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Synthetic analogues of daidzein, having more potent osteoblast stimulating effect

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Abstract

A series of daidzein derivatives were synthesized and assessed for stimulation of osteoblast differentiation using primary cultures of rat calvarial osteoblasts. Data suggested that three synthetic analogues, **1c**, **3a** and **3c** were several folds more potent than daidzein in stimulating differentiation and mineralization of osteoblasts. Further, these three compounds did not show any estrogen agonistic activity; however had mild estrogen antagonistic effect. Out of the three compounds, **3c** was found to maximally increase the mineralization of bone marrow osteoprogenitor cells. **3c** also robustly increased the mRNA levels of osteogenic genes including bone morphogenetic protein-2 and osteocalcin in osteoblasts. Unlike daidzein, **3c** did not inhibit osteoclastogenesis. Collectively, we demonstrate osteogenic activity of daidzein analogues at significantly lower concentrations than daidzein.

Keywords

Phytoestrogen, Daidzein, Deoxybenzoin , Formylation,Mineralization.,Osteogenic , Osteoblast Proliferation

With the discontinuation of hormone replacement therapy for bone maintenance after menopause, many postmenopausal women are looking for prophylactic alternatives, particularly from natural source.[1,2] As a result, there is a growing interest in assessing the role of plants and plant-derived compounds in prevention of postmenopausal osteoporosis.[3] Many bioactive compounds have been identified from plants including a large class of flavonoids, the so called phytoestrogens, which exhibit antioxidant properties and may act as estrogen receptor (ER) agonists with beneficial outcomes in postmenopausal osteoporosis.[4]

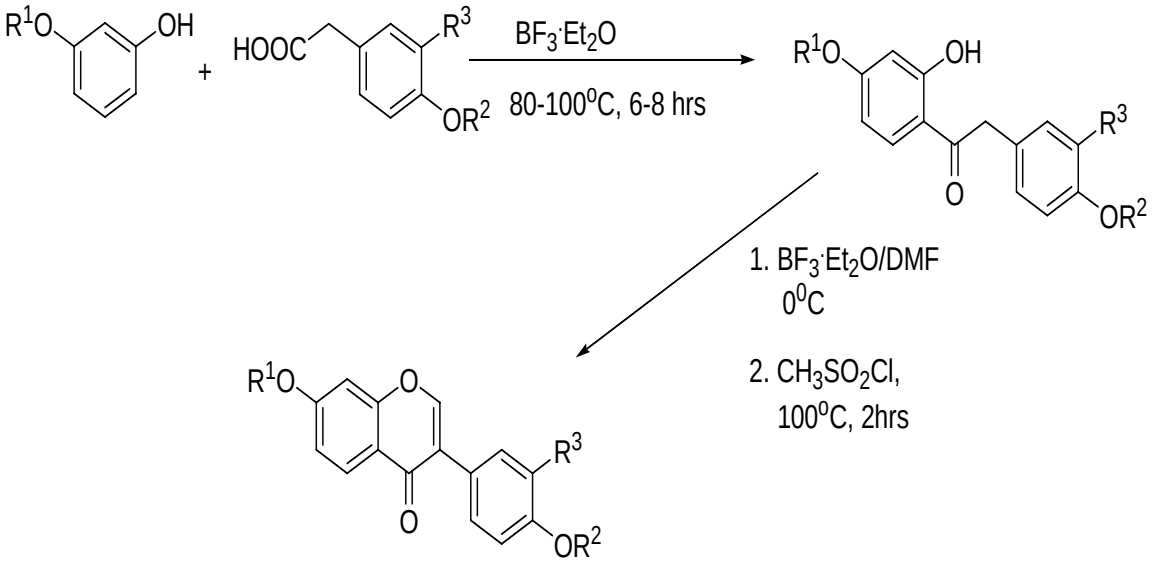
Daidzein is an extensively studied phytoestrogen with respect to its skeletal effects. Daidzein affords bone-protective action by stimulation of osteoblast[5-7] and inhibition of osteoclast functions[8] through the ERs. Besides its ER mediated effect, daidzein has non-genomic effect on bone wherein it stimulates protein synthesis in osteoblasts by activating amino acyl tRNA synthetase.[9] Daidzein also enhances bone morphogenetic protein-2 production in osteoblast[5]. Ten weeks of daily injection of daidzein at 16.6 $mg.kg^{-1}$ dose to growing ovariectomized (Ovx) rats exhibited significant bone forming effect.[10] In adult OVx mice on high calcium diet, daidzein at 100 $mg.kg^{-1}.day^{-1}$ oral

dose for 12 weeks favorably influenced both trabecular and cortical bone.[11] Besides, high dietary intake of isoflavones like daidzein and genistein has been reported to increase BMD in lumbar spine of Japanese[12], Chinese[13] and American[14] postmenopausal women. However, uterine estrogenic action of daidzein precludes its osteoprotective use in postmenopausal women. The uterine estrogenic effect of daidzein is partly contributed by its highly estrogenic metabolite, equol. [15-16] Because of its intestinal biotransformation to equol, daidzein has low oral bioavailability.

