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Synthesis and biological evaluation of isoflavone fatty acid esters with potential weight loss and hypolipidemic activities

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ABSTRACT

A series of isoflavone fatty acid esters were designed on the basis of endogenous oleoyl-estrone using estrogen moiety modification strategy. Ten new compounds were synthesized, and their body weight loss and hypolipidemic bioactivities were assayed. Some of these novel compounds could effectively inhibit the differentiation of 3T3-L1 preadipocytes in vitro. The most potent compound **1a** significantly decreased the body weight and white adipose tissue weight in a high-fat diet-induced rat model. Also, compound **1a** showed good hypolipidemic activity and low toxicity.

1. Introduction

Obesity has come to be recognized as a critical global health problem. It is a serious risk for the development of non-insulin dependent diabetes, cardiovascular disease, non-alcoholic fatty liver disease, endocrine problems and various types of cancer.[1] Overall mortality due to any cause increased with the prevalence of obesity. The most serious threaten associated with obesity (particularly central adiposity) is metabolic disturbances, including hypertension, type 2 diabetes and atherogenic dyslipidaemia such as increased triglycerides and small dense low-density lipoprotein (LDL) cholesterol, and decreased high-density lipoprotein (HDL) cholesterol.[2,3] The rapidly expanding obesity problem is leading to an enormous burden on global health care and welfare systems.[4] Despite the helpful dietary intervention exercise therapy, and their combined approach in relieving symptoms of obesity, some anti-obesity drugs have been shown a significant effects on treatment of metabolic disorders. There are data demonstrated that modest weight loss (5–10%) reduces risk factors for diabetes, cancer and cardiovascular disease.[5,6]

Oleoyl-estrone (OE) is a naturally occurring fatty acid monoester of estrogen that has been reported to produce rapid and sustained weight loss (Fig. 1).[7] Endogenous OE is a common form of the estrogenic hormone estrone in lipoproteins and its fatty acid moiety of the molecule is a crucial factor in its effects.[8] OE is synthesized in white adipose tissue and later released into the blood stream.[9,10] In animal studies, OE reduced body weight in obese Zucker rats.[11] Also, it was reported that OE could decrease circulating plasma lipids, reduce plasma insulin levels and improve insulin resistance.[12,13]

Studies on rats indicated that OE has no estrogenic effects by itself, but the estrone it contains may eventually result in some estrogenic effects when OE is hydrolyzed in tissues.[14] Free estrone and its metabolite 17β-estradiol have notable estrogenic effect. The data of Phase 2a clinical trials from Manhattan Pharmaceuticals showed oral administration of OE may lead to limited and dose-dependent increases in estrone and estradiol.

Isoflavones are found at high levels in soy plants and structurally similar to estrogens.[15] They are suggested to be a potential alternative to estrogen therapy in the treatment and prevention of menopausal symptoms, osteoporosis, breast cancer and cardiovascular disease. Genistein, daidzein, glycitein, formononetin and biochanin A were considered to be the most important and hence most studied isoflavone phytoestrogens (Fig. 1).[16,17]

It is also reported that isoflavones have healthful benefits in human obesity and have a positive influence on plasma cholestrol.[18] They produce weaker estrogenic effects when endogenous estrogen level is inadequate. In contrast, they can inhibit the efficacy when estrogens are adequate.[19] With the dual-effects, isoflavones are considered to be effective and safer estrogen replacements.

In view of the structure–activity relationship of OE, we changed the steroidal skeleton of OE into a benzopyrone isoflavone moiety. The rationale behind the transformation is that new compounds

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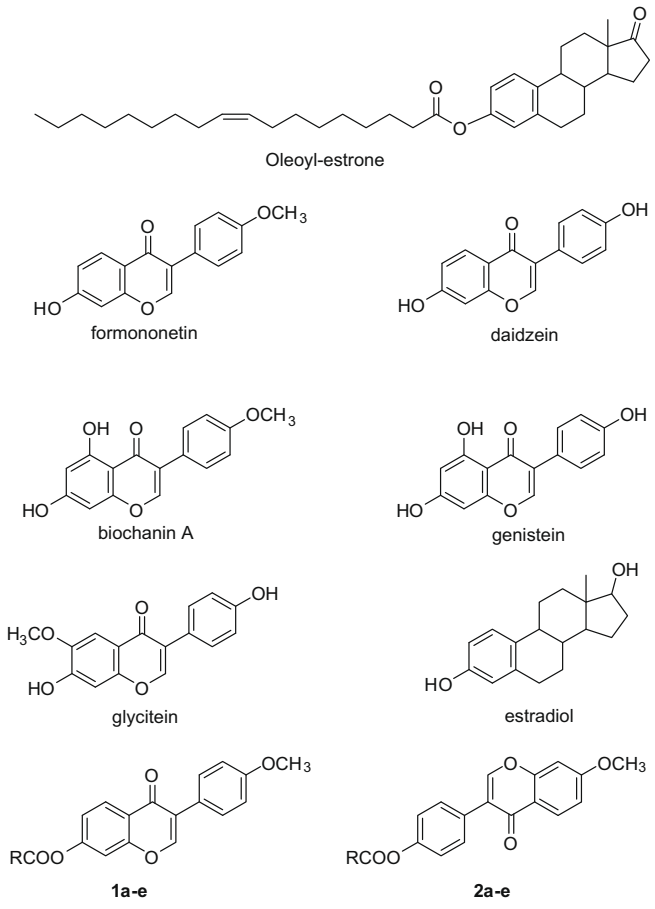


Figure 1. Structures of compounds mentioned or synthesized in this study.

may retain some of the characteristics of OE but eliminate probable estrogenic side effects. By using substituted daidzein as skeletons, 4′-methoxy daidzein-7-fatty acid esters **1a–e** and 7-methoxy daidzein-4′-fatty acid esters **2a–e** (Fig. 1) were designed and synthesized, and their bioactivities were assayed.

