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RESEARCH ARTICLE

Novel diether compounds inhibiting differentiation of osteoclasts

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Abstract Osteoporosis is a disorder in which bone mass decreases and is responsible for many degenerative bone diseases. The excessive formation and activity of osteoclasts results in pathological disorders of the bone. Receptor Activator of Nuclear Factor κB Ligand (RANKL) is regarded as a key regulator of osteoclast activity and as a new therapeutic target for treating osteoporosis. Herein, we have synthesized several new small molecules and tested their inhibition activity on RANKL-induced osteoclast formation. The active compounds **2c** and **4d** showed inhibitory activity against RANKL-induced osteoclast differentiation (IC_{50} = 1.56 and 2.20 μM, respectively). The most active compound **2c** prevented LPS-induced osteoclastogenesis in vivo. These data imply that the compound may be the potential candidate for a new therapeutic drug for treatment of bone resorption-associated diseases.

Keywords Osteoporosis · Osteoclast · Differentiation · RANKL

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Introduction

Osteoporosis is a common disease around the world, with about two hundred million persons are affected. In osteoporosis, bone mass is reduced and, at the same time, the bone microarchitecture deteriorates, which results in the weakened bones. The balance between osteoclasts and osteoblasts is important for bone remodeling and maintaining bone mass. Imbalance in this event can results in bone disorders such as osteoporosis, rheumatoid arthritis, and periodontal disorders (Poole and Compston 2006). Excessive formation and/or activity of osteoclasts is the main character of these pathological diseases (Kim et al. 2012).

Osteoclasts are large, multinucleated cells that are formed from monocyte/macrophage hematopoietic lineage cells (Boyle et al. 2003). The differentiation and activation of osteoclasts require the presence of receptor activator of nuclear factor ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) (Suda et al. 1999). In the presence of RANKL and M-CSF, osteoclast precursors like bone marrow-derived macrophages (BMM) differentiate into osteoclasts, expressing osteoclast specific markers such as tartrate-resistant acid phosphatase (TRAP), cathepsin K (CATK), calcitonin receptor (CTR) and integrin receptors (Teitelbaum and Ross 2003). Binding of RANKL to its receptor RANK leads to the activation of MAPK signaling pathways including p38, JNK, and ERK, which triggers the expression of key transcription factors like NF-κB, c-fos, and NFATc1 (Ishida et al. 2002; Shinohara and Takayanagi 2007; Takayanagi et al. 2002).

RANKL also promotes the bone resorbing activity of osteoclasts and prolongs their survival. The RANKL-RANK system is not only involved in physiological bone development, but regulates the bone remodeling cycle, playing an important role in pathological bone destruction.

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RANK-RANKL signaling therefore can be a potent target of the therapeutic intervention for various bone diseases (Sakae et al. 2005).

Medicines currently used for osteoporosis, including bisphosphonates agents (alendronate, etidronate), hormonal agents (raloxifene), vitamin D agents, calcitonin agents, and calcium agents are not likely to elicit patient compliance and persistent medicine intake. Oral bisphosphonates can induce upper gastrointestinal side effects, so that patients must take them together with a sufficient amount of water on an empty stomach and sit upright for 30–60 min after the uptake of the medication, without ingesting foods or beverages. Thus, there are a lot of precautions for administration of medication. Hormonal agents cause side effects as large as the therapeutic effects. PTH is required to be taken through a subcutaneous route every day and is expensive. Intake of calcium and vitamin D alone does not guarantee reliable pharmaceutical efficacy. Osteoporosis therapy requires the administration of medication over a long period of time. Therefore, a need for a novel medication that guarantees excellent pharmaceutical efficacy without serious side effects on long-term use. The cathepsin K inhibitors, relacatib, balicatib, ONO-5443, and odanacatib were evaluated in preclinical and clinical studies (Zerbini and McClung 2013).

A distinctive role of the leukotriene B_4 receptor BLT_1 in osteoclastic activity during bone loss was reported (Hikiji et al. 2009). LY293111 and SC-53228 are potent and selective antagonists of the leukotriene B_4 receptor BLT_1 (Fig. 1). Another leukotriene B4 receptor antagonist, DW 1350 was reported as an antiosteoporotic agent. (Suh et al. 2003; Lee et al. 2003, Lee et al. 2006; Wierzbicki et al. 1998). Based on these leukotriene B4 antagonists contained diether structure, we modified our 5-lipoxygenase inhibitor contained benzoxazoles previously reported (Song et al. 2010) to design new agent for osteoporosis. In this paper, we report the synthesis of novel small chemical compounds which have benzothiazole moiety and a diether linker between two aromatic rings in the structures. The prepared compounds showed inhibitory activity against osteoclast differentiation via RANKL inhibition without any associated cytotoxicity.

Materials and methods

Synthesis

All melting points were taken in Pyrex capillaries using electrothermal digital melting point apparatus (Büchi). ^{1}H-NMR spectra were recorded on a 400 MHz Varian FT-NMR spectrometer using tetramethylsilane as an internal standard. Samples were dissolved in acetone-d_6 or DMSO-d_6. Mass spectra data were obtained on a Jeol JMS 700 high resolution mass spectrometer at the Korea Basic Science Institute (Daegu). Most of the reagents were purchased from Sigma-Aldrich Chemical Company.

Synthesis of 2-(5-phenoxypentyloxy)benzo[d]thiazole derivatives

*Synthesis of 2-(5-Chloropentyloxy)benzo[d]thiazole (**1**)* K_2CO_3 (0.274 g, 1.98 mmol) was added to a solution of 2-hydroxybenzothiazole (0.2 g, 1.32 mmol) in 10 mL of acetonitrile, followed by stirring at 50–55 °C for 30 min. 1-Bromo-5-chloropentane (0.245 g, 1.32 mmol) was added to the reaction mixture and reacted at 80–90 °C for 3 h. The reaction mixture was cooled to room temperature, and diluted with 10 mL of EtOAc, washed with distilled water and saturated with NaCl aqueous solution. It was dried over $MgSO_4$ and the solvent was removed by concentration under reduced pressure to yield compound **1** (0.333 g, 98 %).

Transparent oil; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.579 (d, $J = 7.8$, 1H), 7.393–7.295 (m, 2H), 7.194 (t, $J = 7.5$, 1H), 4.015 (t, $J = 7.2$, 2H), 3.602 (t, $J = 6.6$, 2H), 1.869–1.743 (m, 4H), 1.574–1.496 (m, 2H); HR-FABMS Calcd for $C_{12}H_{15}ClNOS$ (M^++H): 256.0563, Found: 256.0565.

*Synthesis of 2-(5-Phenoxypentyloxy)benzo[d]thiazole (**2a**)* Phenol (0.018 g, 0.20 mmol) in 3 mL of DMF was added NaOH (0.012 g, 0.29 mmol), followed by stirring at 50–55 °C for 30 min. The solution was mixed and reacted with compound **1** (0.05 g, 0.20 mmol) in 3 mL of DMF at 80–90 °C for 3 h. The reaction mixture was cooled to room temperature, diluted with 6 mL of EtOAc, washed with

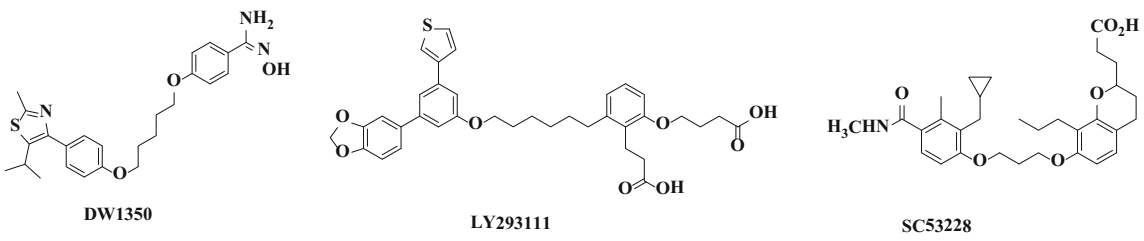


Fig. 1 The leukotriene B4 antagonists contained diether structure

distilled water, and saturated in NaCl aqueous solution. It was dried over $MgSO_4$ and the solvent was removed by concentration under reduced pressure. Recrystallization in ethanol was done to yield compound **2a** (0.014 g, 24 %).

White powder, mp 73–74 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.60 (d, $J = 7.9$, 1H), 7.40–7.32 (m, 2H), 7.28–7.24 (m, 2H), 7.20 (t, $J = 7.5$, 1H), 6.92–6.88 (m, 3H), 4.05 (t, $J = 7.2$, 2H), 4.00 (t, $J = 6.4$, 2H), 1.88-1.80 (m, 4H), 1.62-1.57 (m, 2H); ^{13}C NMR (400 MHz, DMSO-d_6, δ, ppm): δ 168.64, 158.57, 136.86, 129.41 (×2), 126.62, 123.04, 122.88, 121.45, 120.34, 114.37 (×2), 111.40, 67.06, 42.08, 28.27, 26.87, 22.75; HR-FABMS Calcd for $C_{18}H_{20}NO_2S$ (M^++H): 314.1215, Found: 314.1219.

