Unusual Cladiellin-type Diterpenoids from the South China Sea Soft Coral Cladiella krempfi: Structures and Struc-ture-Activity Relationship with EGFR Postprint

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Abstract

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Full Text

Unusual Cladiellin-type Diterpenoids from the South China Sea Soft Coral *Cladiella krempfi*: Structures and Structure-Activity Relationship with EGFR

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Abstract

Two new cladiellin-type diterpenoids (1 and 2) and four known related compounds (3–6) were isolated from the South China Sea soft coral *Cladiella krempfi*. Compound 2 represents the third example of cladiellins bearing an unusual peroxy group at the C-6 position from *C. krempfi*. The structures and absolute configurations of the new compounds were established through extensive spectroscopic analysis, X-ray diffraction, and/or chemical correlation. In bioassays, all compounds were evaluated for cytotoxicity and EGFR inhibitory activity. Molecular docking experiments were conducted to investigate the structure-activity relationship of cladiellin-type diterpenoids regarding EGFR inhibitory activity.

Keywords: soft coral; *Cladiella krempfi*; cladiellin-type diterpenoid; X-ray diffraction; structure-activity relationship

1. Introduction

Soft corals of the genus *Cladiella* (order Alcyonacea, family Alcyoniidae) are widely distributed throughout the tropical Indo-Pacific region and represent a rich source of diverse and complex diterpenoids, particularly 2,11-cyclized cembranoids known as cladiellin- or eunicellin-type diterpenoids [1]. Literature surveys indicate that cladiellins isolated from *Cladiella* species, primarily *Cladiella krempfi*, can be classified into various categories, including those with a single ether bridge (C2–C9 or C2–C6), those with two ether bridges (C2–C9/C3–C7 or C2–C9/C12–C17), etc. [2]. Some of these diterpenoids exhibit broad biological activities, such as anti-inflammatory [3], antifouling [4], antiproliferative [5], and cytotoxic effects [6]. Notably, sclerophytin A demonstrated significant cytotoxicity against mouse lymphocytic leukemia L1210 cells at concentrations as low as 1 ng/mL (Figure 1 [Figure 1: see original paper]) [7]. Moreover, epidermal growth factor receptor (EGFR), which is associated with the inhibition of tumor cell proliferation, angiogenesis, tumor invasion, metastasis, and apoptosis, represents the only target protein identified to date through which cladiellin-type diterpenoids can exert their effects. For instance, Sayed and co-workers reported that pachycladin A exhibited promising EGFR inhibitory activity with an IC_{50} value of 0.5 M (Figure 1). Consequently, cladiellins have attracted considerable attention from chemists and pharmacologists worldwide for further investigation [8,9].

In our ongoing efforts to discover novel bioactive marine natural products, we have isolated numerous secondary metabolites with diverse biological activities from marine invertebrates [10–14], including cladiellins [5,15]. To obtain new and bioactive cladiellins analogous to sclerophytin A and pachycladin A, specimens of *C. krempfi* were collected off Ximao Island, Hainan Province, China, and chemically investigated, leading to the isolation and characterization of two new cladiellin-type diterpenoids, namely litophynols C and D (1 and 2) (Figure 1), along with four known compounds (3–6). Herein, we report the isolation, structure elucidation, and structure-activity relationship (SAR) analysis of these isolates assisted by molecular docking experiments.

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2. Results and Discussion

Standard workup procedures [16–18] of the Et_2O-soluble portion of the acetone extract from the soft coral *C. krempfi* yielded pure compounds 1 (1.4 mg), 2 (5.7 mg), 3 (2.2 mg), 4 (2.0 mg), 5 (2.1 mg), and 6 (1.0 mg). The known compounds 3–6 were readily identified as litophynol A (3) [19], (1R*,2R*,3R*,6R*,8R*,9S*,10R*,14R*)-3,8-dibutanoyloxycladiell-7(16),11(17)-dien-6-ol (4) [15], kremfielin B (5) [3], and litophynin E (6) [20] by comparing their NMR spectroscopic data and optical rotations with literature values.

