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NF-κB Activation and PPAR Transactivational Effects of a New Aliphatic Acid Amide from Pericarps of *Zanthoxylum piperitum*

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A new aliphatic acid amide, ZP-amide F (**1**), and eight known compounds, including bungeanumamide A (**2**), tumuramide C (**3**), ZP-amide A (**4**), ZP-amide B (**5**), ZP-amide D (**6**), hyperin (**7**), quercitrin (**8**), and (−)-sesamin (**9**), were isolated from pericarps of *Zanthoxylum piperitum*. The effects of these compounds on TNFα-induced NF-κB activation and transactivational activity of PPARs, including PPARα, PPARβ(δ) and PPARγ subtypes, were evaluated. Compounds **7** and **9** exhibited potent inhibitory effects on TNFα-induced NF-κB activation with IC_{50} values of 5.50 and 8.10 μM, respectively. Aliphatic acid amide compounds **3**, **4** and **6** displayed enhanced effects on PPAR transactivational activity with EC_{50} values of 47.12, 19.13 and 12.02 μM, respectively. Among them, compound **4** demonstrated an increase in PPARα transactivational activity, compound **3** showed a moderate increase on all PPAR subtypes, whereas compound **6** displayed weak PPAR transactivational activity.

Key Words : ZP-amide F, *Zanthoxylum piperitum*, Anti-inflammation, NF-κB, PPAR

Introduction

Nuclear factor kappa B (NF-κB) is a transcription factor involved in the regulation of several important processes, such as cell proliferation, apoptosis, the immune response, and the inflammatory response.[1-3] Its main functions as a regulator in the immune response, inflammation, cell proliferation and survival have been well-established.[4] NF-κB regulates the expression of a wide variety of genes in the immune response, adhesion molecules, and pro-inflammatory cytokines in inflammatory cells. Activation of NF-κB by various stimuli, including inflammatory cytokines such as tumor necrosis factor alpha (TNFα) and interleukin-1 (IL-1), T-cell activation signals, growth factors, and stress inducers, causes transcription at κB sites that is associated with a number of diseases, such as inflammatory disorders and cancer.[5,6] Therefore, inhibition of the NF-κB signaling pathway is a potent target for the prevention and/or treatment of cancers and inflammatory diseases.

Ligand-activated transcription factors belonging to the peroxisome proliferator-activated receptor (PPAR) family have recently been of much interest due to their possible regulatory role in the inflammatory and immune responses.[7,8] Their mode of action is indicated *via* formation of heterodimers with the nuclear receptor RXR, followed by binding to specific DNA-response elements in target genes known as peroxisome proliferator response elements (PPREs).[9] To date, three types of PPARs, including PPARα, PPARβ(δ) and PPARγ, have been identified. PPARα is expressed predominantly in liver, heart, kidney, intestinal mucosa, and brown adipose tissue (tissues with high catabolic rates for fatty acids and peroxisome metabolism).[10,11] PPARβ(δ) has become a pharmacological target for the treatment of metabolic disorders associated with metabolic syndromes, including dyslipidemia, obesity and insulin resistance.[12] PPARγ is the molecular target for insulin-sensitizing thiazolidinedione drugs such as troglitazone, pioglitazone and rosiglitazone, which have been approved for use in the treatment of type 2 diabetes.[13,14] Both PPARα and PPARγ have been reportedly implicated in the regulation of macrophage and endothelial cell inflammatory responses.[7] Discovery of PPAR agonists has become a target for the prevention and treatment of obesity, insulin resistance, metabolic syndromes, inflammation, and cardiovascular disease.[15]

Zanthoxylum piperitum DC. (Rutaceae) is commonly known as Japanese pepper. It is a deciduous aromatic spiny shrub widely distributed in Eastern Asian countries, including China, Japan and Korea. In several folk medicine remedies, *Z. piperitum* is used for treatment of helminth infections and digestive organs. Fresh young leaves and dried fruits of *Z. piperitum* have been used as a pungent spice and bitter seasoning.[16-18] Phytochemical investigation of this plant has led to the isolation of terpenoids,[17,19] aliphatic acid amides,[16,20] alkaloids, phenolics, flavonoids, and lignans.[21-23] These compounds have reportedly shown various pharmacological effects, including antibacterial, anti-oxidant, anti-inflammatory, and repellent activities.[24-27] The present study describes the isolation and structural elucidation of a new aliphatic acid amide from a methanol extract of the pericarps of *Z. piperitum.* The effects of isolated compounds on transcriptional activity of TNFα-induced NF-κB and PPARs, including PPARα, PPARβ(δ) PPARγ subtypes, were evaluat-

