A Novel Bone Resorption Inhibitor, A-75943 Isolated from *Streptomyces* sp. SANK 61296

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A novel bone resorption inhibitor, A-75943, was isolated from *Streptomyces* sp. SANK 61296. Its structure was determined to be (1″*S*,2′*R*,3″*S*,4*S*′,5″*S*)-2-[(3″,5″-dimethyl-2″-oxocyclohexan-1″-yl)-6′-oxotetrahydropyran-4′-yl]acetamide by spectral analyses and chemical conversion of cycloheximide. A-75943 inhibited bone resorption *in vitro* in a concentration-dependent manner with an IC_{50} of 0.35 μM, and also displayed an inhibitory effect on bone resorption in thyroid and parathyroid-extracted rats.

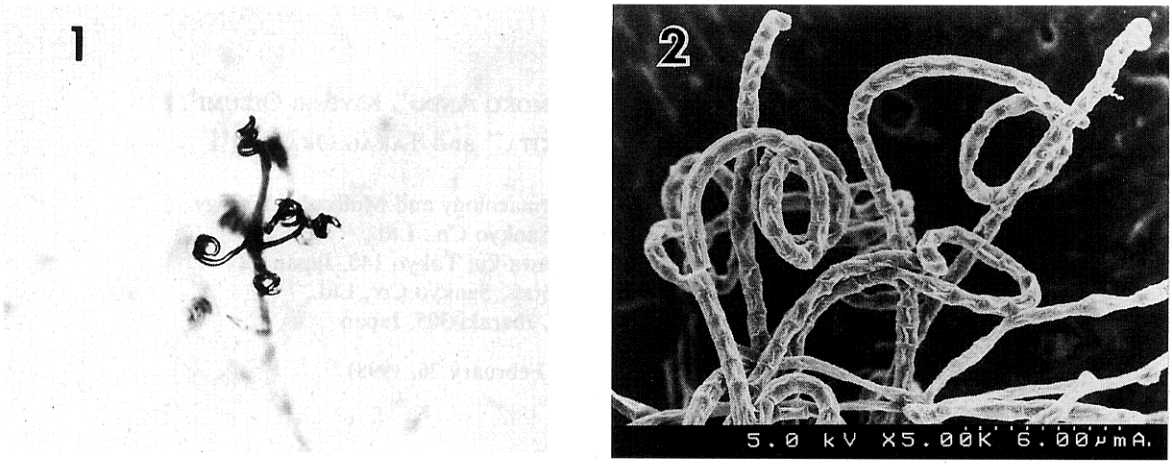
Bone sustains not only weight, but also plays an important role as a reservoir for minerals such as calcium and phosphorus. Bone, after formation, is not a permanent structure, but is constantly undergoing formation and resorption under strict control, resulting in bone integrity and homeostasis. An imbalance in formation and resorption, therefore, results in bone diseases such as osteoporosis, osteopetrosis, hypercalcemia, and hypocalcemia[1]. Osteoporosis is a chronic bone disease, frequently occurring in postmenopausal women, whom bone resorption by osteoclasts is aberrant; first the bone mass decreases and finally the bone breakes easily. The inhibition of bone resorption may, therefore, prevent the onset and the progress of osteoporosis.

In the course of a program to discover novel drugs or lead compounds thereof, for prevention and treatment of osteoporosis and hypercalcemic diseases, we extensively tested fermentation broths or their lipophilic extracts for *in vitro* inhibition of pit formation in an osteoclast-induced bone resorption assay using mouse unfractionated bone cells and found that a lipophilic extract of a fermentation supernatant of *Streptomyces* sp., SANK 61296, potently inhibited bone resorption. We herein report on fermentation, isolation, structure elucidation, and *in vitro* and *in vivo* bone resorption inhibitory activities of A-75943 (**1**) and also describe the chemical conversion of cycloheximide to **1**.

Identification of the Producing Strain

The producing organism, strain A-75943, was isolated from a soil sample collected on Mt. Tsukuba, Ibaraki Prefecture, Japan. Methods and media described by the International Streptomyces Project (ISP)[2] and WAKSMAN[3] were used to determine most of the cultural and physiological characteristics. For morphological observation, a light microscope and a scanning electron microscope (S-4500, Hitachi) were used. Whole-cell hydrolysates were analyzed by the method of HASEGAWA *et al.*[4]. Strain A-75943 grew relatively well on both natural and synthetic media, and formed spiral spore chains at the tips of the aerial mycelia. Spore surfaces were smooth. The mature spore chains comprised more than 50 spores each (Fig. 1). Neither melanoid pigment nor soluble pigment was produced. The cultural characteristics in various agar media are shown in Table 1. The physiological properties are shown in Table 2. L,L-Diaminopimelic acid was found in the cell wall, and no characteristic sugar component was detected in the whole-cell hydrolysates, indicating that the cell wall type was I/NC. Based on the taxonomic characteristics described above, strain A-75943 was identified as *Streptomyces* sp. SANK 61296 and was deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology,

Fig. 1. Photographs of strain A-75943.