*Synthesis of 4-(5-(Benzo[d]thiazol-2-yloxy)pentyloxy)benzonitrile (**2b**)* 4-Cyanophenol (0.047 g, 0.39 mmol), NaOH (0.024 g, 0.59 mmol), and compound **1** (0.1 g, 0.39 mmol) were reacted in the same manner as compound **2a**, followed by recrystallization in ethanol to yield compound **2b** (0.070 g, 53 %).

White powder, mp 98–101 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.68 (d, $J = 9.2$, 2H), 7.60 (d, $J = 7.7$, 1H), 7.40–7.31 (m, 2H), 7.20 (t, $J = 7.5$, 1H), 7.09 (d, $J = 9.2$, 2H), 4.13 (t, $J = 6.6$, 2H), 4.05 (t, $J = 7.2$, 2H), 1.91–1.83 (m, 4H), 1.63-1.55 (m, 2H); ^{13}C NMR (400 MHz, DMSO-d_6, δ, ppm): δ 168.60, 162.07. 136.83. 134.13 (×2), 126.59, 123.02, 122.88, 122.84, 121.44, 115.49, 111.39 (×2), 102.60, 67.85, 42.04, 27.96, 23.23, 22.57; HR-FABMS Calcd for $C_{19}H_{19}N_2O_2S$ (M^++H): 339.1167, Found: 339.1162.

*Synthesis of 4-(5-(Benzo[d]thiazol-2-yloxy)pentyloxy)-N,N-diisopropyl-3-methoxybenzamide (**2c**) Synthesis of 4-Hydroxy-N, N-diisopropyl-3-methoxybenzamide.* A solution of 4-hydroxy-3-methoxybenzoic acid (0.1 g, 0.59 mmol) in 5 mL of dichloromethane was mixed with $SOCl_2$ (0.637 g, 5.35 mmol) and 0.025 mL of DMF in that order and refluxed for 1 h. Following concentration under reduced pressure using a rotary evaporator, the residue was dissolved in 5 mL of dichloromethane, and then mixed with diisopropylamine (0.301 g, 2.97 mmol) in an ice bath. The mixture was stirred at room temperature for 2 h, diluted with 5 mL of EtOAc and washed with 1 N HCl aqueous solution, 1 N NaOH aqueous solution and a saturated NaCl aqueous solution. It was dried over $MgSO_4$ and the solvent was removed by concentration under reduced pressure to afford **4-**hydroxy-*N*, *N*-diisopropyl-3-methoxybenzamide (0.087 g, 58 %).

Pale yellow powder; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.27 (d, $J = 8.0$, 1H), 7.07 (s, 1H), 6.92 (d, $J = 8.2$, 1H), 3.88 (s, 3H), 3.73 (brs, 2H), 1.32 (brs, 12H).

*Synthesis of 4-(5-(Benzo[d]thiazol-2-yloxy)pentyloxy)-N,N-diisopropyl-3-methoxybenzamide (**2c**)* NaOH (0.010 g, 0.24 mmol) was added to a solution of 4-hydroxy-*N*, *N*-diisopropyl-3-methoxybenzamide (0.041 g, 0.16 mmol) in 6 mL of DMF and stirred at 50–55 °C for 30 min. Compound **1** (0.041 g, 0.16 mmol) was added to the reaction mixture in 3 mL of DMF and reacted at 80–90 °C for 4 h. The reaction mixture was cooled to room temperature, diluted with 6 mL of EtOAc and washed with distilled water and a saturated NaCl aqueous solution. It was dried over $MgSO_4$ and the solvent was removed by concentration under reduced pressure. Recrystallization in ethanol was done to afford compound **2c** (0.069 g, 91 %).

Yellow oil; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.60 (d, $J = 7.9$, 1H), 7.40–7.31 (m, 2H), 7.20 (t, $J = 7.4$, 1H), 6.94 (d, $J = 8.0$, 1H), 6.88 (s, 1H), 6.82 (d, $J = 8.0$, 1H), 4.07–4.01 (m, 4H), 3.80 (s, 3H), 3.74 (brs, 2H), 1.89–1.83 (m, 4H), 1.63–1.59 (m, 2H), 1.32 (brs, 12H); ^{13}C NMR (400 MHz, acetone, δ, ppm): δ 169.88, 168.69, 149.45, 149.06, 137.36, 132.21, 126.47, 122.90, 122.64, 122.17, 118.22, 112.82, 111.06, 110.24, 68.44, 55.34 (×3), 42.29, 27.15 (×2), 23.14, 20.09 (×4); HR-FABMS Calcd for $C_{26}H_{35}N_2O_4S$ (M^++H): 471.2318, Found: 471.2321.

*Synthesis of 4-(5-(Benzo[d]thiazol-2-yloxy)pentyloxy)-N,N-diethyl-3-methoxybenzamide (**2d**)* Vanillic acid diethylamide (0.044 g, 0.20 mmol), NaOH (0.012 g, 0.29 mmol), and compound **1** (0.05 g, 0.20 mmol) were reacted in the same manner as compound **2a**, to afford compound **2d** (0.083 g, 95 %).

Yellow oil; ^{1}H NMR (400 MHz, acetone-d_6) 7.60 (d, $J = 7.7$, 1H), 7.40–7.32 (m, 2H), 7.20 (t, $J = 7.5$, 1H), 6.97–6.95 (m, 2H), 6.90 (d, $J = 8.0$, 1H), 4.07–4.02 (m, 4H), 3.81 (s, 3H), 3.40–3.39 (m, 4H), 1.89–1.84 (m, 4H), 1.63–1.61 (m, 2H), 1.15 (t, $J = 7.2$, 6H); ^{13}C NMR (400 MHz, acetone, δ, ppm): δ 171.04, 169.68, 150.46, 150.30, 138.32, 131.30, 127.45, 123.89, 123.63, 123.60, 123.15, 120.15, 113.55, 112.04, 69.40, 56.38 (×2), 43.26, 43.08, 28.14, 24.51, 24.10, 14.01 (×2); HR-FABMS Calcd for $C_{24}H_{31}N_2O_4S$ (M^++H): 443.2005, Found: 443.2008.

*Synthesis of 2-(5-(3,5-dimethylphenoxy)pentyloxy)benzo[d]thiazole (**2e**)* 3,5-Dimethylphenol (0.40 g, 0.33 mmol), NaOH (0.020 g, 0.50 mmol), and compound **1** (0.085 g, 0.33 mmol) were reacted for 4 h in the same manner as compound **2a**, and recrystallization in ethanol was done to afforded compound **2e** (0.108 g, 96 %).

White powder, mp 61–63 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.59 (d, $J = 7.7$, 1H), 7.39–7.31 (m, 2H), 7.20 (t, $J = 7.5$, 1H), 6.54 (s, 1H), 6.52 (s, 2H), 4.04 (t, $J = 7.4$, 2H), 3.94 (t, $J = 6.4$, 2H), 2.22 (s, 6H), 1.84–1.78 (m, 4H), 1.59–1.54 (m, 2H); ^{13}C NMR (400 MHz, DMSO-d_6, δ, ppm): δ 168.63, 158.62, 138.47 (×2), 136.86, 126.60, 123.03, 122.86 (×2), 121.96, 121.46, 112.11, 111.38, 66.91, 42.09, 28.32, 26.85, 22.75, 21.01 (×2); HR-FABMS

Calcd for $C_{20}H_{24}NO_2S$ (M^++H): 342.1528, Found: 342.1530.

*Synthesis of 2-(5-(4-(1H-1,2,4-thiazol-1-yl)phenoxy)pentyloxy)benzo[d]thiazole (**2f**)* 4-(1,2,4-Triazol-1-yl)phenol (0.032 g, 0.20 mmol), NaOH (0.012 g, 0.29 mmol), and compound **1** (0.05 g, 0.20 mmol) were reacted in the same manner as compound **2a**, and recrystallization in ethanol was done to afforded compound **2f** (0.055 g, 74 %).

White powder, mp 103–104 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 8.89 (s, 1H), 8.05 (s, 1H), 7.74 (d, J = 9.2, 2H), 7.60 (d, J = 7.8, 1H), 7.40-7.32 (m, 2H), 7.20 (t, J = 7.5, 1H), 7.09 (d, J = 8.8, 2H), 4.09 (t, J = 6.4, 2H), 4.06 (t, J = 7.4, 2H), 1.90–1.84 (m, 4H), 1.62–1.61 (m, 2H); ^{13}C NMR (400 MHz, DMSO-d_6, δ, ppm): δ 168.66, 158.00, 152.04, 141.87, 136.86, 130.09, 126.62, 123.04, 122.88, 121.46, 121.05 (×2), 115.26 (×2), 111.40, 67.68, 42.07, 28.17, 26.85, 22.68; HR-FABMS Calcd for $C_{20}H_{21}N_4O_2S$ (M^++H): 381.1385, Found: 381.1386.