2. Results and discussion

2.1. Chemical synthesis

The Isoflavone fatty acid esters can be obtained by direct condensation of the derivatives of daidzein and fatty acid chloride in methylene chloride at boiling temperature, and anhydrous pyridine as catalyst (Scheme 1).

4′-Methoxy daidzein was accomplished by one-pot method under mild conditions.[20] 2,4-Dihydroxy-4′-methoxydeoxybenzoin was prepared from resorcinol and 4-methoxybenzoic acid 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 85 °C and completed in about one and a half hours. The reaction mixture was then directly treated with Vilsmeier reagent *N*,*N*′-dimethyl(chloromethylene) ammonium chloride which was freshly generated separately by treating phosphorous pentachloride with *N*,*N*′-dimethylformamide. Then the intermediate 4′-methoxy daidzein was obtained by pouring the reaction mixture into boiling dilute HCl (A of Scheme 1).

7-Methoxy daidzein was synthesized by a two-step process. The 2,4′-dihydroxy-4-methoxy-deoxybenzoin was prepared in a similar strategy mentioned for the synthesis of 2,4-dihydroxy-4′-methoxy-deoxybenzoin. Besides boron trifluoride etherate, catalytic amounts of *p*-toluenesulfonic acid were added. The cyclization reaction was accomplished using dimethylformamide-dimethylacetal (DMF-DMA) in toluene (B of Scheme 1).[21]

To investigate the effect of the fatty acid moiety to bioactivities, five different fatty acids were selected, and the respective fatty acid chlorides were obtained in the presence of PCl_3 (C of Scheme 1).

2.2. Animal experiments

Compounds **1a–c**, **2a–c** were selected for evaluation of weight loss and hypolipidemic activities in SD rats. Sibutramine, which could attenuate diet-induced obesity and produce promising outcomes in the overall management of high-risk patients with metabolic syndrome, was selected as positive control drug. Before feeding the test compounds, rats were fed with either low fat diet (LFD) or high-fat diet (HFD) ad libitum for 8 weeks. The LFD-fed lean control rats showed much less body weight gain compared to the HFD-fed obese control group. Then the rats were treated with vehicle, Sibutramine (10 mg/day/kg of rat weight) or compounds (50 mg/day/kg of rat weight) by intragastric administration once daily for 30 days. As shown in Table 1, **1a** and **1b** notably reduced the concentrations of total cholesterol (TC), triglyceride (TG), free fatty acids (FFA), body fat mass and body weight, compared with the HFD control group. The decreases of body weight, body fat mass and contents of three lipoprotein fractions in rats treated with **1a** or **1b** were more pronounced than those with **1c** or **2a–c**. The result suggested that compounds containing 4′-methoxy daidzein skeleton and unsaturated fatty acid side chain led to better activity than their derivatives of 7-methoxy daidzein and saturated fatty acid.

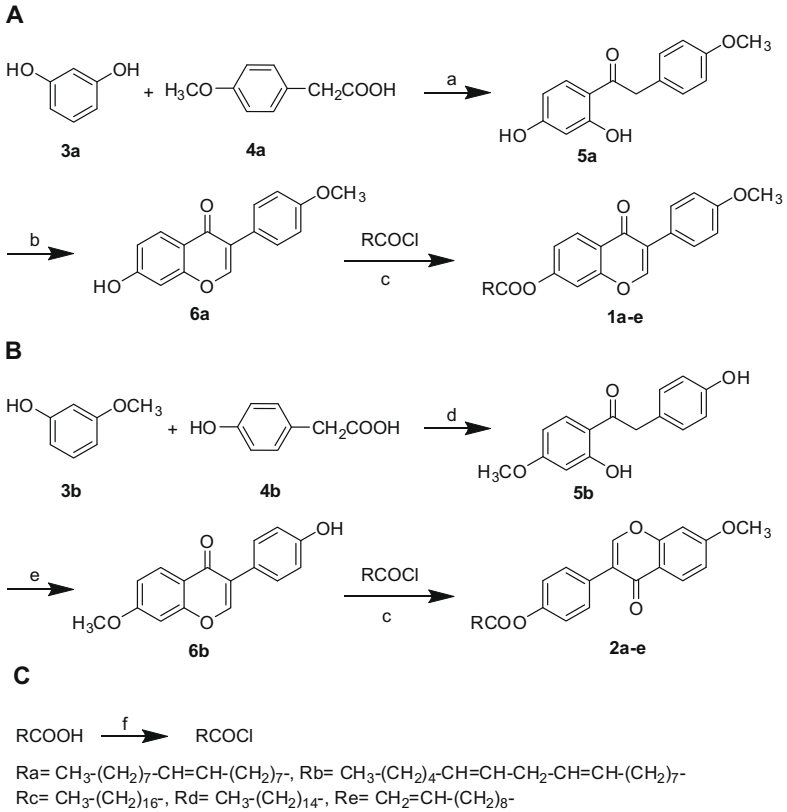
Prompted by the above result, **1a** was further investigated for its hypolipidemic effect in male C57BL/6 mice. Inositol Hexanicotinate was used as positive control drug, which could be hydrolyzed and release free nicotinic acid to reduce lipid levels. Fifty mice were fed with either LFD or HFD ad libitum for 4 weeks. The HFD-fed mice were separated into four groups (n = 10, each group). Each of the groups were then given HFD, HFD plus **1a** (50 mg/kg/d, 100 mg/kg/d) or HFD plus Inositol Nicotinate (200 mg/kg/d). This experiment with different oral doses of **1a** indicated that it significantly lowered TG, TC, low-density lipoproteins (LDL) cholesterol and increased high-density lipoproteins (HDL) cholesterol in plasma (Fig. 2). The hypolipidemic activities were dose-dependent and the HDL cholesterol levels at higher dose group of **1a** were higher by approximately fourfold compared with the HFD. On the other hand, intragastric administration of **1a** reduced plasma insulin obviously (Fig. 3).