From these reports, it appear that daidzein could be a suitable therapeutic 'lead molecule' for postmenopausal osteoporosis if issues pertaining to its metabolism, estrogenicity, and most importantly improvement of its potency in promoting osteoblast function are addressed.

Here, we focused on modifying the structure of daidzein to achieve improved osteogenic effect and eliminate uterine estrogenicity. To that effect, we synthesized novel daidzein derivatives and assessed osteoblast functions (differentiation and mineralization) *in vitro* using rat calvarial and bone marrow osteoblasts. We also studied uterine estrogenicity of active compounds.

Daidzein and methoxylated daidzein derivatives were prepared by reacting appropriate substituted phenyl acetic acid with properly substituted resorcinols in the presence of BF_3.etherate produced deoxybenzoins, deoxybenzoins on formylation gave isoflavones (**1-4**)[17] Scheme 1.



1: $R^1=R^2=R^3=H$
2: $R^1=CH_3$, $R^2=R^3=H$
3: $R^2=CH_3$, $R^1=R^3=H$
4: $R^1=H$, $R^2=CH_3$, $R^3=OCH_3$

1a: $R^1=R^2=$-CH_2CH_2pyrrolidine, $R^3=H$
1b: $R^1=R^2=$-CH_2CH_2piperidine, $R^3=H$
1c: $R^1=R^2=$-$CH_2CH_2N(C_2H_5)_2$, $R^3=H$
1d: $R^1=R^2=$-CH_2CH_2morpholine, $R^3=H$
1e: $R^1=R^2=$Allyl, $R^3=H$
1f: $R^1=R^2=$Isopropyl, $R^3=H$
1g: $R^1=$-$CH_2CH_2N(C_2H_5)_2$, $R^2=R^3=H$
1h: $R^1=R^2=$-CH_2oxiran, $R^3=H$
1i: $R^1=$-CH_2CH_2Br, $R^2=R^3=H$
1j: $R^1=R^2=$-CH_3, $R^3=H$

2a: $R^1=CH_3$, $R^2=$-CH_2CH_2pyrrolidine, $R^3=H$
2b: $R^1=CH_3$,$R^2=$-CH_2CH_2piperidine, $R^3=H$
2c: $R^1=CH_3$,$R^2=$-$CH_2CH_2N(C_2H_5)_2$, $R^3=H$
2d: $R^1=CH_3$,$R^2=$-CH_2CH_2morpholine, $R^3=H$
2e: $R^1=CH_3$,$R^2=$Allyl, $R^3=H$
2f: $R^1=CH_3$,$R^2=$Isopropyl, $R^3=H$
2g: $R^1=CH_3$,$R^2=$Isobutyl, $R^3=H$
2h: $R^1=CH_3$,$R^2=$-CH_2oxiran, $R^3=H$
2i: $R^1=CH_3$,$R^2=$-CH_2CH_2Br, $R^3=H$
2j: $R^1=CH_3$,$R^2=$-$CH=CH_2$, $R^3=H$

3a: $R^1=$-CH_2CH_2pyrrolidine, $R^2=CH_3$, $R^3=H$
3b: $R^1=$-CH_2CH_2piperidine, $R^2=CH_3$, $R^3=H$
3c: $R^1=$-$CH_2CH_2N(C_2H_5)_2$, $R^2=CH_3$, $R^3=H$
3d: $R^1=$-CH_2CH_2morpholine, $R^2=CH_3$, $R^3=H$
3e: $R^1=$Isopropyl, $R^2=CH_3$, $R^3=H$
3f: $R^1=$-CH_2oxiran, $R^2=CH_3$, $R^3=H$
3g: $R^1=$-CH_2CH_2Cl, $R^2=CH_3$, $R^3=H$
3h: $R^1=$-$CH_2CH_2CH_2Br$, $R^2=CH_3$, $R^3=H$
3i: $R^1=$-$CH=CH_2$, $R^2=CH_3$, $R^3=H$

