Phenolic Constituents of *Amorpha fruticosa* That Inhibit NF-κB Activation and Related Gene Expression

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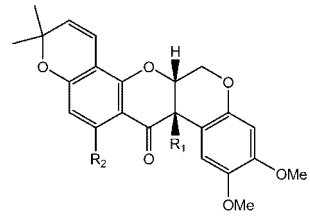
Received June 26, 2008

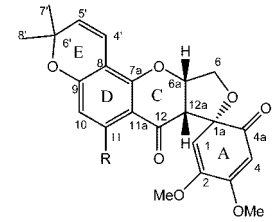
NF-κB is known to play a crucial role in the regulation of genes controlling the immune system, apoptosis, tumor cell growth, and tissue differentiation. Bioassay-guided fractionation of the *n*-hexane-soluble fraction of a methanol extract of *Amorpha fruticosa* afforded four new compounds, **5**, **7**, **8**, and **9**, and eight known compounds. Their structures were elucidated by spectroscopic methods. All compounds inhibited NF-κB activity, and tephrosin (**1**), 11-hydroxytephrosin (**2**), and deguelin (**3**) were the most active, with IC_{50} values of 0.11, 0.19, and 0.22 μM, respectively, in TNF-α-stimulated HeLa cell-based reporter gene assays. Further investigations showed that compounds **1**, **5**, and **6** blocked NF-κB/DNA binding activity and suppressed the expression of NF-κB target genes.

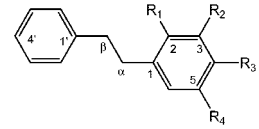
Nuclear transcription factor-κB (NF-κB) is a family of Rel domain-containing proteins consisting of RelA (p65), RelB, c-Rel, NF-κB1 (p50 and p105), and NF-κB2 (p52 and p100). The activation of NF-κB is known to relate to multiple pathophysiological conditions such as cancer, arthritis, asthma, inflammatory bowel disease, and other inflammatory conditions.[1] Under normal conditions, NF-κB is present in the cytoplasm in an inactive state by incorporation with its inhibitor protein IκBs.[2] On activation induced by various agents including TNF-α, LPS, IL-1, H_2O_2, and cigarette smoke, NF-κB is released and translocated into the nucleus and binds to its specific DNA sequences to activate transcription of a variety of genes encoding proteins such as COX-2, iNOS, IL-1, MMP-9, TRAF2, and VEGF, which are involved in immune response, cellular proliferation, angiogenesis, and apoptosis.[1−4] Therefore, NF-κB and the signaling pathways that regulate its activity have become a focal point for intense drug discovery and development efforts.

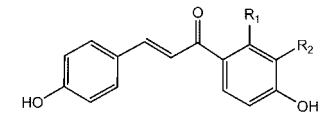
Amorpha fruticosa L. (Leguminosae) is a shrub that originated in North America and that was introduced to Korea through China in 1930s.[5] Previous studies of this plant reported a number of phenolic compounds including stilbenes, flavonoids, and rotenoids[6−10] as well as their antimicrobial,[6] antitumor,[10] and TNF-α inhibition activities.[11]

In our continuing search for NF-κB inhibitors of natural origin, a methanol extract of leaves and twigs of *A. fruticosa* was found to strongly inhibit NF-κB activation in a human cervical cancer (HeLa) cell-based reporter assay. Phytochemical investigation of the *n*-hexane-soluble fraction of the methanol extract led to the isolation of four new and eight known compounds (**1**−**12**) that inhibited NF-κB activation. The structures of the new compounds (**5**, **7**−**9**) were elucidated by spectroscopic methods. The known compounds were identified through the interpretation of their physical and spectroscopic data in comparison with those reported.[6,8,12−15] Herein, we describe the isolation, structure elucidation, and biological characterization of these compounds.