*Synthesis of 2-(5-(4-Fluorophenoxy)pentyloxy)benzo[d]thiazole (**2g**)* 4-Fluorophenol (0.022 g, 0.20 mmol), NaOH (0.012 g, 0.29 mmol), and compound **1** (0.05 g, 0.20 mmol) were reacted in the same manner as compound **2a**, and recrystallization in ethanol afforded the compound **2g** (0.019 g, 30 %).

White powder, mp 69–70 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.60 (d, J = 7.8, 1H), 7.40–7.31 (m, 2H), 7.20 (t, J = 7.5, 1H), 7.05–7.00 (m, 2H), 6.93–6.89 (m, 2H), 4.05 (t, J = 7.4, 2H), 3.98 (t, J = 6.4, 2H), 1.87–1.82 (m, 4H), 1.59–1.58 (m, 2H); ^{13}C NMR (400 MHz, acetone, δ, ppm): δ 169.71, 159.16, 156.82, 156.49, 138.35, 127.47, 123.92, 123.67, 123.18, 116.62, 116.58, 116.38, 112.07, 69.03, 43.23, 28.13 (×2), 24.03; HR-FABMS Calcd for $C_{18}H_{19}FNO_2S$ (M^++H): 332.1121, Found: 332.1115.

*Synthesis of 2-(5-(3-chlorophenoxy)pentyloxy)benzo[d]thiazole (**2h**)* 3-Chlorophenol (0.025 g, 0.20 mmol), NaOH (0.012 g, 0.29 mmol), and compound **1** (0.05 g, 0.20 mmol) were reacted in the same manner as compound **2a**, to afford compound **2h** (0.041 g, 60 %).

Yellow oil; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.59 (d, J = 7.8, 1H), 7.39–7.24 (m, 3H), 7.20 (t, J = 7.5, 1H), 6.95–6.92 (m, 2H), 6.87 (d, J = 8.4, 1H), 4.06-4.01 (m, 4H), 1.88–1.80 (m, 4H), 1.62–1.56 (m, 2H); ^{13}C NMR (400 MHz, acetone, δ, ppm): δ 169.71, 161.13, 138.33, 135.29, 131.52, 127.46, 123.91, 123.65, 123.17, 121.34, 115.58, 114.26, 112.03, 68.82, 43.22, 28.09 (×2), 23.97; HR-FABMS Calcd for $C_{18}H_{19}ClNO_2S$ (M^++H):348.0825, Found: 348.0824.

*Synthesis of 6-(5-(Benzo[d]thiazol-2-yloxy)pentyloxy)benzofuran-3(2H)-one] (**2i**)* 6-Hydroxy-3-coumaranone (0.029 g, 0.20 mmol), NaOH (0.012 g, 0.29 mmol), and compound **1** (0.05 g, 0.20 mmol) were reacted in the same manner as compound **2a**, and recrystallization in EtOAc and diethyl ether was done to afford compound **2i** (0.013 g, 18 %).

Brown powder, mp 75–78 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.59 (d, J = 7.9, 1H), 7.49 (d, J = 9.2, 1H), 7.39–7.31 (m, 2H), 7.20 (t, J = 7.5, 1H), 6.70–6.67 (m, 2H), 4.64 (s, 2H), 4.15 (t, J = 6.4, 2H), 3.05 (t, J = 7.2, 2H), 1.94-1.81 (m, 4H), 1.64–1.56 (m, 2H); ^{13}C NMR (400 MHz, acetone, δ, ppm): δ 197.46, 177.19, 169.73, 168.45, 138.32, 127.46, 125.39, 123.92, 123.66, 123.17, 115.18, 112.60, 112.03, 97.86, 76.17, 69.42, 43.18, 28.06 (×2), 23.90; HR-FABMS Calcd for $C_{20}H_{20}NO_4S$ (M^++H): 370.1113, Found: 370.1111.

*Synthesis of 2-(5-(2-Methoxyphenoxy)pentyloxy)benzo[d]thiazole (**2j**)* Guaiacol (0.024 g, 0.20 mmol), NaOH (0.012 g, 0.29 mmol), and compound **1** (0.05 g, 0.20 mmol) were reacted in the same manner as compound **2a**, and recrystallization in diethyl ether afforded compound **2j** (0.064 g, 96 %).

Yellow oil; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.59 (d, J = 8.0, 1H), 7.39–7.31 (m, 2H), 7.20 (t, J = 7.4, 1H), 6.95-6.92 (m, 2H), 6.90–6.82 (m, 2H), 4.05 (t, J = 7.2, 2H), 3.99 (t, J = 6.4, 2H), 3.77 (s, 3H), 1.88–1.81 (m, 4H), 1.63–1.58 (m, 2H); ^{13}C NMR (400 MHz, acetone, δ, ppm): δ 169.68, 150.95, 149.97, 138.35, 127.45, 123.89, 123.63, 123.17, 121.92, 121.78, 114.83, 113.46, 112.04, 69.45, 43.29, 56.25, 28.17 (×2), 24.16; HR-FABMS Calcd for $C_{19}H_{22}NO_3S$ (M^++H): 344.1320, Found: 344.1317.

*Synthesis of 2-(5-(2-Fluorophenoxy)pentyloxy)benzo[d]thiazole] (**2k**)* 2-Fluorophenol (0.022 g, 0.20 mmol), NaOH (0.012 g, 0.29 mmol), and compound **1** (0.05 g, 0.20 mmol) were reacted in the same manner as compound **2a**, to afford compound **2k** (0.057 g, 88 %).

Yellow oil; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.59 (d, J = 7.6, 1H), 7.40-7.32 (m, 2H), 7.20 (t, J = 7.5, 1H), 7.14–7.06 (m, 3H), 6.94–6.90 (m, 1H), 4.10–4.03 (m, 4H), 1.90–1.81 (m, 4H), 1.63–1.58 (m, 2H); ^{13}C NMR (400 MHz, acetone, δ, ppm): δ 169.68, 154.72, 152.30, 148.12, 138.30, 127.44, 125.48, 123.75, 123.16, 121.77, 116.75, 115.96, 112.00, 69.66, 43.21, 28.12 (×2), 23.95; HR-FABMS Calcd for $C_{18}H_{19}FNO_2S$ (M^++H): 332.1121, Found: 332.1125.

*Synthesis of 4-(5-(Benzo[d]thiazol-2-yloxy)pentyloxy)-N′-hydroxybenzamidine (**2l**)* Triethylamine (0.032 g, 0.31 mmol) was added to compound **2b** (0.053 g,

0.16 mmol) in 10 ml of ethanol with stirring. The solution was mixed with $NH_2OH{\cdot}HCl$ (0.022 g, 0.31 mmol) and refluxed overnight. Then, the reaction was cooled to 40 °C and distilled water was slowly added to form crystal. It was washed with distilled water and ether to afford compound **2l** (0.029 g, 49 %).

White powder, mp 144–147 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 8.70 (s, 1H), 7.63 (d, J = 8.8, 2H), 7.60 (d, J = 7.6, 1H), 7.40–7.31 (m, 2H), 7.20 (t, J = 7.6, 1H), 6.90 (d, J = 8.8, 2H), 5.34 (s, 2H), 4.07–4.01 (m, 4H), 1.90–1.80 (m, 4H), 1.61–1.57 (m, 2H); ^{13}C NMR (400 MHz, DMSO-d_6, δ, ppm): δ 168.65, 159.14 (×2), 150.54 (×2), 136.85, 126.64 (×2), 125.61, 123.04, 122.88, 121.45, 113.90, 111.40, 67.28, 42.08, 28.22, 26.85, 22.71; HR-FABMS Calcd for $C_{19}H_{22}N_3O_3S$ (M^++H): 372.1382, Found: 372.1377.

*Synthesis of 4-(5-(benzo[d]thiazol-2-yloxy)pentyloxy)benzamidine hydrohloride (**2m**)* 0.2 mL of 1 M $LiN(SiMe_3)_2$ in THF was placed in a flask. A solution of compound **2b** (0.05 g, 0.15 mmol) in 2 mL of dry THF was added to the flask and purged with nitrogen gas and stirred at room temperature for 4 h. 0.1 mL of 6 N HCl in *i*PrOH was added to the reaction mixture before storage in freezer for overnight. Crystal was formed and it was washed with ether. Filtration under reduced pressure was done to afford compound **2m** (0.008 g, 14 %).

Pale yellow powder, mp 245–247 °C; ^{1}H NMR (Free base, 400 MHz, DMSO-d_6) δ 9.10 (brs, 3H), 7.81 (d, J = 8.4, 2H), 7.66 (d, J = 8.0, 1H), 7.38 (d, J = 4.0, 2H), 7.23–7.20 (m, 1H), 7.12 (d, J = 8.8, 2H), 4.07 (t, J = 6.4, 2H), 3.98 (t, J = 7.2, 2H), 1.80–1.70 (m, 4H), 1.48–1.46 (m, 2H); ^{13}C NMR (400 MHz, DMSO-d_6, δ, ppm): δ 168.68, 164.71 (×2), 162.99, 136.85, 130.13 (×2), 126.64, 123.07, 122.90, 121.45, 119.22, 114.73, 111.42, 67.91, 42.05, 28.01, 26.82, 22.59; HR-FABMS Calcd for $C_{19}H_{22}N_3O_2S$ (M^++H): 356.1433, Found: 356.1435.

