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Substituted Benzothiophene or Benzofuran Derivatives as a Novel Class of Bone Morphogenetic Protein-2 Up-Regulators: Synthesis, Structure−Activity Relationships, and Preventive Bone Loss Efficacies in Senescence Accelerated Mice (SAMP6) and Ovariectomized Rats

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In this work, substituted benzothiophene and benzofuran compounds were found to be a new class of potential anabolic agents by enhancing BMP-2 expression. A series of benzothiophene and benzofuran derivatives have been synthesized, and their activities of up-regulating BMP-2 and bone loss prevention efficacies in SAMP6 mice and OVX rats have been studied. Benzothiophenes **1**, **3**, **14**, **4a**, **7a**, **8a**, and benzofuran analogue **2** showed higher BMP-2 up-regulation rates in vitro. Compound **8a** was found to significantly affect the bone formation rate of tested SAMP6 mice. Compound **1** showed an improved bone quality in SAMP6 mice and also showed activity in OVX rats. We have demonstrated that substituted benzothiophene and benzofuran derivatives, especially compounds **1** and **8a**, enhance BMP-2 expression in vitro and in vivo and stimulate bone formation and trabecular connectivity restoration in vivo. The compounds represent potential leads in the development of a new class of anabolic agents.

Introduction

Osteoporosis is a worldwide health problem, observed most frequently in postmenopausal women and in aging men. The number of people at risk is growing as the population of elderly increases. Agents for the treatment of osteoporosis are classified as either antiresorptive or anabolic. Antiresorptive agents, a basic treatment for osteoporosis in the clinic, prevent bone loss by inhibiting the activity of osteoclasts and, therefore, reducing bone resorption. Anabolic agents act by stimulating the formation of new bone and thus provide an additional option for osteoporosis patients and represent a major advance in the treatment of osteoporosis.[1−6] Anabolic agents are capable of increasing bone mass to a greater degree than antiresorptive agents and also have the capacity to improve bone quality and increase bone strength.[1] The recombinant human PTH (teriparatide) is the only FDA-approved anabolic agent, which is currently available in the U.S. and Europe in the forms of PTH (1−34) and PTH (1−84), respectively. Because of the paucity of available anabolic agents for osteoporosis treatment, there is an urgent need to develop small molecular compounds to treat this disease that are nontoxic, cost-effective, and easy to administer.[7]

Bone morphogenetic protein-2 (BMP-2) is expressed by normal osteoblasts and is a crucial regulator of osteogenic differentiation that has been shown to stimulate osteoblast differentiation and osteogenic nodule formation in vitro, as well as bone formation in vivo. BMP-2 plays an important role in osteoblast differentiation and bone generation and regeneration.[3,8] Human BMP-2 induces structurally sound orthotopic bone in a variety of experimental systems, including femoral defects in rats, tibial and ulnar defects in rabbits, femoral defects in sheep, mandibular defects in dogs, spinal fusion in dogs, and porous in-growth in rats.[9] Recently, results indicate the high potency of BMP-2 as an inducer of osteogenesis and that BMP-2 may be a novel therapeutic agent for diseases associated with bone loss and requiring bone repair.[10] Transfer of BMP-2 cDNA with a recombinant adenovirus into an established osteoporotic sheep model enhanced fracture healing.[11] Recombinant human bone morphogenetic protein-2 (rhBMP-2) can accelerate the repair of bone defects and has three FDA approvals for use in orthopedics.[12]

An alternative approach to exploit the beneficial actions of BMP-2 on bone formation is to discover agents that activate BMP-2 gene expression in bone cells. Mundy et al.[13] reported an assay to search for small molecules that activate the promoter of the *BMP-2* gene. In this report, it was demonstrated that the effects of statins to enhance new bone formation in vitro and in rodents were associated with an increase in the expression of the *BMP-2* gene in bone cells. Following this report, many known bioactive compounds have been reported to increase the gene expression and/or protein expression of BMP-2. Estrogen replacement therapy is one of the most common and effective strategies used to prevent osteoporosis in postmenopausal women. Estrogens are believed to work exclusively by inhibiting bone resorption. The results of Zhou[10] demonstrated that estrogens increase mouse BMP-2 mRNA, suggesting that estrogens may also enhance bone formation. Resveratrol, a naturally occurring compound possessing estrogenic activity, is thought to have considerable potential as a therapy against osteoporosis. The results of Chang[14] indicate that piceatannol stimulates osteoblast differentiation at various stages (from maturation to terminally differentiated osteoblasts), and the induction of differentiation by piceatannol

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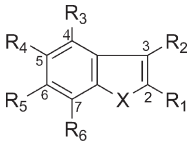
Table 1. Structures and the Up-Regulated Activities of BMP-2 Expression (UpR) of the Tested Benzothiophene and Benzofuran Analogues[a] in Vitro



compd	X	R_1	R_2	R_3	R_4	R_5	R_6	UpR (%)
1	S	COMe	H	H	H	H	H	35.6
2	O	COMe	H	H	H	H	H	33.5
3	S	COMe	H	H	COOMe	H	H	41.4
14	S	COMe	H	H	OMe	OMe	H	37.1
1c	S	*2E*-3-phenylpropenone	H	H	H	H	H	15.4
1d	S	2*E*-3-(2-chloro-phenyl)propenone	H	H	H	H	H	2.0
1e	S	*2E*-3-(4-methoxyphenyl)propenone	H	H	H	H	H	27.3
2c	O	*2E*-3-phenyl-propenone	H	H	H	H	H	24.4
2d	O	2*E*-3-(2-chloro-phenyl)propenone	H	H	H	H	H	1.8
3c	S	*2E*-3-phenylpropenone	H	H	$COOC_2H_5$	H	H	12.6
3d	S	*2E*-3-(2-chlorophenyl)propenone	H	H	$COOC_2H_5$	H	H	24.0
3e	S	*2E*-3-(4-methoxyphenyl)propenone	H	H	$COOC_2H_5$	H	H	1.7
3f	S	*2E*-3-(3,4-dimethoxyphenyl)propenone	H	H	$COOC_2H_5$	H	H	−9.6
3g	S	*2E*-3-(2,3,4-trimethoxyphenyl)propenone	H	H	$COOC_2H_5$	H	H	−21.5
4a	S	$COOC_2H_5$	H	H	COOMe	H	H	49.8
5a	S	$COOC_2H_5$	H	H	H	H	H	8.2
6a	S	$COOC_2H_5$	H	Cl	H	H	H	−0.6
7a	S	$COOC_2H_5$	H	H	OMe	OMe	H	49.9
8a	S	$COOC_2H_5$	H	H	-OCH_2O-		H	64.2
9a	O	$COOC_2H_5$	H	H	H	H	H	7.1
10a	O	$COOC_2H_5$	H	H	Cl	H	Cl	−1.6
11a	O	$COOC_2H_5$	H	H	H	H	F	−18.1
12a	O	$COOC_2H_5$	H	H	H	H	OMe	12.4
5b	S	$COCH_2SOMe$	H	H	H	H	H	19.7
6b	S	$COCH_2SOMe$	H	Cl	H	H	H	18.5
7b	S	$COCH_2SOMe$	H	H	OMe	OMe	H	13.5
8b	S	$COCH_2SOMe$	H	H	-OCH_2O-		H	0.6
9b	O	$COCH_2SOMe$	H	H	H	H	H	3.9
5c	S	$COCH_2SO_2Me$	H	H	H	H	H	5.1
8c	S	$COCH_2SO_2Me$	H	H	-OCH_2O-		H	−9.3
Lov[b]								20.9

[a] Compound concentrations were 4.0 μM. [b] Concentration of Lov was 0.4 μM.

was associated with an increase in BMP-2 production. Flavonoids[15−17] and coumarin derivatives,[18−20] present in many medicinal plants, have a direct stimulatory effect on the proliferation and differentiation of cultured human osteoblast cells in vitro. This proliferative and differentiation effect is mediated by increasing production of BMP-2 in the human osteoblasts.

The BMP pathway is a promising new area to target therapeutic agents for the treatment of low bone mass. In our research, a new class of small molecules, substituted benzothiophene or benzofuran derivatives, were found to be potential anabolic agents targeting BMP-2. We have previously established a novel cell-based high-throughput screen assay for the identification of BMP-2 up-regulators.[21] The assay used murine calvarial MC3T3-E1 cells that were stably transfected with pGL4-BMP2, a firefly luciferase reporter gene fused downstream of the 5′-flanking promoter region of the mouse *BMP-2* gene. We tested more than 13 000 samples from a synthetic and fermentation products collection and identified that 1-(benzo[*b*]thiophen-2-yl)thanone (**1**) and 1-(benzo[*b*]furan-2-yl)thanone (**2**) specifically enhanced the expression of *BMP-2* in cultured cells, which increased promoter activity in a dose-dependent manner and stimulated the expression of BMP-2 at both the RNA and protein levels.

