Journal of Antibiotics (2012) 65, 565–569

www.nature.com/ja

ORIGINAL ARTICLE

Trichocyalides A and B, new inhibitors of alkaline phosphatase activity in bone morphogenetic protein-stimulated myoblasts, produced by *Trichoderma* sp. FKI-5513

Takashi Fukuda[1,2], Ryuji Uchida[1], Satoshi Ohte[3], Hiroyo Inoue[1], Hiroyuki Yamazaki[1], Daisuke Matsuda[1], Kenichi Nonaka[2], Rokurou Masuma[2], Takenobu Katagiri[3] and Hiroshi Tomoda[1]

Two new butenolides, designated trichocyalides A and B, were isolated along with the known compound harzianolide, from the culture broth of *Trichoderma* sp. FKI-5513 by solvent extraction, ODS column chromatography and HPLC. Their structures were elucidated by several spectral analyses, showing that they have the common skeleton of butenofuranone. Trichocyalides A and B inhibited alkaline phosphatase (ALP) activity, a typical marker enzyme of osteoblastic differentiation (IC_{50}: 83.0 and 187 μM, respectively), in bone morphogenetic protein (BMP)-stimulated C2C12 myoblasts mutant cells, which stably express BMP receptor activity, whereas harzianolide showed no inhibitory activity against ALP even at 500 μM.
The Journal of Antibiotics (2012) **65,** 565–569; doi:10.1038/ja.2012.70; published online 5 September 2012

Keywords: bone morphogenetic protein signaling; C2C12 myoblasts; fibrodysplasia ossificans progressiva; trichocyalide; *Trichoderma* sp. FKI-5513

INTRODUCTION

Bone morphogenetic proteins (BMPs), members of the transforming growth factor-β superfamily, are active proteins responsible for ectopic bone formation.[1,2] The BMP signal starts with binding to heterotetrameric transmembrane complexes of type I and type II BMP receptors. Four type I receptors, ALK1, ALK2, ALK3/BMPR-IA and ALK6/BMPR-IB, and three type II receptors, BMPR-II, ActR-II and ActR-IIB, have been reported.[3] Following ligand binding, serines and threonines in the glycine/serine domain of type I receptor are phosphorylated by the activated type II receptor. Thus, the activated BMP type I receptor transmits downstream to BMP pathway-specific Smad1/5/8 and p38 MAPK.[4] Furthermore, phosphorylated Smad1/5/8 forms complexes with Smad4, and they move to the nucleus and work as transcription factors to express early responsive genes, such as Id1 (an inhibitory protein for myogenesis).[4]

Fibrodysplasia ossificans progressiva (FOP) is a congenital disorder of progressive and widespread postnatal ossification of soft tissues.[5] Patients with classical FOP have congenital malformations that result in profoundly decreased mobility.[6,7] Recently, a recurrent heterozygous mutation in the *ACVR1/ALK2* gene was identified by Shore *et al.*[8] at 617 G→A, which causes an amino acid substitution of Arg to His at codon 206 (R206H) of the ALK2 receptor in both familial and sporadic patients with FOP. Because this mutation induced the activation of BMP constitutively, BMP inhibitors could offer therapeutic benefits for FOP.[4,9] In 1994, Katagiri *et al.*[10] reported that C2C12 myoblasts, derived from murine thigh muscle, expressed an osteogenic phenotype in the presence of high concentrations of recombinant BMP and that alkaline phosphatase (ALP) activity was induced by BMP in the clonal myoblasts; therefore, ALP activity was used as a typical marker of BMP signaling in the C2C12 cell line.

Based on these findings, we established the screening method by using a stable ALK2(R206H)-expressing C2C12 cell line (abbreviated as C2C12(R206H) cells).[11] With this method, inhibitors of BMP-induced ALP activity in C2C12(R206H) cells were screened as potential osteoblastic differentiation inhibitors. During the course of this screening program, we reported pyrrolidine-containing compounds[12–14] produced by fungal strain *Fusarium* sp. B88 as candidates for osteoblastic differentiation inhibitors.[11] Continuing this screening, two new active butenolides, designated trichocyalides A and B, were isolated from the culture broth of fungal strain *Trichoderma* sp. FKI-5513 along with the known harzianolide (Figure 1).[15]

In this study, we describe the taxonomy of the producing fungus, fermentation, isolation, structure elucidation and biological activity of these trichocyalides.