6: R1 = COOH, R2 = OH, R3 = prenyl, R4 = OMe
7: R1 = COOH, R2 = OMe, R3 = prenyl, R4 = OH
8: R1 = COOH, R2 = OMe, R3 = geranyl, R4 = OH
9: R1 = COOH, R2 = R4 = OH, R3 = geranyl
10: R1 = H, R2 = R4 = OH, R3 = geranyl, $\Delta^{\alpha-\beta}$

11: R1 = OH, R2 = geranyl
12: R1 = OMe, R2 = H

Results and Discussion

Compound **5** was obtained as yellow powder, and its UV spectrum revealed maxima similar to those of amorphispironone (**4**).[8] The HRESIMS spectrum of **5** revealed a $[M + H]^+$ peak at *m/z* 427.1413, corresponding to the molecular formula $C_{23}H_{23}O_8$. The ^1H and ^{13}C NMR data of **4** and **5** showed similar signals for the A, B, C, and E ring except for the appearance of a singlet at δ_H 5.96 in the ^1H NMR spectrum of **5**, suggesting that C-10 or C-11 of ring D was substituted. A downfield proton signal at δ_H 11.97 due to a chelated OH group indicated that **5** was an 11-hydroxylated derivative of **4**. The HMBC spectrum showed the correlations from H-10 to C-8, C-9, C-11, and C-11a and from 11-OH to C-10, C-11, and C-11a (see the Supporting Information). The NOESY spectrum of **5** revealed coupling between H-6a and H-12a, indicating a *cis* B−C ring junction. The similar CD spectra of **4** and **5**, with positive Cotton effects at 210 and 290 nm and a negative Cotton effect at 360 nm, indicated identical absolute configurations of **4** and **5**. Thus, **5** was determined to be 1a*R*,6a*S*,12a*R*-11-hydroxyamorphispironone.

Compound **7** was obtained as a yellow, amorphous solid, and its UV spectrum was consistent with a bibenzyl structure.[6] The HRESIMS spectrum of **7** revealed a $[M + Na]^+$ peak at *m/z* 363.1574, corresponding to the molecular formula $C_{21}H_{24}O_4Na$. The ^1H NMR spectrum of **7** showed an aromatic singlet at δ_H 5.94, a cluster of benzyl signals around δ_H 6.93−7.07, an OCH_3 signal at δ_H 3.62, and signals characteristic of a prenyl group. The ^{13}C NMR and DEPT spectra of **7** showed the presence of three methyl, three methylene, and seven methine groups and eight quaternary carbon

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10.1021/np800383q CCC: $40.75
Published on Web 10/08/2008

Table 1. ^{1}H NMR Data (δ) of **6**−**9**[a]

no.	**6**	**7**	**8**	**9**
6	6.24, 1H, s	5.94, 1H, s	6.01, 1H, s	6.27, 1H, s
α	3.30, 2H, m	3.13, 2H, m	3.16, 2H, m	3.22, 2H, m
β	2.95, 2H, m	2.68, 2H, m	2.73, 2H, m	2.89, 2H, m
2′−6′	7.20−7.34, 5H, m	6.93−7.07, 5H, m	6.99−7.01, 5H, m	7.19−7.30, 5H, m
1″	3.38, 1H, d (6.8)	3.21, 1H, d (6.8)	3.26, 1H, d (6.8)	3.45, 1H, d (6.8)
2″	5.23, 1H, t (6.8)	5.23, 1H, t (6.8)	5.03, 1H, t (6.8)	5.08, 1H, t (6.8)
4″	1.71, 3H, br s	1.56, 3H, br s	1.87, 2H, m	2.08, 2H, m
5″	1.81, 3H, br s	1.67, 3H, br s	1.99, 2H, m	2.12, 2H, m
6″			5.16,1H, t (6.8)	5.29,1H, t (6.4)
8″			1.53, 3H, br s	1.61, 3H, br s
9″			1.62, 3H, br s	1.69, 3H, br s
10″			1.71, 3H, br s	1.82, 3H, br s
OCH_3	3.81, 3H, br s	3.62, 3H, br s	3.66, 3H, br s	
3-OH	11.59, 1H, br s			11.64, 1H, br s

[a] Values in parentheses: *J* in Hz.

Table 2. ^{13}C NMR Data (δ) of **6**−**9**

no.	**6**	**7**	**8**	**9**
1	146.0	144.1	144.5	146.1
2	103.9	109.2	108.4	103.8
3	163.1	159.6	160.0	163.7
4	115.6	114.2	114.5	112.2
5	162.4	160.8	161.2	160.5
6	106.7	105.1	108.3	108.4
α	38.5	37.6	37.8	38.2
β	39.4	37.6	37.9	38.7
1′	142.0	142.7	142.7	142.2
2′	128.5	128.3	128.3	128.5
3′	128.7	128.6	128.5	128.6
4′	126.1	125.6	124.7	126.0
5′	128.7	128.6	128.5	128.6
6′	128.5	128.3	128.3	128.5
1″	22.2	22.3	22.3	22.3
2″	122.4	123.3	123.1	121.5
3″	131.9	131.5	134.9	139.1
4″	26.0	25.8	40.0	39.9
5″	18.0	17.9	27.0	26.6
6″			124.7	124.0
7″			131.2	132.1
8″			17.8	17.9
9″			25.8	25.8
10″			16.2	16.4
COOH	176.4	177.0	176.8	176.2
OCH_3	55.7	55.3	55.4	

