HETEROCYCLES, Vol. 104, No. 8, 2022, pp.1461 - 1468. © 2022 The Japan Institute of Heterocyclic Chemistry
Received, 3rd June, 2022, Accepted, 17th June, 2022, Published online, 21st June, 2022
DOI: 10.3987/COM-22-14692

CONIFERAINS C AND D, NEW EUNICELLIN-BASED DITERPENOIDS FROM *CLADIELLA CONIFERA*

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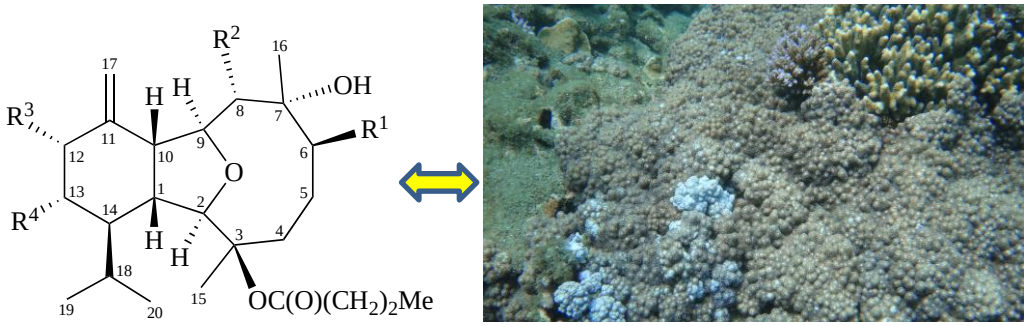
Abstract – Chemical examination of *Cladiella conifera*, collected in the waters of Taiwan, led to the isolation of two new eunicellin-based diterpenoids, coniferains C (**1**) and D (**2**). The structures of **1** and **2** were established by spectroscopic methods. Eunicellins **1** and **2** were found to promote cell viability in DLD-1 cells at a concentration of 20 μM.

INTRODUCTION

The octocorals belonging to the genus *Cladiella* (family Alcyoniidae) were found to be a rich source of eunicellin-based (2,11-cyclized cembranoid) diterpenoids,[1] and the compounds of this type were found to exhibit extensive biomedical using potentially activities.[2,3] *Cladiella conifera* (Tixier-Durivault, 1943) is one of the representative species found in the shallow reef of Taiwan,[4–7] an area with high biodiversity at the intersection of Kuroshio current, South China Sea surface current, and Mainland Coastal current, and several interesting eunicellins and butanolide were isolated from this interesting species.[8,9] In the current study, we report herein the isolation, structure determination, and bioactivities of two new eunicellins, coniferains C (**1**) and D (**2**) (Chart 1).



1: $R^1 = R^3 = OH$, $R^2 = OAc$, $R^4 = H$
2: $R^1 = R^3 = OH$, $R^2 = R^4 = H$
3: $R^1 = OH$, $R^2 = R^3 = OAc$, $R^4 = H$
4: $R^1 = OMe$, $R^2 = R^4 = H$, $R^3 = OH$
5: $R^1 = R^3 = OH$, $R^2 = H$, $R^4 = OAc$

C. conifera

Chart 1. Structures of coniferains C (**1**) and D (**2**), krempfielins O (**3**) and N (**4**), klymollin P (**5**), and a picture of *Cladiella conifera*

RESULTS AND DISCUSSION

Coniferain C (**1**) was obtained as colorless oil and had a molecular formula $C_{26}H_{42}O_8$ from HRESIMS at *m/z* 505.27722 (Calcd for $C_{26}H_{42}O_8$ + Na, 505.27719) (index of hydrogen deficiency, IHD = 6). Analysis of the ^1H, ^{13}C NMR (Table 1), HSQC, and HMBC spectra together with the molecular formula, suggested that there must be three exchangeable protons, requiring the presence of three hydroxy groups. From 1D- and 2D-NMR spectra, revealed the presence of an *n*-butyroxy group (δ_H 1.00, 3H, t, J = 7.2 Hz; 1.68, 2H, t, J = 7.2 Hz; 2.50, 1H, dt, J = 16.2, 7.2 Hz; 2.60, 1H, dt, J = 16.2, 7.2, Hz/δ_C 14.1, Me; 18.4, CH_2; 36.7,

