Wei Xiang[1, 2]
Leng Chee Chang[1]

Calyculatine: a New Dolabellane Diterpenoid from the Marine Sponge *Eunicea calyculata*

Abstract

A new dolabellane diterpenoid, 7(*S*)-hydroperoxy-13-keto-(1*S*,11*R*)-dolabella-3(*E*),8(17),12(18)-triene (**1**), and two known compounds, 7(*S*),8(*S*)epoxy-13-keto-1(*S*),11(*R*)-dolabella-3(*E*),12(18)-diene (**2**) and 13-keto-1(*S*),11(*R*)-dolabella-3(*E*),7(*E*),12(18)-triene (**3**), were isolated from the marine sponge *Eunicea calyculata*. The structure of **1** was elucidated by 1D ^{1}H- and ^{13}C-NMR spectra and 2D HMQC, HMBC, and the relative stereochemistry was established by analysis of coupling constants and NOESY spectra. In addition, an X-ray crystallographic analysis on a single crystal of **2** was carried out. Compounds **1** and **2** were inactive in the hyphae formation inhibition assay (HFI) in *Streptomyces* 85E while compound **3** showed inhibitory activity at a concentration of 20 μg/20 μL in the HFI assay.

Key words
Eunicea calyculata · dolabellane diterpenoid · calyculatine · hyphae formation inhibition · *Streptomyces* 85E

Introduction

Marine sponges have proven to be a prolific and valuable source of structurally unique secondary metabolites, which have shown a variety of bioactivities, such as anticancer and antibiotic. In the Caribbean Sea, the genus *Eunicea* (family Plexauridae) sponge is abundant. Previous phytochemical investigations reported some sesquiterpenes and diterpenes from this genus. In general, five main structural types of diterpenoids from the genus *Eunicea* were reported including cembrane, cubitane, dilophol and fuscoside classes, and dollabellane [1]. In particular, dollabellane diterpenoids were reported from *E. calyculata* [2] and *E. laciniata* [3]. At present, the dolabellane skeleton has been isolated from many other marine animals and plants, such as *Dolabella californica*, *Clavularia inflata*, *Eunicea calyculata*, *E. laciniata*, *E. tourneforti*, *Glossophora galapagensis*, *Dictyota dichotoma*, *D. pardarlis*, *Dilophus fasciola*, *Aplysia dactylomela*, *Odontoschisma denuatum*, *Barbilophozia floerkei*, *B. lycopodioides, B. attenuata* and others [4], [5], [6], [7], [8], [9], [10], [11], [12], [13], [14], [15]. Most of these compounds showed cytotoxic bioactivities [16], and thus received wide attention in the period following 1977.

Protein phosphorylation plays a pivotal role in cellular processes such as proliferation, differentiation, secretion and apoptosis; abnormal phosphorylation is often the cause of disease. Recent studies have shown that both protein kinases and phosphatases in signal transduction are attractive drug targets for cancer treatment [17], [18]. For example, the recent approvals of Gleevec™ and Iressa® by the FDA have justified the development of tyrosine kinase inhibitors in cancer treatments [19]. In this investigation, a prokaryotic whole cell assay using hyphae formation inhibition is proposed as an effective eukaryotic model in the search for protein kinase inhibitors. The eukaryotic signal transduction

Affiliation
[1] Department of Chemistry and Biochemistry, College of Science and Engineering, University of Minnesota Duluth, Duluth, MN, U.S.A.
[2] Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Yunnan, P. R. China

Correspondence
Dr. Leng Chee Chang · Department of Chemistry and Biochemistry · 246 Chemistry Building · 1039 University Drive · University of Minnesota Duluth · Duluth · Minnesota 55812–3020 · U.S.A. · Phone: +1-218-726-7346 · Fax: +1-218-726-7394 · E-mail: lcchang@d.umn.edu

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pathways are regulated by protein kinases and phosphatases that are phosphorylated at specific serine/threonine/tyrosine residues. However, recently, these serine/threonine-phosphorylating-enzymes were also found in *S. coelicolor* A3 (2) [20], [21], [22]. Aerial hyphae formation requires the activity of kinase cascades. It has been shown that a variety of kinase inhibitors block this process [23] (and unpublished data from Professor Julian Davies). For example, staurosporine, a eukaryotic protein kinase inhibitor, strongly inhibited several phosphorylations of cellular proteins and the formation of aerial hyphae with *S. griseus* [24]. Thus, this assay has been adopted to evaluate natural product extracts from the Natural Products Repository of the National Cancer Institute in the search for protein kinase inhibitors.

