

Authorversion: *Can. J. Chem.*, vol.89(1); 2011; 57-60

(-)-6 α -Hydroxy Polyanthellin A, a novel antifouling diterpenoid from the Indian soft coral *Cladiella krempfi* (Hickson)

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Abstract

Bioassay guided purification of methanol extract of the soft coral, *Cladiella krempfi* (Octocorallia: Alcyonacea) yielded a novel eunicellin-type diterpenoid, (-)-6 α -hydroxy Polyanthellin A **1**, active against the cyprids of the fouling barnacle, *Balanus amphitrite* (EC₅₀ 9.02 μ g/ml; LC₅₀ 36 μ g/ml and therapeutic ratio = 4). The structure of compound **1** was determined from its spectral data including IR, NMR (1D & 2D NMR) and Mass Spectra.

Keywords: Biofouling; antifouling; *Cladiella krempfi*; *Balanus amphitrite*; diterpenes

Introduction

The genus *Cladiella* belongs to the order Alcyonacea, class Anthozoa. These soft corals, which grow as thick carpet-like colonies, extending upto several square meters are widely distributed in the tropical and subtropical shallow-water habitats (1). Several bioactive cembrane / eunicellane diterpenes, sesquiterpenes, sterols and glycolipids have been reported from this genus by different groups of workers (2). These include Cladidiol and polyanthellin A, an acetylcholinesterase-inhibiting sesquiterpene and an antibacterial diterpene respectively from a *Cladiella* sp found in Andaman & Nicobar area, a new hemiketal steroid, cladiellin A with antioxidant activity from China, cladiellaperoxide, a diterpenoid (toxic to brine shrimps) from *C. sphaeroides* and several pregnane glycosides from *C. krempfi* (antitumour) collected from Miyazaki Prefecture, Japan (2-6).

Despite their abundance in Indian waters, only limited studies on their chemical / biological properties are available in literature (7). Herein we report isolation and structure determination of a novel diterpenoid, (-)-6 α -hydroxy Polyanthellin A **1**, active against the cyprids of the fouling barnacle, *Balanus amphitrite* from *Cladiella krempfi* from Lakshadweep Islands (India).

Experimental

General: NMR spectra were recorded in CDCl₃ solution in BRUKER Avance 300 and 500 MHz NMR spectrometer, using a standard 5mm probe while HRMS analysis was performed in an Applied Biosystem instrument (QTOF type) and IR spectrum was recorded using Jasco FTIR 4100 IR spectrometer. For column chromatography, silica gel (60-120 mesh size) from s.d. Fine Chem Limited was used. All solvents were procured from s.d. Fine Chem Limited (AR Grade) and used as such.

Cladiella krempfi was collected from the shallow coastal waters of Kavaratti Island, (Lakshadweep, Lat 10°34'0 N; Long 72°37'0 E). The thick carpet-like colonies were light brown-in colour. The specimen (1 Kg wet weight) was washed with fresh water and soaked in methanol before transporting to the laboratory. In the laboratory, the animals were cut into smaller pieces and repeatedly extracted with fresh MeOH. The combined MeOH supernatants were concentrated under reduced pressure using rotavapor. The crude extract was then partitioned into petroleum ether, ethyl acetate, and aqueous fractions in the usual way. The active Petroleum ether fraction (Fr 1, 2 gm) was purified on Sephadex

LH-20 (eluant: chloroform-methanol 1:1) into four broad fractions (Fr 1-1 to 1-4). Bioassay of the fractions indicated the fraction 1-3 (100 mg) to be active which was further purified on silica gel columns (Gradient acetone-hexane), yielding the active compound as a single spot on TLC ($R_f = 0.52$). The structure of the purified compound (10 mg) was determined based on its IR, MS and NMR spectral data.

The antibacterial screening of different fractions were carried out against seven strains of fouling bacteria (*Bacillus cereus*, *B. pumilus*, *B. megaterium*, *Pseudoalteromonas haloplanktis*, *Pseudomonas chlororaphis*, *P. putida* and *P. aeruginosa*) using Disc diffusion method, with 100 μg of compound per disc (8). The settlement inhibition activity was performed against *Balanus amphitrite* (= *Amphibalanus amphitrite*) cyprids at 50 $\mu\text{g}/\text{ml}$ (9, 10). The Effective and Lethal Concentrations (EC_{50} & LC_{50}) of the pure compound were determined using serial dilution method from 100 to 6.25 $\mu\text{g}/\text{ml}$ (8,11-128). The data were analyzed by one-way ANOVA, followed by Tukey's multiple comparison test at 95% confidence level (13). One-way ANOVA was performed with SPSS, Version 10. EC_{50} and LC_{50} values were calculated using Probit analysis (14, 15). The experiment was repeated twice with different batches of larvae.