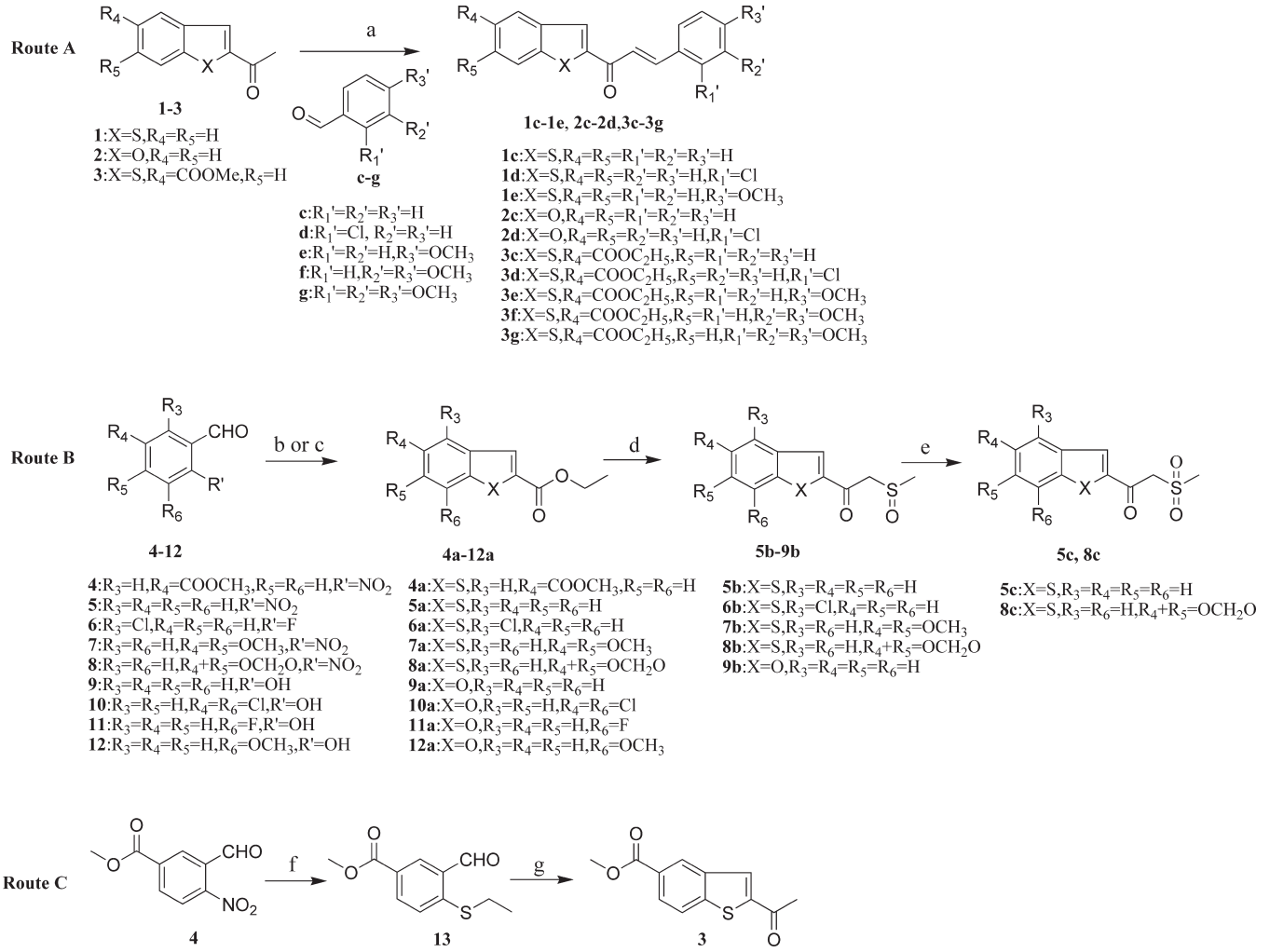
In this work, to identify whether the substituted benzothiophene or benzofuran derivatives have the potency to be developed as new clinical anabolic agents by increasing BMP-2 expression, we synthesized a novel series of benzo[*b*]thiophen and benzo[*b*]furan derivatives and evaluated their ability to enhance *BMP-2* expression in vitro. Moreover, we tested the activity of the compounds in preventing bone loss efficacy in SAMP6 mice and in OVX rats. The in vitro structure−activity relationships of the compounds were also investigated.

Chemistry

Our previous study revealed that the C-3 methyl group in the benzothiophene or benzofuran moiety did not enhance BMP-2 expression.[22] In the present study, we retained the hydrogen atom at the C-3 position and focused an SAR analysis on the variations of the C-2 groups as well as substituents at the 4, 5, 6, and 7 positions of the phenyl ring. The effect of changing the sulfur atom to an oxygen atom in the five-membered heterocycles was also investigated. On the basis of this strategy, 20 substituted benzothiophene analogues and 7 substituted benzofuran analogues were designed and synthesized (Table 1). The synthetic routes are described in Scheme 1. Compound **14** was synthesized using the method in ref 22.

Scheme 1 includes three synthetic routes. Desired compounds (**1c**−**e**, **2c**,**d**, **3c**−**g**) with different cinnamoyl groups

Scheme 1. Synthesis of Substituted Benzothiophene and Benzofuran Derivatives[a]



[a] Reagents and conditions: (a) C_2H_5ONa, C_2H_5OH, room temperature; (b) $HSCH_2COOC_2H_5$, K_2CO_3, DMF, 60−100 °C; (c) $BrCH_2COOC_2H_5$, K_2CO_3, DMF, 60−100 °C; (d) DMSO, NaH, THF, 70−75 °C; (e) 30% H_2O_2, acetic acid, 0 °C; (f) $HSCH_2CH_3$, K_2CO_3, DMF, room temperature; (g) $ClCH_2COCH_3$, CaO, DMF, 90 °C.

at the C-2 position and diverse substituents on the phenyl ring were synthesized according to route A, using **1**, **2**, and **3** (compounds **1** and **2** are commercially available; **3** was synthesized by route C) as the starting materials. Compounds **1**−**3** reacted with substituted benzaldehyde in the presence of sodium ethoxide to afford the desired products (**1c**−**e**, **2c**,**d**, **3c**−**g**). Compounds **2c** and **2d** were derivatives of **2**, while compounds **1c**−**e** and **3c**−**g** were derivatives of **1**.

In route B, compounds **4**−**12** were commercial available starting materials. Desired products **4a**−**8a** were obtained through the reaction of **4**−**8** with ethyl thioglycolate, respectively, in the presence of anhydrous potassium carbonate at 60−100 °C.[23] Compounds **9a**−**12a** were obtained using a similar method employed to produce **4a**−**8a**, with the starting materials **9**−**12** and ethyl bromoacetate.[24,25] Compounds **5b**−**9b** were obtained with **5a**−**9a** and methylsulfinyl carbanion ($CH_3SOCH_2^-$), which was prepared from dimethyl sulfoxide and sodium hydride (NaH).[26] The reaction should be conducted with rigorous exclusion of oxygen, carbon dioxide, and water, since the methylsulfinyl carbanion reacts rapidly with these substances. Oxidation of **5b** and **8b** with 30% H_2O_2 in acetic acid at 0 °C afforded **5c** and **8c**.

Compound **3** was synthesized by route C using commercially available **4** as the starting material, which reacted with ethyl mercaptide in dimethylformamide (DMF) at room temperature in the presence of anhydrous potassium carbonate to yield intermediate **13**. Compound **13** was then reacted with chloroacetone in DMF at 90 °C in which the reaction was catalyzed using calcium oxide to yield the desired compound **3**.

Results and Discussion

In Vitro Activities of Up-Regulated BMP-2 Expression and SAR Analysis. All of the 28 synthesized benzothiophene or benzofuran derivatives were examined for the up-regulation of the *BMP-2* promoter activities in vitro at 4.0 μM. Lovastatin (Lov, 0.4 μM) was the positive control, and the negative control was 0.1% DMSO. The in vitro assay was performed following the reported method.[21] BMP-2 up-regulation rates (UpR[a]) were defined as $(RLU_{\text{test compound}} - RLU_{\text{negative control}}/RLU_{\text{negative control}}) \times 100$, in which RLU is the relative luminescence units. Structures of the tested

[a] Abbreviations: UpR, up-regulation rate; TBV%, trabecular bone volume percentage; TFS%, trabecular formation surface percentage; TRS%, trabecular resorption surface percentage; OSW, average osteoid width; MAR, mineral apposition rate; mAR, osteo cortex mineralization rate; ROI, regions of interest.

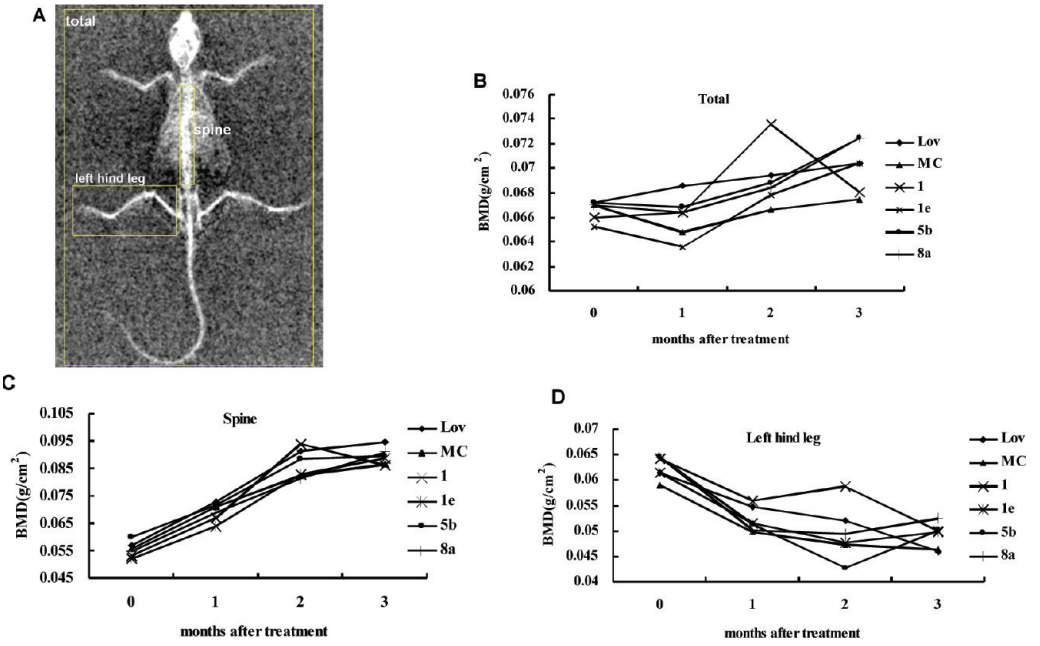


Figure 1. ROIs in which BMD values were measured (A). The BMD data of whole body (total, B), spine (C), and left hind leg (D) of each group at 0, 1, 2, and 3 months after treatment were collected.

compounds and their up-regulation activities are shown in Table 1. Six compounds (**8a**, **7a**, **5a**, **3**, **2**, and **1**) showed higher UpR (over 30%) than Lov (UpR was about 21%), of which the activities of compounds **8a**, **7a**, **5a**, and **3** (UpR over 40%) were higher than that of compound **1** (UpR was 35.6%).