CH_2; 173.1, *n*-butyrate carbonyl), an acetoxy group (δ_H 2.10, 3H, s/δ_C 21.4, acetate methyl; 170.7, acetate carbonyl), and an exocyclic carbon-carbon double bond (δ_H 4.74 and 5.00, each 1H × s, H_2-17/δ_C 148.4, C-11; 111.9, CH_2-17), which accounted for three out of six degrees of unsaturation thus required **1** to be a metabolite with three rings. Moreover, signals of two tertiary methyls (δ_H 1.08, 3H, s/δ_C 17.9, Me-16; δ_H 1.44, 3H, s/δ_C 23.1, Me-15) correlated to oxygenated quaternary carbons, and five oxymethines (δ_H 3.69, 1H, s/δ_C 91.6, CH-2; δ_H 4.43, 1H, br s/δ_C 71.8, CH-12; δ_H 4.49, 1H, dd, J = 9.6, 6.6 Hz/δ_C 80.1, CH-9; δ_H 4.67, 1H, d, J = 6.0 Hz/δ_C 77.4, CH-6; δ_H 5.22, 1H, d, J = 9.6 Hz/δ_C 79.3, CH-8) were observed. Two methyl doublets at δ_H 0.99 (3H, d, J = 7.2 Hz, H_3-19) and 0.81 (3H, d, J = 7.2 Hz, H_3-20) were deduced to be from two methyls of an isopropyl group.

Table 1. ^{1}H (600 MHz, $CDCl_3$) and ^{13}C (150 MHz, $CDCl_3$) NMR data for eunicellins **1** and **2**

	1		**2**	
C/H	δ_H (J in Hz)	δ_C, type[a]	δ_H (J in Hz)	δ_C, type[a]
1	2.28 dd (8.4, 8.4)	45.1, CH	2.29 m	44.2, CH
2	3.69 s	91.6, CH	3.72 d (2.4)	90.5, CH
3		85.7, C		86.4, C
4	1.89 m	35.6, CH_2	1.94 ddd (14.4, 9.6, 1.2)	34.8, CH_2
4′	2.64 dd (15.6, 7.2)		2.52 dd (14.4, 9.6)	
5α/β	1.65 m; 1.50 m	28.7, CH_2	1.68 m; 1.44 m	30.4, CH_2
6	4.67 d (6.0)	77.4, CH	4.58 d (6.6)	79.1, CH
7		79.5, C		76.5, C
8	5.22 d (9.6)	79.3, CH	1.82−1.90 m	45.9, CH_2
9	4.49 dd (9.6, 6.6)	80.1, CH	4.51 ddd (10.2, 6.0, 5.4)	79.4, CH
10	3.31 dd (6.6, 6.6)	49.8, CH	2.90 dd (6.6, 6.0)	51.0, CH
11		148.4, C		148.0, C
12	4.43 br s	71.8, CH	4.38 br s	70.8, CH
13/13′	1.40 m; 1.89 m	31.5, CH_2	1.44 m; 1.88 m	31.1, CH_2
14	1.86 m	36.2, CH	1.84 m	36.4, CH
15	1.44 s	23.1, Me	1.47 s	23.3, Me
16	1.08 s	17.9, Me	1.16 s	22.6, Me
17a/b	5.00 s; 4.74 s	111.9, CH_2	5.06 s; 4.88 s	111.9, CH
18	1.82 m	28.9, CH	1.82 m	28.9, CH
19	0.99 d (7.2)	21.7, Me	1.00 d (6.6)	21.9, Me
20	0.81 d (7.2)	15.8, Me	0.84 d (6.6)	16.4, Me
n-OC(O)Pr-3		173.1, C		172.3, C
	2.49 dt (16.2, 7.2)	36.7, CH_2	2.32 dt (16.2, 7.2)	37.4, CH_2
	2.62 dt (16.2, 7.2)		2.25 dt (16.2, 7.2)	
	1.68 sext (7.2)	18.4, CH_2	1.67 sext (7.2)	18.4, CH_2
	1.00 t (7.2)	13.5, Me	0.98 t (7.2)	13.7, Me
OAc-8		170.7, C		
	2.10 s	21.4, Me		

[c] Multiplicity deduced by HSQC, HMBC, and DEPT spectra.