Compound **1a** was further evaluated for acute toxicity. Its mice median lethal dose (LD_{50}) after intragastric administration (ig) was 2163.8 mg/kg and **1a** exhibited very low toxicity.

2.2.1. 3T3-L1 cell culture experiment

Compounds **1a–d** were selected to evaluate the effects on proliferation and differentiation of 3T3-L1 preadipocytes, and the ability to interact with the expression of PPARγ2-mRNA. The testing compounds were compared with Formononetin (4′-methoxy daidzein).

The experiment of preadipocytes proliferation was assessed using the standard MTT assay, and the result indicated that **1a** suppressed the proliferation of preadipocytes significantly (P <0.05) (Fig. 4). The effect of **1a** on preadipocytes differentiation was as-



Scheme 1. Reagents and conditions for the synthesis of compounds: (a) BF_3/Et_2O, 75–85 °C/90 min; (b) DMF, PCl_5, rt/1 h; (c) CH_2Cl_2, C_5H_5N, 40 °C/3 h; (d) BF_3/Et_2O, *p*-toluenesulfonic acid, 75–85 °C/90 min; (e) DMF–DMA, toluene, 110 °C/2 h; (f) PCl_3, 70 °C/4 h.

Table 1
The effect of some synthetic compounds on body weight, body fat mass, triglyceride, total cholesterol and free fatty acids (mean ± SD, $n = 10$)

	Body weight after treatment (g)	Body fat mass (g/100 g)	Triglyceride (TG) (mmol/L)	Total cholesterol (TC) (mmol/L)	Free fatty acids (FFA) (μmol/L)
Normal	275 ± 34	4.6 ± 1.9	0.61 ± 0.16	1.44 ± 0.19	604.2 ± 57.4
HFD	355 ± 36$^{\#\#}$	6.4 ± 1.4$^{\#\#}$	1.25 ± 0.23$^{\#\#}$	1.86 ± 0.31$^{\#\#}$	785.6 ± 175.8
Sibutramine	301 ± 41*	4.3 ± 1.7*	0.97 ± 0.31*	1.32 ± 0.29*	609.6 ± 138.3*
1a	309 ± 36*	5.0 ± 1.6*	0.91 ± 0.22*	1.31 ± 0.27**	606.7 ± 144.7*
1b	307 ± 32*	4.3 ± 1.5*	0.99 ± 0.21*	1.28 ± 0.26**	603.8 ± 163.5*
1c	320 ± 34	5.4 ± 1.2	1.19 ± 0.30	1.57 ± 0.30	730.2 ± 186.0
2a	328 ± 32	5.6 ± 1.6	1.01 ± 0.24*	1.65 ± 0.25	696.3 ± 205.4*
2b	318 ± 28	5.1 ± 1.3*	1.03 ± 0.21*	1.67 ± 0.23	658.8 ± 201.2*
2c	352 ± 47	6.0 ± 2.1	1.35 ± 0.35	1.71 ± 0.34	738.1 ± 196.0

Date points represent means ± SEM. $^{*}P$ <0.05, $^{**}P$ <0.01 for comparisons against the HFD group; $^{\#}P$ <0.05, $^{\#\#}P$ <0.01 for comparisons against the Normal group.

sessed by Oil-Red-O staining. Figure 5 shows the morphological changes in cells from day 0 to day 8. During 3T3-L1 cells differentiation, the numbers of larger lipid droplets in cells treated with **1a** decreased, while cells without **1a** became round in shape and larger in size with the increase in number. The triglyceride contents of 3T3-L1 cells treated with **1a**–**d** were then measured and all the four compounds decreased the triglyceride content of differentiated adipocytes and notably inhibited the differentiation of preadipocytes (Fig. 6).

To uncover the molecular mechanisms by which our compounds inhibit the fat accumulation and reduce plasma insulin, we next examined whether the peroxisome proliferator-activated receptor-γ (PPAR-γ) signaling pathway was influenced by these compounds. PPARγ is a major regulator of adipogenesis, and is also involved in glucose homeostasis, cell differentiation and inflammation.[22] The expression of PPARγ2-mRNA was assessed using RT-PCR. With pioglitazone as positive control group, the expression of PPARγ2-mRNA in the 3T3-L1 cell treated with **1a** or **1b** was upregulated, while it was downregulated when treated with **1c** or **1d** during the differentiation of 3T3-L1 preadipocytes at concentrations of 100 μmol/L of all testing compounds (Fig. 7). The fact suggested that the fatty acid moiety might play an important role in biological effects, and the unsaturated fatty acid induced a better activity than the saturated fatty acid.

