Activation of PPARγ by Metabolites from the Flowers of Purple Coneflower (*Echinacea purpurea*)

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Thiazolidinediones are insulin sensitizing drugs that target the peroxisome proliferator-activated receptor (PPAR) γ. An *n*-hexane extract of the flowers of *Echinacea purpurea* was found to activate PPARγ without stimulating adipocyte differentiation. Bioassay-guided fractionations yielded five alkamides, of which one was new, and three fatty acids that all activated PPARγ. The new alkamide hexadeca-2*E*,9*Z*,12*Z*,14*E*-tetraenoic acid isobutylamide (**5**) was identified by analysis of spectroscopic data and found to activate PPARγ with no concurrent stimulation of adipocyte differentiation. Compound **5** was further shown to increase insulin-stimulated glucose uptake. The data suggest that flowers of *E. purpurea* contain compounds with potential to manage insulin resistance and type 2 diabetes.

The nuclear receptor PPARγ belongs to the superfamily of ligand-dependent transcription factors.[1] PPARγ is predominantly found in adipose tissue and has been shown to regulate adipocyte differentiation as well as glucose homeostasis. This regulatory effect is induced by ligands that bind to and activate the receptor. Natural ligands of PPARγ are fatty acids as well as eicosanoids. Among a large variety of synthetic ligands are thiazolidinediones (TZDs) and some nonsteroidal anti-inflammatory drugs.[2] Especially, the TZD-type ligands have received much attention, as some ligands of this type are approved as insulin sensitizing drugs for the treatment of type 2 diabetes. The mechanism of action for the TZDs is, at least partly, related to the fact that they promote the differentiation of new small adipocytes in adipose tissues. These small adipocytes are more insulin sensitive and thereby cause the release of free fatty acids to be reduced, the release of adiponectin to be increased, and the expression of inflammatory mediators that promote the insulin resistant state to be inhibited. However, TZDs may cause unwanted side-effects such as fluid retention, weight gain, and hepatotoxicity.[3] Hence, ligands for PPARγ that do not procure these unwanted side-effects are sought after. It has been suggested that PPARγ partial agonists fulfill these requirements, as they maintain their insulin sensitizing activity without having a strong adipogenic potential.[4]

PPARγ agonists isolated from natural sources are few and include structurally diverse compounds from various sources such as fruits, vegetables, and medicinal plants.[5−8] In this study, we have investigated the flowers of *Echinacea purpurea* (L.) Moench. (Asteraceae) for potential partial PPARγ agonists. *E. purpurea* is one of the most used plants in herbal remedies and is mainly used to prevent and treat upper respiratory tract infections. Alkamides, caffeic acid derivatives, and polysaccharides are regarded as the bioactive constituents that contribute to the immunomodulatory, antiviral, and anti-inflammatory activities of this medicinal plant.[9,10] The alkamides have been demonstrated to be detectable in human blood at relevant concentrations after oral administration of *Echinacea* preparations, and the immunomodulatory effects of the alkamides are most likely due to their ability to bind to the cannabinoid receptors.[11]

From a previous screening of several medicinal plants for potential partial PPARγ agonists, flowers of *E. purpurea* were found to exhibit promising bioactivities.[12] Six different extracts (*n*-hexane, dichloromethane (DCM), MeOH, H_2O, EtOH, and EtOAc) were tested in a PPARγ transactivation assay, and especially the *n*-hexane extract was found to significantly activate PPARγ compared to the vehicle (Figure 1A). Furthermore, the *n*-hexane extract was found to have no adipogenic potential in an adipocyte differentiation assay (Figure 1B).

