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Anti-resorptive and Anabolic Activity of 3-(3,5-Dimethoxyphenyl)-6-methoxybenzofuran-4-carboxylate

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Bone remodeling is sustained by the balance between the osteoclast-mediated bone resorption and the osteoblast-mediated bone formation.[1] Old bone is resorbed by matured osteoclasts that are derived from hematopoietic stem cells of the monocyte/macrophage lineage by the receptor activator of NF-κB ligand (RANKL) and then the resorbed area is newly filled with new bone matrix that is synthesized by osteoblasts. Osteoblast-mediated bone formation is also the highly regulated and sequential process. Mesenchymal stem cells (MSCs) initially commit to pre-osteoblasts by bone morphogenetic proteins (BMPs) and subsequently those are matured into osteoblasts, the essential for bone mineralization.

However, the imbalance in bone remodeling caused by the over-activation of osteoclasts and/or the reduced activity of osteoblasts leads to the loss of bone mass that is a major cause of several bone disorders such as osteoporosis. The loss of bone mass in the elder is not directly mortal, but it can increase the risk of fractures that can threat the quality of life by serious problems such as substantial skeletal deformity, pain, increased mortality, and severe economic burden for the treatment.[2] Therefore, including the combination therapy of anabolic agents with *anti*-resorptive agent, the development of *anti*-osteoporotic small molecules with dual mode of action, decreasing bone resorption (*anti*-resorptive activity) and increasing bone formation (anabolic activity) has become a growing area of interest.[3]

Interestingly, in this study, we found that methyl 3-(3,5-dimethoxyphenyl)-6-methoxybenzofuran-4-carboxylate (**1**; Fig. 1) inhibited the RANKL-induced osteoclastogenesis and enhanced the BMP-2-induced osteoblastogenesis by using phenotype-based screening assays. Compound **1** was synthesized as one of intermediates for the total synthesis of oligostilbenoid natural products that exhibit a wide variety of pharmacological activities.[4]

Murine RAW264.7 macrophage cells used in this study have been shown to retain the ability to differentiate into multinucleated osteoclasts in the presence of RANKL. Additionally, when treated with RANKL, RAW264.7 cells express high levels of osteoclast-associated genes such as tartrate-resistant acid phosphatase (TRAP) that is widely

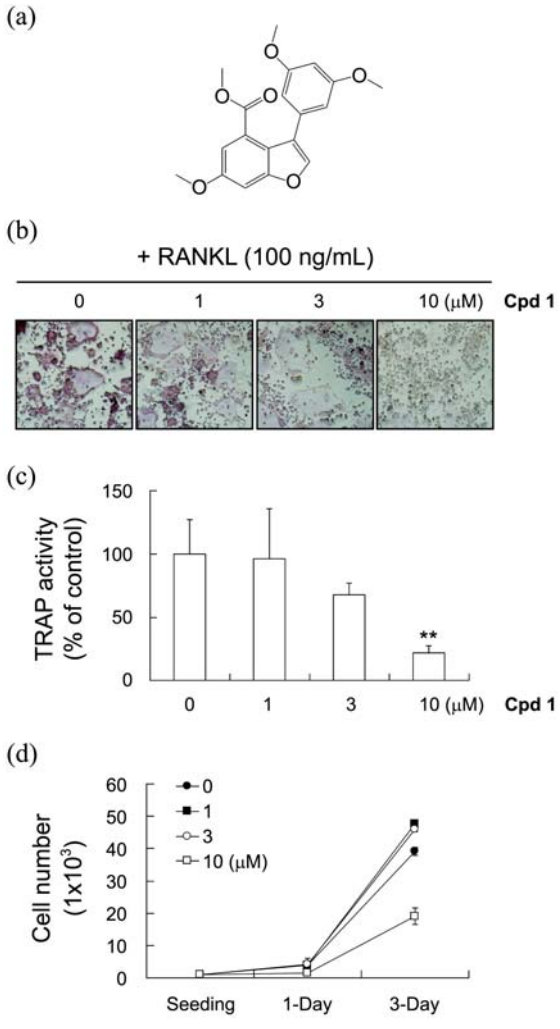

Figure 1. Inhibitory effect of **1** on RANKL-induced osteoclast differentiation in RAW 264.7 cells. (a) Structure of compound **1** (**Cpd 1**). The effects of compound **1** on RANKL-induced formation of TRAP-positive multinucleated osteoclasts (b) and TRAP activity (c) were evaluated. The effect of compound **1** on cell viability was evaluated by CCK-8 assay (d). **, $P < 0.01$

