Kaurane Diterpenoids from *Isodon excisus* Inhibit LPS-Induced NF-κB Activation and NO Production in Macrophage RAW264.7 Cells

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As part of an ongoing search for plant-derived compounds that inhibit the activation of NF-κB, the methanol extract of the aerial parts of *Isodon excisus* was found to have significant inhibitory effects on the activation of NF-κB in murine macrophage RAW264.7 cells. Bioactivity-guided isolation of the extract yielded five new diterpenoids, excisusin A−E (**1**−**5**), along with seven known compounds, inflexarabdonin I (**6**), inflexarabdonin G (**7**), inflexin (**8**), inflexanin A (**9**), inflexanin B (**10**), inflexinol (**11**), and inflexarabdonin A (**12**). The structures were determined by analysis of the spectroscopic data including 2D NMR. All of the isolates were evaluated for their inhibitory effects on LPS-induced NF-κB activation and nitric oxide production in RAW264.7 cells.

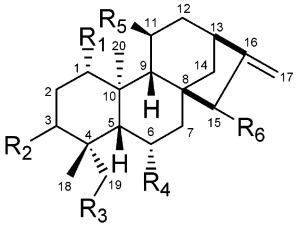
Isodon excisus (Max.) Kudo (Labiatae) is a perennial herb that is distributed widely in Korea, China, and Japan. The aerial parts of this plant have been used for detoxification and to treat gastrointestinal disorders.[1] Several diterpenoids with C-20-nonoxygenated *ent*-kaurane, C-20-oxygenated *ent*-kaurane, 6,7-seco-*ent*-kaurane, 8,9-seco-*ent*-kaurane, *ent*-kaurane dimer, abietane, and labdane skeletons have been isolated and characterized from the genus *Isodon*.[2−6] Among these previously known isolates, some C-20-nonoxygenated *ent*-kauranes and C-20-oxygenated *ent*-kauranes were found to inhibit NF-κB activation.[7−9]

NF-κB plays a key role in the regulation of genes mediating various inflammatory and immune responses, notably the iNOS genes.[10,11] Various inflammatory diseases including rheumatoid arthritis, autoimmune disease, chronic inflammation, and atherosclerosis are associated with the excess production of nitric oxide (NO), which is produced by iNOS in macrophages.[12] Due to the apparent involvement of NF-κB in a variety of human diseases, inhibitors of NF-κB activation are of considerable pharmacological and therapeutic interest in areas focused on inflammation and cancer.[13,14] A number of NF-κB inhibitors such as sesquiterpene lactones, *ent*-kaurane diterpenes, and triterpenoids have been isolated from well-known natural products.[15,16]

In this study, bioactivity-guided fractionation of the MeOH extracts of the aerial parts of *I. exisus* followed by repeated column chromatography led to the isolation of five new diterpenoids, excisusin A−E (**1**−**5**), as well as seven known compounds. We report herein the isolation, structure elucidation, and inhibitory effects of these compounds on the activation of NF-κB and the production of nitric oxide in murine macrophage RAW264.7 cells.

Results and Discussion

The MeOH extract of the aerial parts of *I. excisus* was partitioned successively between *n*-hexane and aqueous MeOH, followed by CH_2Cl_2 and water. The CH_2Cl_2-solubles exhibited potent inhibition of NF-κB activation (IC_{50}: 4.5 μg/mL) in LPS-stimulated murine macrophage RAW264.7 cells. Activity-guided isolation of the CH_2Cl_2-soluble fraction afforded five new kauranes, excisusin A−E (**1**−**5**), as well as seven known *ent*-kauranes, inflexarabdonin I (**6**),[17] inflexarabdonin G (**7**),[18] inflexin (**8**),[19] inflexanin A (**9**),[20] inflexanin B (**10**),[20] inflexinol (**11**),[19] and inflexarabdonin A (**12**).[21]



	R_1	R_2	R_3	R_4	R_5	R_6
1	H	β-OAc	OH	H	OH	= O
2	OH	β-OAc	H	H	OH	= O
3	H	α-OH	OAc	H	OH	β-OH
4	OH	β-OH	H	OH	OAc	= O
5	H	= O	OAc	H	OH	= O
6	H	= O	H	OH	OH	= O
7	OH	β-OAc	H	H	OAc	= O
8	OH	β-OAc	H	= O	OAc	= O
9	H	β-OAc	H	OH	OH	= O
10	OH	β-OAc	H	OH	OH	= O
11	OH	β-OAc	H	OH	OAc	= O
12	OH	β-OAc	H	OH	OAc	β-OH

