Eudesmane Sesquiterpenes from the Aquatic Fungus *Beltrania rhombica*

Vatcharin RUKACHAISIRIKUL,*[,a] Chutanat KAEWBUMRUNG,[a] Souwalak PHONGPAICHIT,[b] and Zubaidah HAJIWANGOH[b]

[a] *Department of Chemistry, Faculty of Science, Prince of Songkla University; Songkhla 90112, Thailand: and* [b] *Department of Microbiology, Faculty of Science, Prince of Songkla University; Songkhla 90112, Thailand.*
Received September 2, 2004; accepted November 9, 2004

From the ethyl acetate extract of the culture broth of *Beltrania rhombica*, two new eudesmane sesquiterpenes, named rhombidiol (1) and rhombitriol (2), were isolated and characterized along with five enantiomers of known sesquiterpenes: (−)-β-eudesmol, (−)-pterocarpol, (−)-chrysanthemol, (−)-longilobol and (−)-5β-hydroxy-β-eudesmol. Their structures were determined by analysis of 1D and 2D NMR data and comparison of spectral data and physical data with those previously reported.

Key words *Beltrania rhombica*; aquatic fungi; eudesmane sesquiterpene

In the course of our ongoing research for bioactive fungal secondary metabolites, the ethyl acetate extract from the culture broth of the freshwater fungus *Beltrania rhombica* was found to exhibit interesting antibacterial and anifungal activities against *Staphylococcus aureus* ATCC25923 and *Candida albicans* with MIC values of 0.98 and 15.6 μg/ml, respectively. This fungus was collected at the Ton-Nga-Chang Wildlife Sanctury, Songkhla Province, southern Thailand.[1)] To our knowledge, no chemical investigation of *B. rhombica* has been reported. We describe herein the isolation and structural elucidation of two new sesquiterpenes and five enantiomers of known eudesmane sesquiterpenes. They were tested for antimicrobial activity against *S. aureus* ATCC25923 and *C. albicans*.

Rhombidiol (**1**) possesses the molecular formula $C_{15}H_{26}O_2$ as determined by HR-MS. The IR spectrum displayed absorption bands at 3360 and 1644 cm^{-1} due to a hydroxyl group and a carbon–carbon double bond, respectively. The ^{1}H-NMR spectrum (Table 1) displayed three singlets [δ 0.73 (Me-14), 1.21 (Me-12) and 1.22 (Me-13)], two quartets of exocyclic methylene protons [δ 4.51 (H-15) and δ 4.71 (H-15), J=1.5 Hz] and a triplet of an oxymethine proton (δ 3.58, J=2.0 Hz, H-9). The ^{13}C-NMR spectrum contained 15 carbons (Table 1). The DEPT spectra indicated the presence of three methyl (δ 16.8, 27.0, 27.5), six methylene (δ 23.3, 24.9, 29.7, 34.8, 36.7, 115.3), three methine (δ 42.1, 42.4, 75.2) and three quaternary (δ 40.0, 72.6, 151.1) carbons. These results established a hydroxylated β-eudesmol skeleton for **1**.[2)] The fragments in the mass spectrum at *m/z* 159, 145, 105 and 59 clearly confirmed a diol with an eudesmane skeleton.[3)] All methyl and the geminal olefinic protons were used as starting points to assign other protons and carbons by analysis of HMBC (Table 1) and HMQC correlations. In addition, the establishment of the proton connectivity was deduced from the COSY spectrum. The angular methyl protons (Me-14) gave ^3J cross peaks in the HMBC spectrum with the methylene, methine and oxymethine carbons at δ 34.8 (C-1), 42.4 (C-5) and δ 75.2 (C-9), respectively, while H-9 showed ^3J HMBC correlations with C-5 and the methine carbon at δ 42.1 (C-7). In addition, ^3J cross peaks between the gem-dimethyl protons (Me-12 and Me-13) and C-7 as well as those between the geminal olefinic protons (H-15) and C-3 (δ 36.7) and C-5 were observed. These results established the location of the hydroxyisopropyl and hydroxyl groups at C-7 and C-9 of the β-eudesmane skeleton, respectively. These assignments were further supported by ^{1}H–^{1}H COSY correlations of H-7 (δ 1.80, m) with Hab-6 (δ 1.20, m and δ 1.63, m) and Hab-8 (δ 1.66, tm, J=12.0 Hz and δ 1.79, dm, J=12.0 Hz) and those of H-9 with Hab-8. The geminal olefinic protons which appeared as two quartets showed cross peaks in the ^{1}H–^{1}H COSY spectrum with each other and with the Ha-3 (δ 2.00, t br d, J=13.5, 6.0 Hz) and H-5 (δ 2.28, dm, J=12.0 Hz). From the large coupling constant of H-5, the presence of a *trans*-decalin framework was deduced.[4)] Irradiation of H-5 affected the signal enhancement of Ha-1, Ha-3 and H-7, but not H-9 and Me-14 (Fig. 1). These results confirmed the *trans*-relationship between H-5 and Me-14 and also established the spatial arrangement of H-7 and H-9 in axial β- and equatorial α-positions, respectively. The triplet signal of H-9 with a small coupling constant of 2 Hz due to coupling with both axial and equatorial neighboring protons (H_aH_b-8) supported its equatorial α-position. From these results, rhombidiol (**1**) was assigned as eudesm-4(14)-ene-9β,11-diol.

