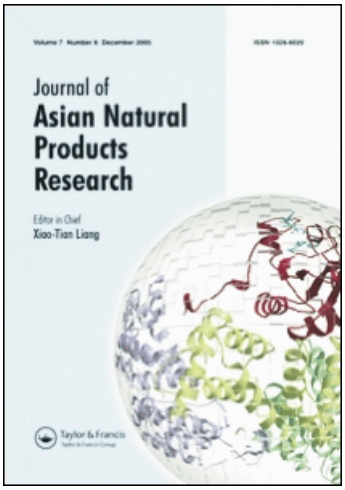

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Three new eudesmanoids from the Formosan soft coral *Nephthea erecta*

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Chemical investigations on the organic extract of the Formosan soft coral *Nephthea erecta* led to the isolation of two new eudesmanoids, (4*R**,5*S**,6*Z*,10*R**)-8-oxo-eudesm-6-en-5α,11-diol (**1**) and (6*E*,10*R*)-4,5-dioxo-11-methoxy-eudesm-6-ene (**2**), together with one new tri-*nor*-eudesmane sesquiterpenoid, (4*S**,5*E*,10*R**)-7-oxo-tri-*nor*-eudesm-5-en-4β-ol (**3**). The structures of metabolites **1**–**3** were elucidated through extensive spectroscopic analyses and by comparison with those reported in the literature. The anti-inflammatory activity using RAW 264.7 macrophages and their cytotoxicity against selected cancer cells of **1**–**3** were evaluated *in vitro.*

Keywords: *Nephthea erecta*; eudesmanoids; tri-*nor*-eudesmane; anti-inflammatory activity; RAW 264.7 macrophages; cytotoxicity

1. Introduction

Sesquiterpenoids and their analogs, especially those of the genus *Nephthea*, constitute a large family of secondary metabolites endowed with a range of structural diversity [1–10]. Some of these secondary metabolites have exhibited an array of biological activities such as insecticidal [2], cytotoxic [3–8], anti-inflammatory [8,9], and antibacterial properties [8]. In the course of our ongoing endeavor to discover bioactive substances from marine organisms, chemical investigations of the Formosan soft coral *Nephthea erecta* Kükenthal were undertaken exhaustively. Two new eudesmane-type sesquiterpenoids, characterized as (4*R**,5*S**,6*Z*,10*R**)-8-oxo-eudesm-6-en-5α,11-diol (**1**) and (6*E*,10*R*)-4,5-dioxo-11-methoxy-eudesm-6-ene (**2**), and a new tri-*nor*-eudesmane sesquiterpenoid, (4*S**,5*E*,10*R**)-7-oxo-tri-*nor*-eudesm-5-en-4β-ol (**3**) (Figure 1), were isolated from the organism. The details of isolation and structural elucidation of these secondary metabolites are discussed in this paper. The *in vitro* anti-inflammatory activity using RAW 264.7 macrophages of **1** (10 μM) exhibited the observed activity against iNOS protein expression, but no discernible activity against COX-2 protein expression. However, metabolites **2** and **3** inconspicuously reduced the levels of the iNOS and COX-2 proteins.

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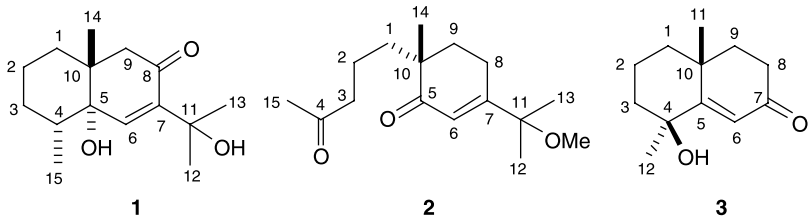


Figure 1. Structures of metabolites **1**–**3**.

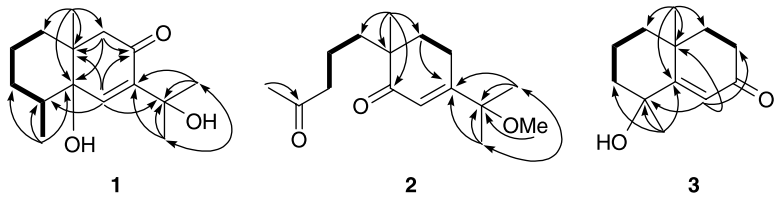
2. Results and discussion

(4*R**,5*S**,6*Z*,10*R**)-8-Oxo-eudesm-6-en-5α,11-diol (**1**) was obtained as a colorless, viscous oil. The HR-ESI-MS of **1** exhibited a pseudomolecular ion peak at *m/z* 275.1621 $[M+Na]^+$, consistent with the molecular formula of $C_{15}H_{24}O_3$, requiring four degrees of unsaturation. The UV spectrum showed the λ_{max} (MeOH) value at 232 nm, indicating the presence of a cyclohex-2-enone moiety, as well as from a strong IR absorption at 1682 cm^{-1} (conjugated carbonyl group). The ^1H and ^{13}C NMR spectra (Table 1) of **1** also proved to contain resonances for the above functionality at δ_H 6.86 (1 H, s, H-6) and δ_C 141.7

