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Iridoids from *Fraxinus excelsior* with Adipocyte Differentiation-Inhibitory and PPAR α Activation Activity

Naisheng Bai,[†] Kan He,^{*,†} Alvin Ibarra,[†] Antoine Bily,[†] Marc Roller,[‡] Xiaozhuo Chen,[§] and Ralph Rühl^{⊥,||,▽}

Naturex, Inc., 375 Huyler Street, South Hackensack, New Jersey 07606, Naturex SA, Site d'Agroparc BP 1218, 84911 Avignon Cedex 9, France, Department of Biomedical Sciences and Edison Biotechnology Institute, Ohio University, Athens, Ohio 45701, Department of Biochemistry and Molecular Biology, Medical and Health Science Center, University of Debrecen, Hungary, Apoptosis and Genomics Research Center, Hungarian Academy of Sciences, Debrecen, Hungary, and Paprika-Bioanalytics BT, Debrecen, Hungary

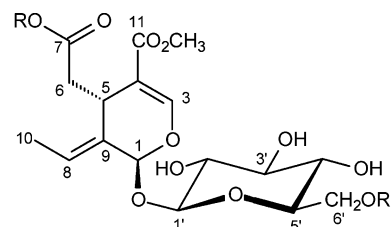
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Two new secoiridoid glucosides, excelsides A (**1**) and B (**2**), were isolated from the seeds of *Fraxinus excelsior*. Their structures were elucidated as (2*S*,4*S*,3*E*)-methyl 3-ethylidene-4-(2-methoxy-2-oxoethyl)-2-[(6-*O*- β -D-glucopyranosyl)- β -D-glucopyranosyl]oxy]-3,4-dihydro-2*H*-pyran-5-carboxylate and (2*S*,4*S*,3*E*)-methyl 3-ethylidene-4-{2-[2-(4-hydroxyphenyl)ethyl]oxy-2-oxoethyl}-2-[(6-*O*- β -D-glucopyranosyl)- β -D-glucopyranosyl]oxy]-3,4-dihydro-2*H*-pyran-5-carboxylate, respectively, on the basis of NMR and MS data. Eight known compounds were identified as nuzhenide (**3**), GI3 (**4**), GI5 (**5**), ligstroside (**6**), oleoside 11-methyl ester (**7**), oleoside dimethyl ester (**8**), 1'''-*O*- β -D-glucosylformoside (**9**), and salidroside (**10**). Compounds **1**–**9** inhibited adipocyte differentiation in 3T3-L1 cells. Dilutions of the aqueous extract of *F. excelsior* (1:10 000) as well as compounds **2**, **3**, **4**, **5**, and **8** activated the peroxisome proliferator-mediated receptor- α (PPAR α) reporter cell system in the range of 10^{-4} M, compared to 10^{-7} – 10^{-8} M for the synthetic PPAR α activator, WY14,643. Both biological activity profiles support the hypothesis that inhibition of adipocyte differentiation and PPAR α -mediated mechanisms might be relevant pathways for the antidiabetic activity of *F. excelsior* extract.

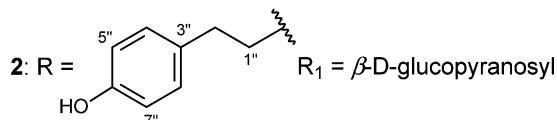
The plant *Fraxinus excelsior* L. (Oleaceae) is known as “common ash” or “European ash” in temperate Asia and Europe.^{1,2} The plant is also widely distributed throughout the southeast of Morocco (Tafilalet), where it is locally known as “Lissan Ettir” and its seeds as “l'ssane l'ousfour”. This region is a rich source of ethnobotanicals and an area in which phytotherapy has been well developed.^{2–4} Aqueous seed extract of *F. excelsior* (FE) has been shown to be highly potent in the reduction of blood glucose levels without significantly affecting insulin levels.^{3–5} The phlorizin-like effect of inhibiting renal glucose reabsorption is a potential mechanism for the hypoglycemic effect of FE.⁵ Previous investigations on the chemical composition of FE led to the characterization of several compound classes including secoiridoid glucosides, coumarins, flavonoids, phenylethanoids, benzoquinones, indole derivatives, and simple phenolic compounds.^{6–9} However, bioactivity studies have not been reported.

