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Genistein derivatives as selective estrogen receptor modulators: Sonochemical synthesis and in vivo anti-osteoporotic action

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Abstract—Genistein derivatives were synthesized from genistein through a facile sonochemical approach in high yields. The bioassay was performed on ovariectomized (OVX) rats in terms of bone mineral density (BMD) and the weight of bone ash (WBA) to lead to the discovery of eight novel genistein-based selective estrogen receptor modulators. Attention to the structure–activity relationship disclosed that the newly introduced 2-hydroxyethylthio scaffolds were essential for the anti-osteoporotic activity. Moreover, the anti-osteoporotic action of genistein, deprivable by methylation, could be restored and enhanced by subsequent sulfonation. The most promising compound was 4′,5,7-tri[3-(2-hydroxyethylthio)propoxy]isoflavone, displaying 24% (or 8%) increment in BMD and 31% (or 11%) increase in WBA of the femora relative to those discerned with the OVX (or genistein) group. Acute toxicity test showed that none of the active compounds was acutely toxic.

1. Introduction

Osteoporosis, presently standing as a major public health problem,[1,2] is characterized by low bone mass and microarchitectural deterioration of bone tissue resulting in the bone fragility and a consequent increment in fracture risks.[3,4] It is anticipated that the cost for the hip fracture therapy in the United States will approach 250 billion US dollars by 2050.[5] Furthermore, the incidence of fractures continues to increase remarkably as the aged population is growing worldwide.[6,7] Concerning the management of osteoporosis, the hormone replacement therapy (HRT) by prescribing estrogen hormone(s) has been widely accepted for relieving climacteric symptoms of postmenopausal women since the regulative role of some estrogen hormones in maintaining bone mass in women has been unambiguously ascertained.[8–11] Moreover, this HRT practice is believed to improve women's quality of life owing to its prevention of spinal deformity or hip fracture.[12–15] In spite of the confirmed curative evidence of the therapeutical approach, severe side effects of the repeated HRT have already emerged including the increased risk in the secondary incidence of endometrial cancer, breast tumor, and venous thrombosis.[16–19] Accordingly, the current major emphasis in this regard has been re-put on searching for novel lead compounds, which can selectively regulate bone metabolism with negligible estrogenic action on uterus even after long-term administrations. Raloxifene can be reckoned as a success under this notion. However, a recently found neuronal toxicity of raloxifene at higher concentrations could be its drawback[20] necessitating continuous research and clinic attention to phytoestrogens such as isoflavones and their derivatives for more reliable selective estrogen receptor modulators (SERMs).

Genistein (4′,5,7-trihydroxyisoflavone, **1**), a major isoflavone phytochemical in some plants belonging to the Leguminosae family, is known as a phytoestrogen that is capable of binding to the estrogen receptor.[21] Much attention has been focused on the role of genistein in preventing bone loss resulted at least in part from the estrogen deficiency.[22–24] Furthermore, the finding that genistein acts as an SERM on osteoblastic cells[25] and in ovariectomized (OVX) mice[26] highlights the possibility that this isoflavone phytochemical hardly affect the uterus in some dose ranges at which it does exert the estrogen-like regulative action on bone and bone marrow

Keywords: Genistein derivatives; SERMs; Synthesis; Osteoporosis; Ovariectomized rat.
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metabolisms. However, few reports have been dedicated to the improvement of the SERM activity and the structure–activity understanding of genistein derivatives. We therefore started a project aiming at the recognition of novel genistein derivatives possessing more intensive efficacy in preventing bone loss. And during the study, special attention was paid as well to the development of a facile sonochemical approach for the selective synthesis of genistein derivatives without any protecting group strategy. We hereby wish to present the results regarding the synthesis and anti-osteoporotic evaluation of novel genistein derivatives as SERMs in vivo.

2. Results and discussion

2.1. Chemistry

Previous attempts at the regioselective preparation of genistein derivatives were limited to the phase transfer catalyzed synthesis and sequential silylations/acylations.[27] Although regioselective acylation can be carried out by taking the advantage of the subtle difference in acidity of the phenolic hydroxyls or by using *tert*-butyldimethylsilyl as protecting group,[28,29] the poor solubility of the intermediates (genistein salts) in organic solvents and/or the removal of the protecting groups often complicate or shallow the selective reactivity in most cases. Moreover, its acylated derivatives cannot be accepted as intermediates for further synthesis since the acylation usually blocks the reactivity of hydroxyls in genistein (**1**). We have very recently reported that ultrasonic irradiation could accelerate hydrolyzation of genistein esters and improve the stability of pyrone ring of genistein esters.[30] In order to investigate the application of ultrasound in the selective structure modification of natural polyphenols, ultrasound was applied hereby for the semi-synthesis of other genistein analogs. Alkyl dibromides such as 1,2-dibromoethane or 1,3-dibromopropane were used to introduce scaffolds through nucleophilic substitution reaction into the parent compound with the rest bromine 'active' enough for further derivations. As illustrated in Table 1, the sonochemical synthetic approach could, under the same or similar reaction conditions, reduce remarkably the reaction time with discernible improvements in the regioselectivity. It is noteworthy that the application of ultrasound in the modification of natural products is of particular interest since this physical technique is more convenient, economic and manageable, and gives good to excellent yields and selectivities, as exemplified by the present preparation of genistein derivatives (Schemes 1 and 2) and other reports.[31,32]

Starting from genistein (**1**), novel compounds **2**–**11** were synthesized as depicted in Scheme 1. Thus, derivatives **2**–**6** were prepared through 'step **a**' under ultrasound irradiation by treating **1** with excessive amounts of 1,2-dibromoethane or 1,3-dibromopropane. Accordingly, the 7-mono-, 4′,7-di- and 4′,5,7-tri-substituted analogs of genistein were obtained in high yields by optimizing the molar rate of **1** to K_2CO_3 as well as duration and temperature of the ultrasonic irradiation whose magnitude was set constantly at 40 kHz, at which the reaction underwent better in the study. The ideal condition for the present preparation of new genistein derivatives was detailed in Table 1 and Scheme 1. In Scheme 1, the five novel intermediates **2**–**6**, bearing the active bromine at the scaffold termini of the molecules, can readily react subsequently with nucleophiles to give multifariously designed novel analogs carrying would-be pharmacophores with nitrogen, sulfur and oxygen atoms. Thus, mercaptoethanol was allocated to react at 48 °C separately with genistein derivatives **2**–**6** in ethanol in the presence of differentiated amounts of triethylamine to give the corresponding sulfur-containing genistein derivatives **7**–**11** (Scheme 1, step **b**). Furthermore, differently controlled methylations of genistein with MeI in the presence of K_2CO_3 were performed to afford compounds **12**–**14** (Scheme 2, step **c**) which were transformed subsequently in high yield at 0 °C under ultrasonic irradiation into the corresponding target sulfonated products **15**–**17** (Scheme 2, step **d**) using concentrated sulfuric acid (98%). Technically, the separation of the obtained genistein sulfonic acids (**15**–**17**) was accomplished by taking the advantage of the difference in their solubilities in H_2SO_4 solutions. Specifically, the majority (>85%) of sulfonic acids **15**–**17** was able to precipitate from 60% H_2SO_4, and the pure products were afforded after recrystallizations of precipitated powders in water. These three sulfonic acids were transformed quantitatively by titration with NaOH into their sodium sulfonates for the bioassay.

All of the semi-synthetic products gave satisfactory analytical and spectroscopic data, which were in the full accordance with their formulated structures.