*Synthesis of Methyl 4-(5-(benzo[d]thiazol-2-yloxy)pentyloxy-3-methoxybenzoate (**2n**) Synthesis of methyl 4-hydroxy-3-methoxybenzoate.* To a solution of 4-hydroxy-3-methoxybenzoic acid (0.1 g, 0.59 mmol) in 10 mL of methanol was added 0.3 mL of conc. Sulfuric acid, and refluxed overnight. The pH of the solution was shifted into an alkaline zone by addition of 5 % aqueous $NaHCO_3$ solution. Following extraction with EtOAc, the extract was washed with a saturated NaCl aqueous solution. It was dried over $MgSO_4$ and the solvent was removed by concentration under reduced pressure to afford the methyl 4-hydroxy-3-methoxybenzoate (0.107 g, 98 %).

Yellow oil; ^{1}H NMR (400 MHz, acetone-d_6) δ 8.390 (s, 1H), 7.561 (d, J = 8.0, 1H), 7.538 (s, 1H), 6.911 (d, J = 8.4, 1H), 6.516 (s, 2H), 3.904 (s, 3H), 3.831 (s, 3H).

*Synthesis of Methyl 4-(5-(benzo[d]thiazol-2-yloxy)pentyloxy)-3-methoxybenzoate (**2n**)* Methyl 4-hydroxy-3-methoxybenzoate (0.043 g, 0.39 mmol) in 3 mL of acetonitrile was mixed with K_2CO_3 (0.027 g, 0.20 mmol) at 50–55 °C for 30 min by stirring. To this solution was added compound **1** (0.05 g, 0.20 mmol) in 3 mL of acetonitrile, followed by reaction at 80 °C for 2 days.

The resulting mixture was cooled to room temperature, diluted with 6 mL of diethyl ether, and washed with distilled water and a saturated NaCl aqueous solution. It was dried over $MgSO_4$ and the solvent was removed by concentration under reduced pressure. Crystallization in diethyl ether and n-hexane afforded the compound **2n** (0.017 g, 21 %).

White powder, mp 68–70 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.60 (d, J = 8.4, 2H), 7.51 (s, 1H), 7.39–7.32 (m, 2H), 7.20 (t, J = 7.6, 1H), 7.03 (d, J = 8.4, 1H), 4.10 (t, J = 6.4, 2H), 4.05 (t, J = 7.2, 2H), 3.84 (s, 6H), 1.93–1.82 (m, 4H), 1.64–1.58 (m, 2H); ^{13}C NMR (400 MHz, DMSO-d_6, δ, ppm): δ 168.65, 165.95, 152.30, 148.47, 136.85, 126.61, 123.10, 123.03, 122.87, 121.63, 121.46, 112.01, 111.82, 111.41, 68.11, 55.52, 51.84, 42.08, 28.15, 26.85, 22.69; HR-FABMS Calcd for $C_{21}H_{24}NO_5S$ (M^++H): 402.1375, Found: 402.1377.

*Synthesis of 4-(5-(Benzo[d]thiazol-2-yloxy)pentyloxy)-3-methoxybenzoic acid (**2o**)* Compound **2n** (0.017 g, 0.42 mmol) was stirred and mixed overnight at room temperature in 7 mL of 2 N NaOH. Distilled water and diethyl ether were added to the reaction mixture. The aqueous layer was separated from the organic layer and acidified with 10 % aqueous solution of HCl to pH 2, followed by extraction with diethyl ether. The extract was washed with saturated NaCl aqueous solution and dried over $MgSO_4$. The solvent was removed by concentration under reduced pressure. Recrystallization in diethyl ether was done to afford compound **2o** (0.037 g, 48 %).

White powder, mp 130–131 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.63 (d, J = 8.4, 1H), 7.60 (d, J = 7.8, 1H), 7.54 (s, 1H), 7.40–7.32 (m, 2H), 7.20 (t, J = 7.6, 1H), 7.03 (d, J = 8.4, 1H), 4.10 (t, J = 6.4, 2H), 4.06 (t, J = 7.2, 2H), 3.85 (s, 3H), 1.94–1.82 (m, 4H), 1.65–1.57 (m, 2H); ^{13}C NMR (400 MHz, acetone, δ, ppm): δ 167.48, 153.90, 150.18, 138.37, 127.48 (×2), 124.53, 123.92, 123.78, 123.66, 123.19, 113.72, 112.95, 112.08, 69.40, 56.31, 43.27, 28.15 (×2), 24.08; HR-FABMS Calcd for $C_{20}H_{22}NO_5S$ (M^++H): 388.1219, Found: 388.1213.

Synthesis of 2-(5-phenoxybutyloxy)benzo[d]thiazole derivatives

*Synthesis of 2-(4-Chlorobutoxy)benzo[d]thiazole (**3**)* 2-Hydroxybenzothiazole (0.2 g, 1.32 mmol) and 1-bromo-4-

chlorobutane (0.227 g, 1.32 mmol) were reacted in the same manner as compound **1** to yield compound **3** (0.302 g, 94 %).

Transparent oil; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.60 (d, J = 7.6, 1H), 7.41–7.33 (m, 2H), 7.21 (t, J = 7.5, 1H), 4.07 (t, J = 7.0, 2H), 3.69 (t, J = 6.4, 2H), 1.92–1.86 (m, 4H); HR-FABMS Calcd for $C_{11}H_{13}ClNOS$ (M^++H): 242.0406, Found: 242.0408.

*Synthesis of 2-(4-Phenoxybutoxy)benzo[d]thiazole (**4a**)* Phenol (0.020 g, 0.21 mmol), NaOH (0.012 g, 0.31 mmol), and 2-(4-Chlorobutoxy)benzo[*d*]thiazole **3** (0.05 g, 0.21 mmol) were reacted in the same manner as compound **2a**, to yield compound **4a** (0.056 g, 90 %).

Brown oil; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.60 (d, J = 7.9, 1H), 7.40–7.33 (m, 2H), 7.29–7.16 (m, 3H), 6.92 (d, J = 8.4, 2H), 6.84–6.81 (m, 1H), 4.10 (t, J = 7.0, 2H), 4.06 (t, J = 6.2, 2H), 1.98–1.86 (m, 4H); ^{13}C NMR (400 MHz, acetone, δ, ppm): δ 169.76, 160.08, 138.29, 135.55, 130.31 (×2), 127.47, 123.95, 123.67, 123.19, 121.40, 115.40 (×2), 112.03, 67.92, 43.05, 27.29, 25.17; HR-FABMS Calcd for $C_{17}H_{18}NO_2S$ (M^++H): 300.1058, Found: 300.1061.

*Synthesis of 4-(4-(Benzo[d]thiazol-2-yloxy)butoxy)benzonitrile (**4b**)* 4-Cyanophenol (0.025 g, 0.21 mmol), NaOH (0.012 g, 0.31 mmol), and compound **3** (0.05 g, 0.21 mmol) were reacted in the same manner as compound **2a**, to afford compound **4b** (0.059 g, 88 %).

Transparent oil; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.68 (d, J = 9.2, 2H), 7.60 (d, J = 7.8, 1H), 7.40-7.33 (m, 2H), 7.21 (t, J = 7.7, 1H), 7.10 (d, J = 9.2, 2H), 4.19 (t, J = 6.0, 2H), 4.11 (t, J = 6.8, 2H), 1.97–1.86 (m, 4H); ^{13}C NMR (400 MHz, acetone, δ, ppm): δ 169.82, 163.39, 138.26, 135.07, 134.90, 127.48, 123.98, 123.66, 123.14, 119.69, 116.42, 112.03, 104.67, 68.66, 42.95, 27.00, 25.60, 24.99; HR-FABMS Calcd for $C_{18}H_{17}N_2O_2S$ (M^++H): 325.1011, Found: 325.1015.

*Synthesis of 4-(4-(Benzo[d]thiazol-2-yloxy)butoxy)-N'-hydroxybenzamidine (**4c**)* Compound **4b** (0.04 g, 0.12 mmol), triethylamine (0.025 g, 0.25 mmol), and NH_2OH·HCl (0.0172 g, 0.25 mmol) were reacted in the same manner as compound **2a**, to afford compound **4c** (0.018 g, 41 %).