The *n*-hexane extract of *E. purpurea* was investigated by bioassay-guided chromatographic fractionation in order to isolate metabolites responsible for the observed bioactivities. Separation of the *n*-hexane extract by flash column chromatography (CC) resulted in two active fractions, which were further separated by semipreparative HPLC, resulting in the identification of palmitoleic acid, α-linolenic acid, linoleic acid, dodeca-2*E*,4*Z*,10*Z*-trien-8-ynoic acid isobutylamide (**1**),[13,14] dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide (**2**),[14,15] dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide (**3**),[14,15] dodeca-2*E*,4*E*-dienoic acid isobutylamide (**4**),[13] and the new hexadeca-2*E*,9*Z*,12*Z*,14*E*-tetraenoic acid isobutylamide (**5**). The identification of compounds was conducted by analysis of UV−vis, ^{1}H NMR, ^{13}C NMR, and mass spectroscopic data. The known compounds were identified by comparison of spectroscopic data with reported values in the literature. The ability to activate PPARγ was investigated for all eight compounds. For compounds **4** and **5**, effects on adipocyte differentiation were assessed, and for the latter the effect on insulin-stimulated glucose uptake was furthermore assessed.

Compound **5**, obtained as a light yellow oil, showed a molecular ion peak at *m*/*z* 304.2638 $[M + H]^+$ (calcd for 304.2640) in the HRESIMS spectrum, corresponding to a molecular formula of $C_{20}H_{33}NO$, indicating that this compound was an alkamide. Signals at δ_H 3.15 (2H, dd, $J = 7$ Hz, H-1′), 1.80 (1H, m, H-2′), 0.92 (6H, d, $J = 7$ Hz, H-3′ and H-4′), and 5.55 (1H, br s, N−H) in the ^{1}H NMR spectrum of compound **5** indicated the presence of an isobutylamino moiety,[12,13,15] which was confirmed by the ^{1}H−^{1}H COSY spectrum. COSY cross-peaks between H_3-3′, H_3-4′, and H-2′ and between H_2-1′ and H-2′ as well as between H_2-1′ and the signal at δ_H 5.55 clearly established the presence of an isobutylamino

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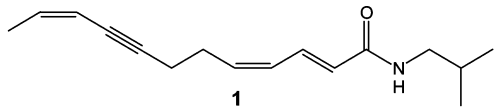

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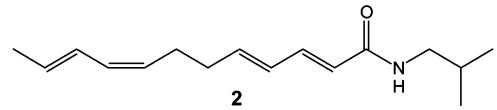

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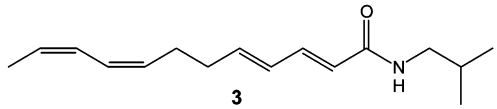

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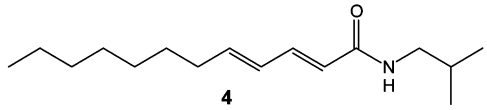

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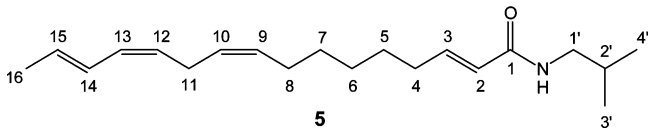

5

moiety in **5**. The ^{13}C NMR spectrum of **5** revealed 20 signals, of which the signals at δ_C 20.1 (C-3′,C-4′), 28.6 (C-2′), 46.9 (C-1′), and 166.1 (C-1) were assigned to the isobutylamide moiety,[16] whereas the rest of the signals consisting of one CH_3, six CH_2, and eight olefinic CH groups suggested that **5** was a hexadeca-tetraenoic acid isobutylamide. The presence of four double bonds was confirmed by the olefinic proton signals at δ_H 5.23 (dd, $J = 7$, 10 Hz, H-12), 5.36 (dt, $J = 6$, 10 Hz, H-9), 5.38 (dt, $J = 6$, 10 Hz, H-10), 5.70 (dq, $J = 7$, 14 Hz, H-15), 5.78 (1H, dt, $J = 1$, 15 Hz, H-2), 5.95 (dd, $J = 10$, 10 Hz, H-13), 6.33 (dd, $J = 10$, 14 Hz, H-14), and 6.82 (dt, $J = 7$, 15 Hz, H-3). The low-field shift of the methine proton at C-3 (δ_H 6.82) suggested that one of the double bonds was attached next to the amide moiety.[13,16] The positions of the other double bonds were determined from the $^1H-^1H$-COSY spectrum. COSY cross-peaks between H-15 and H_3-16, H-14; H-14 and H-15, H-13; H-13 and H-14, H-12; H-12 and H-14, H_2-11; and H-10 and H_2-11, H-9, and COSY cross-peaks between H-9 and H-10, H_2-8 established the positions of the four double bonds in positions 2, 9, 12, and 14, respectively. The double bonds in positions 2 and 14 were assigned an *E* configuration on the basis of olefinic proton couplings of 15 Hz ($J_{2,3}$) or 14 Hz ($J_{14,15}$), and the double bonds in positions 9 and 12 were assigned a *Z* configuration on the basis of olefinic proton couplings of 10 Hz. Therefore compound **5** was established as hexadeca-2*E*,9*Z*,12*Z*,14*E*-tetraenoic acid isobutylamide.