The ^{1}H–^{1}H COSY spectrum of **1** identified the separate proton spin systems among H_2-4/H_2-5/H-6, H-9/H-10/H-1/H-14/H_2-13/H-12, H-14/H-18/H_3-19, and H-18/H_3-20 (Figure 1a). These data, together with the

key ^{2}J- or ^{3}J-^{1}H–^{13}C long-range HMBC correlations between protons and non-protonated carbons such as H-2, H$_2$-4, H-5′ (δ_H 1.65) to C-3; H-5 (δ_H 1.50) to C-7; and H-10 to C-11, permitted elucidation of the major carbon skeleton of **1** (Figure 1a). An exocyclic carbon-carbon double bond at C-11 was confirmed by the HMBC from H$_2$-17 to C-10 and C-12. An HMBC correlation from the oxymethine proton at δ_H 5.22 (H-8), to the acetate carbonyl at δ_C 170.7, placed the acetoxy group on C-8. Furthermore, an HMBC correlation from the oxymethine proton H-2 (δ_H 3.69) to an oxygen-containing methine at δ_C 80.1 (CH-9) suggested the presence of a C-2/9 ether linkage in **1** for forming a tetrahydrofuran ring located between C-2 and C-9. The *n*-butyroxy group was positioned at C-3, an oxygenated quaternary carbon, as indicated by analysis of key ^{13}C NMR signal resonating at δ_C 85.7. Since five of the eight oxygen atoms and four of the seven oxygenated carbons were accounted for by two esters and a tetrahydrofuran moiety; the remaining oxygen atoms must be presented at C-6, C-7, and C-12 as hydroxy groups.

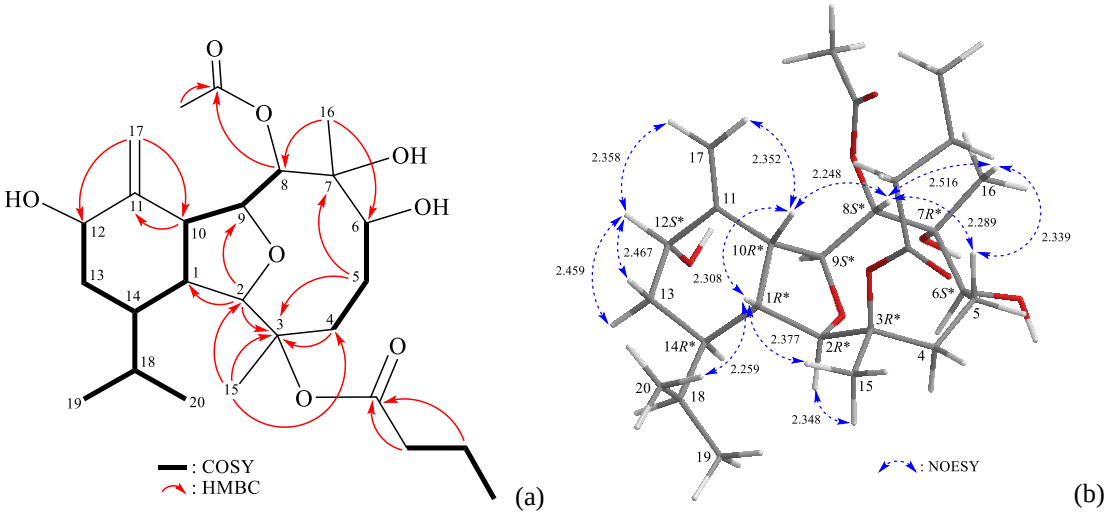


Figure 1. (a) Key COSY, HMBC, and (b) stereoview of **1** and calculated distances (Å) between selected protons with key NOESY correlations

