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Synthesis and Biological Evaluation of Heterocyclic Ring-Fused Betulinic Acid Derivatives as Novel Inhibitors of Osteoclast Differentiation and Bone Resorption

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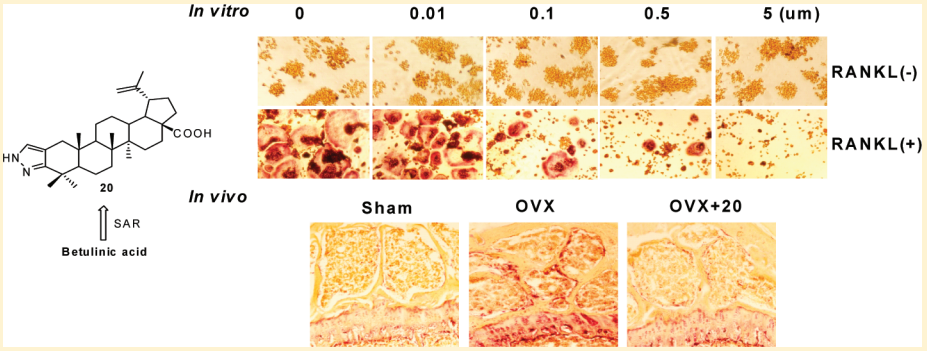
Supporting Information



ABSTRACT: A series of betulinic acid (BA) derivatives were designed and synthesized by introducing various fused heterocyclic rings at C-2 and C-3 positions. Their inhibitory effects of RANKL-induced osteoclastogenesis were evaluated by using a cell-based tartrate-resistant acid phosphatase (TRAP) activity assay. To our delight, most of these compounds exhibited a dramatic increase in inhibitory potency, compared with BA. The most potent compound, **20**, showed 66.9% inhibition even at the low concentration of 0.1 μM, which was about 200-fold more potent than the lead compound BA. What's more, the cytotoxicity assay on RAW264.7 suggested that the inhibition of **20** on osteoclast differentiation did not result from its cytotoxicity. The primary mechanistic study indicated that **20** could inhibit osteoclastogenesis-related marker gene expression levels of cathepsin K and TRAP. More importantly, **20** could attenuate bone loss of ovariectomy mouse in vivo. Therefore, these BA derivatives could be used as potential leads for the development of a new type of antiosteoporosis agent.

INTRODUCTION

Osteoporosis is a serious public health problem, particularly among postmenopausal women, which is characterized by reduced bone mass and increased risk of fractures. Human bone is a continuously renewed organ that maintains its quality through a delicate balance between osteoclast-mediated bone resorption and osteoblast-mediated bone formation.[1] Abnormally increased osteoclastic bone resorption plays a key role in the pathogenesis of osteoporosis, which is associated with postmenopause,[2] advancing age,[3] tumors,[4] infections,[5] and inflammatory response.[6] Efforts have been primarily concentrated on the development of drugs that block bone resorption through decrease of the formation and activity of osteoclasts or promotion of bone formation through increase of the formation and activity of osteoblasts. Although various agents are available for the treatment of osteoporosis, few are ideal for the treatment of associated pathologies. Estrogen loss in women is associated with elevated bone resorption casused by a rise in osteoclast numbers. Estrogen-replacement therapy (ERT) has long been considered the first line therapy for preventing postmenopausal osteoporosis in women.[7] However, ERT increases the risk of breast cancer, stroke, heart attack, and clots.[8] Because of these estrogen-like side effects, ERT is only used for short durations (less than 3 years). Bisphosphonates (BPs) are widely used agents in the management of osteoporosis by prevention of excessive osteoclast-mediated bone resorption, owing to their ability to inhibit osteoclast activity.[9] However, taking BPs can cause severe renal toxicity and osteonecrosis of the jaw; moreover, oral administration of

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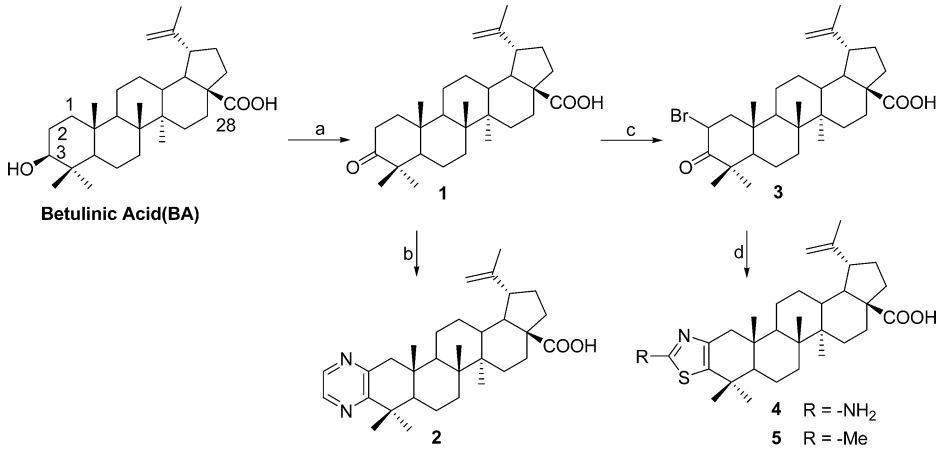
Scheme 1. Synthesis of Pyrazine and Thiazole Derivatives[a]



[a](a) IBX, DMSO, rt, 0.5 h (90%); (b) sulfur, ethylenediamine, morpholine, reflux, 4 h (31%); (c) 5,5-dibromomeldrum's acid, Et_2O, rt, 12 h; (d) thiourea or thioacetamide, EtOH, reflux, 3 h (57% for **4** and 55% for **5** over two steps).

BPs is complicated by poor bioavailability and poor gastrointestinal tolerability.[10] Parathyroid hormone (PTH), a polypeptide, was noted to stimulate bone formation by increasing osteoblast numbers and activity.[11] However, the recommended duration of PTH therapy is relatively short (2 years in the United States and 18 months in Europe) because of its increased incidence of osteosarcoma.[12] Thus, the development of new alternative agents for the treatment of osteoporosis is in considerable demand.

Nowadays, a great variety of antiosteoporosis drugs are available; however, the most relatively successful treatments of osteoporosis are primarily based on antiresorptive agents by inhibition of osteoclast differentiation.[13] In recent years, researchers have clearly uncovered that the receptor activator of NF-κB (RANK), its ligand (RANKL), and the decoy receptor osteoprotegerin (OPG) are key regulators of osteoclastic bone resorption in vitro and in vivo.[14] When the key cytokine RANKL (secreted by osteoblastic cells) binds to RANK (expressed on the surface of osteoclasts and osteoclast precursors), it promotes the differentiation of osteoclasts precursors into full mature, multinucleated, and functional osteoclasts. The antiosteoporosis drugs development has been improved greatly by discovery of RANK/RANKL/OPG signaling pathway in recent decades.

Natural products play a major role in drug discovery, and nearly half of the new drugs introduced into the market in the past 2 decades are natural products or their derivatives.[15] In searching for new types of antiosteoclast formation chemical entities, researchers have found that some natural compounds, especially triterpenoids, could inhibit osteoclastogenesis, such as acetyl-11-keto-β-boswellic acid (AKBA) from ayurvedic therapeutic plant *Boswellia serrata*,[16] 25-acetylcimigenol xylopyranoside (ACCX) from black cohosh,[17] oleanolic acid derivatives,[18] and ganoderic acid DM.[19] Recently, we also found maslinic acid, a natural pentacyclic triterpene acid that can suppress osteoclastogenesis and prevent ovariectomy-induced bone loss by regulating RANKL-mediated NF-kB and MAPK signaling pathways.[20] Betulinic acid (BA) is also a natural pentacyclic triterpenoid, which is found in many plant species and is particularly abundant in plants of the genus *Sambucus*, for example, *Sambucus williamsii* Hance (SWH). In China, SWH is a folk medicine with a long history of being used for treatment of bone fractures and joint diseases, and the present study investigates that SWH extract can increase bone mass and bone stress in ovariectomized rats by increasing the OPG/RANKL mRNA ratio.[21] BA has also been reported to exhibit a variety of biological activities, such as anti-inflammatory,[22] anti-HIV,[23] anticancer,[24] and antioxidant activities, etc.[25] Recently, Chiou et al. also reported that BA increases alkaline phosphatase (ALP) activity and calcium nodule formatiom. Furthermore, it increases the OPG/RANKL ratio to repress bone catabolism without obvious effect on osteoblastogenesis.[26]

Considering the positive effects of SWH and BA on bone fracture and bone loss, BA is very likely to exhibit inhibitory effects on osteoclast differentiation. To our best knowledge, the inhibitory effects of BA on osteoclast cells have not been investigated to date. Thus, we initially screened the activity of BA in osteoclastogenesis and we were pleased to find that BA is a moderate osteoclast differentiation inhibitor ($IC_{50} \approx 20\ \mu M$). Therefore, we considered BA as a lead compound for further development of novel antiosteoporosis agents. It is well-known that pentacyclic triterpenes including BA are too hydrophobic to have reasonable water solubility and related pharmacokinetic properties. Thus, in order to find more potent osteoclastogenesis inhibitors with better water solubility, we designed and synthesized a series of heterocyclic ring-fused BA derivatives at C-2 and C-3 and biological activities were evaluated in this report. The inhibition of RANKL-induced osteoclast differentiation in RAW264.7 cells of BA derivatives were tested in vitro. Results showed that compound **20** was the most potent inhibitor. The effects on cytotoxicity and reduction of marker genes on osteoclasts precursor of **20** were then investigated. The osteoprotective effect of the most promising BA derivative **20** was also evaluated by bone antiresoption in ovariectomized rats in vivo.