Results & Discussion

Structure determination

IR absorptions of compound **1** (yield =10 mg, $[\alpha]_D^{27} = -20^\circ$ (0.17%, CHCl_3), at 3450, 1730 and 1050 cm^{-1} indicated the presence of hydroxyl and ester groups in the molecule. Among this, the ester group could be an acetate, as confirmed by the characteristic NMR peaks at δ 2.01(3H,s, CO-CH₃), 22.6(CO-C₃H₇) and 170.1(CO-CH₃) and facile loss of 60 mass units in the mass spectrum (Table 1). The ¹³C NMR confirmed the presence of 22 carbons, distributed as 4 quaternary, 7 CH, 5 CH₂ and 6 methyls as confirmed by DEPT 135 and DEPT 90 spectra. Noteworthy among this was the presence of six oxygenated carbons (90.8d, 82.9s, 80.3d, 77.2s, 75.5d, 74.6s), but relatively lower polarity of the compound (*i.e.*, eluted with hexane-acetone=60:40 from SiO₂ column, $R_f=0.52$ with hexane- acetone= 70:30 and its easy solubility in CHCl_3), indicating possible presence of one or more cyclic ether / epoxides in the molecule. HRMS revealed the accurate mass of its $[M+H]^+$ pseudomolecular ion to be 381.2630, corresponding to an elemental composition of C₂₂H₃₇O₅ (calculated: 381.2635), indicating its diterpenoid nature. The above molecular

formula indicated a degree of hydrogen deficiency of 5. One of this could be accounted for by the acetate group. NMR spectra ruled out the presence of any unsaturations, indicating the molecule to be tetracyclic.

Comparison of its NMR spectral data with those of different soft coral and gorgonian diterpenoids indicated its close structural similarity to polyanthellin A, a diterpenoid isolated from the gorgonian *Briareum polyanthes* (Table 2) (16). The major differences were the observed downfield shift of C-4 to C-6 signals from δ 36.2, 18.1 and 39.6 in polyanthellin A to δ 40.1 (CH₂), 29.2(CH₂) and 80.3(CH) respectively in compound **1**. Comparison of these values with those of related compounds briarellin M and O (16), as well as examination of COSY, HSQC and HMBC data indicated the presence of the additional hydroxyl group at C-6 (Table 2; Fig.1). The large downfield chemical shift of C-6 at δ 80.26 indicated the presence of a hydroxyl group at this position. This was confirmed by the expected downfield shifts of C-4 and C-5 by 4 and 11 ppm respectively.

The similarities in ¹H & ¹³C chemical shifts, except for C-4, C-5 and C-6 of the isolated compound and polyanthellin A indicated their relative stereochemistry also to be identical. The relatively small coupling constant observed for the 6-H (J = 6.3Hz) indicated this proton to be equatorial. The observed strong nOe cross peaks H1 (δ 2.20) / H10 (δ 2.84) & H2 (δ 3.54), H10/H9 (δ 4.05) & 20 Me (δ 1.47), H9/19 Me (δ 1.23) and 19 Me/H6 (δ 4.55), as well as the medium size cross peaks between H2/H9, H2/H19 and H6/H9 (Fig. 2) further confirmed the stereochemistry. The observed optical rotation of compound **1** ($[\alpha]_D^{27} = -20^\circ$ 0.17%, CHCl₃) is also comparable to that of polyanthellin A ($[\alpha]_D^{20} = -9.9^\circ$ c1.0, CHCl₃) and other related eunicellin diterpenoids, indicating the absolute stereochemistry to be the same. From the above results, the structure of compound **1** could be established as (-)-6 α -hydroxy polyanthellin A (Fig.3).

