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One step synthesis of 2-hydroxymethylisoflavone and their osteogenic activity ☆

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ABSTRACT

An efficient one step synthesis of new 2-hydroxymethylisoflavone is reported. A series of deoxybenzoin was subjected to cyclization with glyoxal in the presence of basic condition (KOH/EtOH) to afford the 2-hydroxymethyl isoflavone. The structures of compounds **5a–g** were confirmed by NMR experiments including ^{1}H, ^{13}C, HMBC, HSQC and COSY. These compounds were assessed for stimulation of osteoblast function using primary culture of rat calvarial osteoblasts in vitro. Compounds **5a**, **5d**, **5f** and **5g** were potent in stimulating differentiation of osteoblasts as assessed by measuring alkaline phosphatase (ALP) activity. Besides, effect of these analogs was also seen on the transcript levels of osteogenic genes like Runx-2, osteocalcin and Bone morphogenetic protein-2 (BMP-2), involved in osteoblast differentiation and mineralization. Based on quantitative PCR data, compound **5f** was found to be the potent followed by **5d**. Compound **5f** robustly increased the mRNA levels of Runx-2 (8.0 fold), BMP-2 (~2 fold) and osteocalcin (~2.0 fold) in osteoblasts. Collectively, we demonstrate osteogenic activity of the novel 2-hydroxymethyl isoflavones with **5f** having the most potent activity.

Isoflavonoids are a group of phenolic compounds produced by various higher plants to protect themselves from environmental stress and are present in foods such as beans, cabbage, soya beans, grains and hops.[1] Some of the most popular isoflavones are glycitein, genistein, formononetin, biochanin A and daidzein.

Isoflavones and its derivatives (Fig. 1) possess a diverse range of biological activities including antimicrobial, antioxidant, stimulating nerve growth, insecticidal activities,[2] anti osteoporotic,[3] hypolipidemic activities[4] etc. It is also reported that isoflavones have healthful benefits in human obesity and have a positive influence on plasma cholestrol.[5] They produce weaker estrogenic effects when endogenous estrogen level is inadequate. In contrast, they can inhibit the efficacy when estrogens are adequate.[6] With the dual-effects, isoflavones are considered to be effective and safer estrogen replacements. These observations inspired several groups to synthesize various derivatives of isoflavone with the intention to either improve biochemical and pharmacokinetic characteristics of the parent drug or to obtain compounds containing essential elements of the parent substance but having novel properties and/or affecting novel molecular targets.

Recently we have reported the osteogenic activity of *Butea monosperma* crude extract. Subsequent studies led to the isolation of several methoxyisoflavones like isoformononetin and formononetin.[3] These methoxyisoflavones were shown to have in vitro

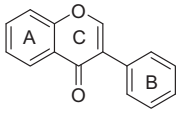

Figure 1. General structure of isoflavone.

osteogenic activity and also increase bone mass in growing rats. In this study we have synthesized analogs of isoformononetin and validated its osteogenic activity in vitro by assessing effect on osteoblast differentiation by measuring ALP activity. Our studies show that these analogs have potent bone forming activity at concentrations ~100 fold lower than isoformononetin.

The literature synthesis for isoflavones in general involves two steps wherein a phenol is reacted in the presence of a Lewis acid with phenylacetic acid to generate an intermediate deoxybenzoin[7] which is then cyclized with a one carbon electrophile. A convenient approach is that of Baker et al.[8] who used oxalyl chloride and pyridine as the cyclizing agent, whereas others have used ethyl formate,[9] triethylorthoformate[10] or carbon disulfide[11] and some other strategy are also reported in the literature.[12] To the best of our knowledge, the synthesis of 2-hydroxymethyl isoflavone using glyoxal has not been reported. Herein, we report on an efficient, synthetic pathway to 2-hydroxymethylisoflavone, which was accomplished through the condensation of deoxybenzoin with glyoxal in basic condition. Key step of our synthesis is base catalyzed enolization of 1,4-diketone intermediate followed by 1,5-hydride shift to afford 2-hydroxymethylisoflavone.