Photograph 1: Light micrograph on potato extract-carrot extract agar at 28°C for 7 days.
Photograph 2: Scanning electron micrograph on potato extract-carrot extract agar at 28°C for 7 days.

Ibaraki, Japan with the accession number of FERM BP-5505.

Fermentation and Isolation

Streptomyces sp. SANK 61296 was cultured in two 30 liter-jar fermentors at 28°C for 7 days. The culture filtrate (23 liters) was extracted with EtOAc to give a dark brown oil (23.43 g), which was subjected to silica gel chromatography. Separation was monitored using pit formation assay on dentine slices. On stepwise elution with *n*-hexane-EtOAc, 1:1 (v/v), EtOAc, MeOH-EtOAc, 1:3 (v/v), MeOH-EtOAc, 1:1 (v/v), and MeOH, the bone resorption activity remained in the fraction (6.27 g, IC_{50} 0.2 µg/ml) eluted with *n*-hexane-EtOAc, 1:1 (v/v). The active fraction (5.2 g) was further subjected to Lobar column chromatography (RP-18 and DIOL) to give A-75943 (**1**, 46.2 mg) as a colorless amorphous powder. The lipophilic mycelial extract (50.1 g) gave a small amount of cycloheximide (4.2 mg).

Structure Determination

A-75943 (**1**) (Fig. 2) was analyzed for $C_{15}H_{23}NO_4$ by HR-EI-MS spectrum ($[M]^+$ *m/z* 281.1597, Δ +3.0 mmu). The IR spectrum [ν_{max} cm^{-1} (KBr)] indicated the presence of an amino (3528, 3411), carbonyl (1710), and amide carbonyl (1686) groups. The ^1H and ^{13}C NMR spectra (Table 3) somewhat resembled those of cycloheximide; a relative chair-formed cyclohexanone ring of cycloheximide was kept intact, but its glutarimide moiety changed in A-75943 (**1**). The HMQC and HMBC (Fig. 3) revealed that the proton ($H_{2'}$, δ_H 4.78 in Py-d_5) at $C_{2'}$ resonated in lower field than that of cycloheximide. This down-field shift was compatible with a lactonization at this position, which was supported by a long-range coupling between $H_{2'}$ and a lactone carbonyl carbon ($C_{6'}$, δ_C 173.3) in the HMBC. The HMBC also connected methylene protons (H_2, 2.45, d, J=7.0 Hz) and $H_{4'}$ (δ_H, 2.78, m) with the amide carbon (C_1, δ_C 170.7), constituting an acetamide moiety. The NOE between $H_{2'}$ and $H_{4'}$, and the coupling constants were compatible with a chair-formed *cis* δ-lactone (Fig. 4). These data, however, could not assign the stereochemical relationship between the cyclohexanone and δ-lactone moieties. The chemical conversion of commercially available cycloheximide to A-75943 (**1**) was, therefore, attempted (Fig. 5).

As it is known that cycloheximide easily undergoes retro-aldol cleavage under basic conditions, dihydrocycloheximide[5], obtained by catalytic hydrogenation of cycloheximide with PtO_2, was a good choice as a starting material. On treatment with K_2CO_3 or $NaHCO_3$ in aq EtOH at 50°C, dihydrocycloheximide gave compound **2a** in 37% yield with K_2CO_3 and in 48.9% yield with $NaHCO_3$, together with its acid (**2b**). In contrast, dihydrocycloheximide, on long-term refluxing with a large excess of triethylamine in MeOH, would not be consumed completely, giving a 1:1 mixture of compound **2a** and the starting material. Compound **2b** was recycled to compound **2a**, in 57.8% yield, through the reaction with diethyl cyanophosphonate [$(EtO)_2P(O)CN$] and ammonia gas. Compound **2a** was oxidized to A-75943 (**1**) with PDC in 87% yield. Consequently, A-75943 (**1**) could be depicted as (1″*S*,2′*R*,3″*S*,4*S*′,5″S)-2-[(3″,5″-

Table 1. Cultural characteristics of strain A-75943.

