ent-Kaurane Diterpenoids from *Croton tonkinensis* Inhibit LPS-Induced NF-κB Activation and NO Production

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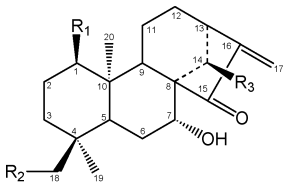
Four *ent*-kaurane diterpenoids including two known, *ent*-7α,14*β*-dihydroxykaur-16-en-15-one (**1**) and *ent*-18-acetoxy-7α-hydroxykaur-16-en-5-one (**2**), and two new, *ent*-1*β*-acetoxy-7α,14*β*-dihydroxykaur-16-en-15-one (**3**) and *ent*-18-acetoxy-7α,14*β*-dihydroxykaur-16-en-15-one (**4**), were isolated from the leaves of *Croton tonkinensis* in a search for inhibitors of NF-κB activation and nitric oxide production. These *ent*-kauranoids inhibited LPS-induced NF-κB activation in murine macrophage RAW264.7 cells at IC_{50} values between 0.07 and 0.42 μM. Consistently, the *ent*-kauranoids markedly reduced LPS-induced NO production in a comparable concentration-dependent manner.

Nuclear factor-κB (NF-κB) is a dimeric transcription factor that activates the expression of many genes involved in the inflammatory process, e.g., the cytokines IL-1*β*, IL-2, and TNF-α, adhesion molecules, or enzymes such as iNOS, cyclooxygenase-II, and 5-lipoxygenase.[1] NF-κB is inactive without stimulation, and it is activated by extracellular signals such as TNF-α, IL-1, lipopolysaccharide (LPS), UV light, and phorbol esters. In unstimulated cells, NF-κB is retained in the cytoplasm via interaction with its inhibitor IκB. In response to various proinflammatory stimuli, IκB is phosphorylated by IκB kinase complex. This leads to the ubiquitination and subsequent proteasome-mediated degradation of IκB, allowing NF-κB to enter the nucleus. NF-κB is highly activated at the site of inflammation of diverse diseases such as rheumatoid arthritis, atherosclerosis, asthma, inflammatory bowel disease, and *Helicobacter pylori*-associated gastritis[1] and associated with cancer, cachexia, diabetes, euthyroid sick syndrome, and AIDS.[2] With its apparent involvement in a variety of human diseases, NF-κB has been shown to be the target of several anti-inflammatory and anticancer drugs.[2] A potential source for NF-κB inhibitors is medicinal plants used in indigenous traditional medicine. The genus *Croton* L. (Euphorbiaceae) consists of 800 species mainly distributed in tropical regions, among which 31 species are cultivated or grow wild in Vietnam.[3] *Croton tonkinensis* Gagnep., commonly named in Vietnamese as "Kho sam Bac Bo", is a tropical shrub native to Northern Vietnam. Its dried leaves (*Folium tonkinensis*) have been used in Vietnamese traditional medicine to treat burns (boils), abscesses, impetigo, abdominal pain, dyspepsia, and gastric and duodenal ulcers. Moreover, it is a component of recipes applied to cure urticaria, leprosy, psoriasis, vaginitis due to trichomonas, and genital organ prolapse.[3,4] Very few investigations on the phytochemicals from *C. tonkinensis* revealed the presence of sterols and an *ent*-kaurane diterpenoid;[5,6] however, the presence of *ent*-kaurane diterpenoids and anti-inflammatory activity of the plant prompted us to investigate chemical constituents inhibiting NF-κB activity in the plant. We herein describe the structure elucidation of **3** and **4** and the effect of compounds **1**−**4** on LPS-induced NF-κB activation in murine macrophage RAW264.7 cells transfected with NF-κB-mediated reporter gene construct and on nitric oxide (NO) production in LPS-stimulated RAW264.7 cells.

