C3_C0039: LABDANE-TYPE DITERPENES FROM THE LEAVES OF *Enicosanthum fuscum* (ANNONACEAE)

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Abstract: Chemical investigation of the hexane extract of *Enicosanthum fuscum* (Annonaceae) resulted in the isolation and identification of four known labdane-type diterpenoids, copalic acid (**1**), 5α,8α-(2-oxokolavenic acid) (**2**), rhinocerotinoic acid (**3**), and labd-13*E*-en-8-ol-15-oic acid (**4**). Their structures were confirmed by spectroscopic data.

Introduction: The genus *Enicosanthum* belongs to Annonaceae family. Only four species are found in Thailand including *E. cupulare* (king) Air-Shaw, *E. Fuscum* (king) Air-Shaw, *E. membranifolium* J. Sinclair and *E. praestigiosum* J. Sinclair. *Enicosanthum fuscum* (King) Airy Shaw which is one of the 4 species, commonly called "nangnaapondting" in Thai.[1] Only a few species of the genus have been chemically investigated. Previous phytochemical studies of plants in this genus have revealed the presence of alkaloids and diterpenes.[2]

Methodology:

General Experimental Procedures: Melting points were determined on the Fisher-Johns melting point apparatus. The optical rotations were determined on a JASCO P-1020 polarimeter. UV spectra were recorded with a Shimadzu UV-VIS 2001S spectrophotometer. The IR spectra were recorded as KBr disk on a Shimudzu FTIR-8900 IR spectrophotometer. The NMR spectra were recorded using a Bruker AVANCE 400 (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR) spectrometer. Chemical shifts are recorded in parts per million (δ) in $CDCl_3$ with TMS as an internal standard. Quick column chromatography (QCC) and column chromatography (CC) were carried out on silica gel 60 H (Merck, 5−40 μm) and silica gel 100 (Merck, 63−200 μm), respectively. Precoated plates of silica gel 60 F254 were used for analytical purposes.

Plant Material: The leaves of *E. fuscum* were collected in Trang Province, Thailand in 2010. The plant was identified by Dr. Piya Chalermglin, Thailand Institute of Scientific and Technological Research, Thailand. A voucher specimen (PKRU2010028) was deposited at the Laboratory of Natural Products Chemistry, Faculty of Science and Technology, Phuket Rajabhat University, Phuket, Thailand.

Extraction and Isolation: The dried leaves of *E. fuscum* (1.6 kg) were powdered and extracted with ethanol at room temperature. The extract was filtered and evaporated to dryness under reduced pressure to give a dark-green viscous gum (130.95 g). The crude extract was partitioned between dichloromethane and water (1.94 L:2.30 L) to give a dichloromethane extract (77.1 g, dark-green viscous gum) and a water extract (20.3 g, dark-brown viscous gum) after removal of solvent. The dichloromethane extract (77.1 g) was subjected to vacuum liquid chromatography over a sintered glass filter column of silica gel using increasing amount of EtOAc in hexane (1% EtOAc to 100% EtOAc) and increasing amount of MeOH in EtOAc (1% MeOH to 100% MeOH)) to yield nine fractions (A-I). Fraction B (3.90 g) was separated by flash CC over silica gel using hexane/CH_2Cl_2 (50:50, 30:70) and methanol to give six fractions (Fractions B-1−B-6). Fraction B-5 (140 mg) was further separated by Lichroprep RP-18 and eluted with MeOH/H_2O (41:1) to give **1** (33 mg). Fraction B-4 (1.56 g) was separated on CC over silica gel using hexane/CH_2Cl_2 (70:30) to give **4** (20 mg). Fraction F (16.2 g) was separated by flash CC over silica gel eluted with

hexane/CH_2Cl_2 (90:10, 80:20, 30:70, 50:50) and CH_2Cl_2 to give six fractions (Fractions F-1–F-6). Fraction F-3 (2.5 g) was further separated by CC over silanized silica gel using hexane/CH_2Cl_2 (30:70, 50:50, 60:40, 70:30, 90:10) and CH_2Cl_2 to give six fractions (Fractions F-3-1–F-3-6). Fraction F-3-3 (30 mg) was further purified by CC on silanized silica gel using CH_2Cl_2/EtOAc (90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 90:10) and EtOAc to give **2** (5 mg). Fraction D (2.3 g) was separated by flash CC on silica gel eluted with hexane and hexane/EtOAc (90:10, 20:80, 30:70, 50:50,) to give six fractions (Fractions D-1–D-6). Fraction D-6 (55.9 mg) was purified by flash CC over silica gel using hexane/EtOAc (90:10, 80:20, 30:70, 50:50) and EtOAc to give five fractions (Fractions D-6-1–D-6-5). Fraction D-6-4 (23 mg) was separated by CC over silica gel using hexane/CH_2Cl_2/acetone (5:2:1, 4:2:1, 3:2:1, 2:2:1) and acetone to give **3** (11 mg).