RESULTS AND DISCUSSION

Synthesis of Inhibitors. A series of BA derivatives with heterocyclic rings (pyrazine, pyrimidine, indole, thiazole, isoxazolone, isoxazole, thiadiazole, and pyrazole) fused at C-2 and C-3 positions were synthesized (Scheme 1−3) based on our previous research.[27]

The synthesis of pyrazine and thiazole derivatives is outlined in Scheme 1. Oxidation of BA with 2-iodoxybenzoic acid (IBX)

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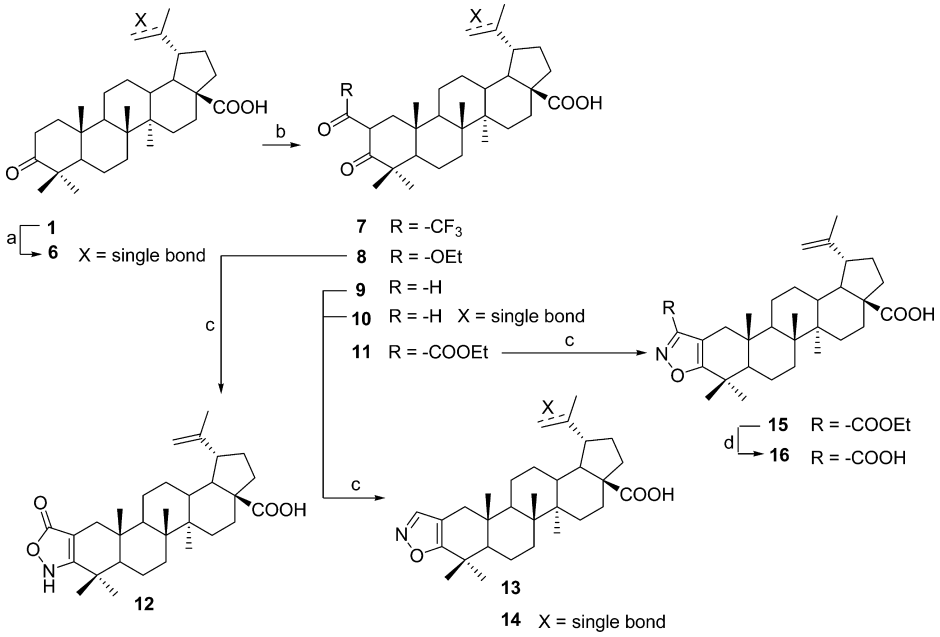
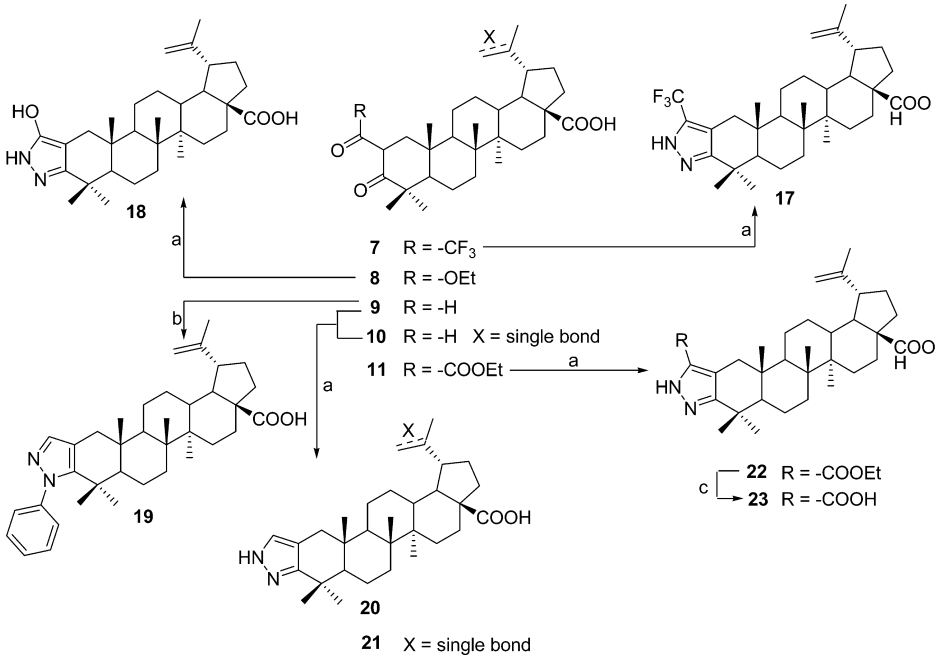
Scheme 2. Synthesis of 5(4*H*)-Isoxazolone and Isoxazole Derivatives[a]



[a]The X shown is a double bond unless otherwise stated. Reagents and conditions: (a) H_2 (1 atm), Pd/C, THF, MeOH, rt, 12 h (94%); (b) RCOOEt (R = −CF_3, −H, −COOEt), MeONa, THF, rt, 5 h (**7**, **9**, **10**, **11**) or RCOOEt (R = −OEt), NaH, THF, 60 °C, 5 h (**8**); (c) $NH_2OH{\cdot}HCl$, EtOH, reflux, 3 h (50% for **12**, 75% for **13**, and 70% for **14** and **15** over two steps); (d) LiOH, EtOH, rt, 15 h (80%).

Scheme 3. Synthesis of Pyrazole Derivatives[a]



[a]The X shown is a double bond unless otherwise stated. Reagents and conditions: (a) $NH_2NH_2{\cdot}HCl$, EtOH, reflux, 3 h (52% for **17**, 50% for **18**, 70% for **20**, 70% for **21**, and 62% for **22** over two steps); (b) $PhNHNH_2{\cdot}HCl$, EtOH, reflux, 3 h (73%); (c) LiOH, EtOH, rt, 15 h (81%).

gave intermediate **1**, which was then converted to pyrazine derivative **2** in the presence of ethylenediamine and sulfur in morpholine.[28] Thiazole derivatives of **4** and **5** were obtained by bromination of **1** with 5,5-dibromomeldrum's acid, followed by heterocyclization with thiourea or thioacetamide in EtOH.

The synthesis of 5(4*H*)-isoxazolone and isoxazole derivatives is outlined in Scheme 2. Hydrogenation of **1** with Pd/C under H_2 afforded **6**. Intermediates **7**, **8**, **9**, **10**, **11** were obtained by Claisen condensation of **1** or **6** with corresponding esters in the presence of MeONa. 5(4*H*)-Isoxazolone derivative **12** was prepared by reaction of **8** with hydroxylamine hydrochloride in EtOH. Treatment of **9**, **10**, and **11** with hydroxylamine hydrochloride in EtOH gave isoxazole derivatives **13**, **14**, and **15**, respectively. Hydrolysis of **15** with LiOH in EtOH afforded **16**.

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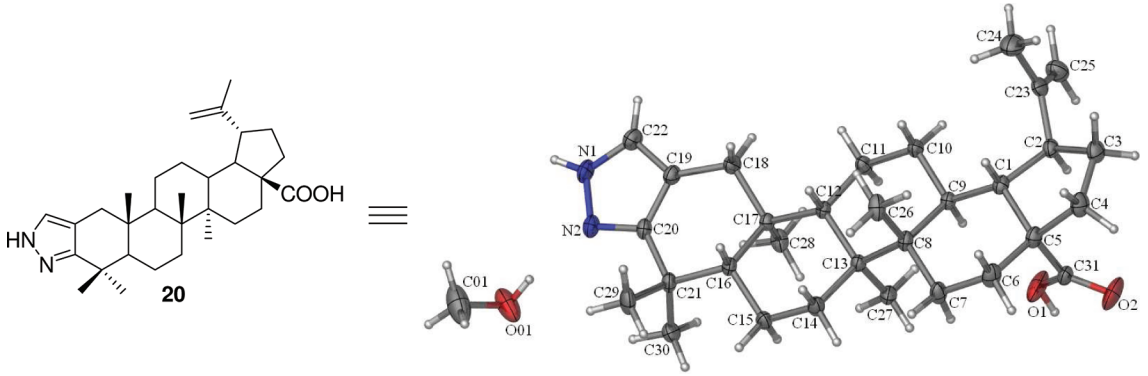
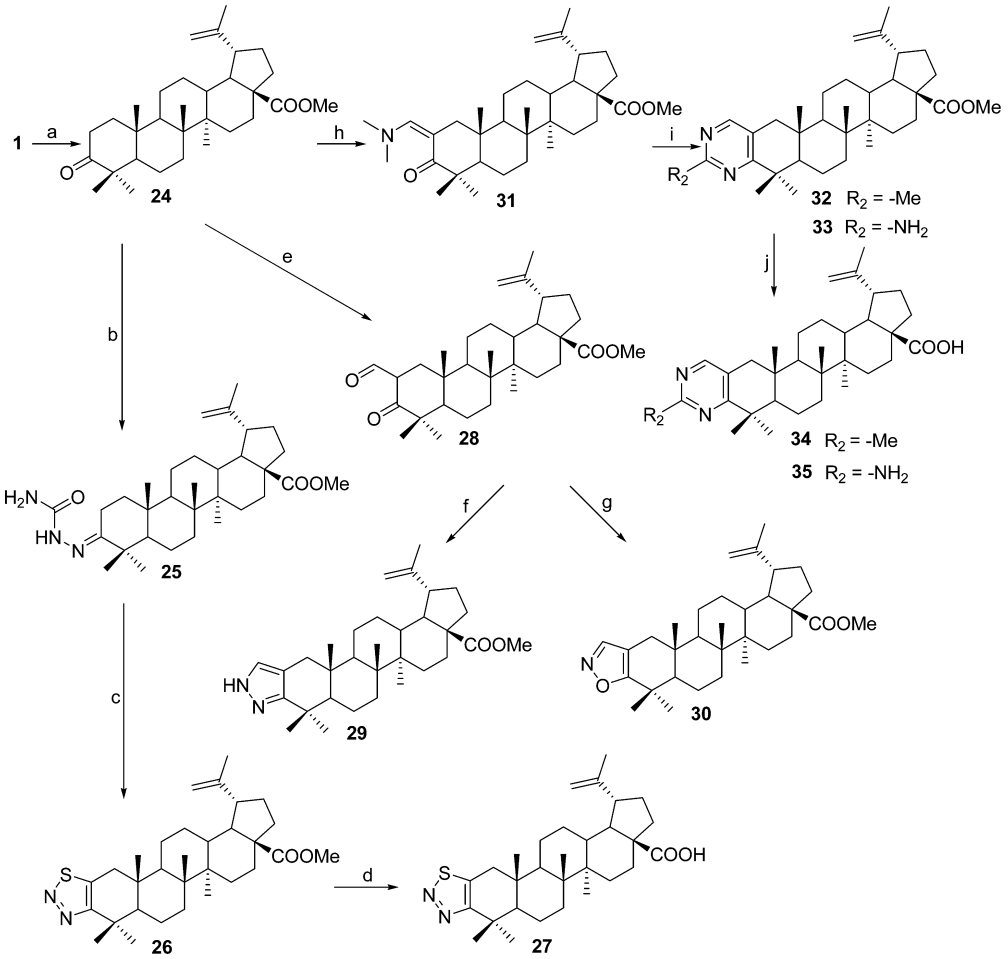
Chart 1. Single-Crystal X-ray Diffraction Analysis of 20



Scheme 4. Synthesis of 1,2,3-Thiadiazole, Pyrimidine, and Methyl Esterification Derivatives[a]



[a]Reagents and conditions: (a) CH_3I, K_2CO_3, DMF, 40 °C, 5 h (90%); (b) $NH_2NHCONH_2$.HCl, Et_3N, THF, rt, 15 h; (c) $SOCl_2$, reflux, 4 h (45% over two steps); (d) LiI, DMF, 140 °C, 16 h, 71%; (e) HCOOEt, MeONa, THF, rt, 5 h; (f) NH_2NH_2·HCl, EtOH, reflux, 3 h (66%); (g) NH_2OH·HCl, EtOH, reflux, 3 h (64%); (h) *N*,*N*-dimethylformamide dimethyl acetal, toluene, reflux, 12 h; (i) ethanimidamide hydrochloride or guanidine hydrochloride, NaOMe, EtOH, reflux, 3 h (44% for **32** and 48% for **33** over two steps); (j) LiI, anhydrous DMF, reflux, 48 h (52% for **34**) or LiI, *n*-octylamine, anhydrous DMF, reflux 48 h (65% for **35**).