atoms including a carboxylic group at δ_C 177.0. This NMR data (Tables 1 and 2) were almost identical to those of amorfrutin A (**6**);[6] however, the chelated OH signal (δ 11.59) in the ^{1}H NMR spectrum of **6** was not present in the spectrum of **7**. The C-3 resonance (δ 159.6) of **7** was shifted upfield 3.5 ppm, while the C-5 resonance (δ 161.2) appeared at lower field compared to those of **6**. These observations as well as HMBC correlations indicated that the OCH_3 group was attached to C-3. The prenyl moiety was attached to C-4, as observed in the HMBC spectrum. Thus, **7** was identified as 2-carboxy-3-methoxy-5-hydroxy-4-prenylbibenzyl.

Compound **8** was obtained as a yellow, amorphous solid, and its UV spectrum was similar to those of **6** and **7**. The HRESIMS spectrum of **8** revealed a $[M + Na]^+$ peak at *m*/*z* 431.2220, corresponding to the molecular formula $C_{26}H_{32}O_4Na$. The NMR data of **8** (Tables 1 and 2) were similar to those of **7** except for the replacement of a prenyl group by a geranyl group, suggesting **8** could be amorfrutin B.[6] However, the ^{1}H NMR spectrum of **8** showed no signal for a chelated OH group, as observed in the structure of **6**, and the HMBC spectrum showed a correlation between the OCH_3 group (δ_H 3.66) and C-3 (δ_C 160.0), indicating that **8** was 2-carboxy-5-hydroxy-3-methoxy-4-geranylbibenzyl.

Compound **9** had a UV spectrum similar to those of **6**−**8**. The HRESIMS spectrum of **9** revealed a $[M + Na]^+$ peak at *m*/*z* 417.2576, corresponding to the molecular formula $C_{25}H_{30}O_4Na$. The NMR data of **8** and **9** (Tables 1 and 2) were almost identical except for the replacement of an OCH_3 signal by a chelated OH group. The chelated OH group at C-3 with the carbonyl group at C-2 led to a downfield shift of C-3 (δ 163.7) compared to **7** and **8**. The HMBC correlations revealed the coupling from 11-OH (δ 11.64) to C-3 (δ 163.7). Thus the structure of **9** was determined as 2-carboxy-3,5-dihydroxy-4-geranylbibenzyl.

Table 3. IC_{50} Values[a] (μM) of Various Solvent Extracts and Pure Compounds of *A. fruticosa* on NF-κB Activation to HeLa and RAW264.7 Cells

compound	HeLa	RAW264.7
MeOH	3.5 ± 0.96[b]	nd
n-hexane	1.42 ± 0.64[b]	nd
$CHCl_3$	>30[b]	nd
H_2O	>30[b]	nd
1	0.11 ± 0.12	2.0 ± 0.32
2	0.19 ± 0.06	4.21 ± 1.67
3	0.22 ± 0.04	1.75 ± 0.31
4	7.39 ± 1.45	13.9 ± 0.84
5	2.12 ± 0.83	7.65 ± 1.34
6	0.84 ± 0.03	4.17 ± 0.23
7	1.75 ± 0.31	3.02 ± 0.65
8	1.13 ± 0.14	2.44 ± 0.87
9	1.56 ± 1.02	3.14 ± 1.07
10	0.26 ± 0.07	0.68 ± 0.19
11	1.03 ± 0.65	1.83 ± 0.84
12	>50	24.7 ± 1.03
Celastrol	0.15 ± 0.47	0.30 ± 0.62

[a] Values are means ± SD from three separate experiments. [b] Data are in μg mL^{-1}; nd = not determined.