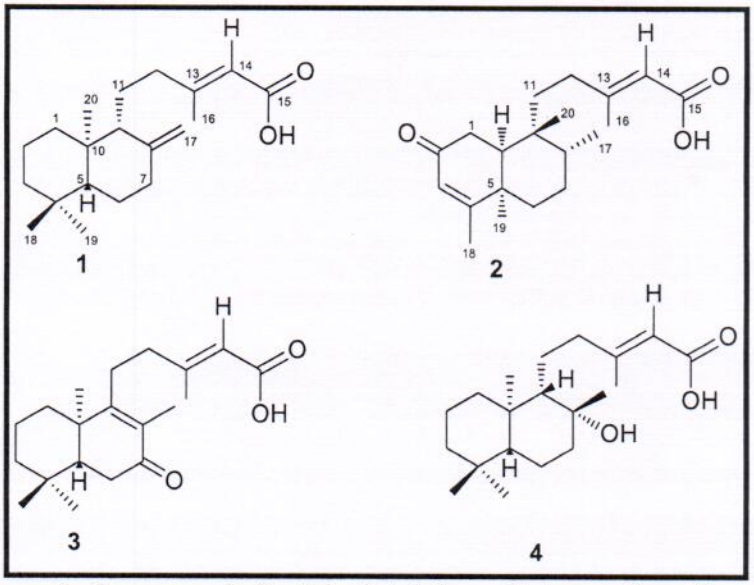

Figure 1. Compounds (**1-4**) isolated from *Enicosanthum fuscum*.

Copalic acid **(1)**

White solid; mp 105-106 °C; $[\alpha]_D^{25}$ -15.0 (*c* 0.07, MeOH); UV (MeOH) λ_{max} nm: 225; IR (KBr) λ_{max}: 1720, 1620, 1160 cm^{-1}; ^{13}C NMR data see Table 1; ^{1}H NMR data see Table 2.

5α,8α-2-Oxokolavenic acid **(2)**

White solid; mp 195-164 °C; $[\alpha]_D^{25}$ -21.0 (*c* 0.03, MeOH); UV (MeOH) λ_{max} nm: 238; IR (KBr) λ_{max}: 1735, 1695, 1650 cm^{-1}; ^{13}C NMR data see Table 1; ^{1}H NMR data see Table 2.

Rhinocerotinoic acid **(3)**

White solid; mp 187-189 °C; $[\alpha]_D^{25}$ +40.0 (*c* 0.02, MeOH); UV (MeOH) λ_{max} nm: 279, 342; IR (KBr) λ_{max}: 1735, 1696, 1654, 1158 cm^{-1}; ^{13}C NMR data see Table 1; ^{1}H NMR data see Table 2.

Labd-13*E*-en-8-ol-15-oic acid **(4)**

White solid; mp 133-135 °C; $[\alpha]_D^{25}$ -14.0 (*c* 0.01, MeOH); UV (MeOH) λ_{max} nm: 283, 354; IR (KBr) λ_{max}: 3400, 1720, 1600, 1219, 1146 cm^{-1}; ^{13}C NMR data see Table 1; ^{1}H NMR data see Table 2.