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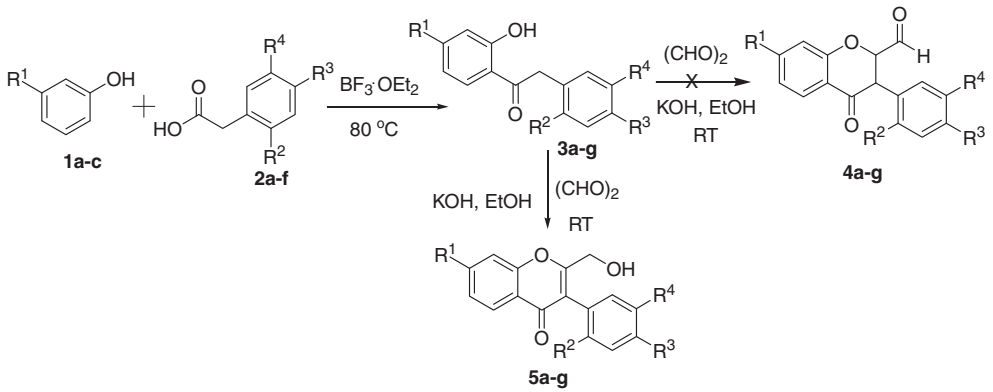

Scheme 1. Synthesis of compounds **5a**–**g**.

We anticipated that glyoxal could be more effective to synthesize 2-carbaldehydeisoflavone (**4**) using Aldol condensation with deoxybenzoin (**2a**–**f**) and followed by intra molecular Michael addition (Scheme 1). 2-Carbaldehydeisoflavone (**4**) could be used to synthesize various derivative of isoflavone utilising aldol condensation reaction with various acetophenones. Deoxybenzoins (**3a**–**g**)[13] were prepared by the published procedure from hydroxy-phenols (**1a**–**c**) and phenylacetic acids (**2a**–**f**) by Friedel–Crafts acylation using boron trifluoride etherate as solvent and also as the Lewis acid for the acylation. This reaction was carried out at 80 °C and completed in about one and a half hours[13] (Scheme 1). When deoxybenzoin was treated with the glyoxal in ethanol using potassium hydroxide as a base at RT, afforded 2-hydroxymethyl isoflavones (**5a**–**g**)[14] in a respectable yield of 50–80%. Proposed mechanism of this step is depicted in Figure 2. It is assumed that condensation of deoxybenzoin with glyoxal undergoes intra molecular ring cyclization to the formation of 2-carbaldehyde dihydroisoflavone and dienol intermediates, followed by 1,5-hydride shift to afford 2-hydroxymethylisoflavones (**5a**–**g**).

The structures of compounds **5a–g**[15–21] were confirmed by 1D and 2D NMR experiments including ^{1}H, ^{13}C, HMBC, HSQC, and COSY. Selected HMBC correlations of compound **5a** are given in Figure 3. This newly developed procedure was applied to the synthesis of a number of isoflavone derivatives, afforded in good to excellent yield depicted in Table 1.

Alkaline phosphatase (ALP),[22] which is one of the important markers of osteoblast differentiation, was measured spectrophotometrically and optical density was determined at 405 nm. Compounds **5a–g** were evaluated for ALP activity stimulation at concentrations ranging from 10^{-12} to 10^{-6} M (Fig. 4). Isoformononetin (10^{-8} M) was used as a positive control. Treatment of calvarial rat osteoblast cells led to increased ALP activity by compounds **5a**, **5d**, **5f** and **5g** with compounds **5d** and **5f** being

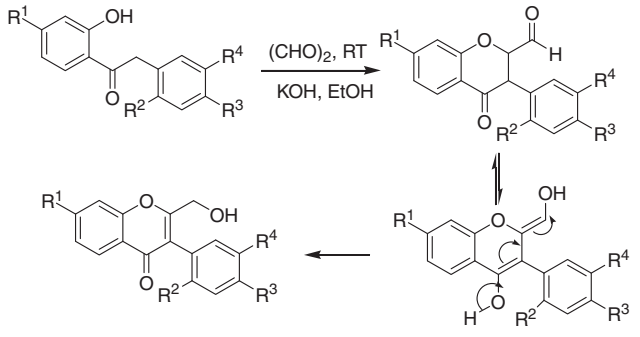

Figure 2. A plausible reaction pathway of formation of 2-hydroxymethyl isoflavone.