Litophynol C (1) was isolated as an optically active colorless oil. Its molecular formula was established as $C_{26}H_{40}O_7$ by HR-ESIMS (m/z 487.2671 $[M + Na]^+$, calcd. 487.2666), indicating seven degrees of unsaturation. The ^{13}C NMR, DEPT, and HSQC spectra of 1 revealed 26 carbon signals comprising five sp^3 methyls, five sp^3 methylenes, nine sp^3 methines (five oxygenated at δC 91.1, 79.2, 83.7, 71.5, and 68.4), one oxygenated sp^3 quaternary carbon (δC 84.7), two sp^2 methylenes, and four sp^2 quaternary carbons (including two ester carbonyls at δC 172.7 and 170.8). Diagnostic ^1H and ^{13}C NMR resonances, along with coupling constants of the coupled protons (Table 1), indicated the presence of two disubstituted terminal double bonds [δH 5.37, 5.64 / δC 120.2 (CH_2), δC 149.4 (qC); δH 4.89, 5.15 / δC 116.4 (CH_2), δC 146.1 (qC)]. These two double bonds and two ester carbonyls accounted for four of the seven degrees of unsaturation, leaving a tricyclic ring system.

The planar structure of 1 was determined through ^1H-^1H COSY and HMBC experiments (Figure 2 [Figure 2: see original paper]). Four structural fragments (a–d) were rapidly identified by careful analysis of the ^1H-^1H COSY spectrum: H-12 (δH 4.46)/H_2-13 (δH 1.27, 1.94) (a); H-8 (δH 5.28)/H-9 (δH 4.68)/H-10 (δH 2.94)/H-1 (δH 2.23)/H-14 (δH 1.87)/H-18 (δH 1.89)/H_3-19 (δH 0.99)/H_3-20 (δH 0.76) (b); H_2-4 (δH 1.79, 2.23)/H_2-5 (δH 1.74, 2.20)/H-6 (δH 4.71) (c); and H_2-2 (δH 2.14)/H_2-3 (δH 1.58)/H_3-4 (δH 0.92) (d). The connections between fragments a–d were established through detailed interpretation of the well-resolved HMBC spectrum (Figure 2). HMBC correlations from H-10 to C-11/C-12, from H_2-17 to C-10/C-11/C-12, and from H-12 to C-14 revealed

a cyclohexane ring (ring A) bearing a terminal double bond at C-11 and an isopropyl group at C-14. Cross peaks from H_3-15 to C-2/C-3/C-4 and from H-2 to C-4/C-9/C-10/C-14 connected ring A with fragment c via C-2 and C-3. Additional cross peaks from H_2-16 to C-6/C-7/C-8 suggested a cyclodecane ring fused with ring A at C-1 and C-10. The strong correlation from H-2 to C-9 and the remaining degree of unsaturation indicated an ether bridge between C-2 and C-9, dividing the cyclodecane ring into a tetrahydrofuran ring B and an oxocane ring C. Finally, the acetyl group at C-8 was deduced from HMBC correlations from H-8 to C-1 and from H_3-2 to C-1 , suggesting that the remaining butyryl group should be connected at C-3. Thus, the planar structure of 1 was established as shown in Figure 1.

The relative configuration of 1 was determined by analysis of its NOESY spectrum (Figure 2). Since the orientation of H-1 in all reported cladiellin-type diterpenoids has been assigned as β [20], that of compound 1 was arbitrarily designated as β-configuration. NOE correlations between H-1/H-10 and H-10/H-8 suggested that H-8 and H-10 were also β-oriented. In addition, cross peaks between H-6/H_3-15, H_3-15/H-2, H-2/H-9, and H-9/H-14 indicated α-configuration for H-6, Me-15, H-2, H-9, and H-14. Based on these observations, the relative configuration of 1 was unambiguously deduced as 1R,*2R*,3R,*6R*,8R,*9S*,10R,*14R*. Due to overlapping NOE correlations between adjacent proton signals, the orientation of H-12 remained difficult to define.

To confirm the absolute configuration of 1, extensive crystallization attempts were undertaken, and fortunately a single crystal was obtained from recrystallization in methanol, enabling successful X-ray crystallographic analysis using Cu Kα radiation (λ = 1.54178 Å). Analysis of the X-ray data unambiguously confirmed the planar structure of 1 and established its absolute configuration as 1R,2R,3R,6R,8R,9S,10R,12S,14R [Flack parameter = 0.04(13)] (Figure 3 [Figure 3: see original paper]). Thus, the structure of 1 was fully determined and named litophynol C (Figure 1).