Rhombitriol (**2**), with a molecular formula of $C_{15}H_{26}O_3$ by HR-MS, exhibited IR absorption bands at 3441 (O–H stretching) and 1645 (C=C stretching) cm^{-1}. The ^{1}H-NMR spectrum (Table 1) was similar to that of (−)-longilobol (**3**). However, the angular methyl protons (Me-14) in **2** were shifted to lower field than those found in **3**, a *trans* 5-hydroxyeudesmane (δ 0.87), suggesting that the C-5 hydroxyl group was *cis* to Me-14.[5)] This was confirmed by the following difference NOE data. For *trans*-fused decalins, such as **1**, enhancement of the axial H-2, H-6 and H-8 signals should be observed when Me-14 is irradiated (Fig. 1). In our case, the axial H-1 signal (δ 1.79, td, J=13.5, 4.5 Hz), instead of the axial H-2 signal (δ 1.74, m), and the axial H-6 signal (δ 2.23, t, J=13.5 Hz) were affected on irradiation of Me-14 (Fig. 1). These results also indicated that an axial α-position of C-8 was occupied by a hydroxyl group, not a hydrogen atom. The methine proton, H-7 (δ 1.96, ddd, J=13.5, 6.5, 3.5 Hz), was located at the axial β-position according to its signal enhancement upon irradiation of the equatorial H-8β. Thus, rhombitriol (**2**) was assigned as 5α, 8α-dihydroxy-β-eudesmol which differed from **3** at the stereochemistry of C-5 and C-8.

* To whom correspondence should be addressed. e-mail: vatcharin.r@psu.ac.th
© 2005 Pharmaceutical Society of Japan