Antifouling activity

The crude extract, partially purified fractions / compounds were inactive against the seven strains of fouling bacteria. Duplicate experiments with varying dosages between 6.25 to 100 μ g/ml confirmed the anti-settlement properties of compound **1** against *B. amphitrite* cyprids in a dose-dependent manner [Fig.4; $p = 0.05$; $F_{5,30} = 184.818$, $EC_{50} 8.82 \mu$ g/ml, $LC_{50} 35.72 \mu$ g/ml (batch 1) and $p = 0.05$; $F_{5,30} = 322.760$, $EC_{50} 9.23 \mu$ g/ml, $LC_{50} 36.22 \mu$ g/ml (batch 2); one-way ANOVA]. The therapeutic ratio was calculated to be 4 from the above results.

Marine invertebrates, especially soft corals and gorgonians are a rich source for novel and bioactive secondary metabolites (17, 18). Since the first isolation of eunicellin, a diterpene from *Eunicella stricta* in 1968, over 50 eunicellin and related cladiellins have been isolated so far (19, 20). Many of these metabolites possess promising cytotoxic properties (17, 19), while a few are known to play a functional role as antifeedants, as indicated by mollusc and fish lethality studies (21).

To the best of our knowledge, no studies have been reported so far on antifouling properties of cladiellin diterpenes. Our studies indicate (-)-6 α -hydroxy polyanthalin A **1** to be active against cyprids of *Balanus amphitrite*. With an LC₅₀ of ~36 μ g/ml and favourable EC₅₀ value of ~ 9 μ g/ml, its therapeutic ratio works out to be 4, which agrees well with the recommended value (i.e. >1) for environmentally compatible antifouling coatings (22). Further studies on structure-activity relationships of this compound using different synthetic analogs/derivatives may lead to an environmentally benign antifouling agent of commercial potential.

Acknowledgements

Our sincere thanks are due to CMLRE, Ministry of Earth Sciences, New Delhi for partial financial support; Director, National Institute of Oceanography for the facilities provided; Dept. of Science and Technology, Lakshadweep Islands for the collection of samples and Dr. Yehuda Benayahu for taxonomic identification. One of the authors (VPL) is thankful to CSIR, Govt. of India for the grant of Senior Research Fellowship.

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Table 1. MS Fragmentation pattern of (-)-6 α -hydroxy polyanthellin A

Observed m/z	Calculated m/z	Molecular formula of the fragment ion	Stable fragment expelled from the parent ion
381.2630	381.2635	C ₂₂ H ₃₇ O ₅	[M+H].+
321.2371	321.2424	C ₂₀ H ₃₃ O ₃	[M-CH ₃ COOH+H] ⁺
303.2319	321.2318	C ₂₀ H ₃₁ O ₂	321-H ₂ O
293.2527	293.2475	C ₁₉ H ₃₃ O ₂	321-CO
285.2221	285.2212	C ₂₀ H ₂₉ O	[M-AcOH- 2H ₂ O+H] ⁺
267.2091	267.2107	C ₂₀ H ₂₇	285-H ₂ O
225.1668	225.1637	C ₁₇ H ₂₁	267-C ₃ H ₆
201.1611	201.1637	C ₁₅ H ₂₁	

Table 2. Comparison of the ^1H and ^{13}C Chemical shifts of (-)-6 α -hydroxy polyanthellin A and polyanthellin A

C-	(-)-6 α -hydroxy polyanthellin A		Polyanthellin A		
	No	δ_c	δ_h	δ_c	δ_h
	1	41.9	2.20(1H, dd, 7.2,10.5, H $_{\alpha\beta}$)	41.6	2.30(1H,m)
	2	90.8	3.54(1H, brs, Heq/ β)	93.7	3.54(1H, brs)
	3	74.6	--	75.5	--
	4	40.1	1.72(1H,dd,7,1.5,eq/ β),1.85(1H, m, ax/ α),	36.2	1.41(1H, m);1.79(1H, m)
	5	29.2	2.05(1H, m, eq/ α); 1.55(1H, m, ax/ β)	18.1	1.49(1H, m);2.32(1H, m)
	6	80.3	4.55(1H, brd, 6.3, eq/ β)	39.6	1.42(2H, m)
	7	77.2	--	74.3	--
	8	46.9	2.35(1H,dd,14.7,11, eq/ β); 1.76(1H,dd, 14.7, 3.5, ax/ α)	47.5	1.86(1H, brd, 14.2); 2.19(1H, dd, 4.9, 14.2)
	9	75.5	4.05(1H, ddd, 11, 7.2, 3.5, ax/ β)	77.2	3.89(1H, t, 5.3)
	10	54.2	2.84(1H, t, 7.2, eq/ β)	51.0	3.21(1H, t, 6)
	11	82.9	--	83.1	--
	12	30.6	2.38(1H, m, eq/ β);1.4 (1H, m, ax/ α)	29.7	2.41(1H, m); 1.25(1H, m)
	13	17.9	1.4 (1H, m, eq/ α);1.22(1H, m, ax/ β)	17.5	1.14(1H, m); 1.40(1H, m)
	14	42.5	1.25 (1H, m, ax/ α)	42.3	1.15(1H, m)
	15	29.1	1.7(1H, m,)	29.6	1.67(1H, m)
	16	21.8	0.96 (3H, d, 6.9)	21.7	0.92 (3H, d, 6.9)
	17	15.6	0.83(3H, d, 6.9)	15.5	0.79(3H, d, 6.9)
	18	30.28	1.14(3H, s, β -Me)	27.5	1.07(3H, s)
	19	23.16	1.23(3H,s, β -Me)	35.6	1.33(3H, s)
	20	24.4	1.47(3H,s,ax/ β -Me)	24.0	1.48(3H, s)
	21	170.1s	--	170.3	--
	22	22.6q	2.01(3H, s)	22.5	1.99(3H, s)