Compound **1** was obtained as an amorphous powder, and its molecular formula was determined as $C_{22}H_{32}O_5$ by HRFABMS (*m*/*z* 399.2159 $[M + Na]^+$; calcd 399.2147). The UV and IR spectra of **1** showed absorption bands characteristic for a five-membered ring ketone conjugated with an *exo*-methylene (242 nm; 1714, 1644 cm^{-1}). The ^1H NMR spectrum of compound **1** showed two singlets at δ_H 5.23 (1H, s) and 6.00 (1H, s) that were assigned to an *exo*-methylene, a pair of AB doublets at δ_H 3.70 (1H, d, $J = 11.0$ Hz) and 4.02 (1H, d, $J = 11.0$ Hz) that were assigned to an oxygenated methylene, two signals at δ_H 4.24 (1H, br d, $J = 4.0$ Hz) and 5.62 (1H, br s) that were assigned to two oxygen-bearing methines, and three singlets at δ_H 1.02 (3H, s), 1.26 (3H, s), and 1.96 (3H, s) due to two tertiary methyl groups attached to quaternary carbons and an acetyl group, respectively. The ^{13}C NMR and DEPT spectra confirmed compound **1** to contain 22 carbons including one conjugated ketone (δ_C 208.8), an acetyl group (δ_C 21.1 and 170.4), a methine (δ_C 73.8) bearing an acetoxyl group, an *exo*-methylene (δ_C 111.1 and 151.7), six methylenes, and three quaternary carbons.

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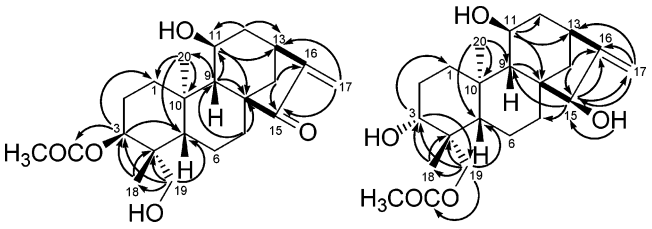

1 **3**

Figure 1. Selected HMBC correlations for compounds **1** and **3**.

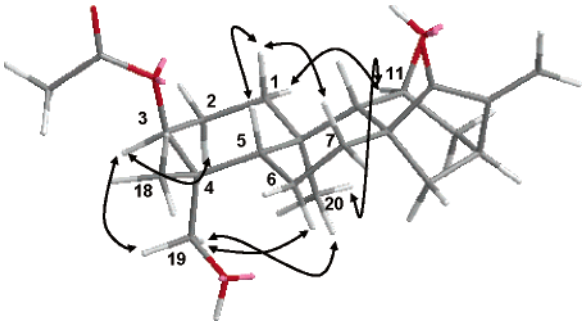

Figure 2. Key correlations of compound **1** in the NOESY spectrum.

On the basis of the structures of diterpenoids previously isolated from the genus *Isodon*,[22,23] compound **1** was presumed to have a kaur-16-en-15-one structure as a basic skeleton with an acetoxyl and three hydroxyl groups. The locations of these functionalities were assigned by the observed HMBC correlations from H-3 (δ_H 5.62, br s) to C-1 (δ_C 33.4), C-5 (δ_C 50.8), and an acetoxyl carbonyl carbon (δ_C 170.4), from H-11 (δ_H 4.24, d) to C-8 (δ_C 50.8) and C-13 (δ_C 37.8), and from H-19 (δ_H 4.02 and 3.70, d) to C-3 (δ_C 73.8), C-4 (δ_C 43.1), and C-18 (δ_C 22.9) (Figure 1). These correlations indicated an acetoxyl group at C-3 and two hydroxyl groups at C-11 and C-19, respectively. The assignment of an oxygenated methylene was further supported by the chemical shift at C-19 because the axial C-19 *C*H_2OH signal (δ_C 64.2) is more upfield than the equatorial C-18 *C*H_2OH signal (above δ_C 70.0).[24] The relative configuration of compound **1** was established by an analysis of the NOESY experiment. The hydroxyl groups at C-3, C-11, and C-19 were shown to be in the β, β, and α orientations, respectively, as deduced from the NOESY correlations of δ_H 5.62 (H-3) with δ_H 1.66 (H-2α) and 4.02 (H-19α) and the NOESY correlations of δ_H 4.24 (H-11) with δ_H 1.70 (H-1α) and 1.02 (H-20α), as well as those of δ_H 4.02 and 3.70 (H-19) with δ_H 1.38 (H-6α) and 1.02 (H-20α) (Figure 2). Therefore, compound **1** was determined to be 11β,19-dihydroxy-3β-acetoxy-*ent*-kaur-16-en-15-one and was named excisusin A. The absolute configuration of **1** was determined from the CD spectrum, which showed first negative, second positive, and third negative Cotton effects. The first negative Cotton effect (λ_{max} 345 nm) corresponding to an enone system was the same as that of *ent*-kaurenes inflexarabdonin (**6**) (λ_{max} 342 nm: -0.77)[17] and *ent*-20-acetoxy-11α-hydroxy-16-kauren-15-one (λ_{max} 342 nm: -0.68).[25]