The RT-PCR result exhibited that **1a** activated PPARγ notably even stronger than Pioglitazone. However, the result that **1a** could inhibit the differentiation of 3T3-L1 preadipocytes indicates that they didn't exert as full PPARγ agonist. It is supposed that **1a** might be act as selective PPARγ modulator (SPPARγM). The activation of PPARγ by **1a** resulted in the reduction of plasma insulin and the inhibition of the preadipocytes differentiation. SPPARγMs promoted selective gene transcription in a gene-specific manner, with

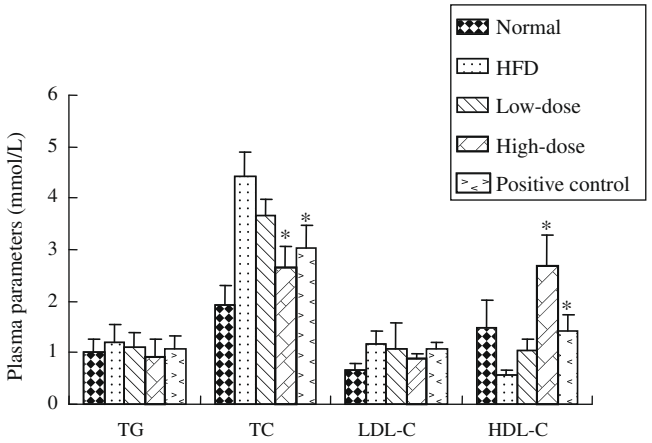


Figure 2. The biochemistry analysis of C57BL/6J mice treated with **1a** for 4 weeks (ig). Normal: normal control group; HFD: high-fat diet-fed group; low-dose: HFD mice treated with low-dose **1a** (50 mg/kg/d); high-dose: HFD mice treated with high-dose **1a** (100 mg/kg/d); Positive control: HFD mice treated with Inositol Hexanicotinate (200 mg/kg/d). $^{*}P \leqslant 0.05$, High-dose **1a** group versus Positive control group (Inositol Hexanicotinate).

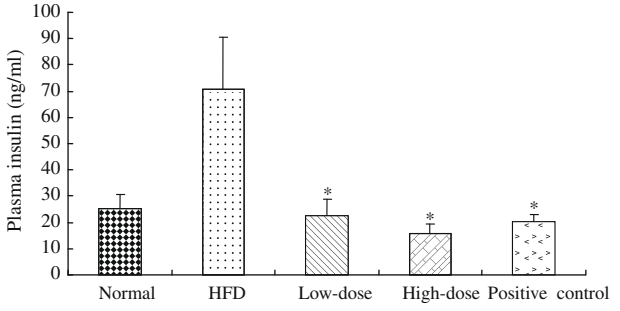


Figure 3. Effect of **1a** on plasma insulin in C57BL/6 J mice treated with **1a** for 4 weeks (ig). $^{*}P \leqslant 0.05$, High-dose **1a** group versus Positive control group (Inositol Hexanicotinate).

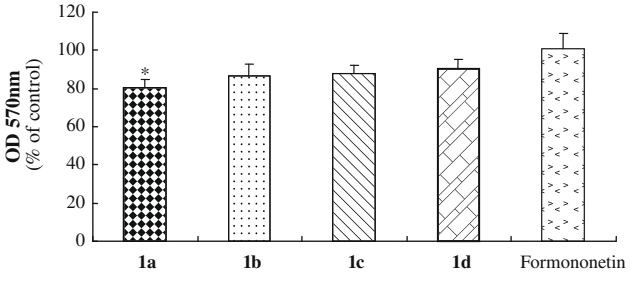


Figure 4. Effect of some synthetic compounds on preadipocytes proliferation by MTT assay. Cells were treated with the synthetic compounds for 48 h. Values represent % of the control (the control group, absence in synthetic compounds). $^{*}P \leqslant 0.05$ versus control group.

some genes induced to similar levels compared with those obtained with a full agonist, whereas others exhibit restricted activation.[22] The effect that SPPARγM induced selective regulation of target genes may contribute to the bioactivities of **1a**.

The concrete mechanism of **1a** or **1b** as selective PPARγ modulator needs to be demonstrated by additional research, so does the mechanism of weight loss. In view of the structural similarity between PPARs, further studies are required to detect whether **1a** or **1b** affects PPARα and PPARβ/δ, which modulate the transcription of genes implicated in lipid and lipoprotein metabolism in liver and muscle.

3. Conclusions

We have synthesized and evaluated a series of isoflavone fatty acid eaters. Compounds, **1a** and **1b**, decreased the concentrations of TC, TG, FFA and body weight notably. And **1a** decreased LDL cholesterol, increased HDL cholesterol and reduced plasma insulin significantly. We also tested the activity of compound **1a**–**d** in vitro experiment. All the four compounds inhibited the differentiation of 3T3-L1 preadipocytes (P <0.05) and **1a** suppressed the proliferation of 3T3-L1 preadipocytes particularly (P <0.05). **1a** and **1b** could up-regulated 3T3-L1 PPARγ-mRNA expression notably, while **1c** and **1d** downregulated the expression. In acute toxicity test of **1a**, it revealed low toxicity (LD_{50} = 2163.8 mg/kg).

Taken together, compound **1a** lowered body weight gain and adiposity, improved plasma lipid profiles, and reduced plasma insulin. Compound **1a** may provide a template to design new novels for the treatment of obesity and metabolic syndrome. Further studies and optimization of **1a** are required to demonstrate its molecular mechanism and to acquire better biological activities.

4. Experimental

4.1. Chemical synthesis

4.1.1. Materials

Most chemicals and solvents were of analytical grade and, when necessary, were purified and dried by standards methods. Reactions were monitored by thin-layer chromatography (TLC) using precoated silica gel plates (silica gel GF/UV 254), and spots were visualized under UV light (254 nm). Melting points (uncorrected) were determined on a Mel-TEMP II melting point apparatus and are uncorrected. Elemental analysis was determined on a Elementar Vario EL III elemental analyser. Infrared (IR) spectra (KBr) were recorded on a Nicolet Impact 410 instrument (KBr pellet). ^{1}H NMR spectra were recorded with a Bruker Avance 500 MHz spectrometer at 300 K, using TMS as an internal standard. MS spectra were recorded on a Shimadzu GC–MS 2010 (EI) or a Mariner Mass Spectrum (ESI), or a LC/MSD TOF HR-MS Spectrum. Chemical shifts (d) are expressed in parts per million relative to tetramethylsilane, which was used as an internal standard, coupling constants (J) are in hertz (Hz), and the signals are designated as follows: s, singlet; d, doublet; t, triplet; m, multiplet; br s, broad singlet.

