Bioactive Chemical Constituents of *Cladiella* Species

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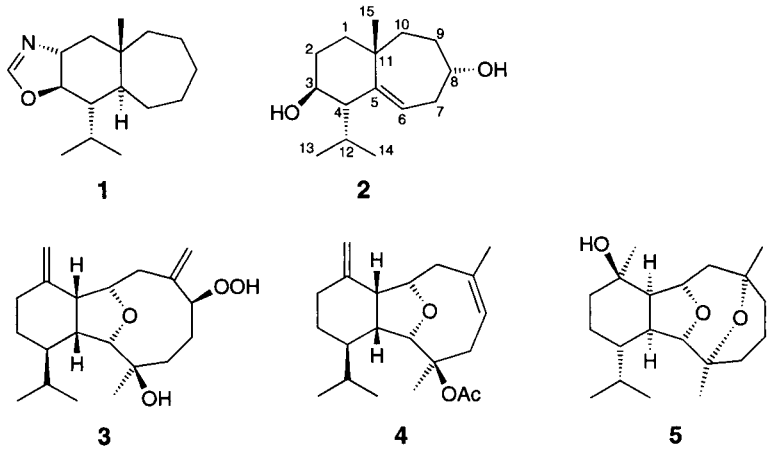
From the methanolic extract of *Cladiella* sp., collected from the Andaman Island, India, a new sesquiterpene, cladidiol (**2**), and three known diterpenes, cladiellaperoxide (**3**), (6*E*)-2α,9α-epoxyeunicella-6,11(12)-dien-3β-ol (**4**), and polyanthellin A (**5**) were isolated. The structures of these compounds were established by extensive spectroscopic studies. Compound **2** exhibited modest acetylcholinesterase-inhibition activity, and compounds **3**–**5** showed antibacterial activities against *Streptococcus pyogenes*, *Escherichia coli*, and *Pseudomonas aeruginosa*.

Introduction. – Chemical investigations on marine soft corals have yielded several novel bioactive natural products for potential biomedical applications. For instance, eleutherobin, isolated from *Erythropodium caribaeorum* and *Eleutherobia* sp., is an extremely potent anticancer agent [1–4]. The AcOEt extract of *Cladiella australis* has shown promising hypoglycaemic activity in rats at a dose of 250 mg/kg [5]. The genus *Cladiella* is a rich source of bioactive eunicellane- and cembrane-type diterpenes [6–10]. Previously, we have reported a novel sesquiterpene, cladioxazole (**1**) from *Cladiella* sp., collected form the Andaman Island, India [11]. The crude AcOEt extract of *Cladiella* sp. exhibited antibacterial activity against *Streptococcus pyogenes*, *Staphylococcus aureus*, *Escherichia coli*, *Enterococcus faecalis*, and *Pseudomonas aeruginosa* at a concentration of 50 μg/ml. Our recent detailed chemical studies on this crude extract has resulted in the isolation of a new sesquiterpene, cladidiol (**2**), and three known diterpenes, cladiellaperoxide (**3**), (6*E*)-2α,9α-epoxyeunicella-6,11(12)-dien-3β-ol (**4**), and polyanthellin A (**5**). Their structures were established by means of extensive spectroscopic studies. Cladidiol (**2**) represents the second example of a sesquiterpene with a caldioxazole-like structure [11]. It was inactive in our antibacterial assay, but exhibited acetylcholinesterase (AChE) inhibition activity. Compounds **3**–**5** showed antibacterial activities against *Streptococcus pyogenes* (ATCC19615), *Escherichia coli* (ATCC 25933), and *Pseudomonas aeruginosa* (ATCC 27853) at a dose of 30 μg/ml, but were inactive in our AChE-inhibition assay.

Results and Discussion. – *Structure Elucidation of Cladidiol.* The UV spectrum of cladidiol (**2**) showed a terminal absorption, indicating the lack of a conjugated chromophore in the molecule. Its IR spectrum displayed intense absorption bands at 3410 (OH), 2904 (CH), and 1602 (C=C) cm^{-1}. The high-resolution electron-impact mass spectrum (HR-EI-MS) of **2** showed the molecular-ion peak at *m/z* 238.1936,



which corresponds to the molecular formula $C_{15}H_{26}O_2$, with three degrees of unsaturation.

The ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) of **2** showed two *d* each integrating for three H-atoms, at δ_H 0.87 and 0.93 ($J = 6.5$), corresponding to Me(13) and Me(14). A *s* at δ_H 1.35 (3 H) was assigned to Me(15). A *ddd* (1 H) at δ_H 3.85 ($J_{3\alpha,4\beta} = 10.6$, $J_{3\alpha,2\beta} = 10.3$, $J_{3\alpha,2\alpha} = 4.2$) was assigned to the H–C(3) methine atom. Its downfield chemical shift indicated the presence of a geminal OH group. Another downfield methine H-atom, geminal to an oxygenated functionality, resonated at δ_H 3.55, and this signal was later assigned to H–C(8). The olefinic H-atom at C(6) resonated δ_H 5.53.

A COSY-45° spectrum of **2** was recorded to assign the ^{1}H-NMR signals and to establish a partial structure of this new metabolite. The interpretation of COSY-45° and TOCSY spectra helped us to establish the connectivity shown in *Fig. 1*. The molecular structure was traced starting from H–C(3) (δ_H 3.85), which showed cross-peaks with CH_2(2) (δ_H 1.57 and 1.41). The latter showed vicinal couplings with CH_2(1) (δ_H 1.95 and 1.73). H–C(3) also exhibited interactions with H–C(4) (δ_H 2.55), which, in turn, showed ^{1}H/^{1}H spin correlations with the H–C(12) (δ_H 2.15). The latter showed cross-peaks with Me(13) (δ_H 0.87) and Me(14) (δ_H 0.93). H–C(4) also showed long-range allylic couplings with H–C(6) (δ_H 5.53) in the TOCSY spectrum (100 ms), and the latter cross-peaks with the CH_2(7) (δ_H 2.33 and 2.12), which further showed vicinal couplings with the H–C(8) (δ_H 3.55). The methine H–C(8) atom showed cross-peaks

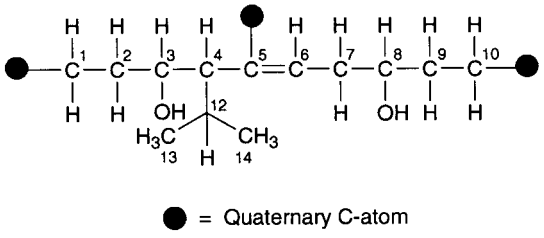


Fig. 1. *Partial structure of cladidiol* (**2**) *derived from COSY-45° and TOCSY spectra*

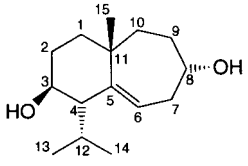
with $CH_2(9)$ (δ_H 1.95 and 1.49). COSY-45° Interactions of $CH_2(9)$ with $CH_2(10)$ (δ_H 2.00 and 1.67) were also observed in the spectrum.

The ^{13}C-NMR spectrum ($CDCl_3$, 125 MHz) of **2** showed 15 C-atoms, and a DEPT experiment was performed to establish the multiplicity of each signal, revealing the presence of three Me, five CH_2, and five CH groups. Subtraction of the DEPT spectrum from the broadband ^{13}C-NMR spectrum indicated the presence of two quaternary C-atoms at δ_C 124.3 and 32.1, which were assigned to C(5) and C(11), respectively. Their ^{13}C-NMR chemical shifts suggested that C(5) and C(11) were sp^2 and sp^3-hybridized, respectively. Two downfield aliphatic signals at δ_C 69.8 and 70.5 were assigned to C(3) and C(8), respectively. Their downfield chemical shifts indicated that both were O-bearing C-atoms. In the HMQC spectrum, H−C(3) (δ_H 3.85) and H−C(8) (δ_H 3.55) showed cross-peaks with C(3) (δ_C 69.8) and C(8) (δ_C 70.5), respectively. HR-EI-MS indicated that C(3) and C(8) were both substituted with an OH group, as supported by the molecular formula $C_{15}H_{26}O_2$, which indicated the presence of three degrees of unsaturation. Two of them were accounted for a bicarbocyclic skeleton, and the third was rationalized by a C=C bond in ring B. These observations indicated that both O-atoms were free OH groups on two different C-atoms. This assumption was further supported by the presence of two peaks in the mass spectrum at *m/z* 220 ($[M - H_2O]^+$) and *m/z* 202 ($[M - 2\ H_2O)^+$]. The interpretation of the data obtained from IR, ^{1}H- and ^{13}C-NMR, COSY, HMQC, and mass spectra suggested that C(3) and C(8) were both substituted with OH groups. This was confirmed by recording the ^{1}H-NMR spectrum of **2** in (D_5)pyridine, which gave rise to an induced paramagnetic shift for H−C′(3) from δ_H 3.85 to 4.12, and, for H−C(8), from 3.55 to 3.78, respectively, which is typical for OH groups [12]. The complete ^{13}C-NMR-chemical-shift assignments and ^{1}H/^{13}C shift correlations for **2**, as determined from the HMQC spectrum, are given in *Table 1*.

