# Chemical synthesis and biological study of 4β-carboxymethyl-epiafzelechin acid, a component from the osteoprotective traditional Chinese medicinal herb, Gu-Sui-Bu [*Drynaria fortunei* (kunze) J. Sm]

*Man Chun Law, Ka Chun Wong, Wai Ying Pang, Man Sau Wong,* Tak Hang Chan**

Department of Applied Biology and Chemical Technology and Laboratory of the Institute of Molecular Technology for Drug Discovery and Synthesis, The Hong Kong Polytechnic University, Hong Kong SAR; and State Key Laboratory in Chinese Medicine and Molecular Pharmacology, Shenzhen, China.

AUTHOR EMAIL ADDRESS. <bcchanth@polyu.edu.hk> (T. H. C.); bcmswong@polyu.edu.hk (M. S. W.)

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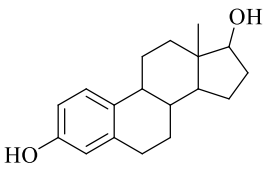
TITLE RUNNING HEAD. Osteoprotective effect of 4β-carboxymethyl-*epi*afzelechin acid

ABSTRACT. The active component of the anti-osteoporotic traditional Chinese medicine Gu-Sui-Bu [*Drynaria fortunei* (kunze) J. Sm], 4β-carboxymethyl-*epi*afzelechin acid (**12**), has been synthesized. The proliferative effects of **12** and a cyclized ester **13** on UMR-106 osteoblast-like cells were determined by cell proliferation assay. UMR-106 cells proliferation and differentiation assays were found to show activity in a dose-dependent manner. Compound **12** showed a more potent proliferative activity than compound **13**, and the cell proliferations were significantly increased by over 50% in UMR-106 cells at 10 nM. The cell proliferation was totally abolished by co-treating cells with ICI 182,780, an estrogen antagonist. The results suggested that the effects of both compounds might be mediated through ER-dependent pathway. Transient transfection study indicated that both compounds could activate ERE-dependent gene transcription mediated by ERα but not ERβ in relative high concentration (μM). These results suggest that compound **12** is a phytoestrogen that stimulates bone cells through ERs.

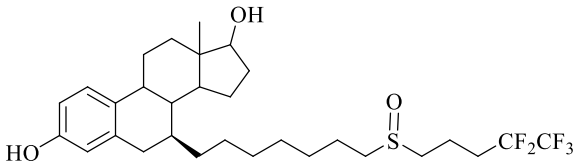
KEYWORDS. Selective Estrogen Receptor Modulator, Osteoporosis, Gi-Sui-Bu [*Drynaria fortunei* (kunze) J. Sm], 4β-carboxymethyl-*epi*afzelechin acid, cells proliferation and differentiation.

## Introduction

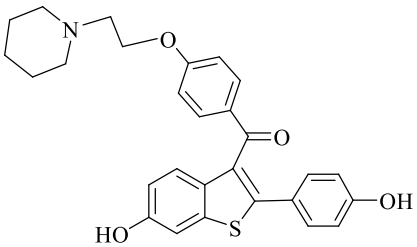
The coordinated activities of bone forming osteoblasts and bone resorbing osteoclasts are essential for bone health. Osteoporosis is a metabolic bone disease where the amount of bone resorbed typically exceeds the bone forming replacement capacity leading to a net decrease in bone mineral density. While both men and women have normal age-dependent loss of bone mass, estrogen deficiency is known to cause osteoporosis in postmenopausal women. It is estimated that approximately half of all women aged >50 years may suffer from osteoporotic fracture. Estrogen replacement therapy (ERT) has long been used to relieve postmenopausal osteoporosis[1] but has recently been ruled out as the first-line therapy due to the possible incidence of breast cancers with long-term use.[2] Estrogen receptors (ER) are the pharmaceutical target for developing therapies for menopausal women as the cause of postmenopausal osteoporosis is mainly due to insufficient regulation of ER in bone cells. The ER's natural ligand, 17β-estrodiol (**1**, $E_2$) acts as receptor agonist.[3] Other compounds, such as ICI 182,780 (**2** also known as Fulvestrant), function as antagonist and can abolish the estrogenic effect of estrogen.[4] Selective ER modulators (SERMs),[5] on the other hand, can act as both agonists and antagonists, depending on the cellular context as well as the ER isoform targets. SERMs such as raloxifene (**3**) has also been used for osteoporosis prevention in postmenopausal women.[6] Many compounds from plant source such as isoflavones, lignans, and coumestans, referred to as phytoestrogen since they exhibit estrogen-like activities, have been considered as potential alternatives treatment for postmenopausal osteoporosis.[7] Genistein (**4**) and daidzein (**5**)} are the most intensively studied phytoestrogens.[8] There is evidence that isoflavones may have beneficial effects on bones in ovariectomized rat model of osteoporosis.[9] Human studies also suggested significant increases in both the bone mineral content and density in the lumbar spine of postmenopausal women with supplementation of soy isoflavones for 6 months.[10] On the other hand, concern about potential genotoxicity and selective endocrine system toxicity of isoflavones has been expressed.[11] Some European authorities have taken the position of not allowing promotion of the health claims of isoflavones.[12]