Eunicea calyculata was selected for further study (collection C000981, Natural Products Repository, NCI) for two reasons. First, the crude organic extract displayed a 16 mm bald phenotype (80 μg/20 μL) in our hyphae formation inhibition assay (HFI). Second, previous literature indicated no reports for protein kinase activities. Thus, bioassay-guided fractionation using the HFI assay resulted in the isolation of three dolabellane diterpenes, 7(*S*)-hydroperoxy-13-keto-1(*S*),11(*R*)-dolabella-3(*E*),8(17),12(18)-triene (**1**), 7(*S*),8(*S*)epoxy-13-keto-1(*S*),11(*R*)-dolabella-3(*E*),12(18)-diene (**2**), and 13-keto-1(*S*),11(*R*)-dolabella-3(*E*),7(*E*),12(18)-triene (**3**). The structure elucidation of **1** and the biological evaluation of compounds **1 – 3** (Fig. **1**) are the subject of this report.

Materials and Methods

General experimental procedures

The optical rotations were measured on a JASCO P-1010 automatic polarimeter. UV and IR spectra were recorded with an HP 8453 UV-Visible spectrophotometer and a Perkin-Elmer BX FT-IR spectrophotometer. Mass spectra and high-resolution mass spectra were taken with a Thermo-Finnigan Advantage Max ion-trap mass spectrometer. 1D-NMR and 2D-NMR spectra were recorded in acetone-d_6 on Varian Mercury Plus (300 MHz) and INOVA Unity (500 MHz) spectrometers equipped with an xyz-shielded gradient triple resonance probe, respectively, ^{1}H- and ^{13}C-NMR chemical shifts were referenced to the central peak of acetone-d_6 (δ_H = 2.05 and δ_C = 29.92). Reversed-phase HPLC was carried out on a Beckman Coulter Gold-168 system equipped with a photodiode array detector using a semi-preparative Econosil C_{18} column (10 μ, 10 × 250 mm) run with a flow rate of 1.5 mL/min. X-ray crystallographic data were obtained with a Bruker-AXS platform diffractometer. Chromatographic fractions and pure compounds were monitored by TLC, detected by UV light at 254 nm and a color reaction by spraying with a solution of vanillin/perchloric acid/EtOH followed by 5 min heating at 100 °C. Column chromatography was carried out on Merck silica gel 60 (70 – 230 mesh).

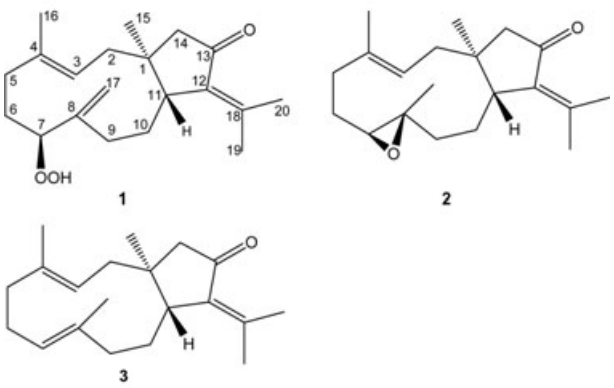

Fig. **1** Chemical structures of compounds **1**, **2** and **3**.

Sponge material

The soft coral *Eunicea calyculata* (352 g collection, C00981) was collected in deep waters (–33.5 m) adjacent to the Caribbean Sea of the Western Atlantic, in March 1987. The sponge was frozen immediately upon collection and shipped to the NCI (National Cancer Institute, Frederick, MD, U.S.A.). After aqueous extraction of the frozen sponges at 4 °C, the extracts were lyophilized and extracted subsequently with CH_2Cl_2-MeOH (1 : 1) and MeOH. The combined organic extracts were evaporated under vacuum and stored at –30 °C. A voucher specimen was deposited at the Smithsonian Sorting Center, Suitland, MD, U.S.A.

