M. H. Haroon[1, 2] and H. Ranjith W. Dharmaratne[1, 3]

New brominated sesquiterpenes from the red alga Laurencia hetroclada Harvey, and their immunosuppressive activity studies

(1) Natural Products Programme, Institute of Fundamental Studies, Kandy, Sri Lanka.

(2) Faculty of Applied Sciences, South Eastern University of Sri Lanka, Sammanthuri, Sri Lanka. (email: haroonmh@fas.seu.ac.lk)

(3) Present Address: National Center for Natural Products Research, School of Pharmacy, University of Mississippi, University, MS 38677, USA

Abstract: Red algae of the genus *Laurencia* Harvey (Ceramiales, Rhodomelaceae) is a complex genus, encompassing a large variety of morphologically-complex algal species. In continuation of our work on chemistry and biological activity studies of some Sri Lankan seaweeds, we examined the chemistry of MeOH extract of *Laurencia hetroclada* collected from Tangalle coast. Above extract was subjected to column chromatography followed by PTLC gave a new brominated sesquiterpene **1** along with two known compounds, algoane **2,** and caulerpin **3.** The ^{13}C NMR spectrum along with the DEPT experiments of **1** revealed only 15 carbon signals corresponding to four methyls, five methylene, one methine and five quaternary carbons. The ^{1}H NMR (CD_3OD, 500 MHz) spectrum of **1** showed signals due to three methyls, four methylenes, and two methine protons. The singlets resonated at 1.25 (3H, s), 1.27 (3H, s), and 1.85 (3H, s) were assigned to Me-8', Me-6', and Me-9', respectively. The characteristic downfield proton at 4.83 (dd, J = 4.2,15 Hz) was assigned to H-4, geminal to the Br atom, whereas the proton that resonated at 3.96 (d, J = 1.7 Hz) was assigned to the oxymethine H-1. Furthermore, the olefinic singlet resonated at 5.26 was assigned to Ha-7' and Hb-7. The down field carbon atoms resonated at 110.2 and 165.2 were assigned to olefinic carbon atoms. The positive CI MS of **1** showed the molecular ion peak $[M+H]^+$ at m/z 349 along with isotopic peak at m/z 351 (1:1), indicating the presence of a Br atom in the molecule. The above MS data were found to be consistent with the molecular formula $C_{15}H_{25}O_4Br$ with 3 degrees of unsaturation. A comparison of the NMR spectroscopic data of **1** with those of **2** suggested a similar skeleton with differences in substitution pattern and unsaturation sites. From the above spectral data, the structure of **1** was confirmed as a new natural product 4-bromo-5-methyl-2-(3'-hydroxy-1',3'-dimethyl-2' methylenecyclopentyl) cyclohexane-1,2,5-triol. Caulerpin (**3**) showed a significant dose suppressive effect with an IC_{50} 5.8 ± 1.0 µg/mL on T-cell proliferation assay.

Keyword: Seaweeds, *Laurencia hetroclada*, T-cell proliferation assay

Introduction:

Red algae of the genus *Laurencia* Harvey (Ceramiales, Rhodomelaceae) is a complex genus, encompassing a large variety of morphologically-complex algal species with worldwide distribution and ambiguous taxonomy (Lyakhova EG, Kalinovsky A.I, Dmitrenok A.S, Kolesnikova S.A, Fedorov S.N, Vaskovsky V.E, 2006).

In continuation of our work on the chemistry and biological activity studies of Sri Lankan seaweeds[2], we investigated *Laurencia hetroclada* which was collected in Tangalle of the southern coast of Sri Lanka, and isolated a new brominated sesquiterpenoids, 4-bromo-5-methyl-2-(3'-hydroxy-

1',3'-dimethyl-2'methylenecyclopentyl) cyclohexane-1,2,5-triol (**1**), along with two known compounds, algoane (**2**), caulerpin (**3**).