Agar media		
Yeast extract - malt extract (ISP-2)	G[a]:	Good, flat, pale yellowish brown
	AM[a]:	Abundant, velvety, brownish gray
	R[a]:	Grayish yellow brown
	SP[a]:	None
Oatmeal (ISP-3)	G:	Good, flat, pale yellowish brown
	AM:	Abundant, velvety, brownish gray
	R:	Grayish yellow brown
	SP:	None
Inorganic salts - starch (ISP-4)	G:	Good, flat, yellowish brown
	AM:	Abundant, velvety, brownish gray
	R:	Grayish yellow brown
	SP:	None
Glycerol - asparagine (ISP-5)	G:	Good, flat, pale yellowish brown
	AM:	Abundant, velvety, brownish gray
	R:	Pale yellowish brown to brownish gray
	SP:	None
Peptone - yeast extract - iron (ISP-6)	G:	Moderate, flat, pale yellowish brown
	AM:	None
	R:	Yellowish brown
	SP:	None
Tyrosine (ISP-7)	G:	Moderate, flat, pale yellowish brown
	AM:	Abundant, velvety, brownish gray
	R:	Brownish gray
	SP:	None
Sucrose - nitrate	G:	Poor, flat, brownish white
	AM:	Poor, velvety, pale yellowish orange
	R:	Pale yellowish brown
	SP:	None
Glucose - asparagine	G:	Moderate, flat, pale yellowish brown
	AM:	Moderate, velvety, brownish white
	R:	Pale yellowish brown
	SP:	None
Nutrient (Difco)	G:	Moderate, flat, pale yellowish brown
	AM:	None
	R:	Pale yellowish brown
	SP:	None
Potato extract - carrot extract	G:	Poor, flat, pale brown
	AM:	Moderate, velvety, brownish gray
	R:	Light brownish gray
	SP:	None
Water	G:	Poor, flat, brownish white
	AM:	Poor, velvety, light brownish gray
	R:	Pale brown
	SP:	None

[a] G: Growth, AM: Aerial mycelium, R: Reverse, SP: Soluble pigment.

dimethyl-2″-oxocyclohexan-1″-yl)-6′-oxotetrahydropyran-4′-yl]acetamide.

Biological Activities

A new bone resorption inhibitor, A-75943 (**1**), isolated from the fermentation broth of *Streptomyces* sp. SANK 61296, was found to be structurally related to cycloheximide, from which **1** was prepared chemically. A-75943 (**1**) inhibited *in vitro* bone resorption in a concentration-dependent manner, with maximal non-toxic inhibition greater than 90% at 5 μg/ml and an IC_{50} of 0.35 μM. At higher concentrations, A-75943 (**1**) exhibited cytotoxicity and significantly decreased the survival of osteoclasts on bone slices; but at lower concentrations it did not affect the survival or morphology of osteoclasts. The *in vitro* inhibition test against protein synthesis[6] indicated that A-75943 (**1**) was 1000-fold less toxic than cycloheximide (**1**: IC_{50} 153 μM;

cycloheximide: IC_{50} 149 nM).

Ro-136298[7] treatment induces an increase in plasma Ca^{2+} level and a decrease in bone mineral density (BMD) in thyroid and parathyroid-extracted rats. Subcutaneous concomitant treatment with A-75943 (**1**) in these rats suppressed these changes; in the experiment-1, the serum Ca^{2+} concentrations in each dose were lowered in a dose-dependent manner as shown in Table 4 and Fig. 6, and the decreases of BMD in each dose were also inhibited in a dose-dependent manner as shown in Table

Table 2. Physiological properties of strain A-75943.

Hydrolysis of starch	+
Liquefaction of gelatin	+
Reduction of nitrate	−
Coagulation of milk	+
Peptonization of milk	+
Production of melanoid pigment	−
Decomposition of	
casein	+
xyrosine	−
xanthine	−
Growth temperature	9~33°C
Optimum growth temperature	17~28°C
Sodium chloride tolerance	3%
Utilization of	
D-glucose	+
L-arabinose	±
D-xylose	±
inositol	±
D-mannitol	±
D-fructose	±
L-rhamnose	±
sucrose	±
raffinose	±

Fig. 2. Structure of A-75943 (**1**).

