New Antimycobacterial and Antimalarial 8,9-Secokaurane Diterpenes from *Croton kongensis*

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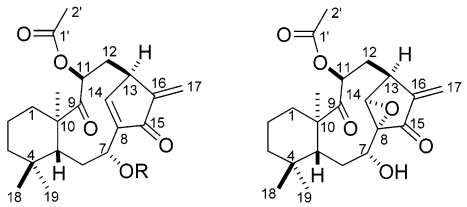
Two new 8,9-secokaurane diterpenes, *ent*-8,9-*seco*-7α,11*β*-diacetoxykaura-8(14),16-dien-9,15-dione (**1**) and *ent*-8,9-*seco*-8,14-epoxy-7α-hydroxy-11*β*-acetoxy-16-kauren-9,15-dione (**2**), together with two known compounds, *ent*-8,9-*seco*-7α-hydroxy-11*β*-acetoxykaura-8(14),16-dien-9,15-dione (**3**) and *ent*-7*β*-hydroxy-15-oxokaur-16-en-18-yl acetate, were isolated from *Croton kongensis*. This is the first report on the presence of 8,9-secokauranes in the plant genus *Croton*. Diterpenes **1**−**3** exhibited antimycobacterial activity with minimum inhibitory concentrations (MICs) of 25.0, 6.25, and 6.25 *μ*g/mL, respectively, and possessed antimalarial activity with IC_{50} ranges of 1.0−2.8 *μ*g/mL. They also demonstrated comparable cytotoxicity toward the Vero (IC_{50} ranged from 0.9 to 3.2 *μ*g/mL), KB (IC_{50} from 1.2 to 13.8 *μ*g/mL), and BC cell lines (IC_{50} from 1.1 to 2.2 *μ*g/mL, except for compound **1**, which was inactive against BC cells).

The genus *Croton* (Euphorbiaceae) is distributed throughout Thailand, and several species have been used as ingredients in Asian traditional medicine for the treatment of dysmenorrhea, gastric ulcers, gastric cancers, and dysentery. The plant *Croton kongensis* Gagnep., commonly known in Thailand as "Plao Ngeon" or "Plao Noi", is frequently used in folk medicine for dysmenorrhea. Our preliminary activity screening showed that a crude CH_2Cl_2 extract of *C. kongensis* exhibited antimalarial (IC_{50} 0.9 *μ*g/mL) and antimycobacterial (MIC 12.5 *μ*g/mL) activities. This prompted us to investigate the active principles in this plant for which no chemical analyses have been reported. In this paper, we report the isolation, structural elucidation, and biological activities of three 8,9-secokauranes from *C. kongensis*.

The CH_2Cl_2 extract of *C. kongensis* leaves was separated by Sephadex LH-20 column chromatography and HPLC (C_{18} reversed-phase column), yielding the two new 8,9-secokauranes **1** and **2**, together with the known *ent*-8,9-*seco*-7α-hydroxy-11*β*-acetoxykaura-8(14),16-dien-9,15-dione (**3**) and *ent*-7*β*-hydroxy-15-oxokaur-16-en-18-yl acetate. Spectral data of compounds **3** and **4** were in good agreement with those published.[1,2]



1 R = Ac
3 R = H

2

Compound **1** was assigned the molecular formula $C_{24}H_{32}O_6$, as revealed by ESITOFMS. The ^1H and ^{13}C NMR spectra of **1** were almost identical to those of the known 8,9-secokaurane **3**,[1] except for the additional acetate signal in **1**. Analysis of the ESITOFMS and ^1H and ^{13}C NMR spectral data readily indicated that **1** is an acetate derivative of **3**. The downfield shift of H-7 (δ_H 5.55) and H-11 (δ_H 5.38) in **1** implied that the hydroxyl groups at C-7 and C-11 were esterified, and the HMBC correlations from H-7 to C-1″ and from H-11 to C-1′ unambiguously placed the acetate groups at C-7 and C-11. On the basis of these data, compound **1** was assigned as an acetate derivative of the known 8,9-secokaurane **3** and identified as *ent*-8,9-*seco*-7α,-11*β*-diacetoxykaura-8(14),16-dien-9,15-dione. The assignment of protons and carbons in **1** was successfully established by analysis of its 2D NMR spectral data (Table 1). Assignments of the partial structures from H-1 to H-3, H-5 to H-7, and H-11 to H-14 were completed by analysis of the $^1H-^1H$ COSY spectral data, while the assignment of quaternary carbons was determined from HMBC correlations.