4.1.2. The Isoflavones

4.1.2.1. 7-Hydroxy-3-(4-methoxyphenyl)-4*H*-chromen-4-one (4′-methoxy daidzein, 6a). A mixture of *m*-dihydroxybenzene (**3a**; 0.72 g, 6.5 mmol), 4-methoxyphenylacetic acid (**4a**; 1.0 g, 6.0 mmol) and BF_3/Et_2O (10 mL) was heated (85 °C) for 90 min with stirring. The mixture was then cooled to 10 °C and DMF (10 mL) was added dropwise. In another flask, DMF (20 mL) was cooled to 10 °C and $PC1_5$ (2 g, 9 mmol) was added in small portions. The mixture was then allowed to stand at 55 °C for 20 min. The pale pink colored solution containing *N*,*N*′-dimethyl(chloromethylene)ammonium chloride was then added to the above reaction mixture slowly. During the addition, the temperature of the reaction mixture was maintained below 30 °C. The mixture was then stirred at room temperature for 1 h. Then the reaction mixture was poured slowly into boiling HCl (0.1 N) and filtering the precipitated product. Recrystallization from aqueous methanol afforded 1.0 g of 4′-methoxy daidzein (**6a**) (62.5%, yield); light-yellow solid; mp 256–258 °C; MS (ESI) *m*/*e*: 269[M+H]$^{+}$.

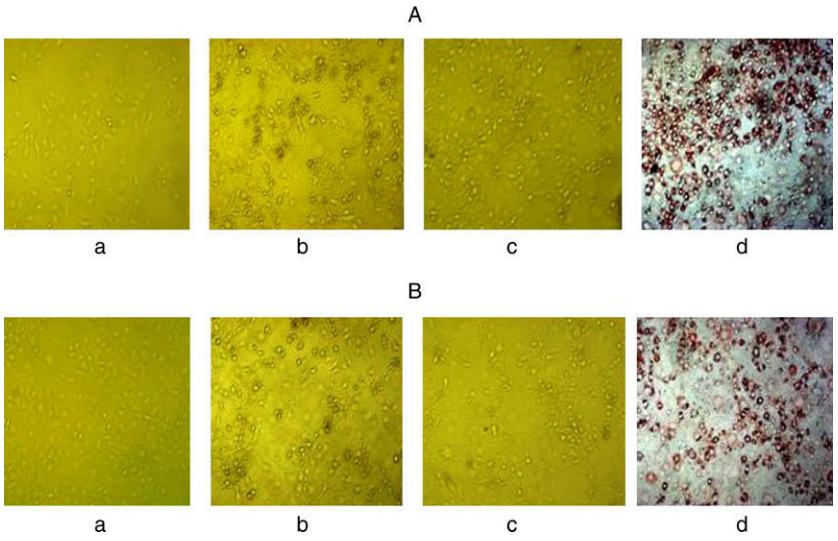


Figure 5. Morphology of differentiated adipocytes. (A) Untreated 3T3-L1 cells. (B) 3T3-L1 cells treated with **1a**. a. Undifferentiated cells. (b) Cells on day 4 of differentiation. (c) Cells on day 8 of differentiation, day 2 after treated with **1a**. (d) Cells on day 8 of differentiation, stained with oil red O.

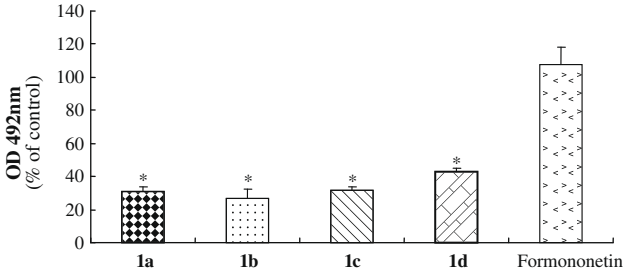


Figure 6. Effect of some synthetic compounds on triglyceride content of differentiated adipocytes Cells were treated with the synthetic compounds for 48 h during differentiation. Values represent % of the control (the control group, absence in synthetic compounds). $^{*}P \leqslant 0.05$ versus control group.

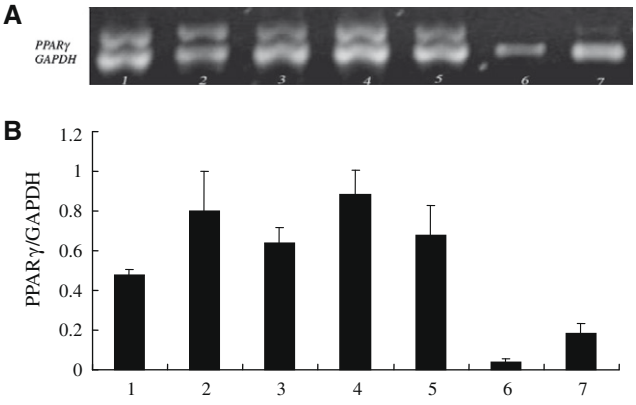


Figure 7. Effect of some synthetic compounds on 3T3-L1 cell PPARγ2-mRNA expression. (1) Blank control group, (2) **1a** (100 μmol/L), (3) Formononetin (100 μmol/L), (4) **1b** (100 μmol/L), (5) pioglitazone (100 μmol/L), (6) **1c** (100 μmol/L), (7) **1d** (100 μmol/L).

