Inhibition of NF-κB Activation and iNOS Induction by *ent*-Kaurane Diterpenoids in LPS-Stimulated RAW264.7 Murine Macrophages

Silvia Aquila,[†] Zhi-Ying Weng,[‡] Yue-Qin Zeng,[‡] Han-Dong Sun,*[,§] and José Luis Ríos*[,†]

Departament de Farmacologia, Facultat de Farmàcia, Universitat de València, Avenida Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain, Department of Pharmacy, Kunming Medical College, Kunming 650031, People's Republic of China, and State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, People's Republic of China

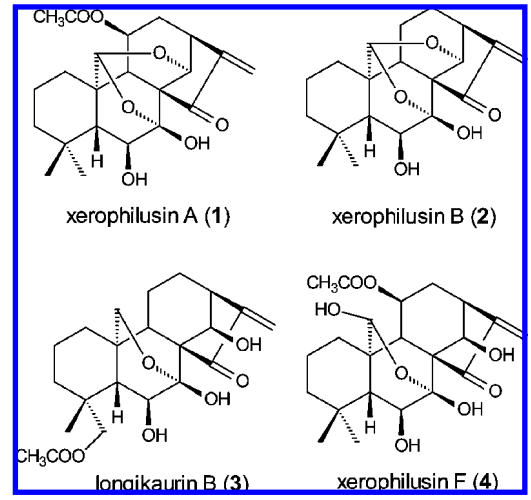
Received March 3, 2009

Xerophilusin A (**1**), xerophilusin B (**2**), longikaurin B (**3**), and xerophilusin F (**4**) from *Isodon xerophylus* inhibit LPS-induced NO production in RAW 264.7 macrophages, with IC_{50} values of 0.60, 0.23, 0.44, and 0.67 μM, respectively, and they all inhibited mRNA production in these same cells. They decreased the luciferase activity in RAW 264.7 cells transiently transfected with the NF-κB-dependent luciferase reporter, with IC_{50} values of 1.8, 0.7, 1.2, and 1.6 μM, respectively. Compounds **1**−**3** reduced NF-κB activation, with compound **4** showing no effect, but p65 translocation from the cytoplasm to the nucleus and the LPS-induced degradation of IκB were inhibited by all four test compounds. These findings indicate that *ent*-kauranes are potential anti-inflammatory agents, with a specific mechanism in which both the inhibition of NF-κB translocation and the consequent decrease of pro-inflammatory mediators are implicated.

Isodon xerophylus (C.Y. Wu et H.W. Li) H. Hara (Lamiaceae) is a perennial shrub with antitumor, anti-inflammatory, and antibacterial properties.[1] Several previous studies have described the chemical composition of this species, in which diterpenoids with an *ent*-kaurane structure are predominant.[2,3]

Naturally occurring diterpenes have been proven to exert various biological activities. Of these compounds, the *ent*-kauranoids comprise a specific group of diterpenoids studied for their cytotoxic activity[2−7] and, to a lesser extent, as potential anti-inflammatory agents.[7−9] In general, many terpenoids,[10] and *ent*-kauranes in particular,[7−9] exhibit anti-inflammatory activity, an effect related to various mechanisms such as the inhibition of the neutrophil response due to the inhibition of cytosolic Ca^{2+} release by 16β,17-dihydroxy-*ent*-kauran-19-oic acid,[9] as well as the inhibition of prostaglandin E_2 production through the suppression of nuclear factor-κB (NF-κB) activation.[8] This latter mechanism seems to be more relevant for the anti-inflammatory effect of *ent*-kauranes, making these compounds good candidates for NF-κB activity modulation through interference with the steps leading up to the release of IκB kinase (IKK). This, in turn, can most likely be accomplished by influencing the activity of NF-κB-inducing kinase (NIK).[11]