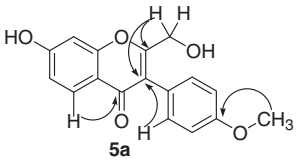

Figure 3. Selected HMBC correlation of compound **5a**.

Table 1
Synthesis of 2-isoflavone derivatives **5a**–**g**

Product	R_1	R_2	R_3	R_4	Yield (%)
5a	OH	H	OCH_3	H	78
5b	OCH_3	H	OH	H	65
5c	OH	H	OCH_3	OCH_3	72
5d	OH	H	H	H	75
5e	OH	OCH_3	OCH_3	H	66
5f	H	OH	OCH_3	H	50
5g	H	H	H	H	80

the most active. These compounds increased osteoblast differentiation at ~100 fold lower concentration than isoformononetin which is known for its osteogenic activity. In order to validate which amongst the four compounds was the most potent osteogenic agent, their activity was further checked by quantitative PCR.

We next studied the effect of compounds on the expression of osteogenic genes (osteocalcin, Runx-2 and BMP-2)[23] in calvarial osteoblasts by quantitative real time PCR (qPCR).[24] Runx-2, a bone-specific transcription factor, is a key regulator of osteoblast differentiation (Fig. 5). Runx-2 expression is induced by BMP-2 stimulation. Thus, Runx-2 is a downstream transcription factor in BMP-2 signaling. Runx-2 transcription is important for expression of osteocalcin, an important biomarker of osteoblast differentiation. The transcript levels of Runx-2, OCN and BMP-2 were assessed by qPCR after treatment with compounds and results were expressed as fold change over untreated cells. Runx-2 gene expression was increased by both **5f** (8.0 fold increase) and **5d** (6.5-fold increase) with no effect of **5a** and **5g**. BMP-2 expression was increased by **5f** (2.0 fold) and **5g** (2.5 fold) with no effect of other two. In case of OCN, mRNA expression was increased by ~2.0 fold by **5f** with no effect of other compounds. Thus based on real time PCR data **5f** was the most active (increased expression of Runx-2, BMP-2 and OCN) followed by **5d**. Taken together, **5f** > **5d** > **5g** > **5a**.

In conclusion, we have reported efficient and operationally simple synthesis of 2-hydroxymethyl isoflavone. This simple one step process provides the first simple entry into a series of isoflavones with different substitution in ring A or B at will and should provide a simple route to substituted analogues for SAR studies. These

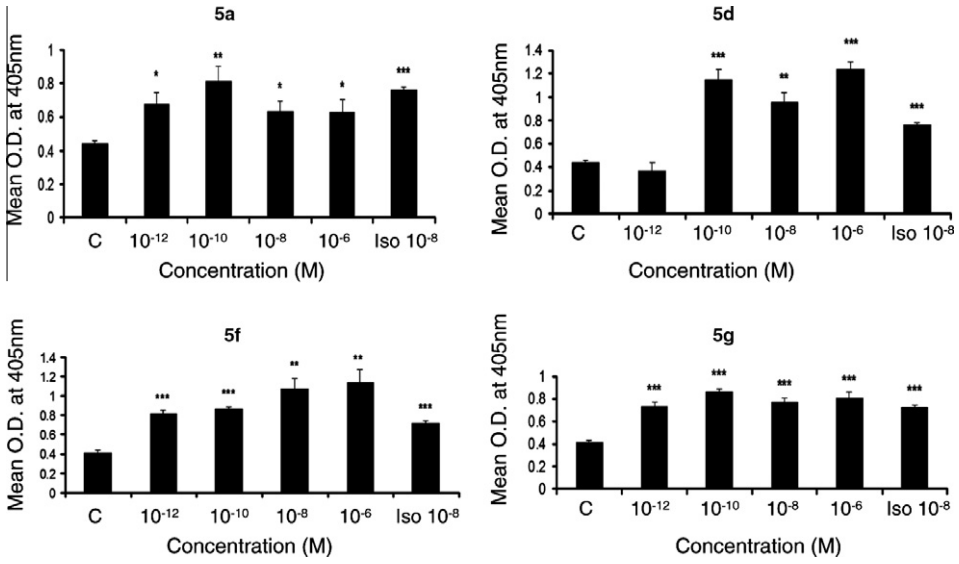

Figure 4. Effect of compounds on osteoblast differentiation. 2×10^3 rat calvarial osteoblasts were seeded in 96 well plates and exposed to various concentrations of compounds ranging from 10^{-12} M to 10^{-6} M for 48 h and ALP activity was determined spectrophotometrically at 405 nm. Isoformononetin (Iso) at 10^{-8} M was used as a positive control. Data shown as mean ± SEM; $n = 6$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with vehicle treated cells.