Table 1. ^1H and ^{13}C NMR spectroscopic data of **1**–**3**.[a]

	1		**2**		**3**	
C/H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H
1	32.8 (t)[b]	α: 1.86 dt (13.2, 4.4)[c] β: 1.17 br d (13.2)	36.6 (t)[b]	a: 1.56 m b: 1.39 m	41.2 (t)[b]	α: 1.71 m β: 1.34 td (13.2, 3.2)[c]
2	20.2 (t)	α: 1.56 m β: 1.61 m	19.3 (t)	a: 1.52 m b: 1.32 m	17.3 (t)	α: 1.56 m β: 2.10 dt (14.0, 4.0)
3	29.9 (t)	α: 1.61 m β: 1.53 m	44.6 (t)	a: 2.43 m b: 2.39 m	40.2 (t)	α: 1.95 m β: 1.52 m
4	32.5 (d)	1.94 m	215.5 (s)		71.5 (s)	
5	72.6 (s)		207.8 (s)		169.8 (s)	
6	141.7 (d)	6.86 s	123.5 (d)	5.92 s	123.2 (d)	6.04 s
7	144.3 (s)		168.7 (s)		201.2 (s)	
8	202.6 (s)		25.7 (t)	2.43 m	34.1 (t)	α: 2.39 br dt (17.6, 3.6) β: 2.61 ddd (17.6, 14.4, 4.8)
9	49.9 (t)	α: 2.93 d (16.8) β: 2.05 d (16.8)	34.0 (t)	α: 1.78 m β: 1.98 m	40.1 (t)	α: 1.74 m β: 1.86 dd (14.4, 4.8)
10	40.6 (s)		43.6 (s)		35.8 (s)	
11	71.8 (s)		73.6 (s)		24.5 (q)	1.46 s
12	28.5 (q)	1.42 s	26.0 (q)	1.34 s	29.0 (q)	1.42 s
13	29.2 (q)	1.43 s	25.8 (q)	1.35 s		
14	21.7 (q)	1.05 s	22.7 (q)	1.08 s		
15	14.9 (q)	1.00 d (6.4)	30.5 (q)	2.13 s		
OMe			51.4 (q)	3.10 s		

[a] Spectra were measured in $CDCl_3$ (^1H, 400 MHz and ^{13}C, 100 MHz).
[b] Multiplicities are deduced by HSQC and DEPT experiments.
[c] *J* values (in Hz) are in parentheses.



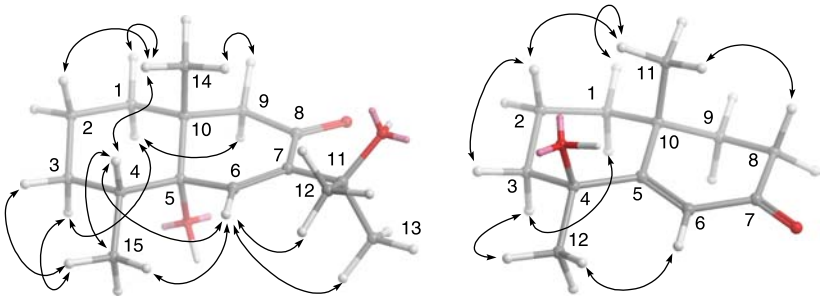
1 2 3

Figure 2. Selected HMBC (→) and $^{1}H-^{1}H$ COSY (—) correlations of **1**–**3**.

(CH, C-6), 144.3 (qC, C-7), and 202.6 (qC, C-8). In addition, its IR spectrum absorption band at 3439 cm^{-1} indicated the presence of a hydroxyl moiety. This assumption was further supported by the ^{13}C NMR signals resonating at δ_C 72.6 (qC, C-5) and 71.8 (qC, C-11). The above functionalities accounted for two of the four degrees of unsaturation, indicating a bicyclic structure for **1**.