Extraction and isolation

A crude organic extract (3.0 g) derived from 352 g wet weight of the initial collection tested positive in the hyphae formation inhibition assay at a concentration of 80 μg/20 μL in our primary screening and was thus subjected to bioassay-guided fractionation. The residue (3.0 g) was chromatographed over silica gel (250 g) using *n*-hexane then *n*-hexane/acetone mixtures of increasing polarity. Elution by *n*-hexane (1.5 L) afforded fraction 1, and elution by *n*-hexane/acetone (9 : 1, 8 : 2, 7 : 3, 0 : 1) (1.6 L, each) afforded fractions 2, 3, 4 and 5, respectively. Compound **2** (98 mg) was crystallized (60 mL, acetone) from the combination of fractions 3 and 4. Fraction 2 was rechromatographed on a silica gel (50 g) employing *n*-hexane/acetone (9 : 1, 8 : 2) (0.6 L and 1.5 L, respectively) to afford fractions 2 – 1 and 2 – 2. Compound **3** (9 mg) was isolated from fraction 2 – 1 by Sephadex LH-20 column chromatography (CH_3Cl/MeOH, 1 : 1, 0.5 L). Compound **1** (9 mg) was further purified by HPLC (C_{18} column with detection at 243 nm) eluting isocratically with MeOH/H_2O (7 : 3) from fraction 2 – 2, with a flow rate of 1.5 mL/min.

Calyculatine (**1**): colorless oil; R_f: 0.5, silica gel 60 F_{254}, hexane/acetone (6 : 4); $[\alpha]_D^{20}$: 64.1° (c 0.10, MeOH); UV (MeOH): λ_{max} (log ε) = 243 (3.87) nm; IR (KBr): ν_{max} = 3055, 2987, 2306, 1711, 1620, 1422, 1265, 896 cm^{-1}; ESI MS (positive ion mode): *m/z* = 341.3 [M + Na]$^+$; HR-ESI-MS: *m/z* = 341.2080 [M + Na]$^+$ (calcd. for $C_{20}H_{30}NaO_3$: 341.2093); ^{1}H- and ^{13}C-NMR data for **1**, see Table **1**.

X-ray crystallographic analysis data of 7(*S*),8(*S*)epoxy-13-keto-1(*S*),11(*R*)-dolabella-3(*E*),12(18)-diene (2)

A colorless crystal was obtained from acetone; crystal size: 0.50 × 0.30 × 0.30 mm; crystal data: a = 8.2112 (6) Å, b = 8.6145 (7) Å, c = 12.7256 (10) Å, β = 99.4260 (10)°; V = 888.00 (12) A^3, space group $P2_1$, Z = 2, D_{calc} = 1.131 g/cm^3, λ = 0.71073 Å, μ(MoKα) = 0.711 mm^{-1}, F(000) = 332, T = 173 (2) K; a total of 2158 reflections ($1.62 < \Theta < 27.52$) were collected on a Siemens SMART platform CCD area detector with a normal focus sealed Mo X-ray tube, The SIR-97 direct methods package was used to locate the non-hydrogen atoms, and the SHELXL-97 (Bruker)