The inhibitory effects **1**−**12** on the NF-κB activation were evaluated in a NF-κB-dependent reporter gene assay in human cervical cancer (HeLa) cells and murine macrophage RAW264.7 cells stimulated by TNF-α and LPS, respectively. As shown in Table 3, all compounds except for **12** showed strong inhibitory effects on the NF-κB activation in both cell lines as compared with the NF-κB inhibitor celastrol.[16] Of these compounds, tephrosin (**1**), 11-hydroxytephrosin (**2**), and deguelin (**3**) were the most active, with IC_{50} values of 0.11, 0.19, and 0.22 μM in the HeLa cell-line-based reporter assay, respectively. Interestingly, **1**−**11** exhibited significantly lower IC_{50} values in HeLa cells than in RAW264.7 cells. The MTT assay showed that all compounds except for **10** had no significant toxicity to HeLa, RAW264.7, and MCF-7 cell lines up to 30 μM, indicating that the inhibitory effects of these compounds on the NF-κB activation were not due to cytotoxicity.

To confirm that these compounds inhibited NF-κB activation in the reporter assay, compounds **1**, **5**, and **6** were selected to investigate their effects on NF-κB/DNA binding activity by electrophoretic mobility shift assay. As shown in Figure 1A, the compounds dose-dependently blocked the DNA-binding activity of NF-κB in MCF-7 cells induced by TNF-α. NF-κB is known to regulate the expression of various genes involving in inflammation (iNOS, IL-1) and tumorigenesis (COX-2, MMP-9, cyclin D1, cIAP).