The SAR study was initially focused on the 1-(benzo-[*b*]thiophen/furan-2-yl)thanone analogues (compounds **1**, **3**, and **14**). Previous studies have indicated that electron donating groups on the phenyl ring cause a higher UpR than electron-withdrawing groups when R_1 was acetyl. The UpR of compound **3** was higher than the lead compounds **1** and **14** with two methoxy groups on the phenyl ring. This implied that the phenyl ring substituted by a methyl carboxylate may be beneficial to the activity of enhancing BMP-2 expression.

All the UpRs of the generated analogues **1c**−**e**, **2c**,**d**, and **3c**−**g** were significantly reduced when the acetyl group at the C-2 position in compounds **1**, **2**, and **3** was replaced with cinnamoyl groups. Compared with **1c** (UpR = 15.4%), the UpR of **1e** (27.3%) was much higher; however, the activity of **1d** (UpR = 2.0%) decreased. The electron donating group was better than the electron-withdrawing group in this system. The UpR of compound **2d** was 1.8%, much less than that of **2c** (24.4%), indicating that the 2′-chloride on the phenyl ring of the cinnamoyl group was not favorable for up-regulating the activity of BMP-2. By comparing **3d** (24.0%), **3e** (1.7%), **3f** (−9.6%), and **3g** (−21.5%) with **3c** (12.6%), we found that the introduction of an ethyl carbonate group at the C-5 position of the phenyl ring was favorable to the activity while the electron-donating methoxy group in the phenyl ring of the cinnamoyl group decreased the activity. As such, the more methoxy groups there are, the lower is the activity.

The SAR was further explored when the acetyl group of **1** and **2** was replaced by an ethylcarboxylate group. The UpRs of **5a** (8.2%) and **9a** (7.1%) were much lower than that of **1** and **2**. Replacing the C-4 hydrogen atom with chlorine (**6a**, −0.6%) decreased the activity. However, compared to **5a** and **1**, the 5,6-dimethoxy group (**7a**, 49.9%), the 5,6-methylenedioxy group (**8a**, 64.2%), and the 5-methyl carbonate group (**4a**, 49.8%) on the benzo[*b*]thiophene phenyl ring significantly improved the activities. The UpR of **8a** was almost 2-fold higher than the rate observed for compound **1**. Compared with **9a** and **2**, the 5,7-dichloro group (**10a**, −1.6%), the 7-fluoro group (**11a**, −18.1%), and the 7-methoxy group (**12a**, 12.4%) on the benzo[*b*]furan phenyl ring led to reductions in activities.

Modification of the C-2 group within the benzo[*b*]thiophene/furan framework, by the introduction of methylsulfinyl and methylsulfonyl groups at the β position of the C-2 carbonyl group, decreased the activities when compared with **1** and **2**. Decreasing activities were observed for **5c** (5.11%) versus **5b**, **7b** (13.5%) versus **7a**, **8b** (0.6%) versus **8a**, **8c** (−9.3%) versus **8b**, and **9b** (3.9%) versus **9a**. However, there was still some augment of the activities when compared to their respective analogues: **5b** (19.7%) versus **5a**, **6b** (18.5%) versus **6a**.

The SAR analysis showed that the structure of **8a** gave optimal up-regulation of *BMP-2* promoter activity in vitro. The activities of **1e** and **5b** were the best among the analogues with C-2 cinnamoyl groups and C-2 methylsulfinyl/methylsulfonyl groups, respectively. Taken together, we selected **8a**, **1e**, and **5b** of each C-2 group category to evaluate their in vivo activities in SAMP6 mice. Compound **1**, initially identified to be active for enhancing expression of *BMP-2* in our work, was chosen for in vivo efficacy evaluation in SAMP6 mice and also in OVX rats.

Effects of the Tested Compounds on Bone Mineral Density in SAMP6 Mice. In vitro efficacies of compounds **1**, **1e**, **5b**, and **8a** were evaluated in SAMP6 mice, using Lov (purchased from Tianzun Chemicals, Nanjing, China) as the positive control. Male and female SAMP6 mice (5-months old) were administered Lov, **1**, **1e**, **5b**, and **8a** orally at doses of 10, 30, 30, 30, and 30 (mg/kg)/day, respectively, for 3 months. The mice of the untreated group (MC) were given the vehicle (0.5% methylcellulose) by gavage.

The effects of the tested compounds on bone mineral density (BMD) of the SAMP6 mice were initially observed. BMD measurements of SAMP6 mice were performed by dual-energy X-ray absorptiometry (DXA) (OTEOCORE 3, MEDILINK, France) at 0, 1, 2, and 3 months after treatment. The whole body (total), spine, and left hind leg BMD values were analyzed using the small animals software package of OTEOCORE 3 as the regions of interest (ROIs, Figure 1A).

The total BMD of all tested animals except the Lov group decreased during the first month (Figure 1B). Then the total BMD of all groups increased in the following 2 months, while there was a reduction in group **1** during the third month. Total BMD of groups Lov, **5b**, and **8a** showed persistent and greater enhancement than the MC group following the first month of treatment. The spine BMD values of all groups were similar (Figure 1C), while the Lov and **8a** groups spine BMD values increased more than the MC group in the last 2 months. Spine BMD values of group **1** were obviously enhanced during the first 2 months but decreased in the third month, which was also observed in the total BMD and left hind leg BMD values (Figure 1D). BMD values of the left hind leg of all groups decreased during the 3 months, while the Lov, **1**, and **8a** groups showed a slower rate of decrease when compared with the MC group.

After 3 months of treatment, the total BMD of the Lov group was enhanced compared with the MC group (Table 2). The total BMD of the **1e** group was almost the same as the Lov group, whereas the **5b** and **8a** groups were higher than those of the MC and Lov groups. The spine BMD of all tested compound groups was enhanced compared with the MC group after 3 months of treatment, and the spine BMD of the Lov group was observed to increase the most. Insignificant variation of the left hind leg BMD values was observed among the six groups; however, there was a slight enhancement in the **8a** group.

Effects of the Tested Compounds on Bone Histomorphometry in SAMP6 Mice. Following treatment for 3 months, the right femora of the tested SAMP6 mice were harvested, and the undecalcified bone sections were obtained (Figure 2). The trabecula of all the treated groups was denser than that of the MC group. Bone histomorphometry was analyzed using a Leica Qwin image analysis system (Table 3). Trabecular bone volume percentage (TBV%) of each treated group increased compared to that of the untreated **MC** group. The **Lov**, **1**, **1e** and **8a** groups increased more substantially suggesting an enhancement of the mice bone masses of these groups. Trabecular formation surface percentage (TFS%) of each treated group was also enhanced and the **Lov**, **1**, **1e** groups had a more obvious rise, indicating an improvement of osteoblast activity in these groups. Trabecular resorption surface percentage (TRS%) of all groups did not significantly vary, which indicated that there were no noticeable differences of the osteoclast activities. Average osteoid width (OSW) of the **Lov** group mice was much larger than that of other groups, indicating that only **Lov** among the 6 compounds can increase the osteoblast activity of the osteoid in SAMP6 mice. Compared with the MC group, the bone formation related histomorphometries (TBV% and TFS%) of all treated groups increased; however, bone resorption related histomorphometry (TRS%) were clearly not affected.

Effects of the Tested Compounds on BMP-2 Expression in SAMP6 Mice. The compounds were selected for in vivo efficacy evaluation mainly according to the UpR value of BMP-2 promoter activity. After 3 months of treatment, to

Table 2. BMD Values of the SAMP6 Mice after 3 Months of Treatment

	BMD (g/cm^2)		
group	total	spine	left hind leg
Lov	0.070 ± 0.003	0.095 ± 0.007[a]	0.046 ± 0.003
MC	0.067 ± 0.002	0.087 ± 0.007	0.046 ± 0.002
1	0.068 ± 0.002	0.086 ± 0.004	0.050 ± 0.001
1e	0.070 ± 0.006	0.089 ± 0.013	0.050 ± 0.007
5b	0.072 ± 0.005	0.090 ± 0.013	0.050 ± 0.004
8a	0.072 ± 0.003[a]	0.091 ± 0.008	0.053 ± 0.008

[a] $p < 0.05$ ($n = 5$) compared to that of the MC group.

