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Synthesis of novel 5,6-dehydrokawain analogs as osteogenic inducers and their action mechanisms

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ABSTRACT

An imbalance between bone resorption by osteoclasts and bone formation by osteoblasts can cause bone loss and bone-related disease. In a previous search for natural products that increase osteogenic activity, we found that 5,6-dehydrokawain (**1**) from *Alpinia zerumbet* promotes osteoblastogenesis. In this study, we synthesized and evaluated series of 5,6-dehydrokawain analogs. Our structure-activity relationships revealed that alkylation of *para* or *meta* position of aromatic ring of **1** promote osteogenic activity. Among the potential analogs we synthesized, (*E*)-6-(4-Ethylstyryl)-4-methoxy-2*H*-pyran-2-one (**14**) and (*E*)-6-(4-Butylstyryl)-4-methoxy-2*H*-pyran-2-one (**21**) both significantly up-regulated Runx2 and Osterix mRNA expression at 10 μM. These osteogenic activities could be mediated by bone morphogenetic protein (BMP) and activation of p38 MAPK signaling pathways. Compounds **14** and **21** also inhibited RANKL-induced osteoclast differentiation of RAW264 cells. These results indicated that novel 5,6-dehydrokawain analogs not only increase osteogenic activity but also inhibit osteoclast differentiation, and could be potential lead compounds for the development of anti-osteoporosis agents.

Osteoporosis, characterized by systemic dysfunction in bone mass and strength that leads to fragility fractures, is a growing medical and socioeconomic issue. Osteoblastic bone formation and osteoclastic bone resorption are tightly coordinated during the remodeling cycle and loss of bone mass results from an imbalance of these fundamental processes.[1,2] Recent therapies for osteoporosis have mainly focused on the suppression of bone resorption by osteoclasts.[2] For example, bisphosphonates and selective estrogen receptor modulators have been used clinically to prevent bone loss.[1,2] However, by the time they initiate such therapies, many osteoporotic patients have already lost substantial amounts of bone. Therefore, anabolic treatments that enhance osteogenesis are needed.[3] In this regard, the ability of some chemical components in food and medicinal plants to promote osteogenesis has been studied.[4–7] In particular, the ability of isoflavones such as daidzein and genistein, which abundant in soybeans, to prevent bone loss has been extensively investigated using various experimental models and in clinical studies.[8–10] Recently, the synthesis and osteogenic activity of daidzein analogs was reported.[11,12] These analogs are expected to be lead compounds for osteoporosis treatments.

As a part of our exploration of natural products that enhance osteogenic activities, we identified the kavalactone 5,6-dehydrokawain (**1**) from *Alpinia zerumbet*,[13] and showed that it has osteogenic activity (Fig. 1).[14] To develop 5,6-dehydrokawain analogs that have more potent osteogenic activity than the natural parent products, and to reveal structure–activity relationships,

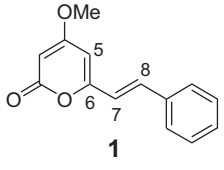

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Fig. 1. Structure of 5,6-dehydrokawain (**1**).

Abbreviations: ALP, alkaline phosphatase; αMEM, alpha minimum essential medium; BMP, bone morphogenetic protein; DMSO, dimethyl sulfoxide; HRMS, high-resolution mass spectrometry; MAPK, mitogen-activated protein kinase; ODM, osteoblast differentiation medium; RANKL, receptor activator of nuclear factor-κB ligand; TRAP, tartrate-resistant acid phosphatase.

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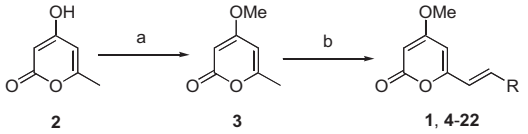
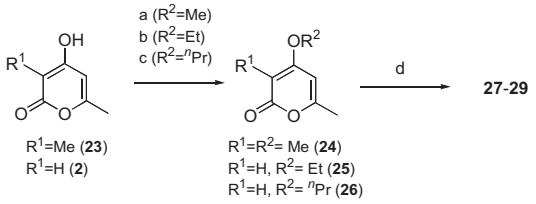
here we synthesized a series of chemically modified 5,6-dehydrokawain analogs and evaluated their osteogenic activity using the mouse osteoblast cell line MC3T3-E1. For those compounds that had potent activities, we investigated the molecular mechanisms underlying the osteogenic activity and the effects against osteoclastogenesis using RANKL-induced RAW264 cells.