NF-κB is responsible for regulating the transcription of a large number of genes involved in inflammation, immunity, cancer, and apoptosis.[12] This transcription factor is present in the cytoplasm of all cells as an inactive protein bound to its inhibitor protein IκB. The process of NF-κB activation begins when a given stimulus activates IKK, which then triggers site-specific phosphorylation of the inhibitor of NF-κBα (IκBα)[13] and is rapidly degraded by the proteasome complex. The free NF-κB is then translocated to the nucleus, where it binds to various target genes.[14] Among its other functions, NF-κB controls the expression of genes encoding pro-inflammatory cytokines and chemokines and also has an effect on the expression of genes that encode inducible enzymes such as cyclooxygenase-2 and inducible nitric oxide synthase (iNOS).[15] Since control of NF-κB activation constitutes a relevant target for the treatment of inflammatory diseases, molecules with the capacity to inhibit the consecutive steps leading to NF-κB activation have attracted increasing interest.[11] While different methods for inhibiting the various NF-κB pathways may also be relevant, NF-κB cytoplasmatic regulation is probably the most effective strategy for controlling NF-κB activation.[16,17] Recently, several investigators have reported the effects of *ent*-kauranes from *Isodon excisus* on NF-κB activation and nitric oxide (NO) production,[18,19] affirming that these mechanisms may be the manner in which these compounds can be developed into effective anti-inflammatory and anticancer agents. In their extensive study of these mechanisms in eriocalyxin isolated from *I. eriocalyx*[16] and other related diterpenoids isolated from *I. rubescens*,[17] Leung et al.[17] demonstrated that these *ent*-kauranes inhibit NF-κB activation by blocking the binding of NF-κB to its response element through a reversible interaction with the p65 and p50 proteins at an allosteric site. In addition, Giang et al.[20] studied several structurally related *ent*-kauranes isolated from *Croton tonkinensis* found to have the same properties. Recently, Weng et al.[7] isolated two new *ent*-kauranes, xerophinoids A and B, which inhibited NO production as well as those of tumor necrosis factor (TNF)-α and interleukin (IL)-1β, all of which could be related to the inhibition of NF-κB activation. In the present study, we have examined the effects of *I. xerophylus* constituents xerophilusin A (**1**), xerophilusin B (**2**), longikaurin B (**3**), and xerophilusin F (**4**) on NF-κB activation, in order to determine the anti-inflammatory potential of these compounds.



* To whom correspondence should be addressed. (H.-D.S.) Tel: +86 871-5223251. Fax: +86 871-5216343. E-mail: hdsun@mail.kib.ac.cn. (J.L.R.) Tel: +34 963544973. Fax: +34 963544498. E-mail: riosjl@uv.es.
[†] Universitat de València.
[‡] Kunming Medical College.
[§] Kunming Institute of Botany, Chinese Academy of Sciences.