4a: $R^1=$-CH_2CH_2pyrrolidine, $R^2=CH_3$, $R^3=OCH_3$
4b: $R^1=$-CH_2CH_2piperidine, $R^2=CH_3$, $R^3=OCH_3$
4c: $R^1=$-$CH_2CH_2N(C_2H_5)_2$, $R^2=CH_3$, $R^3=OCH_3$
4d: $R^1=$-CH_2CH_2morpholine, $R^2=CH_3$, $R^3=OCH_3$
4e: $R^1=$Allyl, $R^2=CH_3$, $R^3=OCH_3$
4f: $R^1=$Isopropyl, $R^2=CH_3$, $R^3=OCH_3$
4g: $R^1=$-CH_2oxiran, $R^2=CH_3$, $R^3=OCH_3$
4h: $R^1=$-CH_2CH_2Br, $R^2=CH_3$, $R^3=OCH_3$

Scheme 1. Synthesis of daidzein analogues.

The compounds **1-4** on Williamson type O-alkylation reaction with different 1-(2-chloro-alkyl) substituted amine hydrochloride in the presence of potassium carbonate in acetone/DMF gave alkyl substituted amino compounds **1a-d,g**, **2a-d**, **3a-d** and **4a-d** in good yield. The compounds **1-4** on reaction with different haloalkyls (allylbromide, isopropylbromide, 2-bromopropane, epichlrohydrine, 1,2-dibromoethane and 1,3-dibromopropane, methyliodide) afforded compounds **1e,f,h-j**, **2e-j**, **3e-h**, **4e-h**.[18] The compounds **2i** and **3g** with NaH in dry DMF afforded **2j** and **3i** in very good yield. All the synthetic products gave satisfactory analytical and spectroscopic data, which were in the full accordance with their formulated structures.

All compounds were tested for osteogenic activity using primary cultures of rat osteoblasts following previously described protocol from our laboratory.[19-21] Production of alkaline phosphatase (ALP) serves as a differentiation marker of osteoblasts.[22-23] We used osteoblast ALP assay to screen the activity of various synthesized compounds following our previously published protocol. For osteoblast mineralization, calvarial osteoblasts and bone marrow cells were cultured for 18-21 days in differentiation media containing 10mM β-glycerophosphate and 50 μg/ml ascorbic acid in presence or absence of compounds. Cells were then stained with alizarin red-S and dye was extracted to quantify the extent of osteoblast mineralization.[19,24] Transcript levels of osteogenic genes (bone morphogenetic protein-2 and osteocalcin) were determined by real time RT-PCR (qPCR) following our previously published protocols.[25] Primer pairs used were; for BMP-2, 5'- CGG ACT GCG GTC TCC TAA-3' (sense); 5'- GGG GAA GCA GCA ACA CTA GA-5' (antisense); for osteocalcin, 5'- GGA CAT TAC TGA CCG CTC C-3' (sense), 5'-TTT TCA GTG TCT GCC GTG AG-3' (antisense); for GAPDH, 5'-CAG CAA GGA TAC TGA GAG CAA GAG-3' (sense), 5'-GGA TGG AAT TGT GAG GGA GAT G-3' (antisense). Data are expressed as mean±SEM. The data obtained in experiments with multiple treatments were subjected to one-way ANOVA followed by post hoc Tukey' test of significance using MINITAB 13.1 software.