2.2. Biological evaluation

All semi-synthetic genistein derivatives except for bromides **2**–**6** (assumed to be highly toxic owing to the presence of 'active bromine') were assessed in vivo for the inhibitory effect on bone resorption on the OVX rat model by inspecting bone mineral density (BMD), the weight of bone ash (WBA), the uterine weight (as a systemic estrogen parameter), body weight and the biochemical parameters in serum.

Success of ovariectomy was confirmed by means of vagina smear (Fig. 1) and by observation of marked atrophy of uterine horns. The vagina smears were evaluated by morphological observation of cell with phase-contrast microscope. Sham-operated rats were standing in oestrus. Many dead keratinocytes were observed in the smear (Fig. 1A). Only epithelia were observed in the vagina smears of OVX rats which demonstrated that the operation of OVX rats was successful (Fig. 1B).

From the radiograms of the femora (Fig. 2) and the results of BMD, WBA (Fig. 3A and B), it could been seen that the 2-hydroxyethylthio side chains in the sulfur-containing derivatives **7**–**11** are important for the inhibition of bone loss since they were all stronger than that of the parent compound (**1**). However, the dependence of the activity on the 2-hydroxyethylthio chain numbers

Table 1. The synthetic conditions and yields for compounds **2**–**17** with and without (data in bracket) ultrasonic irradiation

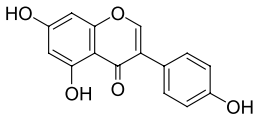
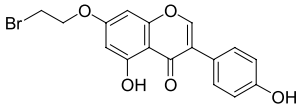
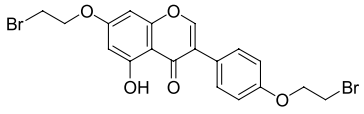
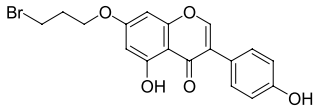
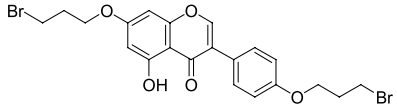
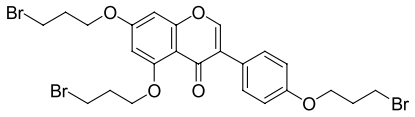
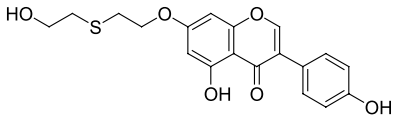
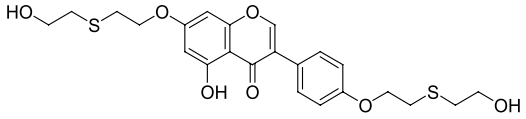
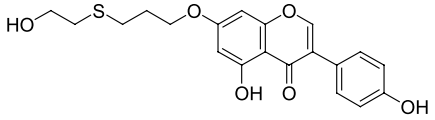
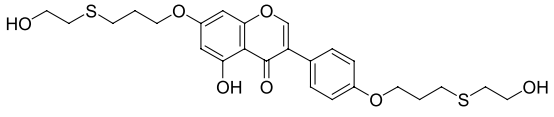
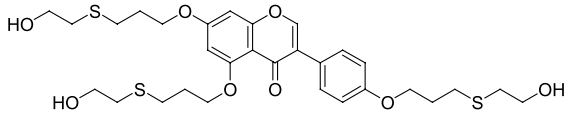
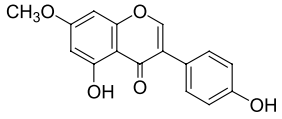
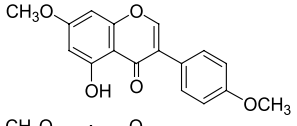
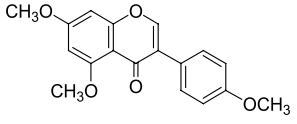
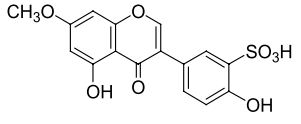
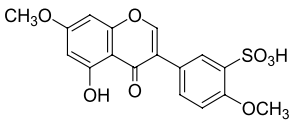
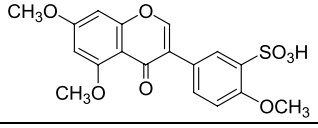
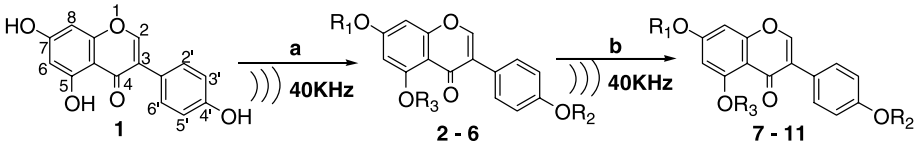
Compounds	Structures	Time (h)	Temperature (°C)	Yield (%)
1		—	—	—
2		1.5 (8)	40 (40)	86 (85)
3		3 (16)	60 (60)	82 (70)
4		1.5 (8)	40 (40)	85 (88)
5		3 (16)	60 (60)	80 (75)
6		4 (48)	80 (80)	90 (86)
7		2 (12)	48 (48)	85 (80)
8		2 (12)	48 (48)	80 (77)
9		2 (12)	48 (48)	85 (80)
10		2 (12)	48 (48)	80 (70)
11		2 (12)	48 (48)	92 (75)
12		1 (8)	50 (50)	78 (68)
13		1.5 (12)	50 (50)	80 (75)
14		3 (24)	50 (50)	85 (80)
15		0.5 (8)	0 (5)	80 (76)

Table 1 (*continued*)

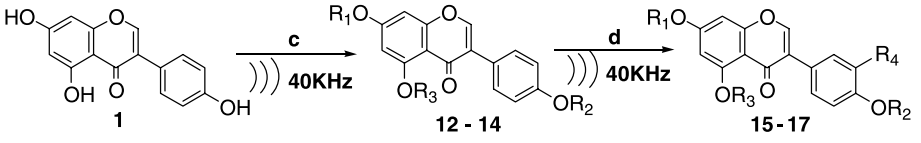
Compounds	Structures	Time (h)	Temperature (°C)	Yield (%)
16		0.5 (8)	0 (5)	85 (70)
17		0.5 (8)	0 (5)	85 (75)



Compounds	R_1	R_2	R_3
2	CH_2CH_2Br	H	H
3	CH_2CH_2Br	CH_2CH_2Br	H
4	$CH_2CH_2CH_2Br$	H	H
5	$CH_2CH_2CH_2Br$	$CH_2CH_2CH_2Br$	H
6	$CH_2CH_2CH_2Br$	$CH_2CH_2CH_2Br$	$CH_2CH_2CH_2Br$
7	$CH_2CH_2SCH_2CH_2OH$	H	H
8	$CH_2CH_2SCH_2CH_2OH$	$CH_2CH_2SCH_2CH_2OH$	H
9	$CH_2CH_2CH_2SCH_2CH_2OH$	H	H
10	$CH_2CH_2CH_2SCH_2CH_2OH$	$CH_2CH_2CH_2SCH_2CH_2OH$	H
11	$CH_2CH_2CH_2SCH_2CH_2OH$	$CH_2CH_2CH_2SCH_2CH_2OH$	$CH_2CH_2CH_2SCH_2CH_2OH$

*Reagents: (**a**) $BrCH_2CH_2Br$ or $BrCH_2CH_2CH_2Br$, K_2CO_3, DMF; (**b**) $HSCH_2CH_2OH$, triethylamine, ethanol.

Scheme 1. The synthetic route for compounds **2**–**11**. Reagents: (a) $BrCH_2CH_2Br$ or $BrCH_2CH_2CH_2Br$, K_2CO_3, DMF; (b) $HSCH_2CH_2OH$, triethylamine, ethanol.