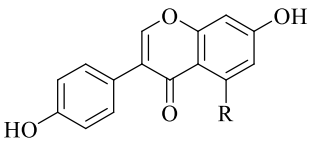

**1** Estradiol 17β




**2** ICI 182,780

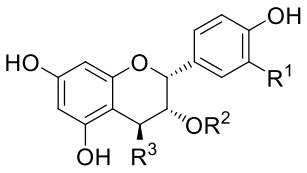



**3** Raloxifene

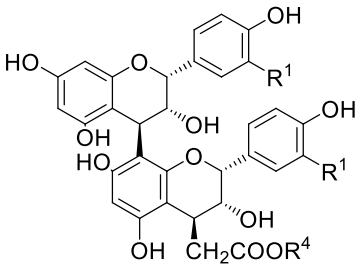



**4** R=OH, genistein
**5** R=H, daidzein

The traditional Chinese medicine Gu-Sui-Bu [*Drynaria fortunei* (kunze) J. Sm] has been commonly used to manage bone disease.[13] The stimulatory effects of Drynaria Rhizoma extracts on the proliferation and differentiation of osteoblastic MC3T3-E1 cells had been investigated.[14] Extract of *D. fortune*i also promoted bone mineral density of ovariectomized (OVX) mice.[15] Recently, aqueous extract of Gu-Sui-Bu was found to have a promoting effect on the deposition of calcium in the bone matrix and on bone calcification by bone tissue culture.[16] Through a bioassay-guided separation, several flavan-3-ols of the *epi*afzelechin (**6**) structure, compounds **7**-**10** and the dimer **11**, were isolated from the rhizomes of *D. fortunei*.[17] Several of these compounds showed potent proliferative effects on ROS 17/2.8 osteoblastic cells. Particular intriguing is the potent effect of compounds **9** and **10**, comparable to that of $E_2$ (**1**), on proliferation of ROS 17/2.8 osteoblastic cells.[17] Because the amounts of these active components from natural source are quite limited (for example, 12.5 mg of **10** was isolated from 6 Kg of dried rhizomes of *D. fortunei*),[17] we are therefore interested in synthesizing 4β-carboxymethyl-*epi*afzelechin acid (**12**), the free acid of **10**, and examine its biological activities and the possible mechanistic pathways with respect to estrogen receptors.




**6** epiafzelechin, $R^1=R^2=R^3=H$
**7** $R^1=R^3=H$, $R^2$=allose
**8** $R^1=R^3=H$, $R^2$=acetylallose
**9** $R^1=R^2=H$, $R^3=CH_2COOMe$
**10** $R^1=R^2=H$, $R^3=CH_2COONa$