10.1021/np9001465 CCC: $40.75
Published on Web 07/02/2009

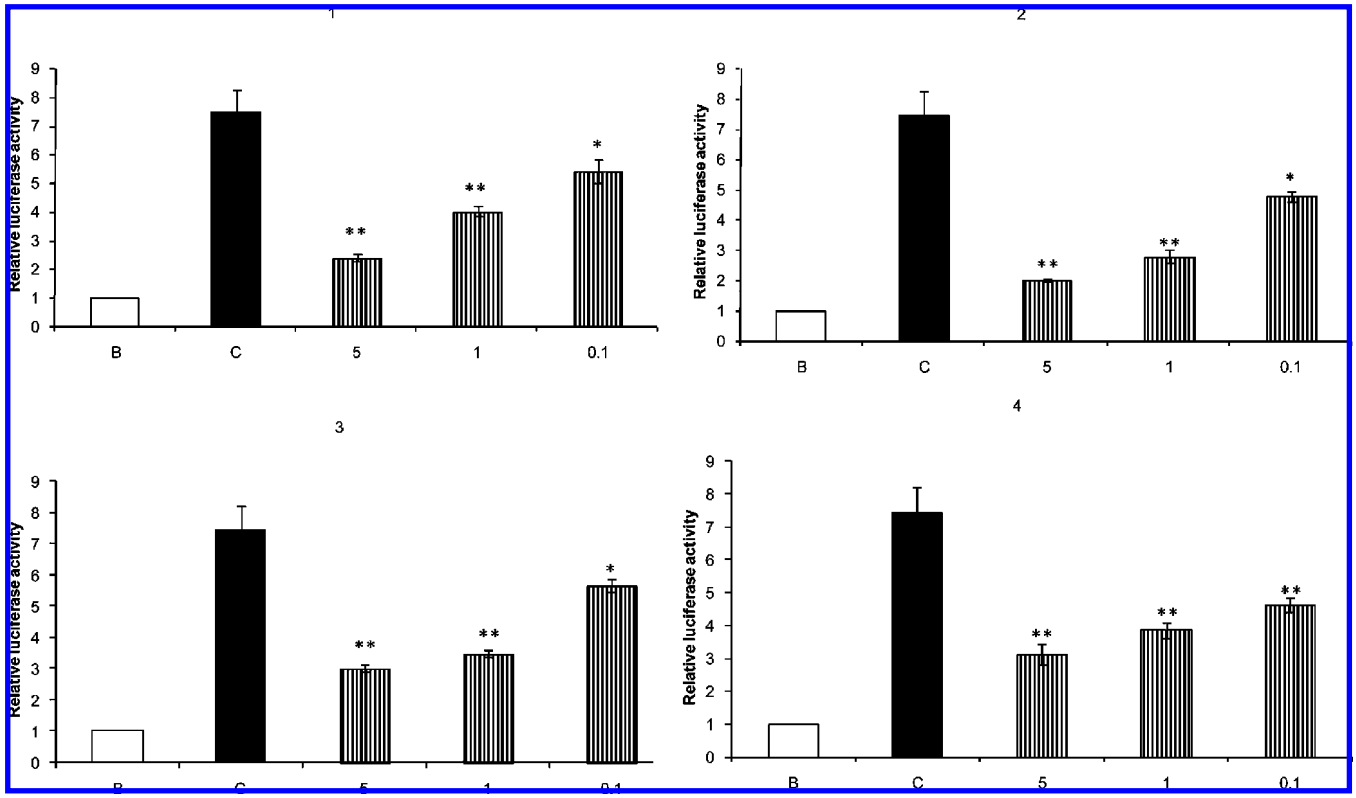


Figure 1. Effects of *ent*-kauranes (5.0, 1.0, and 0.1 μM) on the transcriptional activity of NF-κB in stimulated RAW 264.7 cells transiently transfected with the NF-κB reporter gene. Cells were pretreated with test compounds and stimulated with LPS for 24 h. The transcriptional activity was detected through the luciferase activity and expressed as the comparative degree of control represented by this activity, in arbitrary units, in stimulated cells without the test compounds. Data represent mean ± SD of relative luciferase activity of at least three values. Values were considered statistically significant (versus control) for $p < 0.05$ (*) and $p < 0.01$ (**).

Results and Discussion

Incubation of RAW 264.7 cells with compounds **1** and **2** showed no toxicity at values of 5 μM. In contrast, compound **3** showed no toxicity at 10 μM, while compound **4** was not toxic at 25 μM. At these same concentrations viability was higher than 97.5%, with IC_{50} values of 11.3 μM (50−0.1 μM, $r^2 = 0.9993$), 10.1 μM (50−0.1 μM, $r^2 = 0.9969$), 44.8 μM (50−0.1 μM, $r^2 = 0.9958$), and 52.4 μM (100−10 μM, $r^2 = 1.0000$) for **1**−**4**, respectively.

All four kauranes inhibited the NO production in RAW 264.7 macrophages induced by lipopolysaccharide (LPS, 1 μg/mL) in a concentration-dependent manner. The IC_{50} values obtained for **1**−**4** were 0.600 μM (10−0.1 μM, $r^2 = 0.9649$), 0.225 μM (1−0.05 μM, $r^2 = 0.9627$), 0.435 μM (1−0.05 μM, $r^2 = 0.9930$), and 0.673 μM (10−0.1 μM, $r^2 = 0.9443$), respectively. To determine whether these compounds inhibit NO production through an inhibition of the gene expression, we studied their effects on iNOS induction in RAW 264.7 macrophages using western blot analysis techniques. All four compounds inhibited iNOS expression, as can be seen in Figure S1A, Supporting Information. Moreover, they all inhibited mRNA production in these same cells at a concentration of 5 μM (Figure S1B, Supporting Information).

Since the production of NO in stimulated RAW 264.7 cells is regulated by iNOS induction, which is, in turn, controlled by NF-κB, the effects were studied of **1**−**4** on the activation of this nuclear factor using a luciferase reporter gene assay. Thus, RAW 264.7 cells were transfected transiently with the NF-κB-dependent luciferase reporter and subsequently treated with LPS (1 μg/mL) and then with **1**−**4** (5.0, 1.0, and 0.1 μM) for 24 h. Luciferase activity was reduced in the cells treated with the four test compounds, with IC_{50} values of 1.8, 0.7, 1.2, and 1.6 μM, respectively. A correlation between the effect on NO production and the inhibition of luciferase activity was also observed (Figure 1).

