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"Exploring the chemical diversity in marine organisms: new molecules for pharmaceutical applications"

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INTRODUCTION: *Marine products chemistry*

It has long been recognized that natural product structures have the characteristics of high chemical diversity, biochemical specificity and other molecular properties that make them favourable as lead structures for drug discovery. These qualities make them different from libraries of synthetic and combinatorial compounds.[1]

Moreover, the chemical diversity that characterizes the natural molecules makes the exploration of their biological properties not only a major source of new compounds that could be used for the production of drugs, but also a useful tool for the discovery of new mechanisms of action. For these reasons, the chemistry of natural substances has been making significant progress in recent decades.

In addition, the growing interest in natural molecules has been also favoured by the development of modern biochemical techniques and genetics, the advent of new techniques for purification and structural determination as well as a series of biological assays capable of highlighting the nature and the possible drug activity.

The ocean, which hosts approximately 87% of the Earth's life, offers huge potential for the discovery of pharmaceutical products. The vast ocean, which has an area of about 360 million km^2, possesses incredible resources of novel compounds for investigation by natural product chemists, playing a leading role in drug discovery.

Unlike terrestrial organisms, marine organisms have to adapt to extreme environmental conditions such as high pressure, high salt concentration, low nutrient concentration, low but steady temperature (except the high temperature near underwater volcanoes and the extremely low temperature in polar regions), limited sunlight, and low oxygen content. To acclimatize to these conditions, marine organisms possess unique characteristics that differentiate them from terrestrial organisms in many aspects, such as metabolism, behaviour, information transfer, and adaptation strategy. These differences are responsible for the diversity in the secondary metabolism of marine organisms.[2]

Moreover, the coexistence of an enormous number of species that interact each other and with the environment in different ways has resulted in organisms which produce chemically diverse compounds with a wide variety of possible ecological roles. These include -but are not limited to-: a) toxins, which can reduce predation , larval settlement and overgrowth by neighbouring organisms; b) compounds reducing palatability or nutrient uptake in predators; and c) compounds which direct larval settlement and reproduction. Thus, among marine organisms, the chance of finding bioactive compounds is remarkably higher because many of these compounds are involved in their chemical defence, which is essential for the survival of sessile organisms, often lacking any physical defence from their predators.[3]

The isolation of new substances from marine environment, which often exhibit unusual and complex molecular architecture, never identified in terrestrial organisms, seems to suggest the existence of a separate "Chemistry of the Sea." The diversity of organisms in the marine environment has inspired researchers for many years to identify novel marine natural products that could eventually be developed into therapeutics. By 1974, two marine-derived natural products (cytarabine, Ara-C and vidarabine, Ara-A) were part of the pharmacopeia used to treat human disease.[4]

From 1984 to 2002, the study of marine natural products and the ever-increasing number of new identified metabolites has been well documented in the annual reviews by Faulkner,[5,6] that is presently continued by Blunt and co-workers.[7,8] These reviews provide statistics on new compounds of marine origin broadly grouped by the organisms from which they are isolated, while giving details of reported biological activities. For the year of 2008 alone, marine natural product research resulted in the isolation of 1065 new compounds whereas in 2009, 13 marine natural products were in human clinical trials expecting to be approved as therapeutic agents.[8,9] Much of the research into marine natural products is focused on finding and assessing compounds with exploitable biological activities, for example those with antitumor, antibiotic (as applied to many forms of life) and bio-modulating properties. An excellent

annual review series on the pharmacology of these compounds has been published by Mayer and co-workers for the last 11 years.[10,11]

There are currently three Food and Drug Administration (FDA)-approved drugs in the US Pharmacopeia, namely cytarabine (Cytosar-U®, Depocyt®), vidarabine (Vira-A®) and ziconotide (Prialt®). Currently, trabectedin (Yondelis®) has been approved by the European Agency for the Evaluation of Medicinal Products (EMEA), and is completing key Phase III studies in the US for approval (Figure 1). Concomitantly numerous other marine natural products or derivatives thereof are in different phases of clinical trials (Table 1).

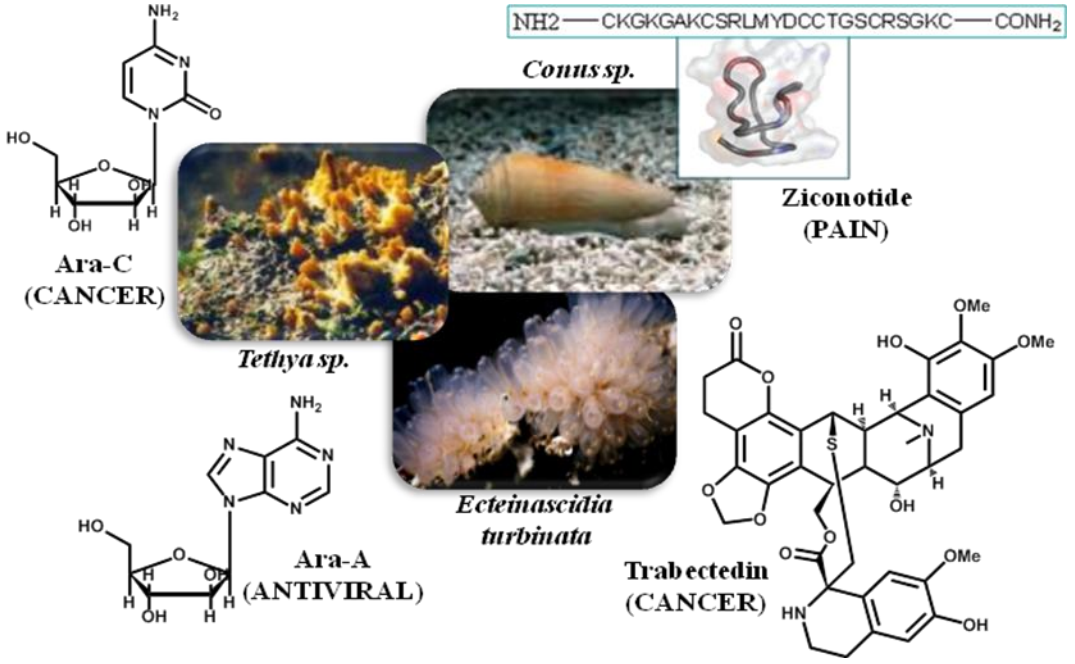


Figure 1: Marine natural products or derivatives thereof approved for use by the FDA or EMEA, their biological source, chemical structures and treatment usage.

Status	Compound name	Trademark	Marine organism	Chemical class	Disease area
Approved	Cytarabine, Ara-C Vidarabine, Ara-A Ziconotide Trabectedin (ET-743) (EU Registered only)	Cytosar-U® Vira- A® Prialt® Yondelis®	Sponge Sponge Cone snail Tunicate	Nucleoside Nucleoside Peptide Alkaloid	Cancer Antiviral Pain Cancer
Phase III	Eribulin Mesylate(E7389) Soblidotin (TZT 1027)	NA NA	Sponge Bacterium	Macrolide Peptide	Cancer Cancer
Phase II	DMXBA (GTS-21) Plinabulin (NPI-2358) Plitidepsin Elisidepsin PM1004 Tasidotin (ILX-651) Pseudopterosins	NA NA Aplidin® Irvalec® Zalypsis® NA NA	Worm Fungus Tunicate Mollusc Nudibranch Bacterium Soft coral	Alkaloid Diketopiperazine Depsipeptide Depsipeptide Alkaloid Peptide Diterpene glycoside	Cognition Schizophrenia Cancer Cancer Cancer Cancer Cancer Wound healing
Phase I	Bryostatin 1 Hemiasterlin (E7974) Marizomib(Salinosporamide A; NPI-0052)	NA NA NA	Bryozoa Sponge Bacterium	Polyketide Tripeptide Beta-lactone-gamma lactam	Cancer Cancer Cancer

Table 1: The odyssey of marine pharmaceuticals: a current pipeline perspective (Alejandro M.S. Mayer *et al*., TRENDS in *Pharmaceuical Sciences 31*, **2010**, 255-265).

Aim of the work

It is increasingly recognized that the oceans preserve a huge number of natural products and novel chemical entities, with biological activities that may be useful in the quest for finding drugs with greater efficacy and specificity for the treatment of many human diseases.[12,13]

In this light, the aim of my project was to isolate and characterize novel molecules from marine organisms with regard to the identification of new "lead compounds" for pharmaceutical applications. The organisms considered for this study were selected by using two different strategies. The first one was based on enhancement of the taxonomic diversity. In this process, an emphasis was placed on collecting specimens related to - but differing from - those known to contain bioactive natural products. The second approach was to evaluate ecological factors such as costumer pressure, growth form (e.g. thin encrusting), level of resource competition, presence or absence of biofouling, etc., and relate this to the expression of the secondary metabolism. Some invasive species have chemical defences, which may enhance their invasion success, so as many marine organisms are soft bodied and have a sedentary life style necessitating chemical means of defence. Therefore, they have evolved the ability to synthesize or to obtain from marine microorganisms bioactive compounds that help them in deterring predators, keep competitors at bay or paralyze their prey.

The work presented in this thesis can be divided in two parts. The first includes Chapters 1 and 2, dealing with the chemical studies of species known to be rich in bioactive natural compounds. The second part including Chapter 3 and 4 is based on the chemical investigation of organisms selected by ecological observations.

Each Chapter is dedicated to a different phylum. In particular,

- Chapter 1 reports the results of the chemical analysis of two organisms of the phylum Cnidaria, the Antarctic soft coral *Alcyonium antarcticum* and the Mediterranean sea anemone *Parazoanthus axinellae*;
- Chapter 2 describes the chemical study of the Mediterranean ascidian *Pseudodistoma crucigaster*, belonging to the phylum Chordata;
- Chapter 3 deals with the chemical investigation of a member of the phylum Porifera, the Mediterranean sponge *Haliclona fulva*;
- Chapter 4 is dedicated to the phylum Mollusca and includes the chemical studies of opisthobranchs *Placida dendritica* and *Aldisa andersoni* from the Indian Ocean and of the nudibranch *Peltodoris atromaculata* from the Mediterranean Sea.

The research work has been conducted at the Institute of Biomolecular Chemistry (ICB) of CNR, Pozzuoli, Naples, and for a limited period of three months at the University of Athens. The biological material has been collected by marine biologists of the ICB research group in the frame of distinct collection campaigns by scuba

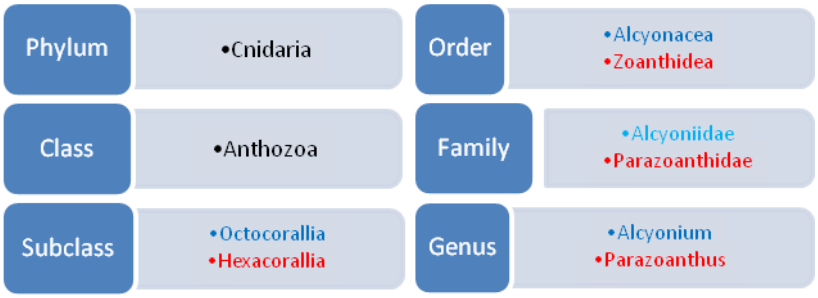
diving. The lipophilic extracts obtained have been fractionated by utilizing chromatographic techniques whereas the structure elucidation of pure compounds has been carried out by an extensive use of spectroscopic methods.

CHAPTER 1: *Phylum Cnidaria*

Cnidaria is a phylum containing over 9,000 species of animals found exclusively in aquatic and mostly marine environments.

The phylum is classified into four main classes: 1) Anthozoa, including sea anemones, corals, sea pens; 2) Scyphozoa, containing jellyfishes; 3) Cubozoa, that comprises box jellies; 4) and Hydrozoa, a diverse group that embraces all the freshwater cnidarians as well as many marine forms.

The class Anthozoa is in turn subdivided into two subclasses Octocorallia and Hexacorallia, encompassing corals (e.g. *Alcyonium antarcticum*) and sea anemones (e.g. *Parazoanthus axinellae*), respectively.



1.1. *Alcyonium antarcticum*

Alcyonium antarcticum is a soft coral belonging to the order Alcyonacea reported from Sub Antarctic and Antarctic zones. The Antarctic benthic community has been regarded with major interest only recently, due to the interest of the scientists. In spite of the low temperature, the pronounced seasonality and limitation of food reserves, the Antarctic ecosystem appears very rich and stable. To date, there are only about 300 natural products (excluding fatty acids and sterols) described from Antarctic marine organisms, many of which are not found in congeners from temperate and tropical regions.

Cnidarians represent an ecologically important group in Antarctic benthic community and they are recognized to be rich in natural products with interesting biological properties.[14] There are about 270 species of Antarctic cnidarians described, but until now only eight of them have been studied. The most studied Antarctic cnidarians belong to the group of the soft corals (Order Alcyonacea) and include *Clavularia frankliniana*, *Alcyonium paessleri* and *Gersemia antarctica*.[15] These three species are chemically defended,[16] although they have structural skeletal elements (sclerites). In fact, chemo-ecological experiments showed that extracted tissues are not ichthyo-deterrent compared to non-extracted tissues, suggesting that sclerites have no apparent effect in deterring potential predatory fish.

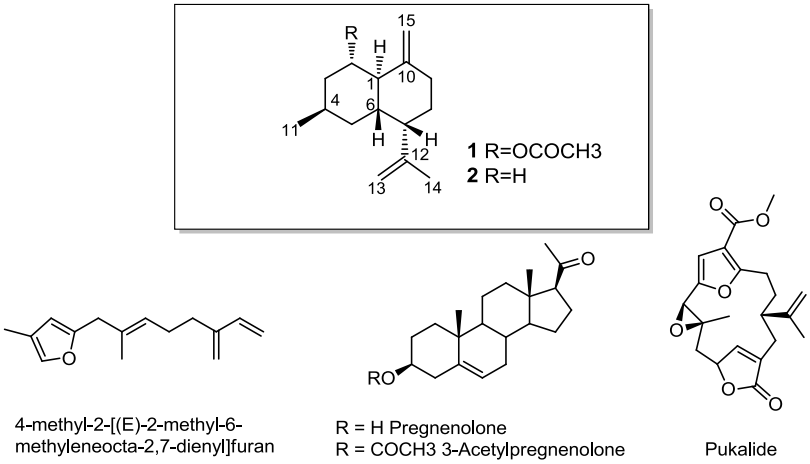
This indicates that chemical compounds, removed during the organic extraction process, are responsible for predator deterrence. Organic extracts of *Alcyonium paessleri* and *Gersemia antarctica* have also been found to possess antifouling and antimicrobial activities.

Chemical studies on soft corals of the genus *Alcyonium* demonstrated that they are especially rich in terpenes and steroids.[17] Recently, the investigation of the lipophilic extract of the soft coral *Alcyonium grandis* led to the isolation of nine new sesquiterpenes belonging to the chemical class of illudalanes, which showed strong ichthyotoxic activity against predators.[17b] Cytotoxic[18,19] and antispasmotic[20] activities have been reported for alcyopterosins, illudalane sesquiterpenes isolated from the sub-Antarctic deep sea soft coral *Alcyonium paessleri*.[18] Moreover, interesting DNA-binding properties have been described for alcyopterosins and their synthetic analogues.[21,22]

In addition, in 2004, Mellado *et al.* have found in the Antarctic octocoral *Anthomastus bathyproctus* polyoxygenated steroids exhibiting cytotoxic activity against some human tumour cell lines.[23]

The work here described is the first chemical investigation of the soft coral *A. antartictum*, collected in Terra Nova Bay Antarctica and has resulted in the isolation of two new bicyclic sesquiterpenes, alcyonicene (**1**) and deacetoxy-alcyonicene (**2**),[24] along with three other known compounds, 4-methyl-2-[(*E*)-2-methyl-6-methyleneocta-

2,7-dienil]-furan[25], pregnenolone and pregnenolone acetate[26], and pukalide[27].



The new compounds **1** and **2** exhibit the rare bulgarane skeleton previously described only for metabolites of essential oils from *Mentha piperita*[28] and *Juniperus oxycedrus*.[29]

1.1.1. Isolation procedure

The frozen soft coral *A. antarcticum* (dry weight, 112 g), collected in January 2002 during the XVII Italian Campaign in Antarctica off Terra Nova Bay (Stazione M. Zucchelli), was chopped and then extracted exhaustively with Me_2CO (400 mL x 4) using ultrasound.

After removing the organic solvent under reduced pressure, the aqueous residue was subsequently extracted with Et_2O (200 mL x 4) to obtain an oily residue of 3.8 g. The ethereal extract was submitted to the first fractionation step, by silica-gel column chromatography, to give five fractions: fr. I (550 mg), fr. II (20 mg), fr. III (890 mg), fr. IV (810 mg), and fr. V (400 mg). These fractions were subsequently purified as described in Table 2 to obtain the pure compounds.

The known metabolites were identified by comparison of their NMR and mass spectral data with those reported in the literature.[25-27] The structures of compounds **1** and **2**, which exhibited a rare bulgarane skeleton[30], never described from the marine environment, were determined by an extensive use of spectroscopic methods.

Table 2: Purification procedures of the various fractions.

Fraction	Method	Compound
I	Silica gel column purification (light petroleum/diethyl ether gradient)	4-methyl-2-[(*E*)-2-methyl-6-methyleneocta-2,7-dienyl]-furan (50 mg) deacetoxy-alcyonicene (**2**, 0.5 mg)
II	HPLC *n*-phase (*n*-hexane/EtOAc, 95:5, Kromasil analytical column, flow rate 1 mL/min)	alcyonicene (**1**, 6 mg)
III	Silica-gel column chromatography (light petroleum/diethyl ether gradient)	pregnenolone-3-acetate (35 mg)
IV	Sephadex LH-20 C/M 1:1	pregnenolone (1.0 mg)
V	Silica-gel column chromatography (light petroleum/diethyl ether gradient, and $CHCl_3$/MeOH 8:2)	pukalide (7.0 mg)

1.1.2. Structure determination

The molecular formula $C_{17}H_{26}O_2$ of compound **1**, named alcyonicene, was established by the analysis of the sodiated molecular peak at *m/z* 285.1824 $[M+Na]^+$, obtained from the HRESIMS spectrum of the sample. This molecular formula indicated five degrees of unsaturations.

Analysis of the ^1H NMR spectrum of **1** (Figure 2), showed a series of methyl signals, at high-field region, that were consistent with the presence of a terpenoid compound.

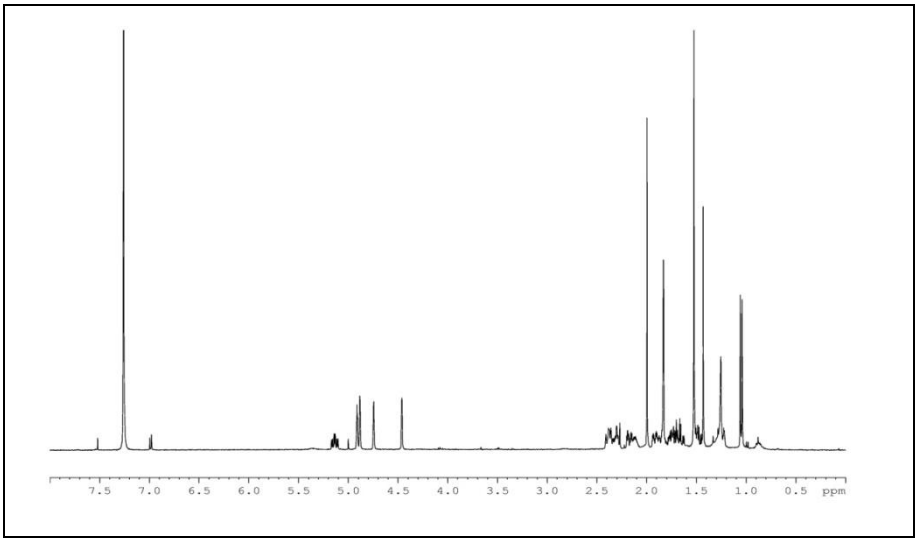


Figure 2: ^{1}H NMR spectrum of alcyonicene **(1)**

In particular, two signals at δ 1.82 (br s, H_3-14) and 1.05 (d, *J* = 7.3 Hz, H_3-11), each integrating for three protons, were attributed to a vinyl methyl and to a secondary sp^3 methyl, respectively. In addition, the low-field region of the ^{1}H NMR spectrum showed a 1H multiplet at δ 5.15 (ddd, *J* = 4.7, 10.8, and 10.8 Hz, H-2) that was attributed to a proton linked to an oxygenated carbon, as confirmed by its HSQC correlation at δ_C 70.2. Moreover, the spectrum contained four 1H broad singlets at δ 4.91 (H-13a), 4.89 (H-13b), 4.75 (H-15a), and 4.46 (H-15b) that suggested the presence of two exomethylene groups.

The ^{13}C NMR spectrum of **1** (Figure 3) disclosed five sp^2 and twelve sp^3 carbons.

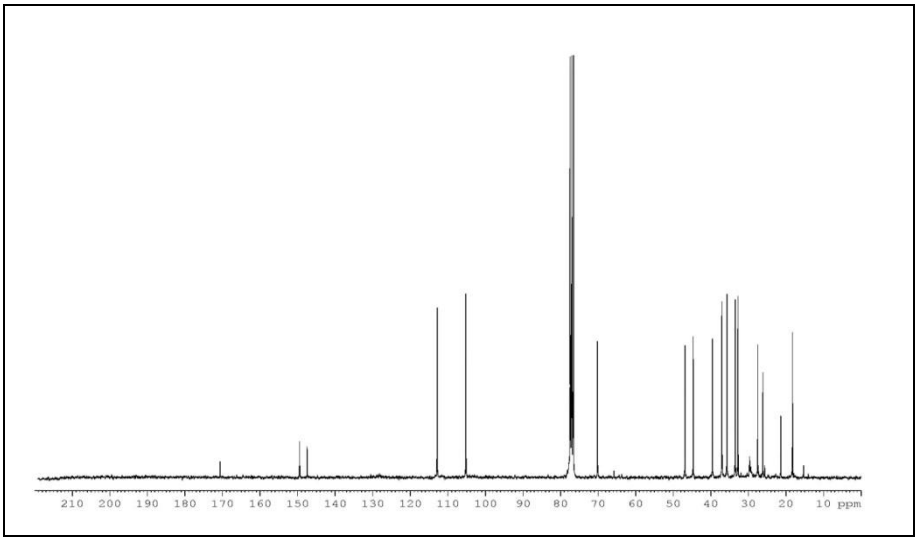


Figure 3: ^{13}C NMR spectrum of alcyonicene (**1**)

Four olefinic carbon signals [(δ 149.5, s, C-10), (147.5, s, C-12), (112.9, t, C-13), (105.2, t, C-15)] were recognized to belong to the two exomethylene groups, whereas one sp^2 carbon was attributed to the carboxyl of the acetyl group, confirmed by an intense IR band at 1731 cm^{-1}. The presence of an acetyl group was also deduced by methyl signals δ 21.5 (CO*C*H_3) in the ^{13}C NMR spectrum and a 3H acetyl singlet at δ 2.01 (COC*H*$_3$) in the ^{1}H NMR spectrum. The remaining two unsaturations required by the molecular formula were thus attributed to two rings. Thus compound **1** had a bicyclic sesquiterpene skeleton.[24,31-33]

The ^{1}H-^{1}H COSY experiment showed the presence in the molecule of a single spin system, H-1/H_2-9 sequence (Table 3),

according to the decaline framework of a cadinene carbon skeleton.[28-34] Analysis of the HMBC spectrum of **1** confirmed this hypothesis and aided us to assign all the proton and carbon values, as reported in Table 3.