Scheme 1. Synthesis of 5,6-dehydrokawain and its analogs. Reagents and conditions: (a) Me_2SO_4, K_2CO_3, DMSO, rt; (b) RCHO, $Mg(OMe)_2$, MeOH, 60 °C.



Scheme 2. Synthesis of 5,6-dehydrokawain analogs **27**–**29**. Reagents and conditions: (a) dimethyl sulfate, K_2CO_3, DMSO, rt; (b) diethyl sulfate, K_2CO_3, DMSO, rt; (c) dipropyl sulfate, K_2CO_3, DMSO, rt; (d) benzaldehyde, $Mg(OMe)_2$, MeOH, 60 °C.

The synthesis of 5,6-dehydrokawain analogs was performed using an aldol reaction (Scheme 1).[15,16] Commercially available 4-hydroxy-6-methylpyrone was methylated by dimethyl sulfate, and condensed with a variety of aldehydes by magnesium methoxide in anhydrous methanol. Only 3-ethylbenzaldehyde was synthesized as previously described.[17] Other aldehydes were obtained from commercial suppliers and used without further purification. For condensation, the mixtures were heated at reflux for 3–6 h and then cooled. The solvent was removed *in vacuo* and the crude product was purified by preparative HPLC.

To prepare pyrones **27**–**29**, commercially available pyrones were reacted with each alkylating reagent and then condensed with benzaldehyde (Scheme 2). The detailed method, synthetic yields, melting points, ^1H and ^{13}C NMR, HRMS data, purity data are described in Supplementary data.

To evaluate the effects of these compounds on osteoblast differentiation, MC3T3-E1 cells were cultured with the synthesized derivatives in osteoblast differentiation medium (ODM) containing ascorbic acid and β-glycerophosphate which were essential for the expression of osteoblast phenotype.[18] After 4 and 10 days, alkaline phosphatase activity and osteoblastic mineralization, respectively, were assessed. Alkaline phosphatase activity is an early-stage osteogenesis marker, whereas mineralization characterizes late-stage osteogenesis.[19]

We first synthesized compounds **1** and **4**–**14** as a small-scale library for modification of the phenyl ring in 5,6-dehydrokawain (**1**) and evaluated osteogenic activities in an initial screen (Fig. 2A). Synthetic **1** (60 μM) enhanced ALP activity by approximately 1.25-