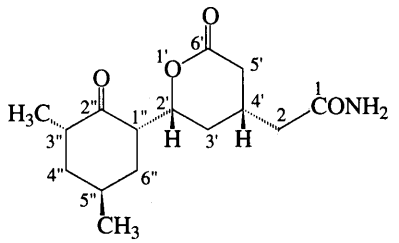


Fig. 3. HMQC and HMBC of A-75943 (**1**).

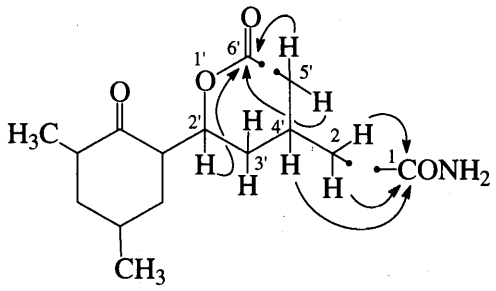
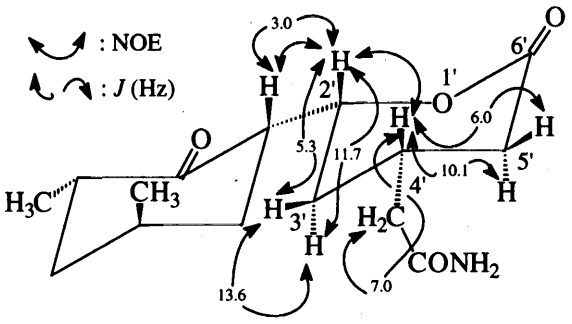


Table 3. ^1H and ^{13}C NMR data of A-75943 (**1**).

#	^{13}C (δ, Multi., 100 MHz)		^1H (δ, Multi., J, 400 MHz)	
	Py-d_5	CD_3OD	Py-d_5	CD_3OD
1	170.7 (s)	173.8		
2	42.26 (t)	42.5[a]	2.45, d, 7.0	2.22, d, 7.0
2′	78.1 (d)	80.0	4.78, ddd, 3.0, 5.3, 11.7	4.72, ddd, 3.0, 5.7, 11.8
3′	32.2 (t)	33.1	1.45, td, 11.7, 13.6	1.38, td, 11.8, 13.6
			2.29, m	2.49, m
4′	29.33 (d)	30.0	2.78, m	2.73, m
5′	36.6 (t)	36.6[b]	2.46, dd, 10.1, 17.3	2.06~2.24, m
			3.01, ddd, 1.3, 6.0, 17.3	2.73, ddd, 2.0, 5.9, 17.5
6′	173.3 (s)	176.4		
1″	49.6 (d)	50.5	2.70, td, 5.5, 12.7	2.86, td, 5.8, 13.2
2″	211.0 (s)	213.4		
3″	27.1 (d)	28.3	1.94, m	1.93, m
4″	42.30 (t)	43.7[a]	1.42, dt, 4.6, 13.1	1.62, dt, 4.8, 13.1
			1.69, m	1.69, m
5″	40.5 (d)	41.5	2.47, m	2.70, m
6″	35.5 (t)	36.1[b]	1.73, dt, 4.7, 13.2	1.78, dt, 4.7, 13.2
			2.00, m	2.06~2.24, m
3″-CH_3	18.0 (q)	18.4	1.09, d, 7.2	1.28, d, 7.1
5″-CH_3	14.7 (q)	14.8	0.98, d, 6.3	0.96, d, 6.5

[a,b] Exchangeable with each other.

Fig. 4. NOE and coupling pattern.



5 and Fig. 7. The experiment-2 was carried out for comparison with cycloheximide. These results *in vivo*, together with inhibition data of protein synthesis *in vitro*, suggest that A-75943 (**1**), compared with cycloheximide, displays a moderate inhibitory effect on bone resorption with less toxicity.

Fig. 5. Conversion of dihydrocycloheximide to A-75943 (**1**).