Compounds	R_1	R_2	R_3	R_4
12	CH_3	H	H	-
13	CH_3	CH_3	H	-
14	CH_3	CH_3	CH_3	-
15	CH_3	H	H	SO_3H
16	CH_3	CH_3	H	SO_3H
17	CH_3	CH_3	CH_3	SO_3H

*Reagents: (**c**) MeI, K_2CO_3, acetone; (**d**) 98% H_2SO_4.

Scheme 2. The synthetic route for compounds **12**–**17**. Reagents: (c) MeI, K_2CO_3, acetone; (d) 98% H_2SO_4.

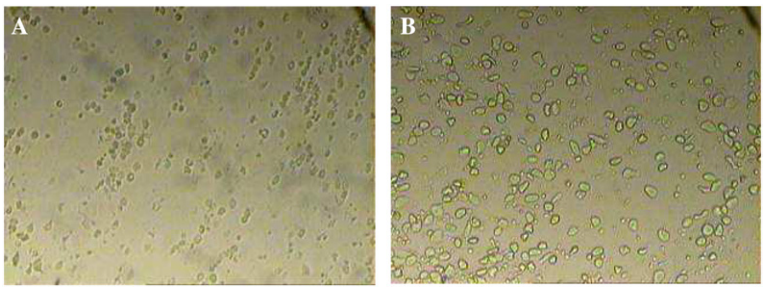


Figure 1. Vagina smears of rats: (A) Vagina smear of sham-operated rat. (B) Vagina smear of OVX rat.

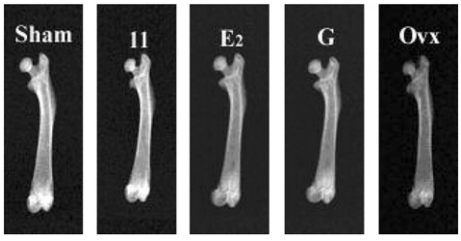


Figure 2. Radiograms of the femora. Sham and OVX expressed the femora taken from sham-operated and OVX rats, and E_2, G and **11** stood for those from OVX rats treated after surgery with 17β-estradiol at 0.5 μmol/kg per day, with genistein at 25 μmol/kg per day, and with compound **11** at 25 μmol/kg per day, respectively. The marked bone loss, observed in the distal metaphysis of the femoral cancellous bone in OVX rat, was prevented by treatments with 17β-estradiol, genistein and compound **11**.

was not very striking although the fully substituted genistein analog **11** was substantially more active than its congeners **7**–**10**. Furthermore, the anti-osteoporotic action of the methylated derivatives **12**–**14** was trivial. Any of them was not as effective as the parent compound (**1**). This observation implied that the methylation at any of the three hydroxyl groups in the starting material would attenuate the bone loss inhibitory activity. As an extreme, permethylation of genistein could deprive the isoflavone of the anti-osteoporotic activity, but the lost activity of methylated derivatives could be restored and substantially enhanced by sulfonation as demonstrated by the anti-osteoporotic data of **15**–**17** (bioassayed as the corresponding sodium salts). It is noteworthy that the direct sulfonation of genistein gave complex mixture (data not shown) suggesting that the

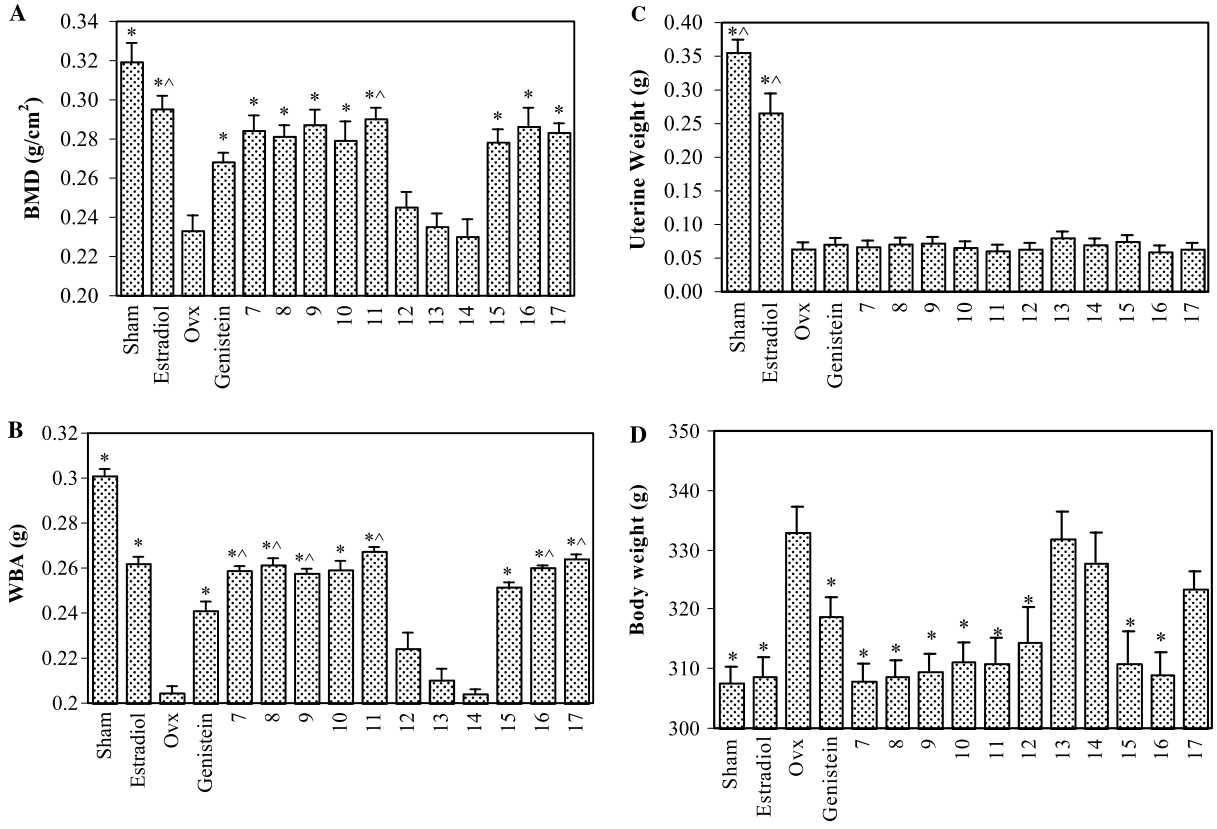


Figure 3. Effect of genistein, its analogs **7**–**17** and 17β-estradiol on BMD (A), WBA (B), uterine weight (C) and body weight (D). BMD, WBA, uterine weight and body weight values were expressed as mean ± SEM based on six independent experiments. The p values were obtained using Student's t-test. (*) $p < 0.01$, compared with the value of OVX group; and (∧) $p < 0.01$, compared with the value of genistein group.

derivation strategy was of very limited practical value. The aforementioned observations highlighted that the sulfur element could be most possibly helpful for intensifying the anti-osteoporotic activity of genistein. The magnitude of **11** in inhibiting bone resorption has a 24% increment in BMD and a 31% increase in WBA of the femora when compared with those of the OVX control ($p < 0.01$). Relative to those of genistein (**1**), compound **11** increased BMD and WBA of femora by 8% and 11% ($p < 0.01$), respectively.