Results and Discussion: Compound **1** was isolated as a white solid and analyzed for $C_{20}H_{32}O_2$. Its IR spectrum exhibited an absorption band at 1720 cm^{-1}, characteristic of an acid carbonyl group, and absorption bands at 1620 and 1160 cm^{-1}, corresponding to carbon-carbon double bond and carbon-oxygen bond, respectively. The UV spectrum (MeOH, λ_{max}) exhibits maxima at 225 nm. The structure was identified by ^1H and ^{13}C NMR data and by comparision of these data with literature data.[3,4] The ^1H NMR spectrum showed four singlets at δ_H 2.16, 0.86, 0.80, and 0.68, corresponding to the methyl groups (Me-16, 18, 19 and 20), the signals at δ_H 4.85 (*s*) and 4.49 (*s*) for two olefinic protons (H_2-17) of an exocyclic double bond and a singlet at δ_H 5.67 (*s*), characteristic of the olefinic proton H-14. In the ^{13}C NMR spectrum, 20 signals were observed and characterized by DEPT 135° as four methyls, eight methylenes, three methines and five quaternary carbons. According to this information in conjunction with HMBC analyses (Figure 2). The relative stereochemistry was established by NOESY correlations (Figure 4), and information from the literature,[5] **1** was identified as copalic acid.

Compound **2** was obtained as a white solid and analyzed for $C_{20}H_{30}O_3$. Its IR spectrum exhibited an absorption band (1735 cm^{-1}), characteristic of an acid carbonyl, and absorption bands (1695 and 1650 cm^{-1}), corresponding to carbon-oxygen double bond and carbon-carbon double bond, respectively. Assignment for the resonances of all the hydrogen and carbon atoms in the molecule was made by one- and two-dimensional NMR experiments (^1H NMR, ^{13}C NMR, DEPT, COSY, NOESY HMQC, and HMBC). The ^1H NMR spectrum showed resonances for five methyl signals at δ_H 0.95 (*d*, J = 7.3 Hz, H-17), 1.24 (*s*, H-19) and 0.82 (*s*, H-20) for two tertiary and one secondary methyl groups on saturated carbons, and at δ_H 1.94 (*d*, J = 1.5 Hz, H-18) and 2.14 (*d*, J = 1.0 Hz, H-16) for two tertiary methyl groups on unsaturated carbons. The latter exhibited an allylic coupling to olefinic protons at δ_H 5.80 (*s*, H-3) and 5.65 (*s*, H-14). The signal at δ_H 2.14 is characteristic of 5α,8α(2-oxokolavenic acid) with the *E*-configuration.[3,6] The ^{13}C NMR and DEPT experiments showed six quaternary carbons including a carbonyl carbon at δ_C 200.2 (C-2), two olefinic methine carbons at δ_C 125.5 (C-3) and 115.1 (C-14), two methine carbons, five methylene carbons and five methyl carbons. The relative configurations of the positions C-5, C-8, C-9 and C-10 were determined based on the NOESY correlations (Figure 4). This information in conjunction with COSY and HMBC analyses (Figure 3), indicated that compound **2** was 5α,8α-(2-oxokolavenic acid).

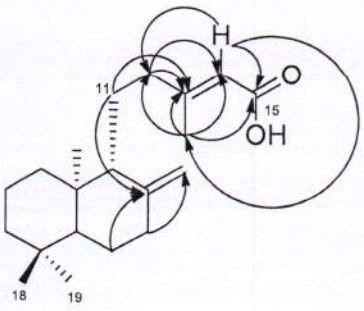

Figure 2. Selected HMBC correlations of **1**.

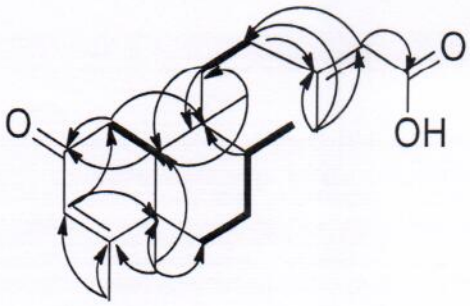

Figure 3. Selected HMBC correlations of **2**.

Compound **3** was obtained as a white solid and analyzed for $C_{20}H_{31}O_3$. Its IR spectrum exhibited absorption bands for carboxyl carbonyl group (1735 cm^{-1}), and ketone carbonyl group (1696 cm^{-1}). The relative stereochemistry was established by NOESY correlations (Figure 4). The ^1H NMR and ^{13}C NMR data (tables 1 and 2) were similar to those of **1,** except that methylene proton on C-7 in **1** was replaced by a carbonyl group and a methylene group on C-17 was replaced by a methyl proton resonating at δ_H 1.78 (*s*) in **3**.

Thus, on the basis of the spectroscopic data and comparison of the ^{1}H and ^{13}C NMR spectral data with the previously reported data,[6,7] compound **3** was identified as rhinocerotinoic acid.