During prescreening, FE was found to activate PPAR α and mildly inhibited adipocyte differentiation in 3T3-L1 preadipocytes. PPAR α - or PPAR γ -mediated pathways have been associated with protection against diabetes.^{10–12} Various synthetic PPAR α / γ -selective agents have been reported to have potent antidiabetic activity.^{12,13} The focus of this study was to isolate and characterize the potential active principle(s) of FE and evaluate their biological activity in adipocyte (3T3-L1 cells) differentiation and PPAR α reporter assays. Sequential combination of normal, reversed-phase, and gel permeation column chromatography led to the isolation of nine secoiridoids including the new excelsides A (**1**) and B (**2**), the known nuzhenide (**3**),¹⁴ GI3 (**4**),¹⁵ GI5 (**5**),^{8,16} ligstroside (**6**),¹⁷ oleoside-11-methyl ester (**7**),¹⁸ oleoside dimethyl ester (**8**),¹⁹ and 1'''-*O*- β -D-glucopyranosylformoside (**9**),²⁰ and the phenylethanoid salidroside (**10**).^{21,22} Herein, we report the structures

of **1** and **2**, the inhibitory effects of the iridoids **1**–**9** on adipocyte differentiation in 3T3-L1 cells, and the activation of PPAR α by **2**, **3**–**5**, and **8**, as potential mechanisms underlying the reported antidiabetic activity of FE.



1: R = CH₃, R₁ = β -D-glucopyranosyl



Results and Discussion

Excelside A (**1**) was obtained as an amorphous powder. Its molecular formula, C₂₄H₃₆O₁₆, was established on the basis of its HRFABMS (*m/z* 603.1890 [M + Na]⁺, calcd for C₂₄H₃₆O₁₆Na, 603.1901) and was supported by NMR data. The IR spectrum of **1** showed hydroxy absorption at 3401 and carbonyl absorptions at 1734 and 1717 cm⁻¹. The ¹H and ¹³C NMR data indicated that **1** was an oleoside-type secoiridoid glucoside based on proton signals at δ 7.51 (s, H-3), 5.94 (s, H-1), 6.09 (q, *J* = 7.2 Hz, H-8), 1.72 (d, *J* = 7.2 Hz, H₃-10), and 4.80 (d, *J* = 7.6 Hz, H-1'), as well as the corresponding ¹³C NMR signals at δ_c 155.2 (C-3), 94.8 (C-1), 124.7 (C-8), 13.6 (C-10), and 100.6 (C-1'). The ¹H NMR signals at δ 3.62 and 3.70 and corresponding ¹³C NMR (gHMQC) resonances at δ_c 51.9 and 52.3 were ascribed to two methoxy groups. The two methoxy groups showed correlations with C-7 (δ_c 173.7) and C-11 (δ_c 168.7) in the gHMBC spectrum, respectively, indicating that **1** possesses a 7,11-

* To whom correspondence should be addressed. Tel: (201) 440-5000. Fax: (201) 342-8000. E-mail: k.he@naturex.us.

[†] Naturex, Inc.

[‡] Naturex SA.

[§] Ohio University.

[⊥] University of Debrecen.

^{||} Hungarian Academy of Sciences.

[▽] Paprika-Bioanalytics BT.

Table 1. NMR Data (400 MHz, methanol-*d*₄) of Excelsides A (**1**) and B (**2**)