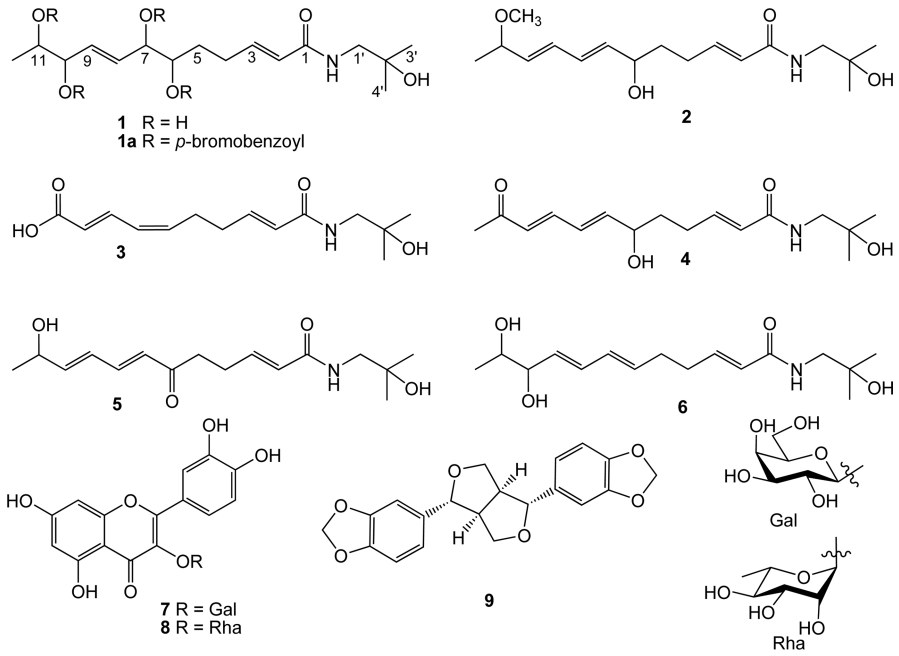

Figure 1. Chemical structure of compounds **1**-**9**.

ed in HepG2 cells.

Results and Discussion

A methanol extract (496.0 g) of *Z. piperitum* was suspended in water and successively partitioned with *n*-hexane, CH_2Cl_2, EtOAc, and *n*-BuOH. These fractions were subjected to various separation procedures, and nine compounds were isolated (Figure 1). Structures of known compounds (**2**–**9**) were elucidated by comparing spectroscopic data to published values, and identified as bungeanumamide A (**2**),[28] timuramide C (**3**),[29] ZP-amide A (**4**),[16] ZP-amide B (**5**),[16] ZP-amide D (**6**),[16] hyperin (**7**),[30] quercitrin (**8**),[31] and (−)-sesamin (**9**).[32]