Compound **2**, a white amorphous powder, had the molecular formula $C_{22}H_{32}O_5$, as determined by positive HRFABMS (*m*/*z* 399.2149 $[M + Na]^+$; calcd 399.2147). Comparison of the ^{13}C NMR and DEPT data of compound **2** with those of the known compound inflexanin B (**10**) revealed the only difference to be the substitution of an oxygenated methine group at C-6 in compound **10** with a methylene group in compound **2**.[20] The HMBC correlations of H-5 (δ_H 1.51, m) and H-7 (δ_H 2.30, m) with C-6 (δ_C 18.6) further confirmed the above deductions. Moreover, the cross-peaks in the NOESY spectrum of compound **2** indicated that the corresponding substituents in compound **2** had the same orientations as those of compound **10**. The CD spectrum of **2** was characteristic of an *ent*-kaurene derivative.[17,25] Hence, compound **2** was determined to be 1α,11β-dihydroxy-3β-acetoxy-*ent*-kaur-16-en-15-one and was named excisusin B.

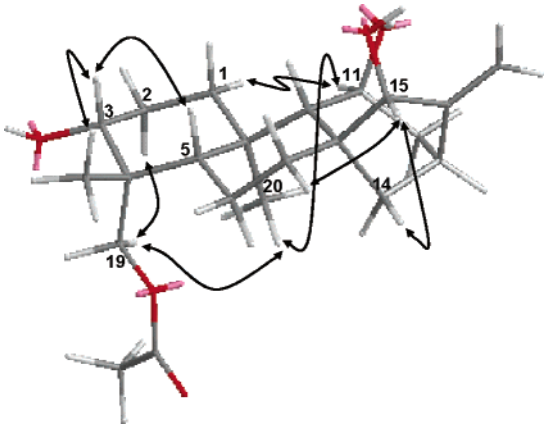

Figure 3. Key correlations of compound **3** in the NOESY spectrum.

Compound **3**, obtained as an amorphous powder, had the molecular formula $C_{22}H_{34}O_5$ according to the positive HRFABMS (*m*/*z* 401.2296 $[M + Na]^+$, calcd 401.2304). This compound showed no UV absorption bands above 230 nm typical of a ketone conjugated with an *exo*-methylene in its spectrum but showed signals of an *exo*-methylene moiety [δ_H 5.38 and 5.17 (each 1H, br s, H-17) in its ^1H NMR and δ_C 159.5, 105.8 in its ^{13}C NMR spectra].[26] Considering structures of diterpenoids previously isolated from the genus *Isodon*, as well as detailed interpretation of the NMR data, compound **3** appeared to be a 15-hydroxy-*ent*-kaur-16-ene with an acetoxyl and two additional hydroxyl groups as substituents. The HMBC correlations from H-19 (δ_H 4.75 and 4.54, d) to C-3 (δ_C 78.0), C-4 (δ_C 42.7), C-18 (δ_C 23.5), and the acetoxyl carbon (δ_C 171.1) indicated that the acetoxyl group was at C-19. Further HMBC correlations from H-3 (δ_H 3.49, d) to C-1 (δ_C 39.0), C-18 (δ_C 23.5), and C-19 (δ_C 66.6) and from H-11 (δ_H 4.13, br s) to C-8 (δ_C 45.3), C-10 (δ_C 37.6), and C-13 (δ_C 40.1) suggested that the hydroxyl groups were present at C-3 and C-11, respectively (Figure 1). The hydroxyl group at C-15 was determined to be β-oriented from the upfield shift of C-9 (δ_C 56.4) of compound **3** compared with that of compound **1** (δ_C 64.4) due to the γ-gauche steric compression effect between 15β-OH and C-9,[27] as well as from observation of NOE correlations between H-15α (δ_H 4.06, d) and H-14β (δ_H 1.08, m). Furthermore, the cross-peaks of H-3 (δ_H 3.49) with H-5β (δ_H 1.04) and CH_3-18 (δ_H 1.35) and of H-11 (δ_H 4.13) with H-1α (δ_H 1.70) and H-12α (1.90) in the NOESY spectrum confirmed that the C-3 and C-11 hydroxyl groups were α- and β-oriented, respectively (Figure 3). Therefore, compound **3** was determined to be 3α,11β,15β-trihydroxy-19-acetoxykaur-16-ene and was named excisusin C. However, the absolute configuration of **3** remains to be clarified.