Compound **5** is an atypical alkamide for *E. purpurea*, as most alkamides from this species are olefinic or acetylenic C_{11}- or C_{12}-alkamides. However, a C_{16}-alkamide hexadeca-2*E*,9*Z*-dien-12,14-diynoic acid isobutylamide closely related to **5** has previously been isolated from roots of *E. angustifolia*.[13] Dodeca-2*E*,4*Z*,10*Z*-trien-8-ynoic acid isobutylamide (**1**) was not reported from the flowers of *E. purpurea* before, but is known to be present in the roots.[14]

Biological activity was assessed using a PPARγ transactivation assay. The active fractions I and K (see Experimental Section) exhibited significant activation of PPARγ at 100 and 50 μg/mL, respectively, compared to the vehicle (data not shown). Fractionation of fraction K resulted in the isolation of palmitoleic acid, α-linolenic acid, and linoleic acid, which were tested at 0.4, 4, and 40 μM in the PPARγ assay. These fatty acids gave significant 10−12-fold activations of PPARγ at 40 μM compared to the control. Fractionation of fraction I resulted in the isolation of compounds **1**−**5**, which were tested in at least three different concentrations in the μM range in the PPARγ transactivation assay (Figure 2). Compounds **1**−**3** gave at 100 μM only up to 2-fold activation of PPARγ compared to the vehicle, which is considered an insignificant activation of PPARγ. A weak activation of PPARγ was observed for compound **4** with a significant 4-fold activation at 80 μM compared to vehicle, whereas at a concentration of 100 μM apparent toxicity affected the results (data not shown). Compound **5** was tested in five different concentrations and gave a significant activation of PPARγ of more than 10-fold at 30 μM compared to the control. However, at a concentration of 100 μM toxicity was observed. The degree of activation for compound **5** was compared to a known partial agonist of PPARγ, nTZDpa,[4] and was found not to be as efficient as this partial agonist (Figure 2). Compounds **4** and **5** were further tested in an adipocyte differentiation assay using two different protocols to determine their adipogenic potential. Compound **4** stimulated adipocyte differentiation using the DEX protocol, which is sensitive to PPARγ agonists. Using the MDI protocol, in which differentiation is independent of the presence of an exogenous agonist, inhibition of adipocyte differentiation was seen. These observations might be attributable to effects separate from the PPARγ activating properties, and compound **4** has previously been shown to signal via cannabinoid receptors known to influence adipocyte differentiation.[17] Alternatively, the inhibition of adipocyte differentiation might reflect a displacement of a more potent adipogenic endogenous ligand. Compound **5** did not stimulate adipocyte differentiation in the above-mentioned protocols even in concentrations above 200 μM. For both the PPARγ transactivation assay and the adipocyte differentiation, the TZD rosiglitazone (Rosi), which is a full PPARγ agonist, was used as positive control. Further, the ability of compound **5** to sensitize adipocytes for insulin was tested in a glucose uptake experiment (Figure 3). Compound **5** did dose dependently increase uptake of glucose in 3T3-L1 adipocytes in response to low insulin concentrations in a manner comparable to the positive control Rosi.