The stimulation of RAW 264.7 cells with LPS activates the transcription factor NF-κB and, as a result, the expression of different genes. The effects were tested of the *ent*-kauranes on NF-κB activation and the translocation of p65, as well as on IκB degradation. NF-κB activation was clearly reduced in the presence of test compounds **1**−**3** (1 μM), whereas compound **4** had no effect (Figure S2, Supporting Information). In contrast, p65 translocation from the cytoplasm to the nucleus was inhibited by all four test compounds, as shown in Figures S3 and S4A, Supporting Information. Compound **4** again exhibited a different behavior in that its effect was dependent on the amount of LPS stimulation time. Finally, all four *ent*-kauranes tested were found to inhibit the LPS-induced degradation of IκB (Figure S4B, Supporting Information).

Inflammation is a complex biological response of tissues to different stimuli or irritants. In this process, cells such as macrophages are implicated directly by the overproduction of mediators such as NO, which has been associated with persistent inflammation and tissue destruction.[21] As such, modulation of the biosynthesis or activity of NO could result in the amelioration of the pathogenesis of inflammatory processes. After stimulation, NO is synthesized by the enzyme iNOS, which is previously induced in the corresponding inflammatory cells, including polymorphonuclear leukocytes and resident macrophages.[22] As mentioned above, the expression of this enzyme occurs upon stimulation of IKK, which triggers site-specific phosphorylation of IκBα and is subsequently degraded by the proteasome complex. The free NF-κB is then translocated to the nucleus, where it binds to its target genes,[14] one of which induces the enzyme iNOS.[15] As a consequence, NO production in the damaged tissue increases significantly. In this context, various researchers have focused their efforts on the search for new compounds that inhibit NF-κB; terpenoids are one

promising group of compounds that exert specific effects on this transcription factor.[11]

Our study examined the effects of four *ent*-kauranes (**1**−**4**) on LPS-induced NO production as well as on iNOS expression in LPS-stimulated RAW 264.7 macrophages. As mentioned above, all four compounds significantly inhibited NO production in these cells, with IC_{50} values in the nM to μM range. To determine whether this effect was related to the gene expression of the enzyme, we tested the effect of the four *ent*-kauranes on LPS-induced iNOS expression, obtaining a good correlation between the expression of the enzyme and NO production. To study the expression of iNOS in LPS-activated RAW 264.7 cells, the effects were tested on NF-κB activation in the same cell system using an NF-κB reporter gene assay, and it was found that there was a correlation between the aforementioned inhibition of iNOS induction and a decrease in NF-κB transcriptional activity. Moreover, we observed that the induced translocation from the cytoplasm to the nucleus was blocked by each of the four compounds, as seen from the immunofluorescent staining of the p65 protein (see explanation in Figure S3, Supporting Information).

The inhibitory activity of *ent*-kauranes toward NF-κB has been accounted for by invoking the presence of reactive centers. Thus, various studies have focused on the exomethylene group conjugated to a carbonyl group in the cyclopentanone ring.[16−18,20] This reactive group interacts with biological nucleophiles such as the sulfhydryl group of the cysteine residue in the DNA-binding domain of the NF-κB subunit through a Michael-type reaction.[20] In this context, Castrillo et al.[11] described several relevant targets through which *ent*-kauranes can inhibit NF-κB activity. For example, the inhibition of NIK with no coordinate activation of p38 and/or of the extracellular signal-regulated kinase (ERK)-1 and ERK2 leads to the abrogation of the inflammatory response of genes, depending on NF-κB activation. Leung et al.[17] established several differences between five *ent*-kauranes, all α,β-unsaturated ketones, against NF-κB activation. Whereas all five compounds interfered with the DNA-binding activity of NF-κB to its response DNA sequence, only two of them, oridonin and ponidonin, had an additional impact on the translocation of NF-κB from the cytoplasm to the nucleus without affecting IκB-α phosphorylation and degradation. Moreover, another related compound, eryocalyxin B, selectively blocked the binding between NF-κB and the response elements in vivo without affecting the nuclear translocation of the transcription factor. Furthermore, in vitro binding assays suggested that eryocalyxin B interferes with the binding of p65 and p50 subunits to the DNA in a reversible and noncompetitive manner.[17] Because the chemical−structure relationship between the compounds tested by Leung et al.[16,17] and compounds **1**−**4** is similar, several analogies can be established. For example, the presence of an α,β-unsaturated ketone seems to be critical for the activity of these compounds, with the presence of a second α,β-unsaturated ketone increasing potency.[17] However, other functionalities must also be taken into account, such as the presence of hydroxy, acetoxy, or epoxy groups. All the compounds tested, albeit to various degrees, reduced both p65 translocation to the nucleus and IκB degradation. These effects could be explained by the suppression of IκBα phosphorylation and degradation through the inactivation of IKK, as Leung et al. demonstrated previously.[17]