Compound **4**, a white amorphous powder, was determined from its positive HRFABMS (*m*/*z* 415.2104 $[M + Na]^+$; calcd 415.2097) to have the same molecular formula ($C_{22}H_{32}O_6$) as inflexanin B (**10**). The ^1H and ^{13}C NMR spectra of compound **4** suggested its structure to be similar to that of inflexanin B (**10**).[20] The only difference between these two compounds was the position of an acetoxyl group. HMBC correlations between H-11 (δ_H 6.92) and an acetoxyl carbonyl carbon (δ_C 170.1) clearly indicated the acetoxyl group to be at C-11 rather than at C-3 as in **10**. A NOESY experiment confirmed the relative configurations of compound **4**, wherein the correlations were observed from H-1β to H-5β and H-9β, from H-3α to H-2α and Me-19, from H-6β to H-5β and H-7β, and from H-11α to H-1α and Me-20. These results indicated that the C-1, C-3, and C-6 hydroxyl groups and the C-11 acetoxyl

Table 1. ^{13}C NMR Data of Compounds **1**−**5** (125 MHz, C_5D_5N, δ in ppm)

position	**1**	**2**	**3**	**4**	**5**
1	33.4	75.9	39.0	76.8	38.4
2	23.0	34.0	28.1	37.5	34.4
3	73.8	78.9	78.0	77.1	212.3
4	43.1	36.8	42.7	39.2	51.3
5	50.8	49.3	55.4	47.7	55.6
6	18.7	18.6	21.5	66.6	19.6
7	35.0	35.2	40.3	43.3	33.5
8	50.8	51.2	45.3	50.0	50.0
9	64.4	65.3	56.4	61.1	62.4
10	38.6	44.8	37.6	45.1	37.7
11	65.0	66.8	65.6	72.1	66.2
12	41.5	41.6	42.8	38.4	41.3
13	37.8	38.2	40.1	38.0	36.7
14	37.2	37.9	36.3	39.0	36.4
15	208.8	209.0	82.8	209.7	208.8
16	151.7	152.5	159.5	151.5	149.7
17	111.1	110.1	105.8	111.3	113.6
18	22.9	27.9	23.5	29.8	21.6
19	64.2	21.7	66.6	24.5	65.7
20	18.1	14.4	17.4	15.2	17.6
$O\underline{C}OCH_3$	170.4	170.3	171.1	170.1	170.9
$OC\underline{O}CH_3$	21.1	20.9	21.2	21.5	20.9

group had α, *β*, α, and *β* orientations, respectively. The absolute configuration of **4** was determined by the measurement of the CD spectrum (see Experimental Section).[17,25] Therefore, compound **4** was determined to be 1α,3*β*,6α-trihydroxy-11*β*-acetoxy-*ent*-kaur-16-en-15-one and was named excisusin D.

Compound **5**, a white amorphous powder, showed a molecular ion peak $[M]^+$ at *m*/*z* 374 in the EIMS, consistent with the formula $C_{22}H_{30}O_5$ deduced from HRFABMS (*m*/*z* 397.1994 $[M + Na]^+$; calcd 397.1991). The ^1H and ^{13}C NMR and DEPT spectra of **5** were similar to those of inflexarabdonin I (**6**)[17] except for the presence of an oxygenated methylene signal (δ_H 3.98 and 4.51, δ_C 65.7) and an oxygenated methine signal (δ_H 4.08, δ_C 66.2) instead of a tertiary methyl and two hydroxyl signals in **6**. In the HMBC spectrum of compound **5**, the CH_3-19 signals at δ_H 3.98 and 4.51 correlated with the acetoxyl carbonyl carbon at δ_C 170.9, indicating that the acetoxyl group was located at C-19. The position of the additional hydroxyl group at C-11 was further determined from the HMBC correlations between H-11 (δ_H 4.08, d) and C-8 (δ_C 50.0), C-9 (δ_C 62.4), C-10 (δ_C 37.7), C-12 (δ_C 41.3), and C-13 (δ_C 36.7). The relative stereochemistry of compound **5** was also revealed by the NOESY spectrum, in which correlations between H-11α and CH_3-20 and H-19α were observed, suggesting that the C-11 hydroxyl group should be in the *β* orientation. The absolute configuration of **5** was determined by the measurement of the CD spectrum (see Experimental Section).[17,25] Hence, compound **5** was determined to be 11*β*-hydroxy-19-acetoxy-*ent*-kaur-16-ene-3,15-dione and was named excisusin E.