[1]Graduate School of Pharmaceutical Sciences, Kitasato University, Tokyo, Japan; [2]Kitasato Institute for Life Sciences, Kitasato University, Tokyo, Japan and [3]Research Center for Genomic Medicine, Saitama Medical University, Saitama, Japan
Correspondence: Professor H Tomoda, Graduate School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108-8641, Japan.
E-mail: tomodah@pharm.kitasato-u.ac.jp
Received 3 February 2012; revised 30 July 2012; accepted 30 July 2012; published online 5 September 2012

RESULTS

Taxonomy

Colonies on potato dextrose agar after 7 days at 25 °C (Figure 2a) were >80 mm in radius, floccose, flat, with white (a) aerial mycelium, covered with moss green (24 pi) conidia, exudate lacking, soluble pigment not produced. Colonies on corn meal dextrose agar after 7 days at 25 °C were 61–62 mm in radius and the surface showed hyaline floccose aerial mycelium. Colonies on synthetic low-nutrient agar after 7 days at 25 °C (Figure 2b) were 53–55 mm in radius, floccose, flat, with hyaline aerial mycelium and covered with hunter green (22 pi) conidia. Colonies on potato dextrose agar at 5 °C and 37 °C were not observed. Conidiophores were highly uniformly branched, branches frequently paired or in threes, arising at or near 90° with respect to the main axis, longer and more profusely branched with distance from the tip (Figure 2c). The phialides arose singly from the main axis and branches or were held at or near 90° in whorls, tending to be conspicuously swollen below the sharply constricted tip, ampulliform, 5.2–10.8 µm long, 2.6–4.1 µm at the widest point, 1.4–2.4 (–3.0) µm at the base. Conidia were ellipsoidal, 5.0–7.2 (−7.8) × 2.3–3.8 µm. The total length of the rDNA internal transcribed spacer (ITS; including 5.8 S rDNA) of FKI-5513 was 572 base pairs. From these findings, the producing strain FKI-5513 was considered to belong to the genus *Trichoderma*.

Fermentation

A slant culture of strain FKI-5513 grown on LcA medium (0.1% glycerol, 0.08% KH_2PO_4, 0.02% K_2HPO_4, 0.02% $MgSO_4 \bullet 7H_2O$, 0.02% KCl, 0.2% $NaNO_3$, 0.02% yeast extract and 1.5% agar, pH 6.0) was used to inoculate 100 ml seed medium (2.0% glucose, 0.2% yeast extract, 0.05% $MgSO_4 \bullet 7H_2O$, 0.5% polypeptone, 0.1% KH_2PO_4 and 0.1% agar, pH 6.0) contained in a 500-ml Erlenmeyer flask. The flask was shaken on a rotary shaker at 27 °C for 3 days. The seed culture (1 ml) was used to inoculate a 500-ml Erlenmeyer flask containing 100 ml production medium (3.0% soluble starch, 1.0% glycerol, 2.0% soy bean meal, 0.3% dry yeast, 0.3% KCl, 0.2% $CaCO_3$, 0.05% KH_2PO_4 and 0.5% $MgSO_4 \bullet 7H_2O$). The fermentation was carried out at 27 °C for 7 days.

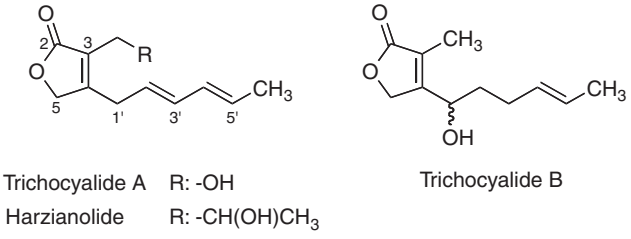

Figure 1 Structures of trichocyalides A, B and harzianolide.