As expected, the weights of uterus in OVX rats were significantly decreased when compared with that in sham-operated rats (Fig. 3C), but on the other hand, OVX animals gained more body weight (Fig. 3D) during the study period than that of sham rats ($p < 0.01$). The gained body weight due to ovarian hormone deficiency was prevented by estrogen, genistein, or its derivatives' administration. The uterine weight of OVX rats was increased by approximately fourfolds after peroral administration of 17β-estradiol at a dose of 0.5 μmol/kg per day. However, the OVX rats treated with genistein or its derivatives at 25 μmol/kg per day did not show any increment in uterine weights. This observation indicates that a big merit of genistein and its derivatives lies in exerting, irrespective of the uterus, the estrogen-like regulation on bone and bone marrow metabolisms.

With regard to the bone metabolic markers, the activity of serum alkaline phosphatase (ALP) associated with bone formation was significantly increased after ovariectomy. Although the serum calcium phosphorus level did not differ significantly among the sham, OVX control group, and the other treated groups (Fig. 4A and B), treatment with 17β-estradiol or the derivatives **9**, **11**, **15**, **16** for two months significantly decreased the level of ALP (Fig. 4C). Serum concentration of ALP, an index of bone formation, was significantly greater in the OVX group than in the sham-operated group.[33,34] Both 17β-estradiol and genistein derivatives decreased serum ALP in the OVX rats in our study. The results from bone metabolic makers, BMD, WBA and X-ray observation indicated that bone turnover was increased after ovariectomy and the incidence of the bone turnover could be decreased by peroral administration of genistein or its derivatives.

To get the knowledge about the acute toxicity of the active materials, compounds **1**, **7**–**9**, **11** and **16** were orally administrated to mice ($n = 10$) at 27 mmol/kg (a 500-fold effective dosage). The tested animals appeared normal immediately after the administration and did not exhibit any indication of acute toxicity for 14 days afterward (Table 2). The body weights in treated mice were similar to that of the control mice dosed with an equal volume of vehicle. The result from this acute toxicity determination demonstrated that the genistein and its derivatives (**7**–**9**, **11** and **16**) are not acutely toxic.

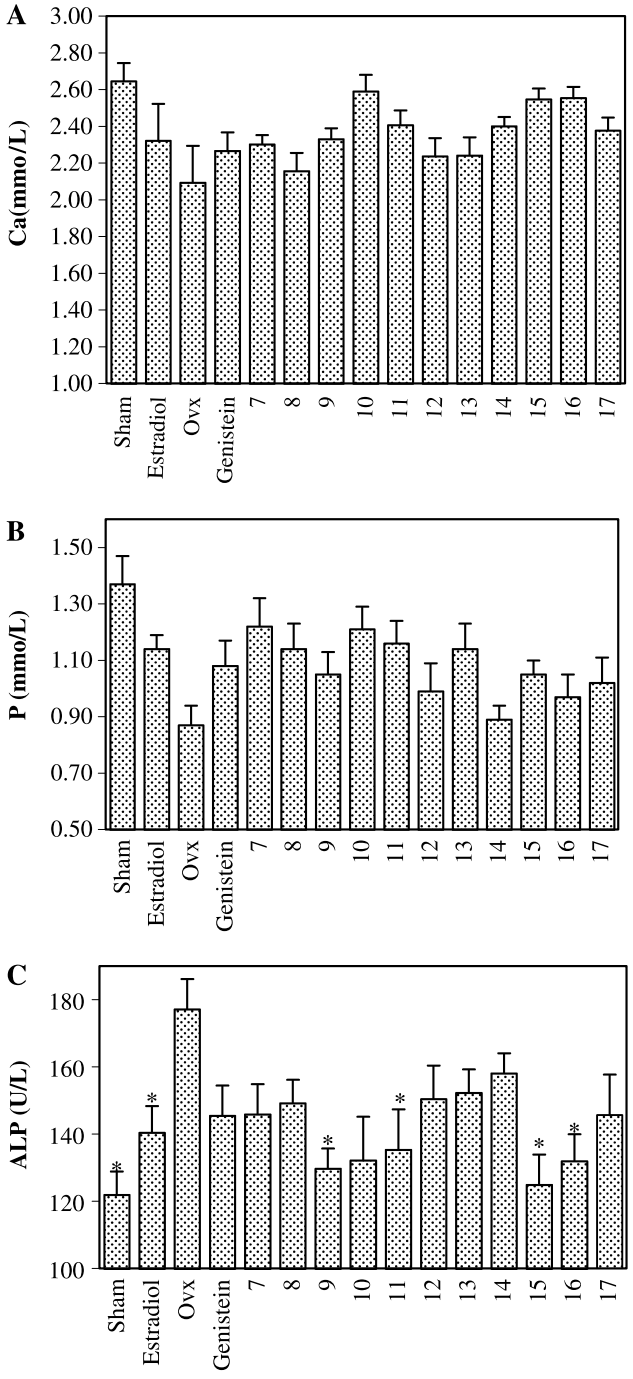


Figure 4. Serum biochemical analysis on calcium (**A**), phosphorus (**B**) and alkaline phosphatase (ALP) (**C**). Values were expressed as mean ± SEM based on six independent experiments. The p values were obtained using Student's t-test. (*) $p < 0.01$, compared with the value of OVX group.

3. Conclusion

A set of novel anti-osteoporotic genistein derivatives have been synthesized through an efficient sonochemical approach with the desired regioselectivity allowed by the subtle difference in acidity and regioaccessibility of hydroxyls co-anchored on the isoflavone skeleton. Another strategic key point lies in the selective introduction of 'active bromine'-bearing scaffolds into the designed key bromides of genistein, from which further derivations can be realized with the target compounds afforded in high yields. These methodological merits may shed a new light on the potent application in the selective struc-

Table 2. The acute toxic determination of compounds **1**, **7**–**9**, **11** and **16**

Tested compounds	Number of mice	Limited (mmol/kg)	Limited (mg/kg)	Body weight[a] on day 1	Body weight[a] on day 14
Genistein (**1**)	10	27	7290	20.0 ± 1.0	29.1 ± 0.3
7	10	27	10,098	19.8 ± 0.8	28.9 ± 0.7
8	10	27	12,906	20.5 ± 0.6	28.5 ± 0.7
9	10	27	10,476	20.1 ± 0.4	28.7 ± 0.4
11	10	27	16,848	19.5 ± 0.5	29.0 ± 1.0
16	10	27	10,800	20.3 ± 0.9	28.6 ± 0.5
Vehicle	10	—	—	20.4 ± 0.7	28.3 ± 0.9

[a] Body weights were mean ± SEM ($n = 10$).

ture modification of natural polyphenols such as resveratrol and fisetin, both being of current special concerns.[35] The anti-osteoporotic activity of the new derivatives was evaluated in vivo to find eight new SERMs (**7**–**11** and **15**–**17**), each being more active than genistein. The attention to the structure–activity relationship of the series of semi-synthetic isoflavone derivatives demonstrated that the newly introduced 2-hydroxyethylthio motif was a key pharmacophore for the inhibition of bone loss.

4. Experimental

4.1. General

Reaction and the resulted products were monitored by thin-layer chromatography (TLC) on Merck pre-coated silica gel F_{254} plates with separated compounds visualized at 254 nm under a UV lamp and/or by spraying with a diluted ferric chloride solution. Melting points (uncorrected) were determined on a XT4 MP apparatus (Taike Corp., Beijing, China). The IR spectra were recorded on a Perkin-Elmer FT-IR spectrometer (KBr pellets). ESI mass spectra were obtained on a Mariner System 5304 high-resolution mass instrument, and ^{1}H NMR spectra were recorded in DMSO-d_6 on a Bruker DPX500 or DPX300 spectrometer with TMS and solvent signals allotted as internal standards. Elemental analyses were performed on a CHN–O-Rapid instrument and were within ± 0.4% of the theoretical values. Sonication was performed in a Kunshan KQ 500E ultrasonic cleaner (Jiangsu, China) with irradiation delivered at 40 kHz and 500 W. The reaction flask was positioned in the maximum-energy area in the cleaner with cycled water running to control the temperature of the water bath. BMD measurements were performed for the whole femur on a Lunar DPX-IQ dual-energy X-ray densitometer. Serum alkaline phosphatase (ALP), serum total calcium (Ca) and phosphorus (P) were measured with spectrophotometer using kits (kits were purchased from Nanjing Jiancheng Bioengineering Institute, China).