Compound **1** was obtained as a colorless wax. Its molecular formula was determined to be $C_{16}H_{29}NO_6$, based on quasi-molecular ion peaks in the HR-ESI-MS at *m/z* 332.2073 $[M+H]^+$ (calcd for $[C_{16}H_{30}NO_6]^+$: 332.2068) and *m/z* 354.1925 $[M+Na]^+$ (calcd for $[C_{16}H_{29}NO_6Na]^+$: 354.1887). The ^{1}H-NMR showed signals of two pairs of olefinic protons [δ_H 6.02 (d, J = 15.6 Hz, H-2)/6.82 (dt, J = 7.2, 15.6 Hz, H-3) and 5.79 (2H, overlapped, H-8 and H-9)], four oxygenated methine protons [δ_H 3.50 (m, H-6), 3.67 (m, H-11), 3.91 (t, J = 4.8 Hz, H-10) and 3.96 (t, J = 4.8 Hz, H-7)], three methylene protons [δ_H 2.41 and 2.27 (m, H_2-4), 1.53 and 1.71 (m, H_2-5), 3.24 (s, H_2-1′)], and three methyl groups [δ_H 1.15 (3H, d, J = 6.0, H-12), 1.17 (6H, s, H_3-3′, H_3-4′)]. The ^{13}C-NMR and DEPT spectra of compound **1** revealed signals for 16 carbons, including a carbonyl carbon [δ_C 169.1 (C-1)], an oxygenated quaternary carbon [δ_C 71.6 (C-2′)], four olefinic methine carbons [δ_C 124.8 (C-2), 132.9 (C-9), 133.0 (C-8), 145.8 (C-3)], four oxygenated methine carbons [δ_C 71.6 (C-11), 74.8 (C-6), 76.6 (C-7), 77.3 (C-10)], three methylene carbons [δ_C 29.4 (C-4), 32.3 (C-5), 51.1 (C-1′)], and three methyl carbons [δ_C 18.7 (C-12), 27.2 (C-3′ and C-4′)]. This evidence suggests that compound **1** is an aliphatic acid amide with a usual skeleton-type from the *Zanthoxylum* species.[16,20,29] Proton assignments of the main chain were indicated by strong COSY correlations H-2/H-3/H-4/H-5/H-6/H-7/H-8/H-9/H-10/H-11/H-12. The position of first double bond, located at C-2/C-3, was confirmed by HMBC correlations of H-2 (δ_H 6.02) and H-3 (δ_H 6.82) with C-1 (δ_C 169.1), and hence, positions of the remaining double bond (C-8/C-9) and four hydroxyl groups (C-6, C-7, C-10, C-11), located in the main chain, were also determined through back-bone COSY correlations. The HMBC correlation between H-1′ (δ_H 3.24) and C-1 (δ_C 169.1), as well as the downfield chemical shift of both H-1′ (δ_C 3.24) and C-1′ (δ_C 51.1) suggest an amide connectivity between C-1 and C-1′. The last hydroxyl group was attached at C-2′, which was confirmed by HMBC correlations of a magnetically equivalent pair of methyl protons at H-3′ and H-4′ (δ_H 1.17) with C-2′ (δ_C 71.6) and C-1′ (δ_C 51.1). Next, configuration of the double bond at C-2/C-3 was determined to be "*E*" by *J* coupling constants of the corresponding olefinic protons ($J_{H\text{-}2/H\text{-}3}$ = 15.6 Hz). Due to overlapping signals between H-8 and H-9, configuration of the double bond at C-8/C-9 was determined through its *p*-bromobenzoyl derivative, **1a**. The *J* coupling constant ($J_{H\text{-}8/H\text{-}9}$ = 16.0 Hz) in ^{1}H-NMR of compound **1a** indicated *E*-configuration of the double bond at C-8/C-9 in both compounds **1a** and **1**. Consequently, the chemical structure of compound **1** was determined to be 6,7,10,11-tetrahydroxyl-*N*-(2-hydroxyl-2-methylpropyl)-2*E*,8*E*-dodecadienamide, and termed ZP-amide F.

Due to four hydroxyl groups in close proximity (C-6, C-7, C-10, C-11), we attempted to identify the absolute configuration of compound **1** by X-ray crystallography. Using the heavy atom method, compound **1** reacted with *p*-bromo-

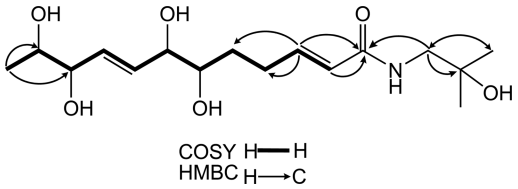

Figure 2. Important HMBC and COSY correlations of compound **1**.

Table 1. The NMR spectroscopic data of compound **1**

No.	δ_C	δ_H (mult., *J* in Hz)	HMBC (H→C)
1	169.1	–	–
2	124.8	6.02 (d, 15.6)	C-1, C-4
3	145.8	6.82 (dt, 7.2, 15.6)	C-1, C-2, C-4, C-5
4	29.4	2.41 (m) 2.27 (m)	C-2, C-3, C-5, C-6
5	32.3	1.71 (m) 1.53 (m)	C-3, C-4, C-6
6	74.8	3.50 (m)	–
7	76.6	3.96 (t, 4.8)	C-6, C-9
8	133.0	5.79[a]	C-9, C-10
9	132.9	5.79[a]	C-7, C-8
10	77.3	3.91 (t, 4.8)	C-8, C-11
11	71.6	3.67 (m)	C-9
12	18.7	1.15 (d, 6.0)	C-10, C-11
1′	51.1	3.24 (s)	C-1, C-2′, C-3′, C-4′
2′	71.6	–	–
3′	27.2	1.17 (s)	C-1′, C-2′, C-4′
4′	27.2	1.17 (s)	C-1′, C-2′, C-3′