Based on previous surveys, all the naturally occurring eunicellins have the H-1 is *cis* to H-10, and these two protons are assigned as β-oriented in eunicellin-based diterpenoids.[10,11] The relative configuration of **1** was elucidated from the interactions observed in a NOESY experiment, CS Chem3D Model, and that obtained from vicinal protons coupling constant analysis. In the NOESY experiment of **1** (Figure 1b), correlations of H-1 with H-10 and H$_3$-20 indicate that H-1, H-10, and the isopropyl group were situated on the same face. They were assigned as β-protons, as H-14 was α-oriented. H-2 showed a correlation with H$_3$-15 with no coupling found between H-1 and H-2, indicating that the dihedral angle between H-1/H-2 is approximately 90° and H-2 and Me-15 should be α-oriented at C-2 and C-3, respectively. H-8 exhibited cross peaks with H-10, one of the methylene protons (δ_H 1.50) at C-5, and H$_3$-16, but not with H-9 and H-6, showing that these protons are β-oriented and the oxymethine protons at C-6 and C-9, acetoxy group at C-8, and hydroxy group at C-7, are α-oriented, respectively. A large coupling constant (*J*) of 9.6 Hz was

noted between H-8 and H-9, showing that the plane between H-8 and H-9 have a dihedral angle of about 180° and demonstrating the α-orientation of H-9. In the methylenecyclohexane moiety of **1**, H-12 exhibited correlations to both H-13α/β and one of the olefin methylene proton (δ_H 5.00, H-17a), revealing that the hydroxy group at C-12 is placed on the axial-down direction in the six-membered ring and assigned as α-oriented. Based on the above findings, the structure, including the relative configuration of **1**, was determined and the chiral carbons of **1** were assigned as (1*R**,2*R**,3*R**,6*S**,7*R**,8*S**,9*S**,10*R**,12*S**,14*R**). It was found that the spectroscopic data of **1** were similar to those of a known eunicellin, krempfielin O (**3**) (Chart 1), that was isolated from a Taiwanese soft coral *Cladiella krempfi*,[12] except that the signals corresponding to an acetoxy group at C-12 in **3** were replaced by a hydroxy group in **1**. On the basis of the above observations, eunicellin **1** was found to be the 12-*O*-deacetyl derivative of **3**.

Coniferain D (**2**) has the molecular formula $C_{24}H_{40}O_6$ as determined by HRESIMS at *m/z* 447.27152 (Calcd for $C_{24}H_{40}O_5$ + Na, 447.27171) (IHD = 5). It was observed that the spectroscopic data of **2** resembled those of **1**. The NMR spectra revealed that the signals corresponding to the C-8 oxymethine in **1** (δ_H 5.22, 1H, d, J = 9.6 Hz/δ_C 79.3, CH-8) were replaced by those of an aliphatic methylene in **2** (δ_H 1.82–1.90, 2H, m/δ_C 45.9, CH_2-8) (Table 1). Interpretation of the 2D NMR spectroscopic data of **2** confirmed the above elucidation, and thus established the planar structure (Figure 2a). The configurations of stereogenic centers in **2** were assigned as (1*R**,2*R**,3*R**,6*S**,7*S**,9*R**,10*R**,12*S**,14*R**), same as those in **1**, according to the NOESY spectrum (Figure 2b). Thus, the structure of **2** was confirmed, and this compound was named coniferain D. It was found that the NMR data of **2** were similar with those of known eunicellin analogues, krempfielin N (**4**)[12] and klymollin P (**5**)[13] (Chart 1), except that the signals corresponding to the methoxy group at C-6 in **4** were replaced by a hydroxy group in **2**; and the acetoxy group at C-13 in **5** were replaced by a proton in **2**, respectively. Thus, coniferain D (**2**) was found to be the 6-*O*-demethyl derivative of **4** and the 13-deacetoxy derivative of **5**, respectively.