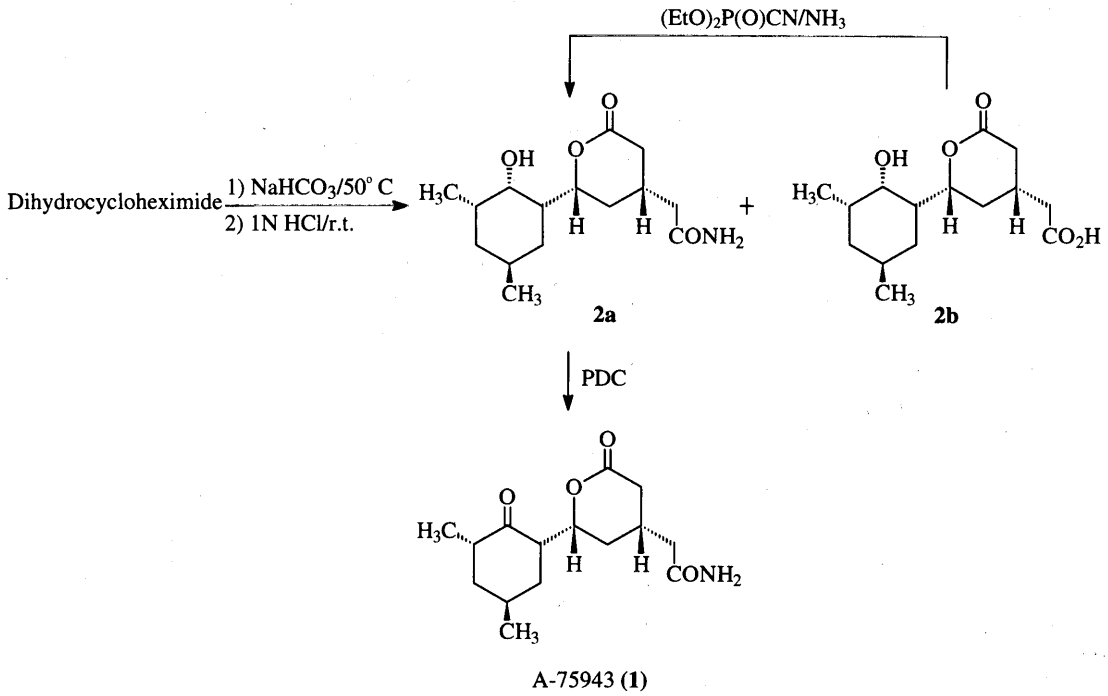
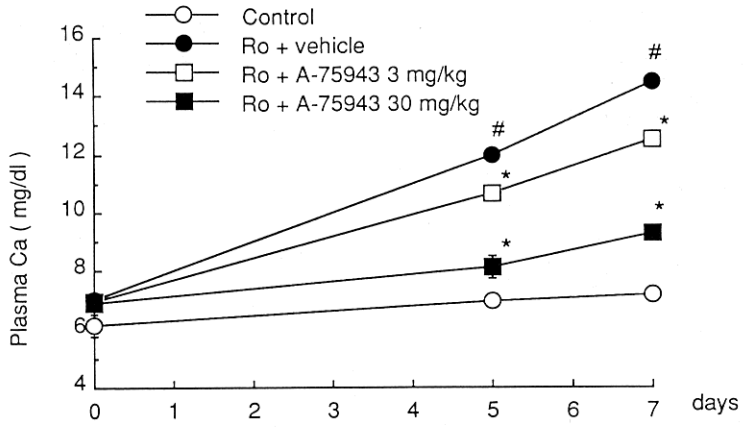


Table 4. Effect of A-75943 (**1**) on serum Ca^{2+} concentration in thyroid and parathyroid-extracted rats.

	Compounds, doses[a]	Serum Ca^{2+} concentration ± SEM (mg/dl)		
		0 (days)	5	7
Exp-1 n=6	Ro-136298	7.03±0.22	11.96±0.15	14.05±0.23
	+**1**, 3 mg/kg	6.99±0.12	10.66±0.22	12.51±0.17
	+**1**, 30 mg/kg	6.92±0.25	8.14±0.4	9.29±0.29
Exp-2 n=6	Control	6.14±0.37	7.00±0.2	7.18±0.18
	Ro-136298	6.16±2.7	9.21±0.41	12.36±0.24
	+**1**, 10 mg/kg	5.71±0.31	8.85±0.09	12.04±0.15
	+Chx[b], 0.1 mg/kg	6.15±0.22	7.74±0.16	10.46±0.24
	+Chx, 0.03 mg/kg	6.32±0.23	9.01±0.17	12.27±0.18

[a] Administered consecutively and subcutaneously.
[b] Cycloheximide.

Fig. 6. Time course of changes in plasma Ca^{2+} concentration.