All of the isolates (**1**−**12**) were examined for their ability to inhibit activation of NF-κB in LPS-stimulated RAW264.7 cells using the NF-κB-mediated reporter gene assay system.[28] As shown in Table 2, all of the compounds, except for excisusin C (**3**) and inflexarabdonin A (**12**), exhibited inhibitory activity, with IC_{50} values ranging from 0.2 to 3.0 μM. Parthenolide was included as the positive control. Since the activation of NF-κB results in expression of inflammatory enzymes such as iNOS, the effects of kauranoids **1**−**12** on LPS-induced NO production were investigated in RAW264.7 cells, with aminoguanidine used as a positive control. The observed IC_{50} values (Table 2) were comparable to those of NF-κB activation. The cell viability measured by the MTT assay showed that none the compounds tested had significant cytotoxicity to the RAW264.7 cells at their effective concentration for the inhibition of NF-κB activation and NO production (data not shown). These results demonstrate that excisusin C (**3**) and inflexarabdonin A (**12**), which lack the carbonyl group at C-15, did not have an inhibitory effect on NF-κB activation and NO production even at 10 μM. These results, along with those previously reported, suggest that the α-methylenecyclopentanone system present in each of the active *ent*-kauranes was the active center for these inhibitory effects.[2,29]

Table 2. Inhibition of NF-κB Activation and NO Production by the Isolated Compounds **1**−**12** [IC_{50} values (μM)][a]

compound	NF-κB activation	NO production
1	0.90 ± 0.01	0.67 ± 0.01
2	0.26 ± 0.01	0.56 ± 0.01
3	>10	>10
4	3.21 ± 0.05	2.89 ± 0.17
5	1.02 ± 0.16	1.36 ± 0.07
6	1.15 ± 0.06	1.24 ± 0.02
7	0.24 ± 0.04	0.48 ± 0.01
8	0.23 ± 0.02	0.69 ± 0.01
9	0.40 ± 0.12	0.63 ± 0.01
10	1.82 ± 0.21	2.52 ± 0.07
11	0.44 ± 0.08	0.94 ± 0.01
12	>10	>10
positive control	1.15 ± 0.01	32.17 ± 0.07

[a] The data are presented as the mean ± SD from three separate experiments. The inhibitory effects are represented as the molar concentration (μM) giving 50% inhibition (IC_{50}) relative to the vehicle control. Positive control for NF-κB activation: parthenolide. Positive control for NO production: aminoguanidine.

In conclusion, these results support the pharmacological basis of this plant being used as a traditional herbal medicine for the treatment of inflammation and cancer. However, further studies will be needed to determine how these *ent*-kaurane diterpenoids inhibit NF-κB activation.

Experimental Section

General Experimental Procedures. The optical rotations were measured using a JASCO DIP-1000 polarimeter. The UV and IR spectra were obtained on JASCO UV-550 and Perkin-Elmer model LE599 spectrometers, respectively. The ^1H and ^{13}C NMR and 2D-NMR spectra were recorded on a Bruker DRX 500 MHz NMR spectrometer using $CDCl_3$, C_5D_5N, or CD_3OD as solvent. High-resolution fast atom bombardment (HRFAB) and electron impact (EI) mass spectra were obtained on JMS 700 (JEOL) and Hewlett-Packard MS 5988 mass spectrometers, respectively. Preparative HPLC was carried out using a Waters 515 pump, a 2996 photodiode array detector, and a YMC J'sphere ODS-H80 column (4 um, 150 × 20 mm). The column was eluted with a mixed solvent system of ACN−H_2O at a flow rate of 6.5 mL/min. Open column chromatography was performed using silica gel (Kieselgel 60, 70−230 mesh, Merck) and Lichroprep RP-18 (40−63 μM, Merck). Thin-layer chromatography (TLC) was performed using precoated silica gel 60 F_{254} (0.25 mm, Merck) plates.

Plant Material. The aerial parts of *Isodon excisus* were collected from Hwacheon, Kangwondo, Korea, in August 2003, and were identified by emeritus professor Kyong Soon Lee, a plant taxonomist at Chungbuk National University. A voucher specimen of this plant was deposited at the Herbarium of the College of Pharmacy, Chungbuk National University, Korea (CBNU0308).

Extraction and Isolation. The air-dried aerial parts of *I. excisus* (1.6 kg) were pulverized and extracted three times with MeOH (3 × 15 L) at room temperature. The extract was combined and concentrated under vacuum at 60 °C. The residue was suspended in 90% MeOH and then partitioned with *n*-hexane (3 × 1.5 L) and CH_2Cl_2 (3 × 1.5 L). The CH_2Cl_2 extract was found to inhibit the activation of NF-κB with an IC_{50} value of 4.5 μg/mL. This extract (13.7 g) was then chromatographed over silica gel (9 × 25 cm), eluted with CH_2Cl_2−MeOH (1:0 to 1:1, then pure MeOH), to give seven fractions (IEA−IEG). Fraction IED (4.1 g) was applied to a silica gel column (3 × 25 cm) and eluted with *n*-hexane−acetone (5:1, 3:1, 3:2, then pure acetone) to yield six fractions (IED1−IED6). Fraction IED6 (0.3 g) was subjected to flash column chromatography on RP-18 (2 × 30 cm) and eluted with ACN−H_2O (10, 20, and 30% ACN), affording seven subfractions (IED61−IED67). Subfractions IED62, IED64, and IED66 were further purified by semipreparative HPLC using ACN−H_2O (50:50) at a flow

