Crotonkinensins A and B, Diterpenoids from the Vietnamese Medicinal Plant *Croton tonkinensis*

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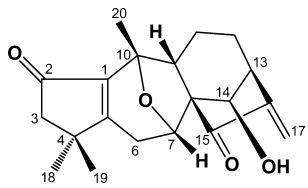
Two new diterpenoids, crotonkinensins A (**1**) and B (**2**), were isolated from the leaves of the Vietnamese endemic medicinal plant *Croton tonkinensis*. Their structures were determined to be 7α,10α-epoxy-14β-hydroxygrayanane-1(5),16(17)-dien-2,15-dione (**1**) and 7α,10α-epoxy-14β-hydroxygrayanane-1(2),16(17)-dien-15-one (**2**) by spectroscopic analysis. Compounds **1** and **2** showed strong anti-inflammatory effects on the LPS-induced COX-2 promoter activity and COX-2 expression in Raw 264.7 cells.

The leaves of *Croton tonkinensis* Gagnep. (Euphorbiaceae), an endemic species in Northern Vietnam, have been used indigenously to treat stomachache and malaria.[1] Previous studies have indicated the presence of alkaloids[2] and diterpenoids[3−7] in the leaves. As part of an ongoing investigation aimed at discovering natural compounds as anti-inflammatory drugs, this study examined the bioactive constituents of the leaves of *C. tonkinensis*. A bioassay-guided investigation of the phytochemical constituents of an EtOH extract of the leaves resulted in the isolation of two new compounds, crotonkinensins A (**1**) and B (**2**), along with eight known *ent*-kaurane diterpenes (see Supporting Information). This paper reports the isolation, structural elucidation, and anti-inflammatory activity of crotonkinensins A and B.



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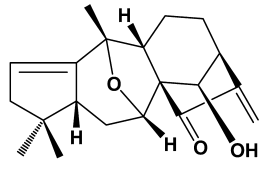

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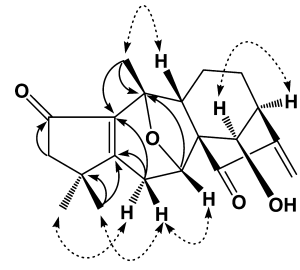
Compound **1** was obtained as an amorphous powder with a negative specific rotation [α_D^{25}] −134.3 (*c* 0.20, MeOH). The IR spectrum of compound **1** revealed the presence of hydroxy, carbonyl, and conjugated ketone groups. The ^{1}H NMR spectrum exhibited two olefinic proton singlets (δ_H 5.41 and 6.05), two oxygenated methine proton signals (δ_H 4.48 and 4.80), 10 aliphatic proton resonances, and three methyl groups (Table 1). The ^{13}C NMR and DEPT spectra of compound **1** revealed the presence of 20 carbon resonances, including two ketone (δ_C 202.3 and 204.0), four olefinic (δ_C 118.4, 142,6 151.0, and 179.1), three oxygenated (δ_C 72.3, 74.0, and 80.3), four aliphatic methylene, two methine, two quaternary, and three methyl carbons. In the HMBC spectrum (Figure 1), the C-15 carbonyl carbon (δ_C 202.3) correlated with two olefinic protons (δ_H 5.41 and 6.05, H_2-17), an oxygenated methine proton (δ_H 4.48, H-14), and two methine protons (δ_H 2.20 and 2.94). The COSY spectrum (see Supporting Information) showed strong ^{1}H−^{1}H connectivities between H-9 (δ_H 2.20) and H_2-11 (δ_H 1.48 and 1.64) and between H-13 and H_2-12 (δ_H 1.59

Table 1. ^{1}H (500 MHz) and ^{13}C NMR (125 MHz) Data for Compounds **1** and **2** in $CDCl_3$