[a]Overlapped signals, assignments were done by HMQC, HMBC, and COSY experiments.

benzoyl chloride and was converted into its *p*-bromobenzoate ester, **1a**.[33] Unfortunately, the product of compound **1a** was obtained as an amorphous powder and was thus unable to recrystallize. To date, nearly all aliphatic acid amides compounds from the *Zanthoxylum* species have been isolated as a racemic, and their absolute configurations have not yet to be determined.[16] Thus, a lack of supporting literature and limited experiments prevented the complete determination of the absolute configuration of compound **1**. A total selective synthetic pathway to identify the absolute configuration of aliphatic acid amide compounds from *Zanthoxylum*, as well as ZP-amide F (**1**), will be required for future studies.

Isolated compounds **1-9** were assessed for their effects on TNFα-induced NF-κB transcriptional activity in human hepatocarcinoma (HepG2) cells using an NF-κB-luciferase assay. First, the cytotoxicity of each compound was examined. Cell viability was measured by the MTS colorimetric assay using the Cell Counting Kit-8 reagent. Compounds **1-9** had no significant cytotoxicity in HepG2 cells up to 50.0 μM (data not shown), and hence, these compounds were used at alternate concentrations (< 50 μM) in subsequent experiments. Among the tested compounds, compounds **7** and **9** significantly inhibited TNFα-induced NF-κB transcriptional activity in a dose-dependent manner (Figure 3) with IC_{50} values of 5.50 and 8.10 μM, respectively; other compounds

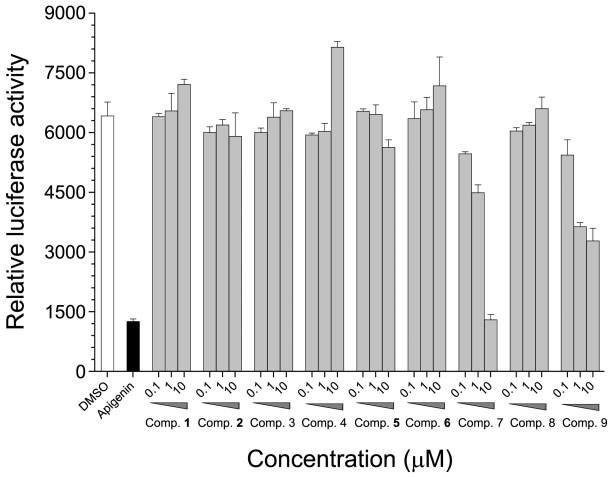

Figure 3. Effects of compounds **1**–**9** on the TNFα-induced NF-κB luciferase reporter activity in HepG2 cells. Data were expressed as mean ± SD of at least three experiments performed in triplicate. Statistical significance is determined by one-way analysis of variance followed by Dunnett's multiple comparison test, $P < 0.05$ versus control.

Table 2. PPARs transactivational activities of compounds **1**–**9**. EC_{50}: the concentration of a tested compound that gave 50% of the maximal reporter activity

Compounds	EC_{50} (μM)
1	> 50[a]
2	> 50
3	47.12 ± 3.57
4	19.13 ± 3.24
5	> 50
6	12.02 ± 1.20
7	> 50
8	> 50
9	> 50
Rosiglitazone	1.60 ± 0.07

[a]A compound was considered inactive with EC_{50} > 50 μM. The values were expressed as mean ± SD (n = 3)

were inactive in this assay. Apigenin (IC_{50} 1.64 μM) was used as a positive control. We continuously investigated the effects of compounds **1**–**9** on PPAR activity using a nuclear transcription PPRE cell-reporter system. The results showed that compounds **4** and **6** greatly enhanced PPAR transcriptional activity with EC_{50} values of 19.13 and 12.02 μM, respectively (Table 2). Compound **3** displayed moderate activity, with an EC_{50} value of 47.12 μM, whereas compounds **1**, **2**, **5**, and **7**–**9** were inactive. Therefore, the PPAR transactivational effects of compounds **3**, **4** and **6** were further examined on individual PPAR subtypes, including PPARα, PPARβ(δ) and PPARγ, using the GAL-4-PPAR chimera assay. As shown in Table 3, compound **4** exhibited increased transactivational activity on PPARα only; at 10 μM, this enhancement was twofold higher compared with the vehicle. However, compound **4** displayed a weak increase on PPARα transactivational activity after dilution to 0.1 μM. Interest-