4.2. Chemistry

1,2-Dibromoethane, 1,3-dibromopropane, triethylamine, mercaptoethanol, acetone, ethanol, DMF, MeI, H_2SO_4 (98%), NaOH and K_2CO_3, all being of A. R. grade, were purchased from Shanghai Chemical Reagent Company (Shanghai, China). Genistein (**1**, >96%) provided by Shanxi Huike Botanical Development Co. Ltd was further refined prior to use by its recrystallization from ethanol as pale yellow needles, mp 298–299 °C; IR ν_{max} cm^{-1}: 3411 (OH), 1650 (C=O); ^{1}H NMR δ: 6.21 (d, $J = 1.9$ Hz, 1H, H-8), 6.37 (d, $J = 1.9$ Hz, 1H, H-6), 6.80 (d, $J = 8.5$ Hz, 2H, H-3′ and H-5′), 7.35 (d, $J = 8.5$ Hz, 2H, H-2′ and H-6′), 8.32 (s, 1H, H-2), 9.64 (s, 1H, 4′-OH), 10.94 (s, 1H, 7-OH), 12.96 (s, 1H, 5-OH); HRMS (ESI) Calcd $C_{15}H_{10}O_5$ $[M-H]^-$ 269.0447. Found 269.0449. Anal. Calcd for $C_{15}H_{10}O_5$: C, 66.67; H, 3.73. Found: C, 66.68; H, 3.87.

The purity of each active compound mentioned in the study was checked to be >99% through elemental analyses plus HRMS and ^{1}H NMR spectra.

4.2.1. 4′,5-Dihydroxy-7-(2-bromoethoxy)isoflavone (2). Genistein (0.27 g, 1 mmol), dibromoethane (4.7 g, 25 mmol) and potassium carbonate (0.07 g, 0.5 mmol) in 60 ml of dry DMF were sonicated. After the completion of reaction, the resultant mixture was cooled to room temperature and filtered. The filtrate was distilled to form yellow solid. Recrystallization of the solid from 15 ml acetone gave compound **2** as yellow needle, mp 175–178 °C; IR ν_{max} cm^{-1}: 3421 (OH), 2922 (CH_2), 1665 (C=O); ^{1}H NMR δ: 3.81 (t, $J = 5.5$ Hz, 2H, –CH_2Br), 4.43 (t, $J = 5.5$ Hz, 2H, –OCH_2–), 6.41 (d, $J = 2.0$ Hz, 1H, H-8), 6.67 (d, $J = 2.0$ Hz, 1H, H-6), 6.82 (dd, $J = 8.5$ Hz, 1.0 Hz, 2H, H-3′ and H-5′), 7.38 (d, $J = 8.5$ Hz, 2H, H-2′ and H-6′), 8.39 (d, $J = 1.0$ Hz, 1H, H-2), 9.64 (s, 1H, 4′-OH), 12.95 (s, 1H, 5-OH); HRMS (ESI) Calcd $C_{17}H_{13}BrO_5$ $[M-H]^-$ 374.9864. Found 374.9886. Anal. Calcd for $C_{17}H_{13}BrO_5$: C, 54.13; H, 3.47. Found: C, 54.06; H, 3.51.

4.2.2. 4′,7-Di(2-bromoethoxy)-5-hydroxyisoflavone (3). Genistein (0.27 g, 1 mmol), dibromoethane (9.4 g, 50 mmol) and potassium carbonate (0.14 g, 1 mmol) in 60 ml of dry DMF were sonicated. After the completion of reaction, the afforded mixture was cooled to room temperature and filtered. The filtrate is distilled to yield yellow solid. Recrystallization of the solid from 15 ml acetone gave compound **3** as pale yellow needle, mp 140–142 °C; IR ν_{max} cm^{-1}: 3447 (OH), 2929 (CH_2), 1663 (C=O); ^{1}H NMR δ: 3.80 (overlapped multiplets, 4H, 2(–CH_2Br)), 4.34 (t, $J = 5.5$ Hz, 2H, –OCH_2–), 4.44 (t, $J = 5.4$ Hz, 2H, –OCH_2–), 6.44 (t, $J = 1.5$ Hz,

1H, H-8), 6.70 (t, $J = 1.5$ Hz, 1H, H-6), 7.03 (d, $J = 8.5$ Hz, 2H, H-3′ and H-5′), 7.50 (d, $J = 8.5$ Hz, 2H, H-2′ and H-6′), 8.42 (s, 1H, H-2), 12.93 (s, 1H, 5-OH); HRMS (ESI) Calcd $C_{19}H_{16}Br_2O_5$ $[M+H]^+$ 482.9437. Found 482.9415. Anal. Calcd for $C_{19}H_{16}Br_2O_5$: C, 47.14; H, 3.33. Found: C, 47.19; H, 3.45.

4.2.3. 4′,5-Dihydroxy-7-(3-bromopropoxy)isoflavone (4). Genistein (0.27 g, 1 mmol), dibromopropane (5.1 g, 25 mmol) and potassium carbonate (0.07 g, 0.5 mmol) in 60 ml of dry DMF were sonicated. After the completion of reaction, the obtained mixture was cooled to room temperature and filtered. The filtrate is distilled to give yellow solid. Recrystallization of the solid from 15 ml acetone gave compound **4** as yellow needle, mp 124–126 °C; IR ν_{max} cm^{-1}: 3423 (OH), 2934 (CH_2), 1663 (C=O); ^1H NMR δ: 2.24 (m, 2H, –$CH_2CH_2CH_2$–), 3.65 (t, $J = 5.5$ Hz, 2H, –CH_2Br), 4.18 (t, $J = 5.5$ Hz, 2H, –OCH_2–), 6.42 (s, 1H, H-8), 6.67 (s, 1H, H-6), 6.82 (d, $J = 8.5$ Hz, 2H, H-3′ and H-5′), 7.38 (d, $J = 8.5$ Hz, 2H, H-2′ and H-6′), 8.40 (s, 1H, H-2), 9.63 (s, 1H, 4′-OH), 12.95 (s, 1H, 5-OH); HRMS (ESI) Calcd $C_{18}H_{15}BrO_5$ $[M-H]^-$ 389.0020. Found 389.0008. Anal. Calcd for $C_{18}H_{15}BrO_5$: C, 55.26; H, 3.86. Found: C, 55.14; H, 3.80.

4.2.4. 4′,7-Di(3-bromopropoxy)-5-hydroxyisoflavone (5). Genistein (0.27 g, 1 mmol), dibromopropane (10.2 g, 50 mmol) and potassium carbonate (0.14 g, 1 mmol) in 60 ml of dry DMF were sonicated. After the completion of reaction, the yielded mixture was cooled to room temperature and filtered. The filtrate is distilled to give yellow solid. Recrystallization of the solid from 10 ml acetone gave compound **5** as pale yellow needle, mp 128–130 °C; IR ν_{max} cm^{-1}: 3447 (OH), 2927, 2875 (CH_2), 1661 (C=O); ^1H NMR δ: 2.24 (m, 4H, 2(–$CH_2CH_2CH_2$–)), 3.65 (overlapped multiplets, 4H, 2 (–CH_2Br)), 4.08 (overlapped multiplets, 4H, 2(–OCH_2–)), 6.41 (br s, 1H, H-8), 6.66 (br s, 1H, H-6), 7.01 (d, $J = 8.5$ Hz, 2H, H-3′ and H-5′), 7.50 (d, $J = 8.5$ Hz, 2H, H-2′ and H-6′), 8.43 (s, 1H, H-2), 12.91 (s, 1H, 5-OH); HRMS (ESI) Calcd $C_{21}H_{20}Br_2O_5$ $[M+H]^+$ 510.9749. Found 510.9790. Anal. Calcd for $C_{21}H_{20}Br_2O_5$: C, 49.24; H, 3.94. Found: C, 49.34; H, 3.88.

