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Synthesis of novel chemical probes for the study of tanshinone binding proteins

Jin-Soo Lee,[a,b] Sun-Young Han,[a] Myong Sang Kim,[a] Chan-Mo Yu,[b] Myung Hee Kim,[a] Seong Hwan Kim,[a] Yong Ki Min[a,*] and Bum Tae Kim[a]

[a]*Laboratory of Chemical Genomics, Bio-Organic Science Division, Korea Research Institute of Chemical Technology, Deajeon 305-600, Republic of Korea*
[b]*Department of Chemistry, Sungkyunkwan University, 300 Cheoncheon-dong, Jangan-gu, Suwon, Gyeonggido 440-746, Republic of Korea*

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Abstract—Novel diazirine or biotin-labeled tanshinone probes were synthesized and evaluated for TRAP inhibitory activity against RANKL-induced osteoclastogenesis in RAW264.7 cells. We found that diazirine-labeled derivatives (**18** and **20**) are potent inhibitors of RANKL-induced osteoclastogenesis. IC_{50} values were 18.02 and 15.00 μM, respectively. These probes will be useful reagents for investigating tanshinone–proteins interactions.

Tanshinones are natural products isolated from *Salvia miltiorrhiza Bunge*, a traditional Chinese medicinal herb, by Nakao and Fukushima in 1934.[1] The major components of tanshinones are tanshinone IIA (**1**), 3-hydroxytanshinone (**2**), tanshinone IIB (**3**), and cryptotanshinone (**4**) (Fig. 1). These compounds have been observed to possess various pharmacological activities including antibacterial, antidermatophytic, antioxidant, anti-inflammatory, antineoplastic, and antiplatelet aggregation activities.[2–9] Recently, tanshinones have been reported to inhibit the osteoclast differentiation and bone resorption by blocking RANKL/RANK signaling pathway, which indicates that tanshinones can be used for the treatment and prevention of osteoporosis and other bone-resorptive diseases.[10,11] Several inhibitors that include estrogens, selective estrogen-receptor modulators (SERMs), and bisphosphonates have been used for the regulation of osteoclast generation and function. However, the effects of these compounds in bone biology are still under investigation.[12] Despite various biological activities, the mechanism of action of tanshinones remains unknown. This led to the design of chemical probes for the study of tanshinone binding proteins, which can provide the useful direct probing method for defining target proteins. Here, we investigated the synthesis of tanshinone derivatives with a photoactive moiety or a biotin group at the A-ring of tanshinone.

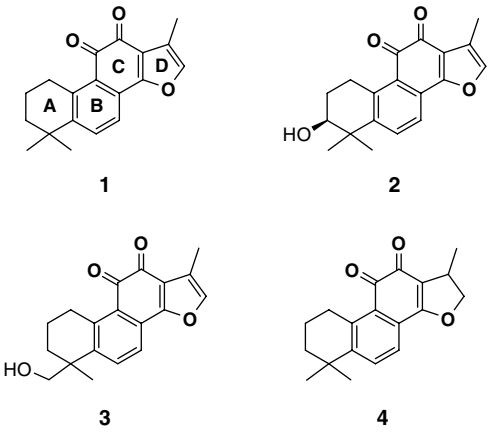

Figure 1. Chemical structures of tanshinone IIA (**1**), 3-hydroxytanshinone (**2**), tanshinone IIB (**3**), and cryptotanshinone (**4**).

Trifluoromethylaryldiazirine was selected as a photophore due to its many useful features; (a) chemical stability in various reaction conditions that may facilitate

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* Corresponding author. Tel.: +82 42 860 7029; fax: +82 42 861 0307; e-mail: ykmin@krict.re.kr