Table 3. Bone Histomorphometry of the SAMP6 Mice after 3 Months of Treatment with the Tested Compounds

group	TBV% (%)	TFS% (%)	TRS% (%)	OSW (μm)
Lov	7.44 ± 2.05[a]	6.22 ± 0.97[a]	6.64 ± 1.64	6.59 ± 0.29[a]
MC	3.88 ± 0.58	4.46 ± 0.47	7.47 ± 1.12	5.93 ± 0.42
1	4.95 ± 0.80[a]	5.89 ± 0.61[b]	7.09 ± 1.31	5.55 ± 0.82
1e	8.94 ± 3.68[a]	6.54 ± 0.81[b]	6.29 ± 1.48	5.50 ± 0.82
5b	5.95 ± 1.93	5.13 ± 0.82	6.45 ± 0.60	5.85 ± 0.64
8a	7.10 ± 2.15[a]	5.55 ± 0.93	6.45 ± 0.82	5.76 ± 0.58

[a] $p < 0.05$ ($n = 5$) compared to that of the MC group. [b] $p < 0.005$ ($n = 5$) compared to that of the MC group.

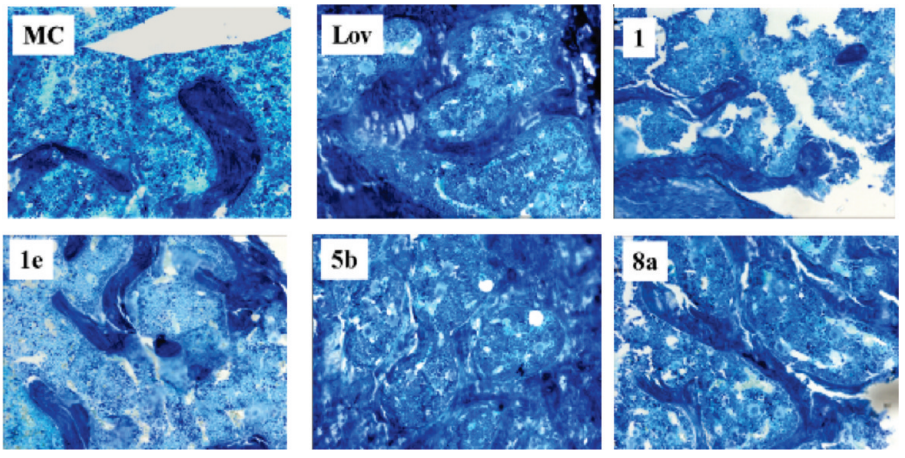


Figure 2. Bone histology of the right femora of the SAMP6 mice after 3 months of treatment with the tested compounds. The dark-blue bands in the pictures (1 × 200) represent the bone trabecular.

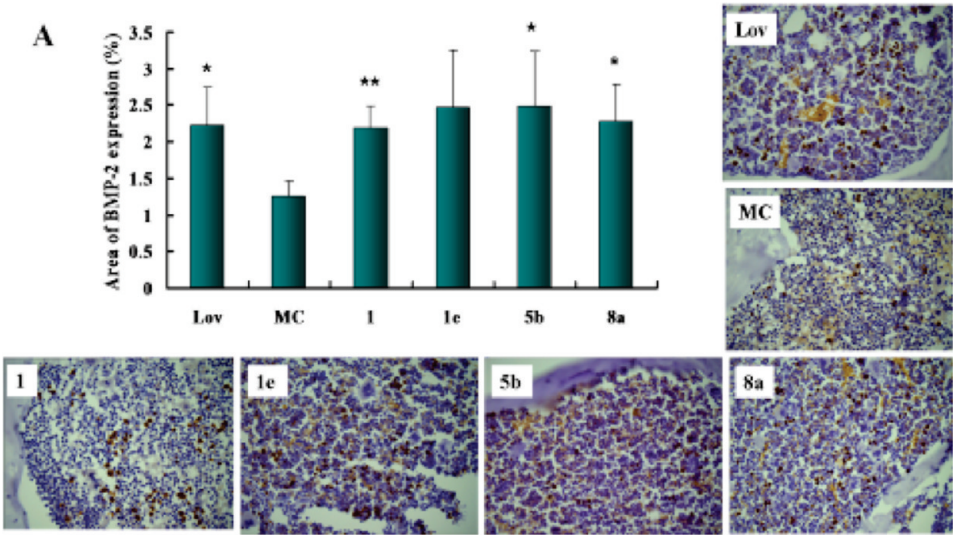


Figure 3. Immunohistochemical analysis of BMP-2 expression in the femora of SAMP6 mice. Brownish-red dots in the pictures (1 × 400) were cells that expressed BMP-2. Five visual fields in the medullary cavity under the epiphyseal plate were uptaken, and the percentage of the area of BMP-2 expression was calculated for each visual field. The percentage of the area of BMP-2 expression was the ratio of the area of cells showing a positive reaction (brownish red dots) to the total bone tissue area in a visual field. (A) Shown is the average percentage of area of BMP-2 expression: (∗) $p < 0.05$ ($n = 5$) compared to that of the untreated group (MC); (∗∗) $p < 0.005$ ($n = 5$) compared to that of untreated group (MC).

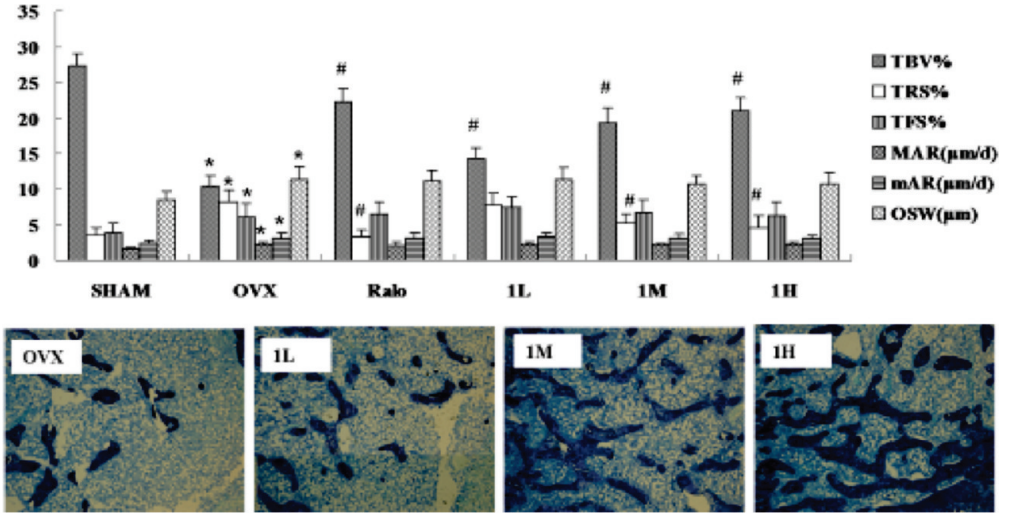


Figure 4. Efficacy of compound **1** preventing bone loss in OVX rats. Dosages of compound **1** are 7.5 (mg/kg)/day (**1L**), 15 (mg/kg)/day (**1M**), and 30 (mg/kg)/day (**1H**). MAR is mineral apposition rate, and mAR is osteo cortex mineralization rate: (∗) $p < 0.05$ ($n = 7$) compared to the SHAM group; (#) $p < 0.05$ ($n = 7$) compared to the OVX group. Dark-blue bands in the pictures illustrate trabecular of left proximal tibia (1 × 50).

determine whether the compounds increased the BMP-2 expression in vivo, the left femora of all SAMP6 mice were harvested to obtain frozen bone section lengthways using a freezing microtome. Immunohistochemistry was performed to observe the expression levels of BMP-2 using a Leica Qwin image analysis system. The positive reaction cells of the osteoblast and marrow stroma stained as brownish red dots (Figure 3). No cartilage cells were found to express BMP-2 in all groups. Moreover, cartilage cells were seldom observed which may be due to the senescence of this senile osteoporotic model. The percentage of area of BMP-2 expression of all treated groups had increased compared with that of the MC group (Figure 3A). The observed increases of the Lov, **1**, **5b**, and **8a** groups were more significant, and the BMP-2 expression areas of the **5b** and **8a** groups were higher than the area of the Lov group.

Compounds **1**, **1e**, **5b**, and **8a** up-regulated the BMP-2 promoter activity in vitro and also increased BMP-2 expression in vivo, which implied these compounds improved bone quality possibly through the BMP-2 pathway.

Bone Loss Preventing Efficacy in OVX Rats. The in vivo bone loss preventing efficacy of compound **1** was also evaluated in OVX rats (OVX) model. Six groups were assigned, including sham-operated (SHAM), OVX with vehicle (OVX), OVX with raloxifene (Ralo, 5 (mg/kg)/day, orally for 3 months), and OVX with compound **1** (**1L**, 7.5 (mg/kg)/day; **1M**, 15 (mg/kg)/day; **1H**, 30 (mg/kg)/day; orally for 3 months, respectively).