The synthesis of pyrazole derivatives is outlined in Scheme 3. Pyrazole derivatives **17**, **18**, **20**, **21**, and **22** were synthesized by condensation of **7**, **8**, **9**, **10**, and **11** with hydrazine hydrochloride in EtOH, respectively. Compound **19** was obtained by reaction of **9** with phenylhydrazine hydrochloride. Compound **23** was prepared in a manner similar to that for **17**, by hydrolyzing with LiOH in EtOH. The structure and configuration of **20** were proven by single-crystal X-ray diffraction analysis[29] (Chart 1).

The synthesis of 1,2,3-thiadiazole, pyrimidine, and methyl esterification derivatives is outlined in Scheme 4. Compound **24** was prepared by methyl esterification of **1** with MeI. Reaction of **24** with semicarbazide hydrochloride produced **25**. 1,2,3-Thiadiazole derivative **27** was obtained by reaction of **25**

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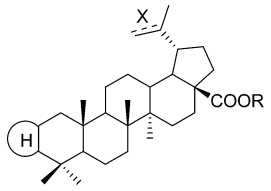
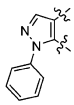
Table 1. Inhibitory Effects of BA and Its Derivatives against RANKL-Induced Osteoclast Differentiation on RAW.264.7 Cells[d]



Compound	H	Inhibition (%)[a] 5μM	10μM	20μM	Compound	H	Inhibition (%)[a] 5μM	10μM	20μM
BA		0	18.0 ± 3.3	44.8 ± 1.3	20		99.0 ± 0.2	100	100
2		58.4 ± 0.3	100	100	21[b]		100	100	100
4		56.2 ± 6.0	86.9 ± 1.8	92.4 ± 0.3	22		100	100	100
5		100	100	100	23		49.6 ± 3.0	100	100
12		32.2 ± 5.4	77.4 ± 1.5	100					
13		98.1 ± 0.5	100	100	27		31.0 ± 5.5	100	100
14[b]		100	100	100	29[c]		0	0	0
15		73.9 ± 3.1	100	100	30[c]		0	1.3 ± 0.6	96.5 ± 0.4
16		23.5 ± 8.3	100	100	32[c]		0	0	100
17		82.3 ± 3.3	100	100	33[c]		0	0	100
18		24.6 ± 1.0	100	100	34		23.2 ± 5.3	100	100
19		0	55.7 ± 2.3	100	35		100	100	100

[a]Inhibition (%) = 100% − osteoclast formation (%) of each tested compound.[30] [b]X is single bond. [c]R is methyl group. [d]Unless otherwise indicated, the X and R are a double bond and H atom, respectively.

with $SOCl_2$, then demethylation of C-28 methyl ester with LiI in DMF. Claisen condensation of **24** with HCOOEt produced **28**, which was further reacted with hydrazine hydrochloride or hydroxylamine hydrochloride to produce **29** or **30**, respectively. Compounds **32** and **33** were obtained by reaction of **24** with *N*,*N*-dimethylformamide dimethyl acetal in toluene and then condensation with ethanimidamide hydrochloride and guanidine hydrochloride, respectively. Demethylation of C-28 methyl ester of compound **32** with LiI in refluxing DMF gave **34**. According to our previous report,[27] the amino group in the guanidine ring was methylated by the MeI generated from the demethylation of C-28 methyl ester of compound **33**. To avoid this undesired reaction, *n*-octylamine was added as a MeI scavenger, and thus, compound **35** was synthesized successfully.

Screening Betulinic Acid and Its Derivatives by Osteoclast Differentiation Assay. The inhibitory effects of all the heterocyclic ring-fused betulinic acid derivatives were evaluated against RANKL-induced osteoclast differentiation on RAW.264.7 cells. RAW264.7 is a mouse monocytic cell line that is used as a standard osteoclast differentiation model. Normally, it will take 3 days for RAW264.7 cells to differentiate into mature osteoclasts with RANKL stimulation. The mature osteoclasts are characterized as tartrate-resistant acid phosphatase (TRAP) positive (red) multinucleated cells. First, RAW264.7 cells were treated with 50 ng/mL RANKL along with the treatment of each tested compound at various concentrations of 5, 10, 20 μM (Table 1). For the compounds with almost full inhibition at 5 μM, further tests at lower concentrations of 0.1, 0.5, 1.0 μM were performed (Table 2). TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts. As shown in Table 1, the inhibitory effect of these heterocyclic ring-fused derivatives was

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Table 2. Inhibitory Effects of Potent Activity of BA Derivatives against RANKL-Induced Osteoclast Differentiation on RAW.264.7 Cells

	inhibition (%)[a]		
compd	0.1 μM	0.5 μM	1.0 μM
5	35.4 ± 9.5	58.3 ± 5.6	73.7 ± 4.0
13	0	66.8 ± 0.6	78.9 ± 0.4
14	0	0	25.4 ± 8.0
20	66.9 ± 0.4	72.5 ± 2.9	85.1 ± 1.5
21	55.4 ± 1.3	82.3 ± 3.0	87.6 ± 1.5
22	0	0	0
35	0	4.9 ± 0.6	14.5 ± 2.7

[a]Inhibition (%) = 100% − osteoclast formation (%) of each tested compound.

much stronger than that of their lead compound BA except the C-28 methyl ester compounds (**29**, **30**, **32**, and **33**). Although methyl esterification at C-28 position led to a significant decrease in the inhibitory activity (**29**, **30**, **32**, **33** vs **20**, **13**, **34**, **35**), hydrogenation of isopropenyl at the C-19 position had only minimal effect on inhibition (**13** vs **14** and **20** vs **21** in Tables 1 and 2). As seen from Tables 1 and 2, the five-membered heterocyclic ring-fused derivatives had more potent effects than the corresponding six-membered analogues in most cases (**5**, **13**, **20** vs **2**, **34**, **35**). The inhibitory potency decreased if the substituents were introduced into the five-membered heterocyclic rings (**13** and **20** vs **12**, **15**, **16** and **19**, **17**, **18**, **22**, **23**). Further, we can see that the inhibition decreased more as hydrophilic substituent groups were introduced into the five-membered heterocyclic rings than that with hydrophobic substituent groups (**4** vs **5**; **16**, **18** vs **15**, **17**; **23** vs **22**). The inhibitory effects of some derivatives were almost 100% at 5 μM as shown in Table 1. Therefore, we tested these compounds at 0.1, 0.5, and 1.0 μM further. The results in Table 2 showed that the inhibition of **14**, **22**, and **35** decreased dramatically, although they inhibited RANKL-induced osteoclastogenesis completely at 5 μM. To our delight, compounds **5**, **13**, **20**, and **21** still possessed potent antiosteoclastogenesis effects even at low concentrations, especially the pyrazole derivative **20**. Its inhibition was 66.9% even at the low concentration of 0.1 μM (IC_{50} = 0.09 μM), which was about 200-fold more potent than the lead compound BA ($IC_{50} \approx 20$ μM, Supporting Information Figure 1). Figure 1 showed that **20** significantly reduced the number of mature osteoclast in RAW264.7 cells, which was confirmed by TRAP staining.

Compound 20 Has Little Cytotoxicity on Osteoclasts within the Effective Concentrations. Compound **20** is the most potent inhibitor among these tested BA derivatives. To examine whether the impaired osteoclastogenesis in the presence of **20** is due to the decrease in viability of the precursor cells, we investigated the cytotoxicity of this compound upon osteoclast precursors RAW264.7 by standard MTS assay (Figure 2). Compound **20** did not show any

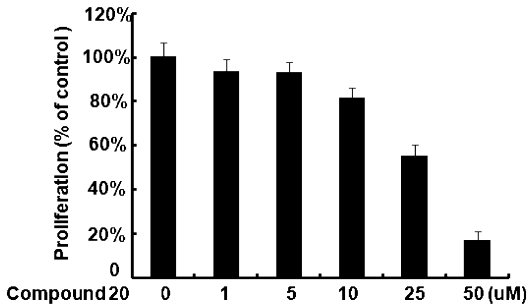

Figure 2. Cytotoxic effect of compound **20** on precursor osteoclasts. RAW264.7 cells (3000/well) were plated on 96-well plates. Cells were incubated with the indicated doses of **20**. After incubation for 72 h at 37 °C, the percentage of cell survival was determined with the MTS/CellTiter 96 aqueous assay (Promega, Madison, WI). The data are the mean of three experiments carried out in triplicate. The bar indicates the SD.

cytotoxic effects at the concentration (0.1 μM) of about half the inhibition (66.9%) of osteoclastogenesis, and only slight effects on cell viability were observed at high concentrations of 5 and 10 μM (the concentration of compound **20** reduction of cell viability to 50% is 35.34 μM). The results suggested that the effects of **20** on osteoclast differentiation were not from its cytotoxicity. The selectivity index was calculated as the ratio of

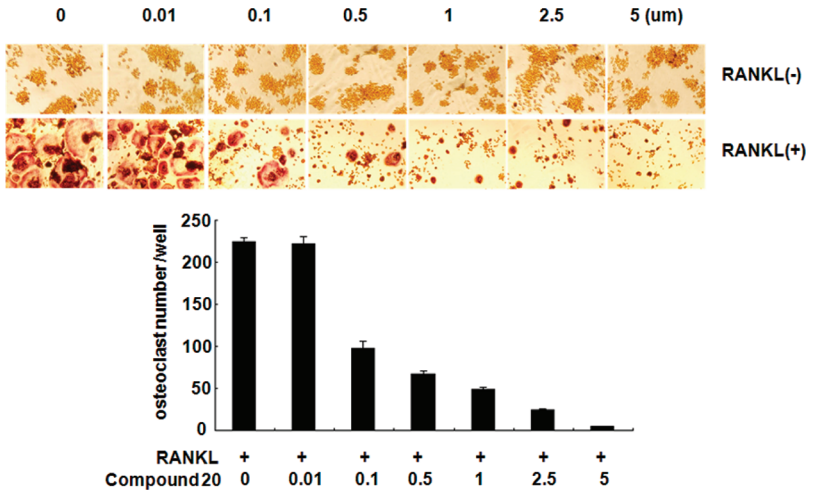

Figure 1. Compound **20** inhibits RANKL-induced osteoclastogenesis in a dose-dependent manner. RAW 264.7 cells (3×10^3 cells/well) were treated with or without RANKL (50 ng/mL), followed by addition of the indicated concentrations of **20** for 3 days and stained for TRAP expression. (Top) Photographs of cells (original magnification 100×). (Bottom) TRAP positive multinucleated (>3 nuclei) osteoclasts were counted. The data are the mean of three experiments carried out in triplicate. The bar indicates the SD.

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the concentration of the compound that reduced cell viability to 50% to the concentration of the compound needed to inhibit the osteoclast differentiation effect to 50% of the control value. The selectivity index of compound **20** was 35.34/0.09 = 392.67.