Litophynol D (2) was isolated as an optically active colorless oil. Its molecular formula was established as $C_{26}H_{40}O_8$ by HR-ESIMS (m/z 503.2618 $[M + Na]^+$, calcd. 503.2615), indicating seven degrees of unsaturation. The ^{13}C NMR, DEPT, and HSQC spectra of 2 revealed 26 carbon signals, including five sp^3 methyls, five sp^3 methylenes, nine sp^3 methines, one oxygenated sp^3 quaternary carbon, two sp^2 methylenes, and four sp^2 quaternary carbons. As shown in Table 1, the NMR data of 2 were remarkably similar to those of 1, indicating they are structural analogs. Careful comparison of 1D and 2D NMR spectra revealed that the primary differences between 1 and 2 involved the downfield shift of C-6 from δC/H 68.4/4.71 in 1 to 81.6/4.93 in 2, along with its neighboring carbons (e.g., C-5 from δC/H 35.9/1.74,2.20 in 1 to 30.4/1.50,2.16 in 2; C-7 from δC 149.4 in 1 to 145.0 in 2), strongly suggesting that the hydroxyl group at C-6 in 1 was replaced by a hydroperoxide group in 2. The 16 Da increase in molecular weight further supported this assignment. Detailed 2D NMR analyses as shown in Figure 2 confirmed the planar structure and relative configuration of 2.

To further verify this assignment, triphenylphosphine was used to reduce the hydroperoxide group of 2 to the corresponding alcohol in $CDCl_3$ (Scheme 1). The reduction product was identical to 1 based on ^1H NMR (Figure 4 [Figure 4: see original paper]) and MS data (Figure S30). Thus, the absolute configuration of 2 was unambiguously determined to be the same as that of 1 (Figure 1).

Given the interesting anticancer activities reported for sclerophytin A and pachycladin A [7,9], all isolates were tested for cytotoxic effects against A549 (human lung cancer) tumor cells and for EGFR inhibitory activity; however, none exhibited significant bioactivity. Comparing the structures of our isolated compounds with those of the highly bioactive sclerophytin A and pachycladin A suggests that esterification of hydroxyl groups, the site of esterification, and the length of the ester chain may influence activity. We therefore conducted intensive molecular docking analysis to study the SAR, aiming to provide insights for future structural modification.

Based on our SAR speculation, sclerophytin A, pachycladin A, and litophynol C (1) were selected for detailed molecular docking analysis. The high-resolution EGFR crystal structure (PDB code: 5X2A, resolution 1.85 Å) was used to investigate potential binding modes of these three compounds within the EGFR catalytic domain using Discovery Studio software (Figure 5 [Figure 5: see original paper]).

Pachycladin A occupied the same region as 7XO, with its C-6 and C-7 hydroxyl groups participating in hydrogen bonds with Cys797, Asn842, Lys745, and Asp855 of the EGFR crystal structure 5X2A (Figure 5B, upper row). Strikingly, these four residues are located in the kinase active site. For sclerophytin A, three hydrogen bonds were formed with Ser720 and Arg841, while the C-8 carbonyl of litophynol C (1) was the only group participating in a hydrogen bond with Lys745 (Figure 5A and C, upper rows). Furthermore, the C-3 acetate and C-11 butyrate of pachycladin A fully occupied the hydrophobic pocket, promoting van der Waals interactions with Leu718, Ser719, Ser720, Gly724, Asp800, Arg841, Asn842, Leu844, Thr854, and Asp855 (Figure 5B, middle and lower rows).

The lower binding affinity of compound 2 compared to pachycladin A may be attributed to the position and length of the ester groups, which could influence interactions with the hydrophobic pocket and explain why our isolates lacked obvious EGFR inhibitory activity. Although sclerophytin A was previously reported to be highly cytotoxic against mouse lymphocytic leukemia L1210 cells, it displayed lower binding affinity than pachycladin A, suggesting it may be less effective against non-small cell lung cancer and warrants further validation and target identification.

3. Discussion

In summary, this study represents the first detailed chemical investigation of *C. krempfi* from Ximao Island in the South China Sea. Two new cladiellin-type diterpenoids, litophynols C and D (1 and 2), and four known related compounds (3–6) were isolated and fully characterized. The stereochemistry of the new compounds was unambiguously determined through extensive spectroscopic analysis, X-ray diffraction, and/or chemical correlation. The discovery of 1 and 2 expands the diversity and complexity of marine diterpenoids. Although the bioassay results were somewhat disappointing as none of the tested compounds showed significant bioactivity, SAR studies using molecular docking analysis of these and previously reported molecules have provided valuable clues for future structural modification of cladiellin-type diterpenoids toward anticancer drug leads.