Results and Discussion

The methanolic extract of the dried leaves of *C. tonkinensis* showed strong inhibition on NF-κB activation (IC_{50} 1.4 μg/mL) in LPS-stimulated murine macrophage RAW264.7 cells. Solvent partition of the methanolic extract resulted in the localization of the active components in *n*-hexane- and CH_2Cl-soluble fractions. Further bioactivity-guided fractionation of combined *n*-hexane and CH_2Cl_2 fractions using the NF-κB reporter gene assay has led to the isolation and characterization of four active *ent*-kaurane dieterpenoids, *ent*-7α,14*β*-dihydroxykaur-16-en-15-one (**1**),[7] *ent*-18-acetoxy-7α-hydroxykaur-16-en-5-one (**2**),[6] *ent*-1*β*-acetoxy-7α,14*β*-dihydroxykaur-16-en-15-one (**3**), and *ent*-18-acetoxy-7α,14*β*-dihydroxykaur-16-en-15-one (**4**). Among them, compounds **3** and **4** are new.



1 R_1= H, R_2 = H, R_3 = OH
2 R_1= H, R_2 = OAc, R_3 = H
3 R_1= OAc, R_2 = H, R_3 = OH
4 R_1= H, R_2 = OAc, R_3 = OH

Compound **3** was obtained as a white amorphous powder, $[\alpha]^{18}_D$ −36.7° ($CHCl_3$). The molecular formula was established as $C_{22}H_{32}O_5$ from the HRFABMS data at *m*/*z* 377.2330 ($[M + H]^+$, calcd 377.2328) and ^{13}C NMR spectroscopic data including DEPT technique. The existence of a cyclopentanone ring conjugated to an *exo*-methylene in **3** was evident from the following data: λ_{max} 231.3 nm (log ε 4); ν_{max} 1730 and 1650 cm^{-1}, δ_H 6.17 and 5.41 (each 1H, s) as well as δ_C 147.3 (s), 118.2 (t), and 207.9 (s). In addition to the above-mentioned signals, the ^1H NMR spectrum showed the presence of three tertiary methyls at δ_H 0.88, 0.97, and 1.15, an acetyl methyl at δ_H 1.99, and three

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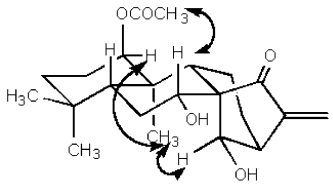

Figure 1. Selected NOESY correlations observed for compound **3**.

oxygenated methines at δ_H 4.38, 4.84, and 4.89. The ^{13}C NMR and DEPT spectra confirmed that the molecule contained 22 carbons including three methyls, six methylenes, six methines, five quartenary carbons, and an acetoxyl group. The presence of three oxygenated methines at δ 4.89 (1H, s), 4.84 (1H, br s), and 4.38 (1H, dd, J = 4.2, 12.3 Hz) was consistent with the ^{13}C NMR data and DEPT multiplicities at δ 74.5 (CH), 72.8 (CH), and 74.8 (CH). By comparison of the spectroscopic data with those of related compounds, it was assumed that **3** had the same skeleton as that of *ent*-kaur-16-en-15-one, with an acetoxyl and two hydroxyl groups. The acetoxyl substituent was placed at C-1 as a result of the comparison of the ^1H and ^{13}C NMR data of **3** with those of **1** and literature[6−8] and the correlation observed between acetyl methyl (δ_H 1.99), H-1 (δ_H 4.84), and the ester carbonyl at δ_C 170.2 in the HMBC spectrum. Two hydroxyl groups were located at C-7 and C-14, respectively, on the basis of $^1H-^1H$ COSY, HMQC, and HMBC correlations. The relative stereochemistry of **3** was elucidated on the basis of NOESY correlations (Figure 1). The β-orientation of the acetoxyl group was deduced from the coupling pattern of H-1, which was a broad singlet (br s), and a strong NOE correlation of H-1 with C-20 methyl protons. The coupling pattern of H-1 is known to be dependent on the orientation of 1-OH, and H-1β appears as a doublet−doublet pattern with a large coupling constant with C-2 methylene protons; however, H-1α shows a broad singlet due to the β-orientation of 1-OH.[9] Furthermore, the NOE correlations between acetoxyl methyl and H-7 and between 20-methyl and H-14 revealed that the hydroxyl groups at C-7 and C-14 had α- and β-orientation, respectively. Therefore, the structure of **3** was determined as *ent*-1β-acetoxy-7α,14β-dihydroxykaur-16-en-15-one.