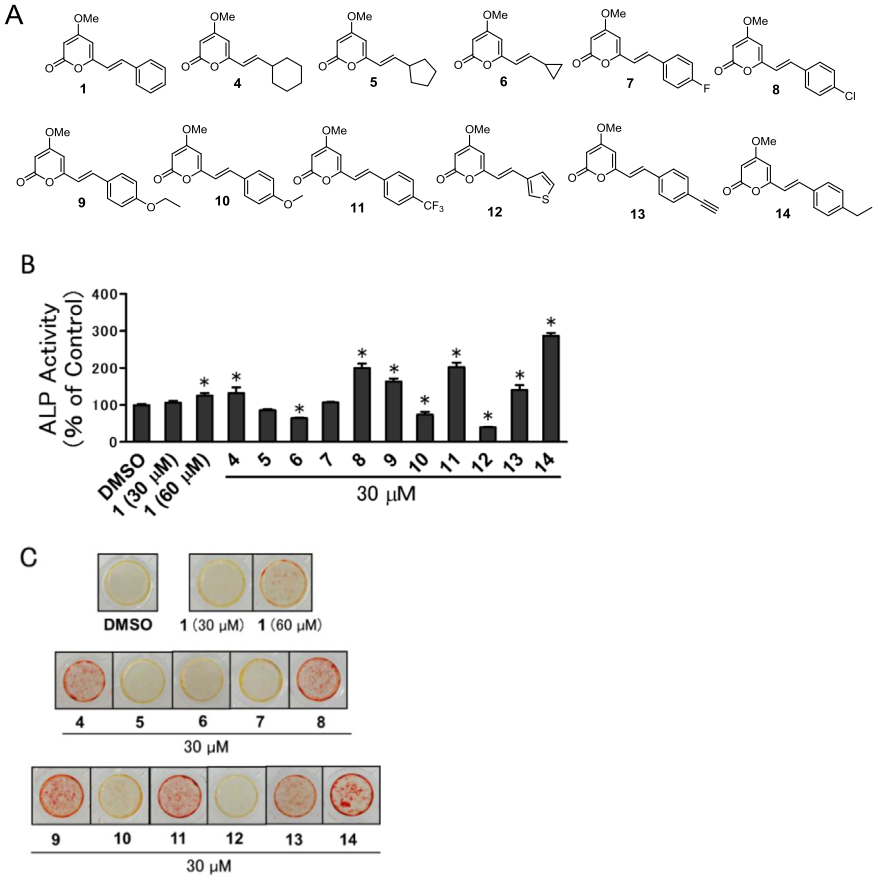


Fig. 2. Structures of synthesized compounds **1** and **4**–**14** (A), and their effects on ALP activity (B) and mineralization (C) in MC3T3-E1 cells. The cells were cultured with or without test compounds in an osteoblast differentiation medium containing ascorbic acid (50 μg/mL) and β-glycerophosphate (10 mM) for 4 days (ALP activity) and 10 days (Mineralization). Mineralization was visualized by Alizarin Red S staining and photographed. The data represent means ± SD of triplicate analysis. The photographs show representative views of triplicate analyses. $^*P < 0.05$ versus the vehicle control by Dunnett's test.

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fold relative to vehicle control (DMSO), which was similar to that seen for the natural products we previously reported (Fig. 2B).[14] Furthermore, 60 μM compound **1** enhanced matrix mineralization (Fig. 2C). Although analogs **5**, **6**, **7**, **10**, **12** did not increase ALP activity and matrix mineralization at 30 μM, six of eleven 5,6-dehydrokawain analogs (**14**, **11**, **8**, **9**, **13** and **4**) at 30 μM increased ALP activity by 1.32- to 2.87-fold relative to vehicle control (Fig. 2B). This concentration was lower than that at which compound **1** had osteogenic activity. At 30 μM these derivatives also facilitated matrix mineralization by the cells (Fig. 2C) These results suggested that six of the 5,6-dehydrokawain analogs promote both early- and late-stage osteogenesis. Interestingly, compound **4,** wherein the phenyl ring of **1** was changed to a cyclohexyl ring, had enhanced osteogenic activity, suggesting that the aromatic ring was not necessary for osteogenic activity. Compounds **14**, **11** and **8** were highly active inducers of osteogenic differentiation, indicating that attachment of hydrophobic substituents at *para* positions on the phenyl ring is important to enhance osteogenic activity.

When the substituent was an ethyl group, marked enhancement of osteogenic activity was observed. To examine how substitutions at various positions affected ALP activity, we next synthesized 5,6-dehydrokawain analogs bearing an ethyl or a methyl group at the *ortho*, *meta*, and *para* positions (Fig. 3A). Compared with the ALP activity seen for cells treated with **14**, cells treated with the *meta* derivative **15** had similar ALP activity, but *ortho* derivative **16-**treated cells had significantly decreased ALP activity (Fig. 3B). We saw a similar trend for the mineralization assay. The dramatically increased mineralization induced by **14** was slightly lower in cells treated with **15**, and significantly decreased in cells treated with **16** (Fig. 3C, D). The methylated analogs **17**–**19** also showed a similar trend, although ALP activity and mineralization of **17-**treated cells were significantly lower than those of **14-**treated cells (Fig. 3). These results demonstrated that the presence of substituents at the *para* or *meta* position are needed for potent osteogenic activity. These findings may thus be helpful for future studies of biological mechanisms of bone formation/resorption using 5,6-dehydrokawain analogs that remain bioactive.

To investigate how alkyl chain length affects the activity of 5,6-dehydrokawain analogs, we further synthesized derivatives **20**–**22** with *n*-propyl, *n*-butyl, and *tert*-butyl groups, respectively (Fig. 4A). The effects on osteogenic activity of these compounds were dependent on the alkyl length (Fig. 4B, C). Of five alkylated 5,6-dehydrokawain analogs, **21**, which had the longest alkyl chain, had the most potent promotion of ALP activity and enhancement of mineralization. When comparing the activities of **21** and **22**, **21** showed significantly stronger ALP and mineralization activity than **22** (at 10 μM), thus highlighting the importance of straight chain expansion for osteogenic activity.