Table 3. PPARα, PPARβ(δ), and PPARγ transactivational activity of compounds **3**, **4**, **6**. Ciprofibrate, L-165041, and rosiglitazone were used as positive controls for PPARα, PPAR β(δ), and PPARγ, respectively

Compounds	Concentration (μM)	Stimulation (%)		
		Gal4/PPARα-LBD	Gal4/PPARβ(δ)-LBD	Gal4/PPAR γ-LBD
3	0.1	101.22 ± 15.58	100.00 ± 11.09	99.47 ± 5.47
	1.0	116.96 ± 5.19	112.25 ± 2.08	108.80 ± 12.23
	10.0	154.72 ± 11.13	156.37 ± 6.24	119.73 ± 2.58
4	0.1	103.32 ± 2.23	100.98 ± 11.09	97.88 ± 14.16
	1.0	118.53 ± 5.93	102.45 ± 10.40	95.14 ± 12.23
	10.0	214.51 ± 8.16	115.69 ± 0.00	102.88 ± 2.58
6	0.1	116.43 ± 5.93	100.00 ± 9.71	106.75 ± 17.06
	1.0	94.41 ± 5.93	105.39 ± 9.71	100.83 ± 8.69
	10.0	104.37 ± 6.68	116.67 ± 0.00	99.47 ± 5.47
Ciprofibrate	1.0	312.59 ± 64.76	–	–
L-165041	1.0	–	291.18 ± 28.93	–
Rosiglitazone	1.0	–	–	294.99 ± 32.85

ingly, compound **3** moderately activated all PPAR subtypes, whereas compound **6** showed a weak enhancement effect. The PPAR agonist ciprofibrate, L-165041, and rosiglitazone, were used as positive controls for PPARα, PPARβ(δ) and PPARγ subtype experiments, respectively (EC_{50} 2.84, 1.58 and 3.89 μM). These results led us to conclude that several aliphatic acid amides (**3**, **4** and **6**) and phenolic compounds (**7** and **9**) from the pericarps of *Z. piperitum* exhibit significant anti-inflammatory effects by enhancing PPAR transactivational activity and inhibiting TNFα-induced NF-κB activation. These results also provide scientific support for the use of *Z. piperitum* in the prevention of inflammatory and metabolic diseases.

Experimental

General Procedures. Optical rotations were determined using a Jasco DIP-370 automatic polarimeter. The FT-IR spectra were measured using a Jasco Report-100 infrared spectrometer. The NMR spectra were recorded using a JEOL ECA 600 spectrometer (^{1}H, 600 MHz; ^{13}C, 150 MHz). High-resolution electrospray ionization mass spectra (HR-ESI-MS) were obtained using an Agilent 6530 Accurate-Mass Q-TOF LC/MS system. Column chromatography was performed using a silica gel (Kieselgel 60, 70-230, and 230-400 mesh, Merck, Darmstadt, Germany), YMC RP-18 resins, and thin layer chromatography (TLC) was performed using pre-coated silica-gel 60 F_{254} and RP-18 F_{254}S plates (both 0.25 mm, Merck, Darmstadt, Germany).

Plant Material. The pericarps of *Z. piperitum* were purchased at Daekwang Farm, Busan, Korea in October, 2012 and were taxonomically identified by one of authors (Prof. Young Ho Kim). A voucher specimen (CNU12111) was deposited at the Herbarium of College of Pharmacy, Chungnam National University, Daejeon, Korea.