Compound **4** was isolated as a white amorphous powder, $[\alpha]^{18}_D$ −20° ($CHCl_3$). Its mass spectrum showed the same molecular formula, $C_{22}H_{32}O_5$ (*m*/*z* 377.2330 $[M + H]^+$, calcd 377.2328), as that of **3**. The ^1H and ^{13}C NMR spectra of **4** were quite similar to those of **3** except the presence of oxygenated methylene signals at δ_C 72.3 (t), δ_H 3.66 (d, J = 10.1 Hz), and δ_H 3.87 (d, J = 10.1 Hz) instead of one tertiary methyl signal in **3**. These data indicated that one of the three tertiary methyl groups in the basic *ent*-kaur-16-en-15-one skeleton in compound **4** was oxidized to a hydroxymethyl group and then further acetylated. Comparing the ^1H and ^{13}C NMR data of compound **4** with those of compound **1**,[7] compound **2**,[6] and candicandiol[10] revealed that the acetoxyl group was located at C-18 because only the signals for Me-19 (δ_H 0.88, δ_C 17.5) and Me-20 (δ_H 1.11, δ_C 18.4) were observed. The location of the acetyoxyl group at the C-18 position was confirmed by HMBC spectra, which showed correlations between 18-CH_2 and C-3, -4, -5, and -19 and the ester carbonyl at δ_C 171.4. Thus, on the basis of the above spectral data, compound **4** was characterized as *ent*-18-acetoxy-7α,14β-dihydroxykaur-16-en-15-one.

Compounds **1**−**4** were examined for their dose−response effect on LPS-induced NF-κB activation using the NF-κB-mediated reporter gene assay system. RAW264.7 cells transfected with a NF-κB-mediated reporter gene construct were stimulated with LPS in the presence of various concentrations of compounds **1**−**4**, and then the expression of reporter gene (secreted alkaline phosphatase gene) in the culture medium was measured in comparison with a known NF-κB inhibitor, parthenolide.[11] Compounds **1**−**4** strongly inhibited the LPS-induced activation of NF-κB in a dose-dependent manner without affecting cell viability in MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based colorimetric assays. The half-maximal inhibitory concentrations (IC_{50}) of compounds **1**−**4** toward NF-κB were in the range 0.07−0.42 μM, indicating that all compounds showed much more potent activity than parthenolide (Table 1**)**. Since the activation of NF-κB results in the expression of inflammatory enzymes such as iNOS, we investigated the effect of *ent*-kauranoids **1**−**4** on LPS-stimulated NO production in RAW264.7 cells. The observed IC_{50} (Table 1) were comparable with those of NF-κB activation and much more potent than the known iNOS inhibitor aminoguanidine. This activity was not due to their potential cytotoxicity since **1**−**4** showed no impairment of cell viability up to a concentration of 3 μM.

Table 1. IC_{50} Values (μM)[a] of Compounds **1**−**4** in NF-κB Activation and NO Production Assays

compound	NF-κB activation	NO production
1	0.11 ± 0.02	0.26 ± 0.02
2	0.10 ± 0.01	0.21 ± 0.04
3	0.42 ± 0.07	0.47 ± 0.03
4	0.07 ± 0.01	0.15 ± 0.02
PTN	2.34 ± 0.04	2.01 ± 0.06
AG		4.06 ± 0.05

[a] Data are means ± SD of six independent experiments. PTN: parthenolide. AG: aminoguanidine.

