Chapter 7
SPECTROSCOPIC AND MOLECULAR CHARACTERIZATION
OF POTENT ACTIVE PRINCIPLES SPONGES

Introduction

Bioactive substances derived from living organisms have found extensive use in the treatment of various diseases. The advent of biotechnology as a powerful and applied branch of biological sciences has accelerated the momentum of search for novel bioactive substances. Among the marine natural organisms, sponges have provided the greatest number of marine natural products and have attracted a considerable synthetic attention.

Over the past 30 years there has been a marked increase in the number of MNPs reported annually. During the period 1996-2000 there was, however, a decline in the number of new compounds were reported and the same trend still persists. Sponges continue to dominate as a source of novel compounds than the other marine sources (38% of the total marine bioactive substances are derived from sponges). Sponges with their chemical defence mechanisms, are one of the most studied organisms for the isolation of NPAs (Thakur and Anil, 2000). The constant threat from competitors, by way of over growth, poisoning, infection or predation has armed sponges with a store house of potent chemical defense agents (Bakus et. al., 1974; Thakur and Muller, 2004). Sponges produce a wide array of secondary metabolites ranging from derivatives of amino acids and nucleosides to macrolides, porphyrins, terpenoids, aliphatic cyclic peroxides and sterols (Faulkner 1993). Also, terpenoids isolated from Acanthella cavernosa
and analogues of the sesquiterpene avaron from *Dysidea avara* are reported to be promising NPAs on account of the low EC50 (<1μg/ml) values against barnacle cyprids.

Lot of published reviews showed the importance of sponges as potential source of pharmaceutical leads (Nakao *et al.*, 2004). Research on active compound isolation and structural determination are comparatively scanty. Since the functional aspects of a compound is in close relation with it's chemical structure, the determination of the chemical structure of the compounds is of utmost importance. Most of the new drugs have been chemical synthesized whose base structure is based on the compounds derived naturally. In the current study, various bioactive and pharmacological potentials of the sponges namely, *H.exigua, Dendrilla nigra, Axinella donnani, Callyspongia sp.* and *Sigmoidocia* were determined. Sponges belonging to the genus *Haliclona* have been a good source for several alkaloids exhibiting diverse pharmacological properties. These include the alkylpyridines from different *Haliclona* sp, ceramides from *H. koremella*, a hexapeptide from *Haliclona* sp etc. The latter two compounds were good inhibitors of the growth and settlement of the green algae *Ulva conglobata* and blue mussel *Mytilus edulis* respectively. In addition, several novel alkaloids have been reported from the species *Haliclona exigua* (Venkateswarlu *et al.*, 2001; Rao *et al.*, 2004) and *H. cribricultis*. In the present study an attempt has been made to purify and also partially characterize the active metabolites of the metabolic extract of the marine sponge, *Haliclona exigua*, which shows more potency in the pharmacological studies.
Materials and Methods

Collection, Extraction and Purification

*H. exigua* was collected and extract based on the earlier method used in (Chapter I) was the condensed extract fractionated into Petroleum ether (Fr. 1), Ethyl acetate, (Fr.2) and Aqueous (Fr. 3) fractions in the usual way. The active compounds were finally purified from the above fraction using Gel Permeation Chromatography (Sephadex LH-20, 30 X 5 cm, CHCl3: MeOH 1:1) followed by HPLC (ODS, MeOH: H2O gradient elution, 2 ml/min).

The structure elucidation of the active fraction was carried out using various spectroscopic techniques such as IR, UV-Vis, NMR and HRMS. NMR spectra were recorded in CDCl3 solution in a BRUKER Avance 300 MHz NMR spectrometer, while HRMS analysis was performed in an Applied Biosystem instrument (QTOF). All solvents were procured from SD. Fine Chem Limited (AR Grade) and used as such. Based on the carbon signals received from NMR, the structure of active principles were predicted using bio-informatic tools.