no.	1			2		
	δ (<i>J</i> in Hz)	δ_C , mult.	HMBC (H to C)	δ (<i>J</i> in Hz)	δ_C , mult.	HMBC (H to C)
1	5.94 s	94.8 d	8, 1'	5.95 s	94.7 d	8, 1'
3	7.51 s	155.2 d	1, 4, 5, 11	7.51 s	155.2 d	1, 4, 5, 11
4		109.3 s			109.3 s	
5	3.98 dd (9.6, 4.4)	31.9 d	1, 3, 4, 6, 7, 8, 9, 11	3.96 dd (9.6, 4.4)	32.0 d	7, 11
6	2.76 dd (14.0, 4.4)	41.1 t	4, 5, 7, 9	2.73 dd (14.0, 4.4)	41.3 t	7
	2.52 dd (14.0, 9.6)		4, 5, 7, 9	2.52 dd (14.0, 9.6)		7
7		173.7 s			173.4 s	
8	6.09 q (7.2)	124.7 d	1, 5, 10	6.06 q (7.2)	124.8 d	1, 5, 9, 10
9		130.4 s			130.1 s	
10	1.72 d (7.2)	13.6 q	8, 9	1.62 d (7.2)	13.6 q	8, 9
11		168.7 s			168.7 s	
OCH ₃	3.70 s	52.3 q	11	3.70 s	51.9 q	11
OCH ₃	3.62 s	51.9 q	7			
1'	4.80 d (7.6)	100.6 d	1	4.82 d (7.6)	100.4 d	1, 2'
2'	3.30 m	77.8 d		3.37 m	77.8 d	
3'	3.52 m	77.6 d		3.53 m	77.5 d	
4'	3.40 m	71.5 d		3.39 m	71.5 d	
5'	3.32 m	74.7 d		3.34 m	74.7 d	
6'	4.16 dd (12.0, 2.0)	70.1 t	1'''	4.16 d (12.0, 1.6)	70.1 t	5', 1'''
	3.76 dd (12.0, 6.8)			3.75 dd (12.0, 6.4)		
1''				4.27 m	67.0 t	7, 2'', 3''
				4.07 m		7, 2'', 3''
2''				2.82 t (6.8)	35.2 t	
3''					130.3 s	
4''				7.04 d (8.4)	131.1 d	2'', 3'', 6''
5''				6.72 d (8.4)	116.4 d	3'', 4'', 6''
6''					157.0 s	
7''				6.72 d (8.4)	116.4 d	
8''				7.04 d (8.4)	131.1 d	
1'''	4.36 d (7.6)	105.2 d	6'	4.31 d (7.6)	105.2 d	6'
2'''	3.18 m	75.2 d		3.17 m	75.1 d	
3'''	3.39 m	77.7 d		3.41 m	77.8 d	
4'''	3.27 m	71.6 d		3.26 m	71.4 d	
5'''	3.25 m	77.8 d		3.16 m	77.6 d	
6'''	3.85 br d (11.6)	62.7 t		3.82 dd (12.0, 2.4)	62.7 t	
	3.66 m			3.62 dd (12.0, 5.6)		

oleoside dimethyl ester unit.^{19,23} The additional ¹³C NMR signals were ascribable to a β -glucopyranosyl moiety (δ_C 105.2, 75.2, 77.7, 71.6, 77.8, and 62.7) attached to C-6' of the oleoside moiety, as evidenced by a 7.5 ppm downfield shift of C-6' and upfield shifts of C-3' and C-5' of 0.8 and 3.2 ppm, respectively, compared to compound **8**. The HMBC cross-peaks between H-1''' at δ 4.36 and C-6' at δ_C 70.1, as well as H-6' (δ 4.16 and 3.76) and C-1''' (δ_C 105.2), supported these conclusions. The 10-methyl group attached to the $\Delta^{8,9}$ -olefinic bond was determined to have an *E*-configuration by the ROESY spectrum, showing a strong correlation between H-10 (δ 1.72) and H-5 (δ 3.98). A ROESY correlation between the signals of H-1 (δ 5.94) and H-6 (δ 2.52) indicated that H-1 is α -oriented. The *D*-configuration of the glucopyranosyl was confirmed via acid hydrolysis (see Experimental Section). Hence, the structure of **1** was determined to be (2*S*,4*S*,3*E*)-methyl 3-ethylidene-4-(2-methoxy-2-oxoethyl)-2-[(6-*O*- β -D-glucopyranosyl- β -D-glucopyranosyl)oxy]-3,4-dihydro-2*H*-pyran-5-carboxylate, for which the name excelside A is suggested. The ¹H and ¹³C NMR data assignments are given in Table 1.