The fatty acids are well-known PPARγ agonists and, thus, have been thoroughly described as having a significant influence on both glucose and lipid metabolism.[18,19] Activation of PPARγ by alkamides has to our knowledge not been reported previously. Furthermore, the new alkamide **5** did not stimulate adipocyte differentiation, but still retained the insulin-sensitizing effects, which makes it a potential beneficial PPARγ partial agonist. Studies including cofactor recruitment assays as well as ligand-binding studies will also be needed to examine the antidiabetic potential of this compound further. One major structural difference between compound **5** and the alkamides **1** to **4** is the length of the carbon chain. For fatty acids the highest affinity for PPARs are seen for chain lengths of 16−20 carbon atoms, and the present results seem to indicate that this is also the case for the alkamides.[18] One study previously investigated *E. purpurea* for its potential as an antidiabetic agent, and in this study aqueous extracts of the roots had no significant effects on in vitro insulin-dependent glucose metabolism in adipocytes.[20] However, the present study indicates that the flowers of *E. purpurea* may contain compounds with potential for the management of insulin resistance and type 2 diabetes.

Experimental Section

General Experimental Procedures. Silica gel aluminum cards (0.2 mm, 20 × 20 cm, F_{254nm}; Sigma-Aldrich Chemie) were used for TLC, and all plates were inspected by UV light followed by visualization with vanillin (15 g vanillin, 250 mL of EtOH, 2.5 mL of conc H_2SO_4). Silica gel (63−200 μm, Merck) was used for flash CC. Semipreparative HPLC was carried out using a Dionex P680 HPLC pump equipped with a Dionex UVD34OU detector and a Develosil ODS-HG-5 RP-18 column (5 μm; 250 × 20 mm, Nomura Chemical Co.). ^1H and ^{13}C NMR data were acquired on a Varian 300 MHz spectrometer using