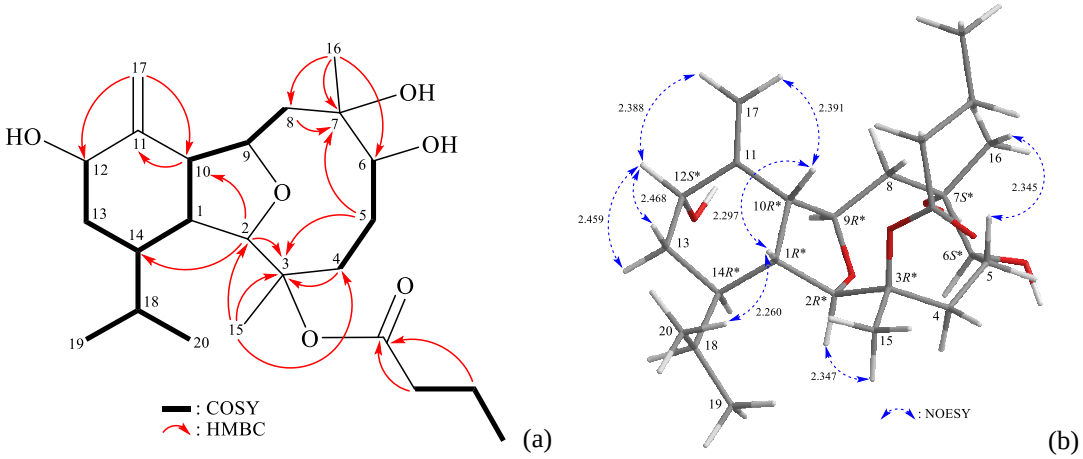


Figure 2. (a) Key COSY, HMBC, and (b) stereoview of **2** and calculated distances (Å) between selected protons with key NOESY correlations

Table 2. Effects of compounds **1** and **2** on cell viability in HT-29 and DLD-1 tumor cells

Compounds	HT-29		DLD-1	
	Viability (%)	[a]IC_{50} (μM)	Viability (%)	[a]IC_{50} (μM)
1	92.11 ± 3.78	> 20	158.81 ± 3.69***	> 20
2	95.29 ± 1.58	>20	145.77 ± 7.12***	> 20
DMSO	100.00 ± 1.52	>20	100.00 ± 2.10	> 20

Percentage of cell viability of 20 μM for 72 h. Resultls are expressed as mean ± S.E.M. (n = 3). ***p < 0.001 compared with DMSO (0.1%) group. [a]Concentration necessary for 50% inhibition (IC_{50}).

The bioactivities of eunicellins **1** and **2** against human colorectal adenocarcinoma cell lines HT-29 and DLD-1 were investigated. The results are shown in Table 2. These two compounds did not exhibit cytotoxic effect but did promote cell viability in DLD-1 cells. It indicated that eunicellins **1** and **2** may be used in the future for regenerative medicine applications including neurodegeneration and bond regeneration.

EXPERIMENTAL

General Experimental Procedures. Optical rotations were measured using a JASCO P-1010 digital polarimeter. IR spectra were measured on a Thermo Scientific Nicolet iS5 FT-IR spectrophotometer. NMR spectra were taken on a 600 MHz Jeol NMR (model ECZ600R) spectrometer operating at 600 MHz for ^{1}H and 150 MHz for ^{13}C in $CDCl_3$ using the residual $CHCl_3$ signal (δ_H 7.26 ppm) and $CDCl_3$ (δ_C 77.1 ppm) as the internal standard for ^{1}H and ^{13}C NMR, respectively; coupling constants (J) are given in Hz. ESIMS and HRESIMS were recorded using a Bruker APEX II FTMS system. Column chromatography was carried out with silica gel (60–230 mesh, Merck) or Sephadex LH-20 (GE, Healthcare). TLC was performed on plates coated with silica gel 60 with fluorescent indicator UV_{254} (0.20 mm, Macherey-Nagel) and/or RP-18 W/UV_{254} (0.15 mm, Macherey-Nagel), and then visualized by spraying with 10% H_2SO_4 and heating on a hot plate. Reverse-phase HPLC (RP-HPLC) was performed using a system comprising a pump (L-2130, Hitachi), a photodiode array detector (L-2455, Hitachi), an injection port (Rheodyne, 7725), and reverse-phase columns (Luna 5 μm, C18(2) 100 Å AXIA Packed, 250 × 21.2 mm; Phenomenex; Supelco Ascentis C18 Cat #:581343-U, 250 × 10 mm, 5 μm).