	1		**2**	
position	δ_C, mult	δ_H, mult (*J* in Hz)	δ_C, mult	δ_H, mult (*J* in Hz)
1	142.6 s		147.7 s	
2	204.0 s		116.3 d	5.21 br s
3	51.9 t	2.26 br s	47.3 t	1.98 dt (15.5, 2.0)
		2.27 br s		2.14 m
4	39.8 s		41.3 s	
5	179.1 s		47.2 d	2.81 t (7.5)
6	26.1 t	2.75 dd (18.5, 5.5)	27.1 t	1.77 m
		3.33 d (18.5)		2.28 dd (7.5, 12.5)
7	74.0 d	4.80 d (5.5)	75.3 d	4.49 d (3.0)
8	66.0 s		65.3 s	
9	57.4 d	2.20 m	53.3 d	2.13 m
10	80.3 s		82.1 s	
11	19.4 t	1.48 m	19.2 t	1.41 m
		1.64 m		1.74 m
12	30.6 t	1.59 m	30.9 t	1.61 m
		2.45 m		2.50 m
13	42.3 d	2.94 d (8.5)	42.1 d	2.99 d (10.0)
14	72.3 d	4.48 br s	71.7 d	4.51 br s
15	202.3 s		202.9 s	
16	151.0 s		151.7 s	
17	118.4 t	5.41 s	117.0 t	5.37 s
		6.05 s		6.07 s
18	26.5 q	1.39 s	28.2 q	1.20 s
19	26.9 q	1.22 s	24.5 q	0.92 s
20	16.1 q	1.50 s	17.7 s	1.36 s



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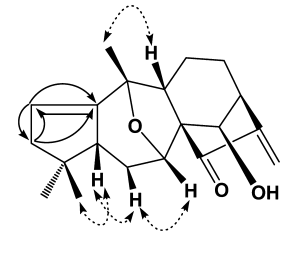

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Figure 1. Key HMBC (H (solid arrow) C) and selected NOESY (H (dotted arrow) H) correlations for compounds **1** and **2**.

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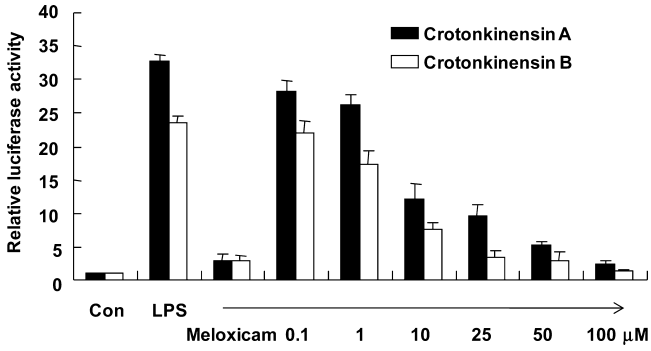

Figure 2. Effects of compounds **1** and **2** on the LPS-induced COX-2 promoter activity in Raw 264.7 cells. Con: control, LPS: lipopolysaccharide. Meloxicam was treated at the concentration of 20 μM.

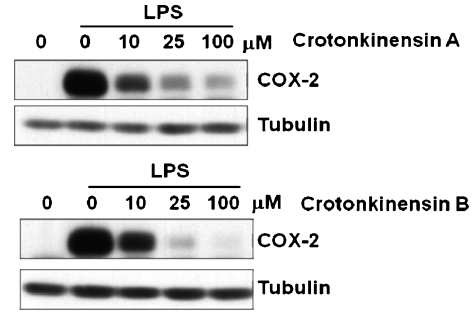

Figure 3. Western blot analysis for measuring the COX-2 expression after exposing Raw 264.7 cells to compounds **1** and **2**.