Following treatment for 3 months, the right left proximal tibia of all rats were harvested and the undecalcified bone sections were obtained (Figure 4). In the **1H** group receiving a dose of 30 (mg/kg)/day and the **1M** group receiving the

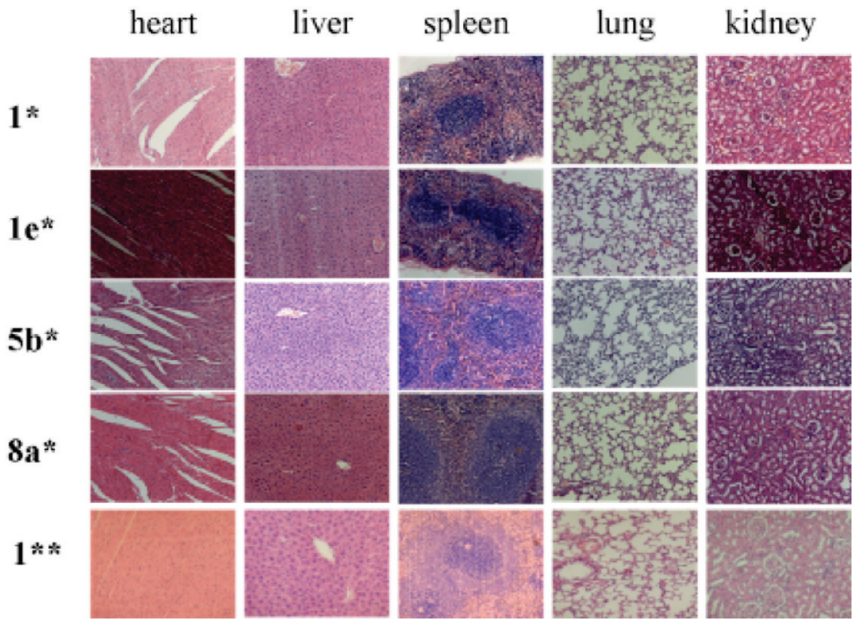


Figure 5. Pathological sections (1 × 200) of the hearts, livers, spleens, lungs, and kidneys after 3 months of treatment. (*) SAMP6 mice were administered tested compounds with a dose of 30 (mg/kg)/day orally. (**) Rats were administered compound **1** as 150 (mg/kg)/day orally.

dose of 15 (mg/kg)/day, the number, thickness, area, and connectivity of trabecula had clearly increased when compared to the OVX group. Ovariectomy (see OVX group) is associated with a lower trabecular bone volume and a higher trabecula resorption surface percentage compared with SHAM (Figure 4). Compound **1** improved the trabecula in a dose-dependent manner. According to the bone histomorphometry analyzed using a Leica Qwin image analysis system, all doses of compound **1** increased TBV% and decreased the trabecular resorption surface percentage (TRS%) when compared to OVX. TBV% of **1H** was consistent with that of the Ralo group.

Safety Evaluation of Tested Compounds. Following the treatment of **1**, **1e**, **5b**, and **8a** for 3 months, the hearts, livers, spleens, lungs, and kidneys of SAMP6 mice were harvested and a histological examination was performed (Figure 5). The histomorphology of the organs of the treated and untreated animals was similar, and no pathological changes were found. Organ indexes (organ weight/body weight) of the six groups were similar to each other. Body weights of all mice increased slowly during the 3 months, and the 24 h consumption of water (g/g body weight) and food (g/g body weight) of the treated groups was similar to the untreated group.

Preliminary acute toxicity in the Kunming mice was performed. Compounds **1e**, **5b**, and **8a** were given orally at doses of 500, 700, and 500 (mg/kg)/day for 3 days, and the animals were closely monitored during the 3 days and the following 14 days. No animal died during the 17 days, indicating that the LD_{50} values of **1e**, **5b**, and **8a** were larger than 500, 700, and 500 mg/kg orally. Rats were given compound **1** orally at a dose of 150 (mg/kg)/day for 3 months; no pathological changes were seen of the main organs (Figure 5). The body weights of the animals increased slowly. The animals were healthy in behavior and had shiny fur. The LD_{50} of **1** on the Kunming mice was over 5 g/kg (orally) and 1.58 g/kg (intraperitoneally). All these data indicated that compounds **1**, **1e**, **5b**, and **8a** were considerably safe in vivo.

Conclusion

The BMP pathway is a promising new target to design therapeutic agents for the treatment of low bone mass. Substituted benzothiophene and benzofuran derivatives were found to be a new class of small molecules that act as potential anabolic agents targeting BMP-2. Twenty-seven substituted benzothiophene and benzofuran derivatives have been synthesized as a novel class of BMP-2 up-regulators and also as a new class of potent antiosteoporosis agents. The synthesis, structure−activity relationships, and bone loss prevention efficacies of these compounds in different animal models have been presented. At concentrations 10-fold higher than that of Lov, compounds **1**, **2**, **3**, **14**, **4a**, **7a**, and **8a** were found to exhibit more potent activities on enhancing BMP-2 expression in vitro, whereas compounds **1e**, **2e**, **3d**, **5b**, and **6b** were found to exhibit similar BMP-2 up-regulating rates as Lov. The SAR information revealed that (i) the phenyl ring of benzothiophene substituted by methyl carboxylate may be good for activity, (ii) the C-2 acetyl group of benzothiophene or benzofuran replaced by a cinnamoyl group decreased the activity in vitro, (iii) the C-2 ethylcarboxylate group made C-5, C-6 methoxyl or methylenedioxy groups more favorable to the activities, and (iv) the introduction of methylsulfinyl and methylsulfonyl groups at the β-position of the C-2 carbonyl group decreased the activities compared with compounds **1** and **2**.

Compounds **1**, **1e**, **5b**, and **8a**, based on the structures and in vitro activities, were chosen to test in vivo efficacy in SAMP6 mice. The four tested compounds significantly enhanced mouse BMP-2 expression in vivo and improved mouse bone quality. Compared with the MC group, the bone formation related histomorphometries (TBV% and TFS%) of all treated groups were increased; however, bone resorption related histomorphometry (TRS%) was not affected. This observation indicated that these types of compounds improve bone quality possibly by stimulating the formation of new bone growth through the BMP-2 pathway.

Groups treated with compounds **1** and **8a** showed higher UpRs in vitro and significantly enhanced mouse BMP-2 expression in vivo when compared with the values observed in the untreated group. Compound **8a** enhanced the total BMD and bone mass of SAMP6 mice at a dose of 30 (mg/kg)/day orally for 3 months. In the SAMP6 mice model, compound **8a** showed much stronger effects on the bone formation rate of the tested mice. Compound **1**, first found to be a BMP-2 up-regulating agent, improved bone quality in SAMP6 mice and also showed evident activity in OVX rats. In addition, histological examination of the main organs of the SAMP6 mice and rats proved that compounds **1** and **8a** were considerably safe in vivo.

Most of the antiosteoporosis agents currently used in the clinic can effectively prevent further bone loss and stabilize bone mass but do not have substantial influence on facilitating new bone formation. A safe, efficacious drug that stimulates the formation of bone and restoration of trabecular connectivity would represent a major therapeutic advance in the osteoporosis field. In this work, it was demonstrated that substituted benzothiophene and benzofuran derivatives, especially compounds **1** and **8a**, enhance BMP-2 expression in vitro and in vivo and stimulate bone formation and trabecular connectivity restoration in vivo. The compounds represent potential leads in the development of a new class of anabolic agents.

Experimental Section

Chemistry. Melting points (mp) were determined with an X6 microscope melting point apparatus and were uncorrected. ^{1}H NMR spectra were recorded on a Varian Mercury-400 spectrometer. HRMS spectra were recorded on an AccuTOF CS mass spectrometer. The area normalization purities of tested compounds were >95% as determined by a high-pressure liquid chromatography (HPLC). HPLC was performed using an Agilent 1200 series, diode array detector. The eluting process was performed on a Dikma PlatisilTM ODS 5 μm, 250 mm × 4.6 mm column using a mobile phase of water−methanol (20:80 v/v for compounds **1**, **2**, **3**, **4a**, **5a**, **6a**, **7a**, **8a**, **9a**, **10a**, **11a**, **5b**, **6b**, **7b**, **8b**, **9b**, **5c**, and **8c**; 10:90 v/v for compounds **1c**, **1d**, **1e**, **2c**, **2d**, **3c**, **3d**, 3e, **3f**, and **3g**), an injection volume of 1 μL controlled by an autosampler, and a flow rate of 1.0 mL/min.

(*E*)-1-(Benzo[*b*]thiophen-2-yl)-3-phenylprop-2-en-1-one (1c). 1-(Benzo[*b*]thiophen-2-yl)ethanone (**1**, 500 mg, 2.8 mmol), sodium ethylate (191 mg, 2.8 mmol), and benzaldehyde (**c**, 361 mg, 3.4 mmol) were added to 25 mL of ethanol. The reaction mixture was stirred at room temperature for 1.5 h and then evaporated under vacuum. The residue was dissolved in chloroform and washed with water. The organic layer was dried over anhydrous magnesium sulfate and then filtered to remove the precipitate and concentrated in vacuo. The residue was purified by flash chromatography over silica gel, affording the title compound (349 mg, 60%). Yellow solid; mp 119−122 °C. ^{1}H NMR ($CDCl_3$) δ: 7.41−7.51 (m, 5H), 7.55 (d, J = 15.6 Hz, 1H), 7.68−7.70 (m, 2H), 7.89−7.94 (m, 3H), 8.12 (s, 1H). HRMS (EI^+) calcd for $C_{17}H_{12}OS$ $[M]^+$ 264.0609; found 264.0595.

(*E*)-1-(Benzo[*b*]thiophen-2-yl)-3-(2-chlorophenyl)prop-2-en-1-one (1d). The title compound was obtained from **1** and 2-chlorobenzaldehyde (**d**) using a procedure similar to that for compound **1c**. Yield, 67%; yellow solid; mp 105−108 °C. ^{1}H NMR ($CDCl_3$) δ: 7.34−7.38 (m, 2H), 7.43−7.51 (m, 3H), 7.52 (d, J = 15.6 Hz, 1H), 7.78−7.81 (m, 1H), 7.91 (d, J = 8.4 Hz, 1H), 7.93 (d, J = 8.0 Hz, 1H), 8.12 (s, 1H), 8.28 (d, J = 15.6 Hz, 1H). HRMS (FAB^+) calcd for $C_{17}H_{11}ClOS$ $[M + H]^+$ 299.0297; found 299.0313.