The HMBC spectrum was very useful to establish a gross structure of compound **2** from the partial structure (*Fig. 1*) deduced from the COSY-45° and TOCSY spectra. The $CH_2(1)$ (δ_H 1.95 and 1.73) and $CH_2(10)$ (δ_H 2.00 and 1.67) H-atoms showed long-range heteronuclear couplings with C(5) (δ_C 124.3) and C(11) (δ_C 32.1). H−C(4) (δ_H 2.55) and H−C(6) (δ_H 5.53) also exhibited cross-peaks with C(5) and C(11). The Me(15) H-atoms (δ_H 1.35) also showed HMBC interactions with C(4) and C(5), which indicated that C(1) and C(10) were linked *via* a quaternary C-atom, *i.e.*, C(11), and C(4) and C(6) *via* C(5), respectively. The connectivity between C(5) and C(10) was also evident from these HMBC experiments. Further important HMBC interactions are shown in *Table 1*. Based on the above data, the structure of cladidiol (**2**) could be derived.

Efforts to establish the absolute configuration of **2** by *Mosher*'s method at C(3) and C(8) were not successful because the compound decomposed under acidic or basic conditions. So, we established the relative configurations at the stereogenic centers with the aid of ^{1}H/^{1}H coupling constants and NOE effects. The C(3) methine H-atom, geminal to an OH group, resonated as a *dd* at δ_H 3.85 and showed diaxial couplings with axial H−C(2) ($J_{3\alpha,2\beta} = 10.3$), axial/equatorial coupling with equatorial H−C(2) ($J_{3\alpha,2\alpha} = 4.2$), and diaxial coupling with axial H−C(4) ($J_{3\alpha,4\beta} = 10.6$). These coupling constants indicated that H−C(3) was in axial position, and we, thus, assumed β-configuration for HO−C(3). The $[\alpha]_D^{20}$ value of **2** was found to be 112, similar to that of

Table 1. *^1H- and ^{13}C-NMR Data* ($CDCl_3$, 500 and 125 MHz, resp.), *and $^1H/^{13}C$ One-Bond-Shift Correlations* (from HMQC and HMBC experiments) *for Cladidiol* (**2**). Chemical shifts δ in ppm, coupling constants J in Hz.



Position	^{1}H-NMR	^{13}C-NMR	HMBC Interactions
1	1.95 (*m*), 1.73 (*m*)	27.3	C(2), C(5), C(11), C(15)
2	1.57 (*m*)	26.5	C(1), C(3), C(11)
	1.41 (*m*)		
3	3.85 (*ddd*, $J = 10.6$, 10.3, 4.2)	69.8	C(2), C(4), C(5)
4	2.55 (*dd*, $J = 10.6$, 9.3)	45.9	C(2), C(5), C(12)
5	–	124.3	–
6	5.53 (br. *d*, $J = 5.6$)	120.2	C(5), C(7), C(8)
7	2.33 (*m*), 2.12 (*m*)	45.4	C(5), C(6), C(7)
8	3.55 (*m*)	70.5	C(6), C(7), C(9)
9	1.95 (*m*), 1.49 (*m*)	26.4	C(8), C(10)
10	2.00 (*m*), 1.67 (*m*)	33.9	C(8), C(9)
11	–	32.1	–
12	2.15 (*m*)	46.0	C(4), C(13), C(14)
13	0.87 (*d*, $J = 6.5$)	14.6	C(4), C(12), C(14)
14	0.93 (d, $J = 6.5$)	19.4	C(4), C(12)
15	1.35 (*s*)	14.1	C(5), C(11)