Compound (**2**), named deacetoxy-alcyonicene, was isolated only in trace amount from the extract. The NMR spectra of **2** showed proton and carbon resonances very similar to those of **1**, indicating the presence of the same carbon framework. The only difference was the lack of the acetoxy substituent at C-2 in compound **2** with respect to compound **1**. Accordingly, in deacetoxy-alcyonicene (**2**), C-2 was a methylene rather than an oxygenated methine (in **2**: δ_C 23.9, δ_H 1.65/1.41; in **1**: δ_C 70.2, δ_H 5.15). Analysis of the EIMS spectrum showed the molecular peak at *m/z* 204, confirming the molecular formula $C_{15}H_{24}$. Careful analysis of 2D NMR experiments as well as comparison of the spectroscopic data of the related main metabolite **1** led us to assign the ^1H and ^{13}C NMR values of **2** (Table 3).

Table 3: NMR spectroscopic data for alcyonicene (**1**) and deacetoxy-alcyonicene (**2**).

	1			**2**	
Position	δ ^{13}C,[a] m[b]	δ ^{1}H,[c] m	HMBC	δ ^{13}C,[a] m[b]	δ ^{1}H,[c] m
1	46.9, CH	2.40, m	H-2, H-6	42.3, CH	2.08, br t (11.5)
2	70.2, CH	5.15*ax*, ddd (4.7,10.8,10.8)	H-1, H_2-3	23.9, CH_2	1.65, m/1.41, m
3	37.0, CH_2	1.92*eq*, m, 1.48*ax*, ddd (4.4,10.8,15.8)	H-2, H_3-11, H-4,H_2-5	n.d.	1.41, m
4	27.5, CH	2.13, m,	H_3-11	27.4, CH	2.04, m
5	35.7, CH_2	1.66*ax*, ddd (4.4,13.1,17.5) 1.25*eq*, m	H_3-11	n.d.	1.25, m
6	39.5, CH	1.78, m	H-1, H-7	38.6, CH	1.70, m
7	44.7, CH	2.31*eq*, br dd (5.3, 5.3)	H-6, H_2-8, H_2-13, H_3-14	45.4, CH	2.20, br t (5.18)
8	32.8, CH_2	1.86, m 1.72, m	H-7	31.9, CH_2	1.83, m 1.71, m
9	33.5, CH_2	2.38, m 2.18, ddd (3.9,13.1,13.1)	H_2-8, H_2-15	32.6, CH_2	2.40, ddd (2.9,13.1,13.1) 2.17, dt (3.9,13.1)
10	149.5, C	---	H_2-15, H-1, H_2-9	151.5, C	
11	18.2, CH_3	1.05, d (7.3)	H-4	17.9, CH_3	0.97, s
12	147.5, C	---	H_2-13, H_3-14	147.7, C	
13	112.9, CH_2	4.91, br s 4.89, br s	H_3-14	112.5, CH_2	4.89 br s 4.85 br s
14	26.6, CH_3	1.82, s	H-7, H_2-13	26.2, CH_3	1.81, s
15	105.2, CH_2	4.75, br s 4.46, br s	H_2-9, H-1	104.3, CH_2	4.69, br s 4.58, br s
OAc	170.7, C 21.5, CH_3	--- 2.01, s	H_3-17, H-2		

[a] Bruker 300 MHz, δ values are reported in ppm referred to *C*DCl_3 (δ_c 77.4); [b] Assignments deduced by DEPT sequence; [c] Bruker 400 MHz, δ values are reported in ppm referred to C*H*Cl_3 (δ_H 7.26)

1.1.3. Stereochemical assignment

Analysis of the vicinal proton coupling constants (Table 3), NOE difference experiments and the ^{13}C NMR values allowed the establishment of the relative stereochemistry of alcyonicene (**1**). In particular, irradiation of the proton at δ 5.15 (H-2), in the ^{1}H-^{1}H homodecoupling experiments, simplified the signal at δ 2.40 (H-1) to a large doublet (11.6 Hz) suggesting the presence of a *trans*-diaxial relationship of the two angular protons according to a *trans*-fused ring.

The relative configuration at C-4 was suggested by the high-field shifted value of C-11 (δ 18.2), consistent with an axial orientation of the methyl at C-4. This was in agreement with the spectroscopic data reported in the literature for related cadinene models exhibiting at C-4 either the equatorial methyl (i.e. cadinane: $\delta_{C\text{-}11}$ 23.05[31]) or the axial methyl (i.e. xenitorin A: $\delta_{C\text{-}11}$ 18.3[32]; 8-*epi*-xenitorin A: $\delta_{C\text{-}11}$ 18.1[33]). Furthermore, diagnostic NOE effects were observed among H_3-11, H-6 and H-2 thus inferring the axial orientation for all of them (Figure 4). The relative configuration of C-7 was deduced by the multiplicity of the H-7 signal (δ 2.31, br dd, J = 5.3 and 5.3 Hz), consistent with its equatorial orientation. This suggestion was further supported by a series of NOE effects observed between H-7 and H-6, H-8*ax* and H-5*eq* confirming the proposed stereochemistry (Figure 4).

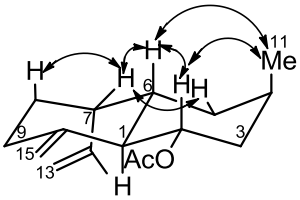


Figure 4: NOE correlations for alcyonicene (**1**)

Alcyonicene (**1**) was thus characterized as possessing a *trans*-fused decaline system with the isopropenyl chain at C-7 axially oriented, as occurs in the bulgarane subgroup of the cadinene sesquiterpene class.[30,34]

Subsequently, with the aim to establish the absolute stereochemistry, compound **1** was hydrolyzed in the corresponding alcohol derivative to which the modified Mosher's method could be applied. Unfortunately, every attempt was unsuccessful due to the rapid degradation of **1** under different hydrolysis conditions. Thus the absolute stereochemistry remained undetermined.

The relative stereochemistry of deacetoxy-alcyonicene **2** was suggested to be the same as **1** by both the similarity of the NMR values and biogenetic considerations.

1.1.4. Biological and ecological activities evaluation

The ecological properties of alcyonicene **1** as well as of known compounds 4-methyl-2-[(*E*)-2-methyl-6-methyleneocta-2,7-dienyl]-furan, pregnenolone, pregnenolone-3-acetate and pukalide were

preliminarily evaluated by conducting assays with *Carassius auratus*[35] and *Gambusia affinis*.[36]

The assay against the mosquito fish *G. affinis* is indicative to establish the ichthyotoxic properties of the samples tested. According to literature procedures,[36] all the isolated metabolites were assayed at 10 ppm, but no significant activity was observed.

In addition, feeding-deterrence tests against the gold fish *C. auratus* were conducted according to literature procedures.[35] Among the compounds tested, pukalide was feeding-deterrent at a concentration of 50 μg/cm^2. A similar ecological activity has been previously reported for a derivative of pukalide, isolated from a soft coral and its prey, the aeolid mollusc *Phyllodesmium guamensis*.[37]

All compounds were also tested in antimicrobial assays against *Escherichia coli* DH5a and *Staphylococcus aureus* ATCC6538P.[38] No significant activity was evidenced at 100 μg/mL.

1.2. *Parazoanthus axinellae*

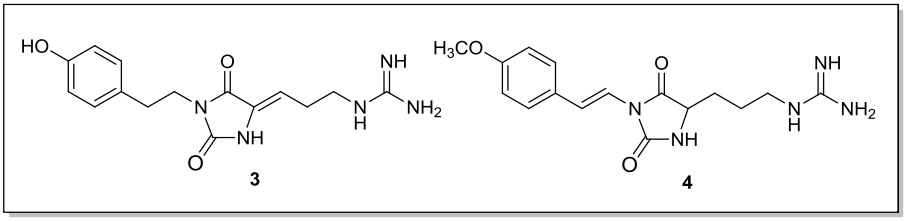
Parazoanthus axinellae is a sea anemone belonging to the order Zoanthidea. The chemical investigation on this animal started during my stage at the University of Athens.

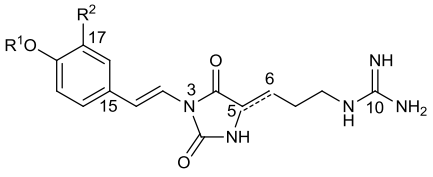
Despite evidence of their rich natural product chemistry,[39] relatively few chemical studies of zoanthids have been so far reported.

Colonial sea anemones of the genus *Parazoanthus* have been identified in almost all the oceans, and they often have been described as epibionts of marine sponges belonging to *Agelas* or *Axinella* genera. As sponges are known to exude toxic compounds, these zoanthids must have developed adaptative tools to minimize effects of such toxins.

P. axinellae has been described to posses three groups of compounds: fluorescent guanidine alkaloids of the zoanthoxanthin families,[40-45] ecdysteroids[46] and hydantoins alkaloids.[47]

The specimens analyzed in this study were collected along the Greek coast. The chemical investigation resulted in the isolation of the known parazoanthines A-E [47] along with two new compounds **3** and **4** also belonging to this class of compounds.





parazoanthine A R^1, R^2=H
parazoanthine B R^1, R^2=H Δ5-6
parazoanthine C R^1=CH3, R^2=H Δ5-6
parazoanthine D R^1=CH3, R^2=Br
parazoanthine E R^1=CH3, R^2=Br Δ5-6

1.2.1. Isolation procedure

The sea anemone *P. axinellae* (dry weight 118 g), collected in 2008, was extracted three times with CH_2Cl_2/MeOH 1:1. An aliquot of the extract (~10 g) was fractionated by VLC, using C_{18}-reverse-phase silica gel and a gradient of MeOH/H_2O until only MeOH. A chromatographic profile (TLC) of the recovered fractions displayed the presence of strong UV-visible spots, mainly in the first fraction. Due to the complexity of this mixture, the purification of compounds was obtained by subsequent chromatographic steps including MPLC and then HPLC (RP-amide column, MeOH/H_2O gradient). parazoanthines –F (**3)**, –A, –B, –G (**4)**, –C, –D and –E were obtained in order of decreasing polarity.

The structures of parazoanthines –F and –G were determined by means of spectroscopic methods whereas the known metabolites were identified by comparison of their NMR and mass spectral data with those reported in the literature.[47]

1.2.2. Structure determination

Analysis of NMR spectra of both compounds **3** and **4** revealed a close resemblance with those of co-occurring known parazoanthines suggesting the same structural framework.

The analysis of the ion peak in the HRESIMS spectrum of parazoanthine F at *m/z* 318.1555 $[M+H]^+$ led us to deduce the

molecular formula $C_{15}H_{19}N_5O_3$. The ^{1}H NMR spectrum (Figure 5) of parazoanthine F contained a series of proton signals, at low-field region, that indicated the presence of an aromatic compound. A para-substitueted phenolic moiety was easily recognized due to the characteristic signals at δ_H 6.69 (2H, d, J □ 8.0 Hz, H-16 and H-20) and 7.00 (2H, d, J = 8.0 Hz, H-17 and H-19) in the ^{1}H NMR spectrum, and at δ_C 126.0 (C, C-15), 132.6 (CH, C-16 and C-20), 116.5 (CH, C-17 and C-19), and 157.5 (C, C-18) (Table 4) in the ^{13}C NMR spectrum.

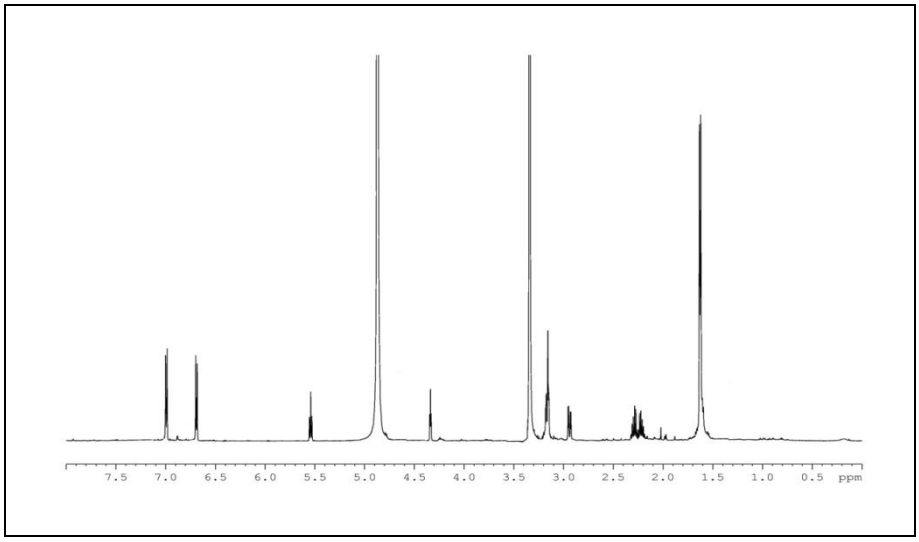


Figure 5: ^{1}H NMR spectrum of parazoanthine F

An additional unsaturation was evidenced by the presence of the ^{1}H NMR signals at δ_H 5.54 (1H, t, J = 7.5 Hz, H-6), and ^{13}C resonances at δ_C 129.4 (C, C-5), and 114.3 (CH, C-6). The NMR data

were very similar to those of known parazoanthine B; the difference was in the presence of two methylenes [C-13 (δ_C 58.2; 2H, δ_H 4.32, t, J = 4.5 Hz, H_2-13) and C-14 (δ_C 40.7; 1H, δ_H 3.10, overlapped; 1H, δ_H 2.95, dd, J = 14.4 and 4.5 Hz, H_2-14)] in parazoanthine F (**3**) rather the double bond [C-13 (δ_C 116.5; 1H, δ_H 6.95, d, J = 15.0 Hz), C-14 (δ_C 121.7; 1H, δ_H 7.42, d, J = 15.0 Hz)] in parazoanthine B.

Table 4: NMR spectroscopic data for parazoanthine F and G (**3**, **4**).

	3		**4**	
Position	δ ^{13}C,[a] m[b]	δ ^{1}H,[c] m	δ ^{13}C,[a] m[b]	δ ^{1}H,[c] m
2	168.1, C	---	174.2	---
4	161.7, C	---	157.2	---
5	129.4, C	---	55.7, CH	4.20, t (5.0)
6	114.3, CH	5.54, t (8.0)	29.7, CH_2	1.92, m
				1.81, m
7	27.8, CH_2	2.25, m	24.9, CH_2	1.75, m
				1.66, m
8	41.3, CH_2	3.15, t (7.5)	41.9, CH_2	3.26, t (7.0)
10	158.2, C	---	162.0	---
13	58.2, CH_2	4.32, t (4.5)	117.1, CH	7.03, d (15.0)
14	40.7, CH_2	3.10, overlapped	121.4, CH	7.48, d (15.0)
		2.95, dd (14.4, 4.5)		
15	126.0, C	---	129.6, C	---
16-20	132.6, CH	6.69, d (8.0)	128.2, CH	7.37, d (8.0)
17-19	116.5, CH	7.00, d (8.0)	115.1, CH	6.92, d (8.0)
18	157.5, C	---	163.1, C	---
CH_3-O			56.9, CH_3	3.78, s

[a] Bruker 300 MHz, δ values are reported in ppm referred to *C*D_3OD (δ_c 49.0);
[b] Assignments deduced by DEPT sequence;
[c] Bruker 400 MHz, δ values are reported in ppm referred to C*H*$_3$OH (δ_H 3.34)

The COSY and HMBC correlations confirmed the sequence of the alkyl chain (Figure 6).

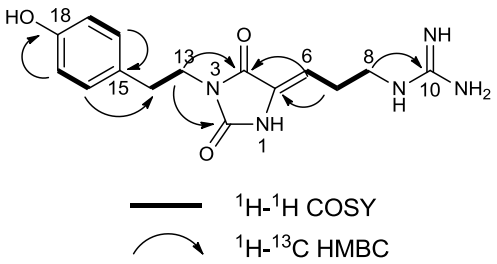


Figure 6: Key COSY and HMBC correlations of **3**

Compound **4**, named parazoanthine G, had the molecular formula $C_{16}H_{21}N_5O_3$, deduced from HRESIMS spectrum (*m*/*z* 332.2881 $[M+H]^+$). This information suggested the addition of a methylene unit compared to parazoanthine A, and the close NMR data (Table 4) suggested a strong similarity between both compounds. The replacement of the hydroxy group in parazoanthine A by a methoxy group in parazoanthine G (**4)** was supported by the ^{1}H NMR signal at δ_H 3.78 (3H, s, CH_3-O) and the ^{13}C NMR resonance at δ_C 56.9, $\underline{C}H_3$, $\underline{C}H_3$-O) and was further confirmed by the key CH_3-O/C-18 HMBC correlation.

1.2.3. Biological and ecological activities evaluation

All compounds were tested on different human cancer cell lines (Hs683, U373, U251, A549, MCF7, SKMEL28, PC3) to evaluate *in vitro* growth inhibitory concentration, but none of them exhibited significant bioactivity.

The main metabolites, the known parazoanthines A and B, were tested *in vitro* Scratch Wound Assay, using different cell lines. Mouse B16F10 melanoma, and human A549 non-small-cell lung cancer (NSCLC) and Hs683 glioma cells were grown until confluence and then a scratch has been performed as detailed in Mathieu V *et al.* 2005.[48] The data indicate that 100 µM of parazoanthine B decreased A549 NSCLC migration, while parazoanthine A did not. Parazoanthine B also induced weak anti-migratory effects on Hs683 glioma cells, while parazoanthine A did not. Parazoanthine B did not display anti-migratory effects on B16F10 melanoma cells because they grew too rapidly in presence of 10% serum. Indeed, the wound healing process was already completed in the control condition at 100% about 12 hrs after having performed the scratch.

New experiments are ongoing with less proliferating B16F10, A549 and Hs683 cancer cells, e.g. in culturing them for 48 hrs in presence of 1% serum only.

Parazoanthines were also analyzed in antimicrobial assays against *Escherichia coli* DH5a and *Staphylococcus aureus* ATCC6538P. The experiments were performed in triplicate at concentration of 100 µg/mL, but no significant activity was evidenced.

CHAPTER 2: *Phylum Chordata*

Chordates are animals which are either vertebrates or one of several closely related invertebrates. This phylum consists of three subphyla: Urochordata, represented by tunicates, Cephalochordata, represented by lancelets, and Craniata, including vertebrata.

Tunicates, also known as ascidians, are a group of underwater saclike marine invertebrate filter feeders, characterized by a tough "tunic" outer made of polysaccharides.

Ascidians are found all over the world and, usually, are sessile animals. They remain firmly attached to substrata such as rocks and shells. There are 2,300 species of ascidians and three main types: solitary ascidians, social ascidians, that form communities through the aggregation of each other at their bases, and colonial ascidians that consist of several small individuals (called zooids) forming colonies up to several meters in diameter.

Tunicates are the natural prey of many animals, including molluscs, flatworms, rock crabs, sea stars, fish and sea otters. They are able to chemical defend themselves by maintaining an extremely high concentration of vanadium in the blood, having a very low pH of the tunic (due to an acid in easily-ruptured bladder cells), and /or producing secondary metabolites harmful to predators and invaders. Some of these metabolites are toxic to cells and are of potential use in pharmaceutical chemistry.

Ascidians are renowned for their great chemical diversity and, during the last 25 years, they have been shown to produce an array of cytotoxic molecules. The high potential of these organisms as a new source of antitumor compounds is demonstrated by the fact that among the first six marine derived compounds that have reached clinical trials as antitumor agents, three are derived from ascidians.[49] Ecteinascidin-743 (ET-743/trabectedin), isolated from the Caribbean tunicate *Ecteinascidia turbinata*, represents a significant milestone in the development of marine derived drugs. Almost 40 years after its discovery and 17 years after the publication of its structure, it became the first marine-derived anticancer drug to reach the market. However, ET-743 was not the unique lead anticancer agent found from marine ascidians.[50] Two closely related compounds - didemnin B and aplidine (Figure 7) isolated from the tropical *Tridemnum solidum* and the Mediterranean *Aplidium albicans*, respectively - have been extensively investigated for more than 20 years; although clinical trials for didemnin B were stopped in the mid-1990s. Differently, phase II clinical trials with aplidine are ongoing in indications that include metastatic melanoma, multiple myeloma, non-Hodgkin's lymphoma, acute lymphoblastic leukaemia, prostate cancer and bladder cancer.

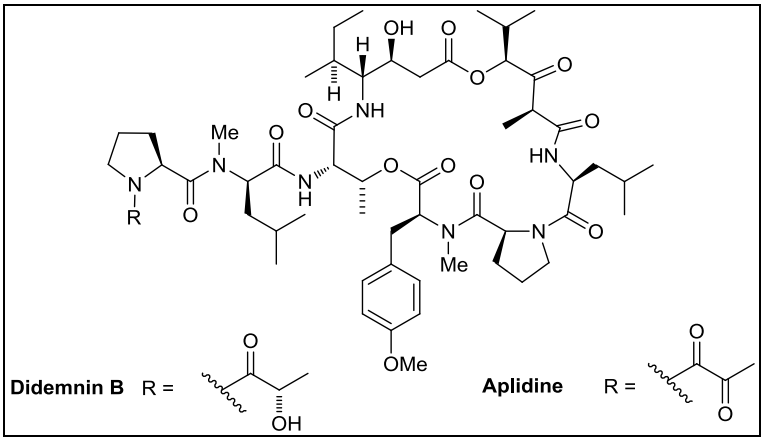


Figure 7: Didemnin B from *Tridemnum solidum* and aplidine (also known as dehydrodidemnin B) from *Aplidium albicans*

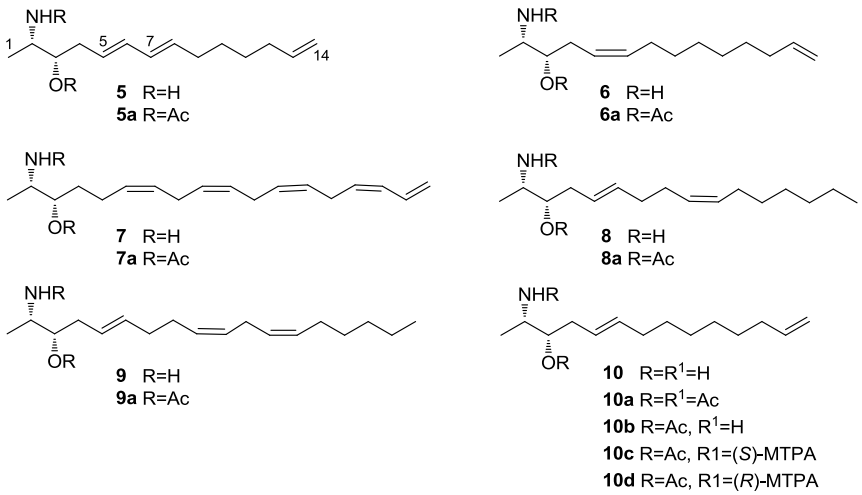
2.1. *Pseudodistoma crucigaster*

Among tunicates, the genus *Pseudodistoma* has been found to be rich in cytotoxic alkaloids,[51] linear[52-54] and cyclic[55-59] amino alcohols, tryptophan-related compounds,[60,61] alkyl amines[62,63] and nucleosides.[64,65]

The Mediterranean *Pseudodistoma crucigaster*[52] has been reported to contain linear *erythro* 2-amino-alken-3-ols, crucigasterins (i.e., crucigasterin 277). These molecules are closely related to the sphingosines,[66] basic building blocks of sphingolipids and glycosphingolipids, that are constituents of cell walls and membranes

and play diverse roles in biological processes such as cell growth, cell differentiation, and the immune response.[67]

In this study, we have analyzed specimens of Mediterranean *P. crucigaster*, collected off the coasts of Sardinia. The chemical investigation has led to the structural elucidation of five novel unsaturated amino alcohols, crucigasterins A-E (**5-9**),[68] isolated along with related known compound **10**, previously reported from South African *Pseudodistoma sp*.[53]



These molecules, which exhibit different carbon chain length and oxidation degree, are all characterised by the *threo*-configuration of the amino alcohol moiety, in contrast with the *erythro*-stereochemistry of previously reported crucigasterins.[52]

All compounds have been characterised as diacetyl derivatives **5a**-**10a**. In addition, a complete NMR assignment of the diacetyl

derivative of known compound **10a**,[53] which previously was not fully characterized, has been also made.