Experimental Section

Test Compounds and Chemicals. Xerophilusin A (**1**), xerophilusin B (**2**), longikaurin B (**3**), and xerophilusin F (**4**) were isolated previously from leaves of *Isodon xerophilus* by Hou et al.[4] Chemicals, biochemicals, and reagents were purchased from Cayman (Ann Arbor, MI), GE Healthcare (Fairfield, CT), Invitrogen (Carlsbad, CA), Panreac (Barcelona, Spain), Promega (Madison, WI), Roche (Mannheim, Germany), Santa Cruz Biotechnologies (Santa Cruz, CA), and Sigma-Aldrich (St. Louis, MO).

Cytotoxicity Assay. The cytotoxicity of the *ent*-kauranes was measured with the aid of Mosmann's colorimetric assay.[23] RAW 264.7 macrophages were exposed to the products (50−0.1 μM, 200 μL) in a microplate for 24 h. After the elimination of the culture medium, 100 μL per well of a 0.5 mg/mL solution of 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) was added, and the cells were incubated at 37 °C until blue deposits were visible. The supernatant was then eliminated, and the colored metabolite was dissolved in DMSO (Sigma-Aldrich, 100 μL per well). This reaction was performed in triplicate. Absorbance was measured at 570 nm with a Labsystems Multiskan MCC/340 plate reader (Helsinki, Finland).

Nitric Oxide Production in RAW 264.7 Macrophages. RAW 264.7 murine macrophages were cultured in a DMEM medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal bovine serum (FBS), all from Invitrogen. The cells were removed from the tissue culture flask with a cell scraper and resuspended until a final relation of 5×10^5 cells/mL in the same culture medium was reached. The macrophages (RAW 264.7) were co-incubated in a 96-well culture plate (200 μL) with 1 μg/mL (final concentration) of LPS (Sigma-Aldrich) at 37 °C for 24 h in the presence of the test compounds (0.05−10 μM), dexamethasone (5 μM), or vehicle (DMEM medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, 0.5% FBS, and ≤2% DMSO). The presence of nitrites in the culture supernatant was determined with the aid of Griess reagent (Sigma-Aldrich). Details of the protocols were previously described by Bas et al.[24] Nitrite production was assessed as the index of NO generation in the induction phase.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). RAW 264.7 cells (5×10^6) were treated with or without LPS (1 μg/mL) and cultured with the test compounds (5 μM) for 8 h. The cells were collected, and the total RNA was extracted with the aid of RNeasy mini kit (50) columns (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions, and then redissolved in diethyl pyrocarbonate (Sigma-Aldrich)-treated H_2O. The concentration of the extracted RNA was calculated by measuring the optical density at 260 nm. The ratio of the optical density at 260 nm to that at 280 nm should be between 1.7 and 1.9. Aliquots of 1 μg of RNA were transformed to first strand cDNA with avian myeloblastosis virus (AMV) reverse transcriptase (Promega Biosciences, San Luis, CA). Then, 2.5 μL of the resulting cDNA was mixed with 0.5 μM primers (Invitrogen, Langley, OK) of iNOS (sense: 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′; antisense: 5′-GGCTGTCAGAGCCTCGTGGCTTTGG-3′) and β-actin (sense: 5′-GCAGAGCAAGAGAGGCATCC-3′; antisense: 5′-CTGTGGTGGTGAAGCTGTAG-3′). The thermocycler conditions were 1 cycle at 94 °C for 4 min for denaturing, 28 cycles at 94 °C for 1 min for denaturing, with an annealing temperature of 60 °C for 1 min and an elongation temperature of 72 °C for 1 min, followed by an elongation temperature of 72 °C for 10 min. After the reaction, the amplified product was removed from the tubes and run on 2% agarose gel (Sigma-Aldrich). The bands were visualized with ethidium bromide and UV radiation in a LAS 3000 mini (Fujifilm, Tokyo, Japan). Quantification was carried out with a Multi Gauge reader (Fujifilm).