Phagocytes like neutrophils and macrophages scavenge microbes and other harmful agents entering the body. They start their action by the release of reactive oxygen species (ROS) to destroy invaders in a process collectively known as oxidative burst. T-cells along with other white blood cells (WBC) subset play a major role on fight against infections (Nielsen M, Gerwien J, Geisler C, Ropke C, Svejgaard A, Odum N, 1998). These cells can cause disorders in the immune system due to changes of stimuli.

In this juncture immunosuppressants are required in the case of a hyperactive immune system, probably caused due to inflammation or a transplant rejection. On the other hand immunostimulating drugs are needed when the immune system stops fighting against infections. As a considerable number of naturally occurring diterpenes has shown immunosuppressant activity, diterpenes **1** and **2** along with bisindole alkaloid caulerpin (**3**) were tested for their immunosuppressant activity. The effect of the above compounds on ROS production, by whole blood phagocytes and in vitro activation and proliferation of T-cells were investigated. Chemiluminescence (CL) of these tests revealed that there is no effect of all the tested compounds **1** and **2**. However, in the case of T-cell proliferation, caulerpin (**3**) showed a significant suppressive effect with IC_{50} 5.8 ± 1.0 that is comparable with the widely used immunosuppressive drug prednisolone.

Methodology

Extraction and isolation procedure

The red alga, *Laurencia hetroclada,* was collected at Tangalle. Processed alga was dried, finely powdered and extracted with methanol using an ultrasonicator. The MeOH extract was subjected to column chromatography (CC) on flash Silica gel (230 -400 mess size, E-Merck) using n-hexane, ethyl acetate, methanol and water as eluants. Further purification of some column fractions using PTLC and gravity column yielded three pure compounds, and their structures were proposed using NMR and MS data.

T-Cell proliferation assay:

The T-cell proliferation assay was performed on isolates as described by Nielson *et al* (1996). In short, lymphocytes were isolated by gradient centrifugation of blood in presence of lymphocyte sedimentation medium. Cell suspension (50 µL) (10^6/mL) with or without compounds (3.1-50 µg/mL) was added to each well of a 96 well round bottom tissue culture plate. The T-cell activator PHA (50 µL) was added in each well to a final concentration of 5 µg/mL. The plates were incubated at 37 ^oC for 72 hours (95 % humidity, 5 % CO_2). Methyl-^3H thymidine 0.5 µCi was added in each well and the plate was incubated for further 18 hours. Cells were harvested on Type G-7 filter paper using cell harvester. The radioactivity was measured using a -scintillation counter. The concentration of compound giving 50% of inhibition was calculated, comparing the activity of compound treated cells measure as the level of radioactivity incorporated in the proliferating cells (CPM) with that of the non-treated cells (control cells).

Results and Discussion

Three compounds have been isolated and the structures of three of them were established using spectral data.

4β-bromo-5β-methyl-2-(3'α-hydroxy-1'β,3'β-dimethyl-2'-methylenecyclopentyl)cyclohexane-1β,2α,5 α -triol (**1)**

The ^1H NMR (CD_3OD, 500 MHz) spectrum of **1** showed signals due to three methyls, four methylenes, and two methine protons. The singlets resonated at 1.25 (3H, s), 1.27 (3H, s), and 1.85 (3H, s) were assigned to Me-8', Me-6', and Me-9', respectively. The characteristic downfield proton at 4.83 (dd, J = 4.2,15 Hz) was assigned to H-4, geminal to the Br atom, whereas the proton that resonated at 3.96 (d, J = 1.7 Hz) was assigned to the oxymethine H-1. Furthermore, the olefinic singlet resonated at 5.26 was assigned to Ha-7' and Hb-7'.