Table 1. NMR Data of Compounds **1** and **2**

Position	C-type	**1** ^{1}H	**1** ^{13}C	**1** HMBC (^{1}H→^{13}C)	**2** ^{1}H	**2** ^{13}C	**2** HMBC (^{1}H→^{13}C)
1	CH_2	H_a: 1.85 (m)	34.8	C-2, C-3, C-10 and C-15	H_a: 1.79 (td, 13.5, 4.5)	34.4	C-2, C-10 and C-15
		H_b: 1.22 (m)			H_b: 1.07 (br d, 13.5)		
2	CH_2	1.65 (m)	23.3	C-1, C-3 and C-10	H_a: 1.74 (m)	21.3	C-1, C-3 and C-10
					H_b: 1.59 (m)		
3	CH_2	H_a: 2.00 (t br d, 13.5, 6.0)	36.7	C-1, C-4 and C-5	H_a: 2.60 (td, 13.5, 6.5)	31.4	C-1, C-4 and C-5
		H_b: 2.30 (dm, 13.5)			H_b: 2.14 (d dm, 13.5, 5.0)		
4	C		151.1			151.5	
5	C					76.0	
	CH	2.28 (dm, 12.0)	42.4	C-1 and C-4			
6	CH_2	H_a: 1.20 (m)	24.9	C-4 and C-11	H_a: 2.23 (t, 13.5)	26.4	C-5, C-7, C-8 and C-11
		H_b: 1.63 (m)			H_b: 1.63 (dd, 13.5, 3.5)		
7	CH	1.80 (m)	42.1	C-5, C-8 and C-9	1.96 (ddd, 13.5, 6.5, 3.5)	43.9	C-5, C-8 and C-9
8	CH				4.30 (m)	69.1	C-6, C-9 and C-10
	CH_2	H_a: 1.66 (tm, 12.0)	29.7	C-6, C-7 and C-9			
		H_b: 1.79 (dm, 12.0)					
9	CH	3.58 (t, 2.0)	75.2	C-5 and C-7			
	CH_2				H_a: 1.87 (dd, 14.5, 4.2)	42.7	C-5, C-8, C-10 and C-14
					H_b: 1.40 (dd, 14.5, 3.1)		
10	C		40.0			37.2	
11	C		72.6			73.3	
12	CH_3	1.21 (s)	27.5$^{a)}$	C-7, C-11 and C-13	1.26 (s)	28.9$^{a)}$	C-7, C-11 and C-13
13	CH_3	1.22 (s)	27.0$^{a)}$	C-7, C-11 and C-12	1.42 (s)	28.7$^{a)}$	C-7, C-11 and C-12
14	CH_3	0.73 (s)	16.8	C-1, C-5, C-9 and C-10	1.13 (s)	22.4	C-1, C-5, C-9 and C-10
15	CH_2	H_a: 4.71 (q, 1.5)	115.3	C-3, C-4 and C-5	H_a: 4.85 (t, 1.5)	107.9	C-3, C-4 and C-5
		H_b: 4.51 (q, 1.5)		C-3, C-4 and C-5	H_b: 4.83 (t, 1.5)		C-3, C-4 and C-5

a) Interchangeable.

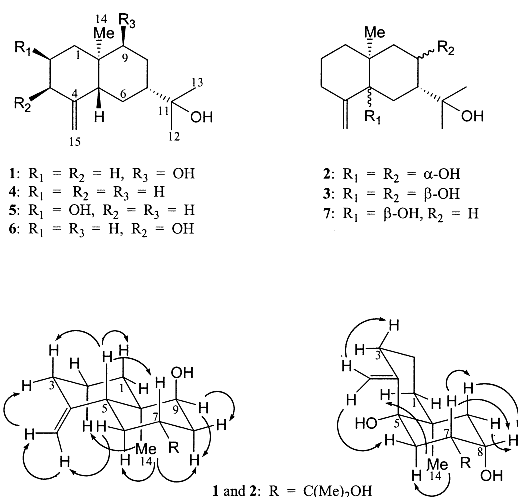

Fig. 1. Selected NOE Enhancements in Compounds **1** and **2**

The other isolated compounds were identified by comparison of physical data, especially $[\alpha]_D$, HR-MS and NMR spectral data, with the reported data to be (−)-longilobol (**3**),[6] (−)-β-eudesmol (**4**),[7,8] (−)-pterocarpol (**5**),[9] (−)-chrysanthemol (**6**)[2] and (−)-5β-hydroxy-β-eudesmol (**7**).[5,10] This is the first report on the isolation of (−)-enantiomers of above sesquiterpenes. All compounds isolated showed weak antibacterial and antifungal activities with similar MIC values >128 μg/ml against *S. aureus* ATCC25923 and *C. albicans* while standard vancomycin and amphotericin B gave MIC values of 1.0 and 0.1 μg/ml, respectively. Further work is required to explain the activity of the crude extract.

Experimental

General Experimental Procedures Melting points were determined on a melting point apparatus Electrothermal 9100 and are uncorrected. Optical rotations were measured in methanol solution at the sodium D line (590 nm) on an AUTOPOL®II automatic polarimeter. Infrared spectra (IR) were obtained on a Perkin Elmer Spectrum GX FT-IR system. ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian UNITY INOVA 500 MHz (^{1}H) and 125 MHz (^{13}C) spectrometer using deuteriochloroform solutions with tetramethylsilane (TMS) as internal standard. EI and HR-EI mass spectra were measured on a ThermoFinnigan MAT95XL spectrometer. Thin-layer chromatography (TLC) and precoated TLC was performed on silica gel 60 GF_{254} (Merck). Light petroleum had bp 40—60 °C.