Extraction and Isolation. The pericarps of *Z. piperitum* (3.0 kg) were extracted with MeOH under reflux for 3 h (5 L × 3 times) to yield 496 g of extract. Crude extract was suspended in water and then partitioned successively with *n*-hexane, CH_2Cl_2, EtOAc, and *n*-BuOH to give *n*-hexane (96.0 g), CH_2Cl_2 (118.0 g), EtOAc (19.0 g), and *n*-BuOH (174.0 g) fractions, respectively. The EtOAc extract (15.0 g) was subjected to silica gel column chromatography, eluted with gradient solvent system of CH_2Cl_2/MeOH/H_2O (1/0/0, 40/1/0.1, 20/1/0.1,10/1/0.1, 5/1/0.1, 1/1/0.1; v/v/v, 1 L for each step) to give 6 fractions EA1–EA7. Fraction EA2 (4.2 g) appeared precipitate which was filtered and washed with MeOH to afford compound **7** (2.0 g). The filtrate was subjected to YMC column chromatography eluting with MeOH/H_2O (2.2/1, v/v) to yield five fractions (EA2A–EA2E). Compound **9** (30.0 mg) was obtained from recrystallization fraction EA2E (350.0 mg) in acetone. Fraction EA3 (2.0 g) was subjected to silica gel column, eluted with EtOAc/MeOH (1/1, v/v) to yield six sub-fractions (EA3A–EA3F). Sub-fraction EA3C (110.0 mg) was separated using YMC column with MeOH/H_2O (1/1, v/v) as eluent to afford compound **8** (10.0 mg). Fraction EA5 (2.2 g) was subjected to silica gel column chromatography, eluted with gradient solvent system of *n*-hexane/acetone (3/1→1/1, v/v) to give four fractions (EA5A–EA5D). Fraction EA5C (500.0 mg) was separated by sephadex LH-20 column eluting with MeOH/CH_2Cl_2 (2/1, v/v) to yield five smaller fractions (EA5C1–EA5C5). Fraction EA5C2 (90.0 mg), and EA5C3 (75.0 mg) were purified through YMC column eluting with MeOH/H_2O (2/3, v/v) to obtain compounds **2** (11.0 mg) and **4** (15.0 mg), and **3** (13.0 mg), respectively. Fraction EA5C5 (110.0 mg) was separated using YMC column, eluted with MeOH/H_2O (1/1.6) to yield compounds **5** (13.0 mg) and **6** (15.0 mg). Finally, fraction EA6 (2.8 g) was subjected to silica gel column, eluted with CH_2Cl_2/MeOH/H_2O (5/1/0.1, v/v/v) to yield seven fractions (EA6A–EA6G). Fraction EA6D (70.0 mg) was further purified on an YMC column eluting with MeOH/H_2O (1/4, v/v) to obtain compound **1** (16.0 mg).

ZP-amide F (1): $C_{16}H_{29}NO_6$, colorless wax, $[\alpha]_D^{18}$ +12.0 (c 0.24, MeOH), IR (KBr) ν_{max} 3305, 2970, 1383, 1179,

1073, 977 cm^{-1}, HR-ESI-MS *m/z* 332.2073 $[M+H]^+$ (calcd for $[C_{16}H_{30}NO_6]^+$: 332.2068), *m/z* 354.1925 $[M+Na]^+$ (calcd for $[C_{16}H_{29}NO_6Na]^+$: 354.1887), ^{1}H-NMR (CD_3OD, 600 MHz) and ^{13}C-NMR (CD_3OD, 150 MHz) data are given in the Table 1.

ZP-amide F tetra-*p*-bromobenzoate (1a): $C_{44}H_{41}Br_4NO_{10}$, white powder, HR-ESI-MS *m/z* (relative intensity) 1085.9287 (100%) $[M+Na]^+$ (calcd for $[C_{44}H_{41}Br_4NO_{10}Na]^+$: 1085.9321) ^{1}H-NMR ($CDCl_3$, 400 MHz) δ_H 7.40-7.80 (H-aromatic), 6.73 (dt, J = 7.2; 15.6 Hz), 6.01 (dd, J = 5.2; 16.0 Hz), 5.95 (dd, J = 5.2, 16.0 Hz), 5.69 (dd, J = 4.0; 5.2 Hz), 5.67 (d, J = 15.6 Hz), 5.62 (dd, J = 4.0; 5.2 Hz), 5.42 (m), 5.29 (dt, J = 4.0; 8.8 Hz), 4.31 (s), 2.02 (q, J = 7.6 Hz), 1.63 (s), 1.62 (overlapped), 1.35 (d, J = 7.2 Hz).