Earlier studies on the biological activities of *ent*-kauranes mainly centered on their cytotoxic activity;[12−14] however, recent investigations on NF-κB inhibitory activity of some *ent*-kaur-16-en-15-ones extended our understanding of the molecular mechanism underlying the anticancer and anti-inflammatory activities of kaurane diterpenes.[15,16] The potent NF-κB inhibitory activity of the *ent*-kaur-16-en-15-ones **1**−**4** could be accounted for by the presence of reactive centers, such as an exomethylene group conjugated to a carbonyl group in the cyclopentanone ring. This functional group can react with biological nucleophiles, especially the sulfhydryl group of the cysteine residue in the DNA-binding domain of the NF-κB subunit by Michael-type reaction, as previously demonstrated with kamebakaurin by us.[16] Therefore this is likely to be the mode of NF-κB inhibition for the diterpenoids **1**−**4**. The secondary metabolites that mediated the anti-inflammatory effects of *C. tonkinensis* are mainly *ent*-kaurane-type diterpenoids with *ent*-kaur-16-en-15-one skeletons. The isolation of substantial amounts of biologically active diterpenoids such as *ent*-18-acetoxy-7α-hydroxy-kaur-16-en-15-one supports the pharmacological basis of this plant, which has been used as an herbal medicine for the treatment of inflammation and makes *C. tonkinensis* an interesting source for lead compounds for anti-inflammatory research.

Experimental Section

General Experimental Procedures. Melting points were measured without correction on an Electrothermal model 9100. UV spectra were obtained on a Shimadzu UV-1601 spectrometer. IR spectra were taken on a JASCO Report-100 spectrometer (KBr pellet). Optial rotations were measured on a JASCO DIP-370 digital polarimeter at 18 °C. ^1H NMR (300 MHz), ^{13}C NMR (75 MHz), DEPT, HMQC, and HMBC were obtained on a Varian Unity NMR spectrometer. HRFABMS were measured

on a JEOL HX 110 mass spectrometer. ESIMS were measured on a Finigan Navigator mass spectrometer. High-performance liquid chromatography (HPLC, Dionex, Dionex Co., Sunnyvale, CA) was carried out on analytical and preparative scales using YMC ODS-H80 (YMC Co., Japan) [150 × 4.6 mm i.d., S-4μm (analytical); 150 × 20 mm i.d., S-4μm (preparative)]. Silica gel 60 (40−63 μm, Merck, Darmstadt, Germany), Sephadex LH-20 (Sigma Chemical Co., St. Louis, MO), and reversed-phase YMC gel (ODS 60-14) were used for column chromatography. TLC was carried out on precoated TLC sheets (silica gel 60 F_{254}, Merck), and the spots were detected by spraying with anisaldehyde-H_2SO_4 and then heating on a hot plate. Fetal bovine serum, media, and supplement materials for cell culture were purchased from GIBCO-BRL (Gaithersberg, MD). Parthenolide and aminoguanidine were purchased from Calbiochem (La Jolla, CA) and Sigma-Aldrich Co., respectively, and used as a positive standard in the assay for NF-κB activation and NO production, respectively.

Plant Material. The air-dried leaves of *C. tonkinensis* were collected in the suburbs of Hanoi, Vietnam, and identified by Prof. Vu Van Chuyen (Hanoi College of Pharmacy) in May 2002. A voucher specimen (No. DHD 2002-5) was deposited in the Herbarium of Hanoi College of Pharmacy, Hanoi, Vietnam.