We also synthesized and evaluated compounds **27**–**29**, which were pyrone ring-modified compounds of **1** (Fig. 5A). These molecules did not increase mineralization at 30 μM (Fig. 5B), and at concentrations of 0.1–60 μM they had no better osteogenic activity than **1** (data not shown). These results suggested that the 4-methoxypyran-2-one structure is important for osteogenic activity.

The effects of all synthesized compounds on cell proliferation were also evaluated. After treating cells with the compounds for 4 days, the cell numbers ranged from 0.85 to 1.25-fold of control values, except for compound **29** (Supplementary Fig. 1). From these results, we concluded that the concentrations of the compounds used in this study (30 μM) were suitable for comparison of osteogenic activities.

To investigate the mechanisms by which the potentially active analogs **14** and **21** facilitate osteogenic differentiation of MC3T3-E1 cells, osteogenic mRNA expression levels of Runx2 and Osterix, as well as Alpl and Bglap, were analyzed by quantitative PCR. Runx2 and Osterix are essential transcription factors that govern osteoblast differentiation[20,21] and regulate the transcription of the osteogenic marker genes Alp1 and Bglap.[22] Relative to untreated MC3T3-E1 cells, cells treated with ODM supplemented

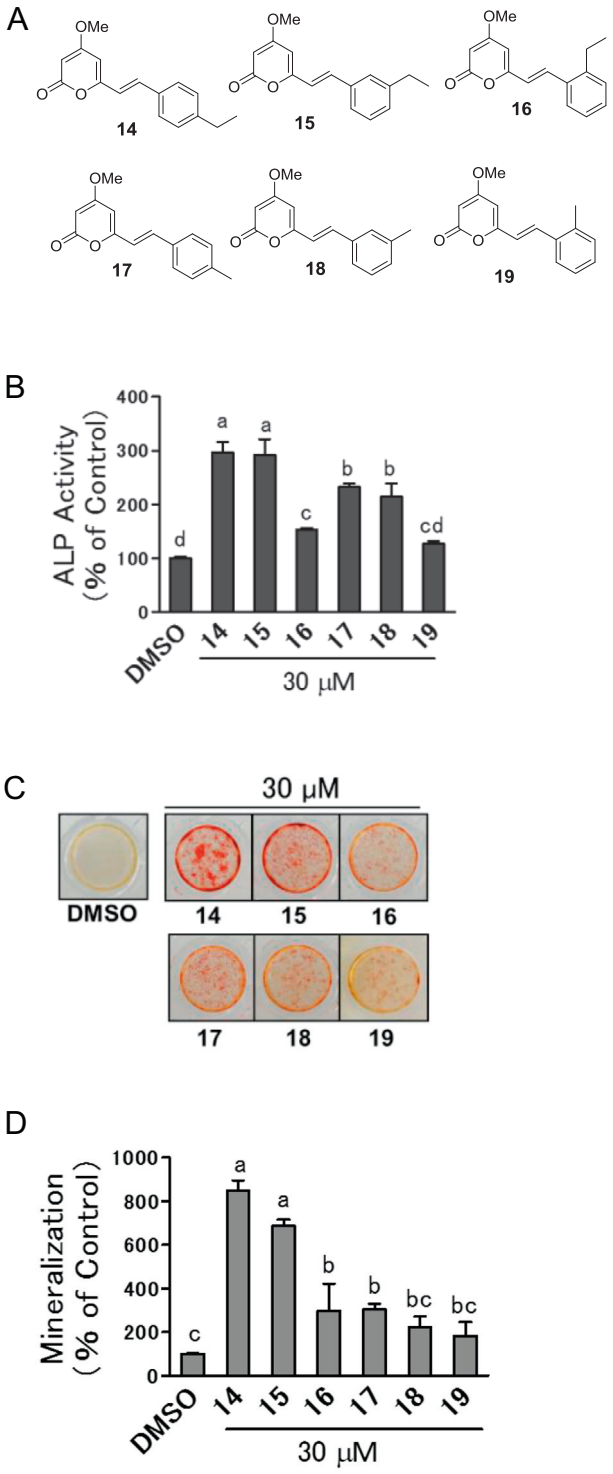

Fig. 3. Structures of synthesized compounds **14**–**19** (A) and their effects on ALP activity (B) and mineralization in MC3T3-E1 cells (C and D). The experimental procedure is the same as that in Fig. 2. For quantification of mineralization, cells were solubilized with cetylpyridinium chloride and the absorbance of the solution was measured at 550 nm. The data represent means ± SD of triplicate analysis. The various letters indicate significant differences in Turkey-Kramer test results ($P < 0.05$).