Compound **1** exhibited a negative rotation similar to that of **3**, so it is therefore reasonable to assume that the absolute configuration of **1** is the same as that of **3**.[1] Moreover, the coupling constants at H-7 (δ_H 5.55, dd, 12.2 and 4.5 Hz for **1**; δ_H 4.71, dd, 12 and 4 Hz for **3**) and H-11 (δ_H 5.38, dd, 5.5 and 1.3 Hz for **1**; δ_H 5.23, dd, 5 and 1 Hz for **3**) of compound **1** were also relatively close to those of **3**.[1] This additional spectral evidence strongly supported the proposed stereochemistry for **1**.

Compound **2** was obtained as a brown oil, and the ESITOFMS established the molecular formula as $C_{22}H_{30}O_6$. The ^1H and ^{13}C NMR spectra of **2** resembled those of the 8,9-secokauranes **1** and **3**, but an olefinic proton signal (δ_H 7.23) in **1** (and **3**) was replaced by an oxygenated methine signal (δ_H 3.84) in **2**. These ^1H and ^{13}C NMR spectral data, together with the evidence from the ESITOFMS, indicated that compound **2** is an oxidized form of **3** in which the double bond C-8/C-14 is epoxidized. The HMBC spectrum helped place an acetate ester at C-11, showing the correlations from H-11 to C-1′ and from the singlet methyl (at δ_H 2.08, H-2′) to C-1′. On the basis of these spectral data, compound **2** was identified as *ent*-8,9-*seco*-8,14-epoxy-7α-hydroxy-11*β*-acetoxy-16-kauren-9,15-dione. The protons and carbons in **2** were completely assigned by analysis of its 2D NMR spectra (Table 1).

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Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Spectral Data ($CDCl_3$) of the 8,9-Secokauranes **1** and **2**

	δ_C, multiplicity[a]		δ_H, multiplicity, J in Hz	
position	**1**	**2**	**1**	**2**
1	32.3, t	32.0, t	1.26, m 1.57, m	1.34, m 1.66, m
2	17.8, t	17.7, t	1.48, m 1.60, m	1.52, m 1.63, m
3	41.4, t	41.5, t	1.51, m 1.59, m	1.51, m 1.56, m
4	34.2, s	34.5, s		
5	40.4, d	39.0, d	1.81, dd, 6.1, 1.2	2.12, dd, 7.2, 0.8
6	32.3, t	34.2, t	1.45, m 1.98, m	1.20, m 1.95, m
7	66.3, d	61.8, d	5.55, dd, 12.2, 4.5	4.73, dd, 11.8, 3.4
8	145.0, s	64.7, s		
9	212.1, s	211.6, s		
10	54.6, s	54.3, s		
11	77.6, d	77.6, d	5.38, dd, 5.5, 1.3	5.39, dd, 6.1, 1.4
12	31.7, t	30.5, t	2.36, ddd, 14.6, 5.4, 2.8 (α) 2.95, ddd, 14.6, 5.0, 1.3 (*β*)	2.27, ddd, 15.1, 6.1, 3.6 (α) 2.94, ddd, 15.1, 4.6, 1.6 (*β*)
13	41.0, d	38.7, d	3.61, brs	3.28, bt, 1.5
14	159.3, d	60.9, d	7.23, d, 2.7	3.84, s
15	193.5, s	195.8, s		
16	147.8, s	146.8, s		
17	112.9, t	118.2, t	5.31, brs 5.94, brs	5.36, d, 1.6 6.01, brs
18	33.8, q	33.9, q	1.14, s	1.12, s
19	22.0, q	21.6, q	0.97, s	1.00, s
20	18.2, q	18.1, q	1.06, s	1.08, s
1′	169.0, s	168.8, s		
2′	20.6, q	20.6, q	2.05, s	2.08, s
1″	169.7, s			
2″	20.9, q		2.02, s	

[a] Multiplicity was determined by analysis of DEPT spectrum.

