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

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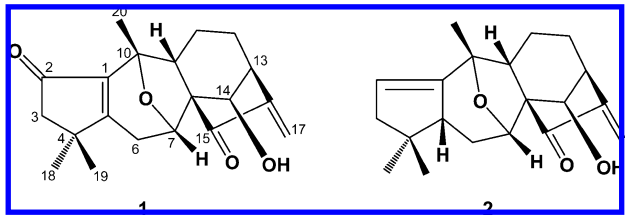
*Received April 6, 2009*

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.



Compound **1** was obtained as an amorphous powder with a negative specific rotation [$\alpha_D^{25}$] −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 ($\delta_H$ 5.41 and 6.05), two oxygenated methine proton signals ($\delta_H$ 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 ($\delta_C$ 202.3 and 204.0), four olefinic ($\delta_C$ 118.4, 142,6 151.0, and 179.1), three oxygenated ($\delta_C$ 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 ($\delta_C$ 202.3) correlated with two olefinic protons ($\delta_H$ 5.41 and 6.05, $H_2$-17), an oxygenated methine proton ($\delta_H$ 4.48, H-14), and two methine protons ($\delta_H$ 2.20 and 2.94). The COSY spectrum (see Supporting Information) showed strong $^1$H−$^1$H connectivities between H-9 ($\delta_H$ 2.20) and $H_2$-11 ($\delta_H$ 1.48 and 1.64) and between H-13 and $H_2$-12 ($\delta_H$ 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 | $\delta_C$, mult | $\delta_H$, mult (*J* in Hz) | $\delta_C$, mult | $\delta_H$, 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 |

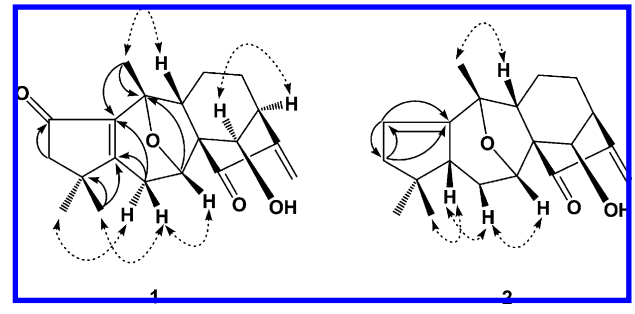


**Figure 1.** Key HMBC (H (solid arrow) C) and selected NOESY (H (dotted arrow) H) correlations for compounds **1** and **2**.

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10.1021/np900215r CCC: $40.75 
Published on Web 11/09/2009

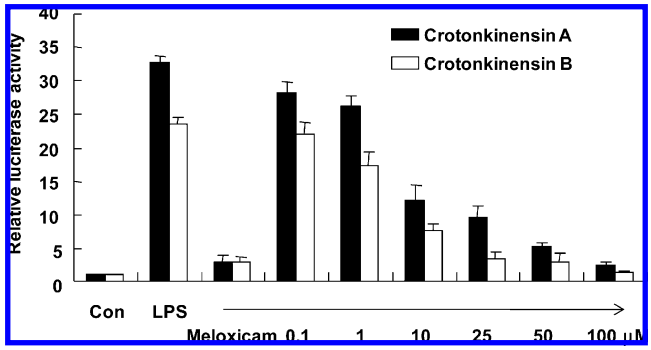


**Figure 2.** Effects of compounds **1** and **2** on the LPS-induced COX-2 promoter activity in Raw 264.7 cells. Con: control, LPS: lipopolysaccharide. Meloxicam was treated at the concentration of 20 $\mu$M.

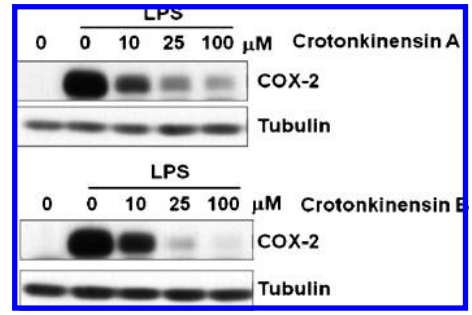


**Figure 3.** Western blot analysis for measuring the COX-2 expression after exposing Raw 264.7 cells to compounds **1** and **2**.