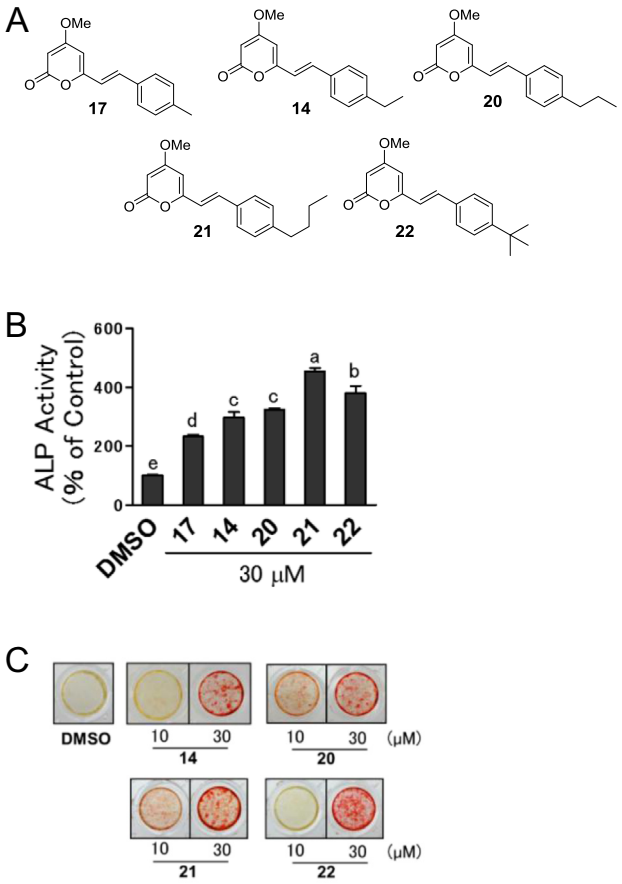

Fig. 4. Structures of synthesized analogs **14**, **17** and **20**–**22** (A), and their effects on ALP activity (B) and mineralization in MC3T3-E1 cells (C). The experimental procedure is the same as that in Fig. 2. The various letters indicate significant differences in Turkey-Kramer test results ($P < 0.05$).

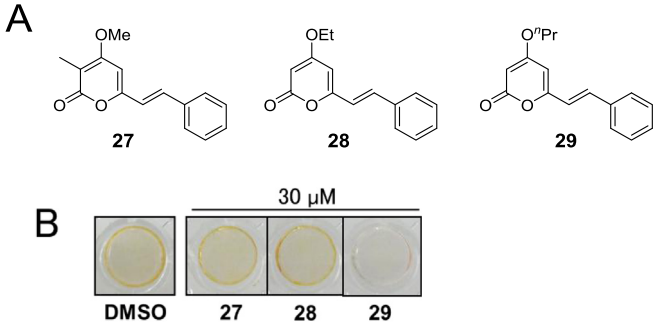

Fig. 5. Structures of synthesized compounds **27**–**29** (A) and their effects on mineralization in MC3T3-E1 cells (B). The experimental procedure is the same as that in Fig. 2. The photographs show representative views of triplicate analysis.

with **14** or **21** for two days showed dose-dependent increases in the expression levels of these four genes. Cells treated with 10 μM **14** expressed higher levels of Runx2 (1.50-fold), Osterix (2.61-fold), Alpl (2.23-fold), and Bglap (1.92-fold) relative to control cells (Fig. 6). Meanwhile, cells treated with 10 μM **21** also expressed higher concentrations of these 4 genes (Runx2, Osterix, Alp1, Bglap, 1.42-, 2.41-, 2.09-, 1.33-fold, respectively) relative to control cells (Fig. 6). The expression levels of Runx2 and Osterix in cells treated with **14** and **21** at 10 μM were higher than the previously reported values for cells treated with 40 μM natural product **1**.[14] These results demonstrated the higher potency of **14** and **21** as osteogenic inducers relative to **1**.