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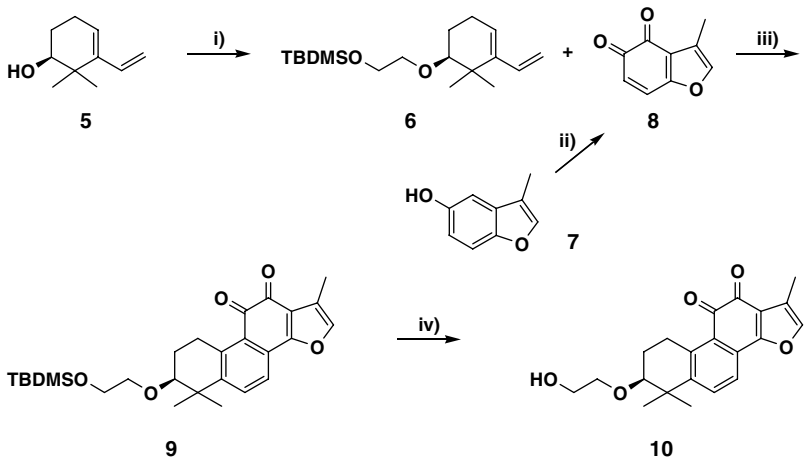
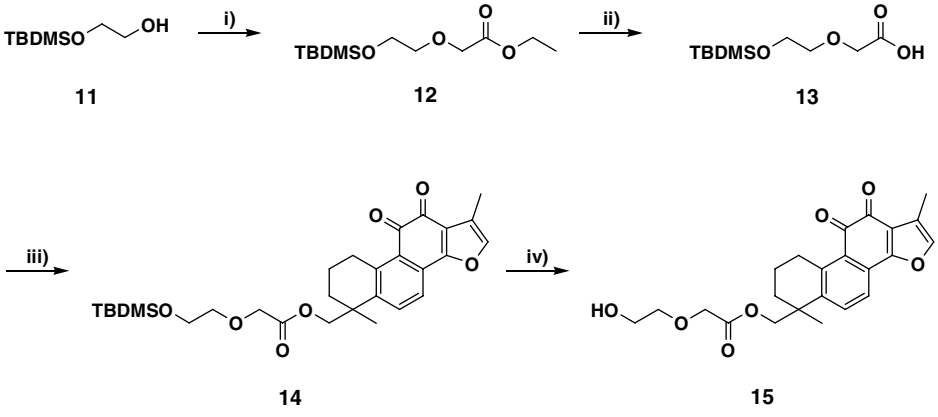
the use of a diazirine group as a carbene precursor; (b) photoactivatability under long-wavelength UV light (wavelength 350–360 nm) that minimize damage to proteins; (c) generation of highly reactive carbene that react with virtually proteins with no intramolecular rearrangements.[13] Biotin labeling is a powerful technique for nonradioactive detection of proteins based on the avidin affinity chromatography because the biotin–avidin interaction is the strongest noncovalent, biological interaction ($K_d = 10^{-15}$ M) between protein and ligand.[14] These methods are especially useful for the identification of ligand-binding sites of target proteins and the investigation of ligand–receptor interactions.[15]

The natural products tanshinones **2** and **3** were prepared as described previously.[16] 3-Hydroxytanshinone (**2**) was synthesized from (*S*)-3-hydroxy-2,2-dimethylcyclohexanone by enantioselective reduction of 2,2-dimethylcyclohexan-1,3-dione with Baker's yeast. Tanshinone IIB (**3**) was prepared from ethyl 2-methyl-1-oxocyclohexane-2-carboxylate as a racemate. Based on the results from previous structure–activity relationships (SAR) study,[17] we synthesized tanshinone derivatives with a biotin or a photoactive moiety at the hydroxyl group of the A-ring of tanshinone. The side chain at 3-hydroxyl group of tanshinone (**2**) was introduced by the reaction of (*S*)-5-hydroxy-6,6-dimethyl-1-vinylcyclohexene (**5**) with $TBDMSOCH_2CH_2Br$ under microwave irradiation at 120 °C for 10 min (Scheme 1). The diene **5** was synthesized from 2,2-dimethyl-1,3-dicyclohexanone, as described in the literature.[16a] The *tert*-butyldimethylsilyl (TBDMS) ether **9** was obtained by the ultrasound-promoted Diels–Alder reaction of diene **6** with *o*-quinone **8**, which was synthesized by the oxidation of **7** using Fremy's salt, followed by the aromatization with DDQ. Finally, the key intermediate **10** was prepared by the deprotection of TBDMS ether **9** using 48% hydrogen fluoride in THF in moderate yield. As illustrated in Scheme 2, we introduced a side chain at hydroxyl group of tanshinone IIB (**3**). Ethyl ester **12** was obtained by the reaction of TBDMS-protected ethylene glycol **11** with ethyl bromoacetate under microwave irradiation. Compound **13** was prepared by the saponification of compound **12** under 1 N NaOH/MeOH condition. The key intermediate **15** was synthesized by the condensation of tanshinone IIB (**3**) and acid



Scheme 1. Reagents and conditions: (i) NaH, $TBDMSOCH_2CH_2Br$, THF, microwave, 120 °C, 10 min, 50%; (ii) Fremy's salt, CH_3OH, 0.07 M KH_2PO_4 (pH 7.0); (iii) a—ultrasound, CH_3OH, 35 °C, 1 h, b—DDQ, benzene, reflux, overnight, 29% in three steps; (iv) 48% HF/THF (1:1), rt, 1 h, 68%.