4.2.5. 4′,5,7-Tri(3-bromopropoxy)isoflavone (6). Genistein (0.27 g, 1 mmol), dibromopropane (15.3 g, 75 mmol) and potassium carbonate (0.49 g, 3.5 mmol) in 60 ml of dry DMF were sonicated. After the completion of reaction, the reaction mixture was cooled to room temperature and filtered. The filtrate is distilled and yellow solid was formed. Recrystallization from 10 ml acetone gave pure compound **6** in colorless needle, mp 110–112 °C; IR ν_{max} cm^{-1}: 3447 (OH), 2930, 2874 (CH_2), 1644 (C=O);^1H NMR δ: 2.22 (overlapped multiplets, 6H, 3(–$CH_2CH_2CH_2$–)), 3.66 (t, $J = 6.4$ Hz, 4H, 2 (–CH_2Br)), 3.82 (t, $J = 6.4$ Hz, 2H, –CH_2Br), 4.08 (overlapped multiplets, 4H, 2(–OCH_2–)), 4.23 (t, $J = 5.9$ Hz, 2H, –OCH_2–), 6.53 (d, $J = 2.1$ Hz, 1H, H-8), 6.69 (d, $J = 2.1$ Hz, 1H, H-6), 6.97 (d, $J = 8.5$ Hz, 2H, H-3′ and H-5′), 7.41 (d, $J = 8.5$ Hz, 2H, H-2′ and H-6′), 8.20 (s, 1H, H-2); HRMS (ESI) Calcd $C_{24}H_{25}Br_3O_5$ $[M+H]^+$ 630.9322. Found 630.9722. Anal. Calcd for $C_{24}H_{25}Br_3O_5$: C, 45.53; H, 3.98. Found: C, 45.48; H, 3.83.

4.2.6. 4′,5-Dihydroxy-7-[2-(2-hydroxyethylthio)ethoxy]isoflavone (7). Compound **2** (0.37 g, 1 mmol), mercaptoethanol (0.15 g, 2 mmol) and triethylamine (0.11 g, 1 mmol) in 50 ml of dry ethanol were sonicated. After the completion of reaction, the resulted mixture was distilled to give pale yellow residue which was washed with water. Recrystallization of the washed solid from 20 ml acetone afforded compound **7** as white needle, mp 132–135 °C; IR ν_{max} cm^{-1}: 3420 (OH), 2926 (CH_2), 1664 (C=O), 1047 (C–OH); ^1H NMR δ: 2.66 (t, $J = 6.5$ Hz, 2H, –SCH_2CH_2O–), 2.91 (t, $J = 6.5$ Hz, 2H, $HOCH_2CH_2S$–), 3.55 (t, $J = 6.5$ Hz, 2H, –CH_2OH), 4.24 (t, $J = 6.5$ Hz, 2H, –OCH_2–), 6.40 (d, $J = 1.5$ Hz, 1H, H-8), 6.66 (d, $J = 1.5$ Hz, 1H, H-6), 6.82 (d, $J = 8.5$ Hz, 2H, H-3′ and H-5′), 7.38 (d, $J = 8.5$ Hz, 2H, H-2′ and H-6′), 8.40 (s, 1H, H-2), 9.62 (s, 1H, 4′-OH), 12.95 (s, 1H, 5-OH); HRMS (ESI) Calcd $C_{19}H_{18}O_6S$ $[M-H]^-$ 373.0740. Found 373.0742. Anal. Calcd for $C_{19}H_{18}O_6S$: C, 60.95; H, 4.85. Found: C, 60.99; H, 4.89.

4.2.7. 4′,7-Di[2-(2-hydroxyethylthio)ethoxy]-5-hydroxyisoflavone (8). Compound **3** (0.48 g, 1 mmol), mercaptoethanol (0.30 g, 4 mmol) and triethylamine (0.22 g, 2 mmol) in 50 ml of dry ethanol were sonicated. After the completion of reaction, the afforded mixture was distilled to give pale yellow residue which was washed with water. Recrystallization of the washed solid from 15 ml acetone gave compound **8** as white needle, mp 66–68 °C; IR ν_{max} cm^{-1}: 3431 (OH), 2924, 2854 (CH_2), 1663 (C=O), 1048 (C–OH); ^1H NMR δ: 2.66 (t, $J = 6.5$ Hz, 4H, 2(–SCH_2CH_2O–)), 2.90 (q, 4H, 2($HOCH_2CH_2S$–)), 3.55 (t, $J = 6.5$ Hz, 4H, 2(–CH_2OH)), 4.15 (t, $J = 6.5$ Hz, 2H, –OCH_2–), 4.25 (t, $J = 6.5$ Hz, 2H, –OCH_2–), 6.42 (d, $J = 2.0$ Hz, 1H, H-8), 6.69 (d, $J = 2.0$ Hz, 1H, H-6), 7.00 (d, $J = 8.5$ Hz, 2H, H-3′ and H-5′), 7.50 (d, $J = 8.5$ Hz, 2H, H-2′ and H-6′), 8.46 (s, 1H, H-2), 12.92 (s, 1H, 5-OH); HRMS (ESI) Calcd $C_{23}H_{26}O_7S_2$ $[M-H]^-$ 477.1033. Found 477.1002. Anal. Calcd for $C_{23}H_{26}O_7S_2$: C, 57.72; H, 5.48. Found: C, 57.51; H, 5.52.

4.2.8. 4′,5-Dihydroxy-7-[3-(2-hydroxyethylthio)propoxy]isoflavone (9). Compound **4** (0.39 g, 1 mmol), mercaptoethanol (0.15 g, 2 mmol) and triethylamine (0.11 g, 1 mmol) in 30 ml of dry ethanol were sonicated. After the completion of reaction, the resultant mixture was distilled to give yellow residue which was washed with water. Recrystallization of the washed solid from 20 ml acetone to yield compound **9** as white needle, mp 152–154 °C; IR ν_{max} cm^{-1}: 3434 (OH), 2942, 2920, 2876 (CH_2), 1664 (C=O), 1042 (C–OH); ^1H NMR δ: 1.95 (overlapped multiplets, 2H, –$SCH_2CH_2CH_2O$–), 2.56 (t, $J = 6.8$ Hz, 2H, –$SCH_2CH_2CH_2O$–), 2.64 (t, $J = 7.1$ Hz, 2H, $HOCH_2CH_2S$–), 3.50 (t, $J = 7.1$ Hz, 2H, $HOCH_2$–), 4.14 (t, $J = 6.2$ Hz, 2H, –CH_2O–), 6.39 (d, $J = 2.0$ Hz, 1H, H-8), 6.65 (d, $J = 2.0$ Hz, 1H, H-6), 6.80 (d, $J = 8.5$ Hz, 2H, H-3′ and H-5′), 7.37 (d, $J = 8.5$ Hz, 2H, H-2′ and H-6′), 8.40 (s, 1H, H-2), 9.62 (s, 1H, 4′-OH), 12.95 (s, 1H, 5-OH); HRMS

(ESI) Calcd $C_{20}H_{20}O_6S$ $[M-H]^-$ 387.0896. Found 387.0872. Anal. Calcd for $C_{20}H_{20}O_6S$: C, 61.84; H, 5.19. Found: C, 61.86; H, 5.20.