Western Blot Analysis. Western blots were performed following the protocol described by Escandell et al.[25] RAW 264.7 macrophages (1.5×10^6 cells per well) were co-incubated in a six-well culture plate (1 mL) with 1 μg/mL (final concentration) of LPS at 37 °C for 24 h (iNOS) and then for 0, 10, 30, and 60 min (p65 and IκB) in the presence of the test compounds (5.0, 1.0, and 0.1 μM for iNOS and 5 μM for p65 and IκB), dexamethasone (5 μM), or vehicle (DMEM medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, 0.5% FBS, and ≤2% DMSO). For the iNOS and IκB analyses, cellular lysates were obtained with lysis buffer: 1% Triton X-100, 1% deoxycholic acid, 20 mM NaCl, 25 mM Tris-HCl pH 7.4, and one tablet of complete mini EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany). After centrifugation (10 000*g*, 10 min), the proteins present in the supernatants were quantified. For p65 analysis, nuclear extracts were obtained as follows: macrophages were washed with cold PBS, and the cells were resuspended in 1.5 mL of cold buffer A (HEPES 10 mM, pH 7.6; EDTA 0.1 mM, pH 8.0; KCl 15 mM; and $MgCl_2$ 2 mM). The lysate was incubated in ice for 10 min and then centrifuged at 4500 rpm (5 min, 4 °C). The pellet was resuspended in 200 μL of cold buffer B (buffer A + 0.2% NP40), incubated, and centrifuged as described above. The pellet was resuspended in 50 μL of buffer C (HEPES 225 mM, pH 7.6; EDTA 0.1 mM, pH 8.0; KCl 15 mM; glycerol 10%; DTT 1 μM; PMSF 5 ppm; aprotinin 0.01 mg/L; and a tablet of antiprotease for each 10 mL of buffer). The mixture was incubated for 5 min in ice, and then 5 μL of 5 M NaCl was added. The

resulting mixture was once again incubated in ice for 30 min with stirring. Finally, the mixture was centrifuged at 14 000 rpm for 15 min at 4 °C, after which the protein content in the supernatant was determined with the aid of the Bradford method. Equal amounts of protein (30 μg) were then loaded on 10% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and kept for 120 min at 250 mA. For iNOS, the membranes were then blocked in phosphate-buffered saline (PBS)-Tween 20 (0.05%) containing 6% defatted milk and incubated with anti-iNOS polyclonal antibody (1:2000 dilution) from Cayman. Blots were washed and incubated with peroxidase-conjugate goat antirabbit immunoglobulin G (1:12000 dilution). For p65 detection, membranes were incubated with anti-p65 polyclonal antibody (1:500 dilution) (SC-7151). Blots were washed and incubated with peroxidase-conjugate goat antirabbit immunoglobulin G (1:12000 dilution; Cayman). For IκB, membranes were incubated with anti-IκB polyclonal antibody (1:500) (SC-1643). Blots were washed and incubated with peroxidase-conjugate goat antimouse immunoglobulin G (1:12000 dilution). All three antibodies were obtained from Santa Cruz Biotechnologies. The immunoreactive bands were visualized with an LAS 3000 mini (Fujifilm, Tokyo, Japan). Western blot quantification was carried out with a Multi Gauge reader (Fujifilm).

Electrophoretic Mobility Shift Assays (EMSA). RAW 264.7 macrophages (5×10^6 cells per dish) were incubated in tissue culture dishes (Ø100 × 20 mm, 5 mL) at 37 °C for 1 h in the presence of the test compounds (1 μM), dexamethasone (5 μM), or vehicle (DMEM medium containing 100 U/mL penicillin, 100 μg/mL streptomycin, 0.5% FBS, and ≤2% DMSO). The cells were then stimulated with LPS for an additional 1 h, after which the nuclear protein was extracted. The protein content of the supernatant was determined with the aid of Bradford reagent, and then 5 μg of nuclear protein was loaded onto a 4% nondenatured polyacrylamide gel. EMSAs were carried out with a DIG-gel shift kit, second generation (Roche Diagnostics, Mannheim, Germany). Bands were visualized with an LAS 3000 mini (Fujifilm), and quantification was carried out with a Multi Gauge reader (Fujifilm).