2.1.1. Isolation procedure

The colonial ascidian *P. crucigaster* (dry weight, 5.2 g), collected along the Sardinia coast, was extracted exhaustively with Me_2CO using ultrasound. After filtration and evaporation *in vacuum* of the organic solvent, the residue was subsequently extracted with Et_2O and BuOH to obtain two gummy residues (0.783 g and 0.976 g, respectively). The ethereal extract was fractionated by a silica gel column chromatography using a gradient eluent system with increasing polarity. A preliminary NMR analysis of the recovered fractions revealed that the most polar fraction was constituted by a complex mixture of unsaturated amino alcohols. Any effort to separate this mixture was unsuccessful, also due to the degradation of the compounds observed during the purification steps.

Thus, the fraction was acetylated with acetic anhydride and pyridine (2 h, room temperature). The acetylated mixture was easily fractionated by reverse-phase HPLC (MeOH/H_2O 8:2) to give six pure metabolites, the diacetyl derivatives **5a**-**10a** of five novel molecules, named crucigasterins A-E (**5**-**9**), and the diacetyl derivative **10a** of known related compound **10**. Compound **10a** was identified by comparison of spectroscopic data with the literature[53] even though only a partial NMR assignment had been reported. However, the

proton and carbon resonances of **10a** were fully assigned, by interpretation of 2D-NMR spectra, in Tables 5 and 6.

2.1.2. Structure determination

Analysis of the NMR spectra of compounds **5a**-**9a** revealed a close structural relationship with compound **10a** indicating the presence in all the molecules of the same 2-amino-3-hydroxy-alkyl residue. The structural differences among them were in either the alkyl chain length or in the number and/or geometry of the double bonds.

Diacetyl crucigasterin A (**5a**) was considered first. The 2-amino-3-hydroxy moiety was easily recognized by sequentially correlated proton signals at δ 1.10 (3H, d, J = 6.7 Hz, H_3-1), due to the terminal methyl, and at δ 4.25 (1H, dq, J = 3.8 and 6.7 Hz, H-2) and 4.85 (1H, dt, J = 3.8 and 6.7 Hz, H-3), that were attributed to two methines linked to the amino and the hydroxyl group, respectively (Figure 8). Moreover, the ^{13}C NMR spectrum displayed carbon signals at δ 18.5 (q, C-1), 46.7 (d, C-2) and 75.9 (d, C-3), that confirmed this assignment.

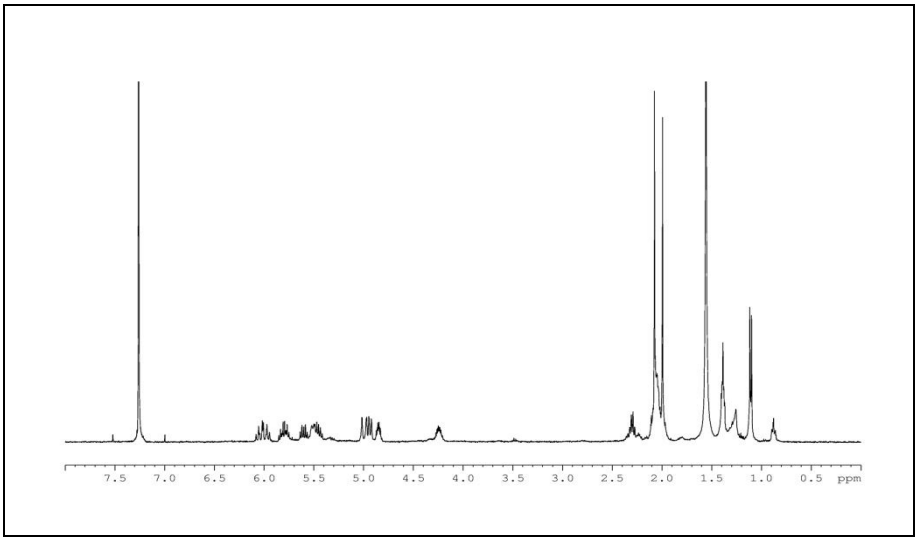


Figure 8: ^{1}H NMR spectrum of diacetyl crucigasterin A (**5a**)

In addition, the carbon resonances indicated the presence of three double bonds (Table 6), one of which had to be in the terminal position. Accordingly, the ^{1}H NMR spectrum displayed in the low-field region seven olefinic signals (Table 5), which were assigned to a conjugated diene system and to the terminal vinyl moiety by analysis of the ^{1}H-^{1}H COSY and HSQC spectra. The geometries of Δ^{5} and Δ^{7} double bonds were suggested to be *E*,*E* by the coupling constants ($J_{5,6}$=$J_{7,8}$=14.6 Hz) and further supported by the ^{13}C chemical shift values of the allylic methylene carbons C-4 (δ 35.0) and C-9 (δ 32.3) (Tables 5 and 6).

Table 5 ^{1}H NMR data[a-c] for Crucigasterins A-E (**5a-9a**) and compound **10a**

Position	**5a** δ_H, mult.	**6a** δ_H, mult.	**10a** δ_H, mult.	**7a** δ_H, mult.	**8a** δ_H, mult.	**9a** δ_H, mult.
1	1.10 (d, 6.7)	1.11 (d, 7.0)	1.10 (d, 6.7)	1.10 (d, 6.7)	1.11 (d, 6.7)	1.11 (d, 6.7)
2	4.25 (dq, 3.8, 6.7)	4.24 (dq, 4.0, 7.0)	4.23 (dq, 4.2, 6.7)	4.22 (m)	4.24 (m)	4.23 (m)
3	4.85 (dt, 3.8, 6.7)	4.85 (ddd, 4.0, 7.6, 6.5)	4.83 (dt, 4.2, 6.7)	4.88 (dt, 3.8, 6.7)	4.84 (dt, 4.1, 6.7)	4.84 (dt, 4.1, 6.7)
4	2.30 (m)	2.25 (ddd ,14.7, 6.5, 7.6) 2.35 (ddd ,14.7, 7.6, 7.6)	2.23 (m)	1.61 (m)	2.23 (m)	2.24 (m)
5	5.46 (m)	5.30 (ddd, 10.0, 7.6, 7.6)	5.31 (dt, 14.9, 6.9)	2.02 (m)	5.41-5.28 (m)	
6	6.03 (dd, 14.6, 10.5)	5.51 (dt, 10.0, 7.6)	5.49 (m)	5.42-5.33 (m)	5.51(dt, 14.0, 7.0)	5.41-5.28 (m)
7	5.99 (dd, 14.6, 10.5)	2.01 (m)	1.98 (m)	5.42-5.33 (m)	2.06 (m)	5.51 (dt,
8	5.60 (dt, 14.6, 7.0)	1.32 (m)	1.26 (m)	2.84 (app. t, 5.6)[c]	2.05 (m)	14.0, 7.0)
9	2.10-1.98 (m)	1.24-1.34 (m)	1.24-1.38 (m)	5.42-5.33 (m)	5.41-5.28 (m)	2.06 (m)
10	1.42-1.36 (m)	1.24-1.34 (m)	124-1.38 (m)	5.42-5.33 (m)	5.41-5.28 (m)	2.10 (m)
11	1.42-1.36 (m)	1.38 (m)	1.38 (m)	2.79 (app. t, 5.6)[c]	2.02 (m)	5.41-5.28 (m)
12	2.10-1.98 (m)	2.06 (m)	2.02 (m)	5.42-5.33 (m)	1.35-1.23 (m)	5.41-5.28 (m)
13	5.80 (ddt, 16.8, 10.1, 6.7)	5.81 (ddt, 16.4, 10.0, 7.0)	5.80 (ddt, 17.2, 10.2, 7.0)	5.42-5.33 (m)	1.35-1.23 (m)	2.76 (app. t,
14	4.99 (br d, 16.8) 4.93 (br d, 10.1)	4.99 (br d, 16.4) 4.93 (br d, 10.0)	4.99 (br d, 17.2) 4.93 (br d, 10.2)	2.97 (app. t, 5.6)	1.35-1.23 (m)	5.8) 5.41-5.28 (m)
15				5.42 (m)	1.29 (m)	5.41-5.28 (m)
16				6.02 (dd, 11.3, 10.8)	0.88 (t, 6.7)	2.02 (m)
17				6.67 (ddd, 16.9, 10.8, 10.2)		
18				5.21 (br d, 16.9) 5.12 (br d, 10.2)		1.38-1.25 (m) 1.38-1.25 (m)
2-NH	5.51 (br d, 9.0)	5.53 (br d, 9.0)	5.51 (overlapped)	5.51 (br d, 9.0)	5.50 (br d, 8.8)	1.38-1.25 (m)
NHAc	1.99 (s)	1.99 (s)	1.99 (s)	1.99 (s)	1.99 (s)	0.89 (t, 6.7)
OAc	2.07 (s)	2.08 (s)	2.07 (s)	2.07 (s)	2.08 (s)	5.50 (br d, 9.0) 2.00 (s) 2.08 (s)

[a] Bruker 400 and 600 MHz; [b]assignments made by COSY, HSQC and HMBC (J =10Hz experiments); [c]values with the same superscript in the same column may be interchanged

Table 6 ^{13}C NMR data[a-c] for crucigasterins A-E (**5a-9a**) and compound **10a**

No.	**5a**	**6a**	**10a**	**7a**	**8a**	**9a**
	δ_C, m	δ_C, m	δ_C, m	δ_C, m	δ_C, m	δ_C, m
1	18.5 q	18.5 q	18.5 q	18.5 q	18.5 q	18.5 q
2	46.7 d	46.9 d	46.7 d	46.9 d	46.8 d	46.8 d
3	75.9 d	76.1 d	76.1 d	75.9 d	76.0 d	76.0 d
4	35.0 t	29.5 t	35.0 t	31.5 t	35.0 t	35.0 t
5	125.1 d	123.2 d	123.9 d	27.0 t	124.4 d	124.5 d
6	134.0 d	133.5 d	134.8 d	128.8[c] d	134.1 d	134.0 d
7	129.9 d	27.3t	32.5 t	128.7[c] d	32.7 t	32.6 t
8	133.9 d	29.0[c] t	29.0[c] t	25.3 t	27.1 t	27.0 t
9	32.3 t	29.5[c] t	29.3[c] t	128.6[c] d	130.5 d	127.8 d
10	28.7[c] t	29.4[c] t	29.0[c] t	128.8[c] d	128.2 d	129.1[c] d
11	28.4[c] t	29.1[c] t	29.0[c] t	25.3 t	27.3 t	25.7 t
12	33.6 t	33.7 t	33.8 t	128.7[c] d	29.7[c] t	128.5[c] d
13	138.9 d	138.9 d	139.2 d	128.7[c] d	29.0[c] t	130.3 d
14	114.3 t	114.1 t	114.1 t	25.6 t	31.8 t	27.2 t
15				128.4[c] d	22.6 t	29.3 t
16				129.2 d	14.1 q	31.5 t
17				131.9 d		22.6 t
18				117.5 t		14.0 q
NHAc	23.4 q	23.4q	23.5 q	23.2 q	23.4 q	23.4 q
	169.2 s	169.3 s	21.0 q	169.3 s	169.3 s	169.3 s
OAc	20.9 q	21.0 q	169.3 s	21.1 q	21.0 q	21.0 q
	170.6 s	170.6	170.6 s	170.7 s	170.6 s	170.6 s

[a] Bruker 300 MHz; [b] Multiplicity deduced by DEPT; [c] Assignments may be interchanged

Diacetyl crucigasterin B (**6a**) had the same molecular formula ($C_{18}H_{31}NO_3Na$) of known metabolite **10a**. Analysis of ^{1}H and ^{13}C NMR spectra (Tables 5 and 6) indicated the presence of an identical structural sequence: they differed only in the geometry of Δ^5 double bond. In particular, the proton coupling constant $J_{5,6}$=10.0 Hz and the carbon values of C-4 (d 29.5) and C-7(d 27.3) clearly indicated that **6a** was the 5*Z*-isomer of **10a**.

Diacetyl crucigasterin C (**7a**), with the molecular formula $C_{22}H_{33}NO_3$, contained a highly unsaturated C_{18} alkyl chain. The ^{1}H NMR spectrum showed signals accounting for 11 olefinic protons (Table 5) according to the presence of five vinyl unsaturations (four

disubstituted and one terminal double bond). The terminal vinyl moiety was conjugated with one of the disubstituted double bonds, whereas the remaining three were isolated. Analysis of the ^{1}H-^{1}H COSY spectrum revealed that the 6H signal at δ 5.42-5.33 was coupled with three bis-allylic methylene groups at δ 2.97 (2H, app. t, *J* = 5.6 Hz, H_2-14), 2.84 (2H, app. t, *J* = 5.6 Hz, H_2-8 or H_2-11) and 2.79 (2H, app. t, *J* = 5.6 Hz, H_2-11 or H_2-8), and to an allylic methylene at δ 2.02 (2H, m, H_2-5). This latter signal was additionally correlated with the methylene multiplet at δ 1.61 (2H, m, H_2-4), which in turn showed cross-peaks with the carbinol proton of 2-amino-3-hydroxy moiety. The ^{13}C NMR values of bis-allylic and allylic methylenes (Table 6) suggested the *Z* geometry of all the disubstituted double bonds.

Diacetyl crucigasterin D (**8a**) was characterized by two additional CH_2 units in the alkyl chain with respect to **6a** and **10a**. The ^{1}H and ^{13}C NMR spectra exhibited signals attributable to two disubstituted double bonds [δ_C 134.1(C-6),130.5 (C-9), 128.2 (C-10), 124.4 (C-5); δ_H 5.51 (H-6), 5.41-5.28 (H-5, H-9 and H-10)], in addition to the signals due to the 2-amino-3-hydroxy moiety (Tables 5 and 6). Analysis of ^{1}H-^{1}H COSY correlations was diagnostic to establish the position of the two double bonds at C-5 and at C-9. The geometry of $\Delta^{5,6}$ double bond was assigned as *E* by the proton coupling constant $J_{5,6}$=14.0 Hz and the ^{13}C NMR chemical shift values of allylic methylene groups [δ 35.0 (C-4) and 32.7 (C-7)], whereas the

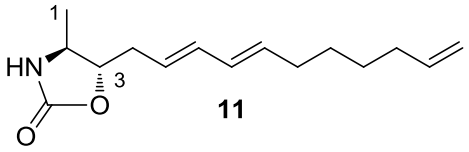
geometry of $\Delta^{9,10}$ double bond was suggested to be *Z* by the chemical shift values of the remaining allylic methylenes [δ 27.1 (C-8) and 27.3 (C-11)].

Diacetyl crucigasterin E (**9a**) had the molecular formula $C_{22}H_{37}NO_3$, consistent with a C_{18} alkyl chain with three double bonds. In fact, the ^{1}H NMR spectrum of **9a** showed three olefinic signals (Table 5), a signal at δ 2.76 (2H, app. t, 5.8 Hz), which was due to a bis-allylic methylene group, and multiplets centred at δ 2.24 (2H, m, H_2-4), 2.10 (2H, m, H_2-8), 2.06 (2H, m, H_2-7) and 2.02 (2H, m, H_2-14), which were attributed to four allylic methylene groups. The ^{1}H-^{1}H COSY experiment helped to locate the three double bonds. The geometry of the three double bonds was suggested by the ^{13}C NMR values (Table 6) of bis-allylic and allylic methylenes resonating at δ 25.7 (C-11), 27.0 (C-8), 27.2 (C-14), 32.6 (C-7), and 35.0 (C-4) consistent with the *Z* stereochemistry for Δ^9 and Δ^{12} double bonds and the *E* stereochemistry for Δ^5 double bond.

2.1.3. Stereochemistry assignment

With the aim to establish the relative configuration of the chiral centers C-2 and C-3 in the novel crucigasterins, a series of NOE difference experiments were recorded on the oxazolidinone derivative (**11**) of crucigasterin A (**5**), according to the procedure applied for related molecules.[53,54] In order to prepare the oxazolidinone, the

diacetyl derivative **5a** (1 mg) was treated with NaOH (4 M) at 70 °C for 20 h, to obtain the free aminoalcohol (0.8 mg, **5**), that was then reacted with 1,10-carbonyldiimidazole (3 mg) in 1 mL of CH_2Cl_2 and 100 mL of DMF at 0 °C for 19 h under argon atmosphere. The solution was extracted with water and the organic layer was dried under nitrogen yielding 0.5 mg of the oxazolidinone **11**.



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The NOE effects observed between H-3 and H_3-1 and between H-2 and H_2-4 suggested that the substituents at both C-2 and C-3 were on the opposite face of oxazolidinone ring. Consequently, H-2 and H-3 were suggested to be in a *threo* relationship in the acyclic starting compound **5**. This suggestion was definitively confirmed by a comparative analysis of NMR data of diacetyl crucigasterin A (**5a**) with those reported in the literature for synthetic *threo* and *erythro* 2-acetamido-3-acetoxy-5*E*,7*E*-tetradecadiene models.[69] A good agreement of carbon NMR resonances was observed between **5a** and the *threo*-isomer, in particular with regards to diagnostic ^{13}C chemical shift value of C-1 (δ_{threo} 18.49; $\delta_{erythro}$ 14.93; $\delta_{\mathbf{5a}}$ 18.5). On this basis, the relative configuration of **5a**, and consequently of **5**, was assigned to be *threo* (2*R**,3*R** or 2*S**,3*S**). Consequently, the *threo*

stereochemistry of the 2-amino-3-hydroxy moiety of all the other crucigasterins was assigned by the indicative ^{13}C NMR value of the terminal methyl C-1.

The next step was to assign the absolute stereochemistry of crucigasterins by applying the Mosher method to suitable derivatives. Unfortunately, during the methanolysis to obtain the hydroxyl substrates, a significant degradation was observed to occur under the reaction conditions for most compounds preventing the formation of the desired hydroxyl derivates. However, the reaction was successfully applied on the known co-occurring compound **10a**, the absolute stereochemistry of which had not been reported in the previous paper.[54]

The O-deacetyl derivative **10b**, obtained by treatment with Na_2CO_3 in anhydrous MeOH of **10a**, was treated with (*R*)- and (*S)*-MTPA chlorides to get the (*S*)- and (*R*)-MTPA esters **10c** and **10d**, respectively. The $\Delta\delta$ ($\delta_{S\text{ester}}$-$\delta_{R\text{ester}}$) values observed (Figure 9) for the signals of protons close to the hydroxyl group at C-3 indicated the *S* configuration as depicted in formula **10b**. Consequently, the absolute stereochemistry of naturally occurring metabolite **10** was assigned as 2*S*,3*S*.

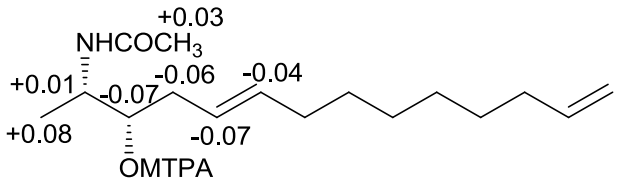


Figure 9: Chemical shifts differences Δδ (δ_{Sester}-δ_{Rester}) between (*S*)- and (*R*)-MTPA derivatives of compound **10a**

The same configuration was suggested for the other crucigasterins based on biogenetic considerations. This suggestion was supported by the optical rotation values of diacetyl derivatives of crucigasterins (**5a**-**10a**), which were all laevorotatory according to the 2*S* absolute configuration.[69] Particularly diagnostic was the comparison of the $[\alpha]_D$ value of diacetyl derivative of crucigasterin A (**5a**) {$[\alpha]_D$ -20.3 (c 0.03, CH_3OH)} with those reported for synthetic (2*R*,3*R*)-{$[\alpha]_D$ +20.7 (c 1.66, CH_3OH)} and (2*S*,3*S*)-{$[\alpha]_D$ -20.6 (c 0.32, CH_3OH)} 2-acetamido-3-acetoxy-5*E*,7*E*-tetradecadiene enantiomers[69,70] further confirming the 2*S*,3*S* absolute configuration for **5a** and for all co-occurring metabolites.

2.1.4. Biological and ecological activities evaluation

Selected diacetyl-crucigasterins have been tested for antibacterial and antifungal activities, exhibiting moderate activity against *E. coli* and *C. albicans*, respectively.

Antifungal assay was performed by the broth macrodilution method following the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) document M27-P.[71, 72] MIC was defined as the lowest concentration of the compound that completely inhibited the growth of the test organism.

The antibacterial assay was performed by using the same method as the antifungal test, only differing in the assay medium and in the incubation temperature.

In particular, the diacetyl-crucigasterins B (**6a**) and E (**9a**) were found to be active at 50 μg/mL against both *E. coli* and *Candida albicans*, and at 100 μg/mL against *E. coli*, respectively.

CHAPTER 3: *Phylum Porifera*

Among the organisms that inhabit the seas, Porifera, commonly known as sponges, comprising the most primitive multicellular animals. These sessile organisms have world-wide distribution and live anchored to substrates, such as rocks, seaweed, shells and crabs. Sponges could have solitary life or make dense colonies that become important habitats for animals and plants: their cavities can host several symbionts as small crustaceans, insect larvae, algae, cyanobacteria, etc.