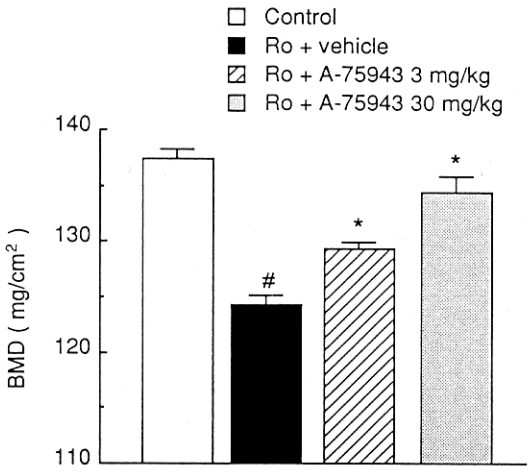
# $p<0.01$ compared with the values of control group. * $p<0.01$ compared with the values of Ro-136298 + vehicle group. The values are mean ± S.E. from 6 rats.

Table 5. Effect of A-75943 (**1**) on bone mineral density (BMD).

	Compounds, doses[a]	Bone density ± SEM (mg/cm^2)
	Ro-136298	124.3 ± 0.9
Exp-1	+ **1**, 3 mg/kg	129.9 ± 0.6
n = 12	+ **1**, 30 mg/kg	134.4 ± 1.4
	Control	137.4 ± 0.9
Exp-2	Ro-136298	126.6 ± 1.1
n = 12	+ **1**, 10 mg/kg	128.3 ± 0.9
	+ Chx[b], 0.1 mg/kg	131.5 ± 0.6
	+ Chx, 0.03 mg/kg	133.6 ± 1.5

[a] Administered consecutively and subcutaneously.
[b] Cycloheximide.

Fig. 7. Inhibitory effect of A-75943 (**1**) on Ro-136298-induced decrease in bone mineral density (BMD) of excized femurs.



# $p<0.01$ compared with the values of control group. * $p<0.01$ compared with the values of Ro-136298 + vehicle group. The values are mean ± S.E. from 11 or 12 femurs.

Experimental

General Procedures

^1H and ^{13}C NMR spectra were recorded on a JEOL JNM-GX400 spectrometer with TMS as an internal standard. IR spectra were recorded on a Nicole 5SXC and JASCO Valor-III spectrometers. UV spectra were recorded on a Shimadzu UV-265FW spectrometer. Mass spectra were recorded on a JEOL JMS-D300 spectrometer. Optical rotations were measured in EtOH at 25°C on a JASCO DIP-370 polarimeter. Diaion HP-20 (Nihon Rensui), silica gel (60~80 mesh; Merck), and packed columns (RP-18, DIOL; Merck) were used for column chromatography.

Fermentation and Isolation

Streptomyces sp. SANK 61296 was inoculated in a 500 ml-buffled Erlenmyer flask containing 100 ml of a seed medium (pH 7.0, sterlized at 120°C for 20 minutes), composed of glucose (1.0 g), glycerol (1.0 g), sucrose (1.0 g), crude yeast (1.0 g), oatmeal (0.5 g), soybean meal (2.0 g), vitamin-free casamino acid (0.5 g), and $CaCO_3$ (0.5 g) in 100 ml of tap water. The medium was precultured on a 200 rpm-rotary shaker at 28°C for one week. Fifteen mililiters each of the preculture was added to two 30 liter-jar fermentors, each containing 7.5 liters

of a production medium (pH 7.0, sterlized at 120°C for 20 minutes), composed of glucose (3.0 g), soybean meal (3.0 g), Sankyo yeast (1.0 g), $CaCO_3$ (4.0 g), and $MgSO_4 \cdot 7H_2O$ (0.2 g) in 100 ml of tap water. The medium was cultured at 28°C for one week. The combined culture filtrate (23 liters), obtained by filtration of a culture broth with the aid of a celite bed (1.2 kg), was twice extracted with EtOAc (40 liters × 2). The combined EtOAc layer was washed successively with satd NaCl soln and water, and was then dried over anhydr Na_2SO_4. Evaporation under reduced pressure to dryness gave a dark red oil (23.43 g). Wet mycelia were successively soaked in 15 liters of acetone - H_2O, 4 : 1 (v/v) and 12 liters of acetone at room temperature. The combined mycelial acetone extract, after the work-up described above, gave a dark brown oil (50.1 g) as a mycelial lipophilic extract. The lipophilic extract of the filtrate was subjected to silica gel chromatography (SiO_2, 300 g). Fractionation by successive elution with 1 liter each of *n*-hexane - EtOAc, 1 : 1 (v/v), EtOAc, MeOH - EtOAc, 1 : 3 (v/v), MeOH - EtOAc, 1 : 1 (v/v), and MeOH, gave an active fraction eluted with *n*-hexane - EtOAc, 1 : 1 (v/v), which was concentrated under reduced pressure, leaving a brown oil (6.272 g, IC_{50} 0.2 μg/ml). The active fraction (5.2 g) was subjected to Lobar column chromatography [RP-18 (C)]. After removal of inactive fractions by elution with 1 liter of a solvent mixture [MeOH - ACN - H_2O, 3 : 1 : 7 (v/v)], the active fraction (246 mg) was eluted with 250 ml of a solvent mixture [MeOH - ACN - H_2O, 3 : 1 : 6 (v/v)]. The active fraction was further purified by Lobar column chromatography [DIOL (B)] with 200 ml of MeOH - $CHCl_3$, 2 : 98 (v/v), reapplied to the same column with 200 ml of MeOH - $CHCl_3$ - TFA, 2 : 98 : 0.3 (v/v) to give A-75943 (**1**, 46.2 mg) as a colorless amorphous powder. $[\alpha]_D^{25}$ −2.7° (*c* 0.52, $CHCl_3$); $C_{15}H_{23}NO_4$; HR-EI-MS $[M]^+$ *m/z* 281.1597, Δ +3.0 mmu; IR [ν_{max} cm^{-1} ($CHCl_3$)] 3528, 3411, 1710, 1686, 1592, 1457, 1450, 1386; UV [λ_{max} nm] end absorption (EtOH).