Excelside B (**2**) was isolated as a white, amorphous powder. Its molecular formula was established as C₃₁H₄₂O₁₇ by HRFABMS (*m/z* 687.2506 [M + H]⁺, calcd for C₃₁H₄₃O₁₇, 687.2500) and was supported by NMR data. The IR spectrum showed a hydroxy absorption at 3400 and carbonyl absorptions at 1701 and 1636 cm⁻¹. The ¹H NMR spectrum displayed typical signals of an oleoside moiety at δ 7.51 (s, H-3), 5.95 (s, H-1), 6.06 (q, H-8), 1.62 (d, H₃-10), and 4.82 (d, H-1'). The corresponding ¹³C NMR data are shown in Table 1. The observed phenylethanoid signals as well as an AA'BB' spin system in the aromatic ring at δ 6.72 (2H, d, *J* = 8.4 Hz) and δ 7.04 (2H, d, *J* = 8.4 Hz) suggested *para*-disubstitution. The long-range ¹H-¹³C correlations (gHMBC) between H-1'' at δ 4.27 and C-7 at δ_C 173.4 suggested that the phenylethoxy moiety was attached at C-7, which related the

structure of **2** to ligstroside (**6**).¹⁷ Six additional ¹³C NMR signals in **2** were assigned to a β -glucopyranosyl moiety (δ 105.2, 75.1, 77.8, 71.4, 77.6, and 62.7). Its attachment at C-6' was confirmed by the observed glycosidation chemical shift effects: a 7.5 ppm downfield shift of C-6' and upfield shifts of 0.8 and 3.1 ppm of C-3' and C-5', respectively, compared to compound **6**. A gHMBC correlation between the anomeric H-1''' (δ 4.31) and C-6' (δ_C 70.1) confirmed this assignment. The position of the methoxy group was assigned to C-11 due to the observed long-range correlations of the *O*-methyl hydrogens (δ 3.70) and C-11 (δ_C 168.7) in the gHMBC spectrum. Acid hydrolysis of **2** yielded *D*-glucose (see Experimental Section). Thus, compound **2** was designated as (2*S*,4*S*,3*E*)-methyl 3-ethylidene-4-[2-[2-(4-hydroxyphenyl)ethyl]oxy-2-oxoethyl]-2-[(6-*O*- β -D-glucopyranosyl- β -D-glucopyranosyl)oxy]-3,4-dihydro-2*H*-pyran-5-carboxylate, for which the name excelside B is suggested. The ¹H and ¹³C NMR data are given in Table 1.

In order to evaluate their antidiabetes and antiadipogenesis activities, compounds **1–9** and FE were evaluated for their glucose transport (GLUT4) stimulatory (GTS) and differentiation inhibitory effects in 3T3-L1 preadipocytes, respectively. GLUT4 is the major insulin-dependent transporter responsible for the uptake of glucose from the bloodstream into muscle and fat tissue, so as to decrease the glucose concentration in the blood. No GTS activity was observed for **1–9** and FE in the glucose uptake activity assay, which excludes the possibility of GLUT4 being a mediator for the effect of secoiridoids in FE on plasma glucose levels. However, **1–9** inhibited adipocyte differentiation in 3T3-L1 preadipocytes as summarized in Table 2. While compound **5** showed significant adipogenesis-inhibitory activity, compounds **2**, **8**, and **9** promoted adipogenesis at low concentrations and inhibited it at higher concentrations. These observations provide a preliminary basis for the observed decreasing fat gain of FE in mice.

Table 2. Inhibitory Activity of the *F. excelsior* Iridoids on Adipocyte Differentiation

compound	concentration (mg/mL)			
	0.05	0.2	0.5	1.0
2	-16.7 ± 1.1 ^a	-30.8 ± 2.9	-22.7 ± 2.5	-24.3 ± 3.9
3	-15.2 ± 1.5	-28.3 ± 1.8	-27.9 ± 2.1	-38.2 ± 4.0
4	2.3 ± 0.4	-8.3 ± 1.0	-10.4 ± 1.7	-2.1 ± 1.1
5	-4.7 ± 2.1	-31.7 ± 4.2	-57.7 ± 5.5	-100

	concentration (mg/mL)			
	0.01	0.05	0.1	0.5
1	102.5 ± 12.2	30.9 ± 5.9	-16.6 ± 2.1	ND ^b
6	-9.3 ± 2.2	-76.4 ± 18.7	-9.8 ± 3.9	ND
7	-7.9 ± 2.1	-34.8 ± 3.3	-85.2 ± 9.4	ND
8	78.2 ± 9.1	-7.3 ± 10.1	-81.0 ± 9.5	ND
9	77.6 ± 7.4	-17.1 ± 3.6	-49.7 ± 7.2	ND