Table 1. Effect of compound **1** on RANKL-induced mRNA expression levels of osteoclastogenesis-related molecules. RAW264.7 cells were treated with compound **1** and/or RANKL, and the mRNA expression levels were evaluated by real-time PCR 1 day or 4 days after the treatment of RANKL. Fold changes relative to each gene level in the control are presented as mean ± standard deviation

RANKL (100 ng/mL)		-	-	+	+
Compound **1** (10 μM)		-	+	-	+
1-Day	TRAP	1.01 ± 0.15	1.01 ± 0.15	12.11 ± 0.59***	6.47 ± 0.73$^{\#\#}$
4-Day	DC-STAMP	1.00 ± 0.11	0.82 ± 0.01**	23.45 ± 1.41***	5.26 ± 0.31$^{\#\#\#}$
	c-Fos	1.00 ± 0.07	0.98 ± 0.11	5.02 ± 0.86**	3.26 ± 0.34$^{\#\#}$
	Fra-1	1.00 ± 0.01	0.87 ± 0.10	2.95 ± 0.22***	1.45 ± 0.06$^{\#\#}$
	Fra-2	1.03 ± 0.28	1.12 ± 0.08	20.59 ± 0.84***	8.25 ± 0.71$^{\#\#\#}$
	c-Src	1.00 ± 0.06	0.88 ± 0.07	9.91 ± 1.36***	8.72 ± 2.01
	ATP6v0d2	1.00 ± 0.11	0.90 ± 0.08	52.87 ± 2.46***	21.38 ± 1.08$^{\#\#\#}$
	Cathepsin K	1.02 ± 0.21	0.44 ± 0.02***	715.04 ± 17.52***	311.75 ± 13.62$^{\#\#\#}$

**, $P < 0.01$; ***, $P < 0.001$ (compared to the control); $^{\#\#}$, $P < 0.01$; $^{\#\#\#}$, $P < 0.001$ (compared to the RANKL-treated group)

used to assess bone resorption.[5] Therefore, the screening based on the RANKL-induced formation of TRAP-positive multinucleated osteoclasts by using RAW264.7 cells was applied in this study. As shown in Fig. 1(b), compound **1** inhibited the RANKL-induced formation of TRAP-positive multinucleated osteoclasts. Additionally, at 10 μM, it significantly inhibited the RANKL-induced activity of TRAP (Fig. 1(c)). To ascertain that the inhibitory effect of compound **1** on the RANKL-induced osteoclastogenesis was not due to its cytotoxicity *per se*, its effect on cell viability was evaluated. Compound **1** attenuated the rate of growth at 10 μM, but it did not show any cytotoxicity in RAW264.7 cells at the concentrations (up to 10 μM) used in this study (Fig. 1(d)). This was also confirmed by caspase-3 activity assay; there was no compound **1**-induced activation of caspase-3 indicating apoptotic cell death (data not shown).

The *anti*-resorptive activity of compound **1** was also evaluated by measuring the mRNA levels of osteoclastogenesis-related molecules. Compound **1** did not inhibit the mRNA expression of TRAP, but consistently with the results described above, the RANKL-induced mRNA expression of TRAP was significantly inhibited by compound **1** (Table 1). Also, on the differentiation day 4, the RANKL-induced mRNA expressions of osteoclastogenesis-related transcription factors such as AP-family members and molecules related with cell fusion such as DC-STAMP were significantly inhibited by compound **1**. Under no differentiation condition (or in the absence of RANKL), the incubation of compound **1** in RAW264.7 cells for 4 days also inhibited the mRNA levels of DC-STAMP and cathepsin K. Among AP-1 family member, c-Fos, Fra-1 and Fra-2 have been suggested to be functionally linked to the process of osteoclastogenesis.[6] DC-STAMP (dendrite cells-specific transmembrane protein) was also reported to be essential for cell fusion in the process of osteoclastogenesis by showing the complete abrogation of osteoclast cell fusion in its knock-out mice.[7] With the exception of c-Src, the mRNA expression levels of molecules related with bone resorption such as ATP6v0d2 and cathepsin K were significantly induced by RANKL, but those inductions were dramatically inhibited by compound **1** (Table 1). ATP6v0d2 is highly expressed in mature osteoclasts and it has been reported to be a key component of the osteoclast-specific proton pump mediating extracellular acidification during bone resorption.[8] Cathepsin K is also highly expressed in osteoclasts and has been well-known to play a critical role in osteoclastic bone resorption.[9]