Table **1** ^{1}H- and ^{13}C-NMR assignments for diterpenoid **1**

No.	*^{13}C*	*DEPT*	*^{1}H*
1	39.5	C	
2	40.3	CH_2	1.75 (1H, dd, *J* = 13.8, 3.6), 2.11 (1H, brdd, *J* = 13.8, 11.0)
3	124.1	CH	5.44 (1H, dd, *J* = 11.0, 3.6)
4	137.5	C	
5	39.2	CH_2	2.35 – 2.42 (2H, m)
6	30.4	CH_2	2.03 – 2.10 (2H, overlap)
7	88.1	CH	4.37 (1H, dd, *J* = 10.5, 3.0)
8	149.7	C	
9	35.5	CH_2	2.18 (1H, m), 2.31 (1H, m)
10	31.6	CH_2	1.58 (1H, m), 1.85 (1H, m)
11	42.7	CH	2.52 (1H, brd, *J* = 10.6)
12	138.0	C	
13	206.0	C	
14	55.0	CH_2	2.06 (1H, d, *J* = 17.3), 2.35 (1H, d, *J* = 17.3)
15	21.8	CH_3	1.26 (3H, s)
16	15.1	CH_3	1.51 (3H, s)
17	115.4	CH_2	5.21 (1H, s), 5.31 (1H, s)
18	147.2	C	
19	23.7	CH_3	1.81 (3H, s)
20	20.4	CH_3	2.09 (3H, s)
OOH			10.6 (1H, brs)

^{1}H- and ^{13}C-NMR spectrum were recorded in acetone-d_6 at 500 MHz, assignments were aided by gHMQC, COSY, gHMBC. The chemical shifts are given in δ units and *J* values are reported in Hz.

package was used to complete the structure determination. Refinement with 1974 reflections (204 with $1 > 2\sigma_1$) led to final *R*, *R* (all), and GOF values of 0.0437, 0.0942, and 1.030. Crystallographic data for the structure in this paper have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication number CCDC 297 501. Copies of the data can be obtained, free of charge, on application to CCDC, 12 Union Road, Cambridge, CB21EZ, UK [fax: +44(0)1223 336033 or email: deposit@ccd.cam.au.uk].

Aerial hyphae formation inhibition assay

Preparation of Streptomyces spore stock: From *Streptomyces* 85E glycerol stock, 5 μL cultures were inoculated onto ISP 4 (inorganic salts starch agar with yeast extract) on an agar plate and incubated in a 30 °C incubator for 5 to 7 days. The aerial mycelia were formed during that time. Once the *Streptomyces* sp. reached the spore stage, spore stock of the cultures was prepared and stored at –20 °C.

Hyphae formation inhibition assay: An assay based on the inhibition of hyphae formation (HFI) in *Streptomyces* 85E [24] was used as our primary bioassay in searching for protein kinase inhibitors. To perform the assay, test compounds or crude natural product extracts of known concentration were dispensed onto disks. *Streptomyces* 85E from spore stock were inoculated in tryptic soy broth and grown overnight with a 30 °C shaker. The mycelial fragments were spread on minimal medium ISP 4 agar plates. The air-dried disks were applied immediately onto the plates, and incubated in a 30 °C incubator. Sporulation usually starts within 24 hours, and the development of aerial hyphae took place in about 36 – 48 hours. Surfactin, a sporulation inhibitor, was employed as a positive control. Solvents such as methanol or DMSO can be used as a negative control. The results are scored after 30 hours. In this assay, an inhibition zone > 9 mm is acceptable and considered active. The crude extracts and subfractions were tested at the concentration of 80 μg/20 μL on 6 mm filter discs. A pure sample was tested at the concentration of 20 μg/20 μL. These were retested again at lower concentrations (10, 5, 1 μg per disk).

Results and Discussion

In our study, two previously reported dolabellanes [2], 7(*S*), 8(*S*)epoxy-13-keto-1(*S*),11(*R*)-dolabella-3(*E*),12(18)-diene (**2**) and 13-keto-1(*S*),11(*R*)-dolabella-3(*E*),7(*E*),12(18)-triene (**3**), were isolated. The structure and stereochemistry of compound **2** were confirmed by the X-ray crystallographic analysis (Fig. **2**).