and 2.45) and weak connectivities from H-13 (δ_H 2.94) to H_2-17 and H-14. The above data suggested a diterpene with a 14β-hydroxykaur-16-en-15-one structure for compound **1**.[4−7] The HMBC spectrum showed correlations from H-20 to C-1 and C-9 and from both H-11 and H-20 to the oxygenated quaternary carbon at δ_C 80.3. Moreover, the HMBC spectrum showed that the C-6 methylene protons (δ_H 2.75 and 3.33) correlated with C-1 (δ_C 142.6) and C-5 (δ_C 179.1), but not with C-10 (δ_C 80.3). These sets of HMBC correlations suggested that the structure of compound **1** comprised a 5/7/6/5 tetracyclic ring system, i.e., a grayanane skeleton.[8−14] The strong long-range correlations from H-18 and H-19 to C-3, C-4, and C-5 indicated a C-1/C-5 double bond. The observation of HMBC correlations between the C-3 methylene protons (δ_H 2.26 and 2.27) and a carbonyl carbon (δ_C 204.0) also indicated the 2-one unit in compound **1**. One oxygenated methine group was assigned at C-7 due to the correlations from H-7 (δ_H 4.80) to C-5, C-8, C-9, and C-14 and the correlation from H-6 to C-7. The strong HMBC correlation between H-7 and C-10 indicated an 7,10-epoxy group, which is supported by the molecular formula $C_{20}H_{24}O_4$ obtained from the molecular ion peak at *m/z* 328.1678 $[M]^+$ (calcd for $C_{20}H_{24}O_4$, 328.1675) in the HREIMS. In the NOESY spectrum, the correlations between H-9/H-20, H-3/H-18 and H-19, H-6β/H-19 and H-7, H-6*a*/H-18, and H-13/H-14 (Figure 1) indicated compound **1** to be 7α,10α-epoxy-14β-hydroxy-1(5),16-dien-2,15-dione-grayanane. The negative specific rotation of compound **1** (MeOH) also supports this stereochemistry.[8−14] Therefore, compound **1** was determined to be 7α,10α-epoxy-14β-hydroxygrayanane-1(5),16-dien-2,15-dione and named crotonkinensin A.

Compound **2** was obtained as a colorless oil with a negative specific rotation $[\alpha_D^{25}]$ −73.1 (*c* 0.31, MeOH). The IR, ^{1}H and ^{13}C NMR, ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY spectra of compound **2** were similar to those of compound **1**, suggesting a grayanane diterpene with a 7α,10α-epoxy-14β-hydroxy-16(17)-en-15-one moiety. Three olefinic proton singlets at δ_H 5.21, 5.37, and 6.07 in the ^{1}H NMR spectrum of compound **2** and four corresponding olefinic carbons (δ_C 116.3, 117.0, 147.7, and 151.7) revealed the disappearance of a carbonyl carbon (δ_C 204.0) in the structure of compound **1**. The HMBC correlations from H-2 to C-3 and C-4 and from H-3 to C-1 and C-2 indicated the presence of a C-1/C-2 double bond. The molecular formula $C_{20}H_{27}O_3$ was deduced for compound **2** from the protonated molecule peak at *m/z* 315.1962 in the HRFABMS spectrum. Therefore, compound **2** was determined to be 7α,10α-epoxy-14β-hydroxygrayanane-1(2),16-dien-15-one and named crotonkinensin B.

The anti-inflammatory effects of crotonkinensins A and B on the COX-2 promoter activity assay were examined by comparing with meloxicam as positive control.[15] The result (Figure 2) shows that both crotonkinensins A and B decreased the LPS-induced COX-2 promoter activity in Raw 264.7 cells after 24 h exposure in a concentration-dependent manner with IC_{50} values of 7.14 ± 0.2 and 5.49 ± 0.2 μM, respectively. Western blot analysis (Figure 3) also showed significant inhibition of COX-2 expression when Raw 264.7 cells were treated with the indicated concentrations of compounds **1** and **2**. These results suggest that grayanane diterpenes **1** and **2** inhibit COX-2 expression through transcriptional regulation.