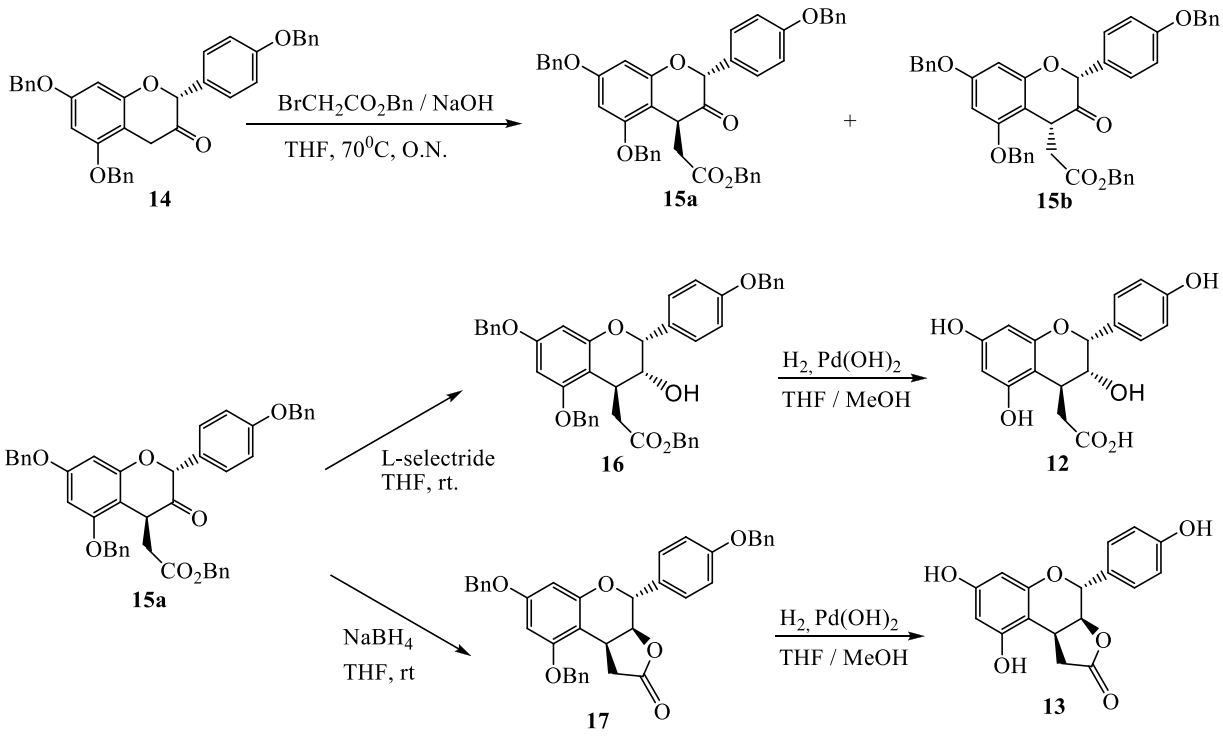

**11** $R^1=H$, $R^4=CH_2COOMe$

## Results and Discussion

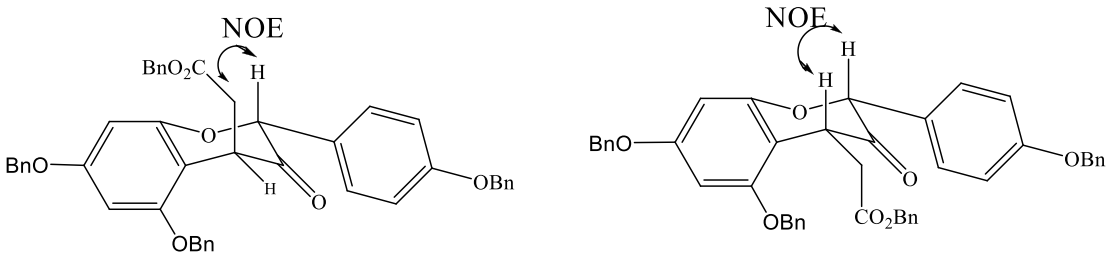
### 1. Chemical Synthesis

Because of our interest in flavonoid chemistry,[18] we have recently completed the total synthesis of afzelechin and *epi*afzelechin (**6**).[19] We have extended a similar protocol in synthesizing 4β-carboxymethyl-*epi*afzelechin acid (**12**) (Scheme 1). Starting from **14** which was prepared according to reported procedures,[19] selective alkylation of the enolate ion of **14** with benzyl bromoacetate using NaOH as base gave the benzyl acetate **15a** with β-stereochemistry together with its diastereomer **15b**. Elevated temperature at 70 °C is required for smooth alkylation giving compounds **15a** and **15b** in good yield. Strong bases such as NaH or LDA gave either low yield or complex mixture at temperature from -70 to 70 °C. The two diasteromers **15a** and **15b** could be separated by careful column chromatography. Their relative stereochemistries were assigned based on NOESY NMR studies (The NOE spectra are given in the Supporting Information, SI). The benzyl ester **15b** was assigned to have the 2,4-*cis* stereochemistry as indicated by the observation of an NOE interaction between the 2-H and 4-H methine hydrogens, whereas for compound **15a** there was no NOE interaction between 2-H and 4-H thus establishing its relative stereochemistry to be 2,4-*trans* (Scheme 2). Reduction of the keto function of **15a** with L-selectride gave a mixture of compounds **16** and **17**. Compound **16** has a hydroxy function resulted from the reduction of the keto group. Its stereochemistry was indicated by the small coupling constants between 2-H, 3-H and 4-H hydrogens, suggesting a 2,3-*cis* and 3,4-*trans* relationship as represented in Scheme 3. The relative stereochemistry is in agreement with the NOESY NMR (SI, Figure 9) in which interactions between 2-H and 3-H, 3-H and 4-H and also between 2-H and the

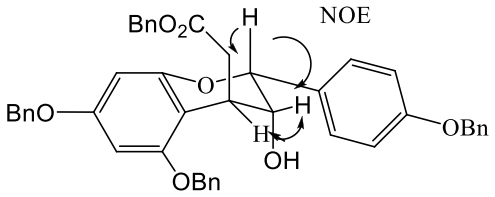
methylene protons of the substituent at C-4 were observed (Scheme 3). Compound **17** is assigned to have the lactone structure as its mass spectrum and its NMR spectrum clearly indicate the loss of the benzyl moiety. The 2,3-*trans* and 3,4-*cis* stereochemistry was supported by NOESY NMR (SI, Figure 11) which show absence of NOE interaction for 2-H and 3-H but strong interaction between 3-H and 4-H. When $NaBH_4$ was used as the reducing agent of **15a**, mainly the lactone **17** was obtained as the major product. Hydrogenolysis of either **16** or **17** individually removed the benzyl protecting group giving the corresponding **12** and **13**. The spectroscopic data of **12** is in agreement with its assigned structure, and in consistent with those reported for **9** and **10** in the literature.[17] Compound **13**, obtained as a side product in the synthesis, can be considered as a dehydrated analog of **12** but with 3,4-*cis* stereochemistry.




**Scheme 1** Synthesis of compounds **12** and **13**




**Scheme 2** NOE of compounds **15a** and **15b**




**Scheme 3** NOE of compound **16**

## 2. Biological Studies

### 2a. Proliferation effects of compounds 12 and 13 on UMR-106 cells

The osteoblast-like UMR-106 cells have been used widely as an osteoblast model because they preserve many properties of osteoblasts including cAMP responsive to PTH, high alkaline phosphatase and synthesis of bone specific collagen.[20] We have examined the proliferation effects of compound **12** on UMR-106 cells. UMR-106 cells were treated with $10^{-14}$ to $10^{-6}$ M of compound **12** for 24 hours. At 24 hour, **12** at all concentrations ($10^{-14}$ to $10^{-6}$ M) could increase osteoblastic cell proliferation in a dose-dependent manner. The optimum concentrations of **12** were found at $10^{-10}$ and $10^{-8}$ M. Specifically, $10^{-10}$ and $10^{-8}$ M of **12** effectively increased cell number by 1.4 to 1.5-fold ($p<0.001$). The proliferation effect was much lower at $10^{-14}$ and $10^{-12}$ M. The activity is comparable to the reported proliferative effect of naturally isolated **9** and **10** on ROS 17/2.8 cells in the concentration range of $10^{-15}$~$10^{-6}$ M.[17] To determine if the proliferation effect of **12** involves estrogen receptor (ER), cells were co-treated with estrogen receptor antagonist, ICI 182,780 (**2**). As shown in Figure 2, the stimulatory effect of 10 nM $E_2$

on cell proliferation could be abolished by co-treatment with ICI 182,780. Similarly, the positive effects of **12** treatments on UMR-106 cell proliferation could be abolished by ICI 182,780 co-treatment. The above results suggested that the stimulatory effect of **12** on cell proliferation is mediated through ER dependent pathway.

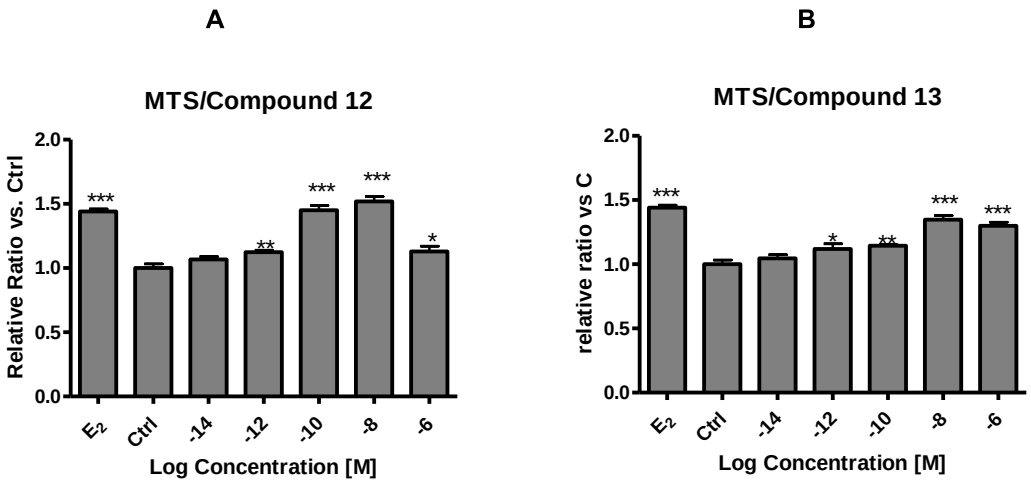



Figure 1. The proliferation effect of compound **12** (Figure 1A) and **13** (Figure 1B) on UMR-106 cells. MTS assay was performed in 96 well microtiter plates seeded with 5000 cells/well using PRE-DMEM with 1% S-FBS and 1% P/S medium. $10^{-8}$ M of 17β-estradiol ($E_2$) was used as a positive control. Concentration from $10^{-14}$M to $10^{-6}$M of compound **12** or **13** was added to cells for 24 hours treatment. The bars represent mean ± SEM value with n=9. The results were obtained from three independent experiments. *$p$<0.05, **$p$<0.01 and ***$p$<0.001 verse control.