cladioxazole (**1**; $[\alpha]_D^{20} = 105$). This similarity in sign and magnitude of the optical rotation suggested that both compounds had the same absolute configurations at C(3), C(4), and C(11) [11]. This assumption was further supported by the NOESY spectrum of **2**, in which H–C(4) (δ_H 2.55) showed a strong NOE interaction with Me(15) (δ_H 1.35) (*Fig. 2*), which indicated a *cis* relationship and a spatial proximity between these two groups. The Me(15) H-atoms also showed cross-peaks with H–C(8), indiating β-orientation for H–C(8) and α-orientation for HO–C(8). Likely conformations of rings A and B of **2**, based on NOE interactions, are shown in *Fig. 2*.

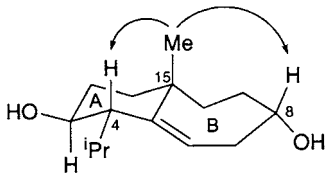


Fig. 2. *Expected main conformations of rings A and B in* **2** *according to the NOESY spectrum*. Important NOE interactions are marked with arrows.

In addition to cladidiol (**2**), we have also isolated three known diterpenes from the crude extract, namely, cladiellaperoxide (**3**), (6*E*)-2α,9α-epoxyeunicella-6,11(12)-dien-3β-ol (**4**), and polyanthellin A (**5**). The UV, IR, ^{1}H- and ^{13}C-NMR, as well as the mass spectra of **3**–**5** were nearly identical to those reported in the literature [13–15].

Compounds **3**–**5** had been previously isolated from marine soft corals, *i.e.*, *Cladiella sphaeroide*, *Heterogorgia uatumani*, and *Briareum polyanthes*, respectively [13–15].

Acetylcholinesterase Inhibition. Alzheimer's disease (AD) is an age-related, chronic neuronal degenerative disorder, occurring in middle or late life. This disease is characterized by a progressive dementia, which is associated with both severe disabilities in performing the activities of everyday life and a reduced life expectancy after onset of the disease. The well-known cholinergic hypothesis of AD is based on accumulated evidence suggesting that enzymes involved in the synthesis and/or hydrolysis of acetylcholine were deficient in the brains of AD patients [16][17]. This breakdown of central cholinergic transmission resulted in an effort for treating AD with cholinomimetic agents that either augment the synthesis or inhibit the hydrolysis of acetlycholine. On this basis, we are interested to identify new marine-natural-product-based acetylcholinesterase (AChE) inhibitors.

Cladidiol (**2**) exhibited AChE-inhibition activity with an IC_{50} value (inhibition concentration to lower enzyme activity by 50%) of 67 μM. Compounds **3**–**5** were also screened, but were inactive in this context.

Antibacterial Activity. Compounds **2**–**5** were tested for their antibacterial activities against *Streptococcus pyogenes* (ATCC19615), *Staphylococcus aureus* (ATCC 25923), *Enterococcus faecalis* (ATCC 19433), *Escherichia coli* (ATCC 25933), and *Pseudomonas aeruginosa* (ATCC 27853) at a concentration of 30 μg/ml, using the disc-agar-diffusion method. Compound **2** was inactive in this bioassay, while compounds **3**–**5** exhibited antibacterial activities against *Streptococcus pyogenes*, *Escherichia coli*, and *Pseudomonas aeruginosa* (*Table 2*).

Table 2. *Antibacterial Activities* (in terms of diameter *d* of inhibition zone) *of Compounds* **3**–**5** *against* Streptococcus pyogenes, Escherichia coli, *and* Pseudomonas aeruginosa. In all cases, 30 μg/ml of test compound was applied to 6-mm discs.