and 2.45) and weak connectivities from H-13 ($\delta_H$ 2.94) to $H_2$-17 and H-14. The above data suggested a diterpene with a 14$\beta$-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 $\delta_C$ 80.3. Moreover, the HMBC spectrum showed that the C-6 methylene protons ($\delta_H$ 2.75 and 3.33) correlated with C-1 ($\delta_C$ 142.6) and C-5 ($\delta_C$ 179.1), but not with C-10 ($\delta_C$ 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 ($\delta_H$ 2.26 and 2.27) and a carbonyl carbon ($\delta_C$ 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 ($\delta_H$ 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-6$\beta$/H-19 and H-7, H-6*a*/H-18, and H-13/H-14 (Figure 1) indicated compound **1** to be 7$\alpha$,10$\alpha$-epoxy-14$\beta$-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 7$\alpha$,10$\alpha$-epoxy-14$\beta$-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 7$\alpha$,10$\alpha$-epoxy-14$\beta$-hydroxy-16(17)-en-15-one moiety. Three olefinic proton singlets at $\delta_H$ 5.21, 5.37, and 6.07 in the $^1$H NMR spectrum of compound **2** and four corresponding olefinic carbons ($\delta_C$ 116.3, 117.0, 147.7, and 151.7) revealed the disappearance of a carbonyl carbon ($\delta_C$ 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 7$\alpha$,10$\alpha$-epoxy-14$\beta$-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 $\mu$M, 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 3$\beta$,5$\beta$,6$\beta$,16$\alpha$-oxygenated grayanane structure.[8−14] However, it is interesting that both **1** and **2** comprise a 7$\alpha$,10$\alpha$-epoxy-14$\beta$-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 $\mu$m particle size) and RP-18 (Merck, 75 $\mu$m 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 $\mu$m 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-7$\beta$-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; [$\alpha_D^{25}$] −134.3 (*c* 0.20, MeOH); IR (film) $\nu_{max}$ 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; [$\alpha_D^{25}$] −73.1 (*c* 0.31, MeOH); IR (film) $\nu_{max}$ 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 ($2 \times 10^6$ 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 $\beta$-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).

**Acknowledgment.** This study was supported by grants from the Plant Diversity Research Center of 21st Frontier Research Program (PF0320903-00) and the National Research Foundation of Korea (NRF) (No. M10642140004-06N4214-00410) funded by the Ministry of Education, Science and Technology (MEST).

**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.

## References and Notes

(1) Loi, D. T. *The Medicinal Plants and Remedies of Vietnam*; Medicinal Publishing House: Hanoi, 2003; p 826.
(2) Ngoc, P. H.; Minh, P. T. H. *Vietnamese J. Pharm.* **1996**, *36*, 15–17.
(3) Minh, P. T. H.; Ngoc, P. H.; Quang, D. N.; Hashimoto, T.; Takaoka, S.; Asakawa, Y. *Chem. Pharm. Bull.* **2003**, *51*, 590–591.
(4) 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.
(5) Giang, P. M.; Son, P. T.; Lee, J. J.; Otsuka, H. *Chem. Pharm. Bull.* **2004**, *52*, 879–882.
(6) Giang, P. M.; Son, P. T.; Hamada, Y.; Otsuka, H. *Chem. Pharm. Bull.* **2005**, *53*, 296–300.
(7) Kuo, P. C.; Shen, Y. C.; Yang, M. L.; Wang, S. H.; Thang, T. D.; Dung, N. X.; Chiang, P. C.; Lee, K. H.; Lee, E. J.; Wu, T. S. *J. Nat. Prod.* **2007**, *70*, 1906–1909.
(8) Shirai, N.; Sakakibara, J. *Phytochemistry* **1980**, *19*, 2159–2162.
(9) Klocke, J. A.; Hu, M. Y.; Chiu, S. F.; Kubo, I. *Phytochemistry* **1991**, *30*, 1797–1800.
(10) Wang, L. Q.; Ding, B. Y.; Qin, G. W.; Lin, G.; Cheng, K. F. *Phytochemistry* **1998**, *49*, 2045–2048.
(11) Terai, T.; Araho, D.; Osakabe, K.; Katai, M.; Narama, I.; Matsuura, T.; Katakawa, J.; Tetsumi, T.; Sato, M. *Chem. Pharm. Bull.* **2000**, *48*, 142–144.
(12) Chen, S. N.; Zhang, H. P.; Wang, L. Q.; Bao, G. H.; Qin, G. W. *J. Nat. Prod.* **2004**, *67*, 1903–1906.
(13) Zhong, G.; Hu, M.; Wei, X.; Weng, Q.; Xie, J.; Liu, J.; Wang, W. *J. Nat. Prod.* **2005**, *68*, 924–926.
(14) Zhang, H. P.; Wang, L. Q.; Qin, G. W. *Bioorg. Med. Chem.* **2005**, *13*, 5289–5298.
(15) Inoue, H.; Tanabe, T.; Umesono, K. *J. Biol. Chem.* **2000**, *275*, 28028–28032.
(16) Salatino, A.; Salatino, M. L. F.; Negri, G. *J. Braz. Chem. Soc.* **2007**, *18*, 11–33.
(17) Iriye, R.; Sakakura, M. *Agric. Biol. Chem.* **1977**, *41*, 2109–2110.
(18) Masutani, T.; Hamada, M.; Kawano, E.; Iwasa, J.; Kumazawa, Z.; Ueda, H. *Agric. Biol. Chem.* **1981**, *45*, 1281–1282.

NP900215R