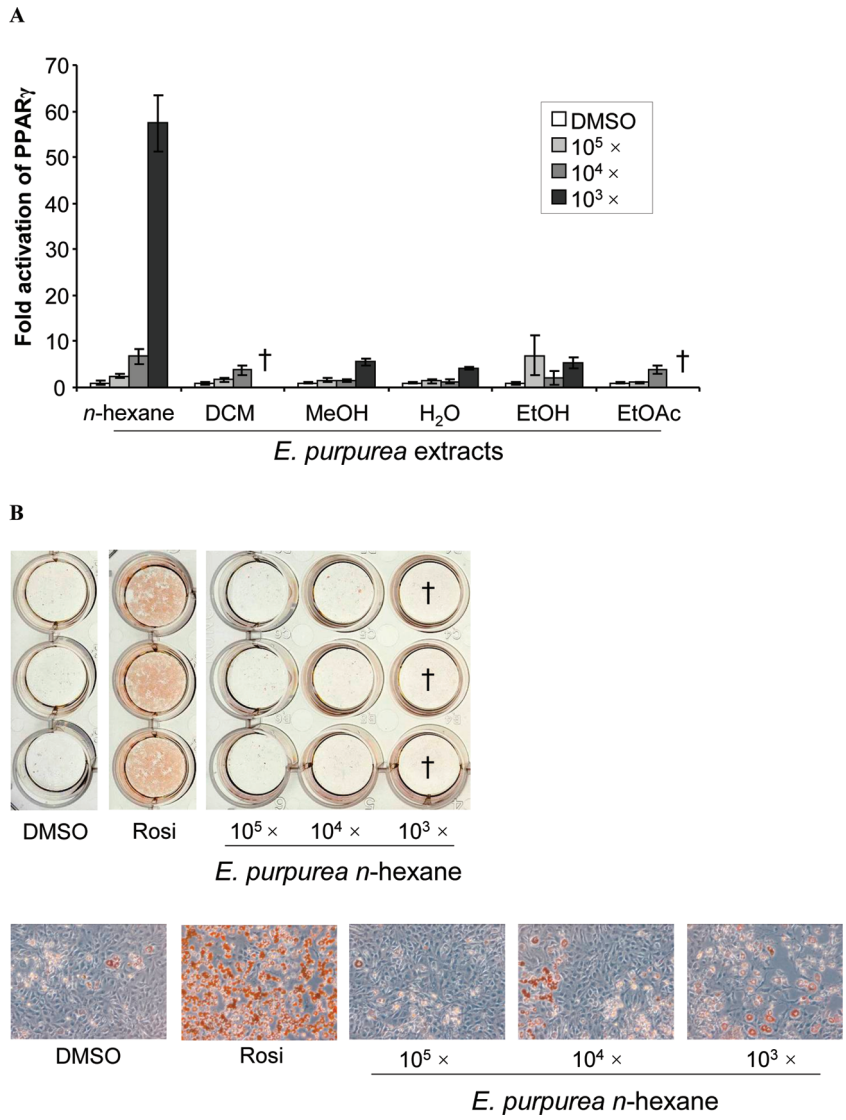

Figure 1. (A) Fold activation of PPARγ by six different extracts of *Echinacea purpurea* (*n*-hexane, DCM, MeOH, H_2O, EtOH, and EtOAc). Dilution factors for the extracts are given in the inset box, activation by the vehicle (DMSO) was set to 1, and the positive control used was Rosi (data not shown). The *n*-hexane extract was the most efficient activator of PPARγ, the DCM and the EtOAc extracts showed moderate activation but were toxic in the highest concentration, and the remaining extracts showed no significant activation. (B) Results obtained for the *n*-hexane extract of *E. purpurea* from an adipocyte differentiation assay. No red staining of the incubated cells indicated no stimulation of adipocyte differentiation.

solvent signals ($CDCl_3$; δ_H 7.26/δ_C 77.7) as references. GC-MS data were obtained using a Varian SATURN GC/MS 2000 equipped with a Varian STAR 3400 CX GC controller. Injection temperature 250 °C; column temperature 40 °C. Method: 40−300 °C with a rate of 5 °C/min and a hold time of 15 min. LC-HRESIMS was performed with LC and MS settings as previously described[21,22] and a gradient from 15−100% MeCN in water for 20 min on a Luna C_{18} column. Luciferase measurements were performed on a LUMIstar BMG luminometer.

Plant Material. *Echinacea purpurea* (L.) Moench. was cultivated at Research Centre Aarslev, Faculty of Agricultural Sciences, University of Aarhus, Denmark. Florets (upper 20 cm of flowering shoot) were harvested in August 2006 and frozen at −22 °C immediately after harvest.

Extraction and Isolation. Florets of purple coneflower (3 kg) were extracted with *n*-hexane (10 L) for 48 h in the dark at 5 °C with periodic shaking, and the extract was dried under vacuum. The extract (5.8 g) was then separated by flash CC (70 mm i.d., 380 g of silica gel) using the following solvent gradient: 100% *n*-hexane (400 mL), from 5 to 50% EtOAc in *n*-hexane in 5% steps (250 mL each), from 50 to 100% EtOAc in *n*-hexane in 10% steps (250 mL each), yielding 45 fractions of 100 mL. The collected fractions were analyzed by TLC with *n*-hexane−EtOAc (60:40) as mobile phase and combined according to TLC into 14 fractions (A−N), which were tested for PPARγ activity, resulting in two active fractions, I and K. Fraction K (226.3 mg) was separated by flash CC (40 mm i.d., 100 g silica gel) using the gradient system 30−80% EtOAc in *n*-hexane in 5% steps (100 mL each), 90% EtOAc in *n*-hexane (100 mL), and 100% EtOAc (100 mL), yielding 57 fractions of 20 mL that were combined according to TLC to give four fractions (K1−K4), which were dried under vacuum and tested for PPARγ activity. The active fraction K2 was further separated by semipreparative HPLC by the solvent gradient A = 0.05% TFA in H_2O, B = MeCN; 0 min (40% B), 100 min (100% B), 120 min (100%, B), 140 min (40% B) to give palmitoleic acid (2 mg), α-linolenic acid (16 mg), and linoleic acid (20 mg). Fraction I (312 mg) was separated by semipreparative HPLC using the solvent gradient A = 0.05% TFA in H_2O, B = MeCN; 0 min (50% B), 80 min (100% B), 100 min (100% B), 115 min (50% B) to give dodeca-2*E*,4*Z*,10*Z*-trien-8-ynoic acid isobutylamide (**1**; 7 mg), dodeca-2*E*,4*E*,8*Z*,10*E*-tetraenoic acid isobutylamide (**2**) and dodeca-2*E*,4*E*,8*Z*,10*Z*-tetraenoic acid isobutylamide (**3**) (**2** and **3** isolated as a mixture; 55 mg), dodeca-2*E*,4*E*-dienoic acid isobutylamide (**4**; 4 mg), and hexadeca-2*E*,9*Z*,12*Z*,14*E*-tetraenoic acid isobutylamide (**5**; 24 mg).