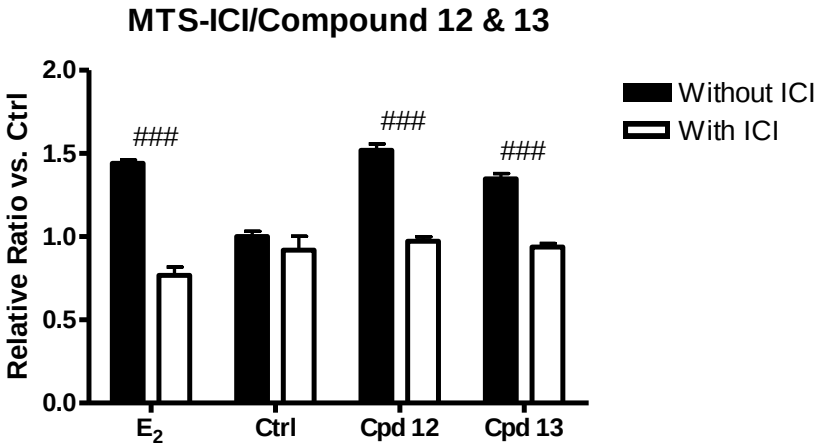


Figure 2. The proliferation effect of compound **12** or **13** on UMR-106 cells. MTS assay was performed in 96 well microtiter plates seeded with 5000 cells/well using PRE-DMEM with 1% S-FBS and 1% P/S medium. $10^{-8}$M of 17β-Estradiol ($E_2$) was used as a positive control. Concentration of $10^{-8}$M of compound **12** or **13** was added to cells for 24 hours treatment. ER-blocker ICI 182,780 was also added to each well at a concentration of $10^{-6}$M. The bars represent mean ± SEM value with n=9. The results were obtained from three independent experiments, triplicate. ###$p$<0.001 versus group with ER blocker.

Figure 1B shows the proliferation effects of compound **13**. Proliferation of the UMR-106 cells also responded in dose-dependent manners in the concentration range of $10^{-10}$ to $10^{-6}$ M with only moderate proliferative activities. The optimum concentration of **13** was found at $10^{-8}$ M. Similarly, the proliferative effects of **13** on UMR-106 cell could be abolished by ICI 182,780 co-treatment (Fig. 2), suggesting that the stimulatory effects of **13** on cell proliferation were also mediated through the ER dependent pathway.

**2b. Differentiation effects of 12 and 13 in UMR-106 cells**

Alkaline phosphatase (ALP) activity is a common marker for assessment of osteoblastic cell differentiation.[21] While the differentiating effect of the pure natural flavan-3-ols had not been previously reported,[17] crude extract of Gu-Sui-Bu at 1 mg/ml can statistically increase the intracellular ALP amount.[22] We demonstrated that the synthetic compound **12** can stimulate alkaline phosphatase (ALP) activity on UMR-106 cells (Figure 3A). The results provide *in vitro* evidence for compound **12** in promoting bone formation. Treatment of UMR-106 cells with **12** and **13** for 24 hours stimulated cell differentiation in a dose-dependent manner (Figure 3A and B). Compound **12**, varied in concentration from $10^{-10}$ to $10^{-6}$ M, as well as 10 nM $E_2$, significantly increased cell differentiation by 18% ($p<0.01$) at $10^{-10}$ M, 14% ($p<0.01$) at $10^{-8}$ M and 18% ($p<0.01$) at $10^{-6}$ M respectively. In a similar approach,

compound **13** was found to have lesser extent of stimulatory effect on differentiation by about 10% at $10^{-10}$ M. Their effects could not be abolished in the presence of ICI 182,780, suggesting the stimulatory effect of both compounds is not mediated through ER (Figure 4).

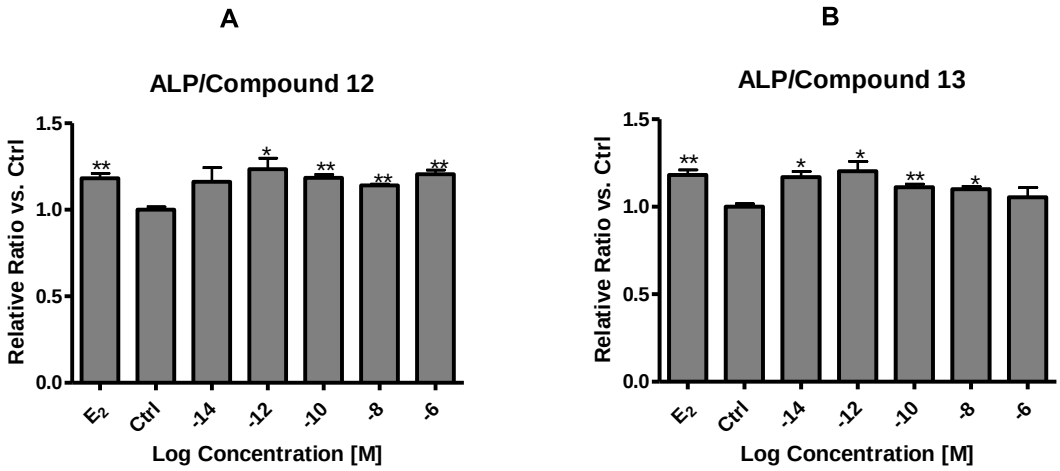



Figure 3A and B. The differentiation effect of compounds **12** and **13** on UMR-106 cells. ALP assay was done in 96 well microtiter plates seeded with 5000 cells/well using PRE-DMEM with 1% S-FBS and 1% P/S medium. $10^{-8}$ M of 17β-estradiol ($E_2$) was used as a positive control. Concentration from $10^{-14}$ M to $10^{-6}$ M of compound **12** or **13** was added to cells for 24 hours treatment. An additional Bradford protein assay was done to normalize the protein content of each well. The bars represent mean ± SEM value with n=9. The results were obtained from three independent experiments *$p$<0.05 and **$p$<0.01 versus control.