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4. Materials and Methods

4.1. General Experimental Procedures 1D and 2D NMR spectra were recorded on Bruker AVANCE III 500 (125 MHz) and Agilent 1260 Prospekt 2 Bruker Ascend 600 (600 MHz) spectrometers in $CDCl_3$ using solvent signals as internal standards ($CHCl_3$, δH 7.26 ppm; δC 77.16 ppm). IR spectra were recorded on a Nicolet 6700 spectrometer (Thermo Scientific, Waltham, MA, USA) with a KBr ATR plate. CD spectra were recorded on a J-815 instrument. Optical rotations were measured on a Perkin-Elmer 241-MC polarimeter (PerkinElmer, Fremont, CA, USA). Melting points were determined on an X-4 digital micromelting point apparatus. LR-ESIMS and HR-ESIMS data were obtained on a Bruker Daltonics Esquire 3000 plus instrument (Bruker Daltonics K. K., Kanagawa, Japan) and a Waters Q-TOF Ultima mass spectrometer (Waters, MA, USA). Commercial silica gel (100–200, 200–300, and 300–400 mesh; Qingdao, China) and Sephadex LH-20 gel (Amersham Biosciences) were used for column chromatography (CC). Precoated SiO_2 plates (HSGF-254, Yan Tai Zi Fu Chemical Group Co., Yantai, China) were used for analytical TLC. Semi-preparative HPLC was performed on an Agilent-1260 system equipped with a DAD G1315D detector at 210 and 254 nm using an ODS-HG-5 column (250 mm $\times$ 9.4 mm, 5 m) eluted with a CH_3CN–H_2O system at 3 mL/min. All solvents used for CC and HPLC were of analytical grade (Shanghai Chemical Reagents Co., Ltd.) and chromatographic grade (Dikma Technologies Inc.), respectively.

4.2. Biological Material The soft coral specimen was identified as *C. krempfi* by Prof. Xiu-Bao Li from Hainan University and collected off the coast of Ximao Island, Hainan Province, China, in 2019 at a depth of -15 m. A voucher specimen (No. 19XD-6) is deposited at the Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

4.3. Extraction and Isolation Freeze-dried animals (96 g, dry weight) were cut into pieces and exhaustively extracted with acetone at room temperature (5 × 1 L). The acetone extract was evaporated to yield a brown residue, which was partitioned between Et_2O and H_2O. The organic layer was concentrated under reduced pressure to afford 3.6 g of brown residue, which was separated by gradient silica gel column chromatography (200–300 mesh, 0% to 100% Et_2O in petroleum ether) to yield ten fractions (A–J). Fraction D (182.8 mg) was subjected to Sephadex LH-20 chromatography with PE/DCM/MeOH (2:1:1) to give five subfractions (D1–D5). Compound 5 (2.1 mg, tR = 9.1 min) was obtained from subfraction D2 by RP-HPLC (CH_3CN-H_2O, 80:20, 3.0 mL/min). Fraction E (63.0 mg) was separated by Sephadex LH-20 (PE/DCM/MeOH, 2:1:1) to obtain four subfractions (E1–E4). Subfractions E2–E4 were further purified by RP-HPLC under the same gradient (CH_3CN-H_2O, 80:20, 3.0 mL/min). Subfraction E2 yielded compound 4 (2.0 mg, tR = 21.5 min), subfraction E3 afforded compound 1 (1.4 mg, tR = 6.8 min), and subfraction E4 gave compound 2 (5.7 mg, tR = 7.4 min). Fraction F (220.2 mg) was analyzed by Sephadex LH-20 column chromatography eluted with CH_2Cl_2 to obtain six subfractions (F1–F6). Compound 6 (1.0 mg, tR = 9.4 min) was obtained from subfraction F3. Compound 3 (2.2 mg, tR = 9.0 min) was isolated from fraction G (363.6 mg) through a series of chromatographic steps.

Litophynol C (1): Colorless crystal, mp 183–184 °C; $[\alpha]D^{24}$ +9.9 (c 0.14, $CHCl_3$); IR (KBr) max/cm^{-1}: 3471, 2924, 2353, 1725, 1254, 1071, 1031; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data, see Table 1; HR-ESIMS m/z 487.2671 $[M + Na]^+$ (calcd. for $C_{26}H_{40}O_7$, 487.2666).

Litophynol D (2): Colorless oil; $[\alpha]D^{24}$ +15.8 (c 0.57, $CHCl_3$); IR (KBr) max/cm^{-1}: 3455, 2950, 1724, 1249, 1069, 1034; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 125 MHz) data, see Table 1; HR-ESIMS m/z 503.2618 $[M + Na]^+$ (calcd. for $C_{26}H_{40}O_8$, 503.2615).