Animal Material. Specimens of *Cladiella conifera* used for this study were collected in May 2017 by hand with SCUBA divers off the coast of Penghu Archipelago, Taiwan (N.23.15.203, E119.30.725). A voucher specimen was deposited in the National Museum of Marine Biology & Aquarium, Taiwan (NMMBA-TW-SC-2017–0504). The coral specimen was identified as *Cladiella conifera* (Tixier-Durvault, 1943) based on its morphology and micrographs of the coral sclerites.[4–7]

Extraction and Isolation. *Cladiella confiera* (171.4 g fresh weight) was collected and freeze-dried. The coral material (58.8 g, dry weight) was minced and extracted exhaustively with a mixture of CH_2Cl_2 and

MeOH (1:1). The extract (6.6 g) was partitioned between EtOAc and H_2O, then the EtOAc layer (3.9 g) was subjected to column chromatography on silica gel (Si. C.C.), and eluted with a gradient solvent system of *n*-hexane, *n*-hexane and EtOAc mixtures, and acetone of increasing polarity to yield 13 sub-fractions A~M. Fraction M was subjected to Sephadex LH-20 with an isocratic solvent system of CH_2Cl_2/MeOH (1:1) to afford 6 sub-fractions M1~M6. Fraction M5 was separated by RP-HPLC, using an isocratic solvent system of a MeOH/H_2O mixture (73:27; flow rate = 5.0 mL/min) to afford **1** (0.6 mg) and **2** (0.9 mg), respectively.

Coniferain C (1): colorless oil; $[\alpha]_D$ –8 (*c* 0.03, $CHCl_3$); ^1H ($CDCl_3$, 600 MHz) and ^{13}C ($CDCl_3$, 150 MHz) NMR data, see Table 1; ESIMS *m/z* 505 $(M + Na)^+$; HRESIMS *m/z* 505.27722 (Calcd for $C_{26}H_{42}O_8$ + Na, 505.27719).

Coniferain D (2): colorless oil; $[\alpha]_D$ +21 (*c* 0.05, $CHCl_3$); ^1H ($CDCl_3$, 600 MHz) and ^{13}C ($CDCl_3$, 150 MHz) NMR data, see Table 1; ESIMS *m/z* 447 $(M + Na)^+$; HRESIMS *m/z* 447.27152 (Calcd for $C_{24}H_{40}O_6$ + Na, 447.27171).

***In Vitro* Cytotoxicity Assay.** The cytotoxicity assay used in this study was performed as described in previous publications.[14,15]

ACKNOWLEDGEMENTS

The authors are grateful to Chao-Lien Ho and Hsiao-Ching Yu, of the High Valued Instrument Center, National Sun Yat-sen University, for obtaining the NMR (NMR 001100) and mass (MS 000600) spectra (MOST 111-2731-M-110-001). This work was mainly funded by grants from the National Museum of Marine Biology and Aquarium; the Ministry of Science and Technology (Grant Nos MOST 107-2320-B-291-001-MY3, 109-2320-B-276-001-MY2, 109-2320-B-291-001-MY3, 110-2320-B-182A-011, and 110-2314-B-242-003); and Chang Gung Memorial Hospital (CMRPG6J0143 and CMRPG6L0311), Taiwan, awarded to Tung-Ying Wu, Shun-Hua Chen, Liang-Mou Kuo, and Ping-Jyun Sung. All funding is gratefully acknowledged.

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