Results

Identification of Secondary Metabolites

1H and 13C NMR spectral data of the active Fr.2-3 indicated it to be related to bis-1- oxaquinolizidines, already reported from this species, while HRMS studies indicated it to be a mixture of at least 5 compounds. Thus, its 1H NMR spectrum had strong signals in the region δ1.0-2.3, indicative of several methylene groups (Fig-10). The signals in the region δ 3.0-4.0 were indicative of the presence of CHOH and CH-N moieties. Its 13C NMR and DEPT.135 spectra (Fig-11) had multiple signals in the region δ 20-45 (t, CH2s), δ 50-55(t,
CH₂'s linked to N atoms), δ 70-95(d, CH linked to O and N atoms). The strong
signals at δ 53.4 (t) and 53.2 (t) could be due to the C-4 and C-6. The peaks at δ
77.3(d), 76.8(d), 76.6(d) and 75.9(d) are expected to be from the C-2 (of the 1-
oxaquinolizidine rings) and other oxygenated carbons. HRMS data revealed the
presence of 5 major compounds (Fig-10), i.e. nor-Araguspongine C (m/z:
465.3686, C₂₇H₄₉N₂O₄); Araguspongine C (m/z: 479.3912, C₂₈H₅₁N₂O₄);
dihydroxy Araguspongine (533.3535, C₂₈H₅₀N₂O₆Na) and mono & dimethyl
derivatives of the latter (547.3781, C₂₉H₅₂N₂O₆Na & 561.3960, C₃₀H₅₄N₂O₆Na).
Absence of vinyl proton signals in the ¹H NMR spectrum and the observed
double bond equivalence of five, as revealed by the elemental compositions of
all above compounds also confirmed the araguspongine-type molecular
structures.

Discussion

Sponges have provided the greatest number of new marine natural
products and have attracted considerable synthetic attention. An acetylated
tetrahydroxy ceramide was isolated from an acetylated extract of Fasciospongia
cavernosa collected on the south east coast of India. Three sulfated ceramides,
calyceramides A-C with neuraminidase inhibition activity were obtained from a
Japanese collection in Discodermia calyx. A sponge of the genus Calyx,
collected in Sulawesi, Indonesia, yielded a ketosphingolipid, calyxoside with
DNA damaging properties. The keto substitution of the calyxoside was located
by reductive amination of a penta-acetate derivative and analysis of MS
fragmentation, while the relative and absolute stereochemistry was proposed
from the CD analysis of the perbenzoyl aglycone. Three glycosphingolipids
were obtained from *Aplysinella rhax* collected in New Caledonia. A presumably new species of *Haliclona* from Queensland contained four unsaturated aminoalcohols with antifungal properties. Plakoside A was synthesized and found to have optical rotation and spectroscopic data identical to plakoside A from *Plakortis simplex*. The spectroscopic data were also identical to the previously synthesized diastereomer; the absolute stereochemistry of the cyclopropyl groups remains unknown.

Three furan- containing fatty acid derivatives, plakorsins A-C, and an epoxide, plakortic acid were isolated from Thaiwanese *P. simplex* specimens. The sponge *Spirastrella abata* collected from Korean waters yielded four phosphatidyl cholines, which showed an inhibitory effect on the biosynthesis of cholesterol. *Callyspongia sp. fallax* collected in the Carribbean was found to contain the methoxylated acids. Two antimicrobial lysoplasmanylinositols were isolated from a Japanese *Theonella swinhoei*. Halicholactone from *Halichondria okadai* was synthesized stereoselectively using chiral (diene)- Fe(CO)₃ complexes. Three dithiocyanates, thiocyanatins A, B and C, were isolated from an *Oceanapia* species collected from South West Australia. These compounds have nematocidal activity and their structures were confirmed by synthesis. *Acarnus bicladotylota* collected from the South West coast of India yielded the acetylenic cycloperoxides, peroxyacarnoic acids C and D which were isolated as their methyl esters. A further series of cytotoxic polyacetylenic alcohols, have been isolated from a Korean *Petrosia* species that has previously yielded similar compounds. The absolute stereo chemistry of (-)-adociacetylene B from *Adocia*
sp. was confirmed from a synthesis of both (+) and (-) isomers employing enzymatic resolution.

The weakly cytotoxic heptapeptide, wainunuamide was isolated from *Stylotella aurantium* collected in Fiji. The total synthesis of cis, cis-ceratospongamide from the red alga *Ceratodictyon spongiosum* and symbiotic sponge *Sigmadocia symbiotica* has been reported. Hymenamide C from *Hymeniacidon* sp. has been synthesised using solid support methodology. A total synthesis of phakellistatin 11, isolated from *Phakelia* sp., revealed that the synthetic product is much less cytotoxic than the originally isolated sample.