Compound 20 Suppresses the Osteoclastogenesis-Related Marker Gene Expression. The TRAP enzyme is abundantly expressed by osteoclasts, and studies on TRAP knockout mice showed disturbed endochondral ossification with decreased resorptive activity of osteoclasts.[31] Cathepsin K is highly expressed in osteoclasts near the ruffled border membrane,[32] and it has been shown to participate in osteoclast-mediated degradation of the subosteoclastic collagenous bone matrix.[33] Increased levels of cathepsin K mRNA have been observed under conditions of enhanced bone resorption.[34] Osteoclast function is executed directly by the expression of a large number of related marker genes, such as TRAP and cathepsin K. We investigated whether **20** can regulate osteoclastogenesis-related marker gene expression by quantitative real-time PCR. Our results (Figure 3 and Supporting Information Figure 2) indicated that the expression of TRAP and cathepsin K was up-regulated with RANKL stimulation, while **20** could inhibit RANKL-induced expression levels of the two genes during osteoclastogenesis in a dose- and time-dependent manner, which was consistent with our previous observation that **20** could suppress osteoclast differentiation.

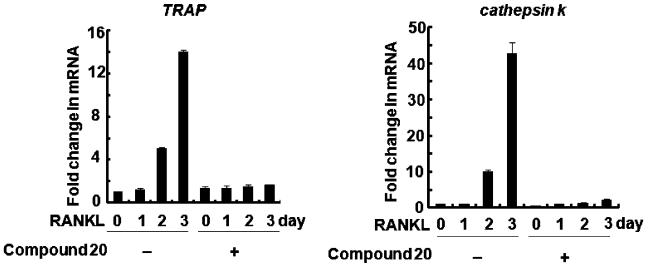

Figure 3. Compound **20** inhibits the expression of osteoclast mark genes. RAW264.7 cells were pretreated with or without 5 μM **20** for 6 h in the presence of 50 ng/mL RANKL for the indicated time periods. Total RNA was isolated and subjected to quantitative real-time PCR for the expression levels of TRAP and cathepsin K. The data are the mean of three experiments carried out in triplicate. The bar indicates the SD.

Compound 20 Prevents OVX-Induced Bone Loss by Suppressing Osteoclast Activity in Vivo. Activation of osteoclasts plays a key role in bone loss, including menopausal osteoporosis, in which estrogen deficiency enhances the genesis and activity of osteoclasts and results in an unbalanced increase in bone resorption. To examine whether compound **20** inhibits osteoclast in vivo, we used the ovariectomy mouse model to mimic menopause-induced bone loss in women. Spine is one of the most severely affected sites by osteoporosis. Figure 4A and Figure 4B showed an obvious decrease in bone volume and trabecular number and an increase in trabecular space at the lumbar spine in OVX mice compared with sham-operated controls. Treatment of OVX mice with **20** (10 mg/kg) significantly prevented the OVX-induced bone loss, as measured by different parameters (Figure 4A,B). To investigate whether **20** would prevent bone loss through inhibiting osteoclastogenesis activity in vivo, TRAP staining was performed on the proximal tibia section of each group. The staining results (Figure 4 C) showed that the activity and area of osteoclasts in OVX mouse notably increased compared with

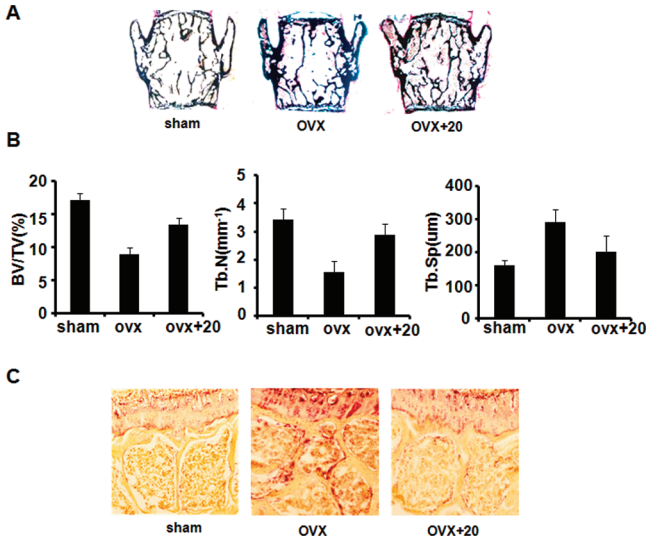

Figure 4. Compound **20** prevented ovariectomy-induced bone loss by suppressing osteoclast activity in vivo. (A) Representative von Kossa stained sections of lumbar vertebrae from sham-operated mice, OVX mice, and OVX + **20** (10 mg/kg) mice. (B) Bone value/total value (BV/TV), trabecular number (Tb.N), and trabecular space (Tb.Sp) were analyzed. (C) Representative osteoclast TRAP stained sections of the proximal tibia from sham-operated mice, OVX mice, and OVX + **20** (10 mg/kg) mice.

sham controls, suggesting that ovariectomy induces osteoclastogenesis. With treatment of OVX-mice by **20**, the OVX-induced osteoclast activity (OVX + **20** vs OVX mice) was dramatically decreased, suggesting that **20** can inhibit ovariectomy-induced osteoclast activity in vivo.

CONCLUSIONS

We synthesized over 20 heterocyclic ring-fused betulinic acid derivatives and evaluated their inhibition on RANKL-induced osteoclast formation in preosteoclast RAW264.7 cells. Among them, compounds **5**, **20**, and **21** exhibited potent inhibitory activity on RANKL-induced osteoclast formation by TRAP assay. Especially **20** (IC_{50} = 0.09 μM) showed about 200-fold more potency than lead compound BA. The cell viability test of **20** on RAW264.7 showed very low cytotoxic effects. The primary mechanism study indicated that **20** could inhibit osteoclastogenesis-related marker gene expression levels of cathepsin K and TRAP during osteoclastogenesis. The experiments of ovariectomy mouse model also showed that **20** could inhibit ovariectomy-induced osteoclast activity in vivo. Further SAR analysis and mechanism studies of these potential leads are ongoing.

In conclusion, we report BA heterocyclic derivatives as a series of new chemical entities for the first time. Especially **20**, which exhibited potent inhibition of osteoclastogenesis both in vitro and in vivo, could be used not only as a chemical tool for the study of osteoporosis biology but also as a promising lead for the development of a new class of antiosteoporosis agents.

EXPERIMENTAL SECTION

General. All reagents and chemicals were purchased from commercial suppliers and used without further purification unless otherwise stated. When needed, the reactions were carried out in flame-dried or oven-dried glassware under a positive pressure of dry N_2. Flash column chromatography was performed on silica gel (QinDao, 200–300 mesh) using the indicated eluents. Thin-layer

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chromatography was carried out on silica gel plates (QinDao) with a layer thickness of 0.25 mm. Melting points were determined using the MEL-TEMP 3.0 apparatus and are uncorrected. ^{1}H (400 and 500 MHz) and ^{13}C (100 and 125 MHz) NMR spectra were recorded on a JEOL-400 and Bruker AM-500 spectrometer with $CDCl_3$ or DMSO-d_6 as solvent and tetramethylsilane (TMS) as the internal standard unless otherwise stated. All chemical shift values were reported in units of δ (ppm). The following abbreviations were used to indicate the peak multiplicity: s = singlet; d = doublet; t = triplet; m = multiplet; br = broad. High-resolution mass data were obtained on a Bruker micrOTOF-Q II spectrometer. Purity of all final analogues for biological testing was confirmed to be >95% as determined by HPLC analysis (for data, see Supporting Information). HPLC analysis was conducted according to the following method with the retention time expressed in min at UV detection of 210, 230, and 254 nm. For HPLC method, an Agilent 1200 series HPLC instrument was used, with chromatography performed on a ZORBAX 150 mm × 4.6 mm, 5 μm C18 column with mobile phase gradient of 0−10% H_2O in MeOH, with a flow rate of 1.0 mL/min.

Compound 1. A solution of BA (2.28 g, 5 mmol) in THF (10 mL) was added dropwise to a stirring solution of IBX (2.1 g, 7.5 mmol) in DMSO (30 mL) at 23 °C. The reaction mixture was stirred for 2 h at 23 °C and then diluted with AcOEt (30 mL) and washed with brine (50 mL). The organic layer was dried over anhydrous Na_2SO_4 and concentrated in vacuo. The residue was purified by flash chromatography (petroleum ether/AcOEt, 8/1 v/v) to give **1** (2.05 g, 90%) as a white solid, mp 251−253 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 4.70 (s, 1H), 4.57 (s, 1H), 3.01−2.96 (m, 1H), 2.46−2.37 (m, 2H), 2.27−2.16 (m, 2H), 1.96−1.86 (m, 3H), 1.66 (s, 3H), 1.62−1.57 (m, 2H), 1.46−1.16 (m, 16H), 1.03 (s, 3H), 0.98 (s, 3H), 0.95 (s, 3H), 0.93 (s, 3H), 0.89 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz): δ 218.3, 182.6, 150.2, 109.7, 56.4, 54.8, 49.8, 49.1, 47.3, 46.8, 42.4, 40.6, 39.5, 38.4, 37.0, 36.8, 34.0, 33.5, 32.0, 30.5, 29.6, 26.6, 25.4, 21.3, 20.9, 19.6, 19.3, 15.9, 15.7, 14.6.

Compound 2. To a solution of **1** (455 mg, 1 mmol) in morpholine (5 mL) was added sulfur (300 mg, 9.4 mmol) and ethylenediamine (0.33 mL, 5 mmol) at 23 °C. The reaction mixture was heated under reflux for 4 h. After cooling, the reaction mixture was poured into water and extracted with AcOEt (30 mL × 3). The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo. The residure was purified by flash chromatography (petroleum ether/AcOEt, 5/1 v/v) and then crystallized from methanol to give **2** (150 mg, 31%) as a white solid, mp 245−247 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 8.37 (d, J = 2.1 Hz, 1H), 8.24 (d, J = 2.2 Hz, 1H), 4.70 (s, 1H), 4.57 (s, 1H), 3.00−2.96 (m, 2H), 2.41−2.37 (m, 1H), 2.26−2.20 (m, 2H), 1.98−1.90 (m, 2H), 1.72−1.69 (m, 1H), 1.65 (s, 3H), 1.55−1.33 (m, 13H) 1.23 (s, 3H), 1.20 (s, 3H), 1.19−1.00 (m, 3H), 0.96 (s, 3H), 0.95 (s, 3H), 0.74 (s, 3H). ^{13}C NMR ($CDCl_3$,100 MHz): δ 181.6, 159.7, 150.7, 150.3, 142.3, 142.2, 109.7, 56.4, 53.0, 49.2, 48.7, 48.4, 46.9, 42.5, 40.5, 39.4, 38.4, 37.0, 36.7, 33.3, 32.1, 31.4, 30.6, 29.7, 25.4, 23.9, 21.3, 20.0, 19.4, 16.0, 15.6, 14.6. ESI-HRMS (m/z): [M + H]$^+$ calcd for $C_{32}H_{47}N_2O_2$, 491.3638, found 491.3634.