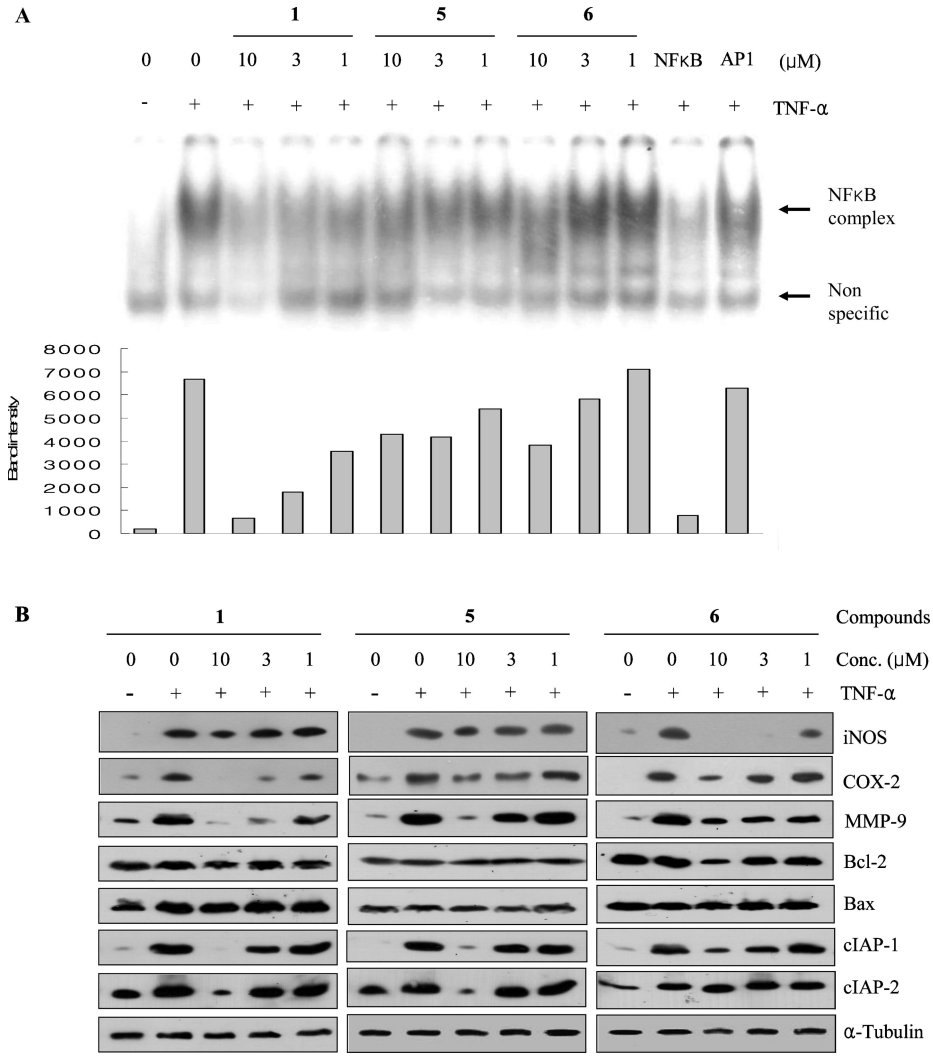

Figure 1. (A) Compounds **1**, **5**, and **6** inhibited NF-κB-DNA binding activity. MCF7 cells were preincubated with compounds for 30 min. Then the cells were stimulated by 20 ng mL^{-1} TNF-α for another 60 min. Nuclear extracts were prepared, and binding activity was examined by EMSA. The amount of NF-κB complex formed was estimated by image scanning and is expressed in arbitrary units at the bottom of panel. (B) Effects of **1**, **5**, and **6** on the expression of NF-κB target genes. MCF7 cells were pretreated with compounds at indicated concentrations for 30 min and then stimulated by TNF-α (20 ng mL^{-1}) for 24 h. Then total cell lysate was analyzed by Western blotting using specific antibodies indicated. α-Tubulin was detected for loading control.

Therefore, the effects of **1**, **5**, and **6** on the expression of NF-κB target genes in TNF-α-induced MCF-7 cells were evaluated by Western blot analysis (Figure 1B). Compounds **1** and **5** moderately suppressed the expression of iNOS, while **6** potently inhibited its expression at 10 and 3 μM. In contrast, **1** and **5** strongly blocked COX-2 induction, and **6** was less effective. Many studies have demonstrated an essential role of NF-κB in preventing apoptosis induced by TNF-α and chemotherapeutic agents. Although **1** and **5** had weak effects on Bcl-2 and Bax protein levels, they strongly down-regulated the expression of antiapoptotic proteins cIAP-1 and c-IAP-2. They also suppressed the expression of MMP-9, which is associated with cancer cell invasion and metastasis.

The rotenoids **1**, **3**, and **4** have been subjects of anticancer studies *in vitro* as well as *in vivo*.[9,10,17−21] The antitumor properties of **3** appear to be executed through PI3K/Akt and NF-κB signaling pathways, which regulate a variety of genes responsible for angiogenesis, apoptosis, invasion, metastasis, and proliferation.[21−24] Thus, other rotenoids (**1**, **2**, **4**, and **5**) exhibiting strong NF-κB inhibitory activity in this study could share this mechanism. The chalcone xanthoangelol (**11**) has been reported to induce apoptosis by activating caspase-3 and to suppress tumor growth and metastasis *in vivo*.[25,26] It is noted that compound **12** was inactive on TNF-α-induced NF-κB activation but weakly active on LPS-induced NF-κB activation, suggesting that geranylation (in the case of **11**) increases the NF-κB inhibitory activity of chalcones.

Experimental Section

General Experimental Procedures. Optical rotations were taken with a JASCO P-1020 digital polarimeter. UV spectra were recorded on an UV-1601 spectrometer and CD spectra were obtained from a JASCO J-700 spectropolarimeter. IR spectra were measured on a JASCO 100 IR spectrophotometer. NMR experiments were performed on a Varian-Unity Ionva-400 instrument. ESIMS were recorded on a Finnigan Navigator LC/MS/DS mass spectrometer and HRESIMS on a Mariner mass spectrometer. HPLC was performed using a Waters system with a 515 pump and a 2996 photodiode array detector.

Plant Material. Leaves and twigs of *Amorpha fruticosa* (20 kg) were collected on the sides of the Gapcheon River, Daejeon, Korea, in July 2006. A voucher specimen (No. 060721) has been deposited in the herbarium of the Center for Molecular Cancer Research, Korean Research Institute of Biosciences and Biotechnology, Daejeon, Korea.

Extraction and Isolation. The air-dried and powdered leaves and twigs of *A. fruticosa* (3.3 kg) were extracted with MeOH (3 × 10 L) at room temperature. The extract was concentrated and suspended in H_2O (3 L). The suspension was successively partitioned with *n*-hexane and $CHCl_3$ (each 3 × 1.5 L), and the organic layer was evaporated *in vacuo* to give brown solids of 280 and 17.5 g, respectively. The *n*-hexane fraction (85% inhibition of NF-κB at 4.0 μg mL^{-1}) was