Scheme 2. Reagents and conditions: (i) NaH, ethyl bromoacetate, THF, microwave, 120 °C, 10 min, 40%; (ii) 1 N NaOH, CH_3OH, rt, 30 min, 67%; (iii) **3**, DCC, DMAP, CH_2Cl_2, rt, overnight, 94%; (iv) 48% HF/THF (1:1), rt, 10 min, 98%.

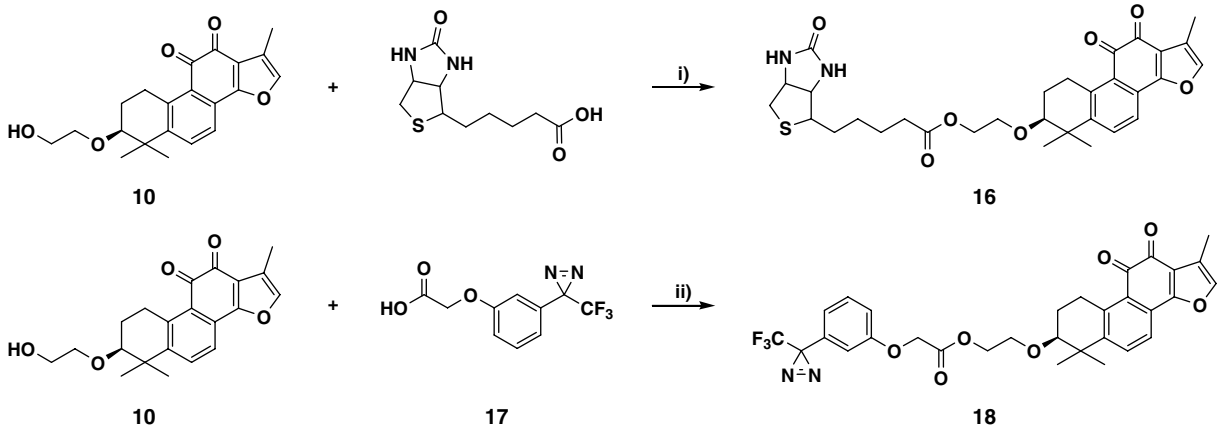
13 in the presence of *N*,*N*-dicyclohexycarbodiimide (DCC) in CH_2Cl_2/DMF (4:1) followed by the deprotection of silyl ether **14** in a solution of 48% HF/THF (1:1, v/v) in good yield. Finally, 3-hydroxytanshinone derivatives (**16** and **18**) were prepared by the condensation of tanshinone derivative **10** with (+)-biotin or 3-(trifluoromethyl)-3*H*-diazirine derivative (**17**)[18] in the presence of DCC in good yields (Scheme 3).[19] Tanshinone IIB derivatives (**19** and **20**) were also obtained from intermediate **15** by the coupling reaction using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDCI) as shown in Scheme 4.[19]

To estimate whether biotin- or diazirine-tagged tanshinone derivatives possess potent biological activity similar to the natural products (**2** and **3**), we determined their inhibitory effects on TRAP (tartrate resistant acid phosphatase; a biomarker of osteoclastogenesis) activity in RANKL-induced osteoclastogenesis in RAW264.7 cells.[20] All compounds (**16** and **18**–**20**) were found to inhibit the TRAP activity as shown in Table 1 and Figure 2. 3-Hydroxytanshinone (**2**) and tanshinone IIB (**3**) inhibited the TRAP activity in RANKL-induced osteoclastogenesis with IC_{50} values of 12.85 and 15.55 μM, respectively. Biotin-tagged derivatives (**16** and **19**) inhibited the TRAP activity, whereas they attenuated the proliferation of RAW264.7 cells with IC_{50} values of 18.72 and 18.34 μM, respectively.[21] It did not mean the cytotoxicity of **16** or **19**, since the number of cells cultured with each compound for 4 day was absolutely higher than that (4000 cells/well) in cell seeding step. On the other hand, diazirine-tagged derivatives (**18** and **20**) were found to be approximately equipotent