Compound	*d* [mm]		
	S. pyogenes	*E. coli*	*P. aeruginosa*
3	7	8	12
4	5	12	7
5	13	6	3

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Experimental Part

General. TLC: *Merck* silica gel GF_{254} (precoated plates). Optical rotation: *Hitachi Polatronic-D* polarimeter. UV Spectra: *Shimadzu UV-240* instrument; λ_{max} in nm. IR Spectra: *Jasco IRA1* spectrophotometer; in cm^{-1}. ^1H- and ^{13}C-NMR-Spectra: *Bruker AM-500* instrument (500 and 125 MHz, resp.); chemical shifts δ in ppm rel. to SiMe4 (= 0 ppm) as internal standard; coupling constants *J* in Hz. EI-MS: *Varian MAT-312* double-focussing mass spectrometer, connected to a *DEC PDP 11/34* computer system; in *m*/*z* (rel. intensity).

Collection of Marine Organism. Cladiella sp. was collected from the Andaman Island, India, from a depth of 1 m by scuba diving. The organism was identified by *P. R.*, and a specimen (MF-VA/39) was deposited at the Department of Pharmaceutical Sciences, Andhra University, Visakhapatnam, Andhra Pradesh, India.

Extraction and Isolation. *Cladiella* sp. (4.5 kg dry weight) was extracted with MeOH. The solvent was evaporated under reduced pressure. This resulting gum-like crude extract was dissolved in H_2O/EtOH 4:1 and extracted with AcOEt. The org. extract was loaded onto a silica-gel column and eluted with hexane, hexane/AcOEt 1:0 → 0:1, and then AcOEt/MeOH 1:0 → 0:1 to get several fractions. The fraction obtained upon elution with hexane/AcOEt 1:1 was subjected to reversed-phase HPLC, using a gradient of MeCN/H_2O 0:1 → 1:0 which afforded cladidiol (**2**; 41 mg)as a colorless gum. This compound was homogenous according to TLC in various solvent systems. Another fraction, obtained on elution with hexane/AcOEt 1:4 was also subjected to reversed-phase HPLC (MeOH/H_2O 0:1 → 1:0), leading to cladiellaperoxide (**3**; 5.8 mg, colorless oil), (6*E*)-2α,9α-epoxyeunicella-6,11(12)-dien-3β-ol (**4**; 7.9 mg, white amorphous solid), and polyanthellin A (**5**; 8 mg, white amorphous solid).

(+)-Cladidiol (=*(1S,4S,6Z,8R,9S)-1-Methyl-8-(1-methylethyl)bicyclo[5.4.0]undec-6-ene-4,9-diol*; **2**). Colorless oil. R_f 0.56 (hexane/AcOEt 7:3). $[\alpha]_D^{20} = 112$ ($c = 0.64$, $CHCl_3$). UV (MeOH): 203. IR ($CHCl_3$): 3410 (OH), 2904 (CH), 1602 (C=C). ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-EI-MS: 238.1936 (M^+, $C_{15}H_{26}O_2^+$, calc. 238.1933), 220.1816 ($[M - H_2O]^+$; calc. 220.1827), 202.1709 ($[M - 2\ H_2O]^+$; calc. 202.1721), 188.1571 ($C_{14}H_{19}^+$; calc. 188.1565), 43.0552 ($C_3H_7^+$; calc. 43.0548).

Acetylcholinesterase (*AChE*) *Inhibition*. For this bioassay, AChE (0.04 U/ml) and acetylthiocholine iodide (ATC; 75 mM) were dissolved in 0.1M phosphate buffer (pH 8). 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) was made up in 10 ml of 0.1M phosphate buffer (pH 7.0), containing 15 mg of $NaHCO_3$. Compound **2** was dissolved in i-PrOH at a conc. of 5% (*v*/*v*). A control probe of similar conc. was also prepared. The colorimetric method was used to determine the inhibition of AChE [18]. Solns. of **2** (50 µl) and AChE (0.5 ml) were mixed in a test tube. DTNB (100 µl) and Buffer (2.4 ml) were added, and the tube was incubated at 25° for 5 min (pre-incubation). The addition of ATC (40 µl) initiated the reaction, and the mixture was again incubated at 25° for 20 min. The absorbance at 412 nm was measured spectrophotometerically, and the data were corrected for the blanks (nonenzymatic hydrolysis). This assay was performed in triplicate.

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