Cell Culture and Reagents. Human hepatocarcinoma HepG2 cells were maintained in Dulbecco's modified Eagles' medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 10 μg/mL streptomycin at 37 °C and 5% CO_2. Human TNF-α was purchased from ATgen (Seoul, Korea).

NF-κB-Luciferase Assay. Human hepatocarcinoma HepG2 cells were maintained in Dulbecco's modified Eagles' medium (DMEM) (Invitrogen, Carlsbad, CA) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 10 μg/mL streptomycin at 37 °C and 5% CO_2. The luciferase vector was first transfected into cells. After a limited amount of time, the cells were lysed, and luciferin, the substrate of luciferase, was introduced into the cellular extract along with Mg^{2+} and an excess of ATP. Under these conditions, luciferase enzymes expressed by the reporter vector could catalyze the oxidative carboxylation of luciferin. Cells were seeded at 2×10^5 cells per well in a 12-well plate and grown. After 24 h, cells were transfected with inducible NF-κB luciferase reporter and constitutively expressing Renilla reporter. After 24 h of transfection, medium was changed to assay medium (Opti-MEM + 0.5% FBS + 0.1 mM NEAA + 1 mM sodium pyruvate + 100 units/mL penicillin + 10 μg/mL streptomycin) and cells were pretreated for 1 h with either vehicle (DMSO) and compounds, followed by 1 h of treatment with 10 ng/mL TNF-α for 20 h. Unstimulated cells were used as a negative control (−), apigenin was used as a positive control. Dual Luciferase assay was performed 48 h after transfection, and promoter activity values are expressed as arbitrary units using a Renilla reporter for internal normalization.

PPRE-Luciferase Assay. Human hepatoma cells (HepG2) were seeded at 1.5×10^5 cells per well in 12-well plates and grown for 24 h before transfection. An optimized amount of DNA plasmid (0.5 μg of PPRE-Luc and 0.2 μg of PPAR-inpCMV) was diluted in 100 μL of DMEM. All cells were transfected with the plasmid mixture using WelFect M Gold (WelGENE Inc.) as described by the manufacturer. After 30 min of incubation at room temperature, the DNA plasmid solution (100 μL) was introduced and mixed gently with cells. After 24 h of transfection, the medium was changed to TOM (Transfection Optimized Medium, Invitrogen) containing 0.1 mM NEAA, 0.5% charcoal-stripped FBS, and the individual compounds (test group), dimethyl sulfoxide (vehicle group), or rosiglitazone (positive control group). The cells were then cultured for 20 h. Next, the cells were washed with PBS and harvested with 1× passive lysis buffer (200 μL). The intensity of emitted luminescence was determined using an LB 953 Autolumat (EG&G Berthold, Bad Wildbad, Germany).[35,36]

PPAR Subtype Specific Transactivational Assay. Human hepatoma cells (HepG2) were seeded at 1.5×10^5 cells per well in 12-well plates and grown for 24 h before transfection. Cells were transfected separately with one pGal4-PPAR subfamily vector [pFAGal4-PPARα-LBD, pFA-Gal4-PPARγ-LBD, or pFA-Gal4-PPARβ(δ)-LBD expression plasmids], together with pFR-Luc using the WelFect M Gold transfection reagent (WelGENE Inc.), as described by the manufacturer. After 24 h of transfection, the medium was changed to TOM (Invitrogen) containing 0.1 mM NEAA, 0.5% charcoal-stripped FBS, and each compound (test group), dimethyl sulfoxide (vehicle group), ciprofibrate (positive control group for PPARα), rosiglitazone (positive control group for PPARγ), or L-165041 (positive control group for PPARβ(δ)). The cells were then cultured for 20 h, after which the cells were washed with PBS and harvested with 1× passive lysis buffer (200 μL). The intensity of emitted luminescence was determined using a Centro LB 960 microplate luminometer (EG&G Berthold) by measuring light emission for 5 s.[37]

Statistical Analysis. All data represent the mean ± standard deviation (SD) of at least three independent experiments performed in triplicate. Statistical significance is determined by one-way analysis of variance followed by Dunnett's multiple comparison test, $P < 0.05$.

Supporting Information. HR-ESI-MS, ^{1}H-, ^{13}C-NMR, HMQC, HMBC and COSY spectra of compound **1**, ^{1}H-NMR data of compound **1a** are available as Supporting information.

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Declaration of Interest. The authors report no conflicts of interest.

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