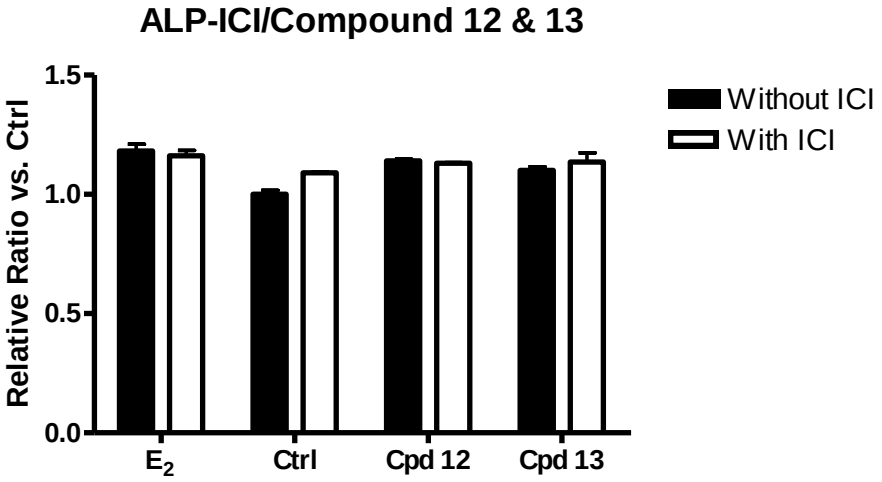
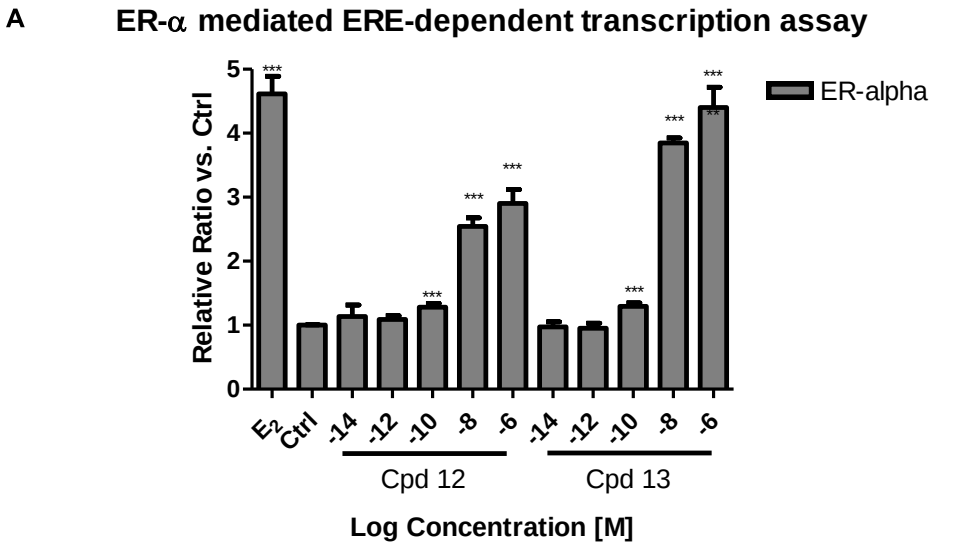



Figure 4. The differentiation effect of compounds **12** and **13** on UMR-106 cells. ALP assay was done in 96 well microtiter plates seeded with 5000 cells/well using PRE-DMEM with 1% S-FBS and 1% P/S medium. $10^{-8}$ M of 17β-estradiol ($E_2$) was used as a positive control. Concentration of $10^{-8}$ M of compound **12** or **13** was added to cells for 24 hours treatment. ER-blocker was also added to each well at a concentration of $10^{-6}$ M. An additional Bradford protein assay was done to normalize the protein content of each well. The bars represent mean ± SEM value with n=9. The results were obtained from three independent experiments

**2c. Effect of compound 12 and 13 on ERα or ERβ-mediated luciferase gene expression in UMR-106 cells.**

There are complex molecular mechanisms underlying the diverse physiological actions of $E_2$ and the many synthetic, dietary and environmental estrogens.[23] The most well-described mechanism of estrogen action is mediated through binding to two nuclear receptors, ERα and ERβ, followed by promotion of specific DNA estrogen response elements (EREs) which can exert either a positive or negative effect on expression of the downstream target gene.[24] In recent years, a membrane-bound estrogen receptor has been described,[25] the G protein-coupled estrogen receptor 1 (GPER1), which appears to play a role in cardiovascular and metabolic estrogen signaling.[26] ICI 182,780 is a potent ERα and ERβ antagonist

but acts as a full agonist on GPER.[25, 27] In view of the observation that the positive effects of **12** and **13** on UMR-106 cell proliferation could be abolished by ICI 182,780 co-treatment (Figure 2), we examined the functional activities of these compounds to induce ER-dependent transcription *in vitro*. To determine if these compounds activate estrogen receptor response element (ERE)-dependent transcription via ERα and ERβ, UMR-106 cells were co-transfected with ERE-luciferase and ER-(α or β) constructs and treated with either **12** or **13**. Our results indicated that 10 nM $E_2$ can activate ERα (Figure 5A, $p<0.001$ vs its vehicle), as well as ERβ (Figure 5B, $p<0.001$ vs its vehicle) and induce the transcription of ERE-dependent luciferase gene. 10 nM of either compounds **12** and **13** induced ERE-dependent luciferase expression significantly through ERα in UMR-106 cells (Fig. 6A). In contrast, neither **12** nor **13** at all concentrations tested activated ERE-dependent transcription through ERβ. It is interesting to note that compounds **12** and **13** show absolute selectivity towards ERα–mediated ERE-dependent transcription.