Previous studies reported the presence of alkaloids, flavonoids, terpenoids, and volatile oils in *Croton* plants.[16] Diterpenes isolated from the *Croton* genus are labdane, clerodane, and kaurane-type diterpenes.[16] Diterpenes have been also isolated from the Vietnamese plant *C. tonkinensis* and identified as kaurane and *ent*-kaurane diterpenes.[3−7] In this study, we isolated two now grayanane-type diterpenes from *C. tonkinensis*. This is the first report of the presence of grayanane-type diterpenes from a *Croton* species as well as the Euphorbiaceae family. Grayanane-type diterpenes are rarely obtained from natural sources, mainly from Ericaceae, and generally occur as the 3β,5β,6β,16α-oxygenated grayanane structure.[8−14] However, it is interesting that both **1** and **2** comprise a 7α,10α-epoxy-14β-hydroxy-16-en-15-one moiety, which is the common structure of *ent*-kaurane-type diterpenes in *C. tonkinensis*.[3−7] Some major *ent*-kaurane diterpenes previously reported from this plant were also isolated in this study (see Supporting Information). Therefore, it appears that the grayanane-type and *ent*-kaurane-type diterpenes are related to each other, and compounds **1** and **2** may be biosynthesized from *ent*-kaurane diterpenes.[12] This finding and previous observations[17,18] suggest that the grayanane diterpenes may be biosynthesized from precursors of *ent*-kaurane diterpenes.

Experimental Section

General Experimental Procedures. Optical rotations were determined on a Rudolph Autopol IV polarimeter using a 100 mm glass microcell. IR spectra (KBr) were recorded on a Nicolet 6700 FT-IR (Thermo Electron Corp.). NMR spectra were obtained on a Varian Inova 500 MHz spectrometer with TMS as the internal standard at Korea Basic Science Institute (KBSI, Gwangju Center, Korea). HRFABMS and HREIMS data were collected on a Micromass QTOF2 (Micromass, Wythenshawe, UK) mass spectrometer. For column chromatography, silica gel (Merck, 63 ≤ 200 μm particle size) and RP-18 (Merck, 75 μm particle size) were used. TLC was carried out with silica gel 60 F_{254} and RP-18 F_{254} plates. HPLC was carried out using a Gilson system with a UV detector and an ODS-H80 column (20 × 150 mm, 4 μm particle size, YMC Co., Ltd., Japan).

Plant Material. The leaves of *C. tonkinensis* were collected in June 2007 in Hanoi, Vietnam. The plant material was identified by Bs. Ngo Van Trai, Department of Plant Resources, National Institute of Medicinal Materials, Hanoi, Vietnam. A voucher specimen (VIET-02) has been deposited at the Herbarium of the National Institute of Medicinal Materials, Hanoi, Vietnam.

Extraction and Isolation. The dry leaves of *C. tonkinensis* (4 kg) were extracted with 90% EtOH (20 L × 2 times) at room temperature for 1 week. The combined EtOH extract was concentrated to yield a dry residue (351 g). This crude extract was subjected to silica gel column chromatography (20 × 20 cm) and eluted with *n*-hexane−EtOAc (49:1, 48:2, 47:3, ... 40:10, 30:20, 25:25, 20:30, 10:40, and 0:50, each 5 L) to yield 10 fractions (F.1: 7.8 g; F.2: 4.6 g; F.3: 6.3 g; F.4: 7.5 g; F.5: 5.0 g; F.6: 3.6 g; F.7: 35.2 g; F.8: 10.2 g;