Porifera are filter-feeders that use flagellated cells to pump water into their canal systems. They can be encrusting or erect, assuming different morphologies according to the environmental characteristics (substrate, currents, waves). Schematically, the body of a sponge may be considered a sort of bag, all perforated (hence the name Porifera) from inhalants thin pores (orifices), with a large opening said exhaling pore or *osculum* and an inner chamber called gastral cavity or *spongocele*.

Depending on the different endoskeleton, phylum Porifera is divided into four classes: 1) Calcispongiae, that are characterized by calcareous skeletons, generally small or tabular; 2) Hexactinellida (Hyalospongiae), also known as vitreous sponges due to their siliceous skeleton, with a funnel or cylindrical shape; 3) Demospongiae (mostly sponges species, 90%), including animals whose skeleton is composed of siliceous spicules, which in some forms are partially or fully

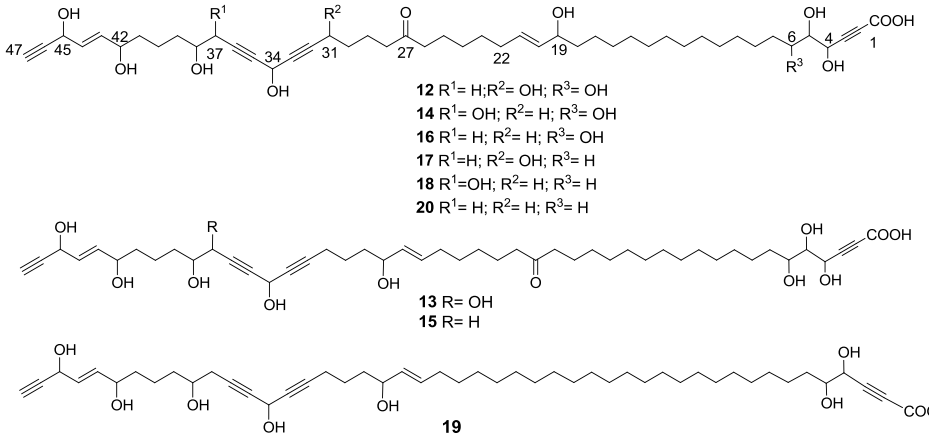
replaced by skeletal elements consisting of a special protein called spongin; 4) Sclerospongiae, a small group of sponges, mostly tropical, whose skeleton consists of calcium carbonate crystals on a network of organic fibbers.

Even sponges are primitive organisms, they have proved to be a rich source of secondary metabolites. It has been repeatedly suggested[73-75] that they are protected by toxic or noxious chemicals, i.e. allomones. Many of these molecules, often characterized by complex molecular architectures, are known to possess interesting biological activities.[76,77]

3.1. *Haliclona fulva*

Haliclona fulva is an encrusting and orange desmosponge with a globular shape. A previous chemical study on this sponge has reported the isolation of a series of bioactive polyacetylenes,[78-80] closely related to petroformines, a family of long chain polyacetylenes isolated from another Mediterranean sponge, *Petrosia ficiformis*.[81] All these compounds display linear alkin chain of 46 carbons with a characteristic 1-yn-3-ol-4-ene moiety at each terminus. In closely related haplosclerid sponges,[82] similar polyacetylenes usually only have a mono-terminal 1-yn-3-ol-4-ene moiety - sometimes modified - and a considerably shorter alkyl chain.[83-87]

The biological interest of polyacetilene molecules prompted us to chemically reinvestigate *H. fulva*. The work here described reports the isolation and spectroscopic elucidation of nine high molecular weight polyoxygenated acetylenes, fulvynes A-I (**12**-**20**),[88] from the butanolic extract of a specimen of the sponge collected in the Gulf of Naples.



The new compounds are characterized by a long linear alkyl chain bearing a residue of propargylic acid, a terminal acetylenic moiety, a diacetylenic carbinol and several hydroxyl and keto groups. Previous isolated metabolites – fulvinol and renierines - have been detected in the ether extract of the sponge under investigation along with a mixture of additional non-polar polyacetylenes. These latter metabolites have been also found in the lipophilic extract of the nudibranch *Peltodoris atromaculata*, which was observed grazing on

H. fulva. The structure elucidation of these compounds will be describe in the next Chapter, dedicated to the phylum Mollusca.

3.1.1. Isolation procedure

The sponge *H. fulva* (dry weight, 42 g), collected along the coast of Procida Island (Gulf of Naples) at a depth of ~ 40 m, was chopped and then extracted with Me_2CO using ultrasound. After filtration and evaporation of the organic solvent *in vacuo*, the residue was subsequently extracted with Et_2O and BuOH. Both organic phases were dried to give two gummy residues (1.46 g and 2.48 g, respectively) which were tested in preliminary antimicrobial and antifungal assays at a fixed concentration (50 µg/mL). The butanol extract showed a good activity against the gram positive *B. subtilis* whereas it showed weak against *E. coli* and *C. albicans* , with respect to the ethereal extract. Therefore, part of the bioactive butanol extract (1.2 g) was thus subjected to Sephadex LH-20 chromatography in MeOH to give eight fractions (I-VIII). Preliminary ^{1}H NMR analysis of these fractions showed that fraction III (0.228 g) contained a mixture of polyacetylenic compounds. An aliquot of this fraction (0.120 g) was further purified on RP-amide semipreparative HPLC column with a gradient of H_2O/MeOH/TFA (from 29:70:0.1 to 100% MeOH, flow 2.0 mL min^{-1}) to afford, in order of retention time, pure fulvynes A-I (**12**-**20**). Preliminary ^{1}H NMR analysis revealed that all isolated molecules were polyhydroxylated acetylenes. In particular,

comparison of the spectra with literature data showed strong similarities of the structural framework of fulvynes with those of osirisynes[89,90] and haliclonyne,[91] polyacetylene compounds isolated from other *Haliclona* species.

The structures of compounds **12**-**20** were determined by an extensive use of spectroscopic techniques.

3.1.2. Structure determination

Among the fulvynes, compound **14**, named fulvyne C, was the most abundant metabolite. As deduced from the sodiated molecular ion peak at *m/z* 851.4879 $[M+Na]^+$ in the HRESIMS, fulvyne C (**14**) had the molecular formula $C_{47}H_{72}O_{12}$, indicating 12 unsaturation degrees.

The ^1H NMR spectrum of **14** (Figure 10) displayed signals due to four olefinic protons [δ_H 5.44, dd (15.2, 7.0); 5.64, dt (15.2, 6.7); 5.79, dd (15.5, 5.4); 5.92, dd (15.5, 6.0)], an acetylenic proton at (δ_H 2.93), nine oxygenated methines in the range δ_H 5.14-347 and a series of methylene groups in the high-field region (δ_H 2.52-1.30) (Table 7).

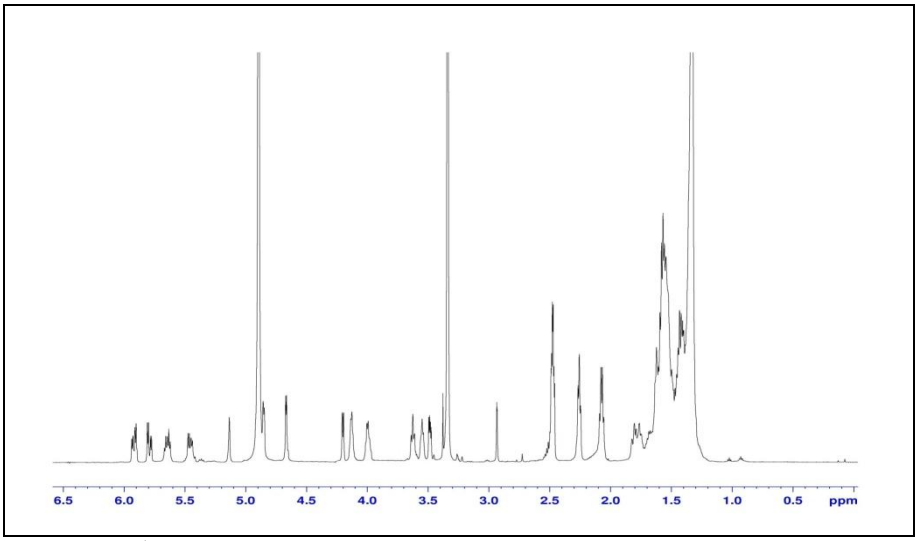


Figure 10: ^{1}H NMR spectrum of fulvyne C (**14**)

The IR spectrum showed bands at 2237, 1696, and 1679 cm^{-1} suggesting the presence of triple bonds and carbonylic functions. The ^{13}C NMR spectrum (Figure 11) showed eight sp carbon resonances in the range at δ_C 85.3-74.8, due to four triple bonds, and C=O signals at δ_C 157.2 and 213.6. Moreover, carbon resonances due to sp^2 carbons of two double bonds in the range δ_C 136.3-130.5, nine oxygen-bearing methines in the region of the field between δ_C 78.4 and 52.5, and several methylene groups were also present (Table 8).

Table 7 ^{1}H NMR data[a-b] for Fulvynes A-E (**12-16**) in CD_3OD

Position	**12** δ_H, m (Hz)	**13** δ_H, m (Hz)	**14** δ_H, m (Hz)	**15** δ_H, m (Hz)	**16** δ_H, m (Hz)
4	4.70, d (4.0)	4.67, d (4.1)	4.67, d (4.2)	4.68, d (4.0)	4.70, d (3.8)
5	3.50, dd (8.0, 4.0)	3.48, dd (8.3, 4.1)	3.47, dd (8.3, 4.2)	3.49, dd (8.0, 4.0)	3.50, dd (8.0, 3.8)
6	3.61, dt (8.0, 7.0)	3.62, t (8.3)	3.65, dt (8.3, 7.9)	3.62, t (8.0)	3.65-3.58, m
7	1.85-1.77, m	1.84-1.80, m	1.84-1.78, m	1.85-1.77, m	1.86-1.76, m
	1.43-1.37, m	1.42-1.38, m	1.46-1.37, m	1.47-1.35, m	1.45-1.38, m
8	1.62-1.56/1.39-1.34, m	1.62-1.57/1.38-1.34, m	1.61-1.56/1.38-1.33, m	1.62-1.56/1.38-1.33, m	1.62-1.57/1.38-1.34, m
9-16	1.40-1.30, m	1.40-1.30, m	1.40-1.30, m	1.40-1.30, m	1.40-1.30, m
17	1.40-1.31, m	1.40-1.30, m	1.38-1.30, m	1.40-1.30, m	1.43-1.38/1.37-1.31, m
18	1.60-1.52/1.50-1.44, m	1.75-1.66, m	1.61-1.51/1.50-1.43, m	1.76-1.66, m	1.60-1.52/1.50-1.43, m
19	4.04-3.95, m	2.48, t (7.0)	3.99, dt (7.0, 6.4)	2.48, t (7.0)	3.98, q (6.6)
20	5.44, dd (15.6, 7.0)	---	5.44, dd (15.2, 7.0)	---	5.43, dd (15.2, 6.6)
21	5.63, dt (15.6, 7.0)	2.47, t (7.0)	5.64, dt (15.2, 6.7)	2.47, t (7.0)	5.63, dt (15.2, 6.6)
22	2.11-2.04, m	1.62-1.53, m	2.07, dt (6.7, 7.0)	1.62-1.54, m	2.13-2.02, m
23	1.48-1.38, m	1.38-1.30, m	1.46-1.38, m	1.37-1.30, m	1.46-1.38, m
24	1.38-1.30, m	1.47-1.38, m	1.38-1.30, m	1.47-1.39, m	1.37-1.31, m
25	1.62-1.55, m	2.07, dt (6.6, 7.0)	1.62-1.54, m	2.07, dt (6.0, 8.0)	1.62-1.56, m
26	2.49, t (10.0)	5.64, dt (15.5, 6.6)	2.49, t (7.3)	5.64, dt (15.0, 6.0)	2.49, t (7.1)
27	---	5.45, dd (15.5, 7.0)	---	5.45, dd (15.0, 6.0)	---
28	2.54, t (10.0)	4.00 , dt (7.0, 6.0)	2.52, t (7.3)	4.00, q (6.0)	2.52, t (7.1)
29	1.76-1.68, m	1.60-1.53/1.52-1.43, m	1.73-1.65, m	1.60-1.52/1.53-1.42, m	1.73-1.65, m
30	1.76-1.72/1.70-1.66, m	1.58-1.53, m	1.56-1.50, m	1.60-1.52, m	1.57-1.49, m
31	4.37, t (8.0)	2.26, t (7.0)	2.27, dt (7.0, 1.6)	2.25, t (7.0)	2.27, t (5.7)
34	5.14, s	5.13, s	5.14, d (1.6)	5.07, s	5.07, s
37	2.41, d (6.0)	4.20, d (6.0)	4.20, d (6.4)	2.40, d (6.0)	2.40, d (6.0)
38	3.75-3.70, m	3.58-3.52, m	3.57-3.53, m	3.76-3.70, m	3.76-3.68, m
39	1.70-1.64/1.58-1.50, m	1.80-1.73/1.57-1.49, m	1.80-1.73/1.57-1.49, m	1.72-1.64/1.58-1.48, m	1.70-1.64/1.57-1.49, m
40	1.56-1.52, m	1.55-1.51, m	1.55-1.51, m	1.58-1.48, m	1.57-1.49, m
41	1.64-1.58/1.58-1.50, m	1.67-1.60/1.58-1.50, m	1.67-1.59/1.61-1.56, m	1.65-1.58/1.54-1.47, m	1.64-1.58/1.57-1.49, m
42	4.16-4.10, m	4.17-4.10, m	4.15-4.11, m	4.12, q (6.0)	4.17-4.08, m
43	5.91, dd (15.0, 6.0)	5.92, dd (15.0, 6.0)	5.92, dd (15.5, 6.0)	5.91, dd (15.0, 6.0)	5.91, dd (15.7, 6.2)
44	5.79, dd (15.0, 6.0)	5.79, dd (15.0, 6.0)	5.79, dd (15.5, 5.4)	5.79, dd (15.0, 6.0)	5.79, dd (15.0, 6.0)
45	4.85[c]	4.85[c]	4.85[c]	4.85[c]	4.85[c]
47	2.93, d (2.0)	2.93, d (1.8)	2.93, d (1.9)	2.93, d (2.0)	2.94, d (1.4)
48					

[a] Bruker 400 and 600 MHz; [b]assignments made by COSY and TOCSY; [c]under water signal

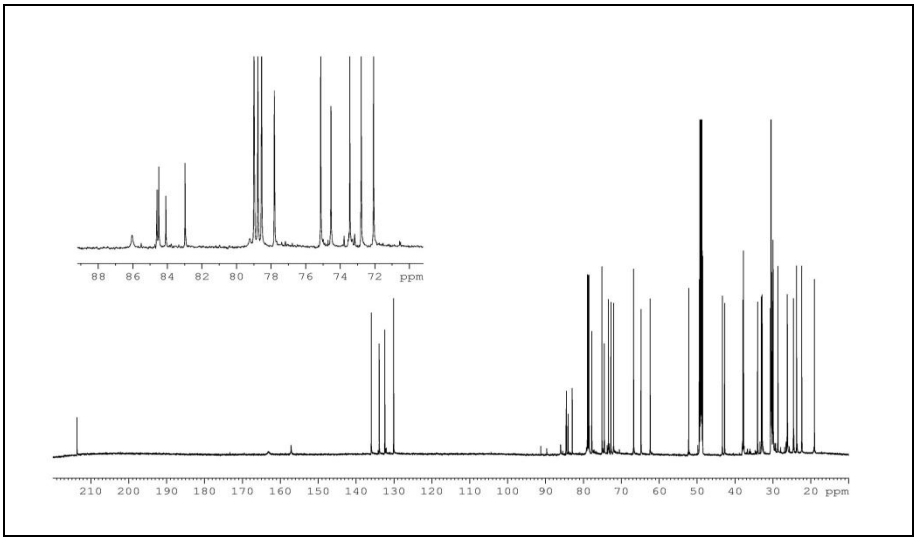


Figure 11: ^{13}C NMR spectrum of fulvyne C (**14**)

All proton-bearing carbons were assigned by the HSQC experiment. Analysis of the ^{1}H-^{1}H COSY spectrum aided us to recognize the partial structures **a**-**e** in the long alkyl chain of **14** (Figure 12).

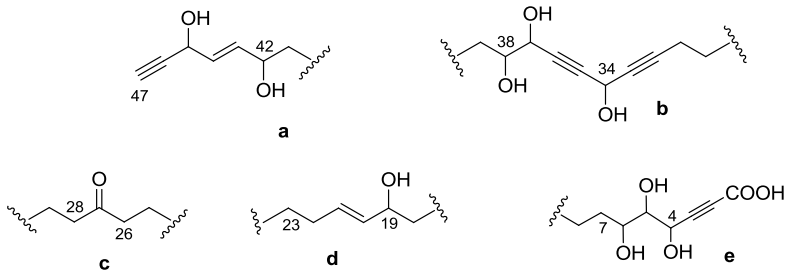


Figure 12. Partial structures of fulvyne C (**14**) as deduced by ^{1}H ^{1}H COSY correlations

The ^{1}H-^{1}H TOCSY experiment was essential to assemble fragments **a**-**e**. In particular, the oxymethine H-42 in partial structure **a** was correlated to the oxymethine H-38 in moiety **b** through an additional methylene H_2-40 (δ_H 1.55-1.51, δ_C 22.6). The α-carbonyl methylene H_2-28 in fragment **c** showed correlations with methylene H_2-31 in fragment **b** through methylene H_2-30 (δ_H 1.56-1.50, δ_C 29.0), whereas the other α-carbonyl methylene H_2-26 had cross-peaks with the allylic methylene H_2-22 in partial structure **d** through an additional methylene H_2-24 (δ_H 1.38-1.30, δ_C 30.4). The observed correlations allowed to connect fragments **a**-**d** accounting for a C30 segment of the alkyl chain of **14**. This implied that fragment **e** had to be linked to the remaining part of the molecule through nine additional methylenes thus satisfying the molecular formula. Analysis of the HMBC spectrum of **14** confirmed the proposed structure and enabled us to assign properly the acetylenic quaternary carbon resonances (Table 8). The geometries of the two double bonds were easily assigned as 20*E*, 43*E* by the coupling constant analysis of the olefinic protons (J = 15.2 and 15.5 Hz, respectively).

Fulvyne A (**12**) had the same molecular formula $C_{47}H_{72}O_{12}$ as fulvyne C (**14**). Comparison of 1D and 2D proton NMR spectra of **12** with those of **14** disclosed a substantial structural analogy between the two compounds suggesting the presence of the same functional groups. In particular, the differences were limited to the partial structure b' where one of the two hydroxyl groups of the vicinal diol

(37-OH) in fulvyne C was shifted on the other side of the di-yne moiety at C-31 in **12** (Figure 13).

The remaining signals appeared to be the same as compound **14** thus confirming that the two polyacetylenes were positional isomers.

Fulvyne B (**13**) was also an isomer of fulvyne A (**12**) and fulvyne C (**14**). The proton and carbon spectra of **13** displayed signals that were strongly reminiscent with those of **14** being consistent with the same partial structures **a-e**. Analysis of 2D NMR spectra, especially TOCSY experiment, clearly demonstrated that the sequence of fragments **b**, **c**, and **d** in fulvyne B (**13**) was different from that set in fulvyne C (**14**). In particular, the keto group was positioned far from the inner triple bonds. In fact, the methylene protons at δ_H 2.26 (H_2-31) were long-range correlated with the allylic oxymethine at δ_H 4.00 (H-28), rather than one of the α-carbonyl methylene as in compound **14**. In addition, the α-carbonyl methylene at δ_H 2.47 (H_2-21) had a clear correlation with the allylic methylene at δ_H 2.07 (H_2-25), consistent with the sequential connection in the chain of fragments **b**, **d**, and **c**, as depicted in formula **13**.

Table 8 ^{13}C NMR data[a-c] for fulvynes A-E (**12-16**) in CD_3OD

	12 δ_C, m	**13** δ_C, m	**14** δ_C, m	**15** δ_C, m	**16** δ_C, m
1	157.3 s	157.2 s	157.2 s	157.2 s	158.4 s
2	80.1 s	79.8 s	80.1 s	79.5 s	79.7 s
3	85.0 s	85.1 s	85.3 s	84.3 s	85.2 s
4	65.1 d	65.1 d	65.1 d	65.1 d	65.1 d
5	78.3 d	78.5 d	78.4 d	78.4 d	78.3 d
6	73.0 d	73.0 d	73.0 d	72.9 d	73.0 d
7	34.2 t	34.2 d	34.2 t	34.2 t	34.2 t
8	26.5 t	26.5 t	26.6 t	26.5 t	26.4 t
17	26.5 t		26.4 t		26.4 t
18	38.3 t	24.9 t	38.3 t	24.7 t	38.3 t
19	73.6 d	43.4 t	73.6 d	43.4 t	73.7 d
20	134.5 d	214.4 s	134.4 d	214.4 s	134.4 d
21	132.5 d	43.5 t	132.5 d	43.5 t	132.5 d
22	33.2 t	24.7 t	33.3 t	24.9 t	33.2 t
23	30.2 t	29.8 t	30.2 t	29.7 t	30.3 t
24	30.4 t	29.6 t	30.4 t	29.6 t	30.4 t
25	24.9 t	33.2 t	24.9 t	33.1 t	24.8 t
26	43.4 t	132.3 d	43.4 t	132.3 d	43.4 t
27	213.6 s	134.5 d	213.6 s	134.5 d	213.9 s
28	42.9 t	73.6 d	42.9 t	73.6 d	42.9 t
29	24.3 t	37.9 t	24.0 t	37.9 t	24.0 t
30	32.9 t	25.9 t	29.0 t	25.9 t	29.0 t
31	62.2 d	19.3 t	19.1 t	19.3 t	19.1 t
32	83.8 s	79.5 s	79.5 s	79.6 s	79.9 s
33	83.2 s	84.8 s	84.8 s	84.4 s	84.3 s
34	52.5 d	52.5 d	52.5 d	52.6 d	52.6 d
35	80.6 s	84.8 s	84.8 s	81.3 s	81.3 s
36	82.2 s	83.2 d	83.3 s	81.5 s	81.6 s
37	28.1 t	67.2 d	67.2 d	28.1 t	28.1 t
38	70.7 d	75.5 d	75.5 d	70.9 d	70.9 d
39	37.0 t	33.2 t	33.3 t	37.0 t	37.0 t
40	22.6 t	22.7 t	22.6 t	22.6 t	22.6 t
41	38.1 t	38.2 t	38.2 t	38.1 t	38.1 t
42	72.4 d	72.4 d	72.4 d	72.4 d	72.4 d
43	136.2 d	136.6 d	136.3 d	136.2 d	136.3 d
44	130.6 d	130.5 d	130.5 d	130.5 d	130.6 d
45	62.7 d	62.7 d	62.7 d	62.7 d	62.7 d
46	84.5 s	84.5 s	84.5 s	84.6 s	84.5 s
47	74.8 d	74.8 s	74.8 d	74.8 d	74.8 d
48					

[a] Bruker 300 MHz; [b] Multiplicity deduced by DEPT; [c] others CH_2 values for compounds **12**, **14**, **16**, are in the range δ_C 30.6-30.9, and for compounds **13**, **15** are in the range δ_C 30.2-30.9.