Extraction and Isolation. The dried leaves of *C. tonkinensis* (2 kg) were powdered and extracted three times with MeOH by percolation at room temperature. The combined MeOH extract was evaporated under reduced pressure to yield a brownish syrup (31 g). The syrup was partitioned between H_2O and *n*-hexane, CH_2Cl_2, EtOAc, and *n*-BuOH successively to afford the corresponding soluble fractions. The *n*-hexane fraction (21.9 g) and the CH_2Cl_2 fraction (5.1 g) were combined on the basis of TLC and their NF-κB inhibitory activity in a NF-κB reporter gene assay. The combined fraction was chromatographed on a silica gel column eluted with *n*-hexane−EtOAc stepwise gradient system (0−100%) to give nine fractions (F1−F9) on the basis of TLC. The active fraction F8 (9.3 g) was fractionated over a silica gel column eluted with hexane−EtOAc (3:1) to give four fractions (F81−F84). Active fraction F82 (1.2 g) was applied to a silica gel column (*n*-hexane−EtOAc, 3:1) to obtain three fractions (F821−F823). Fraction F823 (0.4 g) was subjected to preparative HPLC (ODS-H80, 150 × 20 mm, YMC, MeOH−H_2O, 8:2, flow rate, 6 mL/min) to give *ent*-7α,14β-dihydroxykaur-16-en-15-one (**1**) (10.7 mg, t_R 16.93 min). Repeated silica gel column chromatography of fraction F83 (5.6 g, 2 times, solvent system *n*-hexane−EtOAc, 2.5:1) and further purification on a Sephadex LH-20 column with MeOH afforded *ent*-18-acetoxy-7α-hydroxykaur-16-en-15-one (**2**) (1.2 g). Active fraction F84 (2.1 g) was separated on a silica gel column eluted with *n*-hexane−EtOAc (2:1) to obtain three fractions (F841−F843). Separation of fraction F842 (1.4 g) was performed on a silica gel column (*n*-hexane−EtOAc, 1:1) to afford four fractions (F8421−F8424). Purification of the active fraction F8424 (0.2 g) on preparative HPLC (ODS-H80, 150 × 20 mm, YMC, MeOH−H_2O, 7:3, flow rate, 6 mL/min) yielded *ent*-1β-acetoxy-7α,14β-dihydroxykaur-16-en-15-one (**3**) (12.3 mg, t_R 13.15 min) and *ent*-18-acetoxy-7α,14β-dihydroxykaur-16-en-15-one (**4**) (27.3 mg, t_R 21.31 min).

***ent*-7α,14β-Dihydroxykaur-16-en-15-one (1):** white amorphous powder; mp 200−201 °C; $[\alpha]^{18}_D$ −10° (*c* 0.3, $CHCl_3$); positive-ion FABMS *m/z* 319 $[M + H]^+$, 341 $[M + Na]^+$; the spectral properties of **1** (MS, ^{1}H NMR, and ^{13}C NMR) were identical with those previously reported.[7]

***ent*-18-Acetoxy-7α-hydroxykaur-16-en-15-one (2):** white amorphous powder; mp 119−120 °C; $[\alpha]^{18}_D$ −10° (*c* 0.3, $CHCl_3$); the spectral properties of **2** (MS, ^{1}H NMR, and ^{13}C NMR) were identical with those of our authentic sample.[6]

***ent*-1β-Acetoxy-7α,14β-dihydroxykaur-16-en-15-one (3):** white amorphous powder; mp 110−111 °C; $[\alpha]^{18}_D$ −36.7° (*c* 1.1, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 231.3 (4.0) nm; IR (KBr) ν_{max} 3400, 2950, 1730, 1650, 1460, 1385, 1250, 1240, 1100, 1040, 940 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.17 (1H, s, H-17a), 5.41 (1H, s, H-17b), 4.89 (1H, br s, H-14α), 4.84 (1H, br s, H-1α), 4.38 (1H, dd, J = 12.3, 4.2 Hz, H-7β), 3.07 (1H, br s, H-13), 1.99 (3H, s, OAc), 1.15 (3H, s, H-20), 0.97 (3H, s, H-19); 0.88 (3H, s, H-18); ^{13}C NMR (75 MHz, $CDCl_3$) 72.8 (C-1), 22.6 (C-2), 34.9 (C-3), 32.9 (C-4), 47.2 (C-5), 27.7 (C-6), 74.5 (C-7), 61.3 (C-8), 46.3 (C-9), 42.7 (C-10), 16.8 (C-11), 30.9 (C-12), 45.9 (C-13), 74.8 (C-14), 207.9 (C-15), 147.3 (C-16), 118.2 (C-17), 33.2 (C-18), 21.4 (C-19), 18.5 (C-20), 21.2, 170.2 (1-OAc); positive-ion HRFABMS *m/z* 377.2330 $[M + H]^+$ for $C_{22}H_{32}O_5$ (calcd 377.2328); positive-ion ESIMS *m/z* 399 $[M + Na]^+$, negative-ion ESIMS *m/z* 375 $[M - H]^+$.