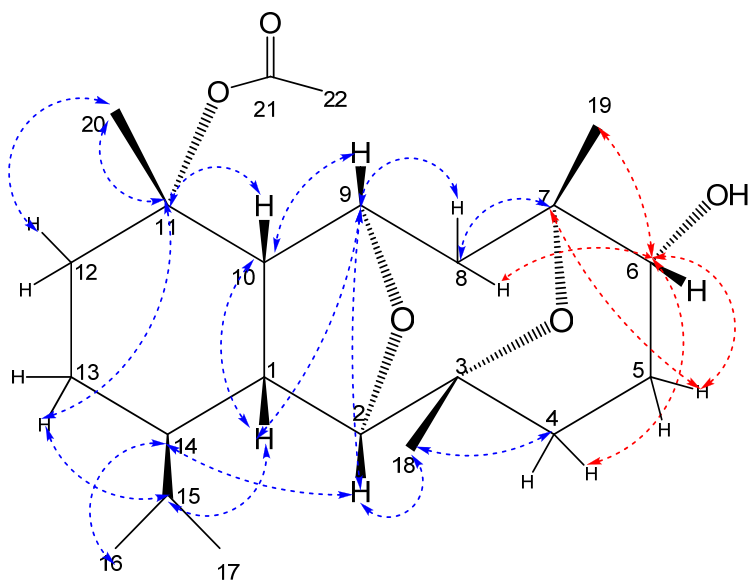


Fig. 1. Schematic representation of major HMBC correlations of (-)-6 α -hydroxy polyanthellin A.

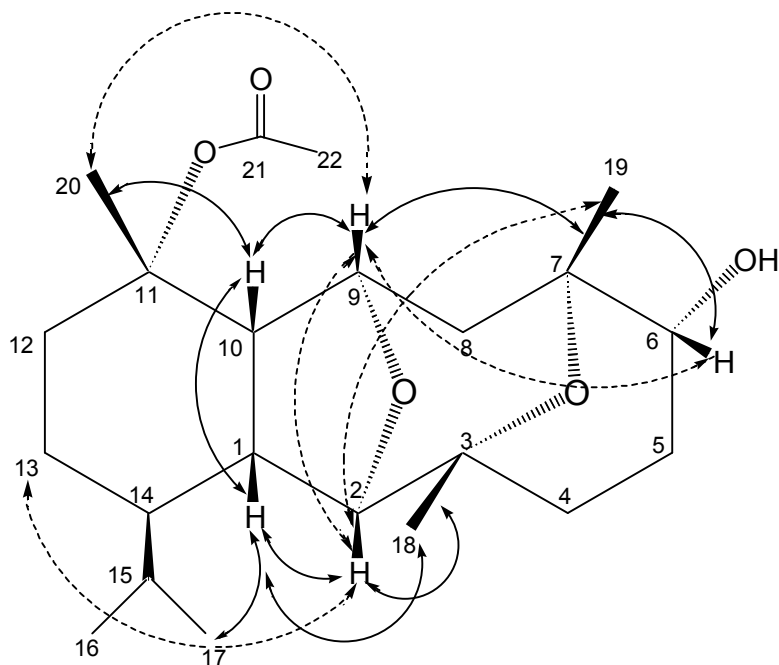


Fig.2. Schematic representation of strong and medium (dotted line) nOe correlations of (-)-6 α -hydroxy polyanthellin A