Table 9 ^{13}C NMR data[a-c] for fulvynes F-I (**17**-**20**) in CD_3OD

	17 δ_C, m	**18** δ_C, m	**19** δ_C, m	**20** δ_C, m
1	157.5 s	157.6 s	158.0 s	157.9 s
2	78.3 s	78.2 s	78.2 s	n.d.
3	85.3 s	85.7 s	85.3 s	85.5 s
4	67.1 d	67.1 d	67.2 d	67.1 d
5	75.0 d	75.1 d	75.3 d	75.1 d
6	33.6 t	33.6 t	33.5 t	33.6 t
7	26.8 t	26.8 t	26.8 t	26.8 t
8				
17	26.5 t	26.4 t		26.4 t
18	38.4 t	38.3 t		38.3 t
19	73.7 d	73.7 d		73.7 d
20	134.4 d	134.4 d		134.4 d
21	132.5 d	132.5 d		132.5 d
22	33.2 t	33.2 t		33.3 t
23	30.2 t	30.2 t		30.2 t
24	30.4 t	30.4 t	26.3 t	30.4 t
25	24.9 t	24.9 t	29.6 t	24.9 t
26	43.4 t	43.4 t	33.2 t	43.4 t
27	213.0 s	213.9 s	132.5 d	213.8 s
28	42.8 t	42.9 t	134.4 d	42.9 t
29	24.3 t	24.0 t	73.6 d	24.0 t
30	32.9 t	29.0 t	37.9 t	29.0 t
31	62.2 d	19.1 t	25.9 t	19.1 t
32	83.8 s	79.4 s	19.3 t	79.9 s
33	83.2 s	84.8 s	79.6 s	83.3 s
34	52.4 d	52.5 d	84.5 s	52.6 d
35	80.6 s	84.8 s	52.6 d	81.3 s
36	82.2 s	83.2 s	81.3 s	81.6 s
37	28.1 t	67.1 d	81.6 s	28.1 t
38	70.8 d	75.5 d	28.1 t	70.9 d
39	37.0 t	33.3 t	70.9 d	37.0 t
40	22.6 t	22.7 t	37.0 t	22.6 t
41	38.1 t	38.2 t	22.6 t	38.1 t
42	72.4 d	72.4 d	38.1 t	72.4 d
43	136.2 d	136.3 d	72.4 d	136.2 d
44	130.5 d	130.5 d	136.2 d	130.5 d
45	62.7 d	62.7 d	130.5 d	62.7 d
46	84.5 s	84.5 s	62.6 d	84.5 s
47	74.8 d	74.8 d	84.6 s	74.8 d
48			74.8 d	

[a] Bruker 300 MHz; [b] Multiplicity deduced by DEPT; [c] others CH_2 values for compounds **17**, **18**, **20** are in the range δ_C 30.6-30.9, and for compound **19** are in the range δ_C 30.2-30.9.

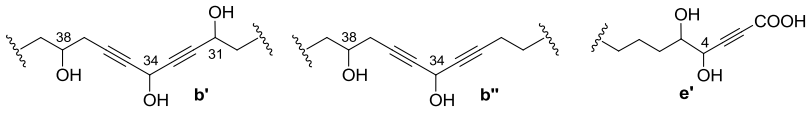


Figure 13. Additional partial structures of fulvynes

The molecular formula $C_{47}H_{72}O_{11}$ of fulvyne D (**15**), as deduced from the sodiated peak at *m/z* 835.4865 in the HRESIMS, indicated that **15** was lacking in an oxygen atom with respect to co-occurring **12-14**. In addition, the ^{13}C NMR spectrum evidenced the presence of eight oxygen-bearing carbons (Table 8) rather than nine oxymethines, such as in **12-14**. Analysis of 2D NMR experiments of fulvyne D indicated that the partial structure **b''** lacked the hydroxyl group in α-position of di-yne moiety present in fulvyne C. In fact, the COSY spectrum showed that the bis propargyl alcohol proton at δ_H 5.07 (H-34) was coupled with two methylene groups at δ_H 2.25 (H_2-31) and δ_H 2.40 (H_2-37), that had to be both linked to internal triple bonds. By TOCSY experiments, the fragments were assembled as in fulvyne B (**13**). Thus fulvyne D (**15**) was the 37-dehydroxy derivative of **13**.

Fulvyne E (**16**) with the molecular formula $C_{47}H_{72}O_{11}$ was isomeric with compound **15**, from which it differs in the sequence of the fragments in the chain. In particular, analysis of the TOCSY spectrum showed a clear correlation of the methylene at δ_H 2.27 (H_2-31) in fragment **b''** with the α-carbonyl methylene at δ_H 2.52 (H_2-28)

in partial structure **c** according to the location of the keto group in proximity of the bis propargylic alcohol as in fulvyne C (**14**). Thus fulvyne E (**16**) was the 37-dehydroxy derivative of **14**.

Last four compounds, fulvynes F-I (**17**-**20**), were all characterized by the lack of a hydroxyl group in fragment **e**, showing a diol moiety close to propargylic acid residue (fragment **e'**) (Figure 13). The molecular formula $C_{47}H_{72}O_{11}$ of fulvyne F (**17**) was deduced from the sodiated molecular ion peak at *m/z* 835.4843 in the HRESIMS. ^{13}C and ^{1}H NMR data of **17** (Table 9 and 10) were quite similar to those of fulvyne A (**12**). Careful analysis of 2D NMR spectra and in particular of both COSY and TOCSY spectra of **17** confirmed the presence of the same sequence of fragments **a**, **b'**, **d**, and **c** as fulvyne A, whereas a difference was detected in the terminal fragment containing the propargylic acid unit missing in one of the three hydroxyl groups. Thus fulvyne F (**17**) was the 6-dehydroxy derivative of fulvyne A (**12**).

Fulvyne G (**18**) was isomeric with fulvyne F (**17**). The NMR spectra displayed strong similarities with those of co-occurring fulvyne C (**14**) except for the spin system in the terminal acid part. As **17**, fulvyne G was characterized by the presence of a di-hydroxyl propargylic acid (fragment **e'**) instead of a tri-hydroxyl moiety.

Table 10: ^{1}H NMR data[a,b] of fulvynes F-I (**17-20**) in CD_3OD

Position	**17** δ_H, m (Hz)	**18** δ_H, m (Hz)	**19** δ_H, m (Hz)	**20** δ_H, m (Hz)
4	4.31, d (4.5)	4.30, d (5.0)	4.26, d (4.8)	4.30, d (4.9)
5	3.65-3.60, m	3.64-3.60, m	3.63-3.58, m	3.65-3.59, m
6	1.67-1.62/1.55-1.48, m	1.71-1.64/1.60-1.50, m	1.74-1.65/1.60-1.50, m	1.72-1.65/1.54-1.48, m
7	1.61-1.54/1.42-1.32, m	1.43-1.33, m	1.42-1.32, m	1.43-1.32, m
8-16	1.40-1.30, m	1.40-1.30, m	1.40-1.30, m	1.40-1.30, m
17	1.39-1.30, m	1.38-1.31, m	1.40-1.30, m	1.37-1.31, m
18	1.58-1.53/1.52-1.43, m	1.60-1.52/1.51-1.45, m	1.40-1.30, m	1.60-1.52/1.49-1.43, m
19	3.99, dt (7.0, 6.0)	3.99, dt (7.0, 6.3)	1.40-1.30, m	3.99, dt (6.5, 7.0)
20	5.44, dd (15.0, 7.0)	5.44, dd (15.0, 7.0)	1.40-1.30, m	5.44, dd (15.0, 6.5)
21	5.63, dt (15.0, 7.0)	5.64, dt (15.0, 7.0)	1.40-1.30, m	5.64, dt (15.0, 6.5)
22	2.10-2.04, m	2.09-2.04, m	1.40-1.30, m	2.10-2.03, m
23	1.47-1.36, m	1.47-1.38, m	1.40-1.30, m	1.46-1.38, m
24	1.38-1.27, m	1.39-1.30, m	1.37-1.31, m	1.37-1.31, m
25	1.65-1.52, m	1.63-1.55, m	1.46-1.38, m	1.62-1.56, m
26	2.49, t (7.0)	2.49, t (7.0)	2.12-2.04, m	2.49, t (7.3)
27	---	---	5.64, dt (15.0, 7.0)	---
28	2.54, t (7.0)	2.51, t (7.0)	5.45, dd (15.0, 7.0)	2.52, t (7.3)
29	1.80-1.68, m	1.73-1.65, m	4.03-3.97, m	1.74-1.66, m
30	1.76-1.69/1.69-1.62, m	1.58-1.48, m	1.70-1.53/1.52-1.40, m	1.56-1.50, m
31	4.37, t (7.0)	2.27, t (7.0)	1.60-1.51, m	2.26, t (7.3)
34	5.14, s	5.14, s	2.25, t (5.5)	5.07, s
37	2.41, t (6.4)	4.20, d (6.5)	5.07, s	2.40, d (5.5)
38	3.75-3.69, m	3.57-3.52, m	2.40, d (5.1)	3.75-3.70, m
39	1.70-1.63/1.56-1.49, m	1.80-1.72/1.59-1.49, m	3.74-3.70, m	1.70-1.65/1.58-1.48, m
40	1.58-1.49, m	1.59-1.49, m	1.71-1.64/1.57-1.49, m	1.58-1.48, m
41	1.64-1.58/1.56-1.49, m	1.68-1.59/1.59-1.49, m	1.57-1.49, m	1.64-1.58/1.57-1.48, m
42	4.15-4.10, m	4.15-4.10, m	1.65-1.59/1.57-1.49, m	4.15-4.10, m
43	5.91, dd (15.0, 6.0)	5.92, dd (15.0, 6.0)	4.15-4.10, m	5.91, dd (15.0,6.0)
44	5.79, dd (15.0, 6.0)	5.79, dd (15.0, 6.0)	5.92, dd (15.0, 6.0)	5.79, dd (15.0, 6.0)
45	4.85[c]	4.85[c]	5.79, dd (15.0, 6.0)	4.85[c]
47	2.93, br d (1.9)	2.93, br d (1.6)	4.85[c]	2.93, br d (1.8)
48			2.93, br d (1.7)	

[a] Bruker 400 and 600 MHz; [b] assignments aided by COSY and TOCSY; [c] under water signal

The molecular formula of fulvyne H (**19**) was deduced as $C_{48}H_{76}O_9$ from the sodiated molecular ion at *m/z* 819.4990 in the HRESIMS. The spectroscopic data of **19** (Table 9 and 10) clearly evidenced that it was lacking in both the ketone and 6-OH groups, whereas the remaining part was identical with fulvyne D (**15**). Thus,

the alkyl chain connecting partial structures **d** and **e'** contained an additional methylene unit with respect to all other co-occurring fulvynes.

Finally, fulvyne I (**20**) had a molecular formula $C_{47}H_{72}O_{10}$ as deduced from the sodiated molecular ion peak at m/z 819.4917. Comparison of NMR data of compound **20** with those of fulvyne E (**16**) indicated the presence of the same fragment sequence **a**, **b"**, **c**, and **d**, whereas the terminal residue was constituted by fragment **e'** characterizing fulvynes F-H (**17**-**19**).

3.1.3. Stereochemical assignment

The relative configuration of contiguous chiral carbons of fulvynes as well as of isolated carbinol centres remained unassigned. Any attempt to obtain suitable derivatives for a stereochemical analysis were unsuccessful due to a rapid degradation of the compounds under different reaction conditions. Even the simple acetylation conducted under mild conditions resulted in the decomposition of the starting material.

3.1.4. Biological and ecological activities evaluation

Due to the antimicrobial activity directed mainly against the gram positive *B. subtilis* of the butanol extract, pure isolated compounds were tested in a more specific assay on a chloramphenicol

resistant *B. subtilis* strain (PY79), showing a good activity against this strain (Table 11). Fulvynes were tested also on a gram-positive human pathogenic strain, *Staphylococcus aureus*, and showed a moderate activity.

Table 11: Summary of the IC_{50}'s of fulvynes **12-20** against *B. subtilis* Cm (PY79)

Compound	IC_{50} (μM)	**Compound**	IC_{50} (μM)
12	12	**17**	12
13	60	**18**	25
14	48	**19**	25
15	12	**20**	12
16	12		

Cytotoxic activity has been reported for polyacetylenes from sponges,[78] thus the main metabolite was also subjected to antiproliferative assays. In particular, fulvyne C (**14**), tested on different tumour cell lines (C6, H9c2, HeLa, 3T3-L1), showed selectivity for mouse pre-adipocyte cells line 3T3-L1, being active with an IC_{50} of 55.7 ± 11 μM (Table 12).

Table 12: Cytotoxicity activity of fulvyne C **14**

Cell lines	IC_{50} (μM)
C6	107.4 ± 3.92
H9c2	120.0 ± 6.4
Hela	109.0 ± 7.2
3T3-L1	55.7 ± 11

CHAPTER 4: *Phylum Mollusca*

Molluscs represent the largest marine phylum comprising about 23% of all marine organisms. Among them the subclass Opisthobranchia is one of most interesting groups from a chemo-ecological point of view. Species of this subclass are recognized to posses secondary metabolites with an extraordinary structural diversity. Opisthobranchs are marine molluscs apparently unprotected by physical constraints of a shell which is either reduced or completely absent in the adults. In spite of this, they are rarely victims of predators. In fact, they have elaborated a series of defensive strategies, which involve cryptic behaviour and use of chemicals. Sometimes, protective substances are accumulated in the anatomical structures located in regions of the body most exposed (*mantle dermal formations*),[92,93] or appendices (*cerata*) that the organisms detach themselves in case of danger.[94, 95]

Another defensive strategy consists in the use of non-toxic compounds, but active as alarm pheromones.[96] These substances are alarm signals, which are issued by an individual in case of predator's attack, indicating to the other members to avoid the dangerous area. An additional alarm signal is represented by the colour of the animals (*warning colouration*). Marine slugs are often very colourful. The brilliant colours warn potential predators of the existence of another primary defensive mechanism such as unpalatability due to repellent or toxic molecules (*aposematism*).

The great variety of chemicals in opisthobranchs is mainly derived from food.[97] It has been proven for some opisthobranch species the ability to make biosynthetic modifications on substrates sequestered from their prey. These chemical transformations often result in considerable variation in the toxicity of the processed compounds. Further, at the highest levels in the evolutionary scale, there are some species able to synthesize their chemical weapons *de novo* from simple precursors.[97]

The study of opisthobranch molluscs and their chemicals has led to the isolation of several bioactive molecules. A representative example is the alkaloid jorumycin, which was isolated from the defensive mucous secretion of the nudibranch *Jorunna funebris*.[98] Jorumycin showed antimicrobial activity against *Bacillus subtilis* and *Staphylococcus aureus* and cytotoxic activity on different tumour cell lines at low concentration.[99] PM-104 – trade name Zalypsis® - is an antitumor derived from jorumycin, that is currently in phase II clinical trial for the treatment of endometrial and uterine cervix cancers by PharmaMar (Figure 14).

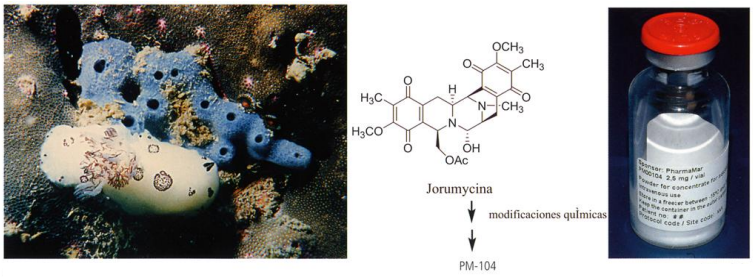


Figure 14. *Jorunna funebris* and Zalypsis®

According to different authors,[100-102] opisthobranchs are divided into a number of orders from 8 to 9 being however sacoglossans and nudibranchs the two largest groups.

Opisthobranchs can be either herbivorous (such as sacoglossans) or carnivorous (such as nudibranchs). They have the extraordinary capability to create new chemistry through either bio-accumulation of selected metabolites from their dietary sources, bio-transformation of dietary compounds , or *de novo* bio-synthesis. A fascinating aspect of the biology of opisthobranchs is related to their gradual evolution from a shelled snail to shell-less slug. Sacoglossans include both shelled and shell-less forms, whereas nudibranch species are all completely naked. The study of these animals offer extraordinary cues to link chemistry to ecology and to evolution.[103]

4.1. *Placida dendritica*

Placida dendritica is a species of the order Sacoglossa, belonging to Limapontioidea superfamily.

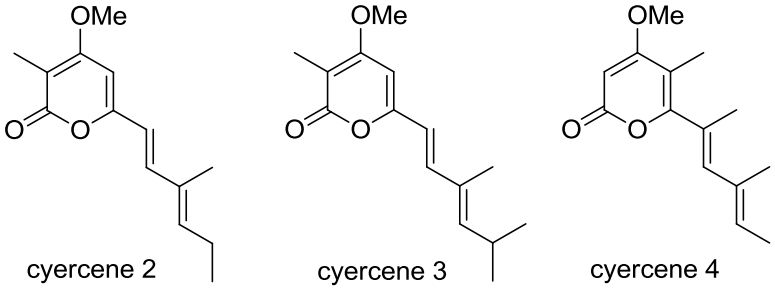
Members of the order Sacoglossa display a complete evolutionary series from shelled molluscs, with a large or reduced shell, to shell-less species.[104] Chemical studies, carried out on both shelled and shell-less sacoglossans, have resulted in the isolation of sesquiterpenoids and diterpenoids, sequestered from green algae and either accumulated or chemically modified, as well as polypropionates biosynthesized *de novo*.

Limapontioidean sacoglossans are shell-less species characterized by a series of dorso-lateral appendages, named cerata, which can be, more or less easily, autotomized. The dorsal surface of this animal is bright green due to the abundant concentration of chloroplasts in the branches of the digestive gland.

The chemistry of limapontioidean sacoglossans is characterized by pyrone-containing compounds.[105] These metabolites, which are formally originated by a polyketide pathway, are associated with specific functions of molluscs, since they may act as mediators in tissue regeneration and chemical defense.[105] Most of these propionates are biosynthesized *de novo* by the sacoglossans, although such aptitude has been rigorously proved only in a few species.[106-110]

The Mediterranean mollusc *P. dendritica* has been reported to possess a number of regular and irregular propionates, named placidenes and isoplacidenes, together with an unusual propionate hydroperoxide.[111,112]

In this study, we have analyzed specimens of *P. dendritica*, which were collected in the Indian Ocean. Three known metabolites, cyercenes 2-4, previously reported from Mediterranean *Cyerce cristallina*,[107] have been isolated and identified by comparison of their NMR and mass spectral data with those reported in the literature.[107]



The polypropionate origin of cyercenes and their *de novo* biosynthesis from ^{14}C-propionic acid had been demonstrated in *C. cristallina* by *in vivo* incorporation experiments.[107,110] Cyercenes were also found to have a different distribution in mantle, cerata and mucous secretion of the mollusc whereas they were absent in the digestive gland, as it was expected from their *de novo* biosynthetic origin. The anatomical compartmentalization of *Cyerce* polypropionates suggested their possible involvement in the mollusc defensive mechanisms, as well as in regenerative processes of cerata.

Interestingly, a subsequent study sets out a possible photochemical link between cyercenes and placidenes, showing that placidene A and isoplacidene A are produced *in vitro* from cyercene A, when the latter is exposed to sunlight (figure 15).[113]

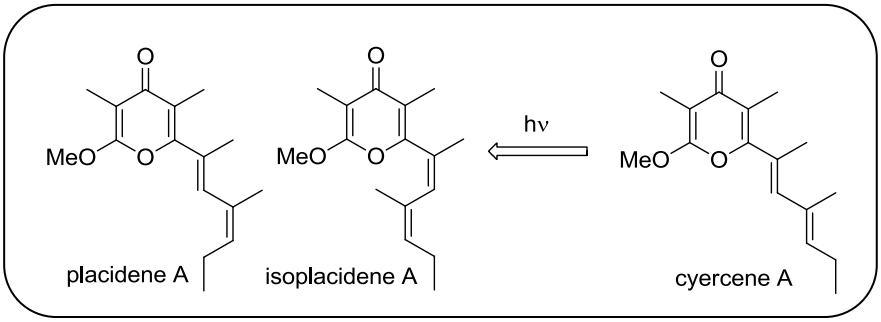


Figure 15. Photoreactions of cyercene A

However, the suggested hypothesis of the origin of placidenes from a photorearrangement *in vivo* of cyercene-like precursors appears not plausible in consideration of the fact that no sacoglossan species has been reported to contain both series of compounds.

4.1.1. Isolation procedure

The mollusc *P. dendritica* (86 individuals), collected in the Indian Ocean, was treated following a standard procedure to obtain separately the extracts of the external and the internal part. In particular, the external extract was obtained by soaking the whole specimens in acetone bath for few minutes. By this procedure,

lipophilic metabolites mainly located in the mantle and in other external parts of the animal are extracted. Then, the solvent was separated from the animals, filtered and evaporated *in vacuum* to give a residue, which was subsequently extracted with Et_2O. This extract (ca. 56 mg) was chromatographed on a silica gel column by eluting with a gradient system of light petroleum ether/Et_2O. The fractions containing polypropionates were further purified on RP-HPLC to give known cyercene 2-4, the structure of which is characterised by an α-pyrone moiety. These compounds were identified by comparison of spectroscopic data with the literature.[107]

4.1.2. Biological and ecological activities evaluation

Due to the selective location of cyercenes in the external part of *C. cristallina*, it was suggested a defensive role for these metabolites which, indeed, were showed to possess a significant ichthyotoxic activity.[107] An analogous ecological role could be hypothesised also for *P. dendritica*.

Based on the consideration that ecologically relevant molecules often show different bioactivities, cyercene 4, the main metabolite isolated, was tested on different human cancer cell lines (Hs683, U373, U251, A549, MCF7, SKMEL28, PC3) to evaluate *in vitro* growth inhibitory concentration, through the MTT colorimetric assay. In the same test, the related α-pyrone aplysiopsene A, which had been previously isolated from the limapontioidean sacoglossan

Aplysiopsis formosa, in the frame of my degree thesis experimental work, [114] was also assayed.

These two molecules are structurally related. They differ only in the position of the methyl group on the pyrone moiety (C-5 or C-3) and in the length of the alkyl chain (figure 16).

