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# Synthetic analogs of daidzein, having more potent osteoblast stimulating effect ☆

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ABSTRACT

A series of didzein derivatives were synthesized and assessed for stimulation of osteoblast differentiation using primary cultures of rat calvarial osteoblasts. Data suggested that three synthetic analogs, **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. Compound **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 analogs at significantly lower concentrations than daidzein.



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.

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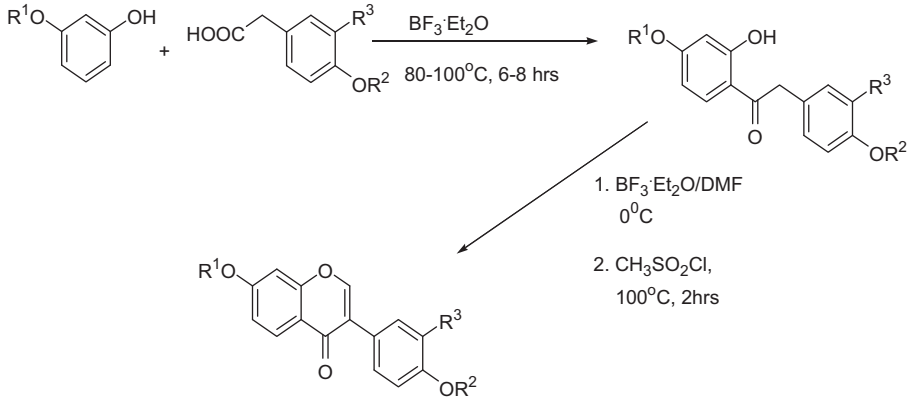
The compounds **1**–**4** on Williamson type *O*-alkylation reaction with different 1-(2-chloro-alkyl) substituted amine hydrochloride in the presence of potassium carbonate in acetone/DMF gave alkyl substituted amino compounds **1a**–**d,g**, **2a**–**d**, **3a**–**d** and **4a**–**d** in good yield. 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 10 mM 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 quantitate 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.




**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: $R^1=R^2$=-$CH_2CH_2$pyrrolidine, $R^3=H$
1b: $R^1=R^2$=-$CH_2CH_2$piperidine, $R^3=H$
1c: $R^1=R^2$=-$CH_2CH_2N(C_2H_5)_2$, $R^3=H$
1d: $R^1=R^2$=-$CH_2CH_2$morpholine, $R^3=H$
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: $R^1=R^2$=-$CH_2$oxiran, $R^3=H$
1i: $R^1$=-$CH_2CH_2Br$, $R^2=R^3=H$
1j: $R^1=R^2$=-$CH_3$, $R^3=H$

2a: $R^1=CH_3$, $R^2$=-$CH_2CH_2$pyrrolidine, $R^3=H$
2b: $R^1=CH_3$,$R^2$=-$CH_2CH_2$piperidine, $R^3=H$
2c: $R^1=CH_3$,$R^2$=-$CH_2CH_2N(C_2H_5)_2$, $R^3=H$
2d: $R^1=CH_3$,$R^2$=-$CH_2CH_2$morpholine, $R^3=H$
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: $R^1=CH_3$,$R^2$=-$CH_2$oxiran, $R^3=H$
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: $R^1$=-$CH_2CH_2$pyrrolidine, $R^2=CH_3$, $R^3=H$
3b: $R^1$=-$CH_2CH_2$piperidine, $R^2=CH_3$, $R^3=H$
3c: $R^1$=-$CH_2CH_2N(C_2H_5)_2$, $R^2=CH_3$, $R^3=H$
3d: $R^1$=-$CH_2CH_2$morpholine, $R^2=CH_3$, $R^3=H$
3e: $R^1$=Isopropyl, $R^2=CH_3$, $R^3=H$
3f: $R^1$=-$CH_2$oxiran, $R^2=CH_3$, $R^3=H$
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: $R^1$=-$CH_2CH_2$pyrrolidine, $R^2=CH_3$, $R^3=OCH_3$
4b: $R^1$=-$CH_2CH_2$piperidine, $R^2=CH_3$, $R^3=OCH_3$
4c: $R^1$=-$CH_2CH_2N(C_2H_5)_2$, $R^2=CH_3$, $R^3=OCH_3$
4d: $R^1$=-$CH_2CH_2$morpholine, $R^2=CH_3$, $R^3=OCH_3$
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: $R^1$=-$CH_2$oxiran, $R^2=CH_3$, $R^3=OCH_3$
4h: $R^1$=-$CH_2CH_2Br$, $R^2=CH_3$, $R^3=OCH_3$

**Scheme 1.** Synthesis of daidzein analogs.