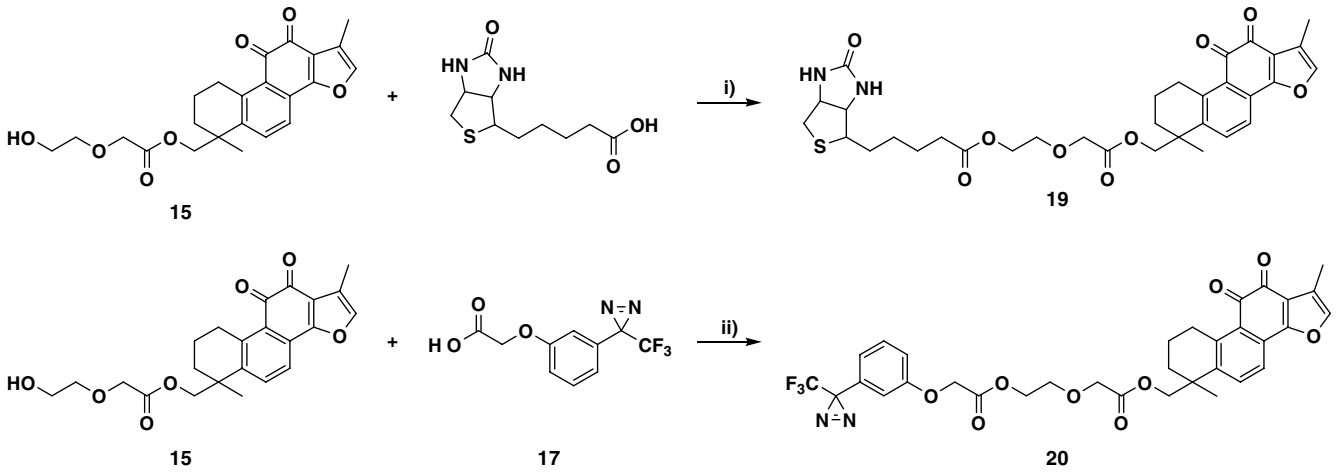
Table 1. The effect of tanshinone derivatives on the cell proliferation and TRAP activity in RANKL-induced osteoclastogenesis in RAW264.7 cells

Compound	Cell proliferation (IC_{50}, μM)[a]	TRAP activity (IC_{50}, μM)[a]
2	>20	12.85
16	18.72	9.56
18	>20	18.02
3	>20	15.55
19	18.34	12.37
20	>20	15.00

[a] IC_{50} values were calculated by using mean values presented as % of control.



Scheme 3. Reagents and conditions: (i) DCC, DMAP, CH_2Cl_2/DMF (1:1), ultrasound, 40 °C, 2 h, 84%; (ii) DCC, DMAP, DMF, rt, overnight, 73%.



Scheme 4. Reagents and conditions: (i) EDCI, DMAP, DMF, rt, overnight, 40%; (ii) EDCI, DMAP, DMF, rt, 3 h, 28%.

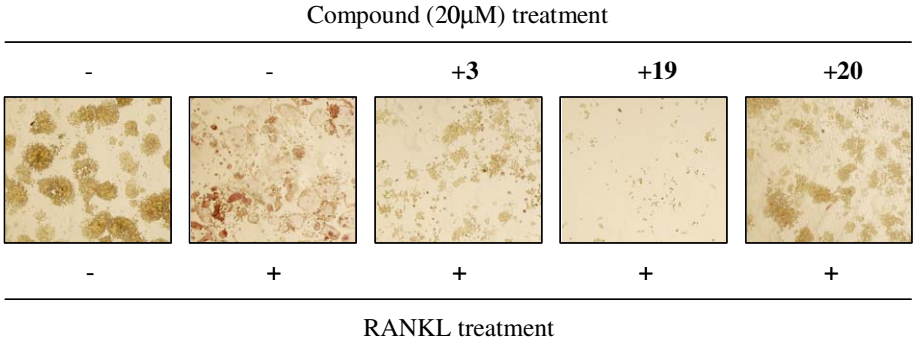


Figure 2. The effect of tanshinone derivatives on the osteoclast formation.

inhibitors of TRAP activity compared to mother compounds (**2** and **3**), with IC_{50} values of 18.02 and 15.00 μM, respectively. We determined that the applied doses of inhibitors (**18** and **20**) did not have any effect on cell proliferation. The results show that diazirine-tagged probes functionalized at the A-ring of tanshinone can maintain the biological activities of the natural products (**2** and **3**) and can be powerful photoaffinity reagents to fish out the target protein(s), which is involved in the cellular process of osteoclastogenesis.