Compound 3. To a solution of **1** (455 mg, 1 mmol) in Et_2O (10 mL) was added 5,5-dibromo-2,2-dimethyl-4,6-dioxy-1,3-dioxane (160 mg, 0.53 mmol) at 23 °C. The reaction mixture was stirred for 12 h at 23 °C and then poured into water and extracted with Et_2O (30 mL × 3). The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated in vacuo to give a crude product **3** (497 mg, 0.93 mmol). The crude product **3** was used without further purification.

Compound 4. To a solution of crude compound **3** (497 mg, 0.93 mmol) in ethanol (10 mL) was added thiourea (152 mg, 2 mmol) at 23 °C. The reaction mixture was heated under reflux for 3 h. After cooling, the mixture was concentrated. The residue was purified by flash chromatography (petroleum ether/AcOEt, 2/1 v/v) to give **4** (291 mg, 57% over two steps) as a white solid, mp 262−264 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 12.10 (brs, 1H), 6.60 (s, 2H), 4.70 (s, 1H), 4.57 (s, 1H), 2.96 (m, 1H), 2.45−2.41 (m, 1H), 2.31−2.25 (m, 1H), 2.14−2.12 (m, 1H), 2.05−2.01 (m, 1H), 1.82−1.80 (m, 2H), 1.65 (s, 3H), 1.58−1.10 (m, 19H), 1.00 (s, 3H), 0.96 (s, 3H), 0.92 (s, 3H), 0.81 (s, 3H). ^{13}C NMR (DMSO-d_6, 100 MHz) δ: 177.3, 165.2, 151.3, 150.3, 112.4, 109.6, 55.4, 52.1, 48.4, 48.4, 46.5, 41.9, 40.1, 38.3, 38.0, 37.6, 36.5, 36.2, 32.9, 31.5, 30.1, 30.0, 29.2, 25.0, 21.9, 20.8, 19.0, 18.9, 15.9, 15.3, 14.2. ESI-HRMS (m/z) [M + H]$^+$ calcd for $C_{31}H_{47}N_2O_2S$, 511.3358, found 511.3353.

Compound 5. To a solution of crude compound **3** (497 mg, 0.93 mmol) in ethanol (10 mL) was added thioacetamide (150 mg, 2 mmol) at 23 °C. The reaction mixture was heated under reflux for 3 h. After cooling, the mixture was concentrated. The residue was purified by flash chromatography (petroleum/AcOEt, 5/1 v/v) to afford **5** (280 mg, 55% over two steps) as a white solid, mp 261−264 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 4.74 (s, 1H), 4.61 (s, 1H), 3.06−3.03 (m, 1H), 2.74−2.70 (m, 1H), 2.63 (s, 3H), 2.28−2.23 (m, 3H), 2.21−2.15 (m, 1H), 2.00−1.98 (m, 2H), 1.75−1.70 (s, 4H), 1.66−1.35 (m, 13H), 1.33−1.27 (m, 5H), 1.18 (s, 3H), 1.00 (s, 3H), 0.97 (s, 3H), 0.78 (s, 3H). ^{13}C NMR ($CDCl_3$,100 Hz) δ: 181.0, 163.3, 156.2, 150.7, 126.0, 109.6, 56.4, 52.7, 49.8, 49.1, 49.0, 47.4, 46.8, 42.4, 40.7, 39.0, 38.5, 38.2, 37.0, 33.4, 32.1, 30.5, 30.3, 29.8, 25.4, 22.3, 19.4, 19.3, 18.6, 16.0, 15.7, 14.5. ESI-HRMS (m/z) [M + H]$^+$ calcd for $C_{32}H_{48}NO_2S$, 510.3406, found 510.3428.

Compound 6. To a solution of betulonic acid **1** (600 mg, 1.32 mmol) in THF (10 mL) and MeOH (5 mL) was added 10% Pd on carbon (60 mg) at 23 °C. The mixture was subjected to 1 atm of H_2 and was stirred for 12 h at 23 °C. The mixture was filtered, and the filtrate was concentrated to give **6** (570 mg, 94%) as a white solid, mp 250−252 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 2.62−2.32 (m, 2H), 2.30−2.15 (m, 3H), 2.02−1.12 (m, 22H), 1.05 (s, 3H), 0.99 (s, 3H), 0.94 (s, 6H), 0.91 (s, 3H), 0.83 (d, J = 6.4 Hz, 3H), 0.73 (d, J = 6.0 Hz, 3H).

Compound 12. To a solution of **1** (227 mg, 0.5 mmol) in THF (10 mL) was added NaH (100 mg, 60% in mineral oil, 2.5 mmol) under N_2 at 23 °C. The reaction mixture was stirred for 0.5 h at 23 °C, and then diethyl carbonate (295 mg, 2.5 mmol) was added dropwise to the mixture. Then the mixture was heated to 60 °C and kept at this temperature for 5 h. After cooling, the reaction mixture was poured into 1 N HCl (30 mL) and extracted with AcOEt (30 mL × 3). The organic layer was isolated and washed with brine, dried with anhydrous Na_2SO_4, and concentrated to afford crude product **8** as an oil, which was used without further purification.

To a solution of **8** in ethanol (6 mL) was added hydroxylamine hydrochloride (69 mg, 1 mmol) at 23 °C. The reaction mixture was heat to 80 °C and kept at this temperature for 3 h. After cooling, the reaction mixture was concentrated. The residue was purified by flash chromatography (MeOH/DCM, 5/95 v/v) to afford **12** (125 mg, 50% over two steps) as a white solid, mp 243−245 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 12.1 (brs, 2H), 4.71 (s, 1H), 4.58 (s, 1H), 2.98−2.93 (m, 1H), 2.30−2.27 (m, 1H), 2.14−2.08 (m, 2H), 1.82−1.78 (m, 2H), 1.68 (s, 3H), 1.63−1.18 (m, 15H), 1.17 (s, 3H), 1.14−1.12 (m, 1H), 1.06 (s, 3H), 1.04−1.02 (m, 1H), 0.96 (s, 3H), 0.91 (s, 3H), 0.74 (s, 3H). ^{13}C NMR (CD_3OD, 100 MHz) δ: 177.3, 171.9, 169.7, 150.3, 109.7, 108.0, 55.5, 52.1, 48.5, 48.3, 46.6, 42.1, 40.2, 37.7(2C), 36.4, 34.2, 34.0, 32.8, 31.7, 30.2, 29.4, 28.8, 25.1, 21.5, 21.0, 19.1, 18.2, 15.9, 15.4, 14.4. ESI-HRMS (m/z) [M + H]$^+$ calcd for $C_{31}H_{45}NNaO_4$, 518.3246, found 518.3274.

Compound 13. To a solution of **9** (prepared with the same procedure and scale as that of **13**) in ethanol (6 mL) was added hydroxylamine hydrochloride (69 mg, 1 mmol). The reaction mixture was heated under reflux for 3 h. After cooling, the mixture was concentrated. And the residue was purified by silica gel chromatography (petroleum ether/AcOEt, 4/1 v/v) to give **13** (181 mg, 75.4% over two steps) as a white solid, mp 249−251 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.99 (s, 1H), 4.77 (s, 1H), 4.65 (s, 1H), 3.08−3.02 (m, 1H), 2.51−2.47 (m, 1H), 2.33−2.26 (m, 3H), 2.21−2.00 (m, 3H), 1.98−1.94 (m, 1H), 1.80 (m, 3H), 1.76−1.50 (m, 14H), 1.29−1.10 (m, 6H), 1.02 (s, 3H), 1.09 (s, 3H), 0.82 (s, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 182.7, 173.3, 150.6, 150.5, 110.0, 109.8, 56.4, 53.0, 49.2, 48.7, 48.4, 46.9, 42.4, 40.5, 39.4, 38.4, 37.0, 36.7, 33.3, 32.1, 31.4, 30.6, 29.7, 25.4, 23.9, 21.3, 20.0, 19.4, 16.0, 15.7, 14.6. ESI-HRMS (m/z) [M + Na]$^+$ calcd for $C_{31}H_{45}NNaO_3$, 502.3297, found 502.3273.

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Compound 14. To a solution of **6** (229 mg, 0.5 mmol) and NaOMe (135 mg, 2.5 mmol) in THF (10 mL) was added dropwise ethyl formate (185 mg, 2.5 mmol) at 23 °C under N_2 . The reaction mixture was stirred at 23 °C for 15 h and then poured into 1 N HCl (40 mL) and extracted with AcOEt (30 mL × 3). The organic layer was washed with brine, dried with anhydrous Na_2SO_4, and concentrated to afford **10** as a colorless oil, which was used without further purification.

To a solution of **10** in ethanol (6 mL) was added hydroxylamine hydrochloride (69 mg, 1 mmol) at 23 °C . The reaction mixture was heated under reflux for 3 h. After cooling, the mixture was concentrated. The residue was purified by silica gel chromatography (petroleum ether/AcOEt, 4/1 v/v) to give **14** (169 mg, 70% over two steps) as a white solid, mp 257−259 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.98 (s, 1H), 2.50−2.47 (m, 1H), 2.29−2.22 (m, 3H), 1.97−1.93 (m, 1H), 1.90−1.18 (m, 22H), 1.17 (s, 3H), 0.98 (s, 6H), 0.86 (d, J = 6.8 Hz, 3H), 0.80 (s, 3H), 0.76 (d, J = 6.8 Hz, 3H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ: 182.9, 173.1, 150.3, 108.9, 56.8, 53.4, 48.8, 48.6, 44.1, 42.5, 40.6, 38.8, 38.2, 37.3, 35.7, 34.7, 33.3, 31.9, 29.7, 29.7, 28.6, 26.7, 22.9, 22.6, 21.3, 21.0, 18.6, 15.9, 15.7, 14.6, 14.5. ESI-HRMS (m/z) $[M + Na]^+$ calcd for $C_{31}H_{47}NNaO_3$, 504.3454, found 504.3462.

Compound 15. Diethyl oxalate (394 mg, 2.7 mmol) was added dropwise to a solution of **1** (228 mg, 0.5 mmol) and NaOMe (135 mg, 2.5 mmol) in THF (10 mL) under N_2. The reaction mixture was stirred for 12 h at room temperature and then poured into 1 N HCl (40 mL) and extracted with AcOEt (30 mL × 3). The organic layer was washed with brine, dried with anhydrous Na_2SO_4, and concentrated to afford crude product **11** as a colorless oil, which was used without further purification.