Isolation

The 7-day-old culture broth (4.0 l) was extracted with 4.0 l acetone. After the acetone extracts were filtered and concentrated, the resulting aqueous solution was extracted with EtOAc (4.0 l). The ethyl acetate layer was dried over Na_2SO_4 and concentrated *in vacuo* to dryness to yield a yellow brown material (2.0 g). The material was dissolved in 30% CH_3CN, applied to an octa decyl silyl (ODS) column (30 g), and eluted stepwise with 30, 50, 70 and 100% CH_3CN (300 ml for each two fractions). The second 30% CH_3CN fraction was concentrated *in vacuo* to dryness to give a brown material (113 mg). The material was finally purified by preparative HPLC (column, PEGASIL ODS, 20 × 250 mm; Senshu Scientific Co. (Tokyo, Japan); solvent, 35% CH_3CN; detection, UV at 210 nm; flow rate, 8.0 ml min^{-1}). Under these conditions, trichocyalide A was eluted as a peak with a retention time of 27 min. The pooled fraction was concentrated *in vacuo* to dryness to give pure trichocyalide A (3.6 mg) as a yellow solid. Additionally, trichocyalide B and harzianolide were purified by preparative HPLC (column, PEGASIL ODS, 20 × 250 mm; Senshu Scientific Co.; solvent, 40% CH_3CN; detection, UV at 210 nm; flow rate, 8.0 ml min^{-1}) from the first tube of 50% CH_3CN (51.3 mg) with retention times of 18 and 24 min. The pooled peaks were concentrated *in vacuo* to dryness to give pure trichocyalide B (6.9 mg) and harzianolide (8.8 mg), respectively.

Physicochemical properties

The physicochemical properties of trichocyalides A and B are summarized in Table 1. On IR spectra, they showed similar absorption maxima at 3440–3444 cm^{-1} and 1743 or 1745 cm^{-1}, suggesting the presence of hydroxy and furanone ring moieties, respectively. The similarity of their spectral data indicated close structural relationships.

Structures elucidation of trichocyalides A and B

Trichocyalide A. The molecular formula of trichocyalide A was determined to be $C_{11}H_{14}O_3$ on the basis of HREI-MS measurement (Table 1). The ^{13}C NMR spectrum (in DMSO-d_6) showed 11 resolved signals, which were classified into one methyl carbon, three sp^3 methylene carbons, four sp^2 methine carbons, two quaternary carbons and one carbonyl carbon by analysis of DEPT spectra. The ^1H NMR spectrum (in DMSO-d_6) showed one methyl signal, three methylene signals and four methine signals. The connectivity of proton and carbon atoms was established by the HMQC spectrum, as shown in Table 2. Analysis of the ^1H–^1H COSY spectrum gave the hexa-2,4-

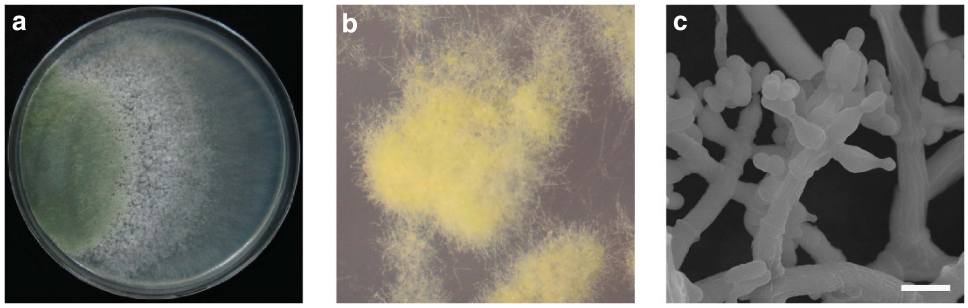

Figure 2 Morphological observation of *Trichoderma* sp. FKI-5513. (**a**) Colonies on potato dextrose agar at 7 days. (**b**) Pustules on synthetic low-nutrient agar at 7 days under a stereoscopic microscope. (**c**) Conidiophore, under a scanning electron microscope (scale bar: 10 µm).