Immunocytochemistry. For studying the localization of NF-κB, cells were grown on chamber slides at a concentration of 40 000 cells/mL (500 μL, 24 h, 37 °C) and then treated first with the test compounds (1 μM, 1 h) and then with LPS (1 μg/mL, 30 min). The cells were subsequently fixed in a methanol/acetic acid (95:5) solution for 20 min at −20 °C. After being washed in PBS, the cells were permeabilized with 0.3% Triton X-100 in PBS at 4 °C overnight and then blocked with 5% bovine serum albumin (BSA) in PBS/0.1% Triton X-100 for 15 min at room temperature. Slides were then incubated with rabbit polyclonal anti-NF-κBp65 (H-286)/SC7151 in a 1:50 dilution. After 2 h of incubation at 37 °C in a humectation chamber, the slides were washed with PBS and incubated with secondary goat antirabbit antibody conjugated to Alexa Fluor 488 (1:1000) (Invitrogen) at 37 °C for 1 h (darkness). Then, 6,4′-diamidino-2-phenylindole dihydrochloride (DAPI) was used to stain the DNA in order to determine whether the NF-κB was localized in the cytoplasm or in the nucleus. Samples were then acquired and examined with a fluorescent microscope (Nikon, Tokyo, Japan).

Transient Transfection and NF-κB-Dependent Reporter Gene Expression Assay. RAW 264.7 cells (2.5×10^5 cells/well) were placed in a 24-well plate. The cells were then transiently transfected with pNF-κB-Luc expression plasmid (0.5 μg/well) and the control plasmid TK-Renilla (0.3 μg/well), both kindly donated by Dr. Lidija Klampfer (Albert Einstein Cancer Center, New York). Transfections were performed using Lipofectamine 2000 in accordance with the instructions of the manufacturer (Invitrogen). After 24 h, the cells were co-incubated with the test compounds (5, 1, and 0.1 μM) and LPS (1 μg/mL) for an additional 24 h. The luciferase assay was performed with the aid of a dual-luciferase reporter assay system in accordance with the instructions of the manufacturer (Promega). Luciferase activity was normalized to TK-Renilla activity to control for transfection efficiency. Light emission was then measured as relative light units in a Fluoroskan Ascent FL (Thermo Labsystems, Franklin, MA).

Statistics. All experiments were performed at least in triplicate. Numerical data are expressed as means ± SD. Statistical analyses were performed with a one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test for multiple comparisons. The difference was considered to be statistically significant for $p < 0.05$. IC_{50} values were calculated from the dose−effect curves of at least four concentrations. Statistical analyses were performed with the aid of a GraphPad Prism 4 statistics package (GraphPad Software, San Diego, CA).

Acknowledgment. S.A. thanks the University of Valencia for a grant received (Beca Jóvenes Investigadores de Países en Vías de Desarrollo). This study was supported in part by grants SAF2006-06726 (Spanish Government through the MIC and FEDER) to J.L.R. and NS-FC30772637 to H.-D.S. The authors also thank Dr. Lidija Klampfer (Albert Einstein Cancer Center, New York) for the kind donation of plasmids.