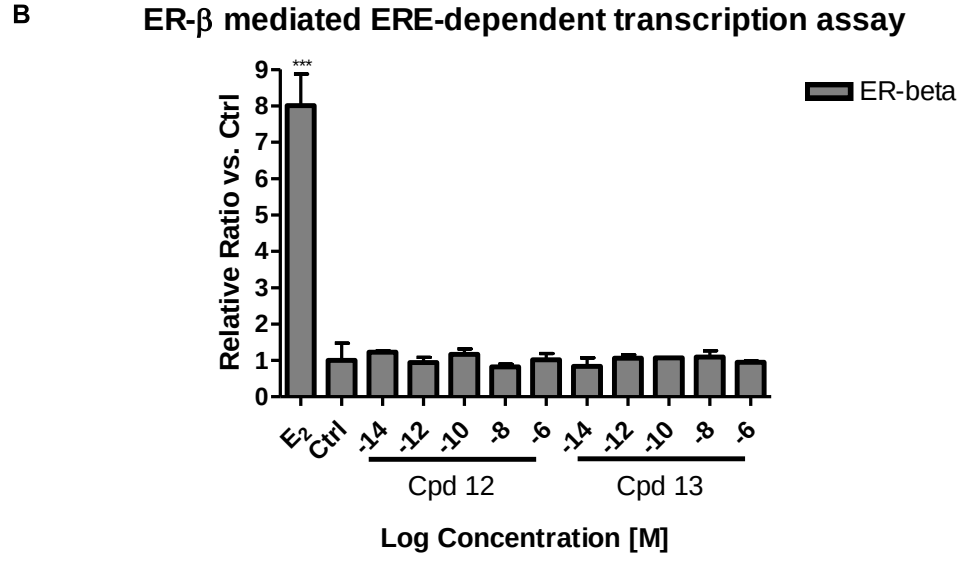




Figure 5. Effect of Compound **12** & **13** on ER transcriptional activity on UMR-106 cells. Cells were co-transfected with plasmid expressing human (a) ERα or (b) ERβ ERE-luciferase reporter and internal control plasmid. $10^{-8}$ M of 17β-estradiol ($E_2$) was used as a positive control. Concentration from $10^{-14}$ M to $10^{-6}$ M of compound **12** or **13** was added to cells for 24 hours treatment. The bars represent mean ± SEM value with n=6. The results were obtained from three independent experiments. ***$p$<0.001 verse control.

## Conclusions

In conclusion, we have synthesized the active flavonoid **12** previously isolated from the traditional Chinese medicine Gu-Sui-Bu [*D.fortunei* (kunze) J. Sm]. The compound **12** and the cyclized compound **13** showed potent proliferation activity as well as differentiating effect on UMR-106 osteoblastic-like cells *in vitro*. Using co-treatment with the ER antagonist ICI 182,780, we were able to demonstrate that compound **12** or **13** exerted its proliferation effect but not the differentiating effect through the ER pathway. Furthermore, compound **12** (or **13**) showed absolute selectivity towards ERα-mediated ERE-dependent transcription and no activity toward ERβ-mediated ERE-dependent transcription, in contrast to 17β-estradiol. Further studies of these compounds would seem warranted.

## Experimental section:

**General for organic synthesis:** All NMR spectra were recorded on a Bruker MHz DPX400 spectrometer and were carried out at room temperature. The chemical shifts are reported as parts per million (ppm) in unit relative to the resonance of $CDCl_3$ (7.26 ppm in the $^1H$, 77.0 ppm for the central line of the triplet in the $^{13}C$ modes, respectively). Low-resolution and high-resolution mass spectra were obtained on a Micromass Q-TOF-2 by electrospray ionization (ESI) mode. Melting points were measured using Electrothermal IA9100 digital melting point apparatus and were uncorrected. All reagents and solvents were reagent grade and were used without further purification unless otherwise stated. The plates used for thin-layer chromatography (TLC) were E. Merck Silica Gel $60F_{254}$ (0.25-mm thickness) and they were visualized under short (254-nm) and long (365-nm) UV light. Chromatographic purifications were carried out using MN silica gel 60 (230 – 400 mesh). 5,7-*Bis*(benzyloxy)-2-(4-benzyloxyphenyl)-chroman-3-one (**14**) was prepared according to reported procedures.[16]

(2,4)-*trans*-5,7-*Bis*(benzyloxy)-2-(4-benzyloxyphenyl)-chroman-3-one-4β-acetic acid, benzyl ester (**15a**). NaOH (20 mg, 0.5 mmol) was added to a stirred solution of **14** (543.2 mg, 1 mmol) in THF (10 mL) at 70 $^{o}$C under an $N_2$ atmosphere. Subsequently, benzyl bromoacetate (86.3 μl, 0.55 mmol) was added dropwise and stirred overnight at the same temperature giving a mixture of the alkylation products **15** with both α and β-stereochemistry. The organic solvent was filtered and evaporated followed by flash chromatography on silica gel (EtOAc/hexane=1:8 v/v) and then recrystallized with hexane and EtOAc to afford the desired *trans*-product **15a** as white solid (81.4 mg, 30% yield). $^{1}$H NMR ($CDCl_3$) δ 7.45–7.18 (m, 20H), 6.87 (J=9 Hz, 2H), 6.39 (br. s, 1H), 6.29 (br. s, 1H), 5.58 (s, 1H), 5.06-4.94 (m, 8H), 3.83 (t, J=4.5 Hz, 1H), 3.31 (dd, J=16.5, 4.5 Hz, 1H), 3.19 (dd, J=16.5, 5.5 Hz, 1H); $^{13}$C NMR ($CDCl_3$) δ 206.28, 171.24, 159.80, 157.38, 154.33, 136.73, 136.53, 136.21, 135.57, 128.62, 128.61, 128.58, 128.44, 128.21, 128.14, 128.12, 128.07, 128.00, 127.69, 127.68, 127.45, 127.40, 127.24, 114.99, 103.87, 96.22, 94.86, 82.13, 70.19, 70.15, 69.94, 66.61, 38.92, 36.77; HRMS (ESI+) calculated for $C_{45}H_{39}O_7$ (M+H) 691.2696, $C_{45}H_{38}O_7Na$ (M+Na) 713.2515, $C_{45}H_{38}O_7K$ (M+K) 729.2255; found $C_{45}H_{39}O_7$ (M+H) 691.2670, $C_{45}H_{38}O_7Na$ (M+Na) 713.2510, $C_{45}H_{38}O_7K$ (M+K) 729.2234.