diene chain (Figure 3). The ^{1}H-^{13}C long-range couplings of ^{2}J and ^{3}J observed in the HMBC experiments (Figure 3) gave the following information. The cross peaks from oxymethylene protons (5-H_2 δ4.73) to C-2 (δ173.8), C-3 (δ125.3) and C-4 (δ163.4) and from 3-CH_2 (δ4.01) to C-2, C-3 and C-4 supported the furane. The cross peaks from 1′-H (δ3.29) to C-3, C-4 and C-5 (δ71.1) supported that the hexa-2,4-diene chain and furanone ring are conjugated at C-4. From the chemical shift and molecular formula, 3-CH_2 should have a hydroxyl methyl group. The structure satisfied the molecular formula and degree of unsaturation. From the ^{1}H coupling constants ($J_{2',3'} = 15.0$ Hz and $J_{4',5'} = 15.0$ Hz), the diene unit was assigned to both *E* geometrical configurations. Taken together, the structure of trichocyalide A was elucidated as shown in Figure 1.

Trichocyalide B. The molecular formula of trichocyalide B was determined to be $C_{11}H_{16}O_3$ on the basis of HRFAB-MS measurement (Table 1). The differences between trichocyalide A and trichocyalide B were the presence of 3-CH_3 and (*E*)-hex-4-en-1-ol chain for trichocyalide B (Figure 4), from the chemical shifts, ^{1}H–^{1}H COSY and HMBC spectra. Cross peaks from 3-CH_3 (δ1.70) to C-2 (δ175.0), C-3 (δ120.3) and C-4 (δ164.4), and from hydroxyl 1′-H (δ4.50) to C-3, C-4 and C-5 (δ69.5) were observed in the HMBC experiments, confirming that the 3-CH_3 and (*E*)-hex-4-en-1-ol chain were linked at C-3 and C-4, respectively (Figure 4). The structure satisfied the molecular formula and degree of unsaturation. From the ^{1}H coupling constants ($J_{4',5'} = 15.0$ Hz), the monoene unit was assigned to *E* geometrical configuration. Taken together, the structure of trichocyalide B was elucidated as shown in Figure 1.

Table 1 Physicochemical properties of trichocyalides A and B

	Trichocyalide A	*Trichocyalide B*
Appearance	yellow solid	yellow solid
MW	194	196
Molecular formula	$C_{11}H_{14}O_3$	$C_{11}H_{16}O_3$
HR-MS (*m/z*)	EI-MS	FAB-MS
Calculated	194.0943 $(M)^+$	197.1178 $(M+H)^+$
Found	194.0941 $(M)^+$	197.1170 $(M+H)^+$
UV λ_{max}^{MeOH} nm (ε)	223 (12 100), 336 (970)	216 (9900), 291(700)
$[\alpha]_D^{26}$	—	+10.2° (*c* = 0.1, MeOH)
IR ν_{max}^{KBr} cm^{-1}	3440, 2929, 1745, 1670 1442, 1346, 1024, 1001	3444, 2931, 1743, 1678 1446, 1086, 1036

Biological properties

Inhibition of ALP activity in osteoblastic C2C12(R206H) cells by trichocyalides. The effects of trichocyalides on ALP activity, a typical marker of osteoblastic differentiation, and on cytotoxicity in C2C12(R206H) cells were tested. As shown in Table 3, trichocyalide A was found to inhibit ALP with an IC_{50} value of 83.0 μM without cytotoxicity even at 500 μM. On the other hand, trichocyalide B exhibited ALP inhibitory activity and cytotoxicity with IC_{50} values of 187 and 354 μM, respectively. Under the same conditions, structurally related herzianolide showed no effect on ALP and cytotoxicity at 500 μM.

Effect of trichocyalides and herzianolide on BMP signaling. The effect of trichocyalides and herzianolide on BMP signaling was investigated using C2C12(Id1-BRE) cells;[14] however, trichocyalides showed no effect on luciferase activity even at 500 μM, indicating that they did not inhibit the early events of BMP signaling. (Table 3).

Antimicrobial activity. Only trichocyalide A showed weak antibacterial activity against *B. subtilis* PCI 219, *K. rhizophila* ATCC 9341 and

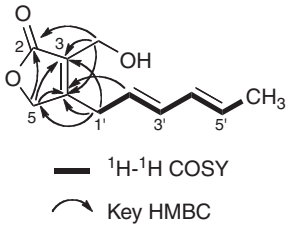

Figure 3 Key correlations in ^{1}H-^{1}H COSY and HMBC spectra of trichocyalide A.