From the COSY spectrum (Figure 2) of **1**, it was possible to establish the proton sequence from H_2-1 to Me-15 through H_2-2, H_2-3, and H-4. The cyclohex-2-enone fragment was confirmed by the HMBC correlations from H-6 to C-8 and C-10, and from H_2-9 to C-5, C-8, and C-10. Additionally, the HMBC spectrum exhibited correlations from Me-14 to C-1, C-5, C-9, and C-10, from Me-15 to C-3 and C-4, and from H-6 to C-4 and C-5, as well as from Me-12 and Me-13 to C-11 and C-7, establishing an eudesmane-type sesquiterpene skeleton of **1**. The above HMBC correlations also led to the position of the two tertiary hydroxyl groups at C-5 and C-11, respectively. Thus, the planar structure of **1** was proposed decidedly.

The relative configuration of **1** was determined by the combination of the NOESY spectrum and a computer-generated lower energy conformation using MM2 force field calculations (Figure 3). The NOE correlations between Me-14 with the following protons of H-1β, H-2β, H-4, and H-9β suggested β-orientation of the above protons, while the methyl group at C-4 was oriented on the opposite side. H-2α was found to show NOE correlations with H-1α and Me-15, indicating α-orientation of Me-15. Moreover, the Δ value ($\delta_{CDCl_3} - \delta_{C_5D_5N}$) of the Me-14 protons (400 MHz) of **1** in two different solvents ($CDCl_3$ and C_5D_5N) is small (− 0.06 ppm), where the dihedral angle between 5-OH and Me-14 is large, indicating α-orientation of 5-OH [11]. On the basis of the above observations, together with other detailed NOESY correlations, the structure of (4*R**,5*S**,6*Z*,10*R**)-8-oxo-eudesm-6-en-5α,11-diol could be determined as shown in Figure 1.



1 3

Figure 3. Key NOESY correlations of **1** and **3**.

The UV absorption maximum at 233 nm, and a strong IR absorption at 1684 cm^{-1}, as well as the observation of the featuring carbon resonances at δ_C 207.8 (qC, C-5), 123.5 (CH, C-6), and 168.7 (qC, C-7) in the ^{13}C NMR spectrum, revealed the presence of an α,β-unsaturated carbonyl functionality in **2**. Furthermore, a keto-carbonyl carbon was recognized as being present in **2** from its ^{13}C NMR signal at δ_C 215.5 (qC, C-4), as well as from a strong IR absorption at 1716 cm^{-1}. The above functionalities accounted for three of the four degrees of unsaturation, suggesting a monocyclic skeleton for **2**. By the assistance of extensive 2D NMR data, including COSY and HMBC correlations (Figure 2), the plane skeleton of **2** was fully proposed. Metabolite **2** is the methoxyl analog of chabrolidione B [7] and its HR-ESI-MS showed a pseudomolecular ion peak at *m/z* 289.1779 $[M+Na]^+$, which is compatible with the molecular composition of $C_{16}H_{26}O_3$. The ^1H NMR spectrum showed an additional proton singlet at δ_H 3.10 corresponding to 11-OMe. The ^{13}C NMR spectrum was comparable to that of chabrolidione B [7], except for an additional methoxyl signal at δ_C 51.4, which is located at C-11 from the HMBC correlations from Me-12, Me-13, and 11-OMe to C-11 (Figure 2). Furthermore, metabolite **2** has the same sign of optical rotation ($[\alpha]_D$ −12) as that of chabrolidione B ($[\alpha]_D$ −9.3). Thus, the absolute configuration of **2** was assumed to be 10*R*. On the basis of the above findings, the structure of (6*E*,10*R*)-4,5-dioxo-11-methoxy-eudesm-6-ene (**2**) was established unambiguously.