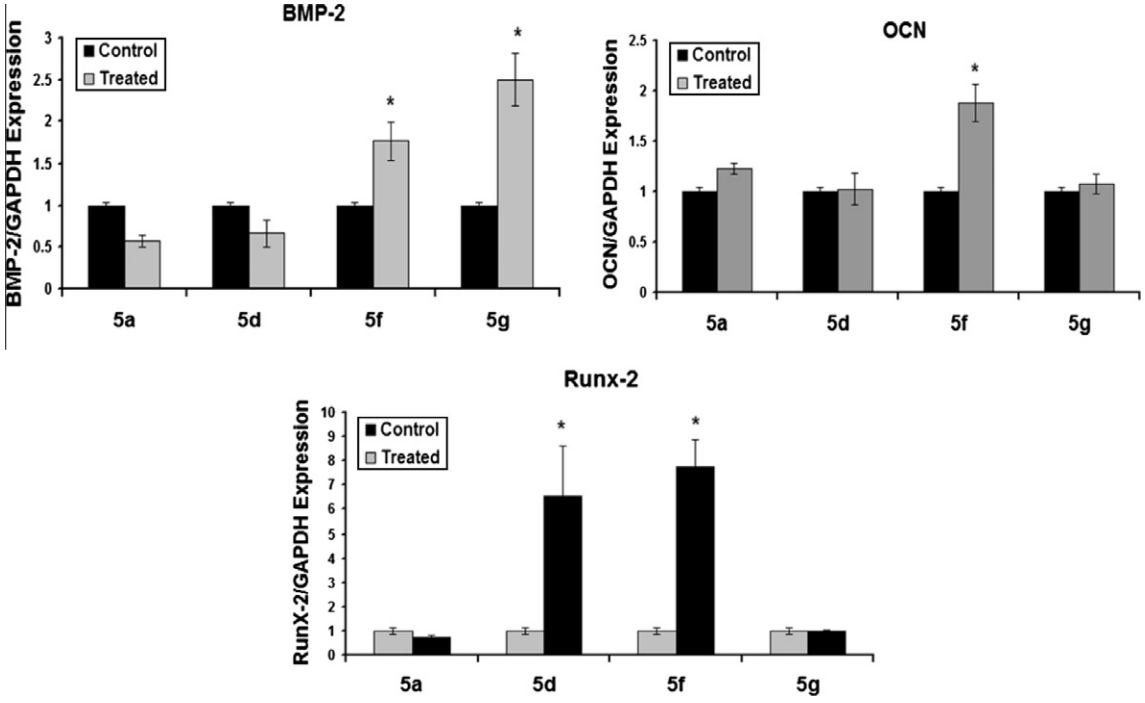

Figure 5. Effects of compounds on mRNA expression of BMP-2, osteocalcin (OCN) and Runx-2. Osteoblast cells were cultured with or without compounds for 48 h. qPCR for osteocalcin, Runx-2 and BMP-2 was performed as described in materials and methods. Data shown as mean ± SEM; $n = 3$; $^{*}P < 0.05$ compared with vehicle treated cells.

2-hydroxymethyl isoflavones are also potential anti-osteoporotic agents. Further studies regarding the biological activities of such derivatives are in progress.