Table 2 ^{1}H and ^{13}C NMR chemical shifts of trichocyalides A and B

	Trichocyalide A			*Trichocyalide B*		
Position	δ_C[a]	δ_H[b]	*HMBC*	δ_C[a]	δ_H[b]	*HMBC*
2	173.8	—	—	175.0	—	—
3	125.3	—	—	120.3	—	—
4	163.4	—	—	164.4	—	—
5	71.1	4.73 s	C-2, 3, 4	69.5	4.80 m	C-2, 3, 4
1′	29.6	3.29 d (*J* = 7.0)	C-3, 4, 5, 3′	65.8	4.50 m	C-3, 4, 5, 2′, 3′
2′	125.4	5.56 m	C-4, 1′	35.5	1.60 m	C-4, 1′, 2′, 4′
3′	133.0	6.12 dd (*J* = 11.0, 15.0)	C-1′, 2′, 6′	27.9	2.00 m	C-1′, 2′, 4′, 5′
4′	131.0	5.99 dd (*J* = 11.0, 15.0)	C-5′, 6′	130.6	5.40 m	C-3′, 6′
5′	128.7	5.64 m	C-3′, 6′	125.0	5.40 m	C-3′, 6′
6′	17.9	1.69 d (*J* = 9.0)	C-4′, 5′	17.8	1.60 m	C-4′, 5′
3-CH_2	52.7	4.01 s	C-2, 3, 4			
3-CH_3				8.7	1.70 t (*J* = 2.0 Hz)	C-2, 3, 4, 5, 1′
1′-OH					5.37 d (*J* = 4.0 Hz)	C-4, 1′, 2′

[a]Chemical shifts are shown with reference to DMSO-d_6 as 39.7 p.p.m.
[b]Chemical shifts are shown with reference to DMSO-d_6 as 2.49 p.p.m.

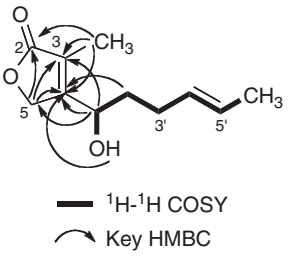

Figure 4 Key correlations in ^{1}H-^{1}H COSY and HMBC spectra of trichocyalide B.

Table 3 Effects of trichocyalides A, B and harzianolide on osteoblastic differentiation, cytotoxicity and BMP signaling

	$IC_{50}(\mu M)$		
Compounds	*ALP*	*Cytotoxicity*	*BMP signaling*
Trichocyalide A	83.0	>500	>500
Trichocyalide B	187	354	>500
Harzianolide	>500	>500	>500

Abbreviations: ALP, alkaline phosphatase; BMP, bone morphogenetic protein.

X. campestris KB 88 on conventional paper disk assay (inhibition zone at 10 μg per 6 mm disk: 11, 10 and 11 mm, respectively).

DISCUSSION

The planar structures of trichocyalides A and B, produced by *Trichoderma* sp. FKI-5513, were elucidated by spectral analyses. All compounds had the common γ-butenolactone moiety. Regarding the stereochemistry of 1′-OH in trichocyalide B, esterification of trichocyalide B with *S*-(+)-alpha-methoxy-alpha-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl)[16] yielded two kinds of esters in a ratio of 2:1 (data not shown). Taking into consideration that trichocyalide B showed a positive value of optical rotation (+10.2°), trichocyalide B was suggested to be a mixture of enantiomorphs, not a racemic mixture. Further study will be necessary to define this point.

As described in this study, trichocyalides A and B are new potential inhibitors of osteoblastic differentiation. Interestingly, trichocyalides A and B showed no inhibition of the luciferase activity of Id1WT4F-luc in C2C12 cells,[14] suggesting that their target does not lie within early events of Smads[9] but within late steps, such as p38 or JNK kinase in the BMP signaling pathway.[4] The target of trichocyalides A and B in osteoblastic differentiation remains to be demonstrated.