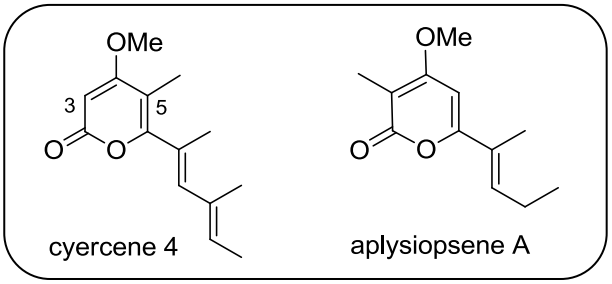


Figure 16. α-pyrones from limapontioidean sacoglossans.

However, these structural differences seem to strongly influence the biological activity. Aplysiopsene A displays significant *in vitro* growth inhibitory activity on various cancer cell lines, while cyercene 4 displays weak activity (Table 13). Further studies are necessary to clarify this structure-activity relationship.

Table 13:

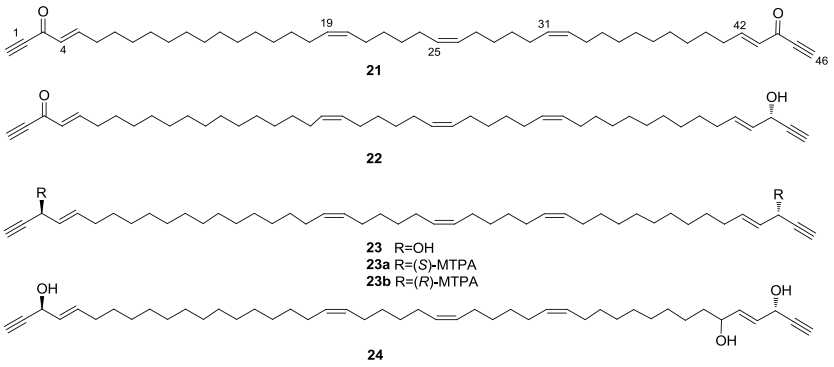
IC_{50} *In vitro* growth inhibitory concentration (μM) after 72 hrs of culture with compound								
Compounds	Human cancer cell lines							
	Hs683 (glioma)	U373 (glioma)	U251 (glioma)	A549 (lung)	MCF7 (breast)	SKMEL28 (melanoma)	PC3 (prostate)	Mean IC_{50}
cyercene 4	>100	94	>100	>100	84	91	>100	>90
aplysiopsene A	2	7	7	2	5	6	2	5±1

4.2. *Peltodoris atromaculata*

Peltodoris atromaculata belongs to the suborder Doridina, the largest nudibranch group (commonly known as "dorids").

Dorids containing the most varied species of nudibranchs[102] are often brilliantly coloured. Most of them are sponge-feeders and an high specialization of feeding upon sponges is recorded for several members of this group. Consequently, the chemistry of dorids is strictly related to that of the sponge diet from which the molluscs derive their secondary metabolites and this is essential to understand some basic biological problems including chemical defence mechanisms.[115]

Previous chemical studies conducted on the nudibranch *P. atromaculata* have displayed the ability of this mollusc to accumulate in the digestive gland a plethora of polyacetylenes compounds, petroformynes, mainly derived from its prey, the sponge *Petrosia ficiformis*.[116,82] In this study, we have analyzed specimens of the Mediterranean *P. atromaculata*, which were observed grazing on another sponge, *Haliclona fulva*.



Very interestingly, a preliminary chromatographic analysis of the lipophilic extract of both the nudibranch *P. atromaculata* and the sponge *H. fulva* revealed a similar secondary metabolite pattern. In particular, the chemical analysis of the mollusc extract led us to isolate and to characterize four new polyacetylenes, named fulvyne 1-4 (**21**-**24**), closely related to fulvinol, the polar polyacetylenic component of *Reniera (=Haliclona) fulva* from Spanish coasts, as well as renierines, short-chain acetylenic compounds previously described from the same animal collected off the Bay of Naples.

It has never been observed any species other than *P. atromaculata* feeding on either of the two sponges. Any predator on *P. ficiformis* and *H. fulva* has to deal with the numerous secondary metabolites that have been detected in these sponges. It is surprising that *P. atromaculata* is believed to feed exclusively on these sponges, which are some of the most cytotoxic Mediterranean sponges. Petroformynes and fulvinol display linear alkyl chains of 46 carbons with a characteristic 1-yn-3-ol-4-ene moiety at each terminus, and

were shown to be cytotoxic in bioassays.[79,80] These metabolites have not been detected in any other Mediterranean sponges. Thus, *Peltodoris* is highly specialized on the two only Mediterranean sponges that contain these specific polyacetylenes.

4.2.1. Isolation procedure

P. atromaculata (3 individuals) was found grazing on the sponge *H. fulva* in Pizzaco off Procida (Italy). The mollusc was collected and dissected into internal glands and mantle. The two tissues were separately extracted in acetone. After filtration and evaporation of the organic solvent in vacuum, the aqueous residues were subsequently extracted with Et_2O to get about 15 mg of mantle extract and 62 mg of internal glands extract. Preliminary TLC analysis showed a different metabolite pattern for the two extracts. In particular, a series of metabolites, most likely of dietary origin, were observed to be concentrated in the extract of internal part also including the digestive gland. This extract was chromatographed on a Sephadex LH-20 column in $CHCl_3$/MeOH, and then further fractionated on silica gel column by eluting with a gradient system of light petroleum ether/Et_2O. Preliminary ^1H NMR analysis revealed the present of a mixture of polyacetilenes that was further purified on RP-HPLC with MeOH to afford, in order of retention time, pure fulvyne 1-4 (**21**-**24**), together with some known renierines. The new molecules were recognized also in the extract of the sponge *H. fulva,* from which

were also purified. The structures were elucidated by interpretation of spectral data.

4.2.2. Structure determination

The NMR spectra of all new compounds **21-24** were reminiscent with that of fulvinol and, with regards to terminal moieties, with those of petroformynes.

Fulvyne 1 (**21**) had a molecular formula $C_{46}H_{72}O_2$ deduced by ESIMS [*m/z* 679 $(M+Na)^+$] and HRFABMS [*m/z* 663.5320 $(M+Li-H)^+$]. Both the ^{1}H- and the ^{13}C-NMR spectra contained a smaller number of signals than those expected from the molecular formula, suggesting a highly symmetric structure. This implied that each resonance of the spectrum was attributable to two magnetically equivalent nuclei. The ^{1}H NMR spectrum contained a singlet at δ 3.20 attributable to the terminal acetylenic protons, a doublet at δ 6.18 (*J*=15.7 Hz, 2H, H-4 and H-45) that correlated in the COSY spectrum with a signal under the solvent at δ 7.25 (Figure 17). The coupling constant of 15.7 Hz indicated an *E* geometry for the double bond belonging to the terminal residue. The remaining signals at δ 1.98-2.08, δ 1.45-1.55 and δ 1.32-1.38 were assigned to an envelope of methylenes constituting the chain.

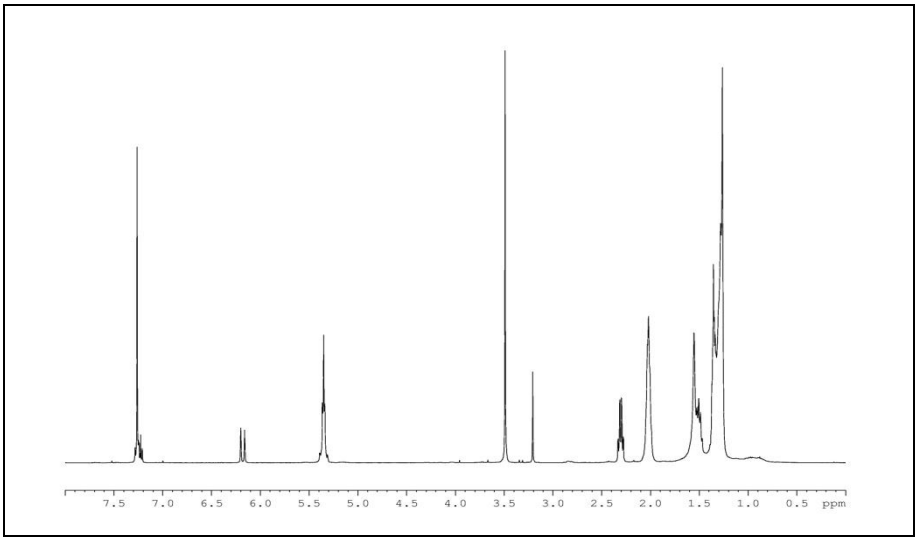


Figure 17: ^{1}H NMR spectrum of fulvyne 1 (**21**)

The presence in the IR spectrum of two bands at 1650 and 2098 cm^{-1} were attributed to a conjugated carbonyl group and of a triple bond, respectively. Moreover, the occurrence in the ^{13}C-NMR spectrum of the resonances at δ 177.8 (s, C-3 and C-44), 155.9 (d, C-5 and C-42), 131.9 (d, C-4 and C-43) and 78.8 (d, C-1 and C-46) suggested that fulvyne 1 contained two (*E*) 1-yn-3-oxo-4-ene moieties. These residues accounted for six of the nine degrees of unsaturation indicated by the molecular formula. The three remaining unsaturations were attributed to three *Z* double bonds as indicated by the ^{1}H NMR signal at δ 5.34 (6H, m) and by the ^{13}C NMR sp^2 signals at δ 130.0, 129.8, and 129.7. The three double bonds along the chain were located at C-19, C-25 and C-31 by analysis of the fragmentation in FABMS/MS.

Fulvyne 2 (**22**) had a molecular formula $C_{46}H_{74}O_2$, deduced from ESIMS [*m/z* 681 $(M+Na)^+$] and HRFABMS [*m/z* 665.6318 $(M+Li-H)^+$], suggesting one less unsaturation. The ^1H and ^{13}C NMR spectra of fulvyne 2 were very similar to those of fulvyne 1. The only difference was due to the presence of a carbinol function rather than the carbonyl group in one of the two terminal moieties of the molecule (Figure 18). In fact, the ^1H-^1H COSY spectrum of **22** disclosed a broad doublet at δ 4.83 (*J*=6.2) that correlated both with a double doublet at δ 5.60 (dd, *J*=15.7, 6.2 Hz), in turn coupled with the signal at δ 5.91 (dt, *J*=15.7, 7.3 Hz), and with a long range coupled alkyne signal at δ 2.56. This was consistent with an (*E*)-1-yn-3-hydroxy-4-ene-moiety (fragment **a**, Figure 19).

The presence of a hydroxy group was further confirmed by the IR band at 3300 cm^{-1}. Furthermore the ^{13}C NMR spectrum showed signals at δ 63.1 (C-44), 128.6 (C-43) and 134.9 (C-42) in agreement with the data reported in literature for petroformynes and fulvinol that possess the same terminus. The pattern of fragmentation observed in FABMS/MS of fulvyne 2 was the same as fulvyne 1 indicating the same position for the inner double bonds.

The ESIMS and HRFABMS spectra of fulvyne 3 (**23**) (ESIMS [*m/z* 683 $(M+Na)^+$] and HRFABMS [*m/z* 667.5205 $(M+Li-H)^+$]) suggested the molecular formula $C_{46}H_{76}O_2$. The ^1H NMR of compound **23** strongly resembled that of known fulvinol. However, analysis of NMR and FABMS/MS data suggested that it differed from

fulvinol in the location of the inner double bonds. In particular the same framework of signal in FABMS/MS - as in fulvynes 1-3 – aided us to located the inner double bonds as depicted in formula.

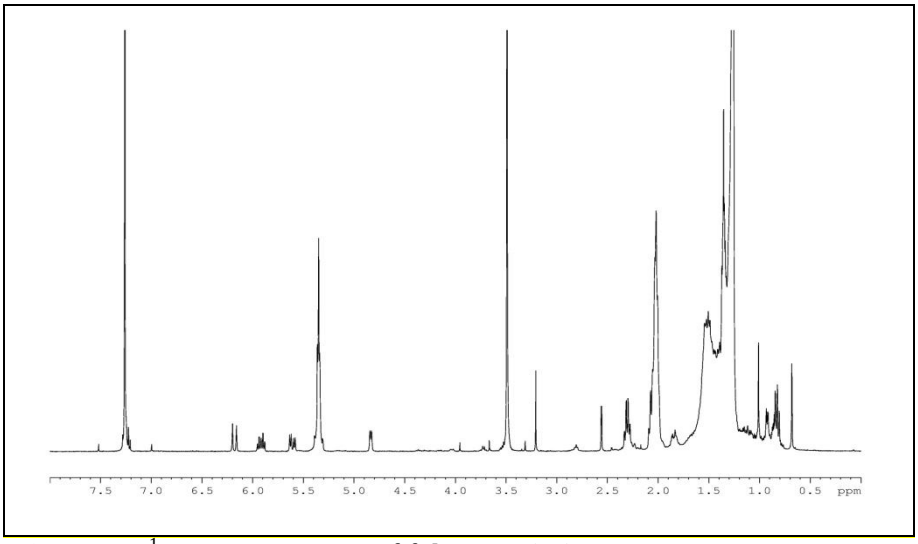


Figure 18: ^{1}H NMR spectrum of fulvyne 2 (**22**)

Fulvyne 4 (**24**) had the molecular formula $C_{46}H_{74}O_3$, as deduced from ESIMS [*m/z* 699 $(M+Na)^+$] and HRFABMS [*m/z* 683.5065 $(M+Li-H)^+$], suggesting the presence of an additional hydroxy group as well as a further unsaturation with respect to fulvyne 3. The ^{1}H NMR spectrum showed two terminal alkyne protons at δ 2.56 (d, *J*=2.3 Hz, H-1) and 2.58 (d, *J*=2.2 Hz, H-46) (Figure 20). The little difference observed in the chemical shift of the two protons was ascribed to the presence of an additional hydroxyl

group in one of the two terminal moieties as easily deduced by ^{1}H-^{1}H COSY experiment (fragment **b**, Figure 19).

Figure 19: fragments **a**-**b** in fulvynes

In particular, H-1 (δ 2.56) was long-range coupled to the alcoholic proton at δ 4.83 (br d, *J*=5.5 Hz, H-3) which was in turn correlated with an olefinic proton at δ 5.60 (dd, *J*=5.5, 15.0 Hz, H-4). This latter proton was connected to the vicinal olefinic proton at δ 5.92 (dt, *J*=6.4, 15.0, H-5) correlating with a methylene signal at δ 2.00 (fragment **a**). On the other hand, the alkyne proton at δ 2.58 (H-46) was long–range coupled with the signal at δ 4.90 (br d, *J*=4.5 Hz, H-44). This carbinolic methine was connected to the olefinic proton at δ 5.82 (dd, *J*=5.5, 15.4 Hz), which was coupled to the vicinal olefinic proton at δ 5.96 (dd, *J*=5.0, 15.4). This latter signal had a cross-peak with the alcoholic proton at δ 4.17 (H-41) that was in turn correlated with the methylene signal at δ 1.40 (fragment **b**). Analysis of the ^{13}C NMR spectrum and 2D NMR experiments confirmed the assignment of the two terminal moieties whereas the FABMS/MS suggested the position of the inner double bonds as depicted in structure **24**.

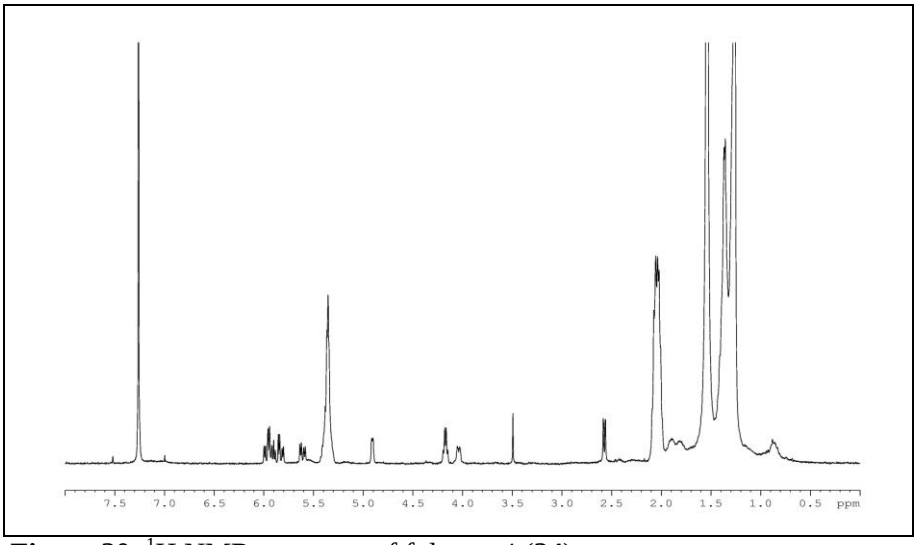


Figure 20: ^{1}H NMR spectrum of fulvyne 4 (**24**)

4.2.3. Stereochemical assignment

With the aim to establish the absolute configuration of fulvynes **1-4**, the Mosher method was applied on compound **23**. Fulvyne 3 was treated with (*R*)- and (*S*)-MTPA chlorides to get the (*S*) and (*R*)-MTPA esters **23a** and **23b**. The $\Delta\delta$ (δ_{Sester}-δ_{Rester}) values observed for the protons close to the hydroxyl groups at C-3 and C-44 indicated the *S* configuration of the two chiral centres (Figure 21).

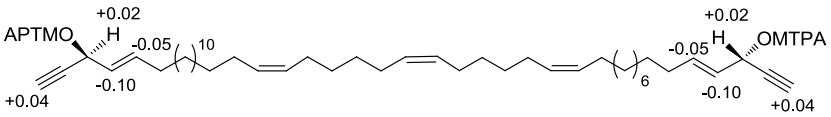


Figure 21: Chemical shifts differences $\Delta\delta$ (δ_{Sester}-δ_{Rester}) between (*S*)- and (*R*)-MTPA derivatives of compound **23**

Based on biogenetic considerations, the *S* configuration was suggested for the carbinol centres of the other co-occurring fulvynes.

4.2.4. Biological and ecological activities evaluation

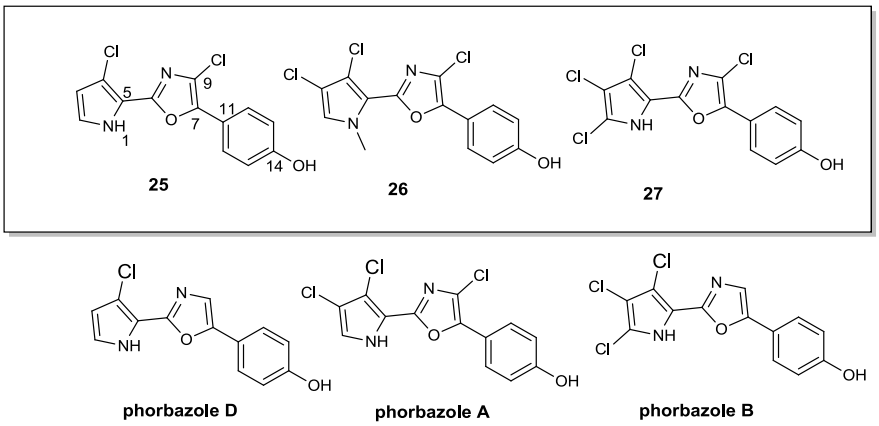
Sponge-derived acetylenes and polyacetylenes are widely recognized for their diverse and potent bioactivities. In fact, they exhibit potent antimicrobial, antiviral, cytotoxic, RNA-cleaving, and enzyme-inhibitory activities as well as brine-shrimp lethality.[79,82,117-127] In addition, some of these compounds have important ecological functions, including metamorphosis-inducing and antifouling effects against larvae of benthic invertebrates.[124]

The *in vitro* growth inhibitory activity of fulvyne 1-4 on various human cancer cell-lines is presently under investigation.

4.3. *Aldisa andersoni*

Aldisa andersoni is a dorid nudibranch that has never been studied to date. Chemical investigations have been conducted for related species of genus *Aldisa* resulting in the isolation of a steroid from *A. smaragdina*[128a] and from *A. sanguinea-cooperi.*[128b]

The nudibranch here analysed has been collected in the Indian Ocean. The chemical study has led to the isolation of three novel chlorinated phenylpyrrolyloxazoles, compounds **25**-**27**. Their structures were strongly reminiscent to known phorbazoles, metabolites previously isolated from the sponge *Phorbas aff. clathrata*[129], that were also detected in the mollusc, suggesting their dietary origin.



4.3.1. Isolation procedure

Because of their small size, 6 individuals of *A. andersoni* were not dissected, but first immersed in acetone and submitted to ultrasound vibration for 1 min. Only the metabolites present in the mantle (external part) were extracted by this procedure. The solvent was removed and the animals were homogenised with a pestle and

again treated with acetone, so as to extract the digestive gland content (internal part).

After filtration and evaporation of acetone in vacuum, the residues were subsequently extracted with Et_2O to obtain two ethereal extracts. The TLC chromatographic analysis showed similar secondary metabolite pattern for the two parts characterised by the presence of a series of UV-sensitive compounds. However, these molecules, clearly present in the digestive gland, were observed to be accumulated in the external part of the animal. Thus, the mantle extract (about 20 mg) was chromatographed on RP-HPLC (MeOH/H_20 gradient), to afford six pure compounds: phorbazoles D (0.7 mg), E (**25**, 4.9 mg), A (4.0 mg), B (2.5 mg), F (**26**, 1.1 mg) and G (**27**, 0.8 mg).

The identification of known phorbazoles A, B and D was made by comparison of their spectroscopic data (^1H and ^{13}C NMR) with the literature (see experimental part).[129]

4.3.2. Structure determination

Analysis of the NMR spectra of novel compounds **25-27**, which we named phorbazoles E, F and G, respectively, revealed a close structural relationship with known phorbazoles. Basically, the structural differences among them relies on the number and on the location of chlorine atoms.

Phorbazole E (**25**) had a molecular formula $C_{13}H_8Cl_2N_2O_2$ deduced by LCMS [*m/z* 295 $(M+H)^+$] and HRESIMS [*m/z* 316.9849 $(M+Na)^+$], that showed a proper cluster for two Cl atoms. The proton NMR spectrum of **25** exhibited only four sp^2 signals at δ 6.27 (d, *J*=2.8 Hz, 1H), 6.93 (d, *J*=8.7 Hz, 2H), 6.97 (d, *J*=2.8 Hz, 1H) and 7.81 (d, *J*=8.7 Hz, 2H), suggesting two separated spin systems. This implied that the two chlorine atoms were located in both the pyrrole and the oxazole rings. The ^{13}C NMR spectrum was more informative. Indeed, it contained 11 sp^2 carbon signals in the range of 111.8-159.6 ppm (Table 14). The two dimensional NMR experiments aided us to fully characterise the molecule. In particular, HMBC experiments, recorded with *J*=5 Hz and *J*=10 Hz, were crucial to attribute the quaternary carbons and to assign the structure as depicted in formula **25**.