Metabolite **3** was isolated as a colorless, viscous oil. According to HR-ESI-MS at *m/z* 217.1206 $[M+Na]^+$ and ^{13}C NMR spectral data, its molecular formula was established as $C_{12}H_{18}O_2$, implying four degrees of unsaturation. The UV spectrum showed λ_{max} (MeOH) value at 235 nm, indicating the presence of a cyclohex-2-enone fragment, as well as from IR absorption at 1681 cm^{-1}. In addition, the ^1H and ^{13}C NMR spectra (Table 1) of **3** further proved to contain resonances for a cyclohex-2-enone moiety [δ_H 6.04 (1 H, s, H-6) and δ_C 169.8 (qC, C-5), 123.2 (CH, C-6) and 201.2 (qC, C-7)]. Since the above functionality accounted for two of the four degrees of unsaturation, metabolite **3** was suggested to be a bicyclic framework. From the COSY spectrum of **3** (Figure 2), it was possible to establish the two partial structures of consecutive proton systems extending from H_2-1 to H_2-3 through H_2-2, and from H_2-8 to H_2-9. The connectivities between C-1 and C-10, C-9 and C-10, C-5 and C-10 were confirmed by HMBC correlations of Me-11 with C-1, C-5, C-9, and C-10 (Figure 2). Furthermore, the HMBC correlations from Me-12 to C-3, C-4, and C-5 suggested that C-3/C-4 and C-4/C-5 were connected, and led the assignment of the tertiary hydroxyl group at C-4. The relative stereochemistry of **3** assigned by the NOESY spectrum was compatible with that of **3** obtained by computer modeling (Figure 3), in which the close contacts of atoms calculated in space were consistent with the NOESY correlations. The cross-peaks between Me-11 and H-1β, H-2β, and H-8β established the β-configuration of Me-11. Furthermore, the observed NOE interactions between H-3α/H-1α and H-3α/Me-12 confirmed the α-orientation of Me-12 and supported the β-orientation of 4-OH. From the aforementioned results, metabolite **3** was fully formulated as (4*S**,5*E*,10*R**)-7-oxo-tri-*nor*-eudesm-5-en-4β-ol.

The determination of absolute stereochemistry of **1** and **3** could not be carried out due to the absence of the secondary hydroxyls or the paucity of the material. It was noteworthy to mention that the absolute configurations of **1** and **3**, as shown in their formulas, were confirmed after the determination of the absolute configuration of **2** on the basis of biosynthetic reasoning.

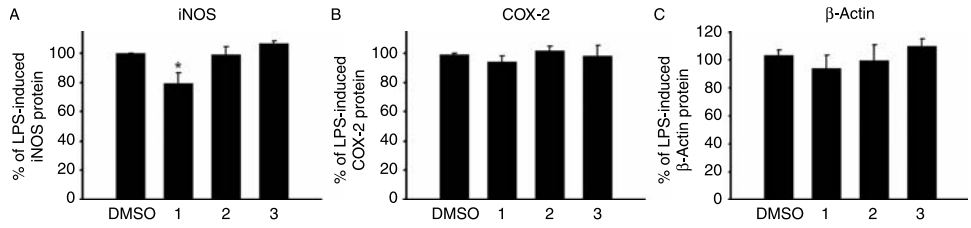


Figure 4. Effect of compounds **1**–**3** at 10 μM on the LPS-induced pro-inflammatory iNOS and COX-2 protein expression of RAW 264.7 macrophages by immunoblot analysis. (A) Immunoblot of iNOS; (B) immunoblot of COX-2, and (C) immunoblot of β-actin. The values are mean ± SEM ($n = 5$). The relative intensity of the LPS-alone stimulated group was taken as 100%. Significantly different from LPS-stimulated (control) group (*$P < 0.05$).

The *in vitro* anti-inflammatory activities of metabolites **1**–**3** were tested using LPS-stimulated cells. Stimulation of RAW 264.7 macrophage cells with LPS resulted in the upregulation of the pro-inflammatory iNOS and COX-2 proteins. At a concentration of 10 μM, metabolites **1**–**3** reduced the levels of the iNOS protein (79.5 ± 7.1, 99.1 ± 5.8, and 106.7 ± 1.8%, respectively) and COX-2 protein (94.2 ± 4.3, 101.9 ± 3.3, and 98.5 ± 7.1%, respectively), comparing with the control cells (LPS alone; Figure 4). The primary anti-inflammatory results of **1** exhibited the observed activity against iNOS protein expression, but no discernible activity against COX-2 protein expression. In addition, metabolites **2** and **3** showed insignificant anti-inflammatory activity against LPS-stimulated RAW 264.7 macrophages. The *in vitro* anti-inflammatory assays were carried out according to the procedure described previously [12].

Metabolites **1**–**3** were tested for cytotoxicity against P-388 (mouse lymphocytic leukemia), HT-29 (human colon adenocarcinoma), and A549 (human lung carcinoma) cancer cell lines. The results showed that metabolites **1**–**3** were not cytotoxic to A549 and HT-29 cells. However, metabolites **1**–**3** exhibited significant cytotoxicity against P-388 cell line with ED_{50} values of 2.60, 2.46, and 2.42 μg/ml, respectively. The anticancer agent mithramycin was used as the positive control and exhibited ED_{50} values of 0.06, 0.08, and 0.07 μg/ml and against P-388, HT-29, and A549 cells, respectively. The experimental details of this assay were carried out according to a previously described procedure [13].