(*E*)-1-(Benzo[*b*]thiophen-2-yl)-3-(4-methoxylphenyl)prop-2-en-1-one (1e). The title compound was obtained from **1** and 4-methoxybenzaldehyde (**e**) using a procedure similar to that for compound **1c**. Yield, 70%; yellow solid; mp 116−119 °C. ^{1}H NMR ($CDCl_3$) δ: 3.87 (s, 3H), 6.96 (d, J = 8.4 Hz, 2H), 7.40−7.49 (m, 3H), 7.64 (d, J = 8.8 Hz, 2H), 7.87 (d, J = 15.2 Hz, 1H), 7.92 (d, J = 8.4 Hz, 2H), 8.09 (s, 1H). HRMS (EI^+) calcd for $C_{18}H_{14}O_2S$ $[M]^+$ 294.0715; found 294.0711.

(*E*)-1-(Benzofuran-2-yl)-3-phenylprop-2-en-1-one (2c). The title compound was obtained from **2** and **c** using a procedure similar to that for compound **1c**. Yield, 69%; yellow solid; mp 98−101 °C. ^{1}H NMR ($CDCl_3$) δ: 7.24−7.28 (m, 5H), 7.37−7.40 (m, 2H), 7.43 (d, J = 7.6 Hz, 1H), 7.47−7.48 (m, 1H), 7.51−7.52 (m, 1H), 7.56 (d, J = 7.6 Hz, 1H), 7.59 (s, 1H), 7.62−7.63 (m, 1H), 7.67 (d, J = 8.0 Hz, 1H), 7.74 (dd, J = 7.6 Hz, 2.8 Hz, 1H), 7.89 (d, J = 15.6 Hz, 1H), 8.27 (d, J = 15.6 Hz, 1H). HRMS (ESI^+) calcd for $C_{17}H_{13}O_2$ $[M + H]^+$ 249.09155; found 249.09089.

(*E*)-1-(Benzofuran-2-yl)-3-(2-chlorophenyl)prop-2-en-1-one (2d). The title compound was obtained from **2** and **d** using a procedure similar to that for compound **1c**. Yield, 61%; yellow solid; mp 108−112 °C. ^{1}H NMR ($CDCl_3$) δ: 7.33 (d, J = 7.6 Hz, 1H), 7.36−7.38 (m, 2H), 7.46−7.49 (m, 1H), 7.51 (d, J = 7.6 Hz, 1H), 7.58 (d, J = 15.6 Hz, 1H), 7.63 (d, J = 8.8 Hz, 1H), 7.67 (s, 1H), 7.74 (d, J = 7.6 Hz, 1H), 7.83 (dd, J = 7.6 Hz, 2.8 Hz, 1H), 8.35 (d, J = 15.6 Hz, 1H). HRMS (EI^+) calcd for $C_{17}H_{11}ClO_2$ $[M]^+$ 283.0526; found 283.0545.

Ethyl 2-Cinnamoylbenzo[*b*]thiophene-5-carboxylate (3c). The title compound was obtained from **3** and **c** using a procedure similar to that for compound **1c**. Yield, 62%; yellow solid; mp 127−128 °C. ^{1}H NMR ($CDCl_3$) δ: 1.44 (t, J = 7.2 Hz, 3H), 4.44 (q, J = 7.2 Hz, 2H), 7.54 (s, 3H), 7.59 (d, J = 15.2 Hz, 1H), 7.68−7.69 (m, 2H), 7.91 (d, J = 15.2 Hz, 1H), 7.94 (d, J = 8.0 Hz, 1H), 8.10 (d, J = 8.0 Hz, 1H), 8.15 (s, 1H), 8.64 (s, 1H). HRMS (EI^+) calcd for $C_{20}H_{16}O_3S$ $[M]^+$ 336.0820; found 336.0796.

Ethyl (*E*)-2-(3-(2-Chlorophenyl)acryloyl)benzo[*b*]thiophene-5-carboxylate (3d). The title compound was obtained from **3** and **d** using a procedure similar to that for compound **1c**. Yield, 62%; yellow solid; mp 91−93 °C. ^{1}H NMR ($CDCl_3$) δ: 1.44 (t, J = 7.2 Hz, 3H), 4.44 (q, J = 7.2 Hz, 2H), 7.35−7.37 (m, 2H), 7.47 (d, J = 8.8 Hz, 1H), 7.50 (d, J = 15.6 Hz, 1H), 7.79 (dd, J = 8.0 Hz, 2.4 Hz, 1H), 7.95 (d, J = 8.4 Hz, 1H), 8.13 (d, J = 8.4 Hz, 1H), 8.17 (s, 1H), 8.30 (d, J = 15.6 Hz, 1H), 8.65 (s, 1H). HRMS (EI^+) calcd for $C_{20}H_{15}O_3SCl$ $[M]^+$ 370.0430; found 370.0418.

Ethyl (*E*)-2-(3-(4-Methoxylphenyl)acryloyl)benzo[*b*]thiophene-5-carboxylate (3e). The title compound was obtained from **3** and **e** using a procedure similar to that for compound **1c**. Yield, 34%; flavogreen solid; mp 146−148 °C. ^{1}H NMR ($CDCl_3$) δ: 1.45 (t, J = 7.2 Hz, 3H), 3.88 (s, 3H), 4.44 (q, J = 7.2 Hz, 2H), 6.97 (d, J = 8.4 Hz, 2H), 7.42 (d, J = 15.2 Hz, 1H), 7.65 (d, J = 8.4 Hz, 2H), 7.89 (d, J = 15.2 Hz, 1H), 7.94 (d, J = 8.4 Hz, 1H), 8.12 (d, J = 8.4 Hz, 1H), 8.15 (s, 1H), 8.64 (s, 1H). HRMS (EI^+) calcd for $C_{21}H_{18}O_4S$ $[M]^+$ 366.0926; found 366.0916.

Ethyl (*E*)-2-(3-(3,4-Dimethoxylphenyl)acryloyl)benzo[*b*]thiophene-5-carboxylate (3f). The title compound was obtained from **3** and 3,4-dimethoxybenzaldehyde (**f**) using a procedure similar to that for compound **1c**. Yield, 10%; yellow oil. ^{1}H NMR ($CDCl_3$) δ: 1.44 (t, J = 7.2 Hz, 3H), 3.94 (s, 3H), 3.98 (s, 3H), 4.45 (q, J = 7.2 Hz, 2H), 6.93 (d, J = 8.4 Hz, 1H), 7.20 (s, 1H), 7.29 (d, J = 8.4 Hz, 1H), 7.40 (d, J = 15.2 Hz, 1H), 7.88 (d, J = 15.2 Hz, 1H), 7.95 (d, J = 8.8 Hz, 1H), 8.12 (d, J = 8.8 Hz, 1H), 8.17 (s, 1H), 8.65 (s, 1H). HRMS (EI^+) calcd for $C_{22}H_{20}O_5S$ $[M]^+$ 396.1031; found 396.1032.

Ethyl (*E*)-2-(3-(2,3,4-Trimethoxylphenyl)acryloyl)benzo[*b*]thiophene-5-carboxylate (3g). The title compound was obtained from **3** and 3,4,5-trimethoxybenzaldehyde (**g**) using a procedure similar to that for compound **1c**. Yield, 30%; yellow solid; mp 113−115 °C. ^{1}H NMR ($CDCl_3$) δ: 1.44 (t, J = 7.2 Hz, 3H), 3.91 (s, 3H), 3.94 (s, 3H), 3.99 (s, 3H), 4.44 (q, J = 7.2 Hz, 2H), 6.75 (d, J = 8.8 Hz, 1H), 7.42 (d, J = 8.8 Hz, 1H), 7.59 (d, J = 15.6 Hz, 1H), 7.94 (d, J = 8.4 Hz, 1H), 8.08 (d, J = 15.6 Hz, 1H), 8.11−8.14 (m, 2H), 8.64 (s, 1H). HRMS (EI^+) calcd for $C_{23}H_{22}O_6S$ $[M]^+$ 426.1137; found 426.1147.

Ethyl 5-Methylbenzo[*b*]thiophene-2,5-dicarboxylate (4a). To a mixture of **4** (2.0 g, 9.6 mmol) and anhydrous potassium carbonate (1.6 g, 11.6 mmol) in DMF at 0 °C, ethyl thioglycolate (1.4 g, 11.7 mmol) was added dropwise.[23] The system was stirred at 0 °C for 30 min and then at 60 °C for 12 h. The solution was filtered to remove the precipitate, and the filtrate was concentrated under vacuum. The residue was dissolved in chloroform and washed with water. The organic layer was dried over anhydrous magnesium sulfate and then filtered to remove the precipitate and concentrated in vacuo. The residue was purified by flash chromatography over silica gel, affording the title compound (2.5 g, 97%) as a white solid; mp 78−79 °C. ^{1}H NMR ($CDCl_3$) δ: 1.43 (t, J = 7.2 Hz, 3H), 3.97 (s, 3H), 4.43 (q, J = 7.2 Hz, 2H), 7.91 (d, J = 8.4 Hz, 1H), 8.10 (d, J = 8.4 Hz, 1H), 8.12 (s, 1H), 8.58 (s, 1H). HRMS (EI$^+$) calcd for $C_{13}H_{12}O_4S$ [M]$^+$ 264.0456; found 264.0452.

Ethyl Benzo[*b*]thiophene-2-carboxylate (5a). The title compound was obtained from 2-nitrobenzaldehyde (**5**) and ethyl thioglycolate using a procedure similar to that for compound **4a**. Yield, 94%; yellow solid; mp 36−38 °C. ^{1}H NMR ($CDCl_3$) δ: 1.42 (t, J = 7.0 Hz, 3H), 4.42 (q, J = 7.0 Hz, 2H), 7.39−7.47 (m, 2H), 7.86−7.89 (m, 2H), 8.06 (s, 1H). HRMS (EI$^+$) calcd for $C_{11}H_{10}O_2S$ [M]$^+$ 206.0397; found 206.0397.