White powder, mp 170–171 °C; ^{1}H NMR (400 MHz, acetone-d_6) δ 8.70 (s, 1H), 7.63 (d, J = 9.2, 2H), 7.60 (d, J = 7.9, 1H), 7.40–7.33 (m, 2H), 7.21 (t, J = 7.6, 1H), 6.91 (d, J = 9.2, 2H), 5.37 (s, 2H), 4.12–4.08 (m, 4H), 1.95–1.87 (m, 4H); ^{13}C NMR (400 MHz, DMSO-d_6, δ, ppm): δ 168.70, 159.06, 150.52, 136.82, 126.65 (×2), 126.63, 125.67, 123.09, 122.91, 121.48, 113.93 (×2), 111.39, 66.95, 41.87, 25.85, 23.85; HR-FABMS Calcd for $C_{18}H_{20}N_3O_3S$ (M^++H): 358.1225, Found: 358.1228.

*Synthesis of 4-(4-(benzo[d]thiazol-2-yloxy)butoxy)-N,N-diisopropyl-3-methoxy benzamide (**4d**)* 4-Hydroxy-*N*, *N*-diisopropyl-3-methoxybenzamide (0.053 g, 0.21 mmol), NaOH (0.013 g, 0.32 mmol), and compound **3** (0.051 g, 0.21 mmol) were reacted in the same manner as in compound **2a**, to afford compound **4d** (0.096 g, 99 %).

Yellow oil; ^{1}H NMR (400 MHz, acetone-d_6) δ 7.60 (d, J = 7.7, 1H), 7.39-7.35 (m, 2H), 7.23–7.17 (m, 1H), 6.96 (d, J = 8.0, 1H), 6.89 (s, 1H), 6.83 (d, J = 8.0, 1H), 4.15–4.09 (m, 4H), 3.81 (s, 3H), 3.75 (brs, 2H), 1.98–1.86 (m, 4H), 1.32 (brs, 12H); ^{13}C NMR (400 MHz, acetone, δ, ppm): δ 170.85, 169.73, 150.45, 149.88, 138.32, 133.30, 127.45, 123.92, 123.62, 123.16, 119.18, 113.92, 112.10, 111.14, 69.19, 56.31, 43.09, 42.79, 27.24, 25.58, 25.25, 21.09 (×4); HR-FABMS Calcd for $C_{25}H_{33}N_2O_4S$ (M^++H): 457.2161, Found: 457.2158.

Biological activity

Cells culture system

Bone marrow cells were obtained from the long bones of 4- to 6-week-old ICR male mice. Bone marrow cells were cultured in the presence of M-CSF (30 ng/ml, R&D) for 3 days to generate the bone marrow–derived macrophages (BMMs). To examine osteoclast formation, BMMs were treated with various concentrations of compounds **2c**, **2h**, **2j**, **2k**, **4a,** and **4d** in the presence of M-CSF (30 ng/ml) and RANKL (100 ng/ml, PeproTech) in 96 well culture plates (CORNING, MA, USA) for 4 days. Cells were fixed for 10 min with 10 % formalin and then re-fixed for 1 min with ethanol/acetone (1:1(v/v)). The cells were stained for TRAP (tartrate-resistant acid phosphatase). TRAP-positive cells having 3 or more nuclei were evaluated as multinucleated osteoclasts by observation under a microscope.

RT-PCR analysis

Total RNA was extracted from BMMs by Easy-Blue (iNtRON Biotechn-ology, Inc.). cDNA was synthesized from total RNA by using RevertAidTM first strand cDNA synthesis Kit (Fermentas, EU) and amplified using polymerase chain reaction (PCR). The PCR program and primers used in this study are given in the following table.

	Primer sequence	PCR condition	Cycle
CTR	F:tttcaagaaccttagctgccagag R:caaggcacggacaatgttgagaag	94 °C 30 s, 58 °C 30 s, 72 °C 30 s	28 cycle
Cath K	F:cttccaatacgtgcagcaga R:acgcaccaatatcttgcacc	94 °C 30 s, 58 °C 30 s, 72 °C 30 s	22 cycle
ATP6v0d2	F:tcagatctcttcaaggctgtgctg R:gtgccaaatgagttcagagtgatg	94 °C 30 s, 59 °C 30 s, 72 °C 30 s	30
DC-STAMP	F:tggaagttcacttgaaactacgtg R:ctcggtttcccgtcagcctctctc	94 °C 30 s, 58 °C 30 s, 72 °C 30 s	30
αv-Integrin	F:cctcagagagggagatgttcacac R:aactgccaagatgatcacccacac	94 °C 30 s, 60 °C 30 s, 72 °C 30 s	28
β3-Integrin	F:gatgacatcgagcaggtgaaagag R:ccggtcatgaatggtgatgagtag	94 °C 30 s, 55 °C 30 s, 72 °C 30 s	32
β-actin	F:tgtgatggtgggaatgggtcag R:tttgatgtcacgcacgatttcc	94 °C 30 s, 58 °C 30 s, 72 °C 30 s	22 cycle

MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetazolium bromide) assay was performed as described by Mosmann, with a modification. BMM cells were seeded at a density of 1×10^4 cells/well onto 96-well plates, treated with samples (compound **2c**, **2h**, **2j**, **2k**, **4a,** and **4d**) and incubated for a predetermined period of time. The culture medium was discarded and the cells were washed with PBS. The MTT solution (0.5 mg/ml) was then added in an amount of 100 μL/well to the plates, the plates were incubated for 5 h with the plates wrapped in foil. Solubilization buffer (10 % SDS in 0.01 M HCl) was added in an amount of 100 μL/well to the plates, which were wrapped in foil again. After the plates were incubated for 16–17 h, absorbance was measured at 570 nm.

Pit formation assay

BMMs were differentiated on dentin slices with M-CSF (30 ng/ml) and RANKL (100 ng/ml) in the presence or absence of **2c** (10 μM) for 7 days. The cells were removed from the dentin slice by wiping the surface of it, and the slices were stained with toluidin blue (J.T. Baker, UK, 1 μg/ml). And the numbers of pits formed by bone resorption on the dentin slices were counted.

Immunoblot analysis

Total cell lysates were isolated, separated by SDS-PAGE, and transferred onto Immobilon-P membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5 % non-fat-milk in PBS-T, and then immunostained with anti-NFATc1 (1:200, Cell Signaling Technology, Beverly, MA, USA), anti-c-Fos (1:1000, Cell Signaling Technology, Beverly, MA, USA), and anti-β-actin (1:4000, Cell Signaling Technology, Beverly, MA, USA) antibodies, followed by secondary horseradish peroxidese-conjugated antibody (1:5000, Cell Signaling Technology, Beverly, MA, USA). The membranes were developed using an advanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK).

In vivo experiment

To study the effects of **2c** on LPS-induced osteoclast formation in vivo, 6-week-old male ICR mice were divided into three groups of 6 and intraperitoneally (i.p.) administrated **2c** (5 mg/kg) or vehicle (PBS) daily. After 1 day, mice were injected subcutaneously with vehicle (PBS) or LPS (0.5 mg) over calvarial bone. The mice were sacrificed 6 days after LPS or vehicle injection, and the whole calvariae were fixed in 4 % paraformaldehyde and stained for TRAP. TRAP + stained area in calvariae were quantified by using image J program (version 1.32; National Institute of Health, Bethesda, MD, USA) according to the manufacturer's protocol. All animal experiments were reviewed and approved by the Sookmyung Women's University Animal Care Committee.

Statistical treatment

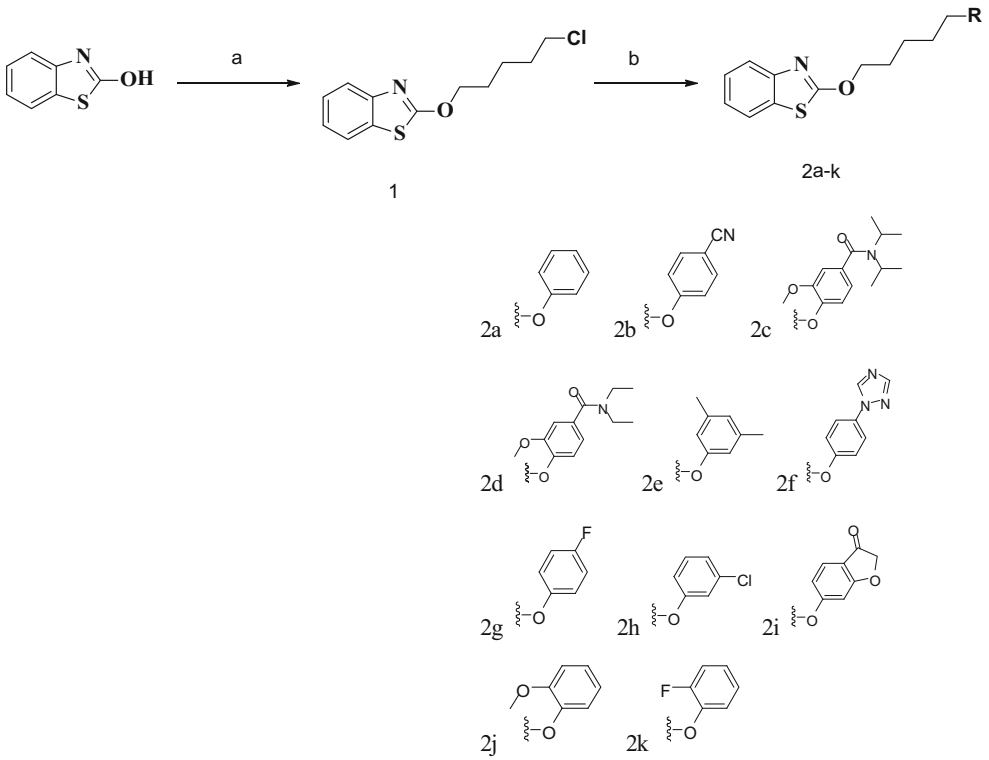
Experimental result is presented as mean ± standard deviation from at least three independent experiments. The difference between the two groups was determined by Student's *t* test and was considered statistically significant when $P < 0.05$.