Reaction of Dihydrocycloheximide to Compound **2a**

a) To a soln of $NaHCO_3$ (400 mg) in 8 ml of water, was added dihydrocycloheximide (174 mg)[5] in 2 ml of MeOH, and the mixture was heated at 50°C for 15 hours. After concentration of the reaction mixture under reduced pressure, the residue was acidified with 1 N HCl, and extracted with EtOAc. The EtOAc layer was successively washed with satd $NaHCO_3$ soln, water, and satd NaCl soln and then dried over anhydr Na_2SO_4. The residue obtained by removal of the solvent under reduced pressure, was recrystallized from EtOAc to yield compound **2a** (68.9 mg) as colorless crystals. The filtrate, after removal of the solvent under reduced pressure, was subjected to silica gel chromatography (SiO_2, 3.0 g). After elution with 20 ml of MeOH - $CHCl_3$, 5 : 95, (v/v), elution with 20 ml of MeOH - $CHCl_3$, 1 : 9, (v/v), followed by recrystallization from EtOAc gave an additional amount of **2a** (14.2 mg). In this experiment, no recovery of compound **2b** was attempted. mp 186 ~ 188°C; $C_{15}H_{25}NO_4$; EI-MS $[M]^+$ *m/z* 283; $[\alpha]_D^{25}$ −8.4° (*c* 0.51, MeOH); IR [ν_{max} cm^{-1} (KBr)] 3523, 3451, 3400, 3339, 3215, 2954, 2932, 1703, 1664, 1445, 1415, 1369, 1336, 1266, 1183, 1161, 1148, 1088, 1044; FAB-MS $[M+H]^+$ *m/z* 284; ^1H NMR (δ, CD_3OD) 0.92 (3H, d, *J* = 6.6 Hz), 1.01 (3H, d, *J* = 7.3 Hz), 1.28 (1H, br d, *J* = 12.5 Hz), 1.32 (1H, td, *J* = 11.6, 13.7 Hz), 1.55 ~ 1.80 (5H, m), 2.05 (1H, m), 2.18 (1H, dd, *J* = 10.4, 17.4 Hz), 2.23 (2H, d, *J* = 7.1 Hz), 2.28 (1H, br d, *J* = 13.7 Hz), 2.43 (1H, m), 2.73 (1H, ddd, *J* = 1.4, 5.8, 11.6 Hz), 3.71 (1H, br s), 4.27 (1H, ddd, *J* = 2.9, 8.7, 11.6 Hz); ^{13}C NMR (δ, CD_3OD) 18.7 (q), 18.9 (q), 28.4 (d), 28.7 (t), 30.0 (d), 32.1 (d), 32.9 (t), 34.5 (t), 36.6 (t), 42.6 (t), 43.4 (d), 71.1 (d), 84.9 (d), 174.2 (s), 176.4 (s); *Anal* Calcd for $C_{15}H_{25}NO_4$: C 63.58, H 8.89; N, 4.94. Found: C 63.53, H 9.15, N, 4.95.

b) To 9 ml of 5% K_2CO_3 soln, was added a soln of dihydrocycloheximide (736 mg) in 14 ml of EtOH - H_2O, 1 : 1 (v/v), and the mixture was heated at 50°C for 70 minutes. According to the procedure described in a), the residue was recrystallized from EtOAc to give compound **2a** (240.5 mg), and the filtrate gave an additional amount of **2a** (33.6 mg). The $NaHCO_3$ layer, after acidification with 1 N HCl, was subjected to HP-20 chromatography (HP-20, 100 ml). After elution with water, compound **2b** was eluted with acetone - H_2O, 1 : 1 (v/v) ~ acetone. The combined eluant, after evaporation of the solvent, was recrystallized from aq MeOH to give compound **2b** (102.7 mg) as colorless crystals.