Fungal Isolation *Beltrania rhombica* was isolated from a foam sample collected at Ton-Nga-Chang waterfall, Songkhla province, southern Thailand, during June–July, 1998.[1] The fungus was identified on the basis of its morphology.[11] *B. rhombica* is a dematiaceous hyphomycetes. It produces 1-celled conidia, brown color with a paler middle band. The conidial body widest at 10 μm with 1 hyaline seta. Colony on potato dextrose agar (PDA) is dark brown with gray aerial mycelium. Culture of *B. rhombica* T031 was stored under sterile mineral oil at room temperature at the Department of Microbiology, Faculty of Science, Prince of Songkla University.

Fermentation and Isolation The fresh mycelium grown on PDA at 25 °C for 5 d was inoculated into 500 ml Erlenmeyer flasks containing 200 ml potato dextrose broth (PDB) on a rotary shaker at 150 rpm at 25 °C for 5 d. The flask cultures were filtered to effect separation into filtrate and wet mycelia. The filtrate was extracted twice with an equal amount of EtOAc. The combined EtOAc solution was dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to obtain a mixture of dark brown viscous oil (830 mg). The extract was fractionated by column chromatography, eluting with solvent mixtures of increasing polarity ($CHCl_3$ to 40% MeOH–$CHCl_3$) to afford 9 fractions (A1—A9). (−)-β-Eudesmol (**4**) [13.4 mg, $[\alpha]_D^{29}$ −37.9° (c=0.09, $CHCl_3$); $[\alpha]_D$ +36.0° (c=1.0, $CHCl_3$)][8] was obtained from fraction A2. Fraction A5 (103.6 mg) eluted with 1—3% MeOH–$CHCl_3$ was subjected to column chromatography, eluting with solvent mixtures of increasing polarity ($CHCl_3$ to 5% MeOH–$CHCl_3$) to afford (−)-longilobol (**3**) [2.8 mg, $[\alpha]_D^{29}$ −105.0° (c=0.08, MeOH); $[\alpha]_D$ +95.0° (c=0.77, MeOH)].[6] Fraction A6 (148.5 mg) eluted with 3—10% MeOH–$CHCl_3$ was fractionated by column chromatography with solvent mixtures of increasing polarity (CH_2Cl_2 to 30% EtOAc–CH_2Cl_2) to yield 6 fractions. (−)-5β-Hydroxy-β-

eudesmol (**7**) [2.5 mg, $[\alpha]_D^{29}$ $-104.4°$ (c=0.09, $CHCl_3$); $[\alpha]_D$ $+122.0°$ (c=1.0, $CHCl_3$)][5] and **1** (2.2 mg) were obtained from the second and sixth fractions while (−)-chrysanthemol (**6**) [3 mg, $[\alpha]_D^{29}$ $-22.6°$ (c=0.12, $CHCl_3$) $[\alpha]_D$ $+20.0°$ (c=0.21, $CHCl_3$)][2] was isolated from the fourth fraction after purification on preparative TLC with 2% EtOAc–CH_2Cl_2 (10 runs). Fraction A8 (133.8 mg, eluted with 10—15% MeOH–$CHCl_3$) was subjected to column chromatography eluted with solvent mixtures of increasing polarity (CH_2Cl_2 to 15% EtOAc–CH_2Cl_2) to afford **2** (5.2 mg) and (−)-pterocarpol (**5**) [4 mg, $[\alpha]_D^{29}$ $-28.8°$ (c=0.26, $CHCl_3$); $[\alpha]_D$ $+34.0°$ (c=0.29, $CHCl_3$)].[9]

Rhombidiol (**1**): White solid, mp 164—165 °C. ^{1}H- and ^{13}C-NMR data see Table 1. IR (film) ν_{max} 3360, 1644 cm^{-1}. HR-EI-MS m/z 220.1828 (Calcd for $C_{15}H_{24}O$ $[M-H_2O]^+$: 220.1824). EI-MS m/z 220 $[M-H_2O]^+$ (11), 202 (42), 187 (28), 159 (100), 147 (41), 145 (45), 105 (35), 91 (36), 59 (52). $[\alpha]_D^{29}$ $-72.0°$ (c=0.05, $CHCl_3$).