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14. *General procedure for synthesis of deoxybenzoin* (**3a**–**g**): The mixture of *p*-methoxy phenyl acetic acid (500 mg, 3.08 mmol) in boron trifluoride etherate (0.6 ml) and resorcinol (280 mg, 3.00 mmol) were heated at 80 °C for 8 h. Reaction mixture was poured in water, extracted with ethyl acetate and dried over anhydrous sodium sulphate. Reaction mixture was concentrated to give crude product. Purification was done by column chromatography using silica gel as adsorbent and hexane, ethyl acetate as eluent to yield compound **3a**.
15. *General procedure for synthesis of 2-hydroxymethyl isoflavone* (**5a**): To a magnetically stirred solution of deoxybenzoin **3a** (500 mg, 2.06 mmol) and potassium hydroxide (169 mg, 3,09 mmol) in ethanol (25 ml) was added the glyoxal (0.15 ml, 3.09 mmol) at room temperature. The reaction mixture was stirred for 4 h. The reaction mixture was poured into water. The organic layer was extracted with ethyl acetate, washed with water, dried over Na_2SO_4 and the solvent was removed under reduced pressure. The residue was purified by column chromatography on silica gel using ethyl acetate/hexane (50:50) or chloroform/methanol (98:2) as the eluting solvents.
16. *Data of compound* **5a**: Pale yellow solid. Mp 197–199 °C; UV (MeOH): λ_{max} 248, 330 nm; IR (KBr): 3178, 1645, 1611, 1557, 1306, 1255, 1089 cm^{-1};^{1}H NMR: (CD_3OD, 300 MHz): 8.18 (1H, d, *J* = 7.47 Hz, H-5), 7.34 (2H, d, *J* = 7.2 Hz, H-2′, 6′), 7.12 (2H, d, *J* = 7.2 Hz, H-3′, 5′), 7.03 (1H, d, *J* = 7.4 Hz, H-6), 7.03 (1H, d, *J* = 7.4 Hz, H-8), 4.51 (2H, s, CH_2), 3.95 (3H, s), ^{13}C NMR: (CD_3OD, 75 MHz): 179.5 (C-4), 164.9 (C-7), 164.8 (C-2), 161.4 (C-4′), 159.6 (C-9), 133.0 (C-2′, 6′), 128.5 (C-5), 125.4 (C-1′), 123.9 (C-3), 116.6 (C-6), 117.4 (C-10), 114.9 (C-3′&5′), 61.0 (CH_2), 55.9 (OCH_3); ESIMS: (*m*/*z*) 299 $[M+H]^+$; Elemental Anal. Calcd for $C_{17}H_{14}O_5$: C, 68.54; H, 4.73. Found: C, 68.47; H, 4.75.
17. *Data of compound* **5b**: Amorphous powder, UV (MeOH): λ_{max} 255, 273, 330 nm; IR (KBr): 3175, 1655, 1600, 1255, 1089 cm^{-1}; ^{1}H NMR (DMSO-d_6, 300 MHz): 7.95 (1H, d, *J* = 8.3 Hz, H-5), 6.82 (2H, d, *J* = 7.8 Hz, H-3′& 5′), 7.10 (2H, d, *J* = 7.8 Hz, H-2′ & 6′), 7.11 (1H, d, *J* = 8.3 Hz, H-6), 7.11 (1H, s, H-8), 4.28 (2H, s, CH_2), 3.91 (3H, s); ^{13}C NMR (DMSO-d_6, 75 MHz): 175.7 (C-4), 163.8 (C-7), 162.9 (C-2), 157.2 (C-4′), 157.0 (C-9), 131.7 (C-2′&6′), 122.2 (C-5), 125.4 (C-1′), 122.2 (C-3), 116.6 (C-6), 116.6 (C-10), 114.7 (C-3′&5′), 59.2 (CH_2), 56.1 (OCH_3); ESIMS: (*m*/*z*) 299 $[M+H]^+$; Elemental Anal. Calcd for $C_{17}H_{14}O_5$: C, 68.54; H, 4.73. Found: C, 68.57; H, 4.68.