The absolute stereochemistry of 8,9-secokaurane **2** was assumed to be the same as that of **1** and **3** due to the similarity of negative rotations observed. The orientation of the epoxide in **2** was evident from the NOESY spectrum, where H-13 showed a more intense cross-peak with H-12α than with H-12*β*, while the H-14 epoxy proton exhibited a cross-peak with H-12*β*. These spectral data implied that H-14 in **2** is *β*; thus the oxirane ring is α-oriented. The stereochemistry of the 8,14-epoxide ring in 8,9-secokauranes has been reported with the same orientation previously.[3]

Although there have been various classes of phytochemicals isolated from the genus *Croton* such as diterpenes,[4−10] a polysaccharide,[11] a flavonoid,[12] a lignan,[13] a benzofuran sesquiterpene,[14] a polyphenol,[15] and an alkaloid,[16] the presence of 8,9-secokauranes has never been before reported. 8,9-Secokauranes have been reported from two liverwort species[1,3] and from several species in the higher plant genus *Rabdosia* (family Lamiaceae).[17] This present work has indicated the genus *Croton* as an additional source of this diterpenoid subclass.

The isolated 8,9-secokaurane diterpenes **1**−**3** exhibited antimycobacterial activity with minimum inhibitory concentrations (MICs) of 25.0, 6.25, and 6.25 *μ*g/mL, respectively. Diterpenes **1**−**3** possessed in vitro antimalarial activity against *Plasmodium falciparum* (K1, multidrug-resistant strain) with IC_{50} ranges of 1.0−2.8 *μ*g/mL (Table S1, Supporting Information). The secokauranes **1**−**3** also demonstrated comparable cytotoxicity toward the Vero cell line (IC_{50} ranged from 0.9 to 3.2 *μ*g/mL), the KB cell line (IC_{50} ranged from 1.2 to 13.8 *μ*g/mL), and the BC cell line (IC_{50} ranged from 1.1 to 2.2 *μ*g/mL, except for compound **1**, which was inactive against BC cells) (Table S1, Supporting Information). The biological activities of *ent*-7*β*-hydroxy-15-oxokaur-16-en-18-yl acetate were not evaluated due to the limited amount of the isolated material. Although it is known that several 8,9-secokauranes possess in vitro cytotoxic activity,[1,3] Perry et al. reported that they were inactive in the NCI's in vivo hollow fiber assay.[1]

Experimental Section

General Experimental Procedures. The optical rotations were measured on a JASCO DIP370 polarimeter. The UV spectra were recorded on a Cary 1E UV−vis spectrophotometer. The IR spectra were measured on a Perkin-Elmer 2000 spectrometer. The ^{1}H, ^{13}C, DEPT, ^{1}H−^{1}H COSY, NOESY, HMQC, and HMBC NMR experiments were carried out on a Bruker DRX 400 NMR spectrometer, operating at 400 MHz for proton and 100 MHz for carbon. The ESITOFMS were obtained using a Micromass LCT mass spectrometer, and the lock mass calibration was applied for the determination of accurate mass.