rate of 6.5 mL/min to yield compounds **6** (2.9 mg), **1** (5.1 mg), and **5** (3.2 mg). Fraction IED4 (1.1 g) was further chromatographed with CH_2Cl_2−MeOH (90:1) to afford four subfractions (IED41−IED44). Subfraction IED43 was subjected to silica gel chromatography (2 × 20 cm) and eluted with CH_2Cl_2−acetone (30:1 to 10:1). The subfraction was then subjected to further chromatography over RP-18 (2 × 30 cm) and eluted with ACN−H_2O (30:70) to provide **9** (8.4 mg), **11** (25.9 mg), and **12** (12.5 mg). Fraction IEF (4.1 g) was applied to a silica gel column (3 × 25 cm) and eluted with *n*-hexane−acetone (5:1, 3:1, 3:2, 1:1, and then pure acetone) to afford six subfractions (IEF1−IEF6). Subfraction IEF3 (0.5 g) was further subjected to flash column chromatography on RP-18 (2 × 30 cm) and eluted with ACN−H_2O (50:50) to give six subfractions (IEF31−IEF36). Subfraction IEF35 was subjected to column chromatography over silica gel (2 × 20 cm) and eluted with CH_2Cl_2−MeOH (50:1, 30:1, 20:1) to afford four fractions (IEF35A−IEF35D). Fractions IEF35A, IEF35C, and IEF35D were further purified by semipreparative HPLC and eluted with ACN−H_2O (23:77) at a flow rate of 6.5 mL/min to yield compounds **2** (13.1 mg), **3** (20.0 mg), and **4** (6.1 mg), respectively. Fraction IEF4 (1.2 g) was subjected to flash column chromatography on RP-18 (2 × 30 cm) and eluted with ACN−H_2O (50:50) to give six fractions (IEF41−IEF46). Fraction IEF41 was subjected to flash column chromatography on RP-18 (2 × 30 cm) and eluted with ACN−H_2O (20:80) to yield inflexanin B (**10**, 550 mg). IEC (1.5 g) was further applied to a silica gel column (3 × 20 cm) and eluted with *n*-hexane−acetone (5:1, 3:1, 3:2, then pure acetone), affording five fractions (IEC1−IEC5). Fractions IEC3 and IEC5 were subjected to flash column chromatography on RP-18 (2 × 30 cm) and eluted with ACN−H_2O (30:70) to give **9** (59.9 mg) and **11** (363 mg). Fraction IEB (0.2 g) was then separated on a silica gel column (2 × 20 cm) and eluted with *n*-hexane−acetone (5:1, 3:1, 3:2, then pure acetone) to yield three fractions (IEB1−IEB3). Fractions IEB1 and IEB3 were further purified by semipreparative HPLC and eluted with ACN−H_2O (70:30) at a flow rate of 6.5 mL/min to yield **8** (9.2 mg) and **7** (3.5 mg).

Excisusin A (1): white amorphous powder; $[\alpha]^{25}_D$ −39 (*c* 0.18, MeOH); UV (MeOH) λ_{max} (log ϵ) 242 (3.85) nm; CD (MeOH) λ_{max} nm ($\Delta\epsilon$) 345 (−0.4), 240 (+0.6), 212 (−2.3); IR (KBr) ν_{max} 3444, 2924, 1714, 1644, 1260, 1046 cm^{-1}; ^{1}H NMR (C_5D_5N, 500 MHz) δ 6.00 (1H, s, H-17a), 5.62 (1H, br s, H-3α), 5.23 (1H, s, H-17b), 4.24 (1H, d, J = 4.0 Hz, H-11α), 4.02 (1H, d, J = 11.0 Hz, H-19a), 3.70 (1H, d, J = 11.0 Hz, H-19b), 3.01 (1H, br s, H-13α), 2.36 (1H, br d, J = 12.0 Hz, H-14α), 2.30 (1H, dd, J = 13.5, 3.5 Hz, H-7α), 2.24 (1H, d, J = 14.0 Hz, H-12α), 2.09 (1H, dt. J = 14.0, 3.5 Hz, H-12β), 1.97 (1H, overlapped, H-9β), 1.96 (3H, s, OAc), 1.70 (1H, m, H-1α), 1.69 (1H, m, H-6α), 1.67 (1H, overlapped, H-5β), 1.66 (1H, overlapped, H-2α), 1.50 (1H, dd, J = 2.0, 3.5 Hz, H-1β), 1.44 (1H, br d, J = 13.5 Hz, H-7β), 1.38 (1H, m, H-6β), 1.35 (1H, m, H-2β), 1.34 (1H, m, H-14β), 1.26 (3H, s, Me-18), 1.02 (3H, s, Me-20); HRFABMS *m*/*z* 399.2159 (calcd for $C_{22}H_{32}O_5Na$, 399.2147).