^a The inhibitory activity was measured using the glucose uptake assay. A negative value indicates an inhibitory activity to adipocyte differentiation. A value of -50 indicates a measured glucose uptake value 50% lower than that of the MDI-treated (fully differentiated) samples, which was arbitrarily set at zero. A value of -100 indicates a measurement equivalent to the value measured by the non-MDI-treated (no differentiation) samples. A positive value indicates an adipocyte differentiation promoting activity. One hundred means a measurement 100% higher than the value measured in the samples treated by MDI (MDI is a combination of IBMX, dexamethasone, and insulin). ^b ND = not determined due to excessive cell death.

Table 3. PPAR α Activation Potential of the *F. excelsior* Iridoids in Reporter Cell Lines

compound/extract	concentration (M)	relative activation in %
DMSO		1
WY14,643	10 ⁻⁵	100
	10 ⁻⁶	62.8 ± 35.2
	10 ⁻⁷	36.7 ± 15.7
	10 ⁻⁸	17.3 ± 13.1
	10 ⁻⁹	13.0 ± 11.0
2	10 ⁻⁴	24.8 ± 9.6
3	10 ⁻⁴	12.2 ± 11.7
4	10 ⁻⁴	21.0 ± 15.7
5	10 ⁻⁴	14.2 ± 13.8
8	10 ⁻⁴	27.9 ± 9.9
FE	1:10,000	34.1 ± 18.7

The biological effects of **2–5** and **8** were also evaluated on the PPAR α reporter cell lines. PPAR α pathways are known to be involved in lipid homeostasis and inflammation.^{24–26} PPAR α is a main target of fibrate drugs for therapy of hyperlipidemia and hyperglycemia.¹¹ In the present study, the synthetic and selective PPAR α activator WY14,643 was used as positive control, exhibiting a concentration-dependent activation (Table 3). Compounds **2–5** and **8** were partly active at a concentration of 10⁻⁴ M, compared to 10⁻⁷–10⁻⁸ M for WY14,643. These preliminary biological profiles suggest that inhibition of adipocyte differentiation and PPAR α -mediated pathways might be relevant mechanisms that can explain the antidiabetic activity of *F. excelsior* extract.

Experimental Section

General Experimental Procedures. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. FT-IR was performed on a Perkin-Elmer spectrum BX system (Perkin-Elmer Instruments, Norwalk, CT). UV spectra were acquired on a Shimadzu UV-1700 UV–visible spectrophotometer. The ¹H and ¹³C NMR spectra were recorded on an Inova-400 (¹H at 400 MHz) instrument (Varian Inc., Palo Alto, CA) with methanol-*d*₄ (reference 3.30 ppm) and D₂O as the solvent (Aldrich Chemical Co., Allentown, PA). The 2D correlation spectra were obtained using standard gradient pulse sequences of Varian VNMR software and performed on 4-nuclei PFG autoswitchable or PFG indirect detection probes. HRFABMS was run on a JEOL HX-110 double focusing mass spectrometer. Both negative and positive ESIMS were obtained on an LCQ ion trap (Thermo-Finnigan, San Jose, CA). GC-MS analysis was carried out on an Agilent HP 6890 Series gas

chromatograph system and Agilent HP 5973 mass spectrometer (Santa Clara, CA) with an Rxi-1ms capillary GC column (60 m × 0.25 mm i.d. × 1.0 μ m). HPLC analysis was performed on an Agilent 1100 LC Series using a Prodigy ODS3 column (5 μ m, 4.6 mm i.d. × 25 cm) with a flow rate of 1.0 mL/min. Solvent system consisted of 0.1% TFA/H₂O (A) and MeCN (B) in the following manner: 0–5 min, 0–20% B; 5–15 min, 20–30% B; 15–25 min, 30–100% B. At the end of the run, 100% of MeCN was allowed to flush the column for 10 min, and an additional 10 min of post-run time was set to allow for equilibration of the column with the starting eluant. The UV detector was operating at 238 nm, and the column temperature was ambient.