The structures of two potent inflammatory peptides, halipeptin A and B, isolated from a member of the genus *Haliclona* from Vanuatu, were from the member of the genus Halilcona from Vauatu were proposed from an analysis of spectroscopic data. Two iron chelating peptides, haliclomamide A and B, isolated from a Haliclona species collected in Palau were proposed to have structures with furan and benzene ring respectively on the basis of spectroscopic analysis.

A potent, neurologically-active aminoacid, neodysiherbaine A has been reported as a minor metabolite of a Micronesian Dysidea. The relative and absolute stereo-chemistries were determined by asymmetric total synthesis. The enantioselective synthesis of both (+)- and (-)- dysibetaine, isolated from *D.herbacea*, has established the absolute configuration as *(S,S)*. Two polychlorinated thiazoles were isolated from Queensland specimens of *D.herbacea*. From the same collection, several polychlorinated dipeptides were reported separately; the absolute stereochemistries were determined by
comparision of optical rotation data. The methyl esters are considered to be artifacts of methanolic extraction. An undescribed species of Dysidea collected in the Philippines yielded the praline-derived dysideaprolines A-F together with the enol-ether containing barbaleucamides A and B.

Two antifungal bromopyrroles, 3-bromomaleimde and 3-4 dibromomaleimide were found on Axinella brevistyla collected in Japan. (-)-Haliclorensin, isolated from Haliclona tulearensis, and assigned the structure, was synthesized by two independent groups and found to be spectroscopically non-identical with the natural product. Subsequently, a re-isolation and re-investigation of the spectroscopic data lead to a revise structure that was confirmed by enantioselective synthesis of both enantiomers. Both enantiomers of stellettadine A from Stelleta sp. were synthesized from (S)- and (R)-citronellal. The (S) isomer was found to have a negative rotation similar to the natural compound which had previously been assigned as (R). (-)-Stellattimide B, originally reported from a Stelletta species with a (6”S) configuration, has now been established as (1S, 4S, 8Ar, 6” R) by total synthesis. (+)-Batzelladine F, originally isolated from Batzella sp., recently re-assigned as Monanchora arbuscula, was originally assigned structure. The structure has been revised on the basis of the enantioselective synthesis of both the revised and putative structures. Mirabilin G was isolated from a South Australian Clathria species.

The Palauan sponge Protophlitaspongia aga yielded 3, 4, 5, 6-tetrahydro-6-hydorxyl methyl-3, 6, dimethyl pyrimidine-4-carboxylic acid that was found to inhibit the settling of larvae of the barnacle Balanus amphitrite. The wondonins A and B were isolated from an association of Poecillastra
wondoensis and a Japsis species from Korea. Naamine B from Leucetta chagosensis has been synthesized.

The sponge H. exigua is a rich source for several bis-1-oxaquinolizidine alkaloids, exhibiting diverse biological properties such as cytotoxic, antifungal, antimalarial, antituberculosis and anti-rat brain nitric oxide synthase activities (Venkateswarlu et al., 2004; Rao et al., 2004). Araguspongine C, belonging to the bis-1-oxaquinolizidine type of alkaloids was first isolated from H. exigua. A number of pharmacological activities have been reported for this compound. However, no reports are available on antifouling activity by this class of compounds. In the present study, a fraction rich in bis-1-oxaquinolizidine alkaloids was found to be active against fouling bacterial strains as well as barnacle cyprids, for the first time.

As several araguspongine analogs have already been successfully synthesized, their use in antifouling paint industry may not be a distant dream. It is obvious from the present studies that the bis-1-oxaquinolizidines in Fr.2-3 significantly inhibited the fish larval growth(Ichthyotoxicity), mosquito larva growth in effective concentration. Apart from this, H. exigua also have some influence over A. donnani, Sigmadosia, Callyspongia, and D. nigra. These may be due to bis-1-oxaquinolizidine alkaloids in the sample.

It was concluded that the bis-1-oxaquinolizidine compound has least toxicity than other sponges and has a vast bioactive and pharmacological potential. This compound may be synthesized and utilized as bio-medicine.
Fig-10 HRMS spectra of Fr 2-3
Fig-11 $^{13}$C NMR and spectra of Fr 2-3
Fig-12 $^1$H NMR of Fr 2-3.
Fig-13 Structure of Araguspongine C