Ethyl 4-Chlorobenzo[*b*]thiophene-2-carboxylate (6a). The title compound was obtained from 2-chloro-6-fluorobenzaldehyde (**6**) and ethyl thioglycolate using a procedure similar to that for compound **4a**. Yield, 12%; yellow solid; mp 55−57 °C. ^{1}H NMR ($CDCl_3$) δ: 1.43 (t, J = 7.0 Hz, 3H), 4.43 (q, J = 7.0 Hz, 2H), 7.35−7.41 (m, 2H), 7.73−7.75 (m, 1H). HRMS (EI$^+$) calcd for $C_{11}H_9O_2SCl$ [M]$^+$ 240.0012; found 240.0014.

Ethyl 5,6-Dimethoxylbenzo[*b*]thiophene-2-carboxylate (7a). The title compound was obtained from 6-nitroveratraldehyde (**7**) and ethyl thioglycolate using a procedure similar to that for compound **4a**. Yield, 63%; white solid; mp 82−84 °C. ^{1}H NMR ($CDCl_3$) δ: 1.405 (t, J = 7.2 Hz, 3H), 3.95 (s, 3H), 3.98 (s, 3H), 4.38 (q, J = 7.2 Hz, 2H), 7.24 (s, 1H), 7.25 (s, 1H), 7.93 (s, 1H). HRMS (EI$^+$) calcd for $C_{13}H_{14}O_4S$ [M]$^+$ 266.0631; found 266.0613.

Ethyl 5,6-Dimethylenedioxybenzo[*b*]thiophene-2-carboxylate (8a). The title compound was obtained from 6-nitropiperonal (**8**) and ethyl thioglycolate using a procedure similar to that for compound **4a**. Yield, 69%; white solid; mp 110−112 °C. ^{1}H NMR ($CDCl_3$) δ: 1.39 (t, J = 7.2 Hz, 3H), 4.37 (q, J = 7.2 Hz, 2H), 6.04 (s, 1H), 7.19 (s, 1H), 7.20 (s, 1H), 7.89 (s, 1H). HRMS (EI$^+$) calcd for $C_{12}H_{10}O_4S$ [M]$^+$ 250.0300; found 250.0305.

Ethyl Benzofuran-2-carboxylate (9a). 9a[24,25] was obtained from salicylaldehyde (**9**) and ethyl bromoacetate using a procedure similar to that for compound **4a**. Yield, 56%; yellow oil. ^{1}H NMR ($CDCl_3$) δ: 1.35 (t, J = 7.2 Hz, 3H), 4.37 (q, J = 7.2 Hz, 2H), 7.22 (t, J = 7.6 Hz, 1H), 7.36 (t, J = 7.6 Hz, 1H), 7.45 (s, 1H), 7.51 (d, J = 8.0 Hz, 1H), 7.60 (d, J = 8.0 Hz, 1H). HRMS (EI$^+$) calcd for $C_{11}H_{10}O_3$ [M]$^+$ 190.0630; found 190.0616.

Ethyl 5,7-Dichlorobenzofuran-2-carboxylate (10a). The title compound was obtained from 3,5-dichlorosalicylaldehyde (**10**) and ethyl bromoacetate using a procedure similar to that for compound **4a**. Yield, 65%; white solid; mp 78−79 °C. ^{1}H NMR ($CDCl_3$) δ: 1.44 (t, J = 7.2 Hz, 3H), 4.46 (q, J = 7.2 Hz, 2H), 7.46 (d, J = 1.6 Hz, 1H), 7.48 (s, 1H), 7.57 (d, J = 1.6 Hz, 1H). HRMS (EI$^+$) calcd for $C_{11}H_8O_3Cl_2$ [M]$^+$ 257.9850; found 257.9840.

Ethyl 7-Fluorobenzofuran-2-carboxylate (11a). The title compound was obtained from 3-fluoro-2-hydroxybenzaldehyde (**11**) and ethyl bromoacetate using a procedure similar to that for compound **4a**. Yield, 47%; yellow oil. ^{1}H NMR ($CDCl_3$) δ: 1.43 (t, J = 7.2 Hz, 3H), 4.45 (q, J = 7.2 Hz, 2H), 7.14−7.25 (m, 2H), 7.43 (d, J = 8.0 Hz, 1H), 7.54 (d, J = 2.8 Hz, 1H). HRMS (EI$^+$) calcd for $C_{11}H_9O_3F$ [M]$^+$ 208.0536; found 208.0531.

Ethyl 7-Methoxylbenzofuran-2-carboxylate (12a). The title compound was obtained from 3-methoxysalicylaldehyde (**12**) and ethyl thioglycolate using a procedure similar to that for compound **4a**. Yield, 58%; white solid; mp 82−84 °C. ^{1}H NMR ($CDCl_3$) δ: 1.42 (t, J = 7.2 Hz, 3H), 4.02 (s, 3H), 4.44 (q, J = 7.2 Hz, 2H), 6.92 (d, J = 7.6 Hz, 1H), 7.21 (d, J = 7.6 Hz, 1H), 7.22 (dd, J = 7.6 Hz, 3.2 Hz, 1H), 7.52 (s, 1H). HRMS (EI$^+$) calcd for $C_{12}H_{12}O_4$ [M]$^+$ 220.0736; found 220.0733.

1-(Benzo[*b*]thiophen-2-yl)-2-(methylsulfinyl)ethanone (5b). Sodium hydride (60%, 280 mg, 7.0 mmol) was washed three times with petroleum ether, dried under vacuum, and then mixed with dry dimethyl sulfoxide (8 mL). The system was protected with nitrogen and heated to 70−75 °C.[26] The reaction system was cooled to 0 °C when effervescence stopped. **5a** (200 mg, 1.0 mmol) in 8 mL of dry tetrahydrofuran was added slowly. The reaction system was then stirred for 30 min at room temperature and quenched with 60 mL of ice cold water. The pH of the solution was adjusted to 3−4 with 2 N HCl. The mixture was extracted with chloroform (80 mL × 2). The combined organic layer was washed with water (160 mL × 3). The organic layer was dried over anhydrous magnesium sulfate and then filtered to remove the precipitate and concentrated in vacuo. The residue was purified by flash chromatography over silica gel, affording the title compound (202 mg, 87%). White solid; mp 120−123 °C. ^{1}H NMR ($CDCl_3$) δ: 2.79 (s, 3H), AB system (4.33, 4.41, J = 13.2 Hz, 2H), 7.43 (t, J = 7.0 Hz, 1H), 7.51 (t, J = 7.0 Hz, 1H), 7.88 (d, J = 7.0 Hz, 1H), 7.94 (d, J = 7.0 Hz, 1H), 8.09 (s, 1H). HRMS (EI$^+$) calcd for $C_{11}H_{10}O_2S_2$ [M]$^+$ 238.0122; found 238.0102.

1-(4-Chlorobenzo[*b*]thiophen-2-yl)-2-(methylsulfinyl)ethanone (6b). The title compound was obtained from **6a** and dimethyl sulfoxide using a procedure similar to that for compound **5b**. Yield, 59%; yellow solid; mp 156−159 °C. ^{1}H NMR ($CDCl_3$) δ: 2.83 (s, 3H), AB system (4.38, 4.45, J = 13.5 Hz, 2H), 7.46 (d, J = 4.5 Hz, 2H), 7.79 (t, J = 4.5 Hz, 1H), 8.24 (s, 1H). HRMS (EI$^+$) calcd for $C_{11}H_9O_2S_2Cl$ [M]$^+$ 271.9733; found 271.9733.

1-(5, 6-Dimethoxylbenzo[*b*]thiophen-2-yl)-2-(methylsulfinyl)-ethanone (7b). The title compound was obtained from **7a** and dimethyl sulfoxide using a procedure similar to that for compound **5b**. Yield, 96%; yellow solid; mp 161−164 °C. ^{1}H NMR ($CDCl_3$) δ: 2.78 (s, 3H), 3.96 (s, 3H), 3.99 (s, 3H), AB system (4.28, 4.35, J = 13.6 Hz, 2H), 7.25 (s, 1H), 7.28 (s, 1H), 7.96 (s, 1H). HRMS (EI$^+$) calcd for $C_{13}H_{14}O_4S_2$ [M]$^+$ 298.0334; found 298.0306.

1-(5, 6-Methylenedioxybenzo[*b*]thiophen-2-yl)-2-(methylsulfinyl)-ethanone (8b). The title compound was obtained from **8a** and dimethyl sulfoxide using a procedure similar to that for compound **5b**. Yield, 90%; yellow solid; mp 182−184 °C. ^{1}H NMR ($CDCl_3$) δ: 2.77 (s, 3H), AB system (4.26, 4.33, J = 13.2 Hz, 2H), 6.08 (s, 1H), 7.22 (s, 1H), 7.24 (s, 1H), 7.91 (s, 1H). HRMS (EI$^+$) calcd for $C_{12}H_{10}O_4S_2$ [M]$^+$ 282.0021; found 282.0002.

1-(Benzofuran-2-yl)-2-(methylsulfinyl)ethanone (9b). The title compound was obtained from **9a** and dimethyl sulfoxide using a procedure similar to that for compound **5b**. Yield, 30%; yellow solid; mp 124−127 °C. ^{1}H NMR ($CDCl_3$) δ: 2.85 (s, 3H), AB system (4.30, 4.39, J = 13.2 Hz, 2H), 7.35 (t, J = 7.2 Hz, 1H), 7.54 (t, J = 7.2 Hz, 1H), 7.61 (d, J = 8.0 Hz,1H), 7.68 (s, 1H), 7.75 (d, J = 8.0 Hz, 1H). HRMS (EI$^+$) calcd for $C_{11}H_{10}O_3S$ [M]$^+$ 222.0351; found 222.0350.