METHODS

General experimental procedures

UV spectra were recorded on a spectrophotometer (8453 UV-Visible spectrophotometer; Agilent Technologies Inc., Santa Clara, CA, USA). IR spectra were recorded on a Fourier transform infrared spectrometer (FT-710; Horiba Ltd., Kyoto, Japan). Optical rotations were measured with a digital polarimeter (DIP-1000; JASCO, Tokyo, Japan). HREI-MS and HRFAB-MS spectra were recorded on a mass spectrometer (JMS-AX505 HA; JEOL, Tokyo, Japan). Various NMR spectra were measured with a spectrometer (XL-400; Agilent Technologies Inc.).

Materials

Dulbecco's modified Eagle's medium was purchased from Nacalai Tesque (Kyoto, Japan). Fetal bovine serum was obtained from HyClone (Waltham, MA, USA). Penicillin/streptomycin was from Invitrogen (Carlsbad, CA, USA). *p*-Nitrophenyl phosphate was purchased from Sigma (St Louis, MO, USA). Recombinant human BMP4 (rhBMP4) was obtained from R&D Systems (Mountain View, CA, USA).

Taxonomic studies of the producing organism

Fungal strain FKI-5513 was isolated from soil collected on Hachijo island, Tokyo, Japan. For determination of the morphological characteristics, the isolate was inoculated as a one-point culture on potato dextrose agar, corn meal dextrose agar and synthetic low-nutrient agar, and grown for 7 days at 25 °C (also at 5 °C and 37 °C on potato dextrose agar) in the dark. Color Harmony Manual 4th Edition (Container Corporation of America, Chicago, IL, USA) was used to determine color names and hue numbers.[17] For the determination of micro-morphological characteristics, the samples were observed under a JSM-5600 scanning electron microscope (JEOL). Genomic DNA of the strain FKI-5513 was isolated using the PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. The rDNA ITS including the 5.8 S rDNA was amplified using primers ITS1 and ITS4.[18] Amplifications were performed in a PCR Verity 96-well thermal cycler (Applied Biosystems) and the PCR products were purified according to the instructions of the QIAquick PCR DNA purification kit protocol (Qiagen, Inc., Valencia, CA, USA). The PCR products were sequenced directly in both directions using primers (ITS1, ITS2, ITS3 and ITS4) and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing products were purified by ethanol/EDTA precipitation, and samples were analyzed on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). Contigs were assembled using forward and reverse sequences with the SeqMan and SeqBuilder programs from the Lasergene8 package (DNAStar Inc., Madison, WI, USA). The ITS sequence of the strain was deposited in the DNA Data Bank of Japan (DDBJ) with accession number AB606411.

Cell culture

A C2C12 myoblasts cell line and C2C12(R206H) cell line,[19] which exhibited ALP activity more quickly and more strongly than the original C2C12 cell line, were cultured in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum and 100 units ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (hereafter referred to as medium A) at 37 °C in 5.0% CO_2. Both cells were subcultured once every three days.

Assay for ALP in BMP-treated C2C12(R206H) cells

ALP activity, a typical marker of osteoblastic differentiation, was measured as described previously.[14] In brief, C2C12(R206H) cells (7.5×10^3 cells per well) in a 96-well plastic plate were cultured at 37 °C in 5.0% CO_2. Following overnight recovery, the culture media were replaced with 100 μl fresh medium A containing rhBMP4 (10 ng ml^{-1}) and a sample (1 μl in MeOH solution). After 48-h incubation, the cells were incubated for 60 min with 100 μl substrate solution (100 mM diethanolamine, 0.5 mM $MgCl_2$ and 1.0 mg ml^{-1} *p*-nitrophenylphosphate) at room temperature. The reaction was terminated by adding 50 μl of 3 M NaOH, and the absorbance at 405 nm was measured with a Power Wave × 340 (BIO-TEK Instruments, Highland Park, IL, USA).