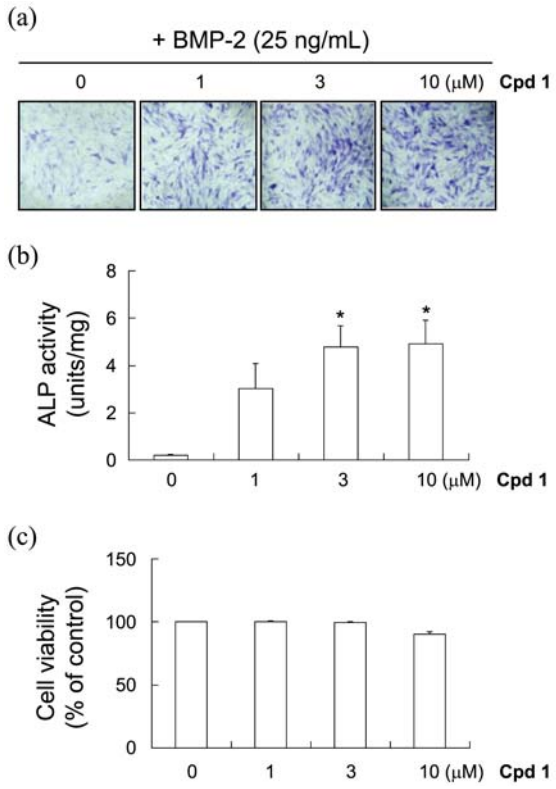

Figure 2. Enhancing effect of compound **1** on BMP-2-induced osteoblast differentiation in C2C12 cells. The effect of compound **1** on ALP induction was evaluated by its staining (a) and activity assay (b). The effect of compound **1** on cell viability was evaluated by CCK-8 assay (c). *, $P < 0.05$

Additionally, compound **1** significantly enhanced the BMP-2-mediated osteoblastogenesis in a dose-dependent manner; its addition enhanced the BMP-2-mediated induction of alkaline phosphatase (ALP, a biomarker of osteoblastogenesis) in murine bi-potential mesenchymal precursor

Table 2. Effect of compound **1** on BMP-2-induced mRNA expression levels of osteoblastogenesis-related molecules. C2C12 cells were treated with BMP-2 and/or compound **1** for 6 days and then the mRNA levels were evaluated by real-time PCR. Fold changes relative to each gene level in the control are presented as mean ± standard deviation

BMP-2 (100 ng/mL)	−	−	+	+
Compound **1** (10 μM)	−	+	−	+
ALP	1.17 ± 0.69	2.03 ± 0.25**	328.18 ± 1.61***	2670.53 ± 300.41##
Msx2	1.00 ± 0.07	1.72 ± 0.03*	1.45 ± 0.16*	2.65 ± 0.15##
Runx2	1.00 ± 0.05	1.22 ± 0.20	2.17 ± 0.21***	2.48 ± 0.22
BMP-4	1.01 ± 0.19	1.62 ± 0.04*	1.76 ± 0.30*	2.50 ± 0.14#

*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (compared to the control); #, $P < 0.05$; ##, $P < 0.01$ (compared to the BMP-2-treated group)

C2C12 cells (Fig. 2(a), 2(b) and Table 2).[10] Compound **1** did not affect the viability of C2C12 cells (Fig. 2(c)).