Results and discussion

Synthesis

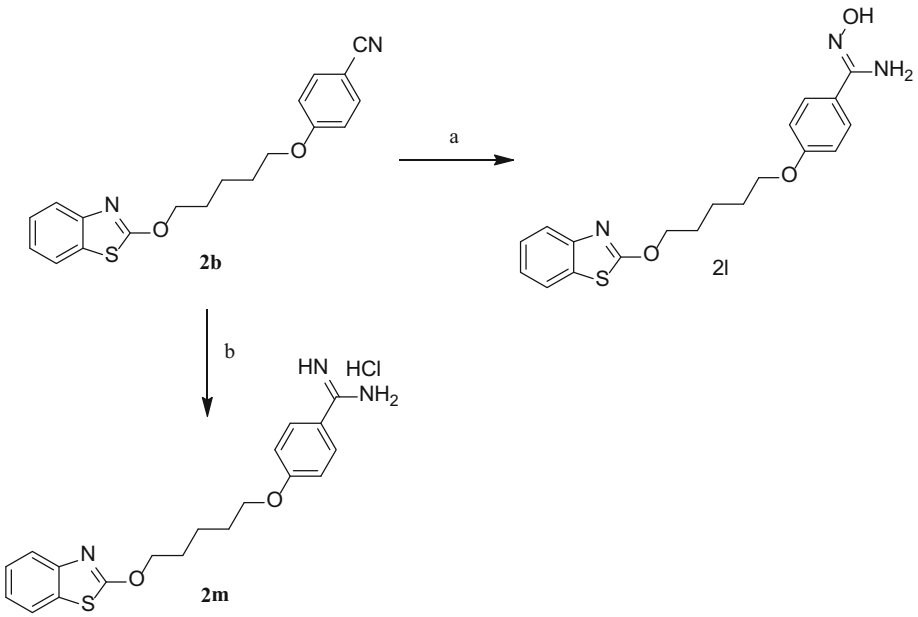
To obtain 2-(5-phenoxypentyloxy)benzo[d]thiazole derivatives **2a–k**, a two-step reaction was performed. 2-Hydroxybenzothiazole was reacted with 1-bromo-5-chloropentane and K_2CO_3 at 80–90 °C to obtain compound **1** in high yield. Then variously substituted phenols were reacted with compound **1**, to yield compounds **2a–k** (Scheme 1). Synthesized compounds were purified by recrystallization.

N-Hydroxybenzamide (**2l**) and benzamidine compound (**2m**) were prepared from cyano compound **2b** (Scheme 2). To obtain the compound **2l**, **2b** was refluxed with NH_2-OH·HCl and triethylamine under anhydrous atmosphere.



Scheme 1 Synthesis of 2-(5-phenoxypentyloxy)benzothiazole derivatives. **a** 1-Bromo-5-chloropentane, K_2CO_3, CH_3CN, 80-90 °C, 3 h; **b** Substituted phenols, NaOH, DMF, 80–90 °C, 3–5 h

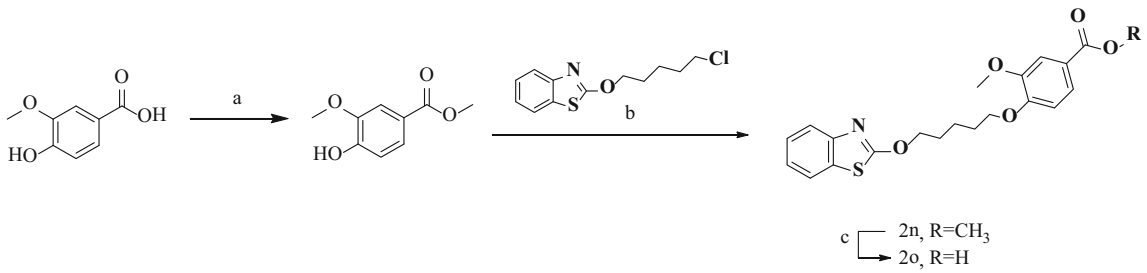
Scheme 2 Synthesis of *N*-hydroxybenzamidine and benzamidine derivatives from cyano compound. **a** $NH_2OH.HCl$, TEA, EtOH, reflux, overnight; **b** (1) $LiN(SiMe_3)_2$ (1 M in THF), THF, rt, 4 h; (2) 6 N HCl in iPrOH, 0 °C, overnight



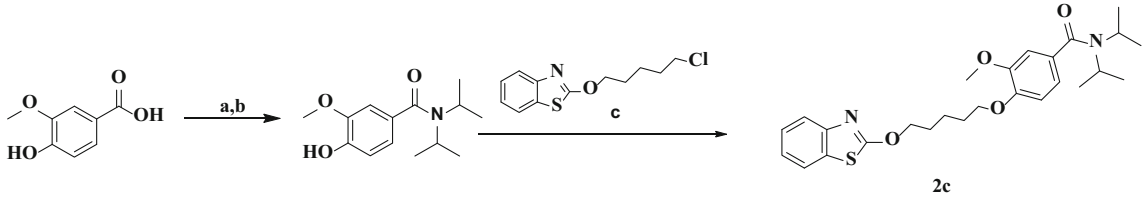
Compound **2m** was obtained by reacting **2b** with $LiN(SiMe_3)_2$.

For the preparation of carboxylic acid **2o**, compound **1** was reacted with methyl 4-hydroxy-3-methoxybenzoate, which was obtained from esterification of 4-hydroxy-3-methoxybenzoic acid with *c*-H_2SO_4 and methanol, to yield compound **2n**. The methyl ester group of compound **2n** was hydrolyzed with 2 *N* NaOH to give compound **2o** (Scheme 3).

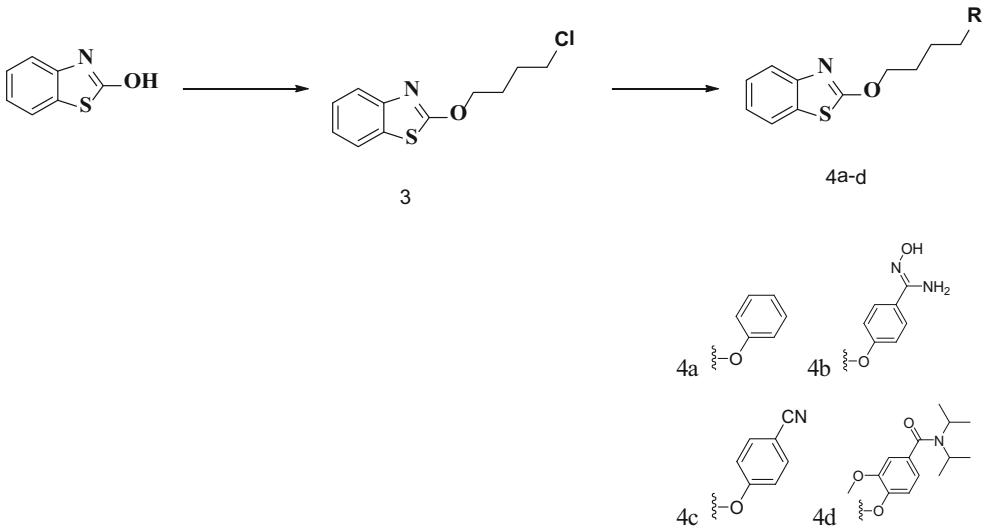
For the preparation of amide **2c,** 4-hydroxy-3-methoxybenzoic acid was reacted with thionyl chloride, and then the acid chloride was reacted with *N,N*-diisopropyl amine to give 4-hydroxy-*N,N*-diisopropyl-3-



Scheme 3 Synthesis of methyl benzoate and benzoic acid substituted 2-(5-phenoxypentyloxy) benzothiazole derivatives. **a** c-H_2SO_4, MeOH, reflux, 18 h, **b** K_2CO_3, CH_3CN, 80 °C, 2 days, **c** 2 N NaOH, rt, overnight



Scheme 4 Synthesis of amide substituted 2-(5-phenoxypentyloxy)benzothiazole derivative. **a** $SOCl_2$, CH_2Cl_2/DMF, reflux, 1 h, **b** Diisopropylamine, CH_2Cl_2, rt, 2 h, **c** DMF, NaOH, 80–90 °C, 3 h



Scheme 5 Synthesis of 2-(4-phenoxybutyloxy)benzothiazole derivatives

methoxybenzamide. The amide was reacted with compound **1** to yield compound **2c** (Scheme 4).