To a solution of **11** in ethanol (6 mL) was added hydroxylamine hydrochloride (69 mg, 1.0 mmol) at 23 °C. The reaction mixture was heated under reflux for 3 h. After cooling, the reaction mixture was concentrated. The residue was purified by silica gel chromatography (petroleum ether/AcOEt, 4/1 v/v) to give the desired compound **15** (193 mg, 70% over two steps) as a white solid, mp 297−299 °C (dec). ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 4.72 (s, 1H), 4.59 (s, 1H), 4.22 (q, J = 7.0 Hz, 2H), 2.99−2.88 (m, 2H), 2.32−2.26 (m, 1H), 2.14−2.12 (m, 1H), 1.99−1.95 (m, 1H), 1.82−1.79 (m, 2H), 1.67 (m, 4H), 1.57−1.22 (m, 19H), 1.11 (s, 3H), 0.97 (s, 3H), 0.92 (s, 3H), 0.70 (s, 3H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 182.7, 176.1, 160.9, 154.2, 150.2, 110.9, 109.8, 61.5, 56.3, 52.9, 49.1, 48.9, 46.8, 42.4, 40.6, 38.5, 38.4, 36.9, 36.0, 35.0, 33.1, 32.0, 30.4, 29.6, 28.4, 25.3, 21.2, 20.9, 19.2, 18.4, 16.1, 15.6, 14.5, 14.0. ESI-HRMS (m/z) $[M + Na]^+$ calcd for $C_{34}H_{49}NNaO_5$, 574.3508, found 574.3559.

Compound 16. To a solution of compound **15** (276 mg, 0.5 mmol) in ethanol (15 mL) was added $LiOH·H_2O$ (42 mg, 1 mmol) at 23 °C. The reaction mixture was stirred at room temperature for 12 h. The reaction mixture was poured into 1 N HCl (40 mL) and extracted with AcOEt (30 mL × 3). The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated. The residue was purified by silica gel chromatography (petroleum ether/AcOEt, 2/1 v/v) to give **16** (212 mg, 81%) as a white solid, mp 284−286 °C (dec). ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 4.71 (s, 1H), 4.57 (s, 1H), 2.97−2.95 (m, 1H), 2.72 (d, J = 16 Hz, 1H), 2.30−2.25 (m, 1H), 2.13−2.11 (m, 1H), 2.03 (d, J = 16 Hz, 1H), 1.81−1.78 (m, 2H), 1.69−1.64 (m, 4H), 1.58−1.53 (m, 3H), 1.45−1.41 (m, 6H), 1.34−1.28 (m, 4H), 1.25 (s, 3H), 1.23−1.16 (m, 1H), 1.14 (s, 3H), 1.04−1.02 (m, 1H), 0.96 (s, 3H), 0.92 (s, 3H), 0.74 (s, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 177.2, 175.2, 161.7, 154.8, 150.2, 110.4, 109.6, 55.4, 52.1, 48.5, 48.2, 46.6, 42.1, 40.2, 38.1, 37.7, 36.3, 35.6, 34.6, 32.8, 31.6, 30.1, 29.3, 28.3, 25.0, 21.1, 21.0, 18.9, 18.0, 16.0, 15.3, 14.3. ESI-HRMS (m/z) $[M - H]^-$ calcd for $C_{32}H_{44}NO_5$, 522.3219, found 522.3185.

Compound 17. To a solution of **1** (227 mg, 0.5 mmol) and sodium methoxide (143 mg, 2.5 mmol) in THF (10 mL) was added ethyl trifluoracetate (355 mg, 2.5 mmol) under N_2 at 23 °C. The mixture was stirred at 23 °C for 12 h. The reaction mixture was poured into 1 N HCl (50 mL) and extracted with AcOEt (30 mL × 3). The organic layer was washed with brine, dried with anhydrous Na_2SO_4, and concentrated to give crude product **7** as a pale yellow oil, which was used without further purification.

To a solution of **7** in ethanol (6 mL) was added hydrazine dihydrochloride (105 mg, 1.0 mmol) at 23 °C. The reaction mixture was heated under reflux for 3 h. After cooling, the mixture was concentrated. The residue was purified by silica gel chromatography (petroleum ether/AcOEt, 2/1 v/v) to give **17** as a white solid (142 mg 52% over two steps), mp 256−258 °C. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 13.2 (brs, 1 H), 12.1 (brs, 1 H), 4.70 (s, 1H), 4.57 (s, 1H), 2.97 (m, 1H), 2.58 (d, J = 15.2 Hz, 1H), 2.28 (m, 1H), 2.13 (d, J = 10 Hz, 1H), 1.99 (d, J = 15.2 Hz, 1H), 1.81 (m, 2H), 1.68 (s, 4H), 1.61−1.27 (m, 13H), 1.24 (m, 5H), 1.12 (s, 3H), 0.98 (s, 3H), 0.94 (s, 3H), 0.78 (s, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 177.4, 150.4, 148.0, 124.0, 121.3, 110.4, 109.7, 55.4, 52.2, 48.5, 48.3, 46.8, 42.1, 39.0, 37.9, 37.6, 36.3, 35.3, 34.2, 32.8, 31.6, 30.1, 29.3, 28.4, 25.1, 24.7, 20.9, 18.9, 18.3, 15.8, 15.3, 14.2. ESI-HRMS (m/z) $[M + Na]^+$ calcd for $C_{32}H_{45}F_3N_2NaO_2$, 569.3331, found 569.3359.

Compound 18. To a solution of **8** (prepared with the same procedure and scale as preparation of **12**) in ethanol (6 mL) was added hydrazine dihydrochloride (105 mg, 1 mmol) at 23 °C. The reaction mixture was heated under reflux for 3 h. After cooling, the mixture was concentrated. The residue was purified by silica gel chromatography (petroleum ether/AcOEt, 1/1 v/v) to give **18** (124 mg, 50% over two steps) as a white solid, mp 243−245 °C. ^{1}H NMR (CD_3OD, 500 MHz) δ: 4.70 (s, 1H), 4.58 (s, 1H), 2.99−2.94 (m, 1H), 2.37−2.26 (m, 2H), 2.13−2.11 (m, 1H), 1.81−1.80 (m, 2H), 1.66−1.65 (m, 4H), 1.61−1.23 (m, 14H), 1.15 (m, 6H), 1.04−1.02 (m, 4H), 0.96 (s, 3H), 0.91 (s, 3H), 0.84−0.82 (m, 1H), 0.72 (s, 3H). ^{13}C NMR (CD_3OD, 125 Hz) δ: 177.2, 158.6, 150.2, 146.7, 109.5, 95.6, 55.4, 52.7, 48.5, 48.4, 46.5, 42.0, 40.2, 38.2, 37.7, 36.3, 35.0, 33.0, 32.9, 31.6, 30.1, 29.3, 25.1, 22.9, 22.7, 20.9, 18.9, 18.4, 15.8, 15.3, 14.3. ESI-HRMS (m/z) $[M + H]^+$ calcd for $C_{31}H_{47}N_2O_3$, 495.3587, found 495.3602.

Compound 19. Ethyl formate (185 mg, 2.5 mmol) was added dropwise to a solution of **1** (227 mg, 0.5 mmol) and NaOMe (135 mg, 2.5 mmol) in THF (10 mL) under N_2 at 23 °C. The reaction mixture was stirred for 15 h at 23 °C and then poured into 1 N HCl (40 mL) and extracted with AcOEt (30 mL × 3). The organic layer was washed with brine, dried with anhydrous Na_2SO_4, and concentrated to afford crude product **9** as a yellow oil, which was used without further purification.

To a solution of **9** in ethanol (6 mL) was added phenylhydrazine hydrochloride (91 mg, 0.63 mmol) at 23 °C. The reaction mixture was heated under reflux for 3 h. After cooling, the mixture was concentrated. The residue was purified by flash chromatography (petroleum ether/AcOEt, 5/1 v/v) to afford **19** (202 mg, 73% over two steps) as a white solid, mp 279−281 °C. ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 12.09 (brs, 1H), 7.49−7.48 (m, 3H), 7.36−7.35 (m, 2H), 7.26 (s, 1H), 4.71 (s, 1H), 4.58 (s, 1H), 2.99−2.96 (m, 1H), 2.62−2.59 (m, 1H), 2.33−2.27 (m, 1H), 2.14−2.12 (m, 1H), 2.03−1.99 (m, 1H), 1.83−1.81 (m, 2H), 1.66 (s, 3H), 1.57−1.09 (m, 16H), 0.96 (s, 3H), 0.95 (s, 3H), 0.92 (s, 6H), 0.79 (s, 3H). ^{13}C NMR (CD_3OD, 100 MHz) δ: 177.5, 150.5, 145.6, 142.4, 137.9, 129.2 (2C), 129.1, 128.7 (2C), 113.6, 109.8, 55.6, 54.2, 48.8, 48.6, 46.6, 42.2, 40.2, 37.9, 37.8, 36.8, 36.4, 34.3, 33.1, 31.7, 30.2, 29.4, 29.0, 25.2, 22.1, 21.1, 19.1, 18.7, 15.8, 15.4, 14.4. ESI-HRMS (m/z) $[M + Na]^+$ calcd for $C_{37}H_{50}N_2O_2Na$, 577.3770, found 577.3798.

Compound 20. To a solution of **9** (prepared with the same procedure and scale as preparation of **19**) in ethanol (6 mL) was added hydrazine hydrochloride (105 mg, 1 mmol) at 23 °C. The reaction mixture was heated under reflux for 3 h. After cooling, the mixture was concentrated. The residue was purified by silica gel chromatography (petroleum ether/AcOEt, 1/1 v/v) to give **20** (168 mg, 70% over two steps) as a yellow powder, mp 257−259 °C. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 12.19 (brs, 2H), 7.10 (s, 1H), 4.70 (s, 1H), 4.57 (s, 1H), 3.00−2.94 (m, 1H), 2.53−2.50 (m, 1H), 2.31−2.26 (m, 1H), 2.14−2.12 (m, 1H), 1.91−1.88 (m, 1H), 1.84−1.78 (m, 2H), 1.65 (s, 3H), 1.62−1.19 (m, 18H), 1.08 (s, 3H), 0.96 (s, 3H), 0.92 (s, 3H), 0.85−0.83 (m, 1H), 0.70 (s, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 177.6, 150.5, 148.3, 133.5, 111.4, 109.8, 55.6, 53.2, 48.7, 48.6, 46.7, 42.1, 40.3, 38.3, 37.8, 36.4, 36.3, 33.1(2C), 31.8, 30.9, 30.2, 29.4,

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25.3, 23.6, 21.1, 19.0, 18.7, 15.8, 15.5, 14.4. ESI-HRMS (m/z) [M + H]$^+$ calcd for $C_{31}H_{47}N_2O_2$, 479.3638, found 479.3671.

Compound 21. To a solution of **6** (457 mg, 1 mmol) and sodium methoxide (270 mg, 5 mmol) in THF (10 mL) was added dropwise ethyl formate (370 mg, 5 mmol) under N_2 at 23 °C. The mixture was stirred at 23 °C for 5 h. The reaction mixture was poured into 1 N HCl (50 mL) and extracted with AcOEt (30 mL × 3). The organic layer was washed with brine, dried with anhydrous Na_2SO_4, and concentrated to give crude product **10** as a pale yellow oil, which was used without further purification.