Since the expression of the early osteogenic factors such as Msx2 (msh homeobox 2), Runx2 (Runt-related transcription factor 2) and BMP-4 as well as the late osteogenic markers such as ALP is important in the BMP-2-induced differentiation of C2C12 into pre-osteoblasts, we further confirmed the anabolic activity of compound **1** by measuring their mRNA expression levels. As shown in Table 2, the mRNA levels of Msx2 and BMP-4 were significantly induced by compound **1** or BMP-2, but those was shown to be additively increased by both. The mRNA expression of Runx2 was significantly induced by BMP-2, but the addition of compound **1** did not enhance the BMP-2-induced mRNA expression of Runx2. The functional role of Msx2 in osteoblastogenesis is still controversial, but several studies have suggested that the temporal and spatial regulation of Msx2 plays critical roles in bone formation.[11] BMP-4 is also osteogenic and the induction of endogenous BMP-4 during BMP-2-induced bone formation has been reported in several studies.[12]

In conclusion, compound **1** exhibited the dual mode of action in bone remodeling, decreasing bone resorption (*anti*-resorptive activity) and increasing bone formation (anabolic activity), suggesting that the chemical structure of compound **1** might be useful to design *anti*-osteoporotic agent with dual activity.

Experimental Section

Materials. RANKL and BMP-2 were purchased from R&D Systems (MN) and PeproTech (Seoul, Korea), respectively. All materials for cell culture were purchased from HyClone (UT, USA).

Osteoclast Differentiation. RAW264.7 cells were purchased from American Type Culture Collection (VA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin with a change of medium every 3 days in a humidified atmosphere of 5% CO_2 at 37 °C. For osteoclast differentiation, RAW264.7 cells were plated in a 96-well plate at the density of 1×10^3 cells/well and cultured in α-minimal essential medium (MEM) supplemented with 10% FBS in the presence of 100 ng/mL RANKL. The following day, serially diluted compound **1** was added into cells.

TRAP Staining and its Activity Assay. On the differentiation day 4, multinucleated osteoclasts were visualized by TRAP staining using a leukocyte acid phosphatase kit 387-A (Sigma, MO). For TRAP activity assay, the multinucleated cells were fixed with 10% formalin for 10 min and 95% ethanol for 1 min, and then dried. To measure TRAP activity, 100 μL of citrate buffer (50 mM, pH 4.6) containing 10 mM sodium tartrate and 5 mM *p*-nitrophenylphosphate (Sigma) was added to the dried cell-containing wells of 96-well plates. After incubation for 1 hr, the enzyme reaction mixtures were transferred into the well of fresh plates containing an equal volume of 0.1 N NaOH. Absorption was measured at 410 nm with Wallac EnVision HTS microplate reader (PerkinElmer, Finland).

Osteoblast Differentiation. C2C12 cells were maintained in DMEM containing 10% FBS, 100 U/mL of penicillin, and 100 μg/mL streptomycin. Cells were seeded and after 1 day, cells were differentiated by replacing the medium with DMEM containing 5% FBS and rhBMP-2 (100 ng/mL). The medium was changed every 3 days.

ALP Staining and Activity Assay. Cells (4×10^3 cells/well) were plated in a 96-well plate. The next day, compound **1** was co-treated with BMP-2 (100 ng/mL) on the differentiation day 0. On the differentiation day 2, cells were washed twice with PBS, fixed with 10% formalin in PBS for 30 sec, rinsed with deionized water, and stained using the Alkaline Phosphatase (ALP) Kit (Sigma) under protection from direct light. Images of stained cells were captured under a microscope equipped with a DP70 digital camera (Olympus Optical, Tokyo, Japan). To measure ALP activity, cells were washed twice with PBS and homogenized in the ice-cold lysis buffer (10 mM of Tris-HCl, pH 7.5, 0.5 mM of $MgCl_2$, and 0.1% Triton X-100). After vortexing for 20 min at room temperature, ALP activity was measured in triplicate using the LabAssay ALP Kit (Wako Pure Chemicals Industries, Osaka, Japan).