18. *Data of compound* **5c**: Yellow solid; Mp 210–211 °C; UV (MeOH): λ_{max} 245, 341 nm; ^{1}H NMR ($CDCl_3$, 300 MHz): 8.03 (1H, d, *J* = 8.8 Hz, H-5), 7.10 (1H, s, H-8), 7.09 (1H, d, *J* = 1.5 Hz, H-2′), 7.09 (1H, d, *J* = 8.8 Hz, H-6), 7.01 (1H, dd, *J* = 7.0 Hz,1.5, H-6′), 6.97 (1H, d, *J* = 7.0 Hz, H-5′), 4.40 (2H, s, CH_2), 3.84 (3H, s), 3.83 (3H, s); ^{13}C NMR ($CDCl_3$, 75 MHz): 174.8 (C-4), 163.8 (C-7), 162.8 (C-2), 157.5 (C-9), 147.6 (C-3′), 147.1 (C-4′), 127.4 (C-5), 123.2 (C-1′), 122.0 (C-3), 119.8 (C-2′), 116.5 (C-6′), 115.3 (C-6), 117.1 (C-10), 113.5 (C-5′), 60.8 (CH_2), 56.1(OCH_3), 56.2 (OCH_3); ESIMS: (*m*/*z*) 329 $[M+H]^+$; Elemental Anal. Calcd for $C_{18}H_{16}O_6$: C, 65.85; H, 4.91. Found: C, 65.77; H, 4.98.
19. *Data of compound* **5d**: Amorphous powder; UV (MeOH): λ_{max} 238, 350 nm; ^{1}H NMR ($CDCl_3$, 300 MHz): 7.22 (3H, m, H-3′,4′ & 5′), 7.80 (1H, d, *J* = 9.0 Hz, H-5), 7.38 (2H, m, H-2′ & 6′), 7.05 (1H, d, *J* = 9.0 Hz, H-6), 7.04 (1H, s, H-8), 4.17 (2H, s, CH_2); ^{13}C NMR ($CDCl_3$, 75 MHz): 176.0 (C-4), 163.3 (C-7), 162.3 (C-2), 126.5 (C-4′), 157.8 (C-9), 128.3 (C-2′&6′), (C-5), 130.9 (C-1′), 123.7 (C-3), 114.3 (C-6), 117.9 (C-10), 129.8 (C-3′&5′), 58.9 (CH_2); ESIMS: (*m*/*z*) 269 $[M+H]^+$; Elemental Anal. Calcd for $C_{16}H_{12}O_4$: C, 71.64; H, 4.51. Found: C, 71.59; H, 4.58.
20. *Data of compound* **5e**: Amorphous powder; UV (MeOH): λ_{max} 251, 278, 330 nm; ^{1}H NMR ($CDCl_3$, 300 MHz): 8.07 (1H, d, *J* = 8.8 Hz, H-5), 6.99 (1H, dd, *J* = 8.8, 1.2 Hz, H-6), 6.90 (1H, d, *J* = 1.2 Hz, H-8), 6.97 (1H, d, *J* = 7.5 Hz, H-6′), 6.30 (1H, d, *J* = 7.5, 1.1 Hz, H-5′), 6.00 (1H, d, *J* = 1.1 Hz, H-3′), 4.17 (2H, s, CH_2), 3.82 (3H, s); ^{13}C NMR ($CDCl_3$, 75 MHz): 175.8 (C-4), 162.7 (C-7), 162.4 (C-2), 157.2 (C-4′), 157.6 (C-9), 108.8 (C-1′), 132.5 (C-6′), 127.2 (C-5), 156.7 (C-2′), 123.4 (C-3), 115.1 (C-6), 116.8 (C-10), 103.0 (C-3′), 106.6 (C-5′), 58.8 (CH_2), 57.2 (OCH_3); ESIMS: (*m*/*z*) 329 $[M+H]^+$; Elemental Anal. Calcd for $C_{18}H_{16}O_6$: C, 65.85; H, 4.91. Found: C, 65.78; H, 4.97.
21. *Data of compound* **5f**: Amorphous powder; UV (MeOH): λ_{max} 247, 341 nm; ^{1}H NMR ($CDCl_3$, 300 MHz): 7.74 (1H, dd, *J* = 8.5, 1.5 Hz, H-5), 7.40 (1H, m, H-7), 7.34 (1H, m, H-8), 7.21 (1H, m, H-6), 6.96 (1H, d, *J* = 7.3 Hz, H-6′), 6.37(1H, dd, *J* = 7.3, 1.2 Hz, H-5′), 6.01 (1H, d, *J* = 1.2 Hz, H-3′), 4.16 (2H, s, CH_2), 3.82 (3H, s); ^{13}C NMR ($CDCl_3$, 75 MHz): 177.2 (C-4), 133.2 (C-7), 162.5 (C-2), 159.0 (C-4′), 156.6 (C-9), 156.7 (C-2′), 132.4 (C-6′), 128.2 (C-5), 108.5 (C-1′), 123.7 (C-3),125.4 (C-6), 123.4 (C-10), 103.1 (C-3′), 106.6 (C-5′), 60.5 (CH_2), 57.9 (OCH_3); ESIMS: (*m*/*z*) 299 $[M+H]^+$; Elemental Anal. Calcd for $C_{17}H_{14}O_5$: C, 68.54; H, 4.73. Found: C, 68.63; H, 4.66.