The 2,4-*cis* isomer **15b** was obtained by recrystallization in hexane/EtOAc (5:1 v/v) to give a white solid (33% yield), mp 105-106 $^{o}$C: $^{1}$H NMR ($CDCl_3$) δ 7.45–7.28 (m, 20H), 7.20-7.18 (m, 2H), 7.01-6.99 (m, 2H), 6.37-6.36 (m, 2H), 5.16 (s, 1H), 5.09-4.99 (m, 8H), 4.07 (t, J=5 Hz, 1H), 3.27 (dd, J=16, 4.5 Hz, 1H), 3.11 (dd, J=15.5, 5.5 Hz, 1H); $^{13}$C NMR ($CDCl_3$) δ 205.99, 171.02, 159.66, 159.17, 157.41, 156.66, 136.91, 136.53, 136.29, 135.62, 129.93, 128.72, 128.65, 128.64, 128.57, 128.42, 128.20, 128.12, 128.10, 127.95, 127.57, 127.43, 127.18, 126.99, 114.86, 105.71, 95.77, 95.39, 84.27, 70.25, 70.18, 69.99, 66.56, 40.96, 36.61; MS (ESI+) calculated for $C_{45}H_{38}O_7Na$ (M+Na) 713.25, $C_{45}H_{38}O_7K$ (M+K) 729.23; found $C_{45}H_{38}O_7Na$ (M+Na) 713.23, $C_{45}H_{38}O_7K$ (M+K) 729.23.

(2,3)-*cis*-5,7-*Bis*(benzyloxy)-2-(4-benzyloxyphenyl)-chroman-3-ol-4β-acetic acid, benzyl ester (**16**). L-Selectride (0.1 ml, 1M solution in THF, 0.1 mmol) was added dropwise to a stirred solution of **15a** (69 mg, 0.1 mmol) in dry THF (5 mL) at r.t. for 1 h under an $N_2$ atmosphere. The organic solvent was filtered and evaporated followed by flash chromatography on silica gel (EtOAc/hexane=1:10 v/v) to afford a mixture of the alcohol **16** (22 mg, 32% yield) and the lactone **17** (20 mg, 34% yield).

Compound **16:** $^1$H NMR ($CDCl_3$) δ 7.46–7.28 (m, 20H), 7.01 (J=8.5 Hz, 2H), 6.23 (s, 2H), 5.01 (m, 9H), 4.02 (d, J=5 Hz, 1H), 3.68 (d, J=10 Hz, 1H), 3.18 (dd, J=16, 3.5 Hz, 1H), 2.43 (dd, J=15.5, 11 Hz, 1H), 1.70 (d, J=5.5 Hz, 1H); $^{13}$C NMR (acetone-$d_6$) δ 172.60, 159.89, 159.29, 159.20, 156.49, 138.40, 138.34, 138.24, 137.43, 132.20, 129.48, 129.45, 129.06, 128.97, 128.84, 128.83, 128.73, 128.59, 128.11, 115.39, 104.85, 95.67, 94.63, 75.17, 70.69, 70.62, 70.58, 69.56, 66.93, 39.41, 36.31; HRMS (ESI+) $C_{45}H_{41}O_7$ (M+H) Calcd: 693.2852, Found: 693.2865, $C_{45}H_{38}O_7Na$ (M+Na) Calcd: 715.2672, Found: 715.2713, $C_{45}H_{38}O_7K$ (M+K) Calcd: 731.2411, Found: 731.2409.

Lactone **17**: $NaBH_4$ (4.2 mg, 0.11 mmol) was added to a stirred solution of **15a** (69 mg, 0.1 mmol) in dry THF (5 mL) at r.t. for 4 h under an $N_2$ atmosphere. The organic solvent was filtered and evaporated followed by flash chromatography on silica gel (EtOAc/hexane=1:10 v/v) to afford lactone **17** (27.6 mg, 40% yield). $^1$H NMR ($CDCl_3$) δ 7.45–7.33 (m, 15H), 7.01 (J=8.5 Hz, 2H), 6.33 (br. s, 1H), 6.27 (br. s, 1H), 5.10-4.99 (m, 6H), 4.81 (t, J=4 Hz, 1H), 4.68 (d, J=9 Hz, 1H), 3.83 (q, J=9 Hz, 1H), 3.16 (dd, J=18, 9 Hz, 1H), 2.58 (dd, J=18, 10 Hz, 1H); $^{13}$C NMR ($CD_3CN$) δ 176.03, 159.58, 159.21, 158.18, 155.39, 136.79, 136.50, 136.24, 128.88, 128.75, 128.63, 128.61, 128.57, 128.30, 128.11, 128.00, 127.51, 127.46, 127.41, 115.07, 104.43, 94.91, 94.52, 78.39, 76.26, 70.33, 70.19, 70.03, 44.75, 35.41, 31.36; HRMS (ESI+) $C_{38}H_{33}O_6$ (M+H) Calcd: 585.2277, Found: 585.2236; $C_{38}H_{33}O_5Na$ (M+Na) Calcd: 607.2097, Found: 607.2169; $C_{38}H_{33}O_5K$ (M+K) Calcd: 623.1836, Found: 623.1866.