Cytotoxicity

The cytotoxicity of a compound to C2C12(R206H) cells was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay.[20] In brief, C2C12(R206H) cells (7.5×10^3 cells per well) were cultured in 96-well plates in the absence or presence of a compound for 48 h at 37 °C in 5% CO_2. After incubation, the cells received 10 μl MTT solution (5.5 mg ml^{-1} in phosphate-buffered saline), and were then incubated at 37 °C for 3 h. A 90 μl aliquot of the lysis solution (40% *N, N*-dimethylformamide, 2.0% CH_3COOH, 20% SDS and 0.03 M HCl) was added to each well, and the plates were incubated for 2 h. The absorbance at 550 nm of each well was read with a Power Wave × 340. Inhibition of cell growth is defined as (absorbance-sample/absorbance-control) × 100. The IC_{50} value is defined as a sample concentration that causes 50% inhibition of cell growth.

Reporter gene assay for monitoring BMP signaling

Recently, a unique mutation, R206H, in the *ALK2* gene was suggested to induce FOP by activating BMP signaling using a constitutively activated BMP type I receptor,[5] indicating that BMP signaling was one of the targets of FOP treatment.[6] The effect of a compound on BMP signaling via Smads (early events in BMP signaling) was examined using a BMP-specific luciferase reporter, Id1WT4F-luc, which is driven by four tandem copies of BRE in the *Id1* gene.[4] In brief, C2C12 cells were inoculated at 1.0×10^4 cells per well in 96-well plates with medium A and incubated for 24 h. The cells were transfected with 200 ng plasmid DNA (40 ng Id1WT4F-luc, 10 ng phRL-SV40 and 150 ng ALK2(R206H)) using 0.5 µl Lipofectamine 2000 (Invitrogen) in OPTI-MEM (GIBCO, Grand Island, NY, USA) according to the manufacturer's protocol. After 2.5-h incubation, the culture medium was replaced with 100 µl fresh Dulbecco's modified Eagle's medium containing 2.5% fetal bovine serum without penicillin/streptomycin. After an additional 3-h incubation, a compound (1 µl methanol solution) was added to each well and cultured for 24 h. Both firefly and Renilla luciferase activities in the cells were determined using the Dual Glo Luciferase assay system (Promega, Madison, WI, USA).

Antimicrobial activity

Antimicrobial activity against 14 species of microorganisms was measured by our established method[21] using *Bacillus subtilis* PCI 219, *Staphylococcus aureus* FDA 209P, *Kocuria rhizophila* ATCC 9341, *Mycobacterium smegmatis* ATCC 607, *Escherichia coli* NIHJ, *Pseudomonas aeruginosa* P-3, *Xanthomonas campestris* pv. *Oryzae* KB 88, *Bacteroides fragilis* ATCC 23745, *Acholeplasma laidlawii* PG 8, *Pyricularia oryzae* KF 180, *Aspergillus niger* ATCC 6275, *Mucor racemosus* IFO 4581, *Candida albicans* ATCC 64548 and *Saccharomyces cerevisiae*. GAM agar (Nissui Seiyaku Co., Tokyo, Japan) for *B. fragilis*; Bacto PPLO agar (Sanko Junyaku Co. Ltd., Tokyo, Japan) supplemented with 15% horse serum, 0.1% glucose, 0.25% phenol red (5 mg ml^{-1}) and 1.5% agar for *A. laidlawii*; Taiyo agar (Shimizu Syokuhin Kaisya Ltd., Shizuoka, Japan) for the other bacteria; a medium composed of 1.0% glucose, 0.5% yeast extract and 0.8% agar for fungi and yeasts. A paper disk (i.d. 6 mm; Toyo Roshi Kaisha Ltd., Tokyo, Japan) containing a 10 µg sample was placed on an agar plate. Bacteria, with the exception of *X. oryzae*, were incubated at 37 °C for 24 h. Yeasts and *X. oryzae* were incubated at 27 °C for 24 h. Fungi were incubated at 27 °C for 48 h. Antimicrobial activity was expressed as the diameter (mm) of the inhibitory zone.

ACKNOWLEDGEMENTS

We wish to thank Dr K Nagai and Ms N Sato (Kitasato University) for measurements of mass and NMR spectra, respectively.