Rhombitriol (**2**): White solid, mp 159—160 °C. ^{1}H- and ^{13}C-NMR data see Table 1. IR (film) ν_{max} 3441, 1645 cm^{-1}. HR-EI-MS m/z 236.1788 $[M-H_2O]^+$ (Calcd for $C_{15}H_{24}O_2$ $[M-H_2O]^+$: 236.1776). EI-MS m/z 236 $[M-H_2O]^+$ (16), 218 (14), 203 (12), 178 (34), 160 (88), 145 (60), 124 (100), 109 (36), 95 (87), 81 (45), 59 (55). $[\alpha]_D^{29}$ $-120.0°$ (c=0.14, MeOH).

Antimicrobial Activity The minimum inhibitory concentrations (MICs) were determined by agar dilution assay.[12] The test microorganisms were *Staphylococcus aureus* ATCC25923 and clinical isolates of *Candida albicans*.[13] The crude extract was dissolved in DMSO. Serial 2-fold dilutions of the test extract were mixed with melted Mueller–Hinton agar (MHA) for bacteria and RPMI glucose medium for yeasts in the ratio of 1 : 50 in microtiter plates with flat-bottomed wells. Final concentrations in agar ranged 500 to 0.49 μg/ml for crude extract and 128—0.25 μg/ml for pure compounds. Inoculum suspensions were spotted on agar- for bacteria filled wells, 10^4 CFU per spot. The inoculated plates were incubatd at 35 °C 18 and 24 h for *S. aureus* ATCC25923 and *C. albicans*, respectively. MICs were recorded by reading the lowest concentrations that inhibited visible growth. Growth control were performed on agar containing DMSO. Vancomycin and amphotericin B were used as positive control for *S. aureus* ATCC25923 and clinical isolates of *C. albicans*, respectively.

Acknowledgments C. K. thanks the Institute for the Promotion of Teaching Science Technology (IPST) for a scholarship. The Postgraduate Education and Research Program in Chemistry (PERCH) is also acknowledged for partial support.

References

1) Sakayaroj J., "A Survey and Study of the Aquatic Fungi at Ton Nga Chang Wildlife-Sanctuary, Songkhla Province," M.Sc. Thesis, Prince of Songkla University, 2000.
2) Raharivelomanana P., Bianchini J.-P., Faure R., Cambon A., Azzaro M., *Phytochemistry*, **41**, 243—246 (1996).
3) Pascual-T. J., Bellido I. S., Gonzalez M. S., *Tetrahedron*, **36**, 371—376 (1980).
4) El-Ghazouly M. G., El-Sebakhy N. A., Seif El-Din A. A., Zdero C., Bohlmann F., *Phytochemistry*, **26**, 439—443 (1987).
5) Kitagawa T., Yamazoe Y., Shibuya H., Takeda R., Takeno H., Yosioka I., *Chem. Pharm. Bull.*, **22**, 2662—2674 (1974).
6) Shafizadeh F., Bhadane N. R., *Tetrahedron Lett.*, **24**, 2171—2174 (1973).
7) Varma K. R., Jain T. C., Bhattacharyya S. C., *Tetrahedron*, **18**, 979—984 (1962).
8) Kumar N., Ravindranath B., Seshadri T. R., *Phytochemistry*, **13**, 633—636 (1974).
9) Nasini G., Piozzi F., *Phytochemistry*, **20**, 514—516 (1981).
10) Xiong Z., Zhou G., Yang J., Chen Y., Li Y., *Tetrahedron Asymmetry*, **9**, 1525—1530 (1998).
11) Ingold C. T., "An Illustrated Guide to Aquatic and Water-Borne Hyphomycetes (Fungi Imperfecti) with Notes on their Biology," Scientific Publication, England, 1975.
12) Lorian V., "Antibiotics in Laboratory Medicine," 4th ed., William and Wilkins, Baltimore, 1996, pp. 28—32.
13) Hajiwangoh Z., "Screening of Antimicrobial Active Metabolites from Filamentous Fungi," M.Sc. Thesis, Prince of Songkla University, 2001.