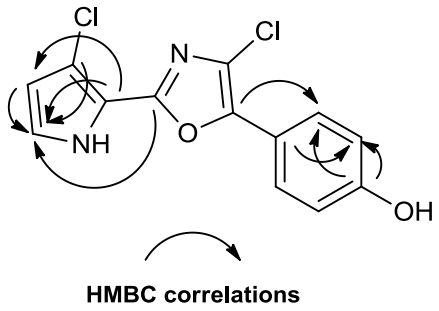


Table 14:

NMR spectroscopic data[a,b] in CD_3OD for phorbazoles E-G (**25**-**27**).

	25			**26**			**27**	
	δ_C, mult.	δ_H (*J* in Hz)	HMBC	δ_C, mult.	δ_H (*J* in Hz)	HMBC	δ_C, mult.	δ_H (*J* in Hz)
2	122.8, CH	6.97, d (2.8)	3	125.2, CH	7.10, s	CH_3-N1	125.2, CH	
3	111.8, CH	6.27, d (2.8)	2	114.7, C		2	114.7, C	
4	111.8, C		2	112.4, C		2	112.4, C	
5	115.8, C		3	115.3, C		CH_3-N1	115.3, C	
6	153.7, C			152.3, C			152.3, C	
8	144.2, C		12	144.2, C		12	144.2, C	
9	116.6, C			117.9, C			117.9, C	
11	119.4, C		13	119.1, C		13	119.1, C	
12,16	127.7, CH	7.81, d (8.7)	13	127.8, CH	7.81, d (8.7)	13	127.6, CH	7.81, d (8.7)
13.15	116.5, CH	6.93, d (8.7)	12	116.9, CH	6.93, d (8.7)	12	116.7, CH	6.93, d (8.7)
14	159.6, C		12,13	159.8, C		12,13	159.2, C	
CH_3-N1				38.4, CH_3	4.00, s			

[a] Bruker 300 MHz and 600 MHz, [b] assignement aided by HMBC

The LCMS analysis of phorbazole F (**26**) showed peaks at *m/z* 343 $(M+H)^+$ with the typical cluster due to the presence of three Cl atoms. The HRESIMS spectrum indicated the molecular formula $C_{14}H_9Cl_3N_2O_2$ as deduced from *m/z* 365.2582 [$(M+Na)^+$]. The NMR spectra of **26** showed proton and carbon resonances very similar to those of phorbazole A, indicating the same substitution pattern in the heterocycle rings. The only difference was in the presence of a 3H signal resonating at δ_H 4.0 (δ_C 38.4), which was attributed to a methyl linked at the pyrrole nitrogen. Thus phorbazole F was the 1-N-methyl phorbazole A.

Phorbazole G (**27**) was obtained in very small amount. The LCMS spectrum provided a protonated molecular peak at *m/z* 363

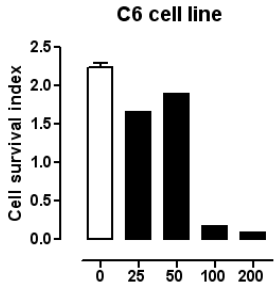
$(M+H)^+$ with a cluster consistent with four Cl atoms. Accordingly, the HRESIMS showed the molecular formula $C_{13}H_6Cl_4N_2O_2$ [*m/z* 384.9183 $(M+Na)^+$]. In agreement with MS data, the ^1H NMR spectrum exhibited only the two doublet signals due to the 4-hydroxyphenyl moiety, indicating that all other positions on the pyrrole and oxazole rings were substituted.

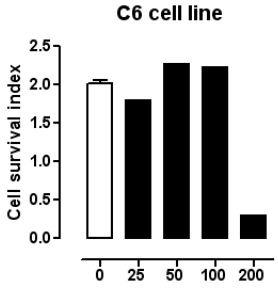
4.3.3. Biological and ecological activities evaluation

Even phorbazoles are most likely sequestered from the sponge diet, the selective accumulation of these molecules in the external part of the mollusc seems to suggest their involvement in the chemical defensive mechanisms of *A. andersoni*. Unfortunately, the small quantity available for each phorbazole metabolite prevented the performing of suitable ecological tests to demonstrate the possible defensive role of phorbazoles.

However, based on the consideration that ecologically relevant molecules often show different bioactivities, main compounds phorbazoles E and F (**25**, **26**) were tested on the cell lines C6 of glioma to evaluate the cytotoxic activity. The cell were treated for 48 h with different concentration of the metabolite (25, 50 , 100 and 200 μM), to obtain the following results:

Compounds **25** **26**





Phorbazole E (**25**) was active at a concentration 100 µM whereas phorbazole F (**26**) was not active.

CHAPTER 5: Experimental Section

5.1. General methods

5.1.1. Biological material

The biological material analyzed in this PhD work was sampled during different research campaigns. The samples were collected using SCUBA, within international collaborative programmes between the Institute of Biomolecular Chemistry of National Council Research of Pozzuoli and foreign Institutes. The material was collected at a depth between 0 and 40 m and suddenly frozen at -20 °C.

5.1.2. Extraction and isolation

The biological material was extracted following a standard procedure (figure 22). In particular, the sample was crushed and sonicated while submerged in a minimum volume of Me_2CO. The organic solvent was removed, filtered through paper, then evaporated under reduced pressure to give an aqueous residue which was then partitioned firstly with Et_2O and secondly with BuOH. The organic layers were removed, dried with anhydrous Na_2SO_4, and concentrated under reduced pressure to give the crude extracts.

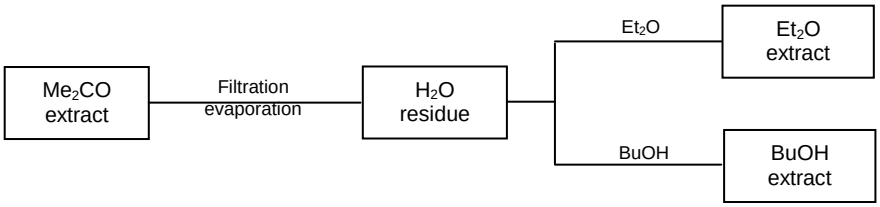


Figura 22: Extraction procedure

Each extract was analyzed by Thin Layer Chromatography (TLC) and ^{1}H NMR. Thin layer chromatography (TLC) was performed by spotting solutions of analyte onto TLC plates using a micropipette. After sufficient time to allow the analyte to dry, the TLC plate was placed into a sealed chamber containing the mobile phase. Upon completion of development, the TLC plate was removed and visualized under 245 nm UV light, then stained using ceric sulfate stain. The stained plates were visualized after heating via a hot air stream. Normal phase thin layer chromatography (NP-TLC) analyses were performed using precoated, glass-backed silica gel plates (Kieselgel 60 F_{254}, 0.25 mm thickness, Merck). Reversed phase thin layer chromatography (RP-TLC) analysis were performed using C18-modified precoated, glass-backed silica gel plates (RP-18 F_{254}, 0.25 mm thickness, Merck).

Thus the extracts were separately fractioned by the suitable chromatographic techniques. Silica-gel chromatography was performed using pre-coated Merck F_{254} plates and Merck Kieselgel 60 powder. Sephadex LH-20 was from Amersham Pharmacia Biotech (Uppsala, Sweden). HPLC separation was performed using a

Shimadzu liquid chromatograph LC-10AD equipped with an UV SPD-10A wavelength detector or HPLC Waters 501 pumps with a refractometer detector. Analytical (250 x 4.6 mm) and semipreparative (250 x 10 mm) columns (Kromasil C18 and Supelco RP-amide) used in RP-HPLC were from Phenomenex and Supelco.

5.1.3. Spectroscopic data

Optical rotations were measured on a Jasco DIP 370 digital polarimeter. IR spectra were recorded on a Biorad FTS 155 FT-IR spectrophotometer. NMR experiments were conducted at ICB-NMR Service Centre. 1D and 2D NMR spectra were acquired in $CDCl_3$ or CD_3OD, DMSOd_6 (shifts are referenced to the solvent signal) on a Bruker Avance-400 operating at 400 MHz, using an inverse probe fitted with a gradient along the Z-axis and a Bruker DRX-600 operating at 600 MHz, using an inverse TCI CryoProbe fitted with a gradient along the Z-axis.^{13}C NMR spectra were obtained on a Bruker DPX-300 operating at 300 MHz using a dual probe and on Bruker DRX-600MHz. High and low resolution LR and HRESIMS were recorded on a Micromass Q-TOF MicroTM coupled with a HPLC Waters Alliance 2695. The instrument was calibrated by using a PEG mixture from 200 to 1000 MW (resolution specification 5000 FWHM, deviation <5 ppm RMS in the presence of a known lock mass). GC-MS was carried out on an ion-trap MS instrument in EI mode (70eV) (Thermo, Polaris Q) connected with a GC system (Thermo, GCQ) by

a 5% diphenyl (30 m x 0.25 mm x 0.25 um) column using helium as gas carrier.

5.2. Experimental section for Chapter 1

5.2.1. *Alcyonium antarcticum*

5.2.1.1. Biological material: The soft coral *Alcyonium antarcticum* was collected in January 2002 during the XVII Italian Campaign in Antarctica off Terra Nova Bay (Stazione M. Zucchelli) and immediately frozen and transferred to ICB. The soft coral was classified by Dr Stefano Schiapparelli (Dipartimento per lo Studio del Territorio e delle sue Risorse, University of Genova). A voucher specimen is stored at ICB under the code AlcyAA.

5.2.1.2. Extraction and isolation: The frozen *A. antarcticum* (dry weight, 112 g) was chopped and then extracted exhaustively with Me_2CO (400 mL x 4) using ultrasound. After filtration and evaporation in vacuo of the organic solvent, the residue was subsequently extracted with Et_2O (200 mL x 4) and n-BuOH (100 mL x 4). The evaporation of Et_2O and n-BuOH extracts gave two oily residues (3.8 g and 2.2 g, respectively). The ethereal extract was subjected to silica-gel column chromatography using as eluent a gradient of light petroleum and Et_2O, $CHCl_3$ and finally MeOH to give five fractions: fr. I (550 mg), fr. II (20 mg), fr. III (890 mg), fr. IV (810 mg), and fr. V (400 mg). Fraction I was subjected to silica gel column purification (light petroleum/diethyl ether gradient) to give the known 4-methyl-2-[(E)-2-methyl-6-methyleneocta-2,7-dienyl]-furan (50 mg) and deacetoxy-alcyonicene (**2**, 0.5 mg). Fr. II was further purified by HPLC n-phase (n-hexane/EtOAc, 95:5, Kromasil analytical column, flow rate 1 mL/min) to give pure alcyonicene (**1**, 6 mg). Fr III was purified by silica-gel column chromatography yielding 45 mg of pregnenolone-3-acetate. Fr.IV gave, after filtration on a Sephadex LH-20 column, pregnenolone (1.0 mg). Finally, the more polar fraction (V) was subjected to silica-gel column chromatography

and TLC to afford pure pukalide (7.0 mg). The known compounds, 4-methyl-2-[(*E*)-2-methyl-6-methyleneocta-2,7-dienyl]-furan, 3-acetylpregnenolone, pregnenolone and pukalide were identified by comparison of the spectroscopic data (^{1}H NMR, ^{13}C NMR, MS and $[\alpha]_D$) with those reported in the literature.

5.2.1.3. Biological assays: Ichthyotoxicity tests against the mosquito fish, *Gambusia affinis* (Baird & Girard, 1853), were conducted according to literature procedures.[36] All the isolated metabolites were assayed at 10 ppm. Feeding-deterrence tests against gold fish, *Carassius auratus*, were conducted according to literature procedures.[35] All the compounds were assayed at 50 μg/cm2.
The antimicrobial activity against *Escherichia coli* DH5a and *Staphylococcus aureus* ATCC6538P was determined. Activity was studied by the broth microdilution method.[38] The test was carried out in a 96-well, flat-bottomed, microtitration plate. Plates were incubated at 37°C without shaking, and reading was performed following 24 h of incubation by optical density (OD) determination with a spectrophotometer; plates were shaken for 5 min and the OD values at 492 nm of each well were read with a microtiter plate reader (Thermolabsystems Multiskan MK3). All experiments were performed in triplicate at concentration of 100 μg/mL.

Alcyonicene (**1**)**:** 6 mg (0.16%), $[\alpha]_D$= +33.9 (*c* 0.6, $CHCl_3$), R_f : 0.5 (light petroleum ether -Et_2O, 95:5), IR (liquid film): 3100, 2938, 2878, 1731, 1645, 1445, 1245, 892 cm^{-1}, ^{1}H NMR and ^{13}C NMR: Table 3, HRMS-ESI: *m/z* [M + Na^+] calcd for $C_{17}H_{26}O_2Na$: 285.1831; found: 285.1824.

Deacetoxy-alcyonicene (**2**): 0.5 mg (0.013%), $[\alpha]_D$= -2.8 (*c* 0.05, $CHCl_3$), R_f : 0.1 (light petroleum ether), IR (liquid film): 3100, 2938, 2878, 1645, 1445, 1245, 892 cm^{-1}, GC-EIMS (EI, 70 eV): *m/z* (%) = 204.2 [M^+] (10), 189.3 (20).

5.2.2. *Parazoanthus axinellae*

5.2.2.1. Biological material: The sea anemone *Parazoanthus axinellae* was collected in June 2008 from Prof. Vassilios Roussis and assistants. A voucher specimen is stored at the University of Athens under the code PAX.

5.2.2.2. Extraction and isolation: The dry *P. axinellae* (118 g) was extracted with CH_2Cl_2/MeOH 1:1 (500 mL x 3). After filtration and evaporation *in vacuo* of the organic solvent, half of the extract (10 g) was subjected to Vacuum Liquid Cromatography (VLC), using reverse phase silica-gel column chromatography and a gradient of H_2O and MeOH to give 13 fractions. Half of Fraction I (1 g) was subjected to Medium Pressure Liquid Cromatography (MPLC), using reverse phase silica gel column and a linear gradient of H_2O(+0.1% TFA)/MeOH from 9:1 to 4:6 in 240 min, and finally H_2O(+0.1% TFA)/MeOH 2:8 to give 12 fraction. Fr. X was further purified by HPLC RP-phase (H_2O/MeOH/TFA, from 24.9:75:0.1 to 49:50:0.1 in 50 minutes, RP-amide semipreparative column, flow rate 2 mL/min) to give pure parazoanthine F (**3**, 1.3 mg) and G (**4**, 1.6 mg), along with parazoanthines A-E. The known compounds were identified by comparison of the spectroscopic data (^{1}H NMR, ^{13}C NMR, MS and $[\alpha]_D$) with those reported in the literature.

5.2.2.3. Biological assays: The antiproliferative activity were tested *in vitro* on seven human cancer cell lines (Hs683, U373, U251, A549, MCF7, SKMEL28, PC3), using the MTT colorimetric assay.
The *in vitro* Scratch Wound Assay were performed using different cell lines. Mouse B16F10 melanoma, and human A549 non-small-cell lung cancer (NSCLC) and Hs683 glioma cells were grown until confluence and then a scratch has been performed as detailed in Mathieu V *et al.* 2005.[48] The cancer cells have been cultured in presence of 10% fetal calf serum. The % of the wound healing process has been quantitatively determined as detailed in Mathieu V *et al.* 2005.[48] All experimental conditions were performed as tetraplicates, excepted for A549 NSCLC cells that was performed in triplicates.

The antimicrobial activity against *Escherichia coli* DH5a and *Staphylococcus aureus* ATCC6538P was determined. Activity was studied by the broth microdilution method.38 The test was carried out in a 96-well, flat-bottomed, microtitration plate. Plates were incubated at 37°C without shaking, and reading was performed following 24 h of incubation by optical density (OD) determination with a spectrophotometer; plates were shaken for 5 min and the OD values at 492 nm of each well were read with a microtiter plate reader (Thermolabsystems Multiskan MK3). All experiments were performed in triplicate at concentration of 100 μg/mL

Parazoanthine F (3): 1.3 mg, UV (MeOH) λ_{max} (log ε) 210 (7.40), 281 (7.41); ^{1}H NMR and ^{13}C NMR in CD_3OD: Table 4, HRMS-ESI: *m/z* $[M + H]^+$ calcd for $C_{15}H_{20}N_5O_3$: 318,1488; found: 318,1555.

Parazoanthine G (4): 1.6 mg, $[\alpha]_D$= +11.4 (*c*, CH_3OH), ^{1}H NMR and ^{13}C NMR in CD_3OD: Table 4, HRMS-ESI: *m/z* $[M + H]^+$ calcd for $C_{16}H_{22}N_5O_3$: 332,3696; found: 332,2881.

5.3 Experimental section for Chapter 2

5.3.1. *Pseudodistoma crucigaster*

5.3.1.1. Biological material: The colonial ascidian *P. crucigaster* Gaill, 1972 was collected in October 2007 off Cala Sgombro (inside part) along the Sardinia coasts by G.V. at a depth of -10 m, immediately frozen and transferred to ICB. The ascidian was classified by Dr. Flavia Greco of Zoologic Department of University of Bari. A voucher specimen is stored at ICB under the code ATC.

5.3.1.2. Extraction and isolation: The frozen ascidian *P. crucigaster* (dry weight, 5.2 g) was chopped and then extracted exhaustively with Me_2CO (400 mL×4) using ultrasound. After filtration and evaporation *in vacuo* of the organic solvent, the residue was subsequently extracted with Et_2O (200 mL×4) and BuOH (100 mL×4). The evaporation of Et_2O and BuOH extracts gave two gummy residues (0.783 g and 0.976 g, respectively). The ethereal extract was subjected

to a silica gel column chromatography using as eluent a gradient of light petroleum ether and Et_2O, $CHCl_3$ and finally MeOH to give eight fractions. Preliminary ^1H NMR analysis of these fractions evidenced that the last fraction (0.207 g) contained a mixture of some interesting compounds. Part of this fraction (0.120 g) was first acetylated with acetic anhydride in pyridine (2 h, room temperature) to prevent degradation of the compounds and then subjected to reverse-phase HPLC column chromatography ($MeOH/H_2O$, 8:2; flow 1 mL/min) to afford in order of decreasing polarity diacetyl crucigasterin A (**5a**) (t_R 9.6 min, 1.4 mg), B (**6a**) (t_R 11.2 min, 1.9 mg), C (**7a**) (t_R 15.2 min, 5.4 mg), D (**8a**) (t_R 25.0 min,3.3 mg), E (**9a**) (t_R 34.0 min, 2.1 mg).

5.3.1.3. Preparation of oxazolidinone derivative (11): In order to prepare the oxazolidinone derivative of crucigasterin A (**5**) the diacetyl derivative **5a** (1 mg) was treated with NaOH (4 M) at 70 °C for 20 h. The free aminoalcohol (0.8 mg, **5**) obtained from this reaction was then reacted with 1,10-carbonyldiimidazole (3 mg) in 1 mL of CH_2Cl_2 and 100 mL of DMF at 0 °C for 19 h under argon atmosphere. The solution was extracted with water and the organic layer was dried under nitrogen yielding 0.5 mg of the oxazolidinone **11**.

5.3.1.4. Preparation of MTPA esters: Diacetyl crucigasterin **10a** (2.1 mg) was treated with Na_2CO_3 in anhydrous MeOH and stirred at room temperature overnight. After filtration and evaporation of the solvent, the reaction product (**10b**) was divided into two parts, which in turn were reacted with *R*-(-)-MTPA chloride and *S*-(+)-MTPA chloride, to give the *S*-(**10c**) and *R*-MTPA-(**10d**)-esters, respectively.
S-MTPA ester was prepared by treating 1 mg of **10b** with 0.005 mL of *R*-(-)-MTPA chloride in dry CH_2Cl_2 (0.5 mL) with catalytic amount of DMAP under stirring for 16 h at room temperature. The ester was purified by chromatography in a Pasteur pipette (SiO_2, $CHCl_3$).
R-MTPA ester was prepared by treating 1 mg of **10b** with 0.005 mL of *S*-(+)-MTPA chloride in dry CH_2Cl_2 (0.5 mL) with catalytic amount of DMAP under stirring for 16 h at room temperature. The

ester was purified by chromatography in a Pasteur pipette (SiO_2, $CHCl_3$).

5.3.1.5 Biological assays: Antifungal assay was performed by the broth macrodilution method following the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS) document M27-P.26,27 The medium used to prepare the 10× drug dilutions and the inoculums suspension was liquid RPMI 1640 with L-glutamine (Sigma Aldrich), 0.165 M morpholinopropane sulfonic acid (MOPS) and 2% glucose (pH 7.0).28,29 The yeast suspension was adjusted with the aid of a spectrophotometer to a cell density of 0.5 McFarland (2×108 CFU/ mL) standard at 530 nm and diluted to 1:4000 (50,000 CFU/mL) in RPMI 1640 broth medium. The yeast inoculum (0.9 mL) was added to each test tube that contained 0.1 mL of 10 twofold dilutions (256×0.05 mg/mL final) of each compound. Broth macrodilution MICs were determined after 48 h of incubation at 35 °C. MIC was defined as the lowest concentration of the compound that completely inhibited the growth of the test organism. The antibacterial assay was performed by using the same method as the antifungal test, only differing in the assay medium (Luria Bertani medium: 10 g/L bactotryptone, 5 g/L bactoyeast, and 10 g/L NaCl, pH 7.5) and in the incubation temperature (37 °C for 24 h). Crucigasterin B (**6**) and E (**9**) were tested for antifungal and antibacterial activities, exhibiting moderate activity against *E. coli* and *C. albicans*, respectively.

Diacetyl crucigasterin A (**5a**). Colourless oil; $[\alpha]_D$ -24.7 (c 0.1, $CHCl_3$); $[\alpha]_D$ -20.3 (c 0.03, CH_3OH); UV (MeOH) λ_{max} (3) 230 (10,216); IR (liquid film) ν_{max} 3418, 2925, 2830, 1715 cm^{-1}; ^1H and ^{13}C NMR data in Tables 5 and 6; HRESIMS *m/z* 330.2042 (M+Na), calcd for $C_{18}H_{29}NO_3Na$ 330.2045.

Diacetyl crucigasterin B (**6a**). Colourless oil, $[\alpha]_D$ -16.8 (c 0.2, $CHCl_3$); IR (liquid film) ν_{max} 3425, 2926, 2837, 1741, 1718, 1651 cm^{-1}; ^1H and ^{13}C NMR data in Tables 5 and 6; HRESIMS *m/z* 332.2204 (M+Na), calcd for $C_{18}H_{31}NO_3Na$ 332.2202.

Diacetyl crucigasterin C (**7a**). Colourless oil, $[\alpha]_D$ -28.0 (c 0.1, $CHCl_3$); IR (liquid film) ν_{max} 3425, 2926, 2837, 1744, 1718, 1643 cm^{-1}; ^1H and ^{13}C NMR data in Tables 5 and 6; HRESIMS *m/z* 382.2341 (M+Na), calcd for $C_{22}H_{33}NO_3Na$ 382.2358.