Hexadeca-2*E*,9*Z*,12*Z*,14*E*-tetraenoic acid isobutylamide (5): light yellow oil; UV λ_{max} (MeOH) 229 nm; ^1H NMR ($CDCl_3$, 300 MHz) δ 6.82 (1H, dt, J = 7, 15 Hz, H-3), 6.33 (1H, dd, J = 10, 14 Hz, H-14), 5.95 (1H, dd, J = 10, 10 Hz, H-13), 5.78 (1H, dt, J = 1, 15 Hz, H-2),

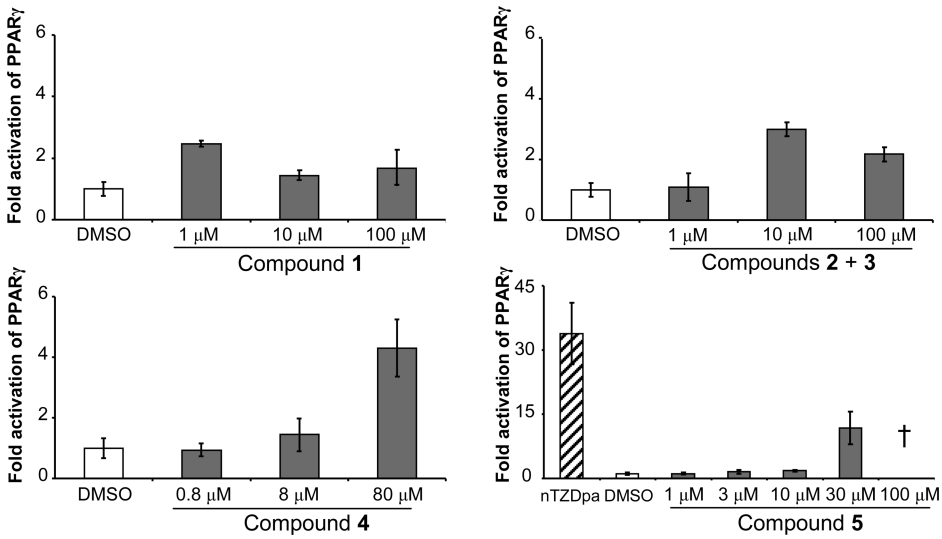

Figure 2. Fold activation of PPARγ by the alkamides (**1**−**5**) isolated from *Echinacea purpurea*. Activation by the vehicle (DMSO) was set to 1, and the positive control used was Rosi (data not shown). The alkamides **1**−**3** were not able to significantly activate PPARγ, whereas alkamides **4** and **5** were activators of PPARγ.