Supporting Information Available: Figure S1 showing the effects of **1**−**4** on expression of iNOS and iNOS mRNA in LPS-stimulated RAW 264.7 cells. Figure S2 and S3 showing the effects of **1**−**4** on NF-κB in LPS-stimulated RAW 264.7 cells. Figure S4 showing the effects of **1**−**4** on the degradation of IκB and the translocation of p65 in RAW264.cells. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Wu, C. Y.; Li, H. W. In *Flora Republicae Popularis Sinicae*; Beijing Academic Press: Beijing, 1987; Vol. 66, p 494.
(2) Hou, A. J.; Li, M. L.; Jiang, B.; Lin, Z. W.; Ji, S. Y.; Zhou, Y. P.; Sun, H. D. *J. Nat. Prod.* **2000**, *63*, 599–601.
(3) Hou, A. J.; Zhao, Q. S.; Li, M. L.; Jiang, B.; Lin, Z. W.; Ji, S. Y.; Sun, H. D.; Zhou, Y. P.; Lu, Y.; Zheng, Q. T. *Phytochemistry* **2001**, *58*, 179–183.
(4) Li, L. M.; Weng, Z. Y.; Huang, S. X.; Pu, J. X.; Li, S. H.; Huang, H.; Yang, B. B.; Han, Y.; Xiao, W. L.; Li, M. L.; Han, Q. B.; Sun, H. D. *J. Nat. Prod.* **2007**, *70*, 1295–1301.
(5) Li, S. H.; Niu, X. M.; Peng, L. Y.; Zhang, H. J.; Yao, P.; Sun, H. D. *Planta Med.* **2002**, *68*, 946–948.
(6) Niu, X. M.; Li, S. H.; Na, Z.; Lin, Z. W.; Sun, H. D. *Helv. Chim. Acta* **2004**, *87*, 1951–1957.
(7) Weng, Z. Y.; Huang, S. X.; Li, M. L.; Zeng, Y. Q.; Han, Q. B.; Ríos, J. L.; Sun, H. D. *J. Agric. Food Chem.* **2007**, *55*, 6039–6043.
(8) Hwang, B. Y.; Lee, J. H.; Koo, T. H.; Kim, H. S.; Hong, Y. S.; Ro, J. S.; Lee, K. S.; Lee, J. J. *Planta Med.* **2001**, *67*, 406–410.
(9) Yeh, S. H.; Chang, F. R.; Wu, Y. C.; Yang, Y. L.; Zhuo, S. K.; Hwang, T. L. *Planta Med.* **2005**, *71*, 904–909.
(10) Ríos, J. L.; Recio, M. C.; Máñez, S.; Giner, R. M. In *Studies in Natural Products Chemistry: Bioactive Natural Products*; Atta-ur-Rahman, Ed.; Elsevier: Amsterdam, 2000; Vol. 22, pp 93−143.
(11) Castrillo, A.; de las Heras, B.; Hortelano, S.; Rodríguez, B.; Villar, A.; Boscá, L. *J. Biol. Chem.* **2001**, *276*, 15854–15860.
(12) Ghosh, S.; May, M. J.; Kopp, E. B. *Annu. Rev. Immunol.* **1998**, *16*, 225–260.
(13) Celec, P. *Biomed. Pharmacother.* **2004**, *58*, 365–371.
(14) Bremner, P.; Heinrich, M. *J. Pharm. Pharmacol.* **2002**, *54*, 453–472.
(15) Baeuerle, P. A.; Henkel, T. *Annu. Rev. Immunol.* **1994**, *12*, 141–179.
(16) Leung, C. H.; Grill, S. P.; Lam, W.; Han, Q. B.; Sun, H. D.; Cheng, Y. C. *Mol. Pharmacol.* **2005**, *68*, 286–297.
(17) Leung, C. H.; Grill, S. P.; Lam, W.; Gao, W.; Sun, H. D.; Cheng, Y. C. *Mol. Pharmacol.* **2006**, *70*, 1946–1955.
(18) Hong, S. S.; Lee, S. A.; Han, X. H.; Jin, H. Z.; Lee, J. H.; Lee, D.; Lee, J. J.; Hong, J. T.; Kim, Y.; Ro, J. S.; Hwang, B. Y. *J. Nat. Prod.* **2007**, *70*, 632–636.
(19) Lee, J. W.; Lee, M. S.; Kim, T. H.; Lee, H. J.; Hong, S. S.; Noh, Y. H.; Hwang, B. Y.; Ro, J. S.; Hong, J. T. *Mediators Inflamm.* **2007**. article ID 93148.
(20) Giang, P. M.; Jin, H. Z.; Son, P. T.; Lee, J. H.; Hong, Y. S.; Lee, J. J. *J. Nat. Prod.* **2003**, *66*, 1217–1220.
(21) McCartney-Francis, N. L.; Song, X.; Mizel, D. E.; Wahl, S. M. *J. Immunol.* **2001**, *166*, 2734–2740.
(22) Ialenti, A.; Ianaro, A.; Moncada, S.; Di Rosa, M. *Eur. J. Pharmacol.* **1992**, *211*, 177–172.
(23) Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55–63.
(24) Bas, E.; Recio, M. C.; Giner, R. M.; Máñez, S.; López-Ginés, C.; Gil-Benso, R.; Ríos, J. L. *Br. J. Pharmacol.* **2007**, *320*, 581–590.
(25) Escandell, J. M.; Recio, M. C.; Giner, R. M.; Máñez, S.; Cerdá-Nicolás, M.; Ríos, J. L. *J. Pharmacol. Exp. Ther.* **2007**, *320*, 581–590.

NP9001465