3T3-L1 fibroblasts were purchased from American Type Culture Collection (ATCC, Rockville, MD). Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's PBS (DPBS) were from Gibco Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was from Atlanta Biologicals (Norcross, GA). Insulin (IS), 3-isobutyl-1-methylxanthine (IBMX), and dexamethasone (DEX) were from Sigma Chemical (St. Louis, MO). 2-Deoxy-D-[³H]glucose and XK 50 columns were from Amersham Pharmacia Biotech (Piscataway, NJ). Chemicals for plasmid preparation and PPAR activity testing were purchased from Sigma-Aldrich KFT (Budapest, Hungary), Certex KFT (Budapest, Hungary), Promega (Bioscience KFT, Budapest, Hungary), or Spectrum 3D (Debrecen, Hungary).

Plant Material. The seeds of *F. excelsior* were collected in Morocco. A voucher specimen (J02/02/A7) was deposited in the Herbarium of Naturex, Inc.

Extraction and Isolation. Air-dried and powdered seeds (2.5 kg) of *F. excelsior* were extracted with H₂O (2 × 15 L) at 95 °C for 2 h. The combined extract was concentrated and dried into powder (500 g). The powder was re-extracted with MeOH (2 × 3.5 L), and the MeOH was evaporated *in vacuo*. The obtained extract (54 g solid) was reconstituted in 0.5 L of H₂O and was loaded on a C-18 (1 L) (Sigma Chemical Co., St. Louis, MO) column (8.0 cm i.d. × 70 cm) eluted with H₂O (5 L) and 10% MeOH/H₂O (3 L). Fractions with similar HPLC chromatograms were combined and concentrated *in vacuo*. The combined water fractions (21 g solid) were separated over silica gel (Sorbent Technologies, Inc.) by column chromatography (500 g, 3.5 cm × 60 cm), eluting with a step gradient consisting of CH₂Cl/MeOH (10:1, 8:1, 5:1, 3:1, 2:1). In each gradient step, 1.5 L of eluent was used and 0.5 L was collected as one fraction. A total of 15 fractions were collected and labeled as W-fractions. These fractions were subjected to column chromatography over MCI gel CHP-20P (Mitsubishi Kasei Co.) (100 mL, 2.5 cm × 40 cm) and/or Sephadex LH-20 (100 mL, 2.5 cm × 40 cm), eluting with a H₂O/MeOH (4:6) system to yield **3** (210 mg from fraction W-9, *t*_R = 12.6 min in HPLC), **6** (46 mg from W-2, *t*_R = 19.3 min), **7** (28 mg from W-11, *t*_R = 9.4 min), **8** (41 mg from W-10, *t*_R = 12.7 min), **9** (21 mg from W-8, *t*_R = 13.4 min), and **10** (22 mg from W-4, *t*_R = 8.1 min). In a similar manner to that above, the 10% MeOH/H₂O eluates (12 g solid) from the C-18 column were chromatographed over a silica gel column using CH₂Cl/MeOH (10:1, 8:1, 5:1, 3:1, 2:1) as solvent system and collecting a total of 15 M-fractions. These fractions were chromatographed over MCI gel CHP-20P and/or Sephadex LH-20 to yield **1** (16 mg from fraction M-8, *t*_R = 10.4 min), **2** (33 mg from M-6, *t*_R = 15.6 min), **4** (238 mg from M-3, *t*_R = 17.9 min), and **5** (36 mg from M-5, *t*_R = 20.4 min).

Excelside A (1): amorphous, white powder; [α]_D²⁵ -106.1 (*c* 0.18, MeOH); UV (MeOH) λ_{max} (log ϵ) 232 (4.61) nm; IR (KBr) ν_{max} 3401, 1734, 1717, 1626 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; HRFABMS *m/z* 603.1890 [*M* + Na]⁺ (calcd for C₂₄H₃₆O₁₆Na, 603.1901).