4.4. X-ray Crystal Structure Analysis of 1 $C_{26}H_{40}O_7$, Mr = 464.58, monoclinic, crystal size 0.15 × 0.08 × 0.05 mm^3, space group $P2_1$, a = 6.3306(2) Å, b = 43.1346(13) Å, c = 10.1469(3) Å, V = 2643.23(14) $Å^3$, Z = 4, calcd = 1.167 g/cm^3, F(000) = 1008.0, 22999 collected reflections, 10102 independent reflections (Rint = 0.0671, Rsigma = 0.0823), final R1 = 0.1189 (wR2 = 0.3028) for reflections with $I \geq 2\sigma(I)$, R1 = 0.1270, wR2 = 0.3080 for all unique data. X-ray measurements were performed on a Bruker D8 Venture X-ray diffractometer with Cu Kα radiation (λ = 1.54178 Å) at 170.0 K. The structure was solved using the ShelXT [21] structure solution program with Intrinsic Phasing and refined with the ShelXL [22] refinement package using Least Squares minimization. Crystallographic data for 1 have been deposited at the Cambridge Crystallographic Data Centre (deposition no. CCDC 2074984). These data can be obtained free of charge via www.ccdc.cam.ac.uk/conts/retrieving.html or from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB21EZ, UK [Fax: (+44) 1223-336-033; E-mail: deposit@ccdc.cam.ac.uk].

5. Bioassays

5.1. EGFR Activity Assays All six compounds were screened for EGFR activity using staurosporine (MedChemExpress, Cat. No. HY-15141; Lot. No. 41248) as the reference compound. EGFR bioassays were performed in 1× kinase base buffer containing 50 mM HEPES (pH 7.5) and 0.0015% Brij-35, with stop buffer containing 100 mM HEPES (pH 7.5), 0.0015% Brij-35, 0.2% Coating Reagent #3, and 50 mM EDTA. For compound preparation, compounds were diluted to 50× the final desired inhibitor concentration in 100% DMSO, and 100 L of each compound dilution was transferred to a 96-well plate. Two empty wells received 100 L of 100% DMSO for no-compound and no-enzyme controls. Then, 10 L of compound from the source plate was transferred to a new 96-well intermediate plate containing 90 L of 1× kinase buffer per well, and the compounds were mixed for 10 min on a shaker. For the assay plate, 5 L from each well of the intermediate plate was transferred in duplicate to a 384-well plate. For the kinase reaction, 2.5× enzyme solution and 2.5× peptide solution were prepared. The assay plate contained 5 L of compound in 10% DMSO, to which 10 L of 2.5× enzyme solution was added and incubated at room temperature for 10 min. Subsequently, 10 L of 2.5× peptide solution was added to each well, incubated at 28 °C for 30 min, and the reaction was stopped with 25 L stop buffer. Percent inhibition was calculated using max-conversion divided by max-min, with inhibition values converted from caliper program data. IC_{50} values were obtained by fitting the data using XLfit excel add-in version 4.3.1.

5.2. Anti-tumor Assays Anti-tumor assays were performed using A549 (human lung cancer) cells following a previously described modification of the MTT colorimetric method [23,24], with 5-fluorouracil as the positive control. To assess the anti-proliferative activity of test compounds, three concentrations were tested in triplicate.

6. Molecular Docking

The EGFR cocrystal structure (PDB code 5X2A) was obtained from the RCSB Protein Data Bank. Water molecules were removed in Discovery Studio (DS), and the binding site for sclerophytin A, pachycladin A, and litophynol C (1) on EGFR was defined as the same region occupied by 7XO. The combined spherical area was centered at x = 3.789, y = 17.478, z = -29.936 with a radius of 10.85 Å. The receptor was prepared for docking using the protein preparation tool in DS. Ligands were drawn in ChemBioDraw and uploaded to DS, then optimized using the prepare ligands and minimize ligands tools. The CDOCKER module was employed for docking, and the simulation with

the highest -CDOCKER_{{{INTERACTION}}_{{ENERGY}}} score was analyzed and visualized in PyMOL [25,26].

Supplementary Materials: Supporting information is available at www.mdpi.com/xxx/s1, including NMR spectra for all compounds and HR-ESI-MS data.

Author Contributions: Y.-W.G. and X.-W.L. conceived and designed the experiments; Y.J. performed the experiments and analyzed the data; L.-G.Y. contributed materials; Y.-W.G., X.-W.L., and Y.J. wrote the paper.

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Note: Figure translations are in progress. See original paper for figures.