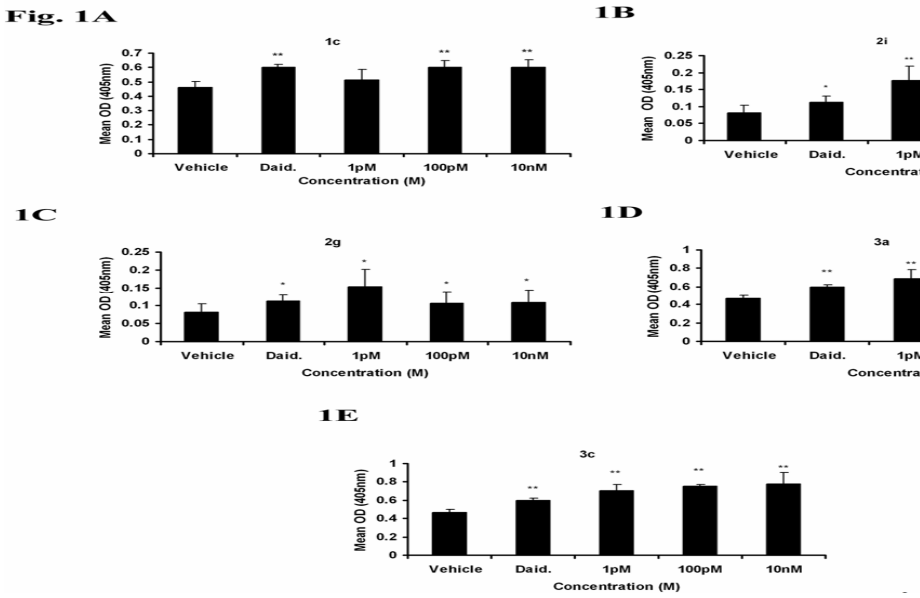


Figure1 Effects of daidzein analogues on osteoblast differentiation. $2x10^3$ rat calvarial osteoblasts were seeded in 96-well plate and exposed to various concentrations of compounds **1c**, **2i**, **2g**, **3a** and **3c** for 48 h. ALP activity was determined spectrophotometrically at 405 nm. Daidzein at 1μM was used as a positive control. Data shown as mean±SD; n=6; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle treated cells

Thirty seven synthetic compounds were screened using osteoblast ALP assay (osteoblast differentiation). As shown in Fig 1A-E, five of out of 37 compounds significantly increased osteoblast ALP activity. These were **1c**, **2g**, **2i**, **3a**, and **3c**. At concentrations ranging from 1.0 pM to 10.0 nM, each of these compounds stimulated osteoblast ALP activity. At these low concentrations, daidzein did not stimulate ALP acitivity (data not shown), and instead required 1.0 μM to significantly stimulate ALP activity in osteoblasts (Fig 1 A-E). From these data, it appears that **1c**, **2g**, **2i**, **3a**, and **3c** are ~100-fold more potent than daidzein in stimulating osteoblast differentiation.

As **1c**, **3a** and **3c** were more active than **2g** and **2i** in stimulating osteoblast ALP activity, we next assessed effect of these three compounds (**1c**, **3a** and **3c**) in stimulating osteoblast mineralization in vitro, using calvarial osteoblasts. **1c**, **3a** and **3c** enhanced formation of mineralized nodules at 10.0 nM concentration (Fig 2).

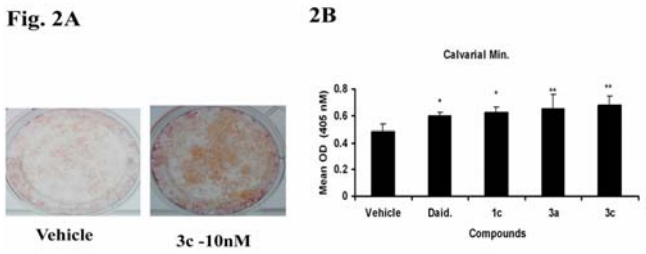


Figure 2. Effects of **1c**, **3a** and **3c** on mineralization of rat calvarial osteoblasts. $2x10^3$ osteoblasts were seeded in 12-well plates and exposed to 10.0 nM **1c**, **3a** and **3c** for 7 days. At the end of the incubation, cells were fixed and stained with alizarin red-S. Stain was extracted and O.D. measured colorimetrically as described in material and method. Data shown as mean±SD; n=4; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle treated cells.

BMCs were cultured in osteoblast differentiation medium for 18 d in presence or absence of compounds (**1c**, **3a** and **3c)**. Mineralized nodules were stained by alizarin red-S. As shown in Fig 3, number of mineralized nodules was significantly more in BMCs treated with 10.0 nM **1c** or **3c**. As **3c** has greater degree of statistical significance than **1c**, we conclude that **3c** is the most effective among the three active compounds in stimulating mineralization of BMCs.