(2,3)-*cis*-4β-Carboxymethyl-*epi*afzelechin acid (**12**). Under an $H_2$ atmosphere, $Pd(OH)_2/C$ (20%, 5 mg) was added to a solution of **16** (69 mg, 0.1 mmol) in a solvent mixture of THF/MeOH (1:1 v/v, 10 mL). The resulting reaction mixture was stirred at r.t. under $H_2$ for 4 h. The reaction mixture was filtered to remove the catalyst. The filtrate was evaporated to afford the *epi*afzelechin acid **12** (8.2 mg, 25% yield). $^1$H NMR ($CDCl_3$) δ 7.70 (dd, J=5, 3.5 Hz 1H), 7.37 (d, J=8.5 Hz, 2H), 6.82 (d, J=8.5 Hz, 2H), 6.01 (d, J=2 Hz, 1H), 5.90 (d, J=2 Hz, 1H), 4.94 (s, 1H), 4.20 (t, J=6 Hz, 1H), 3.41 (dd, J=9.0, 4.5 Hz, 1H), 3.00 (dd, J=16, 4.5 Hz, 1H), 2.43 (dd, J=16, 9.0 Hz, 1H); HRMS (ESI-) $C_{17}H_{15}O_7$ (M-H) Calcd: 331.0818, Found: 331.0827.

(2,3)-*trans*-4β-Carboxymethyl-epiafzelechin lactone (**13**). Under an $H_2$ atmosphere, $Pd(OH)_2/C$ (20%, 5 mg) was added to a solution of **17** (58 mg, 0.1 mmol) in a solvent mixture of THF/MeOH (1:1 v/v, 10 mL). The resulting reaction mixture was stirred at r.t. under $H_2$ for 4 h. The reaction mixture was filtered to remove the catalyst. The filtrate was evaporated to afford the *epi*afzelechin lactone **13** (7.8 mg, 25% yield). $^1$H NMR ($CDCl_3$) δ 7.31 (d, J=8.5, 2H), 6.86 (d, J=8.5 Hz, 2H), 6.10 (br. s, 1H), 6.94 (br. s, 1H), 4.93-4.86 (m, 2H), 3.77 (q, J=9 Hz, 1H), 3.08 (dd, J=17.5, 9 Hz, 1H), 2.62 (dd, J=17.5, 9.5 Hz, 1H); HRMS (ESI-) $C_{17}H_{13}O_6$ (M-H) Calcd: 313.0712, Found: 313.0712.

**Culture of rat osteoblastic-like UMR-106 cell line.** UMR-106 cells (ATCC No. CRL-1661) were grown separately in Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Carlsbad, CA) inside an incubator controlled at 37 °C, 95% humidity and 5% of $CO_2$. Cells were subcultureed every 4-5 days. To remove background hormone for different experiments, medium was changed to phenol-red free DMEM with 1% charcoal-stripped FBS after 70% confluence was reached. After 24 hours, cells were treated with different compounds or 17β-estradiol (Sigma, St. Louis, MO, USA).

**Cell Proliferation Assay.** $5x10^3$ cells were seeded in each well of 96-well microtiter plate. After 24 hours of background hormone depletion, cells were treated with different concentrations of compound

**12** or **13** in $10^{-14}$ M to $10^{-6}$ M and $10^{-8}$ M of 17β-estradiol for another 24 hours in UMR-106 cells. In cell proliferation assay, the viable cells were determined by MTS assay (3-(4,5-dimethylthiazol-2-yl)-5- (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium) (Promega). After completion of MTS assay, the optical densities at 490 nm were read on spectrophotometric plate reader (Bio-Rad model 550, Japan).

**Cell Differentiation Assay.** $5x10^3$ cells were seeded in each well of 96-well microtiter plate. After 24 hours of background hormone depletion, cells were treated with different concentrations of compounds **12** or **13** in $10^{-14}$ M to $10^{-6}$ M and $10^{-8}$ M of 17β-estradiol for another 24 hours in UMR-106 cells. The activity of enzyme alkaline phosphatase (ALP) was measured to indicate the differentiation rate of the cells. ALP converts *p*-nitrophenyl phosphate (*p*-NPP) to *p*-nitrophenol by hydrolysis. Substrate *p*-NPP was added in each well, after 15 or 30 minutes, the optical densities at 405 nm were recorded by spectrophotometric plate reader (Bio-Rad model 550, Japan). In addition, a Bradford protein assay (Bio-rad) was done to normalize the protein content in each sample. Another set of treated cells were lysed with passive lysis buffer (PLB). After 15 minutes, 1 X Bradford reagent was added to each well. Ten minutes later, the optical densities at 405 nm were recorded by spectrophotometric plate reader (Bio-Rad model 550, Japan).

**Transient transfection of UMR-106 cells for ER-α and ER-β-mediated Estrogen Response Element luciferase assay.** UMR-106 cells were grown in 24-well plates with cell density 2.5 x $10^4$ cells per well. After 70% confluence, medium was changed to phenol-red free DMEM with 1% charcoal-stripped FBS. Twenty four hours later, an Estrogen Response Element (ERE)-containing luciferase reporter plasmid (0.4 μg), ERα or ERβ expression construct plasmid (0.4 μg), together with inactive control plasmid pRL-TK (0.1 μg) were co-transfected the cells by Lipofectamine™ 2000 reagent (Invitrogen, Carlsbad, CA). 17β-Estradiol and compounds were added after 6 hours of incubation. After 24 hours, the cells were lysed with passive lysis buffer. The luciferase activity of the cell lysates was measured with Dual-luciferase® reporter assay system (Promega, Madison, WI, USA). The signal was

detected by TD-20/20 luminometer (Turner Design, Sunnyvale, CA, USA). The *firefly* luciferase represents the ERE activity while the *Renilla* luciferase represents the normalization pRL-TK expression.

**Estrogen Receptor Dependency Assay.** UMR-106 cells were co-treated with estrogen receptor antagonist, ICI 182.780, at concentration of $10^{-6}$ M and compounds. The effects of ER antagonist were detected through cell proliferation assay and cell differentiation assay.

**Statistical Analysis.** Results are reported as mean ± standard error mean (SEM). Significant differences between different groups of means were evaluated by student t-test in confidence level at 95%.

**ACKNOWLEDGMENT.** We thank the Research Grants Council of Hong Kong for financial support (PolyU 5003/05P and 5632/09).

**Supporting information available**. Copies of NMR spectra of compounds **12**, **13**, **15a**, **15b**, **16** and **17** are provided as electronic supplementary material.

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