The four carbon linker congeners, 2-(4-phenoxybutoxy)benzo[d]thiazole derivatives (**4a–d**), were prepared by the same methods to yield compound **3**. The reaction started with 2-hydroxybenzothiazole and 1-bromo-5-chlorobutane, then variously substituted phenols were reacted with compound **3**, to afford compounds **4a–b** and **d**. (Scheme 5).

Biological activity

2c inhibits RANKL-induced osteoclast formation and bone resorption in vitro

Inhibition activities on osteoclast formation of the synthesized compounds are shown in Table 1.

Also, we found that the inhibition activities on osteoclast formation was not caused by exposure to chemical

Table 1 Inhibition activities (IC_{50}) on RANKL-induced osteoclast formation of the prepared compounds

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
2a	>50	**2k**	18.40
2b	>50	**2l**	>50
2c	1.56	**2m**	>50
2d	>50	**2n**	>50
2e	>50	**2o**	>50
2f	>50	**4a**	17.68
2g	>50	**4b**	>50
2h	23.30	**4c**	>50
2i	>50	**4d**	2.20
2j	23.35	MK886	3.46

compounds through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetazolium bromide (MTT) assay (Figs. 2, 3).

The optical density (OD) combined with the MTT assay represents a linear correlation with the number of cells. (Freimoser et al. 1999) Figs. 2 and 3 show that the optical density of cells before treating compounds was not decreased compared with the optical density after treatment. This means that the total number of cells was not decreased and the result of the assay should be interpreted as the "inhibition" of osteoclasts, as opposed to their "death".

As briefly presented below, during the course of our study, it became more evident that the effect of the length on of diether linkage is less evident than that of the nature of the end-substituent. Therefore when two compounds with the same substitution group, compound **2c** (IC_{50} = 1.56 μM) was more potent than compound **4d** (IC_{50} = 2.20 μM) which is the four carbon linker congener of **2c**. Likewise, cyano compounds **2l** and **4b**, and *N*-hydroxybenzamidine **3b** and **4c** showed no activity regardless

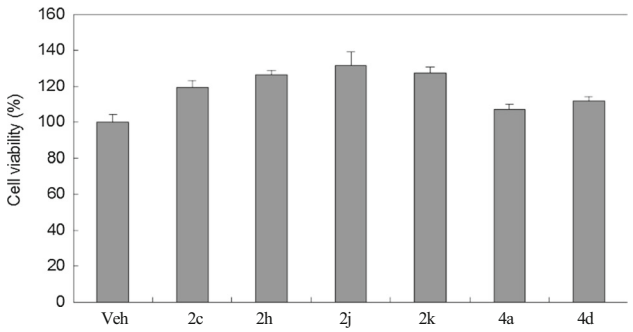


Fig. 2 The effects of the compounds on cell viability. BMMs were cultured with or without **2c** (10 μM), **2e** (50 μM), **2j** (50 μM), **2k** (50 μM), **4a** (50 μM) and **4d** (10 μM) in the presence of M-CSF (30 ng/ml) for 24 h. Cell viability was assessed by MTT assay. Data are expressed as mean ± SD from at least three independent experiments

Fig. 4 The inhibitory effect of **2c** on bone resorption. BMMs were placed on dentin slices and cultured in the absence or presence of the **2c** (10 μM) with RANKL and M-CSF (30 ng/ml) for 6 days. The remained cells were removed and stained with toluidin blue. The resorbed pit numbers were counted. Data are expressed as mean ± SD from at least three independent experiments. $*p < 0.05$ versus RANKL

of the length of diether linker. On the other hand, compound **2a** ($IC_{50} = > 50$ μM) and compound **4a** (IC_{50} = 17.68 μM) were compared, the four-carbon linker congener **4a** showed the activity.

Generally, the compounds with an ortho-substituted group on its phenol moiety showed more potent inhibitory activity, such as **2c** (IC_{50} = 1.56 μM), **2j** (IC_{50} = 23.35 - μM), **2k** (IC_{50} = 18.40 μM), and **4d** (IC_{50} = 2.20 μM). Among these compounds **2c** and **4d,** both compounds have diisopropyl amide moiety, showed the most potent inhibitory activity. Compounds **2g** and **2k** have a fluorine group in their structure, but **2g** is ortho-substituted and **2k** is para-substituted. Compound **2g** didn't show any inhibition activity against osteoclasts, while **2k** showed potent activity. The known 5-LO inhibitor MK886 was used as positive control, and it showed activity with IC_{50} value 3.46 uM.

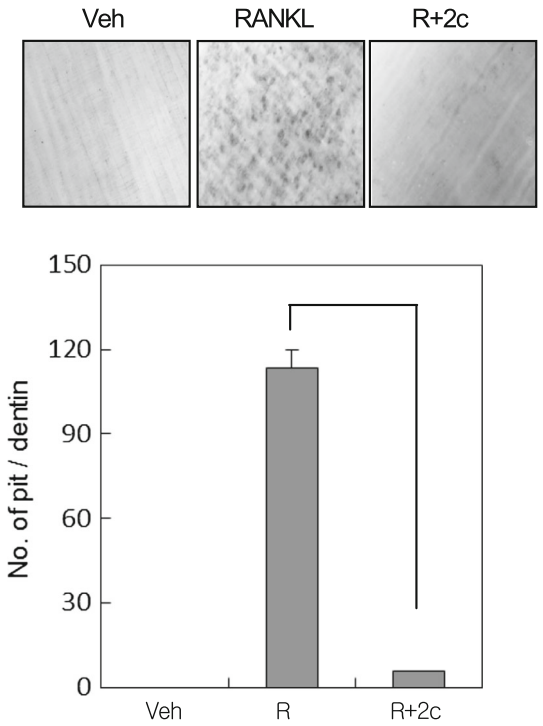
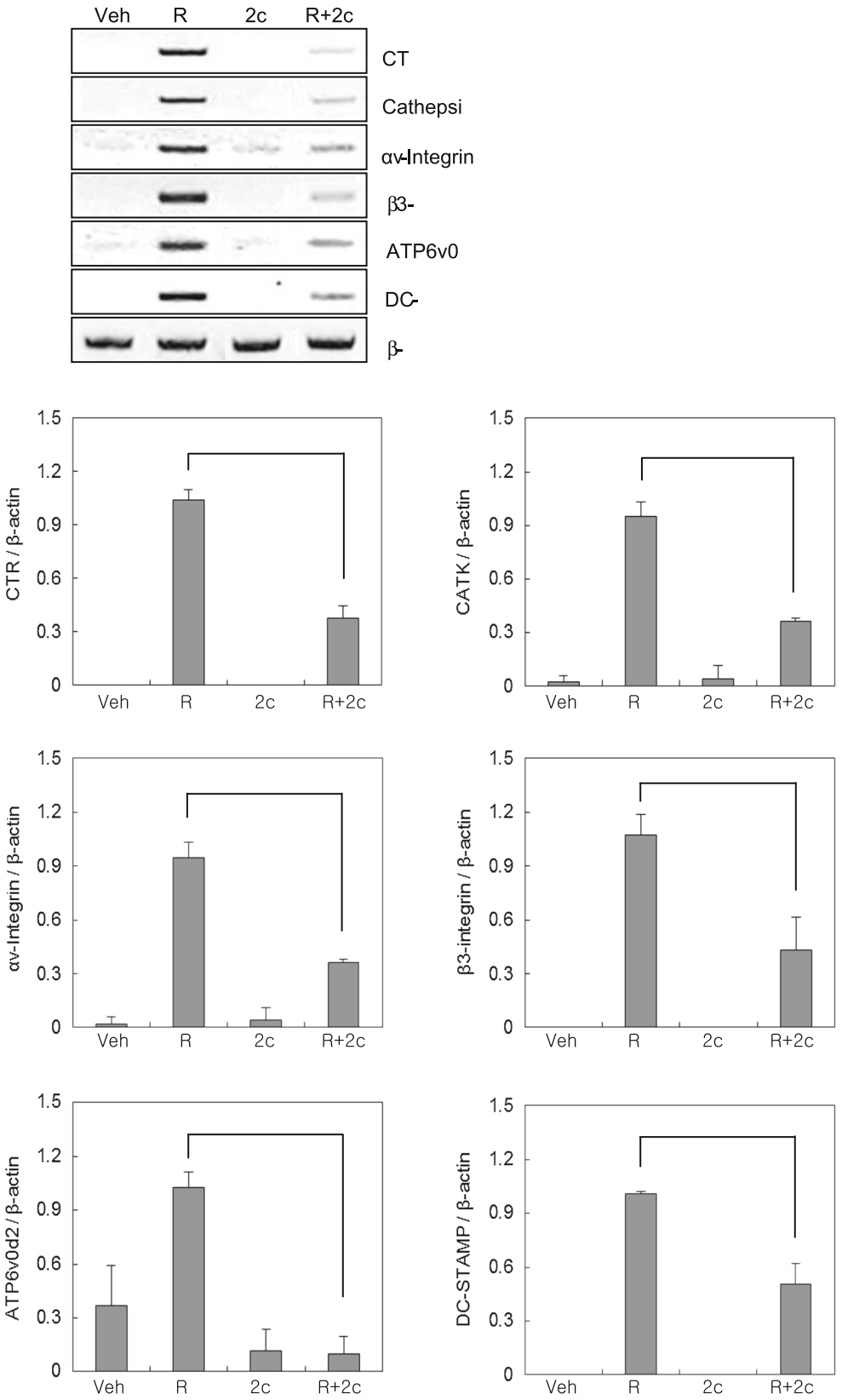