***ent*-18-Acetoxy-7α,14β-dihydroxykaur-16-en-15-one (4):** white amorphous powder; mp 173−175 °C; $[\alpha]^{18}_D$ −20° (*c* 0.6, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 231.6 (3.77) nm; IR (KBr) ν_{max} 3375, 2950, 1750, 1640, 1440, 1385, 1250, 1240, 1100, 1040, 940 cm^{-1}; ^{1}H NMR ($CDCl_3$, 300 MHz) δ 6.17 (1H, s, H-17a), 5.41 (1H, s, H-17b), 4.88 (1H, br s, H-14α), 4.31 (1H, dd, J = 12, 3.9 Hz, H-7β), 3.87 (1H, d, J = 10.1 Hz, H-18a), 3.66 (1H, d, J = 10.1 Hz, H-18b), 3.06 (1H, br s, H-13), 2.09 (3H, s, OAc), 1.11 (3H, s, H-20), 0.88 (3H, s, H-19); ^{13}C NMR (75 MHz, $CDCl_3$) 38.9 (C-1), 17.3 (C-2), 35.2 (C-3), 36.3 (C-4), 47.1 (C-5), 27.9 (C-6), 74.4 (C-7), 61.6 (C-8), 54.1 (C-9), 39.7 (C-10), 17.7 (C-11), 30.9 (C-12), 45.9 (C-13), 74.9 (C-14), 207.8 (C-15), 147.6 (C-16), 117.9 (C-17), 72.3 (C-18), 17.5 (C-19), 18.4 (C-20), 21.0, 171.4 (18-OAc); positive-ion HRFABMS *m/z* 377.2330 $[M + H]^+$ for $C_{22}H_{32}O_5$ (calcd 377.2328).

NF-κB Activity Assay. The NF-κB inhibitory activity assay was carried out according to the established protocols.[11] RAW264.7 cells transfected with a plasmid containing eight copies of κB elements linked to SEAP (secreted alkaline phosphatase) gene 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 compounds tested and LPS (1 μg/mL) for 24 h. Then 100 μL of each culture supernatant was transferred to a new 96-well plate and heated at 65 °C for 5 min. An additional 100 μL of 2× SEAP assay buffer (2 M diethanolamine, 1 mM $MgCl_2$, 20 mM l-homoarginine) was added to each well and incubated at 37 °C for 10 min. The reaction was initiated by the addition of 20 μL of 120 mM *p*-nitrophenyl phosphate dissolved in 1× SEAP assay buffer and incubated at 37 °C. The absorbance of the reaction mixture was measured at 405 nm with a microplate reader (Molecular Devices Co., Menlo Park, CA).

Determination of NO Production. Determination of NO production was carried out according to the established protocols.[11] RAW264.7 cells were transferred in 96-well plates at a density of 1 × 10^5 cells/well. After 3 h incubation, the cells were stimulated with LPS (1 μg/mL) for 24 h in the absence or presence of the compounds tested. As a parameter of NO synthesis, nitrite concentration was measured in the supernatant of RAW264.7 cells by the Griess reaction. Briefly, 100 μL of cell culture supernatant was reacted with 100 μL of Griess reagent [1:1 mixture of 0.1% *N*-(1-naphthyl)ethylenediamine in H_2O and 1% sulfanilamide in 5% phosphoric acid] in a 96-well plate, and absorbance was read with a microplate reader at 570 nm. The nitrite concentration in the supernatants was calculated by comparison with a sodium nitrite standard curve.

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