F.9: 22.3 g, and F.10: 38.5 g). Fraction 7 was repeatedly chromatographed on a silica gel column (7 × 40 cm) eluted with *n*-hexane−EtOAc (9:1, 8:2, 7:3 ... 0.1:9, each 3 L) and separated into six fractions (F.7.1: 0.2 g; F.7.2: 0.3 g; F.7.3: 0.5 g; F.7.4: 0.97 g, F.7.5: 29 g; and F.7.6: 4.1 g). Fraction F.7.5 was subjected to a RP18 column (7 × 25 cm) using MeOH−H_2O (3:1, 4:1 ... 0.10:1) as mobile phase to give six fractions (F.7.5.1−F.7.5.6). Fractions F.7.5.3 and F.7.5.4 were combined, and the major compound in this plant, *ent*-18-acetoxy-7β-hydroxykaur-16-en-15-one, was crystallized from a MeOH solution. The mother liquor was subjected to preparative HPLC [Gilson HPLC, column ODS-H80 (150 × 2 cm, Japan); mobile phase MeOH−H_2O containing 0.1% formic acid (0−30 min: 60% MeOH, 30−32 min: 60−100% MeOH, 32−45 min: 100% MeOH); UV detection at 205 and 254 nm] to give compound **1** (t_R 28 min, 10.6 mg) and a subfraction (t_R 38−40 min). Repeated chromatography of this subfraction using preparative HPLC [Gilson HPLC, column ODS-H80 (150 × 2 cm, Japan); mobile phase MeOH−H_2O containing 0.1% formic acid (0−40 min: 75% MeOH, 40−50 min: 75−100% MeOH, 50−55 min: 100% MeOH); UV detection at 205 and 254 nm] afforded compound **2** (t_R 36 min, 26.5 mg).

Compound 1 (crotonkinensin A): amorphous powder; [α_D^{25}] −134.3 (*c* 0.20, MeOH); IR (film) ν_{max} 3435 (OH), 2960, 2870, 1727 (C═O), 1692 (C═O), 1678, 1641 (C═C), 1631, 1465, 1381, 1341, 1244, 1092, 1063, 912 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m*/*z* 328 $[M]^+$ (43), 310 $[M - H_2O]^+$ (81), 268 (51), 225 (51), 177 (100), 91 (35), 87 (25); HREIMS *m*/*z* 328.1678 $[M]^+$ (calcd for $C_{20}H_{24}O_4$, 328.1675).

Compound 2 (crotonkinensin B): colorless oil; [α_D^{25}] −73.1 (*c* 0.31, MeOH); IR (film) ν_{max} 3426 (OH), 2932, 2868, 1737, 1726 (C═O), 1641 (C═C), 1631, 1463, 1382, 1241, 1102, 1038 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS *m*/*z* 314 $[M]^+$ (2), 296 $[M - H_2O]^+$ (100), 281 (18), 253 (17), 198 (19), 177 (16), 149 (26), 131 (31), 91 (25), 87 (42); HRFABMS *m*/*z* 315.1962 $[M + H]^+$ (calcd for $C_{20}H_{26}O_3H$, 315.1960).

Cell Culture. Raw 264.7 cells were grown in RPMI1640 (HyClone, Logan, UT) containing 10% fetal bovine serum (HyClone) in an atmosphere of 95% air and 5% CO_2 at 37 °C. Cells (2×10^6 cells/mL) were pretreated with indicated concentrations of compounds **1** and **2** for 30 min and then stimulated with lipopolysaccharide (LPS) for 24 h at 37 °C prior to use.

COX-2 Promoter Activity. Cells were cotransfected with COX-2 promoter construct and β-gal plasmid using lipofectamine reagent according to the manufacturer's instructions. Luciferase activity was measured using a microplate luminometer (Berthold, Freiburg, Germany) with a Luciferase Reporter assay kit (Promega).

Western Blot Analysis. For western blot analysis, cells were lysed with RIPA buffer [50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% nonidet-P40 (NP-40), 0.5% deoxycholic acid, and 0.1% sodium dodecyl sulfate (SDS)]. Equal amounts of proteins were separated on a 10% SDS-polyacrylamide gel and transblotted on polyvinylidene difluoride-nitrocelluloase filters. Membranes were incubated with anti-COX-2 (Santa Cruz) and then incubated with horseradish peroxide-conjugated secondary antibody (Santa Cruz). Specific bands were visualized using an ECL detection kit (Millipore).

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Supporting Information Available: The extraction, isolation, and identification of known diterpenes; ^1H and ^{13}C NMR, COSY, HSQC, HMBC, and NOESY spectra for compounds **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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