Compound **1** was isolated as a colorless viscous oil. The molecular formula was established as $C_{20}H_{30}O_3$ from ^{13}C-NMR (including APT) spectra and high-resolution ESI-MS. ^{1}H-, ^{13}C-NMR and APT spectra indicated the presence of four methyls (δ_C = 15.1, 20.4, 21.8, 23.7), six sp^3 methylenes (δ_C = 30.4, 31.6, 35.5, 39.2, 40.3, 55.0), two sp^3 methines (δ_C = 42.7, 88.1), one sp^3 quaternary carbon (δ_C = 39.5), one sp^2 methylene (δ_C = 115.4), one sp^2 methine (δ_C = 124.1), and five sp^2 quaternary carbons (δ_C = 137.5, 138.0, 147.2, 149.7, 206.0). This molecular formula and characteristic spectral data indicated that compound **1** is likely derived from compound **3** and possesses a similar dolabellane skeleton. The infrared absorptions at 1711 and 1620 cm^{-1}, UV absorption at 243 nm and ^{13}C-NMR band at δ_C = 206.0 (s), 147.2 (s) and 138.0 (s) indicated that **1** possessed the same fully substituted α,β-unsaturated ketone chromophore, whereas one non-conjugated trisubstituted endocyclic olefinic bond in **3** changed to a disubstituted exocyclic olefin in **1**. In the HMBC spectrum of **1**, correlations from H_2 – 17 (δ_H = 5.21, 5.31) to C-9 (δ_C = 35.5), C-7 (δ_C = 88.1), C-8 (δ_C = 149.7) and H_2 – 9 (δ_H = 2.18, 2.31) to C-17

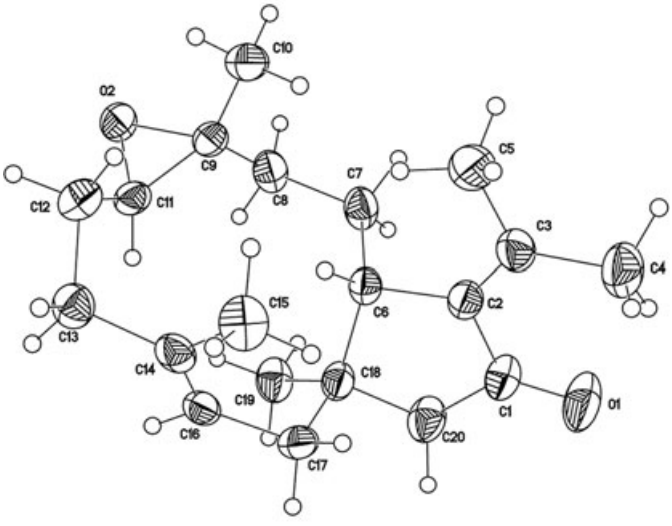

Fig. **2** The X-ray structure of compound **2** (numbering does not follow that of *Chemical Abstracts*).

(δ_C = 115.4), C-7 (δ_C = 88.1), C-8 (δ_C = 149.7), C-10 (δ_C = 31.6) were seen, which revealed that the exocyclic olefinic bond was located at C-8 (δ_C = 149.7, s) and C-17 (δ_C = 115.4, t). This experiment coupled with ^{1}H-^{1}H COSY correlations of CH_2(5)-CH_2(6)-CH(7) and CH_2(9)-CH_2(10)-CH(11) also confirmed an oxygen group attached at C-7. Further consideration of the chemical shift of C-7 (δ_C = 88.1) and the mass spectrum of **1** revealed that this oxygen group was a hydroperoxyl group [25], [26]. Comparison of the NMR spectral data **1** with that of a similar compound, 7-acetoxy-13-keto-dolabella-3(*E*),8(17),12(18)-triene [3] and (1*R*,7*R*)-7-hydroperoxydolabella-4(16),8(17),11(12)-triene-3,13-dione [25] supported this structural elucidation.

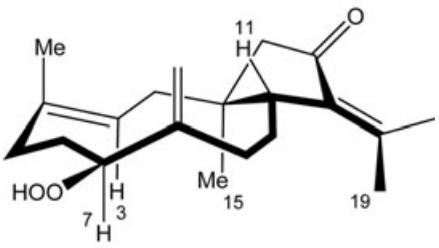

Fig. **3** The stereo-configuration of compound **1**.