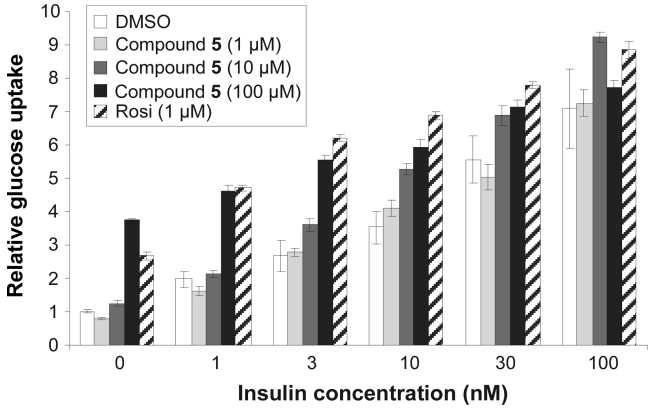

Figure 3. Fold activation of insulin-stimulated glucose uptake by compound **5**. Uptake by the vehicle (DMSO) with no addition of insulin was set to 1, and Rosi was used as positive control. Shown is the average ± SEM (n = 8).

5.70 (1H, dq, J = 7, 14 Hz, H-15), 5.55 (1H, br s, N-H), 5.38 (1H, dt, J = 6, 10 Hz, H-10), 5.36 (1H, dt, J = 6, 10 Hz, H-9), 5.23 (1H, dd, J = 7, 10 Hz, H-12), 3.15 (2H, dd, J = 7 Hz, H_2-1′), 2.90 (2H, m, H_2-11), 2.17 (2H, dt, J = 7, 7 Hz, H_2-4), 2.08 (2H, m, H_2-8), 1.80 (1H, m, H-2′), 1.78 (3H, d, J = 7 Hz, H_3-16), 1.45 (2H, m, H_2-5), 1.40 (2H, m, H_2-7), 1.36 (2H, m, H_2-6), 0.92 (6H, d, J = 7 Hz, H_3-3′ and H_3-4′); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 166.1 (C, C-1), 144.6 (CH, C-3), 130.2 (CH, C-15), 129.6 (CH, C-13), 128.7 (CH, C-12), 127.8 (CH, C-9), 127.5 (CH, C-10), 126.8 (CH, C-14), 123.7 (CH, C-2), 46.9 (CH_2, C-1′), 32.0 (CH_2, C-4), 29.4 (CH_2, C-7), 28.8 (CH_2, C-6), 28.6 (CH, C-2′), 28.2 (CH_2, C-5), 27.1 (CH_2, C-8), 26.0 (CH_2, C-11), 20.1 (CH_3, C-3′,C-4′), 18.3 (CH_3, C-16); assignments are interchangeable for C-9, C-10, and C-12; GC-MS (70 eV, rel int) m/z 304 (74) $[M + H]^+$, 303 (20) $[M]^+$, 274 (26), 260 (24) $[M - C_3H_7]^+$, 247 (10) $[M + H - C_4H_9]^+$, 204 $[M + H - C_5H_{10}NO]^+$ (43), 203 (15) $[M - C_5H_{10}NO]^+$, 154 (61) $[C_9H_{16}NO]^+$, 135 (35), 133 (48), 121 (53), 91 (70), 79 (100), 57 (50) $[C_4H_9]^+$, 41 (83); HRESIMS m/z 304.2638 $[M + H]^+$ (calc for 304.2640).

PPARγ Transactivation Bioassay. For analysis of PPARγ-mediated transactivation, a mouse embryonic fibroblast cell line[23] was transiently transfected at 50−70% confluence using Metafectene (Biontex) as recommended by the manufacturer. For each well a total of 47.5 ng of DNA (2.5 ng of Renilla normalization vector pRL-CMV + 30 ng of the Gal4-responsive Luciferase reporter vector + 15 ng of PPARγ-LBD expression vector pM-hPPARγ-LBD) were used. The media in the plates were changed 6 h after transfection to 200 μL of Dulbecco's modified Eagle's medium (DMEM) with antibiotics (62.5 μg/mL penicillin and 100 μg/mL streptomycin) containing either vehicle (0.1% DMSO), positive control (1 μM rosiglitazone (Rosi), Novo Nordisk A/S), or plant extract or compound dissolved in DMSO (10^3, 10^4, and 10^5 times dilutions of the extract stocks were used). After 18 h of transfection the cells were washed with phosphate-buffered saline (PBS) (200 μL per well) and lysed with lysis buffer (20 μL per well). Assay for Photinus and Renilla activities were measured directly in the plate using a Dual-Glo Luciferase assay system obtained from Promega. All transient transfection experiments were done in triplicate, and double determination for each triplicate was carried out. Photinus activities were normalized to the corresponding Renilla activities to compensate for differences in transfection efficiency.