Plant Material. *Croton kongensis* was collected in January 2002 from Mae-Taeng, Chiangmai Province, Thailand, and identified by N.R. A voucher specimen (no. NR1291951) was deposited at the Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

Extraction and Isolation. Dried leaves of *C. kongensis* (1 kg) were macerated in CH_2Cl_2 (4 L) for 3 days. The extract was filtered and evaporated to dryness, yielding 11.11 g of crude extract. The crude CH_2Cl_2 extract was purified by Sephadex LH-20 column chromatography (MeOH as eluent), from which nine fractions (80 mL each) were collected. Fraction 5 was repeatedly chromatographed on a Sephadex LH-20 column (eluted with MeOH), followed by preparative HPLC (reversed-phased C_{18} column, eluted with MeCN−H_2O (60:40)), furnishing compounds **2** (9 mg) and **3** (20 mg). Fraction 4 was subjected to Sephadex LH-20 column chromatography (eluted with MeOH) to yield *ent*-7*β*-hydroxy-15-oxokaur-16-en-18-yl acetate (4 mg). Fraction 2 was chromatographed on Sephadex LH-20 (eluted with MeOH) followed by silica column chromatography (gradient elution from EtOAc−hexane, 5:95, to EtOAc−hexane, 10:90), yielding compound **1** (12 mg).

***ent*-8,9-*seco*-7α,11*β*-Diacetoxykaura-8(14),16-dien-9,15-dione (1):** brown oil; $[\alpha]^{30}_D$ −147.6° (*c* 0.575, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 241.4 (4.07) nm; IR (neat) ν_{max} 2928, 1743, 1704, 1653, 1624, 1370, 1231, 1022, 932 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESITOFMS *m*/*z* 439.2089 $[M + Na]^+$, calcd for $(C_{24}H_{32}O_6 + Na)^+$, 439.2097.

***ent*-8,9-*seco*-8,14-Epoxy-7α-hydroxy-11*β*-acetoxy-16-kauren-9,15-dione (2):** brown oil; $[\alpha]^{30}_D$ −16.7° (*c* 0.45, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 233.2 (3.48) nm; IR (neat) ν_{max} 3445, 1748, 1733, 1697, 1684, 1646, 1636, 1558, 1540, 1374, 1219 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; ESITOFMS *m*/*z* 413.1969 $(M + Na)^+$, calcd for $(C_{22}H_{30}O_6 + Na)^+$, 413.1940.

***ent*-8,9-*seco*-7α-Hydroxy-11*β*-acetoxykaura-8(14),16-dien-9,15-dione (3):** brown oil; $[\alpha]^{30}_D$ −54.4° (*c* 0.45, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 239.2 (4.46) nm; ESITOFMS *m*/*z* 383 $[M + Na]^+$.

***ent*-7*β*-Hydroxy-15-oxokaur-16-en-18-yl acetate:** colorless oil; $[\alpha]^{30}_D$ −4.0° (*c* 1.0, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 242.8 (3.68) nm; ESITOFMS *m*/*z* 375 $[M + H]^+$.

Bioassays. Cytotoxicity was determined by employing the colorimetric method described by Skehan and co-workers.[18] The reference compound, ellipticine, exhibited activity toward Vero, KB, and BC cell lines with IC_{50} ranges of 0.2−0.3 *μ*g/mL. The antimycobacterial activity was assessed against *Mycobacterium tuberculosis* H37Ra using the Microplate Alamar Blue Assay (MABA).[19] The standard drugs, isoniazid and kanamycin sulfate, used as reference compounds for the antimycobacterial assay showed MIC values of 0.040−0.090 and 2.0−5.0 *μ*g/mL, respectively, in the test systems. The antimalarial activity was evaluated against the parasite *Plasmodium falciparum* (K1, multidrug-resistant strain), which was cultured continuously according to the method of

Trager and Jensen.[20] Quantitative assessment of antimalarial activity in vitro was determined by the microculture radioisotope technique based on the method described by Desjardins et al.[21] The inhibitory concentration (IC_{50}) represents the concentration that causes 50% reduction in parasite growth as indicated by the in vitro uptake of [^{3}H]-hypoxanthine by *P. falciparum*. An IC_{50} value of 1 ng/mL was observed for the standard compound, artemisinin, in this test system.

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Supporting Information Available: ^{1}H and ^{13}C NMR spectra of 8,9-secokauranes **1** and **2**, the NOESY spectrum of **2**, and table of biological test data. This material is available free of charge via the Internet at http://pubs.acs.org.

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