1 Luyten, F. P. *et al.* Purification and partial amino acid sequence of osteogenin, a protein initiating bone differentiation. *J. Biol. Chem.* **264,** 13377–13380 (1989).
2 Wozney, J. M. *et al.* Novel regulators of bone formation: molecular clones and activities. *Science* **242,** 1528–1534 (1988).
3 Macías-Silva, M., Hoodless, P. A., Tang, S. J., Buchwald, M. & Wrana, J. L. Specific activation of Smad1 signaling pathways by the BMP7 type I receptor, ALK2. *J. Biol. Chem.* **273,** 25628–25636 (1998).
4 Katagiri, T. *et al.* Identification of a BMP-responsive element in Id1, the gene for inhibition of myogenesis. *Genes Cells* **7,** 949–960 (2002).
5 Peltier, L. F. A case of extraordinary exostoses on the back of a boy. 1740. John Freke. *Clin. Orthop. Relat. Res.* **346,** 5–6 (1998).
6 Kaplan, F. S. *et al.* Genetic transmission of fibrodysplasia ossificans progressiva. Report of a family. *J. Bone. Joint. Surg. Am.* **75,** 1214–1220 (1993).
7 Glaser, D. L. *et al. In vivo* somatic cell gene transfer of an engineered Noggin mutein prevents BMP4-induced heterotopic ossification. *J. Bone Joint Surg. Am.* **85,** 2332–2342 (2003).
8 Shore, E. M. *et al.* A recurrent mutation in the BMP type I receptor ACVR1 causes inherited and sporadic fibrodysplasia ossificans progressiva. *Nat. Genet.* **38,** 525–527 (2006).
9 Katagiri, T. Heterotopic bone formation induced by bone morphogenetic protein signaling: fibrodysplasia ossificans progressiva. *J. Oral. Biosci.* **52,** 33–41 (2010).
10 Katagiri, T. *et al.* Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage. *J. Cell Biol.* **127,** 1755–1766 (1994).
11 Fukuda, T. *et al.* Fungal pyrrolidine-containing metabolites inhibit alkaline phosphatase activity in bone morphogenetic protein-stimulated myoblastoma cells. *Acta Pharmaceutica Sinica B* **2,** 23–27 (2012).
12 Kakeya, H. *et al.* Lucilactaene, a new cell cycle inhibitor in p53-transfected cancer cells, produced by a *Fusarium* sp. *J. Antibiot.* **54,** 850–854 (2001).
13 Bashyal, B. P., Faeth, S. H. & Gunatilaka, A. A. 13a-Hydroxylucilactaene and other metabolites of an endophytic strain of fusarium acuminatum. *Nat. Prod. Commun.* **2,** 547–550 (2007).
14 Bashyal, B. P. & Gunatilaka, A. A. Tricinonoic acid and tricindiol, two new irregular sesquiterpenes from an endophytic strain of *Fusarium tricinctum*. *Nat. Prod. Res.* **24,** 349–356 (2010).
15 Norman, C., James, R. H., Almaz, T. & Anthony, G. A. Harzianolide, a butenolide metabolite from cultures of *Trichoderma harzianum*. *Pytochemistry* **30,** 3802–3803 (1991).
16 Ohtani, I., Kusumi, T., Kashman, Y. & Kakisawa, H. High-field FT NMR application of Mosher's method. The absolute configuration of marine terpenoids. *J. Am. Chem. Soc.* **113,** 4092–4096 (1991).
17 Jacobson, E., Granville, W. C. & Foss, C. E. *Color Harmony Manual*. 4th ed. (Container of America, Chicago, 1958).
18 White, T. J., Bruns, T., Lee, S. & Taylor, J. W. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. in *PCR Protocols a Guide to Methods and Applications* (eds Innis, M. A., Gelfand, R. H., Sninsky, J. J. & White, T. J.) 315–332 (Academic Press, New York, 1990).
19 Fukuda, T. *et al.* Constitutively activated ALK2 and increased SMAD1/5 cooperatively induce bone morphogenetic protein signaling in fibrodysplasia ossificans progressiva. *J. Biol. Chem.* **284,** 7149–7156 (2009).
20 Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65,** 55–63 (1983).
21 Fukuda, T., Matsumoto, A., Takahashi, Y., Tomoda, H. & Ōmura, S. Phenatic acids A and B, new potentiators of antifungal miconazole activity produced by *Streptomyces* sp. K03-0132. *J. Antibiot.* **58,** 252–259 (2005).