subjected to silica gel column (silica gel 60, Merck, Germany, 230−400 mesh, 3000 g) chromatography using a *n*-hexane−EtOAc gradient to afford eight fractions, A1−A8. Fraction A4 was chromatographed on a silica column (silica gel 60, Merck, Germany, 230−400 mesh, 1500 g) using *n*-hexane−EtOAc (10:1 v/v) to obtain subfractions B1−B7. Fraction B2 was subjected to preparative HPLC (YMC Pack Pro C18 column, 250 × 20 mm, 80% acetonitrile in H_2O) to obtain **9** (2.1 mg) and **10** (38.6 mg). Compounds **3** (8.9 mg), **6** (125 mg), and **7** (7.2 mg) were separated from B5 by preparative HPLC (YMC Pack Pro C18 column, 250 20 mm, 50% acetonitrile in H_2O). Similar HPLC of fraction B6 yielded **1** (223 mg), **2** (262 mg), and **8** (8.4 mg). Fraction A5 was fractionated on a silica gel column (silica gel 60, Merck, Germany, 230−400 mesh, 1000 g) eluted with *n*-hexane−EtOAc (3:1) to afford subfractions C1−C7. Fraction C5 was chromatographed on a reversed-phase column (ODS-60-I40/63, YMC, Japan, 300 g) with a mobile phase of MeOH−H_2O (5:1), giving **11** (11.0 mg). Compounds **4** (172.4 mg), **5** (132.8 mg), and **12** (8.9 mg) were obtained from C6 by preparative HPLC (YMC Pack Pro C18 column, 250 20 mm, 40% acetonitrile in H_2O).

Tephrosin (1): yellow powder; $[\alpha]_D^{25}$ −14.6 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) nm 206 (3.84), 237 (3.52), 272 (4.12), 301(2.92), 315 (2.85); ESIMS *m*/*z* 433.6 $[M - H]^-$; ^1H and ^{13}C NMR data were in agreement with those previously published.[8]

11-Hydroxytephrosin (2): yellow powder; $[\alpha]_D^{25}$ −41.5 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) nm 205 (3.25), 234 (3.01), 273 (3.38), 296 (2.94), 313 (2.90); ESIMS *m*/*z* 425.4 $[M + Na]^+$; ^1H and ^{13}C NMR data were in agreement with those previously published.[12]

Deguelin (3): yellow powder; $[\alpha]_D^{25}$ −50.4 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) nm 205 (3.49), 239 (3.25), 251 (3.28), 271 (3.40), 299 (2.89); ESIMS *m*/*z* 395.5 $[M + H]^+$; ^1H and ^{13}C NMR data were in agreement with those previously published.[8]

Amorphispironone (4): yellow powder; $[\alpha]_D^{25}$ −18.1 (*c* 0.1, MeOH); CD (MeOH) λ_{max} ($\Delta\varepsilon$) nm 210 (+61.5), 290 (+23.6), 360 (−82.3); UV (MeOH) λ_{max} (log ε) nm 209 (3.42), 251 (3.56), 268 (3.64), 317 (3.18); ESIMS *m*/*z* 433.6 $[M + Na]^+$; ^1H and ^{13}C NMR data were in agreement with those previously published.[8]

1a*R*,6a*S*,12a*R*-11-Hydroxyamorphispironone (5): yellow powder; $[\alpha]_D^{25}$ −29.1 (*c* 0.1, MeOH); CD (MeOH) λ_{max} ($\Delta\varepsilon$) 214 (+53.4), 290 (+30.1), 361 (−45.5); UV (MeOH) λ_{max} (log ε) nm 209 (3.37), 274 (3.60), 314 (3.25); IR (KBr) ν_{max} 3400, 1705, 1620, 1475 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.41 (3H, br s, H-7′), 1.43 (3H, br s, H-8′), 3.33 (3H, br s, OCH_3), 3.44 (1H, d, J = 4.8, H-12a), 3.83 (3H, br s, OCH_3), 4.47 (1H, dd, J = 2.6, 10.4, H-6*ax*), 4.59 (1H, d, J = 10.4, H-6*eq*), 5.02 (1H, s, H-1), 5.21 (1H, dd, J = 2.6, 4.8, H-6a), 5.49 (1H, s, H-4), 5.54 (1H, dd, J = 10.0, H-5′), 5.96 (1H, s, H-10), 6.56 (1H, dd, J = 10.0, H-4′) 11.97 (1H, s, 11-OH); ^{13}C NMR (100 MHz, $CDCl_3$) δ 198.7 (H-4a), 191.31 (H-12), 166.5 (H-3), 163.8 (H-9), 163.0 (H-11), 155.4 (H-7a), 148.7 (H-2), 127.0 (H-5′), 115.47 (H-4′), 102.7 (H-11a), 98.1 (H-10), 102.1 (H-8), 105.1 (H-1) 99.7 (H-4), 84.0 (H-1a), 82.3 (H-6a), 78.5 (H-6′), 76.0 (H-6), 59.1 (H-12a), 56.98 (OCH_3), 55.4 (OCH_3), 28.5 (H-7′), 28.3 (H-6′); ESIMS *m*/*z* 427.6 $[M + H]^+$; HRESIMS *m*/*z* 427.1413 (calcd for $C_{23}H_{23}O_8$, 427.1387).