The bromine-bearing carbon resonated at 63.0. Four methylene carbons resonated at 34.7 (C-5'), 40.1 (C-3), 40 (C-4'), and 47.9 (C-6). These assignments of methylene protons were supported by the COSY spectrum of **2.** The COSY spectrum showed correlations between Ha - 3 and Hb - 3, between H - 4 and Ha - 3, between H - 4 and Hb - 3, between Hb - 6 and Ha - 6, between Hb - 6 and H - 1, between Ha - 6 and H – 1, and between Hb - 3 and H – 1. Similarly, correlations were observed between Hb - 5' and Ha - 5', between Hb - 5' and Ha - 4', and between Ha - 5' and Hb - 4'. HMBC correlation of C-8' (1.25) with C-2 (78.9), C-1' (53.2), C-5' (34.7), and C-2' (165.5) clearly indicated the connectivity of the two structural units in the molecule (Figure 2). The relative stereochemistry of **1**was deduced by analysis of the coupling constants and NOESY experiments. The large coupling constants between H-4 and H-3a (15 Hz) suggested that H-4 have an axial orientation (-configuration). As no cross-peak could be detected between Me (9') and H-4, and H-1, in the NOESY experiments, it is concluded that the Me group at the C-5 position should be in the -configuration. Further, the orientation of the exomethylene group in the cyclopentane ring was predicted from the strong NOESY correlations between H-1 and exocyclic olefinic protons (Ha/Hb-7'). The CH_3 group at C-3' was due to have a -configuration whereas the CH_3 group at C-1' has a -configuration, as there were no cross-peaks detected between those groups and selected atoms in the NOESY experiments. From the above spectral data, structure of **1** was deduced as 4-bromo-5-methyl-2-(3'-hydroxy-1',3'-dimethyl-2' methylenecyclopentyl) cyclohexane-1,2,5-triol. According to the literature, compound **1** is a new member of the non aromatic cuparane class of secondary metabolites. The structures of known compounds were identified by comparison of their spectroscopic data with the reported data (McPhail KL, Davies-Coleman MT, Copley RCB, Eggleston D.S,1999, Anjaneyulu A.S.R, Prakash C.V.S, Mallavadhani U.V, 1991;, Govenkar, M.B, Wahidulla, S, 2000, Ali MS, Ahmad VU, Mazhar F, Azhar,I, Usmanghani, K, 1999).

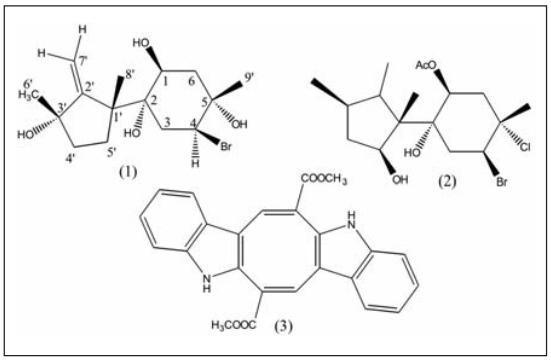

Figure 1: Structures of Compounds 1 - 3.

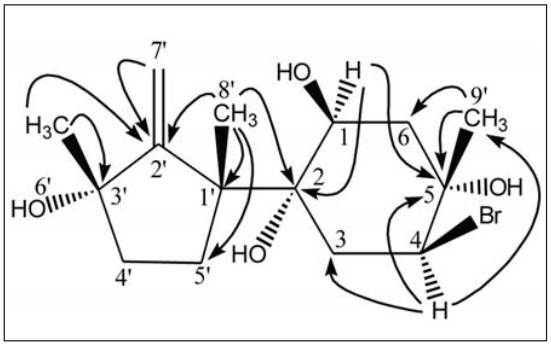

Figure 2: Key HMBC Correlations of 1

T-Cell proliferation assay:

In order to study the immunomodulatory potential of **1, 2** and **3** isolates, their effect on ROS production was analyzed on whole blood phagocytes. In our investigation, no effect on ROS production was noted with compound **1** and **2**, whereas caulerpin (**3)** showed a weak activity with IC_{50} 75.6 ± 3.1 µg/mL. In addition, they were tested for their effect on T-cell proliferation, and only compound **3** showed a significant dose suppressive effect with an IC_{50} 5.8 ± 1.0 µg/mL that is comparable with the positive control prednisolone which is a widely used immunosuppressive drug. Compounds **1** and **2** showed no activity on T-cell proliferation and had the IC_{50} value greater than 50 µg/mL. However, Compound **2** showed a mild suppressive effect at lower concentrations (3.12 and 12.5 µg/mL) when compared with higher concentration 50 µg/mL . This reverse order effect could be attributed to the poor solubility

of **2**. Above observation of the immunosuppressive activity of **3** is in good agreement with the recent report on the anti-inflammatory activity of this bisindole alkaloid **3.**