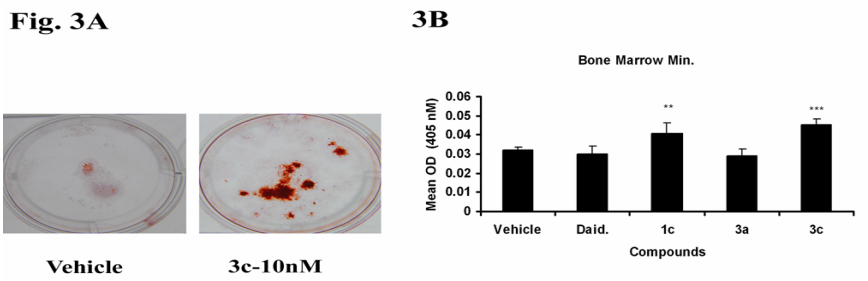


Figure 3. Effect of **1c**, **3a** and **3c** on mineralization of BMCs. BMCs ($2x10^5$ cells) were seeded in 12-well plates and incubated with 10.0 nM **1c**, **3a** and **3c** for 18 days. At the end of the incubation, cells were fixed and stained with alizarin red-S. Stain was extracted and O.D. measured colorimetrically as described in material and method. Data shown as mean±SD; n=4; $^{**}P < 0.01$, $^{***}P < 0.001$ compared with vehicle treated cells.

We next studied the effect of **3c** on the expression of osteogenic genes (osteocalcin and BMP-2) in rat calvarial osteoblasts. qRT-PCR data show that 10.0 nM **3c** robustly stimulated mRNA levels of both osteocalcin (10-fold) and BMP-2 (6-fold) in osteoblasts (Fig. 4).

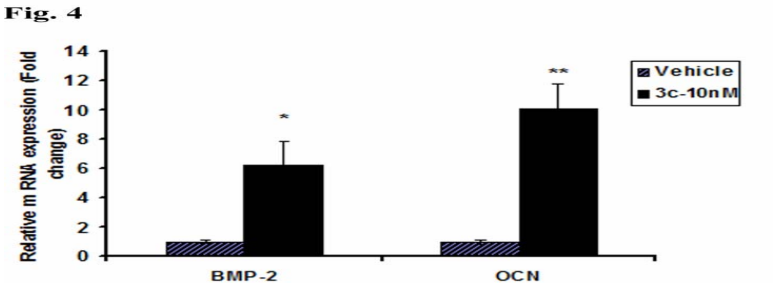


Figure 4. Effects of **3c** on mRNA expression of osteocalcin and BMP-2. Osteoblast cells were cultured with or without **3c** for 24 hrs. qPCR for osteocalcin and BMP-2 was performed as described in materials and methods. Compound **3c** at 10nM increased the osteocalcin mRNA expression (~10 fold) and BMP-2 expression (~6 fold) when compared to vehicle control group. Data shown as mean±SEM; n=4; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle treated cells.

As daidzein is known to have uterine estrogenicity, we studied the effect of **1c**, **3a** and **3c** in rat uterus. Twenty-one day old rats (n=4) were ovariectomized and oral administration of **1c**, **3a** and **3c** was given at 10 $mg.kg^{-1}.day^{-1}$ dose for 3 consecutive days with or without E_2. Raloxifene, a SERM in clinical use was also used as control. Table 1 shows that as expected E2 treatment resulted in a robust increase in uterine weight compared with vehicle treated rats. Raloxifene had modest but significant estrogenic effect. **1c** and **3a** had no effect on uterine weight compared with vehicle, suggesting no estrogenic effect. **3c** however, modestly but significantly reduced uterine weight compared to vehicle, suggesting anti-estrogenic effect. In anti-estrogenicity assay (Table 1), all three compounds inhibited E2-induced increase of uterine weight, suggesting their anti-estrogenic effect.

Table 1. Estrogen agonistic and antagonistic activities of compounds in OVx immature *Sprague Dawley* rats.

Treatment	Dose mg/kg	Estrogenic activity (uterine weight in mg)	Anti-estrogenic activity (uterine weight in mg)
Vehicle	-	35.0±0.6	
Ethynyl estradiol	0.01	82.3^{c}±3.4	
Raloxifene	3	42.3^{a}±1.3	67*±4.1
1c	10	31.3±1.5	57.3^{**}±2.3
3a	10	31.3±4.3	59.3^{*}±5.6
3c	10	28.0^{a}±1.5	63.0*±3.8

Values are mean±SD
$^{a}P < 0.05$, $^{c}P < 0.001$, vs. corresponding vehicle control

$^{*}P < 0.05$, $^{**}P < 0.01$, vs. corresponding ethynyl estradiol group

Daidzein is an extensively studied phytoestrogen with respect to its skeletal effects. It promotes osteoblast functions and inhibits osteoclast functions *in vitro*, albeit at micromolar a concentration that is unlikely to translate *in vivo*.[4] Also, daidzein has been reported to exhibit estrogenicity at the uterine level. Therefore, we synthesized analogues of daidzein with the aim to improve its osteogenic potency and eliminate uterine estrogenicity. In the present work, a total of thirty seven compounds structurally related to daidzein (**1a-j**, **2a-j**, **3a-i**, **and 4a-h**) were evaluated for *in vitro* osteogenic action in the primary cultures of osteoblasts derived from calvaria and BM. In the initial screening using calvarial osteoblasts where ALP production (osteoblast differentiation marker) was measured, five compounds (**1c**, **2g**, **2i**, **3a** and **3c**) significantly increased ALP activity over control. Daidzein was required at several folds higher concentration than these five compounds, suggesting increased osteogenic potency of our synthetic compounds over dadizein.