Excelside B (2): amorphous, white powder; [α]_D²⁵ -115.6 (*c* 0.16, MeOH); UV (MeOH) λ_{max} (log ϵ) 230 (4.33), 275 (4.02), 283 (0.56) nm; IR (KBr) ν_{max} 3400, 1701, 1636, 1518 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; HRFABMS *m/z* 687.2506 [*M* + H]⁺ (calcd for C₃₁H₄₃O₁₇, 687.2500).

Acid Hydrolysis of Compounds 1 and 2 and Sugar Analysis. Solutions of compounds **1** and **2** (2.0 mg each) in 1 N HCl (1 mL) were separately stirred at 85 °C for 3 h. The solution was evaporated under a stream of N₂. The residue was dissolved in 0.1 mL of Tri-Sil Z (*N*-trimethylsilylimidazole/pyridine, 1:4, Pierce Biotechnology, Rockford, IL), and the mixture was allowed to react at 60 °C for 15 min. After drying under a stream of N₂, the residue was dissolved in 1 mL of H₂O and partitioned with 1 mL of CH₂Cl₂. The CH₂Cl₂ layer was analyzed by GC-MS (Rxi-1ms GC column, temperatures for inlet injection, 200 °C; temperature gradient system for the oven, 120 °C for 1 min and then raised to 280 °C at rate of 40 °C/min). D-Glucose

was identified for **1** and **2** by comparison with retention time of authentic D-glucose ($t_R = 9.72$ min) after treatment in the same manner with Tri-Sil Z.

Cell Culture and Adipocyte Differentiation. For differentiation assays, 3T3-L1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) and supplemented with 10% FBS at 37 °C in a 10% CO₂ cell incubator. Preadipocyte 3T3-L1 cells were grown in 12-well plates until 2 days post-confluence. The differentiation was induced as previously described^{27,28} by addition of 1 mg/L insulin (IS), 0.5 mmol/L 3-isobutyl-1-methylxanthine (IBMX), and 0.25 mmol/L dexamethasone (DEX). Two days after induction, the IS/IBMX/DEX-containing medium was replaced with medium containing 1 mg/L IS. The medium was subsequently replaced again with fresh culture medium (DMEM supplemented with 10% FBS) after 2 days and then every other day thereafter. To determine the roles of compounds in adipocyte differentiation, different concentrations of individual compound were added to the medium along with IS/IBMX/DEX (BE/IS/IBMX/DEX). The compound-treated cells were assayed for their glucose uptake activity 9–12 days after the initiation of induction.

Glucose Uptake Activity Assay. Glucose uptake activity was analyzed by measuring the uptake of 2-deoxy-D-[³H]glucose as described previously.^{27,28} Briefly, confluent 3T3-L1 adipocytes grown in 12-well plates were washed twice with serum-free DMEM and incubated with 1 mL of the same medium at 37 °C for 2 h. The cells were washed three times with Krebs-Ringer-Hepes (KRP) buffer and incubated with 0.9 mL of KRP buffer at 37 °C for 30 min. Insulin, FE, or compounds were then added and adipocytes incubated at 37 °C for 15 min. Glucose uptake was initiated by the addition of 0.1 mL of KRP buffer and 37 MBq/L 2-deoxy-D-[³H]glucose and 1 mmol/L glucose as final concentrations. After 10 min, glucose uptake was terminated by washing the cells three times with cold PBS. The cells were lysed with 0.7 mL of 1% Triton X-100 at 37 °C for 20 min. The radioactivity retained by the cell lysates was determined by a scintillation counter. Assays were repeated at least once ($n = 2$), and data were analyzed by comparison of experimental samples of the same treatment conditions as a group with negative control (untreated) samples, positive (insulin-treated) samples, or experimental samples with different treatment conditions. If values were below that of the negative control, they were interpreted as due to inhibitory activity.