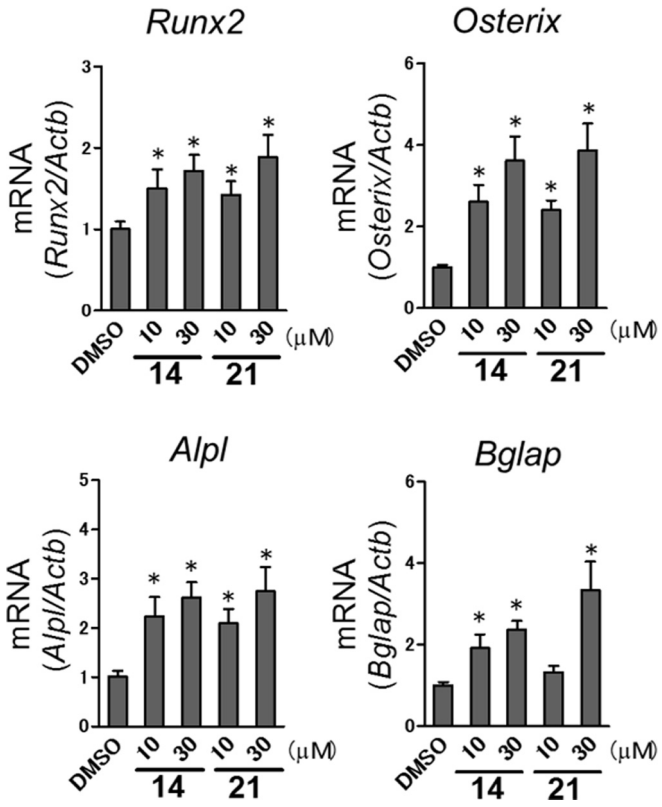

Fig. 6. Effects of potentially active derivatives **14** and **21** on osteoblast-related genes in MC3T3-E1 cells. The cells were incubated for 2 days with compounds in the differentiation medium. Total RNA was isolated and mRNA levels of the genes were measured by real-time PCR and normalized to Actb. The data represent means ± SD of three independent experiments. $^*P < 0.05$ versus the vehicle control by Dunnett's test.

In the qPCR analysis, we revealed that **14** and **21** enhanced mRNA expression of Osterix, which is known to be induced by a bone morphogenetic protein (BMP) via Smad and p38 mitogen–activated protein kinase (MAPK) pathways.[23,24] To demonstrate the involvement of these signaling pathways in the osteogenic activity of **14** and **21**, we investigated phosphorylation of p38 and Smad1/5/8 by western blot analysis. Both **14** and **21** activated p38 MAPK but not Smad/1/5/8 (Fig. 7). On the other hand, BMP-2 used as a positive control activated both p38 MAPK and Smad1/5/8

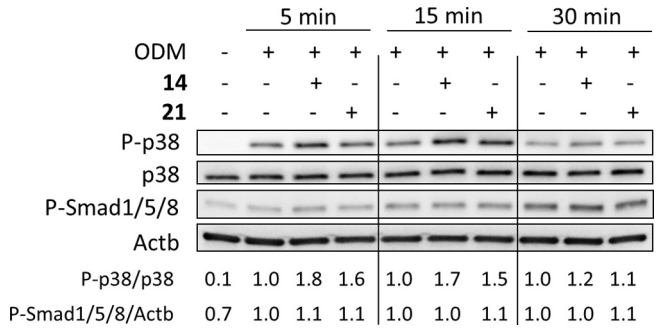

Fig. 7. Effects of potentially active derivatives (**14** and **21**) on phosphorylation of p38 MAPK and Smad1/5/8 in MC3T3-E1 cells. Cells treated with osteoblast differentiation medium (ODM) and test compounds (30 μM) for 5, 15, and 30 min were lysed and analyzed by western blotting. Similar results were obtained in three independent experiments and representative results are shown. The levels of phosphorylated p38 and Smad1/5/8 proteins are shown as a ratio to p38 and Actb respectively.

(Supplementary Fig. 2). These results suggest that p38 MAPK activation is important for the osteogenic activities of **14** and **21**.