Excisusin B (2): white amorphous powder; $[\alpha]^{25}_D$ −56 (*c* 0.21, MeOH); UV (MeOH) λ_{max} (log ϵ) 243 (4.02) nm; CD (MeOH) λ_{max} nm ($\Delta\epsilon$) 345 (−0.6), 240 (+0.8), 212 (−2.6); IR (KBr) ν_{max} 3455, 2945, 1721, 1641, 1245, 1030 cm^{-1}; ^{1}H NMR (C_5D_5N, 500 MHz) δ 6.15 (1H, d, J = 4.5 Hz, 1-OH), 6.01 (1H, br d, J = 4.0 Hz, H-11α), 5.99 (1H, s, H-17a), 5.75 (1H, br s, 11-OH), 5.21 (1H, s, H-17b), 4.92 (1H, br s, H-3α), 4.13 (1H, t, J = 4.5 Hz, H-1α), 3.03 (1H, m, H-13α), 2.47 (1H, d, J = 11.5 Hz, H-14α), 2.33 (1H, br s, H-9β), 2.29 (1H, dd, J = 12.0, 3.5 Hz, H-7α), 2.23 (1H, m, H-12α), 2.20 (1H, m, H-2α), 2.09 (1H, dt, J = 14.5, 4.0 Hz, H-2β), 1.90 (3H, s, OAc), 1.51 (1H, m, H-12β), 1.51 (1H, overlapped, H-5β), 1.43 (1H, m, H-6α), 1.42 (1H, m, H-7β), 1.37 (1H, m, H-6β), 1.37 (1H, m, H-14β), 1.34 (3H, s, Me-20), 0.86 (3H, s, Me-18), 0.85 (3H, s, Me-19); HRFABMS *m*/*z* 399.2149 (calcd for $C_{22}H_{32}O_5Na$, 399.2147).

Excisusin C (3): white amorphous powder; $[\alpha]^{25}_D$ −58 (*c* 0.28, MeOH); UV (MeOH) λ_{max} (log ϵ) 208 (4.32) nm; IR (KBr) ν_{max} 3434, 2940, 1725, 1366, 1214, 1043, 958 cm^{-1}; ^{1}H NMR (C_5D_5N, 500 MHz) δ 5.75 (1H, d, J = 10.0 Hz, 15-OH), 5.38 (1H, s, H-17a), 5.17 (1H, s, H-17b), 4.75 (1H, d, J = 12.0 Hz, H-19a), 4.54 (1H, d, J = 12.0 Hz, H-19b), 4.06 (1H, br d, J = 10.0 Hz, H-15α), 4.13 (1H, br s, H-11α), 3.49 (1H, br d, J = 11.3 Hz, H-3β), 2.64 (1H, br s, H-13α), 2.11 (1H, br d, J = 6.8 Hz, H-12α), 2.06 (3H, s, OAc), 2.01 (1H, br d, J = 12.5 Hz, H-6α), 1.96 (1H, m, H-1α), 1.94 (1H, overlapped, H-14α), 1.94 (1H, br d, J = 8.7 Hz, H-9β), 1.90 (1H, m, H-12β), 1.84 (1H, br d, J = 8.0 Hz, H-2α), 1.62 (1H, br d, J = 12.5 Hz, H-6β), 1.51 (1H, br d, J = 12.5 Hz, H-7α), 1.35 (3H, s, Me-18), 1.30 (1H, br d, J = 12.5 Hz, H-7β), 1.08 (1H, m, H-1β), 1.08 (1H, m, H-14β), 1.04 (1H, m, H-5β), 1.01 (3H, s, Me-20); HRFABMS *m*/*z* 401.2296 (calcd for $C_{22}H_{34}O_5Na$ 401.2304).