Adipocyte Differentiation Assay. Undifferentiated 3T3-L1 preadipocytes were induced to differentiate into adipocytes as described above. The degree of the differentiation of the cells induced by different agents was evaluated by microscopic observation of lipid accumulation, as well as by their glucose uptake activities at the end of the induction. The glucose uptake assay was chosen and performed here for determination of the degree of adipocyte differentiation on the basis of the observation that differentiated adipocytes can be induced by insulin to take up glucose, whereas undifferentiated preadipocytes cannot.^{27,28} In addition, a near-linear relationship was found between the glucose uptake activity of differentiated adipocytes and the triglyceride contents of cells (unpublished data). Glucose uptake and adipocyte differentiation inhibition assays for each compound or fraction were performed at least twice ($n = 2$). The result was normalized and expressed as percentage by considering the activities of the positive (1 nmol/mL of insulin) and negative control (MeOH) as 100% and 0%, respectively. Results were reported as mean \pm standard error of means (SEM). Data were analyzed by comparing compound-treated samples with untreated negative control samples or with insulin-treated positive samples using one-way ANOVA with Tukey's post hoc test. Significance level was set at $p \leq 0.05$.

PPAR Reporter Cell Lines. Preparation of Plasmids. MH100-TK-LUC was utilized as luciferase reporter gene, and β -galactosidase gene was used as internal control. PPAR α and RXR α constructs and β -galactosidase vector were transfected with MH100-TK-LUC. In order to equalize the DNA amount, the VDR⁻¹ vector plasmid was used. All the plasmids contain the ampicillin resistance gene, which are controlled by SV40 promoter, and all plasmids originate from the Nuclear Hormone Receptor Research group of the Department of Biochemistry and Molecular Biology, Debrecen, Hungary. DNA was transformed into *Escherichia coli* DH5- α cells using heat shock transformation. The plasmids were replicated in DH5- α *E. coli* grown in Luria–Bertani (LB) medium supplemented with ampicillin (25 ng/mL). Plasmid extraction was conducted via Wizard Prep Mini Column purification kit.

Cell Culture and Transient Transfection. Human embryonic kidney (HEK) cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin streptomycin, and 2 mM L-glutamine. For experiments, 2×10^6 cells were grown in T-75 flasks at 37 °C with 5% CO₂. In 24-well plates, 80 000 cells were seeded per well to obtain 70–80% confluency, 24 h before transfection. Polyethylenimine (PEI)-based transfection was performed. The protocol was applied for a 24-well plate transfection. Plasmid DNA (1 μ g) was diluted into 50 μ L of 150 mM NaCl per well. PEI solution (2 μ L) was diluted into 50 μ L of 150 mM NaCl for each well. The PEI solution was gently added to the DNA solution, and after mixing, it was incubated at room temperature for 15 to 30 min to permit the formation of PEI/DNA complex. DMEM supplemented with 10% fetal bovine serum (FBS), 1% penicillin streptomycin, and 2 mM L-glutamine was taken out from the transfection plate, and PEI/DNA complex was gently added to each well. The wells were filled with unsupplemented DMEM. The cells were transfected for 4 h, and after changing the medium with supplemented (medium including derivatives) DMEM, the cells were incubated for 2 days to allow luciferase protein expression. After 48 h, cells were rinsed by 1% PBS and lysed with reporter lysis buffer. Plates were shaken for 2 h and kept at –80 °C for 1 h. The luciferase activity of cell lysates was measured with 50 μ L of luciferase assay kit by luminometer (Wallac 1420 Victor, Perform Hungaria KFT, Budapest, Hungary). The results were normalized against β -gal as control.

While FE was diluted 1:10 000 in H₂O, compounds **2–5** and **8** were dissolved in DMSO at 10^{-4} M and applied to the cell culture medium (supplemented DMEM). The cell culture experiments were conducted three times independently and normalized to 100, corresponding to WY14,643 $\times 10^{-5}$ M, a PPAR α agonist used as positive control. The activation of PPAR α by FE, the isolated compounds, and the positive control resulted in the expression of luciferase and consequent increment of the luminescent signals, which were measured by spectrophotometry. Results were expressed as the relative activation of PPAR α proportional to the luminescent signal emitted by the control conditions (DMSO). Results are expressed as mean \pm SD.

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Supporting Information Available: ¹H and ¹³C NMR, COSY, HMQC, HMBC, and ROESY spectra of **1** and **2**. ¹³C NMR data for compounds **6** and **8**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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