Adipocyte Differentiation Bioassay. The 3T3-L1 cells were grown in DMEM supplemented with 10% calf serum and antibiotics (62.5 μg/mL penicillin and 100 μg/mL streptomycin). The cells were seeded in 24-well plates and grown to confluence. Two days postconfluence (designated day 0) the cells were induced to differentiate with DMEM supplemented with 10% fetal bovine serum (FBS), antibiotics, vehicle, or the compound or plant extract tested, and either 1 μM dexamethasone (DEX protocol) or 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine, and 1 μg/mL insulin (MDI protocol).[24] After 48 h, the cells were refed with DMEM supplemented with 10% FBS and the positive control, vehicle, or the test compound (DEX protocol) or including 1 μg/mL insulin (MDI protocol). From day 4, media contained DMEM with 10% FBS and antibiotics and were changed every second day until day 8. All extracts and compounds were tested in three concentrations as indicated. All testing was performed in triplicate. At day 8, cells were washed in PBS and fixed in 3.7% formaldehyde for 1 h. Cellular triacylglycerols were stained with Oil Red O (0.5 g of Oil Red O in 100 mL of 2-propanol and diluted 6:4 with water) for 1 h. After staining, plates were washed twice in water and photographed.

Insulin-Stimulated Glucose Uptake. Insulin-stimulated glucose uptake was analyzed in 3T3-L1 adipocytes differentiated in 96-well plates using the standard MDI protocol (MIX, DEX, and insulin protocol). On day 8 of the differentiation program the cells were treated with vehicle, positive control (1 μM Rosi), or test compound for 48 h. The cells were then washed first with 200 μL/well PBS pH 7.2 containing 1 mM $CaCl_2$ and 1 mM $MgSO_4$, then with 200 μL/well DMEM (1 g/L glucose) containing antibiotics (62.5 μg/mL penicillin and 100 μg/mL streptomycin), and finally incubated in 200 μL/well of the same solution for 2 h at 37 °C. The cells were washed with 200 μL/well Krebs-Ringer-Hepes Buffer (KRHB) pH 7.4 and incubated with 50 μL/well KRHB for 30 min at 37 °C. Fifty μL/well KRHB containing insulin in twice the concentration indicated was added, and the incubation was continued for 15 min at 37 °C. For each plate, 8 wells were incubated with 20 μM cytochalasin B to inhibit carrier-mediated glucose uptake. The counts obtained from cytochalasin B-treated wells were considered as background and subsequently subtracted from the

other measurements. Glucose uptake was initiated by the addition of 50 μL/well KRHB containing 3.0 mM glucose and 0.15 μL of ^{14}C-glucose ([^{14}C] 2-deoxy-D-glucose (5 mCi/L)), yielding a final concentration of 1.0 mM glucose. The cells were incubated for 15 min at 37 °C, and then 50 μL/well 800 mM D-glucose, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid pH 7.5, and 262 mM NaCl were added. The cells were washed three times in 200 μL/well ice-cold PBS. The cells were lysed in 200 μL/well 1% sodium dodecyl sulfate by shaking for 2 h, and radioactivity in the lysates was determined by scintillation counting. Glucose uptake was determined in 8 parallel wells for each sample and for each concentration. The results are presented as the mean ± standard error of the mean.

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Supporting Information Available: Activation of PPARγ by palmitoleic acid, α-linolenic acid, and linoleic acid as well as data regarding effect on adipocyte differentiation by compounds **4** and **5**. This material is available free of charge via the Internet at http://pubs.acs.org.

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