3. Experimental

3.1 General experimental procedures

Optical rotations were determined using a JASCO P1020 digital polarimeter. IR spectra were recorded on a JASCO FT/IR-4100 infrared spectrophotometer. UV spectra were obtained on a JASCO V-650 spectrophotometer. The NMR spectra were recorded on a Varian MR 400 NMR spectrometer (400 MHz for ^1H and 100 MHz for ^{13}C), using $CDCl_3$ with TMS as the internal standard. Chemical shifts are given in δ (ppm) and coupling constants in Hz. ESI-MS were recorded by ESI FT-MS on a Bruker APEX II mass spectrometer. Silica gel 60 (230–400 mesh; Merck, Darmstadt, Germany) and LiChroprep RP-18 (40–63 μm; Merck) were used for column chromatography. Precoated silica gel plates (Kieselgel 60 F_{254}, 0.25 mm; Merck) and precoated RP-18 F_{254s} plates (Merck) were used for analytical TLC analyses. High-performance liquid chromatography (HPLC) was carried out using a Hitachi L-7100 pump equipped with a Hitachi L-7400 UV

detector at 220 nm and a semi-preparative reversed-phase column (Hibar Purospher RP-18e, 5 μm, 250 mm × 10 mm; Merck).

3.2 Animal material

The Formosan soft coral *N. erecta* was collected by hand using scuba at the Green Island located in the southeast coast of Taiwan, in July 2005, at a depth of 10 m, and was stored in a freezer for 5 weeks until extraction. This soft coral was identified by one of the authors (C.-F.D.). A voucher specimen (GN-80) has been deposited at the Department of Marine Biotechnology and Resources, National Sun Yat-sen University.

3.3 Extraction and isolation

A specimen of *N. erecta* was extracted repeatedly with fresh MeOH at room temperature. The combined MeOH extracts were evaporated under reduced pressure at 35°C to yield a brown viscous gum. The resulting MeOH extract (320 mg) was subjected to column chromatography on silica gel using CH_2Cl_2 and MeOH gradient (100:1–0:1) for elution, to give 30 fractions. A fraction eluted with CH_2Cl_2–MeOH (80:1) was further purified by RP-HPLC using 65% MeOH in H_2O to afford **1** (2 mg), **2** (1 mg), and **3** (1 mg).

3.3.1 (4R*,5S*,6Z,10R*)-8-Oxo-eudesm-6-en-5α,11-diol (**1**)

Colorless, viscous oil; $[\alpha]_D^{24}$ + 52 (c = 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε): 232 (3.85); IR (KBr) ν_{max}: 3439, 2932, 1682, 1660, 1461, 1378, 1165, 1126, 979, 747 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, see Table 1; ESI-MS *m/z*: 275 $[M+Na]^+$; HR-ESI-MS *m/z*: 275.1621 $[M+Na]^+$ (calcd for $C_{15}H_{24}O_3Na$, 275.1623). Selected ^1H NMR (C_5D_5N, 400 MHz) δ 6.23 (1H, br s, H-6), 2.63 (1H, d, J = 16.8 Hz, H-9α), 2.19 (1H, d, J = 16.8 Hz, H-9β), 2.16 (1H, m, H-4), 1.88 (1H, m, H-1α), 1.72 (3H, s, Me-13), 1.64 (3H, s, Me-12), 1.12 (3H, d, J = 6.4, Me-15), 1.11 (3H, J = 6.8, Me-14).

3.3.2 (6E,10R)-4,5-Dioxo-11-methoxy-eudesm-6-ene (**2**)

Colorless, viscous oil; $[\alpha]_D^{24}$ − 12 (c = 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε): 233 (3.73); IR (KBr) ν_{max}: 2927, 1716, 1684, 1668, 1457, 1377, 1361, 1262, 1170, 1071, 802, 749 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, see Table 1; ESI-MS *m/z*: 289 $[M+Na]^+$; HR-ESI-MS *m/z*: 289.1779 $[M+Na]^+$ (calcd for $C_{16}H_{26}O_3Na$, 289.1780).

3.3.3 (4S*,5E,10R*)-7-Oxo-tri-nor-eudesm-5-en-4β-ol (**3**)

Colorless, viscous oil; $[\alpha]_D^{24}$ − 6 (c = 0.1, $CHCl_3$); UV (MeOH) λ_{max} (log ε): 235 (3.82); IR (KBr) ν_{max}: 3419, 2926, 1681, 1666, 1458, 1375, 1209, 1124, 876 cm^{-1}; ^1H and ^{13}C NMR spectroscopic data, see Table 1; ESI-MS *m/z*: 217 $[M+Na]^+$; HR-ESI-MS *m/z*: 217.1206 $[M+Na]^+$ (calcd for $C_{12}H_{18}O_2Na$, 217.1204).

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