To reveal participation of BMP signaling pathways in **14** and **21**-mediated effects on osteogenesis, MC3T3-E1 cells were treated with specific inhibitors, treated with **14** and **21**, and mineralization was examined. Treatment with noggin (BMP antagonist) and LDN-193189 (type1 BMP receptor kinase inhibitor) reduced **14** and **21**-promotion of MC3T3-E1 mineralization (Fig. 8). The same results were observed for BMP-2 treated cells. These results suggest that the osteogenic actions of **14** and **21** are mediated by activation of BMP signaling pathways. Furthermore, treatment of cells with SB203580 (p38 MAPK inhibitor) also reduced **14** and **21**-induced mineralization (Fig. 8). The activity of p38 kinase in osteogenesis was reported to be related to its capacity to phosphorylate and increase the activity of fundamental osteogenic transcription factors, including Runx2, Osterix, and Dlx5.[25] Thus, p38-mediated phosphorylation of these transcription factors by p38 may be involved in the effect of **14** and **21** on osteogenesis.

In our previous report, we showed the activation of p38 MAPK by treatment with **1**.[14] Our subsequent research revealed that the osteogenic activity of **1** could be abolished by noggin, LDN-193189, and SB203580 (data not shown). These results suggest that 5,6-dehydrokawain (**1**) and its analogs **14** and **21** act on MC3T3-E1 cells via a similar mechanism. The increased hydrophobicity of these molecules relative to parent compound may enhance cell permeability, and in turn promote osteogenic activity.

Some skeletal disease is induced through excess osteoclastic activity.[2] Osteoclasts differentiate from macrophage cells in response to RANKL stimulation.[26] Thus, we examined the inhibitory activities of **14** and **21** against RANKL-induced osteoclast differentiation using the murine macrophage cell line RAW264. Previously, we showed that natural 5,6-dehydrokawain did not inhibit RANKL-induced TRAP-activity at 20 μM in RAW264 cells. In this study, synthetic **1** also failed to inhibit TRAP activity, but interestingly, analogs **14** and **21** did show inhibitory activity. In particular, **21** had a stronger inhibitory effect that was nearly the same as that seen for ipriflavone, which is used for clinical applications (Fig. 9A). Analogs **14** and **21** also suppressed formation of RANKL-induced multinucleated cells (Fig. 9B). These results suggested that the 5,6-dehydrokawain analogs **14** and **21** not only increase osteogenic activities but also inhibit osteoclastogenesis.

In conclusion, we designed and synthesized 23 5,6-dehydrokawain analogs as potential anabolic agents. Similarly to the natural version, synthetic 5,6-dehydrokawain promoted ALP activity and matrix mineralization. Among synthesized compounds, 14 analogs promoted higher levels of ALP activity and mineralization than 5,6-dehydrokawain. The structure-activity relationships of **1** suggested that alkylation at the *para* and *meta* positions of the

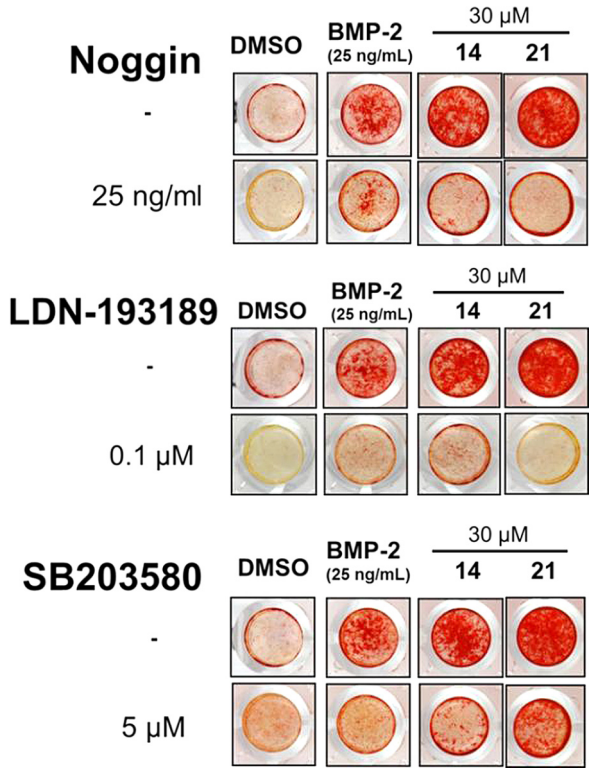

Fig. 8. Effects of BMP-2 inhibitors (Noggin and LDN-193189) and p38 inhibitor (SB203580) on MC3T3-E1 cell mineralization induced by BMP-2 and compounds **14** and **21**. MC3T3-E1 cells were pretreated for 0.5 h with inhibitors and then treated for 10 days with test compounds in an osteoblast differentiation medium. Mineralization was visualized by Alizarin Red S staining. The photographs show representative views of triplicate analyses. Similar results were obtained in three independent experiments and representative results are shown.