Cell Viability Assay. RAW264.7 cells were suspended in α-MEM with 10% FBS and plated in 96-well plates at a density of 1×10^3 cells/well. After 24h, cells were treated with compound **1** and incubated for 1 or 3 days. Cell viability was then measured with the Cell Counting kit-8 according to the manufacturer's protocol. Measured absorbance was converted to cell number with a standard curve. C2C12 cells were seeded in a 96-well plate at 4×10^3

Table 3. Primer sequences used in this study

Target gene	Forward (5'-3')	Reverse (5'-3')
ALP	ATGGGCGTCTCCACAGTAAC	TCACCCGAGTGGTAGTCACA
Msx2	GTCATGGCTTCTCCGACTAA	ATTTTCCGACTTGACCGAGG
Runx2	GCCGGGAATGATGAGAACTA	GGACCGTCCACTGTCACTTT
BMP-4	CCTGGTAACCGAATGCTGAT	AGCCGGTAAAGATCCCTCAT
TRAP	CGACCAGTACGCAGTTCCTC	AACTGCTCAGGTTGGCGTAG
DC-STAMP	CCAAGGAGTCGTCCATGATT	GGCTGCTTTGATCGTTTCTC
c-Fos	CCAGTCAAGAGCATCAGCAA	AAGTAGTGCAGCCCGGAGTA
Fra-1	AGAGCTGCAGAAGCAGAAGG	CAAGTACGGGTCCTGGAGAA
Fra-2	ATCCACGCTCACATCCCTAC	GTTTCTCTCCCTCCGGATTC
c-Src	CCAGGCTGAGGAGTGGTACT	CAGCTTGCGGATCTTGTAGT
ATP6v0d2	AGACCACGGACTATGGCAAC	CGATGGGTGACACTTGGCTA
Cathepsin K	GGCCAACTCAAGAAGAAAAC	GTGCTTGCTTCCCTTCTGG
GAPDH	aactttggcattgtggaagg	acacattgggggtaggaaca

cells/well. After 24 h, cells were incubated with compound **1** for 3 days. Cell growth was then evaluated in triplicate using Cell Counting Kit-8.

Evaluation of mRNA Expression Level. Real-time PCR was performed as described in a previous study.[13] Used primers were presented in Table 3.

Statistical Analysis. Results were expressed as means ± standard error of three independent experiments. Significance was determined using the Student's *t*-test and differences were considered significant when $P < 0.05$.