4.1.2.2. 3-(4-Hydroxyphenyl)-7-methoxy-4*H*-chromen-4-one (7-methoxy daidzein, 6b). A mixture of 3-methoxyphenol (**3b**; 9.6 mL, 88 mmol), 4-hydroxyphenylacetic acid (**4b**; 9.0 g, 59 mmol), *p*-toluenesulfonic acid (0.6 g, 3.5 mmol) and BF_3/Et_2O (36 mL) was heated (85 °C) for 90 min with stirring. The mixture was then poured into saturated $NaHCO_3$ solution and stirred. The product was filtered, and purified by recrystallization from ethanol to yield 7.52 g (48.6%) while solid (**5b**); mp 150–152 °C; MS (EI) *m*/*e*: 258.

2,4′-Dihydroxy-4-methoxydeoxybenzoin (**5b**; 6.0 g, 23 mmol) was dissolved in dry toluene (20 mL) and dimethylformamide-dimethylacetal (8.0 g, 67 mmol) was added. After refluxing for 2 h, the mixture was cooled to room temperature. The precipitated solid was filtered, and purified by recrystallization from methanol to yield 5.11 g (82%) light-yellow solid (**6b**); mp 215–218 °C; MS (ESI) *m*/*e*: 269 $[M+H]^+$.

4.1.3. The isoflavone fatty acid esters

A solution of fatty acid (7.5 mmol) and PCl_3 (2.5 mmol) was stirred at 70 °C for 2 h, and a yellow solution containing fatty acid chloride could be gotten.

A solution of isoflavone (3.7 mmol) and anhydrous pyridine (2 mL) in CH_2Cl_2 (20 mL) was stirred at boiling temperature, and the above reaction mixture was added slowly. Then the reaction continued for 3 h at boiling temperature. Washing with dilute hydrochloric acid (1 mL HCl/20 mL water), saturated $NaHCO_3$ solution, drying and removal of solvent under reduced pressure gives the crude product. Purification by recrystallization from dehydrated alcohol yielded the isoflavone fatty acid ester.

4.1.3.1. 3-(4-Methoxyphenyl)-4-oxo-4*H*-chromen-7-yl (9Z)-oleate (4′-methoxy daidzein-7-oleate, 1a). 80%; white solid; mp 76–80 °C; IR (KBr) 1743 cm^{-1}, 1645 cm^{-1}; ^1H NMR($CDCl_3$) δ 0.88 (t, 3H $-CH_3$), δ 2.60(t 2H $-CO-CH_2-$), δ 3.84 (s 3H $-OCH_3$), δ 5.37 (m 2H $-CH{=}CH-$), δ 7.15 (dd 1H C6-H), δ 7.28 (d 1H C8-H), δ 7.97 (s 1H C2-H), δ 8.31 (d 1H C5-H); MS (EI) *m*/*e*: 532. Anal. Calcd for $C_{34}H_{44}O_5$: C, 76.22; H, 8.03. Found: C, 76.66; H, 8.32.

4.1.3.2. 3-(4-Methoxyphenyl)-4-oxo-4*H*-chromen-7-yl (9Z,12Z)-linoleate (4′-methoxy daidzein-7-linoleate, 1b). 57%; white solid; mp 68–74 °C; IR (KBr) 1761 cm^{-1}, 1638 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (t, 3H $-CH_3$), δ 2.60 (t 2H $-CO-CH_2-$), δ 3.85 (s 3H $-OCH_3$), δ 7.14 (dd 1H C6-H), δ 7.28 (d 1H C8-H), δ 7.97 (s 1H C2-H), δ 8.31 (d 1H C5-H); MS (EI) *m*/*e*: 530. Anal. Calcd for $C_{34}H_{42}O_5{\cdot}0.5H_2O$: C, 76.08; H, 8.03. Found: C, 75.66; H, 8.03.

4.1.3.3. 3-(4-Methoxyphenyl)-4-oxo-4*H*-chromen-7-yl stearate (4′-methoxy daidzein-7-stearate, 1c). 80%; white solid; mp 98–100 °C; IR (KBr) 1762 cm^{-1}, 1638 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88

(t, 3H –CH_3), δ 2.61 (t 2H –CO–CH_2–), δ 3.85 (s 3H –OCH_3), δ 7.15 (dd 1H C6-H), δ 7.29 (d 1H C8-H), δ 7.98 (s 1H C2-H), δ 8.31 (d 1H C5-H); MS (EI) *m*/*e*: 534. Anal. Calcd for $C_{34}H_{46}O_5 \cdot 0.5H_2O$: C, 75.08; H, 8.78. Found: C, 75.10; H, 8.71.

4.1.3.4. 3-(4-Methoxyphenyl)-4-oxo-4*H*-chromen-7-yl palmitate (4′-methoxy daidzein-7-palmitate, 1d). 73%; white solid; mp 102–104 °C; IR (KBr) 1763 cm^{-1}, 1638 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.92 (t, 3H –CH_3), δ 2.65 (t 2H –CO–CH_2–), δ 3.89 (s 3H –OCH_3), δ 7.19 (dd 1H C6-H), δ 7.32 (d 1H C8-H), δ 8.02 (s 1H C2-H), δ 8.35 (d 1H C5-H); MS (EI) *m*/*e*: 506. Anal. Calcd for $C_{32}H_{42}O_5$: C, 75.90; H, 8.67. Found: C, 75.86; H, 8.35.