To a solution of **10** in ethanol (10 mL) was added hydrazine dihydrochloride (210 mg, 2 mmol) at 23 °C. The reaction mixture was heated under reflux for 3 h. After cooling, the mixture was concentrated. The residue was purified by silica gel chromatography (petroleum ether/AcOEt, 1/1 v/v) to give **21** (337 mg, 70% over two steps) as a pale yellow solid, mp 259−261 °C. ^{1}H NMR (CD_3OD, 400 MHz) δ: 7.18 (s, 1H), 2.69−2.65 (m, 1H), 2.43−2.37 (m, 1H), 2.30−2.23 (m, 2H), 2.01−1.98 (m, 1H), 1.86−1.81 (m, 2H), 1.73−1.33 (m, 13H), 1.33−1.20 (m, 7H), 1.17 (s, 3H), 1.03−1.02 (m, 6H), 0.88 (d, J = 6.4 Hz, 3H), 0.82 (s, 3H), 0.79 (d, J = 6.8 Hz, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 180.2, 151.0, 134.4, 113.6, 58.0, 55.0, 50.4, 50.0, 45.6, 43.8, 42.0, 39.8, 39.6, 38.6, 37.8, 34.9, 34.6, 33.3, 31.4, 31.1, 31.0, 28.5, 24.1, 23.8, 23.5, 22.8, 20.3, 16.5, 16.5, 15.2(2C). ESI-HRMS (m/z) [M + H]$^+$ calcd for $C_{31}H_{49}N_2O_2$, 481.3794, found 481.3793.

Compound 22. To a solution of **11** (prepared with the same procedure and scale as preparation of **16**) in ethanol (6 mL) was added hydrazine dihydrochloride (105 mg, 1 mmol) at 23 °C. The reaction mixture was heated under reflux for 3 h. After cooling, the mixture was concentrated. The residue was purified by silica gel chromatography (petroleum ether/AcOEt, 2/1 v/v) to give **22** (169 mg, 61% over two steps) as a white powder, mp 282−284 °C. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 13.13 (brs, 1H), 12.09 (brs, 1H), 4.72 (s, 1H), 4.59 (s, 1H), 4.22 (q, J = 6.5 Hz, 2H), 2.98−2.88 (m, 2H), 2.32−2.27 (m, 1H), 2.14−2.12 (m, 1H), 1.99−1.96 (m, 1H), 1.83−1.81 (m, 2H), 1.67−1.66 (m, 4H), 1.62−1.01 (m, 26H), 0.98 (s, 3H), 0.93 (s, 3H), 0.79 (s, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 177.2, 170.0, 162.1, 150.3(2C), 115.4, 109.6, 59.5, 55.5, 52.4, 48.5, 48.4, 46.6, 42.1, 40.2, 37.9, 37.7, 37.0, 36.3, 32.9, 32.9, 31.7, 30.5, 30.1, 29.3, 25.1, 23.0, 20.9, 19.0, 18.4, 16.0, 15.3, 14.3(2C). ESI-HRMS (m/z) [M + H]$^+$ calcd for $C_{34}H_{51}N_2O_4$, 551.3849, found 551.3849.

Compound 23. To a solution of compound **22** (551 mg, 1 mmol) in ethanol (15 mL) was added LiOH·H_2O (84 mg 2 mmol) at 23 °C. The reaction mixture was stirred at 23 °C for 12 h and then poured into 1 N HCl (40 mL) and extracted with AcOEt (30 mL × 3). The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated. The residue was purified by silica gel chromatography (petroleum ether/AcOEt, 1/1 v/v) to give **23** (422 mg, 81%) as a white solid, mp 292−296 °C (dec). ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 4.72 (s, 1H), 4.58 (s, 1H), 2.99−2.92 (m, 2H), 2.33−2.28 (m, 1H), 2.15−2.13 (m, 1H), 1.99−1.95 (m, 1H), 1.83−1.82 (m, 2H), 1.67 (s, 3H), 1.62−1.01 (m, 24H), 0.97 (s, 3H), 0.94 (s, 3H), 0.72 (s, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 177.4, 163.4, 150.4, 150.3, 136.7, 115.7, 109.7, 55.6, 52.6, 48.6, 46.7, 42.1, 40.4, 37.9, 37.8, 37.2, 36.5, 33.2, 33.1, 33.0, 31.8, 30.7, 30.2, 29.4, 25.3, 23.4, 21.2, 19.1, 18.6, 16.2, 15.5, 14.4. ESI-HRMS (m/z) [M − H]$^-$ calcd for $C_{32}H_{45}N_2O_4$, 521.3379, found 521.3393.

Compound 24. To a suspension of **1** (455 mg, 1 mmol) and K_2CO_3 (280 mg, 2.0 mmol) in DMF (10 mL) was added dropwise CH_3I (1 mL) at 23 °C. The reaction mixture was heated to 40 °C and kept at this temperature for 5 h. The reaction mixture was poured into H_2O (50 mL) and extracted with AcOEt (30 mL × 3). The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated. The residue was purified by silica gel chromatography (petroleum ether/AcOEt, 10/1 v/v) to give **24** (421 mg, 90%) as a white solid, mp 239−241 °C. ^{1}H NMR ($CDCl_3$, 400 MHz) δ: 4.73 (s, 1H), 4.60 (s, 1H), 3.67 (s, 3H), 3.06−3.03 (m, 1H), 2.48 (m, 1H), 2.41(m, 1H), 2.21 (m, 3H), 1.68 (s, 3H), 1.52−1.08 (m, 17H), 1.06 (s, 3H), 1.01 (s, 3H), 0.97 (s, 3H), 0.95 (s, 3H),0.91 (s, 3H).

Compound 26. To a solution of compound **24** (235 mg, 0.5 mmol) in dry THF (20 mL) was added semicarbazide hydrochloride (112 mg, 1 mmmol) and TEA (1 mL) at 23 °C. The reaction mixture was stirred at 23 °C for 12 h and then poured into H_2O (50 mL) and extracted with AcOEt (30 mL × 3). The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated to give crude product **25**, which was used without further purification.

The crude product **25** was dissolved in $SOCl_2$ (10 mL), and the reaction mixture was heated under reflux for 4 h. After cooling, the mixture was concentrated. The residue was purified by silica gel chromatography (petroleum ether/AcOEt, 5/1 v/v) to give **26** (115 mg, 45% over two steps) as a pale yellow solid. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 4.76 (s, 1H), 4.62 (s, 1H), 3.67 (s, 3H), 3.16−3.19 (m, 1H), 2.30−2.27 (m, 3H), 1.90 (m, 2H), 1.70−1.02 (m, 24H), 1.01 (s, 3H), 0.99 (s, 3H), 0.95 (s, 3H), 0.79 (s, 3H).

Compound 27. To a solution of **26** (115 mg, 0.23 mmol) in dry DMF (15 mL) was added anhydrous lithium iodide (308 mg, 2.3 mmol) at 23 °C. The reaction mixture was heated under reflux for 48 h under N_2. After cooling, the mixture was poured into H_2O (40 mL) and extracted with AcOEt (30 mL × 3). The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated. The residue was purified by silica gel chromatography (petroleum ether/AcOEt, 1/1 v/v) to give **27** (80 mg, 71%) as a brown solid, mp 255−257 °C. ^{1}H NMR ($CDCl_3$, 500 MHz,) δ: 4.75 (s, 1H), 4.62 (s, 1H), 3.19−3.02 (m, 2H), 2.30−2.67 (m, 3H), 1.99−1.98 (m, 2H), 1.88−1.04 (m, 25H), 1.00 (m, 6H), 0.80 (s, 3H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 182.5, 165.6, 150.3, 145.5, 109.8, 56.3, 52.4, 49.0, 48.9, 46.8, 42.4, 40.7, 38.6, 38.2, 37.3, 36.9, 36.2, 33.1, 31.9, 31.1, 30.4, 29.6, 25.3, 23.4, 21.3, 19.3, 19.1, 16.0, 15.5, 14.5. ESI-HRMS (m/z) [M + Na]$^+$ calcd for $C_{30}H_{44}N_2NaO_2S$, 519.3021, found 519.3042.

Compound 29. To a solution of **24** (235 mg, 0.5 mmol) and sodium methoxide (135 mg, 2.5 mmol) in THF (10 mL) was added ethyl formate (185 mg, 2.5 mmol) at 23 °C. The mixture was stirred at 23 °C for 5 h and then poured into 1 N HCl (50 mL) and extracted with AcOEt (30 mL × 3). The organic layer was washed with brine, dried with anhydrous Na_2SO_4, and concentrated to afford crude product **28** as a colorless oil, which was used without further purification.

To a solution of **28** in ethanol (6 mL) was added hydrazine dihydrochloride (80 mg, 0.76 mmol) at 23 °C. The reaction mixture was heated under reflux for 3 h. After cooling, the mixture was concentrated. The residue was purified by silica gel chromatography (petroleum ether/AcOEt, 2/1 v/v) to give **29** (162 mg, 66%) as a pale yellow solid, mp 249−251 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.22 (s, 1H), 4.76 (s, 1H), 4.62 (s, 1H), 3.68 (s, 3H), 3.05−3.00 (m, 1H), 2.64−2.61 (m, 1H), 2.26−2.24 (m, 2H), 1.96−1.71 (m, 4H), 1.70 (s, 3H), 1.68−1.60 (m, 1H), 1.58−1.32 (m, 11H), 1.28−1.24 (m, 5H), 1.22−1.19 (m, 1H), 1.18 (s, 3H), 1.10−1.05 (m, 1H), 1.00 (s, 3H), 0.98 (s, 3H), 0.79 (s, 3H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 176.7, 150.6 (2C), 135.2, 112.6, 109.6, 56.6, 53.4, 51.3, 49.4, 49.2, 46.9, 42.4, 40.7, 38.6, 38.3, 36.9, 36.6, 33.4 (2C), 32.1, 31.1, 30.6, 29.8, 25.6, 23.9, 21.4, 19.4, 19.1, 15.9, 15.6, 14.7. ESI-HRMS (m/z) [M + Na]$^+$ calcd for $C_{32}H_{48}N_2NaO_2$, 515.3613, found 515.3619.

Compound 30. To a solution of **28** (prepared with the same procedure and scale as in preparation of **29**) in ethanol (10 mL) was added hydroxyamine hydrochloride (52 mg, 0.75 mmol) at 23 °C. The mixture was heated under reflux for 3 h. After cooling, the mixture was concentrated. The residue was purified by silica gel chromatography (petroleum ether/AcOEt, 5/1 v/v) to give **30** (158 mg, 64%) as a white solid, mp 243−245 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.52 (s, 1H), 4.74 (s, 1H), 4.60 (s, 1H), 3.67 (s, 3H), 3.02−2.92 (m, 2H), 2.27−2.19 (m, 3H), 1.90−1.87 (m, 2H), 1.72−1.68 (m, 4H), 1.61−1.14 (m, 15H), 1.12 (s, 3H), 1.04 (s, 6H), 0.95 (s, 3H), 0.91 (s, 3H). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 176.5, 172.9, 150.4, 150.2, 109.6, 108.8, 56.5, 53.5, 51.2, 49.3, 49.1, 46.8, 42.3, 40.6, 38.8, 38.2, 36.8, 35.7, 34.7, 33.2, 32.0, 30.5, 29.7, 28.6, 25.4, 21.3, 21.1, 19.3, 18.7, 16.0, 15.6, 14.6. ESI-HRMS (m/z) [M + H]$^+$ calcd for $C_{32}H_{48}NO_3$, 494.3629, found 494.3619.