Table 1. ^{13}C NMR data of compounds **1-4** (100 MHz, $CDCl_3$).

Position	1	2	3	4
1	39.1 (CH_2)	35.5 (CH_2)	35.9 (CH_2)	39.2 (CH_2)
2	19.4 (CH_2)	200.2 (C)	18.6 (CH_2)	18.2 (CH_2)
3	42.1 (CH_2)	125.5 (CH)	41.3(CH_2)	42.2 (CH_2)
4	33.6 (C)	172.7 (C)	33.1 (C)	33.2 (C)
5	55.5 (CH)	39.9 (C)	50.3(CH)	55.9 (CH)
6	24.5 (CH_2)	34.9 (CH_2)	39.8 (CH_2)	18.1 (CH_2)
7	38.3 (CH_2)	26.8 (CH_2)	200.2 (C)	42.0 (CH_2)
8	148.3 (C)	36.0 (C)	166.3 (C)	73.7 (C)
9	56.2 (CH)	38.8 (C)	130.6 (C)	58.8 (CH)
10	39.7 (C)	45.7 (C)	41.0 (C)	39.0 (C)
11	21.5 (CH_2)	34.3 (CH_2)	27.7 (CH_2)	23.5 (CH_2)
12	40.1 (CH_2)	35.4 (CH_2)	39.8 (CH_2)	44.8 (CH_2)
13	164.3 (C)	163.0 (C)	161.6 (C)	163.1 (C)
14	114.6 (CH)	115.1 (CH)	115.3 (CH)	115.0 (CH)
15	171.8 (C)	171.6 (C)	177.3(C)	171.8 (C)
16	19.2 (CH_3)	19.5 (CH_3)	19.1 (CH_3)	19.3 (CH_3)
17	106.4(CH_3)	15.7 (CH_3)	11.4 (CH_3)	30.5 (CH_3)
18	33.6 (CH_3)	17.8 (CH_3)	32.8 (CH_3)	33.4 (CH_3)
19	21.7 (CH_3)	18.4 (CH_3)	21.3 (CH_3)	21.7 (CH_3)
20	14.5 (CH_3)	19.0 (CH_3)	18.2 (CH_3)	15.1 (CH_3)