4.1.3.5. 3-(4-Methoxyphenyl)-4-oxo-4*H*-chromen-7-yl 10-undecylenate (4′-methoxy daidzein-7-undecylenate, 1e). 66%; white solid; mp 79–82 °C; IR (KBr) 1744 cm^{-1}, 1646 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.60 (t 2H –CO–CH_2–), δ 3.83 (s 3H –OCH_3), δ 4.95 (dd 2H CH_2=), δ 5.82 (m 1H –CH=), δ 7.14 (dd 1H C6-H), δ 7.27 (d 1H C8-H), δ 7.96 (s 1H C2-H), δ 8.30 (d 1H C5-H); MS (EI) *m*/*e*: 434. Anal. Calcd for $C_{27}H_{30}O_5$: C, 74.53; H, 6.93. Found: C, 74.63; H, 6.96.

4.1.3.6. 4-(7-Methoxy-4-oxo-4*H*-chromen-3-yl)phenyl (9Z)-oleate (7-methoxy daidzein-4′-oleate, 2a). 81%; white solid; mp 76–78 °C; IR (KBr) 1746 cm^{-1}, 1639 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (t, 3H –CH_3), δ 2.56 (t 2H –CO–CH_2–), δ 3.92 (s 3H –OCH_3), δ 5.36 (m 2H –CH=CH–), δ 6.85 (d 1H C8-H), δ 6.99 (dd 1H C6-H), δ 7.94 (s 1H C2-H), δ 8.21 (d 1H C5-H); MS (EI) *m*/*e*: 532. Anal. Calcd for $C_{34}H_{44}O_5$: C, 76.86; H, 8.48. Found: C, 76.66; H, 8.32.

4.1.3.7. 4-(7-Methoxy-4-oxo-4*H*-chromen-3-yl)phenyl (9Z,12Z)-linoleate (7-methoxy daidzein-4′-linoleate, 2b). 69%; white solid; mp 58–62 °C; IR (KBr) 1746 cm^{-1}, 1639 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (t, 3H –CH_3), δ 2.56 (t 2H –CO–CH_2–), δ 3.90 (s 3H –OCH_3), δ 6.85 (d 1H C8-H), δ 6.99 (dd 1H C6-H), δ 7.93 (s 1H C2-H), δ 8.21 (d 1H C5-H); MS (EI) *m*/*e*: 530. Anal. Calcd for $C_{34}H_{42}O_5$: C, 76.64; H, 8.02. Found: C, 76.95; H, 7.98.

4.1.3.8. 4-(7-Methoxy-4-oxo-4*H*-chromen-3-yl)phenyl stearate (7-methoxy daidzein-4′-stearate, 2c). 85%; white solid; mp 98–102 °C; IR (KBr) 1746 cm^{-1}, 1639 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (t, 3H –CH_3), δ 2.57 (t 2H –CO–CH_2–), δ 3.92 (s 3H –OCH_3), δ 6.87 (d 1H C8-H), δ 7.00 (dd 1H C6-H), δ 7.95 (s 1H C2-H), δ 8.21 (d 1H C5-H); MS (EI) *m*/*e*: 534.

4.1.3.9. 4-(7-Methoxy-4-oxo-4*H*-chromen-3-yl)phenyl palmitate (7-methoxy daidzein-4′-palmitate, 2d). 91%; white solid; mp 96–100 °C; IR (KBr) 1745 cm^{-1}, 1638 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.88 (t, 3H –CH_3), δ 2.56 (t 2H –CO–CH_2–), δ 3.92 (s 3H –OCH_3), δ 6.86 (d 1H C8-H), δ 7.00 (dd 1H C6-H), δ 7.94 (s 1H C2-H), δ 8.21 (d 1H C5-H); MS (EI) *m*/*e*: 506.

4.1.3.10. 4-(7-Methoxy-4-oxo-4*H*-chromen-3-yl)phenyl 10-undecenoate (7-methoxy daidzein-4′-undecylenate, 2e). 75%; white solid; mp 88–90 °C; IR (KBr) 1746 cm^{-1}, 1639 cm^{-1}; ^1H NMR ($CDCl_3$) δ 2.56 (t 2H –CO–CH_2–), δ 3.92 (s 3H –OCH_3), δ 4.96 (dd 2H CH_2=), δ 5.81 (m 1H –CH=), δ 6.86 (d 1H C8-H), δ 7.00 (dd 1H C6-H), δ 7.94 (s 1H C2-H), δ 8.21 (d 1H C5-H); MS (EI) *m*/*e*: 434. Anal. Calcd for $C_{27}H_{30}O_5$: C, 74.63; H, 7.04. Found: C, 74.63; H, 6.96.

4.2. Animal experiment

4.2.1. Body weight loss and hypolipidemic activities in SD rats

Male SD rats (Purchased from Experimental Animal Center of Nanjing Medical University, Nanjing, China) were divided into nine groups: normal control rats fed with a low fat diet (normal), high-fat diet-fed control rats treated with vehicle (HFD), high-fat diet-fed rats treated with sibutramine at a dose of 10 mg/kg (sibutramine), high-fat diet-fed rats treated with the selected compound at a dose of 50 mg/kg (**1a**, **1b**, **1c**, **2a**, **2b** or **2c**). The diet was purchased from Qinglongshan Food Service, Nanjing, China. LFD was composed of protein 20%, carbohydrate 70%, and fat 10%, whereas HFD diet was composed of protein 20%, carbohydrate 35%, and fat 45% (of total energy, %kcal). They were allowed free access to tap water and food. Every group of rats, the normal control rats were exception, were fed with high-fat diet for two months. Then every group of rats were given vehicle or compounds by intragastric administration respectively, once daily for one month. After this feeding period, the rats were sacrificed, and blood samples were taken form femoral artery. Plasma was analyzed for triglyceride (TG), total cholesterol (TC) and free fatty acid (FFA) using diagnostic kits following the manufacturer's protocol. Epididymal fat depots and retroperitoneal fat depots were excised immediately following blood collection, washed in cold physiological (isotonic) saline, gently blotted, and then weighed. Also, the body weight was determined.