In summary, we synthesized novel tanshinone probes (**16** and **18**–**20**) and investigated inhibitory effects on TRAP activity in RANKL-induced osteoclastogenesis in RAW264.7 cells. Diazirine-labeled derivatives (**18** and **20**) are potent inhibitors of RANKL-induced osteoclastogenesis. Despite the inhibitory effect of biotinylated tanshinones (**16** and **19**) on the rate of cell proliferation, it can still be valuable bioprobes for investigating ligand–protein interactions. Further studies for the identification of target proteins using these probes are now in progress and results will be published in due course.

Acknowledgments

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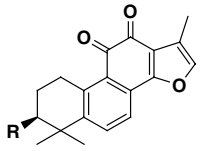
References and notes

1. Nakao, M.; Fukushima, T. *J. Pharm. Soc. Jpn.* **1934**, *54*, 154.
2. Honda, G.; Koezuka, Y.; Tabata, M. *Chem. Pharm. Bull.* **1988**, *36*, 408.
3. Gao, Y. G.; Song, Y. M.; Yang, Y. Y.; Liu, W. F.; Tang, J. X. *Acta Pharmacol. Sin.* **1979**, *14*, 75.
4. Houlihan, C. M.; Ho, C. T.; Chang, S. S. *J. Am. Oil Chem. Soc.* **1985**, *62*, 96.
5. Wu, W. L.; Chang, W. L.; Lee, A. R.; Lin, H. C.; King, M. L. *J. Med. Sci.* **1985**, *6*, 159.
6. Onitsuka, M.; Fujiu, M.; Shinma, N.; Maruyama, H. B. *Chem. Pharm. Bull.* **1983**, *31*, 1670.
7. Lee, A. R.; Wu, W. L.; Chang, W. L.; Lin, H. C.; King, M. L. *J. Nat. Prod.* **1987**, *50*, 157.
8. Luo, H. W.; Hu, X. J.; Wang, N.; Ji, J. *Acta Pharmacol. Sin.* **1988**, *23*, 830.
9. Fang, C. N.; Chang, P. L.; Hsu, T. P. *Acta Chim. Sin.* **1976**, *34*, 197.
10. Kim, H. H.; Kim, J. H.; Kwak, H. B.; Huang, H.; Han, S. H.; Ha, H.; Lee, S. W.; Woo, E.; Lee, Z. H. *Biochem. Pharmacol.* **2004**, *67*, 1647.
11. Lee, S. Y.; Choi, D. Y.; Woo, E. R. *Arch. Pharmacol. Res.* **2005**, *28*, 909.
12. (a) Rodan, G. A.; Martin, T. J. *Science* **2000**, *289*, 1508; (b) Boyle, W. J.; Simonet, W. S.; Lacey, D. L. *Nature* **2003**, *423*, 337; (c) Harada, S.-I.; Rodan, G. A. *Nature* **2003**, *423*, 349.
13. (a) Hatanaka, Y.; Sadakane, Y. *Curr. Top. Med. Chem.* **2002**, *2*, 271; (b) Dorman, G.; Prestwich, G. D. *Trends Biotechnol.* **2000**, *18*, 64; (c) Kotzyba-Hibert, F.; Kapfer, I.; Goeldner, M. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1296; (d) Brunner, J. *Annu. Rev. Biochem.* **1993**, *62*, 483; (e) Bayley, H. In *Photogenerated Reagents in Biochemistry and Molecular Biology*; Work, T., Burdin, R., Eds.; Elsevier: Amsterdam, 1983.
14. (a) Mandal, A. K.; Hines, J.; Kuramochi, K.; Crews, C. M. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4043; (b) Takakusagi, Y.; Ohta, K.; Kuramochi, K.; Morohashi, K.; Kobayashi, S.; Sakaguchi, K.; Sugawara, F. *Bioorg. Med. Chem. Lett.* **2005**, *15*, 4846; (c) van der Veken, P.; Dirksen, E. H. C.; Ruijter, E.; Elgersma, R. C.; Heck, A. J. R.; Rijkers, D. T. S.; Slijper, M.; Liskamp, R. M. J. *ChemBioChem* **2005**, *6*, 2271; (d) Yee, M.-C.; Fas, S. C.; Stohlmeyer, M. M.; Wandless, T. J.; Cimprich, K. A. *J. Biol. Chem.* **2005**, *280*, 29053; (e) Hussey, S. L.; Muddana, S. S.; Peterson, B. R. *J. Am. Chem. Soc.* **2003**, *125*, 3692; (f) Savage, M. D.; Mattson, G.; Desai, S.; Nielander, G. W.; Morgensen, S.; Conklin, E. J. *Avidin–Biotin Chemistry A Handbook*; Pierce Chemical Co: Illinois, 1992; (g) Green, N. M. *Adv. Protein Chem.* **1975**, *29*, 85.
15. (a) Fillion, D.; Deraet, M.; Holleran, B. J.; Escher, E. *J. Med. Chem.* **2006**, *49*, 2200; (b) Riber, D.; Venkataramana, M.; Sanyal, S.; Duvold, T. *J. Med. Chem.* **2006**, *49*, 1503; (c) Hashimoto, M.; Hatanaka, Y. *Anal. Biochem.* **2006**, *348*, 154; (d) Han, S.-Y.; Choi, S. H.; Kim, M. H.; Lee, W. G.; Kim, S. H.; Min, Y. K.; Kim, B. T. *Tetrahedron Lett.* **2006**, *47*, 2915; (e) Han, S.-Y.; Park, S.-S.; Lee, W. G.; Min, Y. K.; Kim, B. T. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 129; (f) Park, J.-J.; Sadakane, Y.; Masuda, K.; Tomohiro, T.; Nakano, T.; Hatanaka, Y. *ChemBioChem* **2005**, *6*, 814.
16. (a) Zhang, J.; Duan, W.; Cai, J. *Tetrahedron* **2004**, *60*, 1665; (b) Haiza, M.; Lee, J.; Snyder, J. K. *J. Org. Chem.* **1990**, *55*, 5008; (c) Lee, J.; Snyder, J. K. *J. Org. Chem.*