Algoane (2):

The compound **2** was isolated as a white crystalline solid with a melting point of 190-192 ˚(C. Its specific rotation was found to be +70˚(c = 0.5, MeOH) which indicated the presence of chirality in the molecule. The negative FAB MS of LH-1 showed its molecular ion peaks at *m/z* 488 corresponding to the $[M-H]^+$ together with isotopic peaks at *m/z* 490, and 492 confirmed the presence of halogen atoms in the molecule and the molecular formula was deduced to be $C_{17}H_{27}Br_2ClO_4$ with three degree of unsaturation. The ^1H NMR spectrum of 2 showed signals due to five methyl, three methylene and four methine protons. The ^1H NMR spectrum exhibited five methyl signals at 0.88 (3H, s), 1.0 (3H, s), 1.4 (3H, s), 1.74 (3H, s), and 2.06 (3H, s). Literature review (McPhail etc.,1999) revealed this structure is a non aromatic, cuprane–type sesquiterpene algoane-1, which has been isolated from South African specimen of the sea hare, *Aplysia dactylomela*.

Caulerpin (3):

The compound **3** was isolated as orange red prisms with a melting point of 313-317 ˚(C. The EI MS of **3** showed a strong molecular ion peak at m/*z* 398 and the molecular formula was deduced to be $C_{24}H_{18}N_2O_4$. The ^1H NMR signals between 7.07 and 7.40 indicated the presence of ortho substituted phenyl rings. The protons resonated at 7.28 (1H, d, $J_{6,7}$ = 8 Hz), 7.16 (1H, t, $J_{6,7}$ =, $J_{6,5}$ = 7.5 Hz), 7.07 (1H, t, $J_{5,6} = J_{5,4}$ 7.5 Hz), 7.40 (1H, d, $J_{4,5}$ = 7.9 Hz) were assigned to H-7/17, H-6/18, H-5/19 and H-4/20 respectively. The methoxy groups resonated at 3.88 were assigned to Me-1' and Me-2' while the NH protons resonated at 9.17. The protons resonated at 8.03 were assigned to H-9/H-13. Based on spectral studies (^1H, ^{13}C NMR and MS) and comparison with the literature values the compound was identified as caulerpin which was previously reported from green alga *Caulerpa racemosa* (Govenkar etc., 2000)

Acknowledgment

M.H. Haroon thanks the Third World Academy of Science for Developing World (TWAS), Trieste, Italy, for providing financial support to conduct a part of his Ph.D. research at the H. E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Pakistan.

References

Ali MS, Ahmad V.U, Mazhar F, Azhar,I, Usmanghani K. Some chemical constituents from marine algae of Karachi coast (Arabian Sea). Turkish Journal of Chemistry 1999; 23(2): 181-183

Anjaneyulu ASR, Prakash CVS, Mallavadhani UV. Two Caulerpin Analogs and a Sesquiterpene from Caulerpa racemosa. Phytochemistry 1991; 30(9): 3041-3042

Govenkar MB, Wahidulla S. Constituents of Chondrin armata. Phytochemistry 2000: 54(8): 979-981

Haroon MH, Premaratne SR, Dharmaratne HRW . Chemistry and Biological activity Studies of Ulva lactuca Linnaeus and Amphiroa anceps. Chemtech, Colombo, Sri Lanka, 2007, July 20-23, Poster 30, pp 79