Amorfrutin A (6): yellow solid; UV (MeOH) λ_{max} (log ε) nm 225 (3.24), 263 (3.07), 305 (2.78); ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$), see Tables 1 and 2; ESIMS *m*/*z* 339.6 $[M - H]^-$; ^1H and ^{13}C NMR data were in agreement with those previously published.[6]

2-Carboxy-3-methoxy-5-hydroxy-4-prenylbibenzyl (7): yellow solid; UV (MeOH) λ_{max} nm (log ε) 226 (3.47), 265 (3.21), 309 (2.95); IR (KBr) ν_{max} 3290, 1690, 1535, 1460, 1210 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$), see Tables 1 and 2; ESIMS *m*/*z* 339.6 $[M - H]^-$; HRESIMS *m*/*z* 363.1574 (calcd for $C_{21}H_{24}O_4Na$, 363.1566).

2-Carboxy-5-hydroxy-3-methoxy-4-geranylbibenzyl (8): yellow solid; UV (MeOH) λ_{max} (log ε) nm 226 (3.52), 265 (3.33), 307 (2.98); IR (KBr) ν_{max} 3300, 1695, 1550, 1470, 1200 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$), see Tables 1 and 2; ESIMS *m*/*z* 407.7 $[M - H]^-$; HRESIMS *m*/*z* 431.2220 (calcd for $C_{26}H_{32}O_4Na$, 431.2192).

2-Carboxy-3,5-dihydroxy-4-geranylbibenzyl (9): yellow solid; UV (MeOH) λ_{max} (log ε) nm 226 (3.25), 265 (3.01), 306 (2.75); IR (KBr) ν_{max} 3350, 1690, 1550, 1460, 1200 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$), see Tables 1 and 2; ESIMS *m*/*z* 393.4 $[M - H]^-$; HRESIMS *m*/*z* 417.2576 (calcd for $C_{25}H_{30}O_4Na$, 417.2559).

Amorphastilbol (10): yellow solid; UV (MeOH) λ_{max} (log ε) nm 209 (4.05), 369 (4.11); ESIMS *m*/*z* 347.6 $[M - H]^-$; ^1H and ^{13}C NMR data were in agreement with those previously published.[13]

Xanthoangelol (11): yellow powder; UV (MeOH) λ_{max} (log ε) nm 208 (3.68), 315 (3.63); ESIMS *m*/*z* 391.6 $[M - H]^-$; ^1H and ^{13}C NMR data were in agreement with those previously published.[14]

2′-Methoxyisoliquiritigenin (12): ellow powder; UV (MeOH) λ_{max} (log ε) nm 212 (3.23), 237 (2.99), 349 (3.28); ESIMS *m*/*z* 269.6 $[M - H]^-$; ^1H and ^{13}C NMR data were in agreement with those previously published.[15]

Cell Culture. Human cervical cancer HeLa cells and murine macrophage RAW.264.7 cells were cultured in DMEM (Invitrogen, Grand Island, NY), and human breast cancer MCF-7 cells were maintained in RPMI1640 (Invitrogen, Grand Island, NY) in a humidified 5% CO_2 atmosphere at 37 °C. All media were supplemented with 10% (V/V) fetal bovine serum (Hyclone, Logan, UT), penicillin, and streptomycin.

NF-κB Reporter Assay. The HeLa and RAW264.7 cells transiently transfected with a plasmid containing eight copies of κB elements linked to the SEAP (secreted alkaline phosphatase) gene were used. SEAP assay was performed as previously described.[27] Briefly, transfected cells were seeded in a 96-well plate at a density of 5×10^4 cells/well. After 3 h incubation at 37 °C, cells were treated with compounds dissolved in DMSO (final concentration of DMSO was 0.01%) and stimulated with 10 ng mL^{-1} TNF-α or 1 μg mL^{-1} LPS for 24 h. Then 100 μL of each culture supernatants was transferred to a 96-well plate and heated at 65 °C for 10 min followed by adding 100 μL of 2× SEAP buffer to each well and incubating for another 10 min. The reaction was initiated by adding 20 μL of 31.6 mg mL^{-1} *p*-nitrophenyl phosphate dissolved in SEAP buffer and incubated at 37 °C for 4 h. The OD values of solutions were measured at 405 nm with a microplate reader (Molecular Devices Co., Menlo Park, CA).