1990, *55*, 4995; (d) Lee, J.; Snyder, J. K. *J. Am. Chem. Soc.* **1989**, *111*, 1522.

17. Tanshinone IIA (**1**) analogues were synthesized and evaluated for the TRAP activity and cell proliferation of RAW264.7 cells in RANKL-induced osteoclastogenesis. The applied doses of inhibitors (**1**, **2**, and **2a**–**c**) did not have any effect on cell proliferation.



1 R=H
2 R=OH
2a R=$CH_3CH_2CO_2$
2b R=$PhCO_2$
2c R=$HOCH_2CH_2O$

Compound	TRAP activity (IC_{50}, μM)[a]
1	18.17
2	12.85
2a	16.68
2b	19.13
2c	10.24

[a]IC_{50} values were calculated by using mean values presented as % of control.

18. Hatanaka, Y.; Hashimoto, M.; Kurihara, H.; Nakayama, H.; Kanoka, Y. *J. Org. Chem.* **1994**, *59*, 383.
19. **Compound 16:** ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.32 (s, 3H), 1.34 (s, 3H), 1.42–1.47 (m, 2H), 1.63–1.77 (m, 4H), 1.97–2.00 (m, 2H), 2.25 (s, 3H), 2.34 (t, J = 7.6 Hz, 2H), 2.74 (d, J = 12.7 Hz, 1H), 2.99 (dd, J = 12.7, 5.0 Hz, 1H), 3.13–3.24 (m, 2H), 3.28–3.35 (m, 2H), 3.61–3.65 (m, 1H), 3.83–3.87 (m, 1H), 4.21 (m, 2H), 4.34 (m, 1H), 4.50 (m, 1H), 5.41 (br s, 1H), 5.85 (br s, 1H), 7.22 (s, 1H), 7.55 (d, J = 8.2 Hz, 1H), 7.61 (d, J = 8.2 Hz, 1H). HRMS-FAB (*m*/*z*) $[M+H]^+$ calcd for $C_{31}H_{37}N_2O_7S$, 581.2321; found, 581.2325.
Compound 18: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.31 (s, 6H), 1.92–2.02 (m, 2H), 3.25 (s, 3H), 3.12–3.21 (m, 1H), 3.30–3.35 (m, 2H), 3.61–3.65 (m, 1H), 3.88–3.92 (m, 1H), 4.33–4.42 (m, 2H), 4.62 (m, 2H), 6.71 (s, 1H), 6.80 (d, J = 7.8 Hz, 1H), 6.91 (dd, J = 8.3, 2.5 Hz, 1H), 7.22 (s, 1H), 7.30 (t, J = 8.3 Hz, 1H), 7.56 (d, J = 8.2 Hz, 1H), 7.61 (d, J = 8.2 Hz, 1H). HRMS-FAB (*m*/*z*) $[M+H]^+$ calcd for $C_{31}H_{28}F_3N_2O_7$, 597.1849; found, 597.1849.
Compound 19: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.33 (s, 3H), 1.45 (m, 2H), 1.62–1.90 (m, 8H), 2.27 (s, 3H), 2.36 (t, J = 7.1 Hz, 2H), 2.74 (d, J = 12.7 Hz, 1H), 2.90 (dd, J = 12.7, 5.0 Hz, 1H), 3.14–3.22 (m, 3H), 3.70 (t, J = 4.7 Hz, 2H), 4.10 (m, 2H), 4.16–4.23 (m, 3H), 4.32 (m, 2H), 4.50 (m, 1H), 5.30 (br s, 1H), 5.75 (br s, 1H), 7.25 (s, 1H), 7.59 (d, J = 8.2 Hz, 1H), 7.62 (d, J = 8.2 Hz, 1H). HRMS-FAB (*m*/*z*) $[M+H]^+$ calcd for $C_{33}H_{39}N_2O_9S$, 639.2376; found, 639.2379.