1-(Benzo[*b*]thiophen-2-yl)-2-(methylsulfonyl)ethanone (5c). 5b (100 mg, 0.42 mmol) was dissolved in 4 mL of acetic acid. Then 2 mL of 30% H_2O_2 was added to the solution at 0 °C. The reaction system was stirred at room temperature for 5 h. The mixture was extracted with chloroform (4 mL × 3). The combined organic layer was washed with water (15 mL × 3). The organic layer was dried over anhydrous magnesium sulfate and then filtered to remove the precipitate and concentrated in vacuo. The residue was purified by flash chromatography over silica gel, affording the title compound (48 mg, 45%). White solid; mp 158−160 °C. ^{1}H NMR ($CDCl_3$) δ: 3.17 (s, 3H), 4.60 (s,2H), 7.45 (t, J = 7.0 Hz, 1H), 7.53 (t, J = 7.0 Hz, 1H), 7.89 (d, J = 8.0 Hz, 1H), 7.96 (d, J = 8.0 Hz, 1H), 8.11 (s, 1H). HRMS (EI$^+$) calcd for $C_{11}H_{10}O_3S_2$ [M]$^+$ 254.0071; found 254.0074.

1- (5,6-Methylenedioxybenzo[*b*]thiophen-2-yl)-2-(methylsulfonyl)-ethanone (8c). The title compound was obtained from **8b** and

30% H_2O_2 using a procedure similar to that for compound **5c**. Yield, 80%; yellow solid; mp 203−206 °C. ^{1}H NMR ($CDCl_3$) δ: 3.15 (s, 3H), 4.53 (s, 2H), 6.09 (s, 2H), 7.22 (s, 1H), 7.24 (s, 1H), 7.94 (s, 1H). HRMS (EI^+) calcd for $C_{12}H_{10}O_5S_2$ $[M]^+$ 297.9970; found 297.9967.

Methyl 2-Acetylbenzo[*b*]thiophene-5-carboxylate (3). Methyl 3-formyl-4-nitrobenzoate (**4**, 1.95 g, 9.3 mmol), ethyl mercaptide (0.7 mg, 11.3 mmol), and anhydrous potassium carbonate (1.55 g, 11.2 mmol) were added to 60 mL of DMF at 0 °C. The reaction system was stirred at 0 °C for 10 min and then at room temperature for 1 h. The solution was filtered to remove the precipitate, and the filtrate was concentrated under vacuum. The residue was dissolved in chloroform and washed with water. The organic layer was dried over anhydrous magnesium sulfate and then filtered to remove the precipitate and concentrated in vacuo. The residue was purified by flash chromatography over silica gel, affording methyl 4-(ethylthio)-3-formylbenzoate (**13**) as a white solid.

13 (obtained above, 1.0 g, 4.5 mmol), calcium oxide (125 mg, 2.2 mmol), and chloroacetone (825 mg, 8.9 mmol) were added to 20 mL of DMF. The reaction mixture was stirred at 90 °C for 10 h. The solution was filtered to remove the precipitate, and the filtrate was concentrated under vacuum. The residue was dissolved in chloroform and washed with water. The organic layer was dried over anhydrous magnesium sulfate and then filtered to remove the precipitate and concentrated in vacuo. The residue was purified by flash chromatography over silica gel, affording the title compound (929 mg, 89%) as a yellow solid; mp 158−161 °C. ^{1}H NMR ($CDCl_3$) δ: 2.69 (s, 3H), 3.98 (s, 3H), 7.93 (d, J = 8.4 Hz, 1H), 8.00 (s, 1H), 8.11 (d, J = 8.4 Hz, 1H), 8.61(s, 1H). HRMS (EI^+) calcd for $C_{12}H_{10}O_3S$ $[M]^+$ 234.0351; found 234.0339.

Biological Methods. In Vitro Assay. Cell Lines. The murine calvarial MC3T3-E1 cells were obtained from the Cell Culture Centre, Institute of Basic Medical Science Chinese Academy of Medical Sciences (Beijing, China). Cells were grown in DMEM medium and supplemented with 10% heat-inactivated FBS at 37 °C in a humidified 5% CO_2 incubator.

Reporter Plasmid Construction.[21] The plasmid pCAT4.5X with the 5′-flanking promoter region −3365 bp to −1658 bp of the mouse BMP-2 gene was kindly provided by Professor Rogers (Biochemistry and Molecular Biology, New Jersey Medical School, UMDNJ). After sequence verification, the 1709 bp product was cloned into the pGL4.17-basic vector (Promega) using SacI and XhoI restriction sites. This constructed vector, containing the mouse BMP-2 promoter region and the neomycin resistance gene, was designated as pGL4-BMP-2.

Animal Experiments. Male and female SAMP6 mice (5-months old) were obtained from the Peking University Health Science Center. Animals were nurtured according to the institutional guidelines of the Chinese Academy of Medical Science and housed in an air-conditioned room with a 12 h light and 12 h dark cycle. There were two female mice per cage and one male mice per cage. The mice were randomly divided into six groups based on the initial BMD. The untreated group (n = 5; 3 males, 2 females) were given the vehicle 0.5% methylcellulose by gavage. The treated groups (n = 5; 3 males, 2 females) were given the study compounds suspended in 0.5% methylcellulose by gavage. The doses of Lov, **1**, **1e**, **5b**, and **8a** were 10, 30, 30, 30, and 30 (mg/kg)/day, respectively, for 3 months. Hearts, livers, spleens, lungs, and kidneys of SAMP6 mice of all groups were harvested and weighed, and histological examinations were performed to detect and observe pathological changes.

Femal rats (205−235 g) were obtained from the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences and the Peking Union Medical College. There were three to four rats per cage. Rats were randomly divided into six groups (n = 7) based on the body weight, including sham-operated (SHAM), OVX with vehicle (OVX), OVX with raloxifene (Ralo, 5 (mg/kg)/day, orally for 3 months), and OVX with compound **1** (7.5, 15, and 30 (mg/kg)/day, orally for 3 months, respectively).

BMD Test. The BMD of mice were measured at 0, 4, 8, and 12 weeks after treatment. Mice were anesthetized with pentobarbital sodium (50 mg/kg, ip) and placed in the prone position. The body was extended and fixed with adhesive tape. BMD was measured by dual-energy X-ray absorptiometry (DXA) (OTEOCORE 3, MEDILINK, France) according to the manufacturer's instructions. BMD values were analyzed using the small animals software package of OTEOCORE 3. The whole body (total BMD), spine, and left hind leg were chosen as the ROIs.

Undecalcified Bone Section and Bone Histomorphometry. Proximal ends ($^1/_3$) of the right femora of mice/rats were harvested and fixed in 4% paraformaldehyde. Femora were dehydrated in ascending concentrations of ethanol (80%, 95%, 100%) and cleared with dimethylbenzene. Samples were immersed in polymer fluids I, II, III for 3 days, respectively. Polymer fluid I contained 100 mL of methyl methacrylate, 35 mL of *n*-butyl methacrylate, 5 mL of methyl benzoate, and 1.2 mL of polyethylene glycol (400). Polymer fluid II contained fluid I and 0.4 g of benzoyl peroxide. Polymer fluid III contained fluid II and 0.8 g of benzoyl peroxide. An amount of 400 μL of *N*,*N*-dimethyl-*p*-toluidine was added to 140 mL of fluid III at 4 °C, and the mixture was stirred for 10 min. The mixture (7 mL) and a sample were placed into a bottle, and the air in the bottle was removed by flushing with nitrogen. The bottle was kept at −20 °C for 1 week. After cropping, the embedded tissue was sliced with a microtome (Reichert-Jung 2040, Oberkochen, Germany) into 5 μm sections. Sections were stained with toluidine blue and analyzed using a Leica Qwin image analysis system for TBV% (trabecular bone volume percentage), TFS% (trabecular formation surface percentage), TRS% (trabecular resorption surface percentage), and OSW (average osteoid width).

Frozen Bone Section and Immunohistochemistry. The proximal ends ($^1/_3$) of the left femora of the mice were harvested and fixed in 4% neutral paraformaldehyde for 24 h. The femora were washed with 0.1 M PBS (pH 7.2−7.4) and decalcified with 10% EDTA·2Na/0.1 M PBS (pH 7.2−7.4) at 4 °C. The liquid was changed every other day for 4 weeks. The bone tissue was washed with 0.1 M PBS (pH 7.2−7.4) and dehydrated using a 15% sucrose solution/0.1 M PBS (pH 7.2−7.4) for 24 h. Frozen bone sections (5 μm) were obtained from a freezing microtome. Immunohistochemistry was performed to observe the expression of BMP-2. The primary and secondary antibodies were a rabbit polyclonal to BMP-2 (Abcam) and a mouse IgG/alkaline phosphatase (PV6001, Zymed), respectively. The expression of BMP-2 was observed using a Leica Qwin image analysis system.

Toxicity Test. Male and female Kunming mice (18−21 g) and female rates were purchased from the Institute of Laboratory Animal Science, Chinese Academy of Medical Sciences and Peking Union Medical College. Mice were divided into three groups with six mice each (three males, three females). The mice of groups **1e**, **5b**, and **8a** were orally given doses of 500, 700, and 500 (mg/kg)/day for 3 days, and the animals were closely monitored during the 3 days and the following 14 days to observe the acute toxicity. Rats (n = 7) were orally given compound **1** at a dose of 150 (mg/kg)/day for 3 months.

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Supporting Information Available: Purities and HPLC traces for the tested compounds in this paper. This material is available free of charge via the Internet at http://pubs.acs.org.

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