4.2.9. 4′,7-Di[3-(2-hydroxyethylthio)propoxy]-5-hydroxyisoflavone (10). Compound **5** (0.51 g, 1 mmol), mercaptoethanol (0.30 g, 4 mmol) and triethylamine (0.22 g, 2 mmol) in 30 ml of dry ethanol were sonicated. After the completion of reaction, the mixture was distilled to give pale yellow residue which was washed with water. Recrystallization of the washed solid from 15 ml acetone afforded compound **10** as colorless needle, mp 102–105 °C; IR ν_{max} cm^{-1}: 3421 (OH), 2923, 2877 (CH_2), 1671 (C=O), 1049 (C–OH); ^1H NMR δ: 1.93 (overlapped multiplets, 4H, 2(–$SCH_2CH_2CH_2O$–)), 2.56 (t, J = 6.8 Hz, 4H, 2(–$SCH_2CH_2CH_2O$–)), 2.64 (t, J = 6.8 Hz, 4H, 2($HOCH_2CH_2S$–)), 3.51 (t, J = 6.8 Hz, 4H, 2($HOCH_2$–)), 4.14 (t, J = 6.0 Hz, 4H, 2(–OCH_2–)), 6.39 (d, J = 2.0 Hz, 1H, H-8), 6.64 (d, J = 2.0 Hz, 1H, H-6), 6.80 (d, J = 8.5 Hz, 2H, H-3′ and H-5′), 7.36 (d, J = 8.5 Hz, 2H, H-2′ and H-6′), 8.39 (s, 1H, H-2), 12.92 (s, 1H, 5-OH); HRMS (ESI) Calcd $C_{25}H_{30}O_7S_2$ $[M+Na]^+$ 529.1293. Found 529.1360. Anal. Calcd for $C_{25}H_{30}O_7S_2$: C, 59.27; H, 5.97. Found: C, 59.19; H, 5.93.

4.2.10. 4′,5,7-Tri[3-(2-hydroxyethylthio)propoxy]isoflavone (11). Compound **6** (0.63 g, 1 mmol), mercaptoethanol (0.30 g, 4 mmol) and triethylamine (0.33 g, 3 mmol) in 30 ml of dry ethanol were sonicated. After the completion of reaction, the reaction mixture was distilled. The pale residue was washed by water and recrystallized from 10 ml acetone to give pure compound **10** in colorless needle, mp 78–80 °C; IR ν_{max} cm^{-1}: 3430 (OH), 2922, 2872 (CH_2), 1639 (C=O), 1051 (C–OH); ^1H NMR δ: 1.96 (overlapped multiplets, 6H, 3(–$SCH_2CH_2CH_2O$–)), 2.56 (m, 6H, 3(–$SCH_2CH_2CH_2O$–)), 2.66 and 2.67 (t, J = 7.0 Hz, 2H each, 2($HOCH_2CH_2S$–)), 2.78 (t, J = 7.0 Hz, 2H, $HOCH_2CH_2S$–), 3.53 (t, J = 7.1 Hz, 4H, 2($HOCH_2$–)), 3.51 (t, J = 7.1 Hz, 2H, $HOCH_2$–), 4.19, 4.12 and 4.07 (t, J = 6.1 Hz, 2H each, 3(–OCH_2–)), 6.50 (d, J = 2.1 Hz, 1H, H-8), 6.64 (d, J = 2.1 Hz, 1H, H-6), 6.95 (d, J = 8.8 Hz, 2H, H-3′ and H-5′), 7.40 (d, J = 8.8 Hz, 2H, H-2′ and H-6′), 8.16 (s, 1H, H-2); HRMS (ESI) Calcd $C_{30}H_{40}O_8S_3$ $[M+H]^+$ 625.1959. Found 625.1962. Anal. Calcd for $C_{30}H_{40}O_8S_3$: C, 57.67; H, 6.45. Found: C, 57.68; H, 6.39.

4.2.11. 4′,5-Dihydroxy-7-methoxyisoflavone (12). Genistein (0.27 g, 1 mmol), iodomethane (0.13 ml, 1 mmol) and potassium carbonate (0.07 g, 0.5 mmol) in 50 ml of dry acetone were sonicated. After the completion of reaction, the afforded mixture was cooled to room temperature followed by filtration. The filtrate was distilled to form a yellow solid. Recrystallization of the solid from 20 ml hot ethanol yielded compound **12** as pale yellow needle, mp 240–242 °C; IR ν_{max} cm^{-1}: 3412 (OH), 2964 (CH_3), 1654 (C=O); ^1H NMR δ: 3.86 (s, 3H, 7-OCH_3), 6.41 (br s, 1H, H-8), 6.65 (br s, 1H, H-6), 6.82 (d, J = 8.0 Hz, 2H, H-3′ and H-5′), 7.38 (d, J = 8.0 Hz, 2H, H-2′ and H-6′), 8.40 (s, 1H, H-2), 9.61 (s, 1H, 4′-OH), 12.95 (s, 1H, 5-OH); HRMS (ESI) Calcd $C_{16}H_{12}O_5$ $[M-H]^-$ 283.0603. Found 283.0599. Anal. Calcd for $C_{16}H_{12}O_5$: C, 67.60; H, 4.26. Found: C, 67.64; H, 4.37.

4.2.12. 4′,7-Dimethoxy-5-hydroxyisoflavone (13). Genistein (0.27 g, 1 mmol), iodomethane (0.26 ml, 2 mmol) and potassium carbonate (0.14 g, 1 mmol) in 50 ml of dry acetone were sonicated. After the completion of reaction, the given mixture was cooled to room temperature followed by filtration. The filtrate was distilled to give a yellow solid. Recrystallization of the solid from 20 ml hot acetone afforded compound **13** as pale yellow needle, mp 138–139 °C; IR ν_{max} cm^{-1}: 3434 (OH), 2958, 2972 (CH_3), 1650 (C=O); ^1H NMR δ: 3.84 and 3.89 (s, 3H each, 4′, 7-OCH_3), 6.40 (d, J = 2.0 Hz, 1H, H-8), 6.42 (d, J = 2.0 Hz, 1H, H-6), 6.99 (d, J = 9.0 Hz, 2H, H-3′ and H-5′), 7.42 (d, J = 9.0 Hz, 2H, H-2′ and H-6′), 7.88 (s, 1H, H-2), 12.89 (s, 1H, 5-OH); HRMS (ESI) Calcd $C_{17}H_{14}O_5$ $[M+H]^+$ 299.0915. Found 299.0899. Anal. Calcd for $C_{17}H_{14}O_5$: C, 68.45; H, 4.73. Found: C, 68.45; H, 4.82.