4.2.2. Bioactivities study in male C57BL/6 mice

Male C57BL/6J mice (Experimental Animal Center of Nanjing University, Nanjing, China) were acclimatized for 3 days, then divided into five groups of 10 mice each, including: normal control group (fed with low fat diet), high-fat diet-fed group (HFD diet: 10% of lard, 2% of cholesterol and 1% of cholate added to LFD), 'low-dose' group (HFD mice treated with **1a** at a dose of 50 mg/kg/d), 'high-dose' group (HFD mice treated with **1a** at a dose of 100 mg/kg/d) and positive control group (HFD mice treated with Inositol Nicotinate at a dose of 200 mg/kg/d). The mice were individually housed and maintained in a 12-h light/dark cycle at 22 ± 2 °C. Food and water were available ad libitum. The body weight and food intake amount were recorded every week. Before feeding the test drug, mice were fed either normal diet or HFD ad libitum for 4 weeks. Then each of the groups were given normal diet, HFD or HFD plus drugs for 4 weeks. On completion of the experiment, after 12 h of fasting, blood samples were taken form vena orbitalis. Plasma was analyzed for triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C) and insulin using diagnostic kits following the manufacturer's protocol.

4.2.3. Acute toxicity test

The toxicity of **1a** was tested on KM mice both male and female in groups of 10 animals per dose. Compound **1a**, dissolved in olive oil, was given orally in doses of 1771, 1968, 2187, 2430 and 2700 mg/kg, respectively. After the administration of the compound, mice were observed for clinical signs of toxicity and number of deaths. After the test, all mice were sacrificed and checked macroscopically for possible damage to the heart, liver, and kidneys. The LD_{50} value was calculated using Bliss method.

4.3. 3T3-L1 cell culture experiments

4.3.1. Cell proliferation assay by MTT

3T3-L1 preadipocytes (5000 cells/well) were seeded in 96-well plates. After two days' conventional culture, the cells were incubated with Dulbecco's Modified Eagle's medium (DMEM, Gibco) containing the selected compounds (100 μmol/L) for 48 h. The culture solution containing 0.1% (V/V) ethanol (96%) was given to the control group. Twenty microliter of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL) was placed in each well for 4 h at 37 °C. Consecutively, 100 μL of dimethyl sulfoxide (DMSO) was added to extract the MTT formazan,

and followed by agitation on a plate shaker for 15 min. The optical density (OD) was then measured on the multiwell enzyme-linked immunosorbent assay automatic spectrometer reader at 540 nm.

4.3.2. Cell differentiation assay by oil red O staining

4.3.2.1. Oil red O staining. To induce cell differentiation, 3T3-L1 preadipocytes were cultivated in the growth medium until completely confluent. Two days after complete confluence was reached (defined as day 0), differentiation was induced using DMEM containing 15% fetal bovine serum (FBS), 0.5 mmol/L 3-Isobutyl-1-methylxanthine (IBMX), 1 μmol/L dexamethasone (DEX), and 10 mg/L insulin for 2 days. On day 2, the medium was replaced with DMEM containing only 15% FBS and 10 mg/L insulin. The medium was replaced with new medium every 2 days. On day 6, insulin was removed, and the medium was replaced with DMEM containing the selected compounds (100 μmol/L).

On day 8, the cells were washed twice with phosphate-buffered saline (PBS) and were then fixed in 10% formaldehyde in PBS for 2 h. Cells were then stained with Oil-Red-O and counter stained with hematoxylin after a wash with distilled water.

4.3.2.2. Measurement of the triglyceride content. 3T3-L1 adipocyte monolayers were washed three times with PBS and then fixed for 30 min with 3.7% formaldehyde in PBS. Oil-Red-O (0.5%) in isopropanol was diluted with 2/3 volumes of water, filtered and added to the fixed cell monolayers for 1 h at room temperature. The cell monolayers were then washed with PBS, and the stained triglyceride droplets in the cells were visualized. The Oil-Red-O-stained triglyceride droplets were then extracted with isopropanol, and the OD at 492 nm was measured.

4.3.3. RT-PCR analysis

The treated 3T3-L1 cells were homogenized in TriZol reagent (Invitrogen) and total RNA was isolated according to manufacturer's protocol. Prior to first strand cDNA synthesis, total RNA was treated with RNase-free DNase (Promega). cDNA was then synthesized using AMV and random hexamer (Invitrogen) as described in the manufacturer's protocol. The primers of PPARγ2 were described as follows:

PPARγ2 Forward: 5′-ACCACTCGCATTCCTTTGAC-3′,
Reverse: 5′-TCAGCGGGAAGGACTTTATG-3′,product size: 567 bp.
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)
Forward: 5′-GTTCCAGTATGACTCTACCC -3′,
Reverse: 5′-AGTCTTCTGAGTGGCAGTGATGGC -3′,
product size: 424 bp.

PCR amplification parameters: PCR was performed in one cycle for 5 min at 94 °C, then 30 cycles of amplification (denaturation for 30 s at 94 °C, annealing for 40 s at 57 °C and extension for 50 s at 72 °C). The last extension step was performed for 10 min at 72 °C. The RT-PCR products were separated on 1.5% agarose gel electrophoresis, and stained with 0.5 μg/ml ethidium bromide. The intensities of the bands were measured using an image documentation system. The house-keeping gene GAPDH was used as internal control for the determination of targeted mRNA levels.

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