As **1c**, **3a** and **3c** were more active ($^{**}P < 0.01$ vs. control) at 10.0 nM than **2g** and **2i** ($^{*}P < 0.05$ vs. control) in osteoblast ALP assay, we next tested the effect of **1c**, **3a** and **3c** on *in vitro* mineralization of calvarial and BM osteoblasts. Our data show that although these three compounds had comparable effects on calvarial mineralization however, in the BM, **3c** was most active in stimulating mineralization ($^{***}P < 0.001$ for **3c** vs. $^{**}P < 0.01$ for **1c**). We further confirmed the osteogenic effect of **3c** in calvarial osteoblasts by quantifying osteogenic gene expression. By qRT-PCR, we demonstrated that **3c** increased mRNA levels of BMP-2 and OCN over control. Collectively, these data indicate that **3c** is a potent osteogenic compound *in vitro*.

Daidzein is a "mixed" agent as it could stimulate osteoblast activity and inhibit osteoclast differentiation, both at μM range.[5,8] Interestingly, unlike daidzein, **3c** did not inhibit osteoclastogenic differentiation of the BM cells in the presence of RANKL and M-CSF (data not shown). From these data, it appears that **3c** is a "pure" osteogenic compound that is much more potent than daidzein and is devoid of uterine estrogenicity. However, significant anti-estrogenic effect of **3c** may find effective use of **3c** against breast cancer (study in progress).

Structure-activity relationship (SAR) studies reveal that the substitution at C-7 and C-4′ appear essential for better *in vitro* osteogenic activity and diminish uterine estrogenicity of daidzein.[19] Keeping this aspect in mind several derivatives of daidzein have been synthesized and screened for *in vitro* osteogenic activity. SAR has been discussed based on the results obtained. Our study reveals that the presence of free hydroxyl at C-4′ with combinations at C-7 of 2-diethylamino-ethoxy and 2-bromo-ethoxy does not appear essential for osteogenic activity, as compounds **1g** and **1i** were found to be inactive in osteogenic assay. In search of most suitable combination for lead optimization some more molecules were designed and synthesized. It appears that methoxy substituent at C-4′ with combinations at C-7 of 2-pyrrolidin-1-yl-ethoxy and 2-diethylamino-1-yl-ethoxy (**3a**, **3c**) led to more potent osteogenic activity than dadizein. Compounds **3b**, **3d-3i**, **4a-4h** possessed methoxy substituent at C-4′ moiety, but were inactive in ALP assay. This could be due to the presence of other substituents. In addition, presence of methoxy group at C-3′ (**4a-4h**) and vinylic, allyloxy, oxiranylmethoxy, heloethoxy, morpholin-ethoxy, piperidin-ethoxy and pyrrolidin-ethoxy moieties at C-7 and C-4′ positions or *vice-versa* also seem non-essential for osteogenic activity since **1a**, **1b**, **1d-1j**, **2a-2f**, **2h**, **2j**, **3b**, **3d-3i**, **4a-4h** had no significant osteogenic effect. Together, these activity data support that the combination of groups at C-7 and C-4′ (**1c**, **2g**, **2i**, **3a**, **3c**) in daidzein skeleton are critical determinants of enhanced osteogenic activity and to eliminate uterine estrogenicity. Enhanced osteogenic activity as exemplified by increased ALP activity, mineralization and transcript levels of osteogenic markers by most active analogue designed as, 7-(2-Diethylamino-ethoxy)-3-(4-methoxy -phenyl)-4H-chromen-4-one (**3c**) led us to conclude that the 7,4′-dihydroxy-isoflavon or 7-hydroxy-3-(4-hydroxy-phenyl)-chromen-4-one or daidzein skeleton is an important one, which could provide some useful clue for further design of an effective bone anabolic agent.

Acknowledgments

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Supplementary data

Experimental procedures, biological evaluation methods and NMR data's of all synthesized compounds are provided. Supplementary data associated with this article can be found, in the online version, at doi:

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