Compound 32. To a solution of **24** (470 mg, 1.0 mmol) in toluene (10 mL) was added *N*,*N*-dimethylformamide dimethyl acetal (0.7 mL, 5.0 mmol) at 23 °C. The reaction mixture was heated under

reflux for 12 h. After cooling, the mixture was concentrated to give crude product **31**, which was used without further purification.

To a solution of **31** and sodium methoxide (54 mg, 1.0 mmol) in absolute ethanol (20 mL) was added acetamidine acetate (118 mg, 1.0 mmol) at 23 °C. The reaction mixture was heated under reflux for 3 h under N_2. After cooling, the mixture was concentrated. The residue was purified by silica gel chromatography (petroleum ether/AcOEt, 10/1 v/v) to give **32** (230 mg, 44% over two steps) as a pale yellow solid, mp 249−251 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 8.16 (s, 1H), 4.76 (s, 1H), 4.62 (s, 1H), 3.68 (s, 3H), 3.01 (m, 1H), 2.69 (d, J = 16 Hz, 1H), 2.65 (s, 3H), 2.29 (m, 2H), 2.18 (d, J = 15.6 Hz, 1H), 1.89 (m, 2H), 1.79 (m, 1H), 1.69 (s, 3H), 1.61−1.28 (m, 22H), 1.27 (s, 3H), 1.22 (s, 3H), 1.14−1.00 (m, 3H), 0.98 (s, 3H), 0.91 (s, 3H), 0.75 (s, 3H). ESI-HRMS (m/z) $[M + H]^+$ calcd for $C_{34}H_{51}N_2O_2$, 519.3945, found 519.3948.

Compound 33. To a solution of **31** (prepared with the same procedure and scale as preparation of **32**) and sodium methoxide (54 mg, 1.0 mmol) in absolute ethanol (20 mL) was added guanidine hydrochloride (96 mg, 1 mmol) at 23 °C. The reaction mixture was heated under reflux for 3 h. After cooling, the reaction mixture was concentrated. The residue was purified by silica gel chromatography (DCM/MeOH, 20/1 v/v) to give **33** (249 mg, 48%), mp 251−253 °C. ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.86 (s, 1H), 4.83 (brs, 2H), 4.75 (s, 1H), 4.61 (s, 1H), 3.68 (s, 3H), 3.02−2.99 (m, 1H), 2.60−2.56 (m, 1H), 2.26−2.24 (m, 2H), 2.09−2,04 (m, 2H), 1.89 (m, 3H), 1.76 (m, 3H), 1.67 (m, 3H), 1.61−1.14 (m, 14H), 1.14 (s, 3H), 0.99 (s, 3H), 0.98 (s, 3H), 0.75 (s, 3H). ^{13}C NMR (CDCl3, 125 MHz) δ: 177.0, 174.0, 162.2, 158.7, 150.8, 117.5, 109.9, 56.8, 53.3, 51.4, 49.6, 49.0, 47.1, 42.6, 42.0, 40.7, 39.4, 38.5, 37.1, 36.5, 33.6, 32.3, 30.9, 30.7, 29.8, 25.7, 23.4, 21.6, 20.2, 19.5, 15.7, 15.5, 14.8. ESI-HRMS (m/z) $[M + H]^+$ calcd for $C_{33}H_{50}N_3O_2$, 520.3903, found 520.3907.

Compound 34. To a solution of **32** (230 mg, 0.44 mmol) in dry DMF (15 mL) was added lithium iodide (590 mg, 4.4 mmol) at 23 °C. The reaction mixture was heated under reflux for 8 h under N_2. After cooling, the mixture was poured into H_2O (20 mL) and extracted with AcOEt (30 mL × 3). The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated. The residue was purified by silica gel chromatography (petroleum ether/AcOEt, 4/1 v/v) to give **34** (115 mg, 52%) as a white solid, mp 256−258 °C. ^{1}H NMR ($CDCl_3$, 500 Hz) δ: 8.21 (s, 1H), 4.76 (s, 1H), 4.62 (s, 1H), 3.08−3.03 (m, 1H), 2.73−2.72 (m, 1H), 2.67 (s, 3H), 2.33−2.31 (m, 2H), 2.21−2.18 (m, 1H), 2.01−1.99 (m, 2H), 1.80−1.77 (m, 1H), 1.71 (s, 3H), 1.68−1.28 (m, 18H), 1.22 (s, 3H), 1.02 (s, 3H), 1.01 (s, 3H), 0.75 (s, 3H). ^{13}C NMR (CDCl3, 125 MHz) δ: 181.1, 172.9, 165.3, 157.2, 150.5, 124.1, 109.7, 56.3, 53.1, 49.2, 48.8, 46.9, 42.5, 42.0, 40.6, 39.3, 38.4, 37.0, 36.2, 33.3, 32.1, 30.8, 30.6, 29.7, 29.6, 25.5, 25.3, 23.3, 21.4, 19.9, 19.3, 15.6, 15.5, 14.6. ESI-HRMS (m/z) $[M + H]^+$ calcd for $C_{33}H_{49}N_2O_2$, 505.3794, found 505.3838.

Compound 35. To a solution of **33** (125 mg, 0.24 mmol) in dry DMF (15 mL) was added lithium iodide (322 mg, 2.4 mmol) and *n*-octylamine (310 mg, 2.4 mmol) at 23 °C. The reaction mixture was heated under reflux for 48 h under N_2. After cooling, the reaction mixture was poured into H_2O (20 mL) and extracted with AcOEt (30 mL × 3). The organic layer was washed with brine, dried over anhydrous Na_2SO_4, and concentrated. The residue was purified by silica gel chromatography (DCM/MeOH, 10/1, v/v) to give **35** (79 mg, 65%) as a yellow solid, mp 265−267 °C. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 12.07 (s, 1H), 7.84 (s, 1H), 6.17 (brs, 2H), 4.71 (s, 1H), 4.58 (s, 1H), 2.99−2.94 (m, 1H), 2.56−2.50 (m,1H), 2.31−2.27 (m, 1H), 2.14−2.12 (m, 1H), 2.05−1.98 (m, 1H), 1.82−1.81 (m, 2H), 1.69−1.66 (m, 4H), 1.59−1.23 (m, 15H), 1.17 (s, 3H), 1.10 (s, 3H), 0.97 (s, 3H), 0.93 (s, 3H), 0.69 (s, 3H). ^{13}C NMR (DMSO-d_6, 125 MHz) δ: 177.2, 171.8, 162.4, 158.5, 150.3, 115.1, 109.6, 55.4, 52.4, 48.5, 48.1, 46.6, 42.0, 40.9, 40.1, 40.0, 39.8, 39.7, 39.5, 39.3, 39.2, 39.0, 38.8, 37.7, 36.3, 35.8, 30.6, 23.1, 19.0, 15.4, 15.2, 14.4. ESI-HRMS (m/z) $[M + H]^+$ calcd for $C_{32}H_{48}N_3O_2$, 506.3747, found 506.3764.

In Vitro Osteoclastogenesis Assay. RAW264.7 cells (kindly provided by Dr. Bryant G. Darnay, University of Texas MD Anderson Cancer Center, Houston, TX, U.S.) were seeded at 3 × 10^3 cells per well using 96-well plates and cultured for 24 h in αMEM with 10% fetal bovine serum (FBS). Thereafter, the cell medium was changed with same medium containing RANKL (50 ng/mL) and various concentrations of tested compounds. After 3 days, cells were fixed and stained for tartrate-resistant acid phosphate (TRAP) (Sigma) activity according to the recommendation of the manufacturer. $TRAP^+$ multinucleated cells with more than three nuclei were counted as osteoclasts.

Cytotoxicity Assay of Compound 20 on Precursor Osteoclasts. The proliferation effect of **20** was determined by the MTS method as described previously[20] with a VersaMax microplate reader (Molecular Devices).

Compound 20 Inhibits the Expression of Osteoclast Mark Gene TRAP and Cathepsin K. For the real-time RT-PCR analysis, total RNA was extracted from cells with TRIzol (Invitrogen). Cathepsin K and TRAP transcripts were quantified on Mx 3005P (Stratagene) using SYBR green dye and normalized with β-actin. The following primer sets were used: mouse TRAP, (forward) 5′-GCTGGAAACCATGATCACCT-3′, (reverse) 5′-GAGTTGCCACA-CAGCATCAC-3′; mouse cathepsin K, (forward) 5′-CTTCCAA-TACGTGCAGCAGA-3′, (reverse) 5′-TCTTCAGGGCTTTCTC-GTTC-3′.

In Vivo Experiments and Bone Histomorphometry. Eight-week-old C57/BL6 mice were purchased from SIBS (Shanghai, China). Five days after ovariectomy, mice were divided into three groups (n = 8): sham-operated mice (Sham) and ovariectomized mice treated with vehicle (OVX) or with **20** (10 mg/kg) every 2 days (OVX + **20**) for 90 days. Sham and OVX groups were intraperitoneally (ip) injected with 50 μL of DMSO. The OVX + **20** group was intraperitoneally injected with **20** that was dissolved in 50 μL of DMSO. We also found that the weight of all the tested mice of the three groups has no big difference after 90 days. After then, all mice were euthanized with excess amounts of anesthetic. Von Kossa staining was performed on the sections of lumbar vertebrae (L3) from sham-operated mice, OVX mice, and OVX + **20** mice as described previously.[20] Bone value/total value (BV/TV), trabecular number (Tb.N), and trabecular space (Tb.Sp) were analyzed by histomorphometric analysis using the OsteoMeasure analysis system (Osteometrics, Atlanta, GA, U.S.) according to standard criteria. For in vivo osteoclast activity, TRAP assay was performed. Tibia bones were fixed in 10% paraformaldehyde (PFA), and decalcification was achieved by 10% EDTA immersion for 2 weeks. The samples were embedded in paraffin and sectioned for TRAP staining (Sigma) as described previously.[20] Mouse maintenance, use, and treatment were in accordance with accepted standards of the Ethics Committee of ECNU.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Further chemical and biological experimental details, including HPLC data and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

BA, betulinic acid; TRAP, tartrate-resistant acid phosphatase; ERT, estrogen-replacement therapy; BP, bisphosphonate; PTH, parathyroid hormone; RANK, receptor activator of NF-κB; OPG, osteoprotegerin; SWH, *Sambucus williamsii* Hance; AKBA, acetyl-11-keto-β-boswellic acid; ACCX, 25-acetylcimigenol xylopyranoside; ALP, alkaline phosphatase; IBX, 2-iodoxybenzoic acid; DMSO, dimethyl sulfoxide; DMF, *N*,*N*-dimethylformamide

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