Compounds **2** and **3** were first isolated from *E. calyculata* in 1982. The absolute configurations of **2** and **3** were determined using Mosher's ^{19}F-NMR method and synthesis [2]. In our study, the structure and relative stereochemistry of **2** were confirmed by the X-ray crystallographic analysis and was identical with the previously isolated dolabellane diterpene [2]. According to biogenetic considerations, the four chiral centers (C-1, -3, -4 and –11) of compound **1** possessed the same configuration as those of compound **3**. In the NOESY spectrum of **1**, correlations of CH_3-15 (δ_H = 1.26) with H-9α (δ_H = 2.31) and H-10α (δ_H = 1.85) were seen. Furthermore, the NOESY correlations of H-3 (δ_H = 5.44, dd) with H-7 (δ_H = 4.37, dd), H-3 (δ_H = 5.44, dd) with CH_3-15 (δ_H = 1.26, s) supported H-7 at α configuration. This ring geometry was consistent with literature findings for the favored conformation of the dolabellane 11-membered ring [3]. Thus, compound **1** was assigned as 7(*S*)-hydroperoxy-13-keto-(1*S*,11*R*)-dolabella-3(*E*),8(17),12(18)-triene, and named calyculatine.

In brief, two phenotypes were observed in the hyphae formation inhibition assay using *Streptomyces* 85E in our laboratory. No zones of inhibition were observed with methanol as a negative control. A clear inhibition zone indicated that the agent tested inhibits both growth and sporulation as is observed with mitomycin C, an anticancer agent (Table **2**). A zone of "bald" cells indicated that the cells in this zone have only substrate hyphae without aerial hyphae as compared to the surrounding colonies. Numerous studies have shown that aerial hyphae formation in *Streptomyces* requires the active participation of kinase cascades, such as tyrosine, threonine, and serine kinases [23], [24], [27]. A variety of kinase inhibitors blocks this process. For example, staurosporine, a eukaryotic protein kinase inhibitor, strongly inhibited several phosphorylations of cellular proteins and the aerial hyphae formation with *S. griseus* [23]. In this study, staurosporine exhibited a 13 mm bald zone with 20 μg/20 μL in the HFI assay. Thus, it is easy to identify compounds cytotoxic to *S.* 85E since they show a clear zone of inhibition that is distinctly different from the "bald" zone indicating aerial hyphae formation inhibition. This supports the rationale that the HFI assay is a potentially powerful tool with which to rapidly screen and identify chemotherapeutic candidate compounds that target cancer cell kinase activity.

The initial crude organic extract of *E. calyculata* showed positive results when evaluated with *S.* 85 E on the HFI assay (16 mm bald zone of inhibition at a concentration of 80 μg/disk), indicating anti-protein kinase activities. Compound **3** inhibited aerial hyphae formation and gave a 13 mm bald zone at a concentration of 20 μg/20 μL, whereas compounds **1** and **2** were inactive. To assess further the biological potential of compound **3**, it will be tested in additional biological systems to characterize its potential as a protein kinase inhibitor. The sulforhodamine B assay will assess the effect of compounds on the cell proliferation. The HFI assay should be able to evaluate general serine/threonine and/or tyrosine activation and further evaluate these in mammalian cells with a mitogen-activated protein kinase (MAP kinase) [28].

Dollabellane diterpenoids were reported as showing moderate cytotoxicity against the P-388 cell line [25] and HeLa cells [9].

Table **2** Effects of different compounds on the growth and sporulation of *Streptomyces* 85E

Compound	*Amount on disc [µg]*	*Zone of inhibition observed [mm]*	*Effect*
Mitomycin C	20.0	30	Clear
Surfactin	4.0	33	Bald
Staurosporine	20.0	13	Bald + clear
Rebeccamycin	20.0	15	Bald + clear
Crude organic extract	80.0	16	Bald
1	20.0	–	Inactive
2	20.0	–	Inactive
3	20.0	12	Bald + clear

* Diameter of disk alone is 7 mm.

All of the stock solutions for this HFI assay were prepared either in DMSO or methanol.

Notes: 1) Surfactin inhibited only sporulation of *Streptomyces* 85E without inhibiting growth. 2) No zones of inhibition were observed with MeOH or DMSO as negative controls. 3) The clear zones of inhibition were observed with mitomycin C.

However, this is the first report of protein kinase activity for dollabellane diterpenoids. Consequently, these compounds will undergo further evaluation with additional biological test systems for protein kinase inhibition.

Acknowledgements

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