Reaction of Compound **2b** with $(EtO)_2P(O)CN$ and NH_3

A soln of compound **2b** (3.375 g) in 25 ml of dry THF was cooled with ice. Through this solution, was passed gaseous NH_3 until colorless precipitates were no longer formed. To this suspension was added $(EtO)_2P(O)CN$ (2.40 g), and the mixture was stirred under ice-cooling for 30 minutes. The usual work-up described in a) gave compound **2a** (1.95 g).

PDC Oxidation of Compound **2a** to A-75943 (**1**)

A soln of compound **2a** (250 mg), PDC (1 g), and a

catalytic amount of pyridinium trifluoroacetate in 12 ml of CH_2Cl_2 and 3 ml of THF was stirred at room temperature for 24 hours. The reaction mixture obtained by the usual work-up, was subjected to silica gel chromatography (SiO_2, 10 g). After elution with acetone-EtOAc, 1:9 (v/v), elution with acetone-EtOAc, 3:7 (v/v), gave A-75943 (**1**) as a colorless amorphous powder (217.5 mg). $[\alpha]_D^{25}$ $-3.1°$ (*c* 0.52, $CHCl_3$).

Cell Preparation

The isolation and culture of osteoclasts for the bone resorption assay were performed according to the method of KITAMURA *et al.*[8]. Briefly, femora and tibiae were removed from 18- to 20-day-old ICR mice (Japan SLC, Japan) and cleaned of soft tissues. Bone cells were released by mincing the bones in DULBECCO's modified essential medium (DMEM, Nissui, Japan) supplemented with 10% fetal bovine serum. Larger bone fragments were then removed after the bone cell suspension had stood for 2 minutes. The cells obtained were washed once and resuspended in DMEM supplemented with 10% FBS. These cells were cultured in this medium in the presence of rat parathyroid hormone [rPTH-(1-34), 5×10^{-8} M, Sigma]. After the cells had been cultured with rPTH-(1-34), they were harvested with PBS containing 0.25% trypsin and 0.1% EDTA using a cell scraper (Sumitomo Bakelite, Japan).

In Vitro Bone Resorption Assay[9]

Dentine slices (6 mm diameter, 0.15 mm thickness) were cleaned by ultrasonication for 10 minutes in EtOH-H_2O, 7:3 (v/v) before use and placed in each well of 96-well culture plates. Aliquots of cultured cells (1×10^5 cells/200 μl DMEM) were then added to each well. The cells were incubated for 2 days at 37°C in a humidified atmosphere of 5% CO_2-air in the absence or presence of a range of concentrations of A-75943 (**1**). Subsequently, the cells were scraped off the slice with a microbrush (Lancelot). The slices were rinsed in water and stained with acid hematoxylin (Sigma) for 4 minutes. Resorbed lacunae were measured under a light microscope by counting the number of pits.

In Vivo Bone Resorption Inhibitory Effect

The *in vivo* bone resorption inhibitory effect of A-75943 (**1**) was examined in Ro-136298-treated thyroparathyroidectomized (TPTX) rats, an experimental model of accelerated bone resorption[8]. Male 8-week-old Wistar Imamichi rats were thyroparathyroidectomized, and animals with plasma Ca^{2+} levels below 8 mg/dl measured on the 3rd day following TPTX were selected for the experiment. These animals were divided into four groups (six rats in each group) and three groups of rats received a subcutaneous injection of Ro-136298, at a dose of 30 μg/body for a total of four times, on the 4th, 5th, 6th, and 8th day following TPTX. Animals in the control group received vehicle alone. Among three Ro-136298-treated groups, two groups received a concomitant daily subcutaneous administration of A-75943 (**1**) (3 and 30 mg/kg), and the remaining group received vehicle from the 4th to the 10th day following TPTX. Blood was taken for plasma Ca^{2+} measurement on the 8th and 10th day. The animals were sacrificed on day 10. The femurs of each animal were removed and cleaned for bone mineral density (BMD) measurement by dual energy X-ray absorptiometry (DCS600R).

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