4.2.13. 4′,5,7-Trimethoxyisoflavone (14). Genistein (0.27 g, 1 mmol), iodomethane (0.62 ml, 3.5 mmol) and potassium carbonate (0.41 g, 3 mmol) in 50 ml of dry acetone were sonicated. After the completion of reaction, the yielded mixture was cooled to room temperature and filtered. The filtrate was distilled to afford a yellow solid. Recrystallization of the solid from 20 ml hot acetone gave compound **14** as colorless needle, mp 161–163 °C; IR ν_{max} cm^{-1}: 2968, 2938 (CH_3), 1633 (C=O); ^1H NMR δ: 3.77, 3.82 and 3.87 (s, 3H each, 4′, 5,7-OCH_3), 6.50 (d, J = 2.3 Hz, 1H, H-8), 6.66 (d, J = 2.3 Hz, 1H, H-6), 6.94 (d, J = 8.8 Hz, 2H, H-3′ and H-5′), 7.40 (d, J = 8.8 Hz, 2H, H-2′ and H-6′), 8.20 (s, 1H, H-2); HRMS (ESI) Calcd $C_{18}H_{16}O_5$ $[M+H]^+$ 313.1071. Found 313.1051. Anal. Calcd for $C_{18}H_{16}O_5$: C, 69.22; H, 5.16. Found: C, 69.25; H, 5.39.

4.2.14. 4′,5-Dihydroxy-7-methoxyisoflavone-3′-sulfonic acid (15). Concentrated sulfuric acid (0.5 ml, 98%) and compound **12** (0.28 g, 1 mmol) in an erlenmeyer flask were sonicated in an ice bath. After the completion of reaction, the mixture was diluted with 0.5 ml of distilled water to give white solid as precipitate. Recrystallization of the solid from 5 ml water afforded compound **15** as white needle, mp 365 °C (dec); IR ν_{max} cm^{-1}: 3419 (OH), 2956 (CH_3), 1655 (C=O), 1179 (S=O); ^1H NMR δ: 3.86 (s, 3H, 7-OCH_3), 6.41 (d, J = 2.1 Hz, 1H, H-8), 6.66 (s, 1H, H-6), 6.84 (d, J = 8.5 Hz, 1H, H-5′), 7.39 (dd, J = 8.5, 1.2 Hz, 1H, H-6′), 7.70 (d, J = 1.2 Hz, 1H, H-2′), 8.43 (s, 1H, H-2); HRMS (ESI) Calcd $C_{16}H_{12}O_8S$ $[M-H]^-$ 363.0170. Found 363.0130. Anal. Calcd for $C_{16}H_{12}O_8S$: C, 52.75; H, 3.32. Found: C 52.61; H, 3.17. Compound **15** was neutralized with NaOH to obtain its corresponding sodium sulfonate for bioassay.

4.2.15. 4′,7-Dimethoxy-5-hydroxyisoflavone-3′-sulfonic acid (16). Concentrated sulfuric acid (0.5 ml, 98%) and compound **13** (0.30 g, 1 mmol) in an erlenmeyer flask were sonicated in an ice bath. After the completion of reaction, the mixture was diluted with 0.5 ml of distilled water to form white solid. Recrystallization of the solid

from 5 ml water yielded compound **16** as white needle, mp 360 °C (dec); IR ν_{max} cm^{-1}: 3421 (OH), 2957 (CH_3), 1654 (C=O), 1199 (S=O); ^1H NMR δ: 3.78 and 3.86 (s, 3H each, 4′, 7-OCH_3), 6.41 (br s, 1H, H-8), 6.67 (br s, 1H, H-6), 7.01 (d, J = 8.6 Hz, 1H, H-5′), 7.45 (dd, J = 8.6, 2.3 Hz, 1H, H-6′), 7.89 (d, J = 2.3 Hz, 1H, H-2′), 8.42 (s, 1H, H-2), 12.92 (s, 1H, 5-OH); HRMS (ESI) Calcd $C_{17}H_{14}O_8S$ $[M-H]^-$ 377.0326. Found 377.0346. Anal. Calcd for $C_{17}H_{14}O_8S$: C, 53.97; H, 3.73. Found: C, 53.88; H, 3.59. Compound **16** was neutralized with NaOH to obtain its corresponding sodium sulfonate for bioassay.

4.2.16. 4′,5,7-Trimethoxyisoflavone-3′-sulfonic acid (17). Concentrated sulfuric acid (0.5 ml, 98%) and compound **14** (0.31 g, 1 mmol) in an erlenmeyer flask were sonicated in an ice bath. After the completion of reaction, the mixture was diluted with 0.5 ml of distilled water to give white solid. Recrystallization of the solid from 5 ml water yielded compound **17** as white needle, mp 360 °C (dec); IR ν_{max} cm^{-1}: 3431 (OH), 2955 (CH_3), 1653 (C=O), 1180 (S=O); ^1H NMR δ: 3.66, 3.72 and 3.81 (s, 3H each, 4′, 5,7-OCH_3), 6.26 (br s, 1H, H-8), 6.36 (br s, 1H, H-6), 7.00 (d, J = 9.0 Hz, 1H, H-5′), 7.32 (dd, J = 9.0 Hz, 2.0 Hz, 1H, H-6′), 7.72 (d, J = 2.0 Hz, 1H, H-2′), 7.94 (s, 1H, H-2); HRMS (ESI) Calcd $C_{18}H_{16}O_8S$ $[M-H]^-$ 391.0482. Found 391.0462. Anal. Calcd for $C_{18}H_{16}O_8S$: C, 55.10; H, 4.11. Found: C, 54.98; H, 4.23. Compound **17** was neutralized with NaOH to obtain its corresponding sodium sulfonate for bioassay.

4.3. Bioassay on the OVX rat model

Ninety-six female Sprague–Dawley rats were obtained from Nanjing University of Traditional Chinese Medicine, aged 2 months, with an average weight of 202 ± 2.5 g (mean ± SEM). Bilateral ovariectomies were performed from a dorsal approach with a small midline dorsal skin incision. The sham-operated rats were subject to sham surgery exposing, but the ovaries were not removed. Success of ovariectomy was confirmed by means of vagina smear and by observation of marked atrophy of uterine horns. All rats were housed in a metal cage on a 12 h light/dark cycle at 22 °C and 60% humidity with free access to tap water. The rats were weighed every week during the experiments.

Genistein derivatives were tested in vivo for their ability to inhibit bone resorption on OVX rat model with the animal aged 2 months. Genistein and its analogs **7**–**17**, as a suspension or uniform solution in DMSO/water (1:9) mixture, were dosed orally to OVX rats at 25 μmol/kg per day through a stomach tube for two months, and 17β-estradiol at 0.5 μmol/kg per day at the same condition. 17β-estradiol, Genistein and **7**–**17** in figures stood for the OVX groups treated respectively by the corresponding compounds. OVX and Sham stood for OVX and sham-operated rat groups in the figures, each animal receiving an equal volume of vehicle (DMSO/water (1:9) mixture). Genistein and 17β-estradiol were accepted as references. OVX rats were fed a low-calcium diet to induce rapid osteoporosis,[4,36] whereas sham-operated animals were given conventional food.

Two months later, OVX (n = 6) and sham-operated (n = 6) rats were sacrificed by bleeding followed by a midline abdominal laparotomy to excise total uterus. Each uterus was put on individual labeled bibulous paper to suck off the liquor on the surface before weighed. Blood was obtained by cardiac puncture and serum was kept frozen at −20 °C for biochemical analysis. After this, the femora were taken out immediately with the accompanying soft tissue cleaned up and stored in a freezer at −80 °C until examination. The right femora were used for the measurement of the bone mineral density (BMD), X-ray analysis and then burnt to ash for the determination of the weight of bone ash (WBA) for evaluating inorganic matter content of bone.

4.4. Acute toxicity assessment

Every mouse (19–22 g) was given orally a single dose (25 ml/kg) of each of **1**, **7**, **8**, **9**, **11**, and **16** at 27 mmol/kg which was 500-fold effective dosage, and the control animal an equal volume of vehicle (DMSO/water (1:9) mixture). Mice were observed for adverse effects immediately, 2, 4, 8, and 24 h after dosing, followed by daily observation till the 14th day.

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