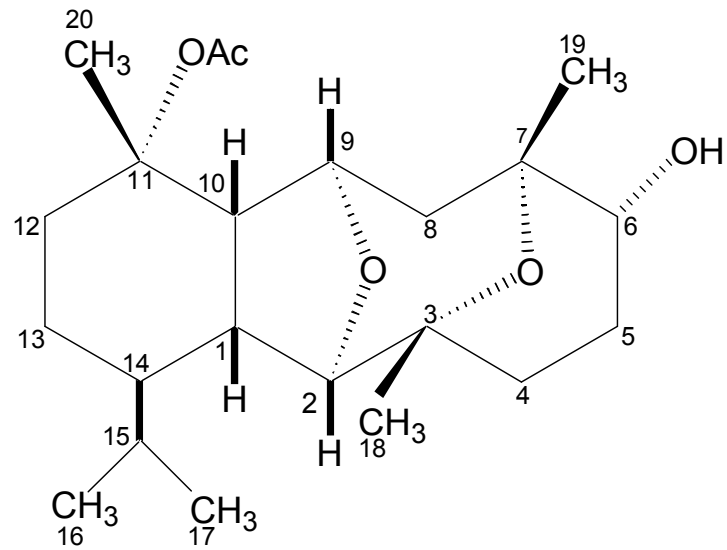


Fig.3. (-)-6 α -hydroxy polyanthellin A

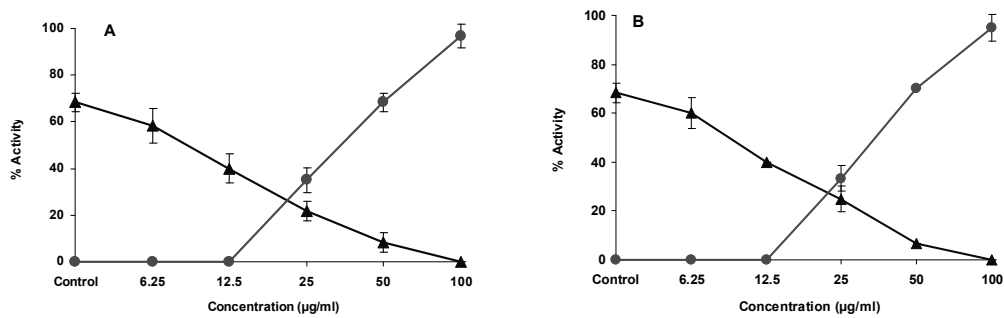
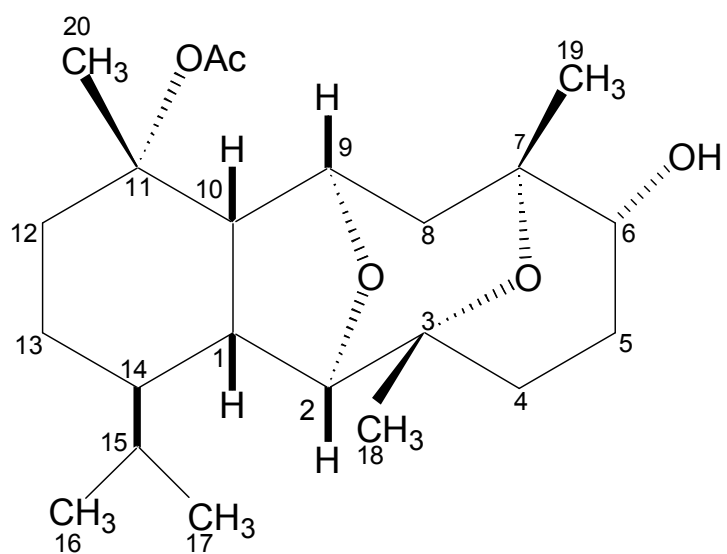


Fig. 4. Effect of (-)-6 α -hydroxy polyanthellin A on the settlement and mortality of cyprids; A: Batch 1 cyprids; B: Batch 2 cyprids. \blacktriangle = %Settlement; \bullet = %Mortality. Data plotted are means \pm SD of six replicates.

Graphical Abstract



A novel eunicellin-type diterpenoid, (-)-6 α -hydroxy polyanthellin A from *Cladiella*

krempfi of Indian waters