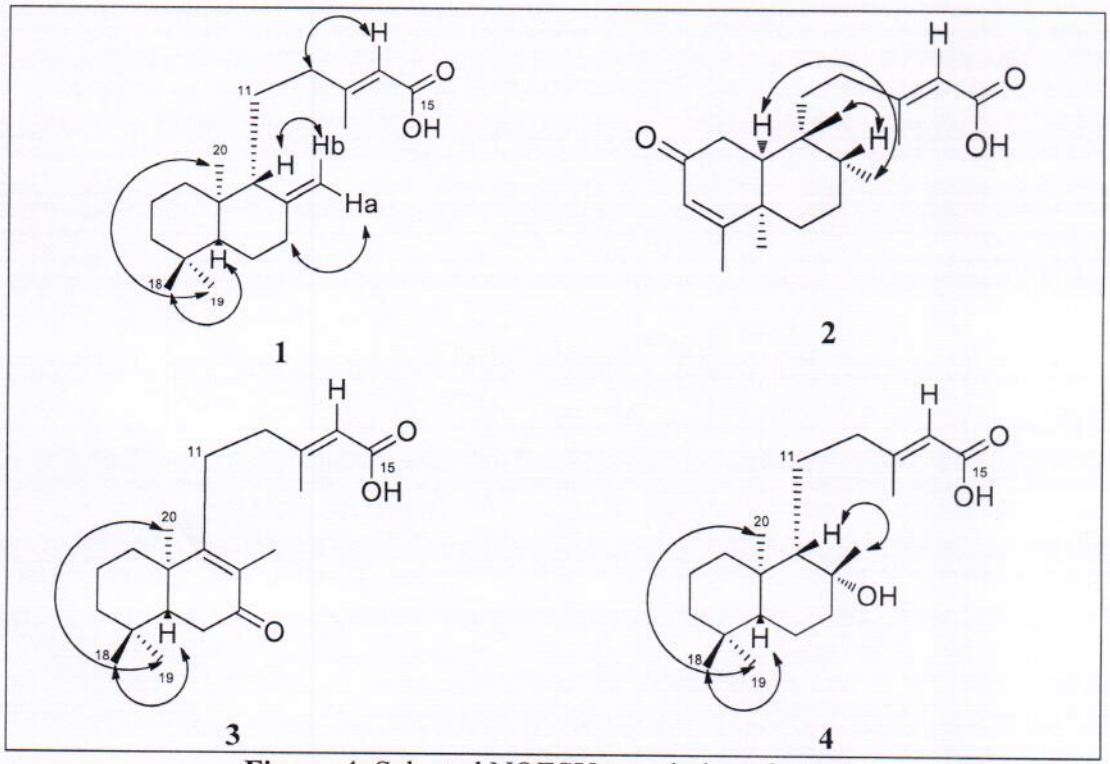

Figure 4. Selected NOESY correlations for **1-4**.

Table 2. ^{1}H NMR data (*J* in Hz) of compounds **1-4** (400 MHz, $CDCl_3$).

Position	1	2	3	4
1	1.00 m	2.56 d (18.7)	1.63 m	1.66 m
	1.73 m	2.82 dd (6.3,18.6)	0.95 m	0.96 m
2	1.50 m		1.57 m	1.59 m
	1.57 m		1.44 m	1.44 m
3	1.17 m	5.70 s	1.38 m	1.38 m
	1.39 m		1.15 m	1.15 m
5	1.08 m		1.60 m	0.93 m
6	1.30 m	1.48 m	1.63 m	1.62 m
	1.55 m	1.84 m	1.27 m	1.27 m
7	1.96 m	1.30 m	1.87 m	1.87 m
	2.38 m	1.60 m	1.85 m	1.85 m
8		1.60 m	1.42 m	1.42 m
9	1.57 m			0.80 s
10		1.42 m		
11	1.50 m	1.18 m	1.56 m	1.58 m
	1.67 m	1.68 m	1.54 m	1.55 m
12	1.98 m	2.06 m	2.02 m	2.04 m
	2.31 m	2.06 m	2.01 m	2.03 m
14	5.67 s	5. 65 s	5. 75 s	5. 71 s
16	2.16 s	2.14 d (1.0)	2.24 s	2.18 s
17	4.49 s	0.95 d (7.3)	1.78 s	1.16 s
	4.85 s		0.87 s	0.87 s
18	0.86 s	1.94 d (1.5)		
19	0.80 s	1.24 s	0.82 s	0.82 s
20	0.68 s	0.82 s	1.10 s	0.95 s

Compound **4** was obtained as a white solid and analyzed for $C_{20}H_{34}O_3$. Its IR spectrum exhibited absorption bands for a hydroxyl group (3400 cm^{-1}) and carboxyl carbonyl group (1720 cm^{-1}). The relative stereochemistry was established by NOESY correlations (Figure 4). The ^{1}H and ^{13}C NMR data (Tables 1 and 2) were comparable to those of **1**, except for the replacement at a methylene group on C-17 in **1** was replaced by a methyl proton resonating at δ_H 1.16 (*s*) in **4** and two hydroxyl group in the molecule. Thus, on the basis of its spectroscopic data and comparison of the ^{1}H and ^{13}C NMR spectral data with the previously reported data,[8,9] compound **4** was identified as labd-13*E*-en-8-ol-15-oic acid.

Conclusion: Purification of the extract from the leaves of *E. fuscum* led to the isolation of four known labdane-type diterpenes **1-4**. Compounds **1-4** were isolated from this genus for the first time. They were identified as copalic acid (**1**), 5α,8α-(2-oxokolavenic acid) (**2**), rhinocerotinoic acid (**3**) and labd-13*E*-en-8-ol-15-oic acid (**4**). Their structures were elucidated by spectral evidences and comparison with reported values.

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Acknowledgements: We are indebted to the Office of the National Research Council of Thailand and Phuket Rajabhat University for financial support. The Bioassay Research Facility of BIOTEC/NASTDA Bangkok, Thailand is gratefully acknowledged for the bioactivity tests. We are grateful to Miss Kittiporn Trisupphakant and Mrs. Nitirat Chimnoi, Chulabhorn Research Institute, Bangkok, Thailand, for recording the UV, IR, MS and HRMS spectra. We also thank Dr. Piya Chaleamklin (Institute of Scientific and Technological Research, Thailand) for the identification of the plant.