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References

1. (a) Boyle, W. J.; Simonet, W. S.; Lacey, D. L. *Nature* **2003**, *423*, 337. (b) Harada, S.; Rodan, G. A. *Nature* **2003**, *423*, 349. (c) Fuller, K.; Wong, B.; Fox, S.; Choi, Y.; Chambers, T. J. *J. Exp. Med.* **1998**, *188*, 997. (d) Cheng, H.; Jiang, W.; Phillips, F. M.; Haydon, R. C.; Peng, Y.; Zhou, L.; Luu, H. H.; An, N.; Breyer, B.; Vanichakarn, P.; Szatkowski, J. P.; Park, J. Y.; He, T. C. *J. Bone Joint Surg. Am.* **2003**, *85-A*, 1544.
2. Harvey, N.; Dennison, E.; Cooper, C. *Nat. Rev. Rheumatol*. **2010**, *6*, 99.
3. (a) Vajda, E. G.; Hogue, A.; Griffiths, K. N.; Chang, W. Y.; Burnett, K.; Chen, Y.; Marschke, K.; Mais, D. E.; Pedram, B.; Shen, Y.; van Oeveren, A.; Zhi, L.; López, F. J.; Meglasson, M. D. *J. Bone Miner. Res*. **2009**, *24*, 231. (b) Arrington, S. A.; Fisher, E. R.; Willick, G. E.; Mann, K. A.; Allen, M. J. *Calcif. Tissue Int.* **2010**, *87*, 263. (c) Reginster, J. Y.; Sarlet, N.; Lejeune, E.; Leonori, L. *Curr. Osteoporos. Rep.* **2005**, *3*, 30. (d) Xie, Y.; Ding, H.; Qian, L.; Yan, X.; Yang, C.; Xie, Y. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 3267.
4. (a) Kim, I.; Choi, J. *Org. Biomol. Chem.* **2009**, *7*, 2788. (b) Kim, K.; Kim, I. *Org. Lett.* **2011**, *12*, 5314.
5. (a) Igarashi, Y.; Lee, M. Y.; Matsuzaki, S. *Biomed. Life Sci*. **2002**, *781*, 345. (b) Kim, M. H.; Ryu, S. Y.; Bae, M. A.; Choi, J. S.; Min, Y. K.; Kim, S. H. *Food Chem. Toxicol*. **2008**, *46*, 3375.
6. (a) Johnson, R. S.; Spiegelman, B. M.; Papaioannou, V. *Cell* **1992**, *71*, 577. (b) Wang, Z. Q.; Ovitt, C.; Grigoriadis, A. E.; Mohle-Steinlein, U.; Ruther, U.; Wagner, E. F. *Nature* **1992**, *360*, 741. (c) Matsuo, K.; Owens, J. M.; Tonko, M.; Elliott, C.; Chambers, T. J.; Wagner, E. F. *Nat. Genet*. **2000**, *24*, 184. (d) Eferl, R.; Hoebertz, A.; Schilling, A. F.; Rath, M.; Karreth, F.; Kenner, L.; Amling, M.; Wagner, E. F. *EMBO J.* **2004**, *23*, 2789.
7. (a) Kukita, T.; Wada, N.; Kukita, A.; Kakimoto, T.; Sandra, F.; Toh, K.; Nagata, K.; Iijima, T.; Horiuchi, M.; Matsusaki, H.; Hieshima, K.; Yoshie, O.; Nomiyama, H. *J. Exp. Med*. **2004**, *200*, 941. (b) Yagi, M.; Miyamoto, T.; Sawatani, Y.; Iwamoto, K.; Hosogane, N.; Fujita, N.; Morita, K., Ninomiya, K.; Suzuki, T.; Miyamoto, K.; Oike, Y.; Takeya, M.; Toyama, Y.; Suda, T. *J. Exp. Med*. **2005**, *202*, 345.
8. Wu, H.; Xu, G.; Li, Y. P. *J. Bone Miner. Res*. **2009**, *24*, 871.
9. (a) Gelb, B. D.; Shi, G. P.; Chapman, H. A.; Desnick, R. J. *Science* **1996**, *273*, 1236. (b) Ishikawa, T.; Kamiyama, M.; Tani-Ishii, N.; Suzuki, H.; Ichikawa, Y.; Hamaguchi, Y.; Moniyama, N.; Shimada, H. *Mol. Carcinog*. **2001**, *32*, 84. (c) Reinheckel, T.; Deussing, J.; Roth, W.; Peters, C. *Biol. Chem*. **2001**, *382*, 735.
10. (a) Collin-Osdoby, P.; Yu, X.; Zheng, H.; Osdoby, P. *Methods Mol. Med.* **2003**, *80*, 153. (b) Katagiri, T.; Yamaguchi, A.; Komaki, M.; Abe, E.; Takahashi, N.; Ikeda, T.; Rosen, V.; Wozney, J. M. *J. Cell Biol*. **1994**, *127*, 1755.
11. (a) Satokata, I.; Ma, L.; Ohshima, H.; Bei, M.; Woo, I.; Nishizawa, K.; Maeda, T.; Takano, Y.; Uchiyama, M.; Heaney, S.; Peters, H.; Tang, Z.; Maxson, R.; Maas, R. *Nat. Genet.* **2000**, *24*, 391. (b) Wikie, A. O.; Tang, Z.; Elanko, N.; Walsh, S.; Twigg, S. R.; Hurst, J. A.; Wall, S. A.; Chrzanowska, K. H.; Maxson, R. E. Jr. *Nat. Genet*. **2000**, *24*, 387.
12. (a) Chen, D.; Harris, M. A.; Rossini, G.; Dunstan, C. R.; Dallas, S. L.; Feng, J. Q.; Mundy, G. R.; Harris, S. E. *Calcif. Tissue Int.* **1997**, *60*, 283. (b) Kawai, M.; Bessho, K.; Maruyama, H.; Miyazaki, J.; Yamamoto, T. *Musculoskelet. Disord*. **2006**, *7*, 62. (c) Bessa, P. C.; Cerqueira, M. T.; Rada, T.; Gomes, M. E.; Neves, N. M.; Nobre, A.; Reis, R. L.; Casal, M. *Protein Expr. Purif*. **2009**, *63*, 89.
13. (a) Kim, M. H.; Ryu, S. Y.; Choi, J. S.; Min, Y. K.; Kim, S. H. *J. Cell. Physiol*. **2009**, *221*, 618. (b) Kim, H. J.; Kim, S. H. *Amino Acids* **2010**, *39*, 1217.