Cytotoxicity Assay. Cell viability was evaluated by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) uptake method. Cells were seeded in a 96-well plate at 4×10^4 cells/well and incubated at 37 °C for 3 h, and then cells were treated with various concentrations of test compounds. After 24 h incubation at 37 °C, 10 μL of 5 mg mL^{-1} MTT solution was added to each well and incubated for another 4 h. After removing the supernatant, formazan crystals were dissolved in 100 μL of DMSO and the optical density values were measured at 570 nm with a microplate reader.

Electrophoretic Mobility Shift Assay (EMSA). An electrophoretic mobility shift assay was performed using a gel shift assay system (Promega), according to the instructions of the manufacturer. A double-stranded oligonucleotide of NF-κB (Promega) was end-labeled with [γ-^{32}P] ATP and purified with a G-25 spin column (Boehringer Mannheim, Mannheim, Germany). Exponentially grown MCF-7 cells at 70% confluence were preincubated with compounds dissolved in DMSO (final concentration of DMSO was 0.001%) for 30 min and then stimulated by TNF-α (20 ng mL^{-1}) or LPS (10 μg mL^{-1}) for another 60 min. Cells were resuspended in 400 μL of cold buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 2.0 μg mL^{-1} leupeptin, and 2.0 μg mL^{-1} aprotinin) by flicking the tube. The cells were allowed to swell on ice for 15 min and then vortexed for 20 s. The homogenate was centrifuged for 30 s in a Microfuge at 4 °C. The pellet was resuspended in 50 μL of ice-cold extraction buffer (20 mM HEPES pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 2.0 μg mL^{-1} leupeptin, and 2.0 μg mL^{-1} aprotinin) and incubated on ice for 20 min with intermittent mixing. Cellular debris was removed by centrifugation for 5 min at 13 000 rpm at 4 °C. The supernatants (nuclear extract) were incubated for 20 min at room temperature with binding buffer and ^{32}P-labeled oligonucleotide. Excess amounts of unlabeled oligonucleotide of NF-κB and AP-1 were used for the competitive test. The DNA−protein complex formed was separated on 4% native polyacrylamide gel. The gel was transferred to Whatman 3 MM paper, dried, and exposed to X-ray film at −70 °C overnight with an intensifying screen. Band intensity was analyzed by scanning densitometry (TINA software, version 2.10; Raytest Isotopenmessgeraete GmbH, Straubenhardt, Germany).

Western Blot Analysis. Exponentially grown cells were plated at 70% confluence and incubated at 37 °C overnight. Various concentrations of test compounds dissolved in DMSO (final concentration of DMSO was 0.001%) were added and incubated for 30 min and then

further incubated with stimuli at indicated times. At the end of the incubation, the cells were harvested by scraping, followed by centrifugation at 1000*g* for 5 min at 4 °C, and washed twice with ice-cold phosphate-buffered saline. Total cell lysates were prepared using a lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg mL^{-1} leupeptin, 150 mM NaCl). Lysates were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a Hybond-C membrane (Amersham Biosciences, Piscataway, NJ). The membrane was blocked with 5% nonfat milk in phosphate-buffered saline containing 0.1% Tween-20 (PBST) at room temperature for 1 h and then incubated overnight at 4 °C with primary antibody (1:1000 dilution in 5% BSA in PBST). Appropriate secondary antibody was then added (1:2500 dilution in 5% nonfat milk in PBST) for 1 h incubation at room temperature, and the antigen−antibody complexes were visualized by enhanced chemiluminescence according to the instructions of the manufacturer (Amersham Bioscience, Piscataway, NJ). α-Tubulin was detected as protein loading control.

Statistical Analysis. Each experiment was performed at least three times, and representative data are shown. Means were checked for statistical differences by using the Student's *t*-test with error probabilities of $p < 0.05$.

Acknowledgment. This work is supported in part by a grant from the Korea Research Institute of Bioscience and Biotechnology Research Initiative Program and a research grant (PF0320701-00) from the Plant Diversity Research Center of the 21st Frontier Research Program funded by the Korean Ministry of Science and Technology. We are grateful to the Korea Basic Science Institute for certain spectroscopic measurements.

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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NP800383Q