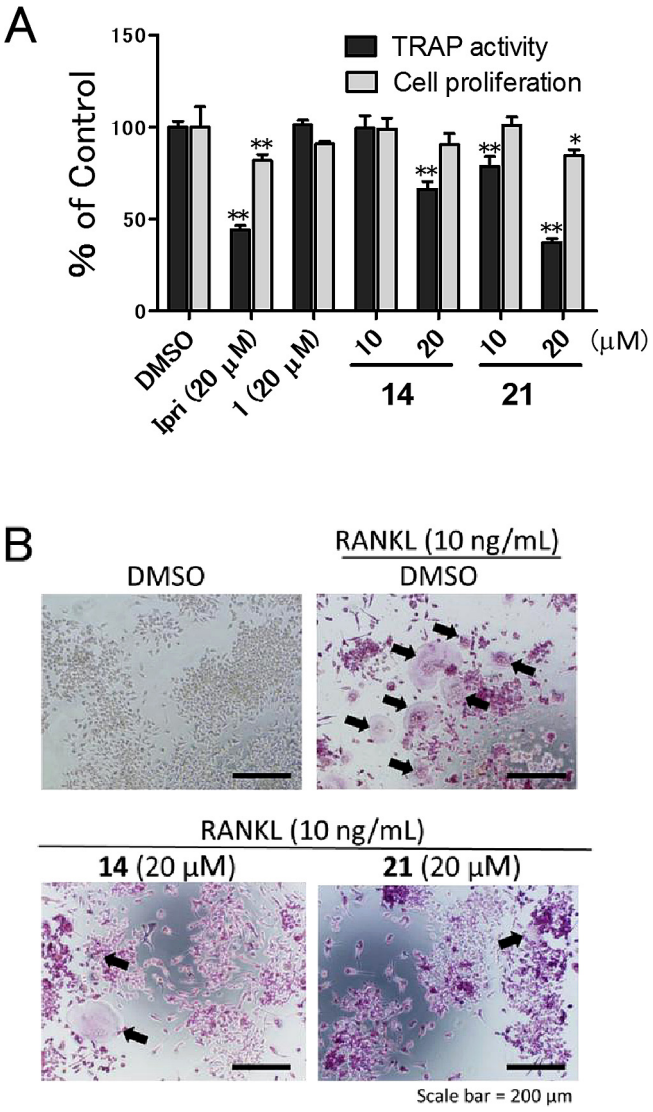

Fig. 9. Effects of 5,6-dehydrokawain (**1**), derivatives **14**, **21** and Ipriflavone (Ipri) on RANKL-induced osteoclast differentiation from RAW264 cells. RAW264 cells were cultured with the test compounds in the presence of RANKL (10 ng/mL) for 3 days. After incubation, TRAP activities and cell numbers were measured. The data represent means ± SD of triplicate analysis. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the vehicle control by Dunnett's test (A). For microscopic analysis, cells were fixed and stained by TRAP. Arrows show TRAP-positive multinucleated cells containing more than five nuclei. Each picture is representative of at least three independent cultures (B). The scale bar shows 200 μm.

phenyl ring was associated with strong osteogenic activity. Among the synthesized analogs, the mechanistic action of analogs **14** and **21** were investigated in detail. These two compounds at 10 μM both up-regulated mRNA expression of Runx2 and Osterix. Further specific inhibitor experiments suggested that the osteogenic action of **14** and **21** was mediated by the BMP signaling pathway and p38 MAPK activation. Interestingly, **14** and **21** also inhibited osteoclastogenesis at 20 μM despite the lack of 5,6-dehydrokawain-mediated inhibition of this process. Although further research is needed to define their action *in vivo*, 5,6-dehydrokawain analogs could be promising therapeutic lead compounds for treatment of osteoporosis.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.04.016.

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