Lyakhova EG, Kalinovsky AI, Dmitrenok AS, Kolesnikova SA, Fedorov SN, Vaskovsky VE. Structures and absolute stereochemistry of nipponallene and neonipponallene, new brominated allenes from the red alga Laurencia nipponica. Tetrahedron Letters 2006; 47(37): 6549-6552

McPhail KL, Davies-Coleman MT, Copley RCB, Eggleston DS. New halogenated sesquiterpenes from South African specimens of the circumtropical sea hare Aplysia dactylomela. Journal of Natural Products 1999; 62(12): 1618-1623

Nielsen M, Gerwien J, Geisler C, Ropke C, Svejgaard A, Odum N. MHC Class II Ligation Induces CD58 (LFA-3)-Mediated Adhesion in Human T Cells, Exp. Clin. Immunogenet 1998; 15: 61-68

U.L. Zainudeen[1] and M.A. Careem[2]

A Cyclic Voltammetry Study on Bi Layer and Multi Layer Conducting Polymer Films

(1) Department of Physical Sciences, South Eastern University of Sri Lanka, Sammanthurai, Sri Lanka. ((email: ulzdeen@seu.ac.lk)

(2) Centre of Ionics, University of Malaya, 50603 Kuala Lumbur, Malaysia.

Abstract: Polypyrrole (PPY) is a prime candidate for a polymer actuator; but has the disadvantage that the electronic conductivity decreases by two or three orders of magnitude as the polymer is reduced. This reduces the actuator performance. A Standard way of overcoming this problem is to add Au or Pt layers as thin patterns or as helices on the polymer. The present work is an attempt to use a second more highly conducting polymer, poly(3,4-ethylenedioxythiophene), (PEDOT), to enhance the electronic conductivity.

Bilayer and multilayers with PEDOT and PPY, each containing dodecyl benzenesulfonate (DBS) as immobile dopant species were synthesized and their electrochemical behaviour was investigated using cyclic voltammetry, optical absorption spectroscopy and electrochemical quartz crystal microbalance (EQCM) techniques. Bilayer results show combined characteristics of each individual polymer. In trilayer films, the reduction of inner and outer PPY layers takes place at two different potentials. The oxidation occurs at one potential only. The pentalayer film confirmed this: three reduction peaks were observed. The separation between the two reduction peaks depends on the thickness of PEDOT layer, the scan rate and the concentration of cycling electrolyte. This separation becomes smaller with the number of cycling, indicating the enhancement of ion diffusion through the PEDOT layer. The optical spectra of the trilayers show clearly distinguishable peaks belonging to PEDOT and PPY polymers, confirming the independent behaviour of the PEDOT in the system. Two important conclusions of relevance for actuator performance were reached: it is possible to make bilayer and multilayer films that do not delaminate – the two polymers are compatible; and both polymers are active in the redox process. Ionic diffusion in the trilayer which is responsible for the actuation can be controlled by choosing appropriate thicknesses for the three layers.

Keywords: Polypyrrole, PEDOT, actuators, EQCM, cyclicvoltammetry

Introduction

In recent years, poly(3,4-ethylene-dioxythiophene) (PEDOT) has emerged as one of the most important materials in the field of conducting polymers. PEDOT is highly insoluble in almost every solvent, exhibits quite a high conductivity, changes in colour depending on the applied potential, is practically transparent in the form of thin oxidized film, is very stable in the doped state and exhibits a low reduced band gap (ca. 1.6-1.7 eV) [1]. All these properties make this conducting polymer useful for many applications. On the other hand, polypyrrole (PPy) has been used as a prime candidate in the field of polymer actuators since it shows great volume changes during the redox cycling. But PPy has the disadvantage that the electronic conductivity decreases by two or three orders of magnitude as the polymer is reduced. This causes a decrease in actuator performance; since only a small part of the polypyrrole film will then be actively contributing to the actuation [2]. A standard solution to this problem involves the addition of an extra electron conductor on the surface of the polypyrrole film, e.g. Au or Pt as helices or as thin patterns [3,4]. The use of a corrugated gold layer on PPy improved the performance of the actuator [5].