Excisusin D (4): white amorphous powder; $[\alpha]^{25}_D$ −55 (*c* 0.14, MeOH); UV (MeOH) λ_{max} (log ϵ) 243 (3.75) nm; CD (MeOH) λ_{max} nm ($\Delta\epsilon$) 348 (−0.4), 278 (+0.1), 227 (−4.3); IR (KBr) ν_{max} 3550, 2933, 1728, 1647, 1326, 1225, 1027 cm^{-1}; ^{1}H NMR (C_5D_5N, 500 MHz) δ 6.92 (1H, br d, J = 4.0 Hz, H-11α), 5.99 (1H, s, H-17a), 5.18 (1H, s, H-17b), 4.75 (1H, br s, H-6β), 4.53 (1H, dd, J = 12.4 Hz, H-1α), 3.73 (1H, br s, H-3α), 3.23 (1H, d, J = 12.6 Hz, H-14α), 2.96 (1H, br s, H-13α), 2.53 (1H, m, H-2α), 2.52 (1H, br d, J = 13.5 Hz, H-7β), 2.28 (1H, m, H-12α), 2.24 (1H, m, H-2β), 2.22 (1H, br s, H-9β), 2.12 (1H, m, H-12β), 2.00 (3H, s, Me-20), 1.97 (1H, br s, H-5β), 1.75 (1H, m, H-7α), 1.75 (1H, m, H-14β), 1.79 (3H, s, OAc), 1.59 (3H, s, Me-19), 1.33 (3H, s, Me-18); HRFABMS *m*/*z* 415.2104 (calcd for $C_{22}H_{32}O_6Na$ 415.2097).

Excisusin E (5): white amorphous powder; $[\alpha]^{25}_D$ −35 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ϵ) 242 (3.55) nm; CD (MeOH) λ_{max} nm ($\Delta\epsilon$) 345 (−0.6), 240 (+1.2), 212 (−3.3); IR (KBr) ν_{max} 3395, 2965, 1729, 1645, 1447, 1370, 1234, 1038 cm^{-1}; ^{1}H NMR (C_5D_5N, 500 MHz) δ 5.90 (1H, s, H-17a), 5.31 (1H, s, H-17b), 4.51 (1H, d, J = 11.4 Hz, H-19a), 4.08 (1H, br d, J = 4.4 Hz, H-11α), 3.98 (1H, d, J = 11.4 Hz, H-19b), 3.10 (1H, br s, H-13α), 2.68 (1H, ddd, J = 16.8, 6.5, 3.6 Hz, H-2α), 2.46 (1H, ddd, J = 16.8, 6.5, 3.6 Hz, H-2β), 2.36 (1H, d, J = 12.0 Hz, H-14α), 2.19 (1H, overlapped, H-1β), 2.19 (1H, overlapped, H-12α), 2.01 (1H, overlapped, H-12β), 2.01 (3H, s, OAc), 2.00 (1H, m, H-7α), 1.60 (1H, br s, H-5β), 1.59 (1H, overlapped, H-1α), 1.47 (1H, br s, H-9β), 1.45 (1H, overlapped, H-7β), 1.21 (3H, s, Me-20), 1.20 (3H, s, Me-18); EIMS *m*/*z* 374 [M]$^+$; HRFABMS *m*/*z* 397.1994 (calcd for $C_{22}H_{30}O_5Na$ 397.1991).

NF-κB Activity Assay. The NF-κB inhibitory activity was determined using a method described elsewhere.[28] The RAW264.7 cells were transfected with a plasmid containing eight copies of the κB elements linked to SEAP (secreted alkaline phosphate) gene. The 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, the cells were treated with various concentrations of the test compounds (0.1, 0.3, 1, 3, and 10 μM) and LPS (1 μg/mL) for 24 h. Then, 100 μL of a 2× SEAP assay buffer (2 M diethanolamine, 1 mM $MgCl_2$, 20 mM L-homoarginine) was added to each well and the incubation continued at 37 °C for 10 min. The reaction was initiated by adding 20 μL of 120 mM *p*-nitrophenyl phosphate dissolved in a 1× SEAP assay buffer and incubated at 37 °C. The absorbance of the reaction mixture was measured at 405 nm using a microplate reader (Molecular Devices Co., Menlo Park, CA).

Determination of NO Production and the Cell Viability Assay. The level of NO production was determined by measuring the amount of nitrite from the cell culture supernatants as described previously.[28] Briefly, the RAW264.7 cells (1×10^5 cells/well) were stimulated with or without 1 μg/mL of LPS (Sigma Chemical Co., St. Louis, MO) for 24 h in the presence or absence of the test compounds (0.1, 0.3, 1, 3, and 10 μM). The cell culture supernatant (100 μL) was then reacted with 100 μL of Griess reagent. The remaining cells after the Griess assay were used to test their viability using a MTT (Sigma Chemical Co., St. Louis, MO)-based colorimetric assay as previously described.[28]

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Note Added after ASAP Publication: There is a change in the IC_{50} value of the CH_2Cl_2 extract in the version posted on March 7, 2007.

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