Fig. 3 The inhibitory effect of **2c** on the mRNA expression of osteoclastogenic gene. BMMs were cultured for 6 days in the absence or presence of the 10 μM of 2c with RANKL (100 ng/ml) and M-CSF (30 ng/ml). Total RNA was then isolated from the cells and cDNA templates prepared. mRNA expression was determined by RT-PCR using specific primers designed for each gene. Data are expressed as mean ± SD from at least three independent experiments. $*p < 0.05$ versus RANKL


Veh
R
2c
R+2c
CT
Cathepsi
αv-Integrin
β3-
ATP6v0
DC-
β-
CTR / β-actin
CATK / β-actin
αv-Integrin / β-actin
β3-integrin / β-actin
ATP6v0d2 / β-actin
DC-STAMP / β-actin
0
0.3
0.6
0.9
1.2
1.5

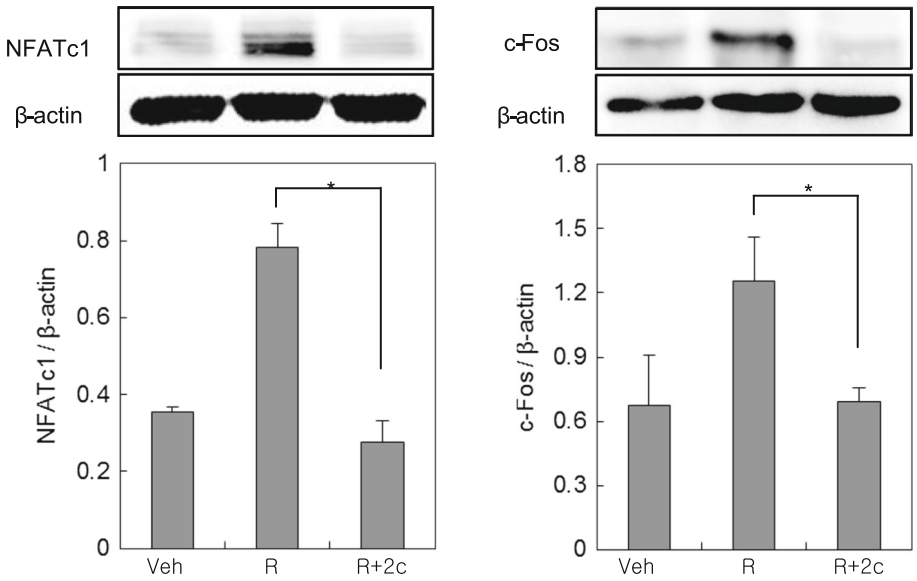


Fig. 5 The inhibitory effect of **2c** on the expression of NFATc1 and c-Fos. BMMs were pre-incubated in the absence or presence of 10 μM of **2c** for 30 min, and then treated with or without 250 ng/ml of RANKL for 24 h. Cell lysates were then subjected to Western blot analysis with the indicated antibodies. Antibodies specific for β-actin was used to normalize the cell extracts, respectively. Data are expressed as mean ± SD from at least three independent experiments. *$p < 0.05$ versus RANKL

Further study on the substituted hydroxybenzothiazoles, like CF_3, F, Cl, Br or NO_2, may be necessary to elucidate the structure activity relationship to design more potent inhibitors of osteoclast differentiation.

2c inhibits the RANKL-induced expression of osteoclastogenic marker genes and bone resorption

Since **2c** showed the most potent anti-osteoclastogenic activity, we further investigated the effect of **2c** on osteoclastogenic marker genes. Osteoclasts largely express calcitonin receptor (CTR), cathepsin K, αv-Integrin, DC-stamp, β3-Integrein, and ATP6v0d2 (Boyle et al. 2003; Suda et al. 1999; Teitelbaum and Ross 2003). Thus, they are reliable markers for the identification of osteoclastogenesis. In accordance with the anti-osteoclastogenic effect of **2c**, RANKL treatment increased the mRNA expression levels of those genes, which are dramatically suppressed by the presence of **2c** (Fig. 3).

We next examined whether the effect of **2c** on osteoclast formation is reflected on the osteoclastic activity. When we performed an in vitro resorption pit assay using a dentine slice, many resorption pits were generated in the wells with RANKL-treated cells (Fig. 4). In contrast, the treatment of **2c** strongly inhibited formation of resorption pits by the RANKL-treated cells (Fig. 4). These results suggest that **2c** exerts its inhibitory effects on osteoclast formation, which leads to reduced bone resorption.

2c Down-regulates RANKL-induced expression of c-Fos and NFATc1

The NFATc1 pathway plays a critical and fundamental role in osteoclast development, and the lack of NFATc1 arrests

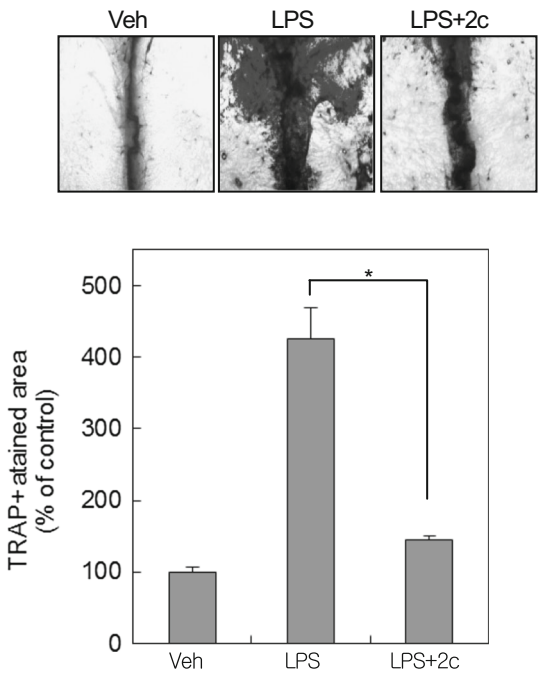


Fig. 6 The inhibitory effect of **2c** on LPS-induced osteoclast formation in vivo. Calvariaes of mice that received vehicle, LPS, or LPS plus **2c** (5 mg/kg) were subjected to TRAP staining. TRAP+ stained area in calvariae were quantified by using image J program. Data are expressed as mean ± SD from at least three independent experiments. *$p < 0.05$ versus LPS

osteoclastogenesis. Thus, we investigated the effects of **2c** on the expression level of NFATc1. RANKL stimulation increased the expression of NFATc1 and the presence of **2c** completely abolished it (Fig. 5). Since c-Fos is known to regulate the expression of NFATc1 by binding to the NFATc1 promoter (Grigoriadis et al. 1994; Asagiri and

Takayanagi 2007), we examined the effect of **2c** on RANKL-induced c-Fos expression. As previously reported, RANKL β-actin increased c-Fos expression, and **2c** treatment decreased it. These results suggest that **2c** suppresses RANKL-induced osteoclast formation via the c-Fos and NFATc1 pathways.

2c prevented LPS-induced osteoclastogenesis in vivo

We finally evaluated the in vivo effect of **2c** on osteoclast formation using a lipopolysaccharide (LPS)-challenged mouse model. LPS has been reported to stimulate bone loss by increasing the number of osteoclasts in vivo. When we injected LPS into the supra-calvarial region of mice with or without **2c**, LPS dramatically increased osteoclast numbers in the calvariae (Fig. 6). In parallel with the effects in vitro, **2c** notably reduced LPS-induced osteoclast formation (Fig. 6). Taken together, we conclude that **2c** has an inhibitory effect on osteoclast formation in vivo.

Conclusions

Nineteen benzothiazole derivatives were synthesized and evaluated for their inhibitory activity against RANKL-induced osteoclast formation. Synthesized compounds have a variously substituted phenol and benzothiazole moiety linked with C4 or C5 chain in their structure. Compounds **2c** and **4d** appeared to be most potent (IC_{50} 1.56 and 2.20 μM, respectively). The two compounds have different linker lengths, but they have a common *N, N*-diisopropyl-3-methoxybenzamide group in the structure. The most active compound **2c** prevented LPS-induced osteoclastogenesis in vivo.

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Compliance with ethical standards

Conflict of interest We declare that we have no conflict of interest.

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