22. *Data of compound* **5g**: Amorphous powder; UV (MeOH): λ_{max} 258, 344 nm; ^{1}H NMR ($CDCl_3$, 300 MHz): 8.21 (1H, dd, *J* = 8.9, 1.7 Hz, H-5), 7.39 (2H, m, H-2′, 6′), 7.68 (1H, m, H-7), 7.46 (1H, d, *J* = 8.0 Hz, H-8), 7.29 (3H, m, H-3′, 4′, 5′), 7.29 (1H, m, H-6), 4.49 (2H, s, CH_2); ^{13}C NMR: ($CDCl_3$, 75 MHz): 177.7 (C-4), 162.3 (C-2), 155.9 (C-9), 133.3 (C-7), 131.4 (C-1′), 130.4 (C-3′, 5′), 128.5 (C-2′, 6′), 128.3 (C-5), 126.3 (C-4′), 125.2 (C-6), 123.7 (C-3), 123.5 (C-10), 60.8 (CH_2); ESIMS: (*m*/*z*) 253 $[M+H]^+$; Elemental Anal. Calcd for $C_{16}H_{12}O_3$: C, 76.18; H, 4.79. Found: C, 76.25; H, 4.67.
23. *Alkaline phosphatase activity*: Calvarial osteoblasts were trypsinized at 70–80% confluence using 0.02% trypsin-EDTA (Sigma). 2×10^3 cells were seeded in α-MEM containing 10% FBS media in 96-well plate with or without IF-01, IF-04, IF-07 and IF-08 (Concentration ranging from 10^{-12} M to 10^{-6} M) for 48 h in presence of 50 μg/ml ascorbate and 10 mM glycerophosphate. At the end of incubation period, total ALP activity was measured with a method using *p*-nitrophenylphosphate (PNPP) as substrate. Isoformononetin was used as a positive control. The reaction mixture contained diethanolamine buffer (1 mol/L, pH 9.8), 0.5 mmol/L $MgCl_2$, and 10 mmol/L PNPP. ALP activity was measured colorimetrically at 405 nm as described previously.
24. *Osteocalcin, Runx-2 and BMP-2 expression by using q-PCR*: Total RNA was extracted from the cultured cells using Trizol (Invitrogen). cDNA was synthesized from 2 μg total RNA with the Revert Aid™ H Minus first strand cDNA synthesis kit (Fermentas, USA). SYBR green chemistry was used for quantitative determination of the mRNAs for Osteocalcin and BMP-2 and a housekeeping gene, GAPDH, following an optimized protocol. The design of sense and antisense oligonucleotide primers was based on published cDNA sequences using the Universal probe library (Roche diagnostics, USA). For real-time PCR, the cDNA was amplified with Light Cycer 480 (Roche diagnostics pvt ltd). The double-stranded DNA-specific dye SYBR Green I was incorporated into the PCR buffer provided in the Light Cycler 480 SYBER green I master (Roche diagnostics pvt ltd) to allow for quantitative detection of the PCR product in a 20-μl reaction volume. The temperature profile of the reaction was 95 °C for 5 min, 40 cycles of denaturation at 94 °C for 2 min, and annealing and extension at 62 °C for 30 s, extension at 72 °C for 30 s. GAPDH was used to normalize differences in RNA isolation, RNA degradation, and the efficiencies of the reverse transcription. Primer pairs used were; for BMP-2, 5′-CGG ACT GCG GTC TCC TAA-3′ (sense); 5′-GGG GAA GCA GCA ACA CTA GA-3′ (antisense); for osteocalcin, 5′-GGA CAT TAC TGA CCG CTC C-3′ (sense), 5′-TTT TCA GTG TCT GCC GTG AG-3′ (antisense); for Runx-2 were, 5′-GCC GGG AAT GAT GAG AAC TAC T-3′ (sense), 5'-TCC GGC CTA CAA ATC TCA GAT C-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).