Compound 20: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.33 (s, 3H), 1.60–1.64 (m, 1H), 1.78–1.91 (m, 3H), 2.26 (s, 3H), 3.18–3.22 (m, 2H), 3.73 (t, J = 4.6 Hz, 2H), 4.07 (m, 2H), 4.17 (d, J = 11.1 Hz, 1H), 4.33 (d, J = 11.1 Hz, 1H), 4.36 (t, J = 4.6 Hz, 2H), 4.66 (s, 2H), 6.72 (s, 1H), 6.81 (d, J = 7.8 Hz, 1H), 6.93 (dd, J = 8.1, 2.0 Hz, 1H), 7.24 (s, 1H), 7.31 (t, J = 8.1 Hz, 1H), 7.57 (d, J = 8.2 Hz, 1H), 7.61 (d, J = 8.2 Hz, 1H). HRMS-FAB (*m*/*z*) $[M+H]^+$ calcd for $C_{33}H_{30}F_3N_2O_9$, 655.1903; found, 655.1906.
20. TRAP staining and activity assay—All materials used for cell culture were purchased from HyClone (UT). RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin with a change of medium every 3 days in humidified atmosphere of 5% CO_2 at 37 °C. To induce the osteoclast formation, 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 (R&D Systems Inc., MN). Next day, chemicals were treated and at day 4, multinucleated osteoclasts were visualized by TRAP staining using a leukocyte acid phosphatase kit 387-A (Sigma, MO) and observed under a microscope. To measure the TRAP activity, the multinucleated cells were fixed with 10% formalin for 10 min and 95% ethanol for 1 min, and then dried. One hundred microliters of citrate buffer (50 mM, pH 4.6) containing 10 mM sodium tartrate and 5 mM *p*-nitrophenylphosphate (Sigma) was added to the dried cells. After incubation for 30 min, the enzyme reaction mixtures were transferred into the well of fresh plates containing 100 μl of 0.1 N NaOH. Absorption was measured at 410 nm with Wallac EnVision HTS microplate reader (Perkin-Elmer, Finland). All experiments were performed in triplicate.
21. Cell proliferation assay—RAW264.7 cells were plated at 96-well plates at the density of 4×10^3 cells/well in α-MEM containing 10% FBS in the presence of 100 ng/ml RANKL. Next day, cells were treated with serially diluted chemicals and incubated for 3 days. Cell proliferation was then measured with a Cell Counting Kit-8 (Dojindo Molecular Technologies, ML) according to the manufacturer's protocol. All experiments were performed in triplicate.