Diacetyl crucigasterin D (**8a**). Colourless oil; $[\alpha]_D$ -12.1 (c 0.3, $CHCl_3$); IR (liquid film) ν_{max} 3425, 2926, 2854, 1744, 1718 cm^{-1}; ^1H and ^{13}C NMR data in Tables 5 and 6; HRESIMS *m/z* 360.2518 (M+Na), calcd for $C_{20}H_{35}NO_3Na$ 360.2515

Diacetyl crucigasterin E (**9a**). Colourless oil; $[\alpha]_D$ -15.2 (c 0.2, $CHCl_3$); IR (liquid film) ν_{max} 3423, 2936, 2861, 1744, 1718cm^{-1}; ^1H and ^{13}C NMR data in Tables 5 and 6; HRESIMS *m/z* 386.2686 (M+Na), calcd for $C_{22}H_{37}NO_3Na$ 386.2671.

Oxazolidinone (**11**): ^1H NMR ($CDCl_3$) δ 6.12 (dd, J=15.3, 10.5 Hz, H-6), 6.00 (dd, J=15.3, 10.5 Hz, H-7), 5.80 (ddt, J=16.4, 10, 6.5 Hz, H-13), 5.65 (ddd, J=15.3, 7.0, 7.0 Hz, H-5), 5.50 (ddd, J=15.3, 7.0, 7.0 Hz, H-8), 5.0 (br d, J=17 Hz, H-14a), 4.95 (br d, J=10 Hz, H-14b), 4.17 (m, H-3), 3.62 (m, H-2), 2.48 (m, H_2-4), 2.05 (m, H_2-9 and H2-12), 1.38 (m, H2-10 and H_2-11), 1.28 (d, J=6 Hz, H_3-1); ESIMS *m/z* 272 $(M+Na)^+$; HRESIMS *m/z* 272.1628 (M+Na), calcd for $C_{15}H_{23}NO_2Na$ 272.1621.

Compound 10a. Colourless oil, $[\alpha]_D$-19.1 (c 0.12, $CHCl_3$); IR (liquid film) ν_{max} 3425, 2926, 2837, 1740, 1718 cm^{-1}; ^1H and ^{13}C NMR data in Tables 5 and 6; HRESIMS *m/z* 332.2204 (M+Na), calcd for $C_{18}H_{31}NO_3Na$ 332.2202.

Compound 10c (*S*-MTPA ester): *S*-MTPA ester was prepared by treating 1 mg of **10b** with 0.005 mL of *R*-(-)-MTPA chloride in dry CH_2Cl_2 (0.5 mL) with catalytic amount of DMAP under stirring for 16 h at room temperature. The ester was purified by chromatography in a Pasteur pipette (SiO_2, $CHCl_3$). Selected ^1H NMR values ($CDCl_3$): δ 5.81 (H-13), 5.49 (H-6), 5.25 (H-5), 5.00 (H-3), 4.98 (H-14a), 4.96

(H-14b), 4.33 (H-2), 2.28 (H_2-4), 2.00 (H_2-7 and H_2-12), 1.95 ($NHCOCH_3$), 1.30 (H_2-8, H_2-9, H_2-10 and H_2-11), 1.12 (H_3-1).

Compound 10d (*R*-MTPA ester): *R*-MTPA ester was prepared by treating 1 mg of 10b with 0.005 mL of *S*-(+)-MTPA chloride in dry CH_2Cl_2 (0.5 mL) with catalytic amount of DMAP under stirring for 16 h at room temperature. The ester was purified by chromatography in a Pasteur pipette (SiO_2, $CHCl_3$). Selected ^1H NMR values ($CDCl_3$): δ 5.81 (H-13), 5.53 (H-6), 5.32 (H-5), 5.07 (H-3), 4.98 (H-14a), 4.96 (H-14b), 4.32 (H-2), 2.34 (H_2-4), 1.92 ($NHCOCH_3$), 1.30 (H_2-8, H_2-9, H_2-10 and H_2-11), 1.04 (H_3-1).

5.4 Experimental section for Chapter 3

5.4.1. *Haliclona fulva*

5.4.1.1. Biological material: The sponge *H. fulva*, was collected in May 2009 in Punta Pizzaco, Procida Island (Naples) by Dr. G. Villani of ICB at a depth of -40 m, and transferred to ICB. A voucher specimen is stored at ICB under the code FUL. Three specimens of *P. atromaculata* found grazing on *H. fulva* were collected as well and transferred to ICB under the code DA.

5.4.1.2. Extraction and isolation: The sponge *H. fulva* (dry weight, 42 g) was chopped and then extracted exhaustively with Me_2CO (500 mL×6) using ultrasound. After filtration and evaporation of the organic solvent in vacuo, the residue was subsequently extracted with Et_2O (500 mL×7) and BuOH (650 mL×2). The evaporation of Et_2O and BuOH extracts gave two gummy residues (1.46 g and 2.48 g, respectively). Part of the butanolic extract (1.2 g) was subjected to a Sephadex LH-20 column chromatography (35×900 mm) using as eluent MeOH, to give eight fractions (I-VIII). Preliminary ^1H NMR analysis of these fractions showed that fraction III (0.228 g) contained a mixture of some interesting compounds. An aliquot of this fraction (0.120 g) was further purified on RP-Amide semipreparative HPLC

column (Supelco, 250 mm×10 mm, 5 mm) with a gradient of H_2O/MeOH/TFA (from 29:70:0.1 to 100% MeOH, flow 2.0 mL min^{-1}) to afford pure compounds **12** (t_R 28.2 min, 4.7 mg), **13** (t_R 31.5 min, 3.1 mg), **14** (t_R 33.0 min, 11.5 mg), **15** (t_R 39.2 min, 4.2 mg), **16** (t_R 41.0 min, 8.7 mg), **17** (t_R 50.0 min, 5.4 mg), **18** (t_R 53.8 min, 5.6 mg), **19** (t_R 55.2 min, 2.4 mg) and **20** (t_R 57.0 min, 2.9 mg).

5.4.1.3. Biological assays: Bacterial strain of *B. subtilis*, chloramphenicol-resistant (PY79), *E. coli* (DH5a) and *C. albicans* were used in the antibacterial tests. The bacteria were grown overnight, diluted 1:1000 in 10 mM sodium phosphate buffer (pH 7.5), and incubated with increasing concentrations of different compounds at a density of 4000 colony forming units (CFUs) per mL. After 4 h at 37 °C, serial dilutions of each proteinebacteria mix were prepared and plated on chloramphenicol (5 μg/mL) containing medium, and colonies formed after each treatment were determined. For each experiment, carried out in duplicate, triplicate assays were performed.

Fulvyne A (12): 4.7 mg, $[\alpha]_D$ +8.2 (c 0.3, CH_3OH); UV (MeOH) λ_{max} (log ε) 205 (3.38); ν_{max} (liquid film) 3309, 2925, 2854, 2237, 1696, 1682, 1593, 1459, 1383, 1205, 1142, 1075, 1020, 971 cm^{-1}; ^1H and ^{13}C NMR data in Tables 7 and 8; ESIMS *m/z* 851 $[M+Na]^+$ and 873 $[M+2Na-H]^+$, positive ion HRESIMS $[M+Na]^+$ *m/z* 851.4862 (calcd for $C_{47}H_{72}O_{12}Na$ 851.4921);

Fulvyne B (13): 3.1 mg, $[\alpha]_D$ +5.0 (c 0.22, CH_3OH); UV (MeOH) λ_{max} (log ε) 206 (3.40); ν_{max} (liquid film) 3400, 2924, 2854, 2237, 1699, 1680, 1559, 1457, 1207 cm^{-1}; ^1H and ^{13}C NMR data in Tables 7 and 8; ESIMS *m/z* 851 $[M+Na]^+$ and 873 $[M+2Na-H]^+$, positive ion HRESIMS $[M+Na]^+$ *m/z* 851.4824 (calcd for $C_{47}H_{72}O_{12}Na$ 851.4921);

Fulvyne C (14): 11.5 mg, $[\alpha]_D$ +16.8 (c 0.5, CH_3OH); UV (MeOH) λ_{max} (log ε) 205 (3.40); ν_{max} (liquid film) 3309, 2933, 2848, 2237, 1696, 1679, 1574, 1459, 1383, 1205, 1142, 1075, 1020, 971 cm^{-1}; ^1H and ^{13}C NMR data in Tables 7 and 8; ESIMS *m/z* 851 $[M+Na]^+$ and

873 $[M+2Na-H]^+$, positive ion HRESIMS $[M+Na]^+$ *m/z* 851.4879 (calcd for $C_{47}H_{72}O_{12}Na$ 851.4921);

Fulvyne D (15): 4.2 mg, $[\alpha]_D$ +3.4 (c 0.4, CH_3OH); UV (MeOH) λ_{max} (log ε) 205 (3.42); ν_{max} (liquid film) 3307, 2924, 2854, 2233, 1696, 1682, 1455, 1384, 1206, 1144, 1073, 1017, 972 cm^{-1}; ^1H and ^{13}C NMR data in Tables 7 and 8; ESIMS *m/z* 835 $[M+Na]^+$, 857 $[M+2Na-H]^+$, 873 $[M+Na+K-H]^+$, positive ion HRESIMS $[M+Na]^+$ *m/z* 835.4865 (calcd for $C_{47}H_{72}O_{11}Na$ 835.4972);

Fulvyne E (16): 8.7 mg, $[\alpha]_D$ +9.4 (c 0.19, CH_3OH); UV (MeOH) λ_{max} (log ε) 206 (3.36); ν_{max} (liquid film) 3306, 2923, 2853, 2233, 1703, 1682, 1585, 1460, 1379, 1205, 1074, 1017, 970 cm^{-1}; ^1H and ^{13}C NMR data in Tables 7 and 8; ESIMS *m/z* 835 $[M+Na]^+$, 851 $[M+K]^+$, 857 $[M+2Na-H]^+$, 873 $[M+Na+K-H]^+$, positive ion HRESIMS $[M+Na]^+$ *m/z* 835.4865 (calcd for $C_{47}H_{72}O_{11}Na$ 835.4972);

Fulvyne F (17): 5.4 mg, $[\alpha]_D$ +6.4 (c 0.38, CH_3OH); UV (MeOH) λ_{max} (log ε) 205 (3.40); ν_{max} (liquid film) 3309, 2925, 2854, 2237, 1696, 1676, 1593, 1383, 1205, 1075, 971 cm^{-1}; ^1H and ^{13}C NMR data in Tables 10 and 9; ESIMS *m/z* 835 $[M+Na]^+$, 857 $[M+2Na-H]^+$, 874 $[M+Na+K]^+$, positive ion HRESIMS $[M+Na]^+$ *m/z* 835.4843 (calcd for $C_{47}H_{72}O_{11}Na$ 835.4972);

Fulvyne G (18): 5.6 mg; $[\alpha]_D$ +7.2 (c 0.31, CH_3OH); UV (MeOH) λ_{max} (log ε) 205 (3.38); ν_{max} (liquid film) 3310, 2920, 2851, 2237, 1701, 1683, 1560, 1459, 1379, 1020, 1074, 1017, 970 cm^{-1}; ^1H and ^{13}C NMR data in Tables 10 and 9; ESIMS *m/z* 835 $[M+Na]^+$, 857 $[M+2Na-H]^+$, 875 $[M+Na+K+H]^+$, positive ion HRESIMS $[M+Na]^+$ *m/z* 835.4865 (calcd for $C_{47}H_{72}O_{11}Na$ 835.4972).;

Fulvyne H (19): .4 mg, $[\alpha]_D$ +12.1 (c 0.24, CH_3OH); UV (MeOH) λ_{max} (log ε) 206 (3.40); ν_{max} (liquid film) 3307, 2924, 2854, 2233, 1683, 1588, 1374, 1206, 1073, 1017, 971 cm^{-1}; ^1H and ^{13}C NMR data in Tables 10 and 9; ESIMS *m/z* 819 $[M+Na]^+$, 841 $[M+2Na-H]^+$, 857

$[M+Na+K-H]^+$, positive ion HRESIMS $[M+Na]^+$ *m/z* 819.4990 (calcd for $C_{48}H_{76}O_9Na$ 819.5387);

Fulvyne I (20): 2.9 mg , $[\alpha]_D$ +8.6 (c 0.38, CH_3OH); UV (MeOH) λ_{max} (log ε) 205 (3.42); ν_{max} (liquid film) 3295, 2919, 2850, 237, 1702, 1683, 1459, 1370, 1080, 1015, 953 cm^{-1}; ^1H and ^{13}C NMR data in Tables 10 and 9; ESIMS *m/z* 819 $[M+Na]^+$, 841 $[M+2Na-H]^+$, positive ion HRESIMS $[M+Na]^+$ *m/z* 819.4917 (calcd for $C_{47}H_{72}O_{10}Na$ 819.5023).

5.5. Experimental section for Chapter 4

5.5.1 *Placida dendritica*

5.5.1.1. Biological material: 86 speciments of *P. dendritica* were collected in November 2008 in the Indo-Pacific Ocean by Dr. G. Villani, and transferred to ICB. The molluscs were identified by Dr. Juan Lucas Cervera (University of Cadiz). A voucher specimen is stored at ICB under the code MA02.

5.5.1.2. Extraction and isolation: The frozen molluscs *P. dendritica* have been treated in order to get extracts of the mantle and the internal glands, separately. In particular, the mantle extract was obtained simply by soaking the whole animals in acetone bath for few minutes, whereas the internal glands extract was obtained by grinding the animal after sonication. After filtration and evaporation *in vacuo* of the organic solvent, the two residues were subsequently extracted with Et_2O (50 mL×4). The evaporation of Et_2O gave two gummy residues (56 mg and 37 mg for the mantle and the gland extract respectively). The mantle extract was fractionated on silica gel column by eluting with a gradient system of light petroleum ether/Et_2O, and finally $CHCl_3$/MeOH 7:3. The fractions containing polypropionates were purified on reverse-phase HPLC column chromatography with a linear gradient of MeOH/H_2O 80:20 until MeOH in 30 minutes (flow 1 mL/min) to afford pure cyercene 2-4.

5.5.2. *Peltodoris atromaculata*

5.5.2.1. Biological material: Three speciments of *P. atromaculata* were collected in May 2009 in Punta Pizzaco, Procida Island (Naples) by Dr. G. Villani at a depth of -40 m. The nudibranch was identified by Dr. G. Villani. A voucher specimen is stored at ICB under the code DA.

5.5.2.2. Extraction and isolation: The frozen molluscs *P. atromaculata* were dissected into internal glands and mantle. The two extract were extracted separately with Me_2CO. After filtration and evaporation *in vacuo* of the organic solvent, the residues were subsequently extracted with Et_2O (20 mL×4). The evaporation of Et_2O gave two gummy residues (15 mg and 62 mg for the mantle and the gland extract respectively). This latter was chromatographed on a Sephadex LH-20 column in $CHCl_3$/MeOH, and then further fractionated on silica gel column by eluting with a gradient system of light petroleum ether/Et_2O, $CHCl_3$ and finally MeOH to give eight fractions. Preliminary ^{1}H NMR analysis revealed the presence of a mixture of polyacetilenes that was further purified on reverse-phase HPLC column chromatography (MeOH; flow 1 mL/min) to afford in order of decreasing polarity fulvyne 1 (**21**, 1.4 mg), 2 (**22**, 1.9 mg), 3 (**23**, 5.4 mg), 4 (**24**, 3.3 mg).

5.5.2.3. Preparation of MTPA esters: Fulvyne 3 (**23**) was divided into two parts, which in turn were reacted with *R*-(-)-MTPA chloride and *S*-(+)-MTPA chloride, to give the *S*-(**23a**) and *R*-MTPA-(**23b**)-esters, respectively.
S-MTPA ester was prepared by treating 1 mg of **23** with 0.005 mL of *R*-(-)-MTPA chloride in dry CH_2Cl_2 (0.5 mL) with catalytic amount of DMAP under stirring for 16 h at room temperature. The ester was purified by chromatography in a Pasteur pipette (SiO_2, $CHCl_3$).
R-MTPA ester was prepared by treating 1 mg of **23** with 0.005 mL of *S*-(+)-MTPA chloride in dry CH_2Cl_2 (0.5 mL) with catalytic amount of DMAP under stirring for 16 h at room temperature. The ester was purified by chromatography in a Pasteur pipette (SiO_2, $CHCl_3$).

Fulvyne 1 (**21**). Colourless oil; UV (MeOH) λ_{max} (log ε) 244 (3.56); IR (liquid film) ν_{max} 2925, 2854, 2098, 1650, 1455, 1232 cm^{-1}; ESIMS *m/z* 679 $[M+Na]^+$ and HRFABMS *m/z* 663.5320 $[M+Li-H]^+$, (calcd for $C_{46}H_{72}O_2Li$ 663,5692).

Fulvyne 2 (**22**). $[\alpha]_D$ -22.9 (c 0.4, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 244 (3.55); IR (liquid film) ν_{max} 3309, 2925, 2854, 2098, 1650, 1455, 1232 cm^{-1}; ESIMS *m/z* 681 $[M+Na]^+$ and HRFABMS *m/z* 665.6318 $[M+Li-H]^+$, (calcd for $C_{46}H_{74}O_2Li$ 665,5849).

Fulvyne 3 (**23**). $[\alpha]_D$ -6.64 (c 0.3, $CHCl_3$); IR (liquid film) ν_{max} 3310, 2925, 2854, 2098, 1455, 1232 cm^{-1}; ESIMS *m/z* 683 $[M+Na]^+$ and HRFABMS *m/z* 667.5205 $[M+Li-H]^+$, (calcd for $C_{46}H_{76}O_2Li$ 667,6005).

Fulvyne 4 (**24**). IR (liquid film) ν_{max} 3310, 2925, 2854, 2098, 1455, 1232. from ESIMS *m/z* 699 $[M+Na]^+$ and HRFABMS *m/z* 683.5065 $[M+Li-H]^+$, (calcd for $C_{46}H_{76}O_3Li$ 683,5955).

5.5.3. *Aldisa andersoni*

5.5.3.1. Biological material: Six speciments of *A. andersoni* were collected in May 2009 in the Indo-Pacific Ocean by Dr. G. Villani, and transferred to ICB. The nudibranches were identified by Dr. Juan Lucas Cervera (University of Cadiz). A voucher specimen is stored at ICB under the code ALDI.

5.5.3.2. Extraction and isolation: The frozen molluscs *A. andersoni* have been treated in order to get extracts of the mantle and the internal glands, separately. In particular, the mantle extract was obtained simply by soaking the whole animals in acetone bath for few minutes, whereas the internal glands extract was obtained by grinding the animal after sonication. After filtration and evaporation *in vacuo* of the organic solvent, the two residues were subsequently extracted with Et_2O (30 mL×4). The evaporation of Et_2O gave two gummy residues (15 mg and 62 mg for the mantle and the gland extract respectively). The mantle extract was purified on reverse-phase HPLC column

chromatography with a linear gradient of MeOH/H_2O 70:30 until MeOH in 40 minutes (flow 1 mL/min) to afford in order of decreasing polarity phorbazole E (**25**) (t_R 11.8 min, 4.9 mg), F (**26**) (t_R 19.8 min, 1.1 mg), G (**27**) (t_R 28.2 min, 0.8 mg).

5.5.3.4. Biological assay: The antiproliferative activity were tested *in vitro* on C6 rat glioma cell line (ATCC Number: CCL-107). DMEM complete growth medium supplemented with fetal bovine serum to a final concentration of 10% (Atmosphere: air, 95%; carbon dioxide (CO2), 5%; Temperature: 37.0°C). The cells were continuously treated for 48 h with various concentrations (25, 50 , 100 and 200 µM) of each compound.

Phorbazole E (25): 4.9 mg, UV (MeOH) λ_{max} (log ε) 208 (6.90), 256 (6.80), 334 (7.01);); IR (liquid film) ν_{max} 1614, 1506, 1449, 1406, 1278, 1176, 837 cm^{-1}; ^1H NMR and ^{13}C NMR in CD_3OD: Table 14, ^1H NMR and ^{13}C NMR in DMSOd_6: 7.73 (d, 8.42 Hz, H-12 and H-16), 7.07 (d, 2.93 Hz, H-2), 6.94 (d, 8.42 Hz, H-13 and H-15), 6.31 (d, 2.93 Hz, H-3); 158.3 (C-14), 151.7 (C-6), 142.1 (C-8), 126.2 (CH-12 and CH-16), 122.5 (CH-2), 122.3 (C-9), 116.9 (C-11), 116.0 (CH-13 and CH-15), 114.7 (C-5), 113.2 (C-4), 110.6 (CH-3). HRESIMS *m/z* 316.9849 $[M+Na]^+$ calcd for $C_{13}H_8Cl_2N_2O_2$, found: 316,9861

Phorbazole F (26): 1.1 mg, ^1H NMR and ^{13}C NMR in CD_3OD: Table 14. ^1H NMR and ^{13}C NMR in DMSOd_6: 7.72 (d, 8.80 Hz, H-12 and H-16), 7.45 (s, H-2), 6.95 (d, 8.80 Hz, H-13 and H-15); 157.6 (C-14), 151.3 (C-6), 142.3 (C-8), 126.1 (CH-12 and CH-16), 124.2 (CH-2), 122.4 (C-9), 116.1 (C-11), 115.7 (CH-13 and CH-15), 115.3 (C-5), 111.2 (C-4), 109.0 (C-3). HRESIMS *m/z* 365.2582 $[M+Na]^+$ calcd for $C_{14}H_9Cl_3N_2O_2$, found: 364.9627.

Phorbazole G (27): 0.8 mg, ^1H NMR and ^{13}C NMR in CD_3OD: Table 14. HRESIMS *m/z* 384.9183 $(M+Na)^+$ calcd for $C_{13}H_6Cl_4N_2O_2$, found: 384.9081

CHAPTER 6: General Conclusion

In the context of drug discovery, the sea has been described as "the last great frontier of Earth". The oceans have an enormous number and diversity of life forms. During the relatively few years that marine organisms have been mined for useful compounds, a large number of bioactive chemicals have been discovered.

Many marine invertebrates are immobile, attached to the ocean floor, and use highly evolved chemical compounds to attract food, block the growth of intruding neighbours or repel predators. It is believed that these survival demands triggered the evolution of a particularly abundant mixture of bioactive compounds. Since its search for anti-cancer drugs in the 1960s the US National Cancer Institute (NCI, Bethesda, MD, USA) has been one of the pioneers in the search for drugs from the sea.

The search for bioactive compounds from marine organisms can be a lengthy, complex and expensive process. Once a useful bioactive compound is discovered, there can be a considerable problem in obtaining sufficient material for clinical studies, let alone for widespread therapeutic use. Often, to overcome this problem, one possibility is to develop the synthesis, even if some compounds have a complicated chemical structure.

The problems associated with the effort are large, but so are the potential impacts on human health. The enormous biodiversity of

the oceans is an irreplaceable resource with many secrets yet to be revealed and exploited for human use.

In the current Ph.D. research work, 7 marine organisms belonging to different phyla have been chemically investigated and 27 new natural products have been isolated and characterised (Figure 23).

In particular, the species analysed, that were collected in distinct geographical areas, from Antarctica to Indo-Pacific Ocean to Mediterranean Sea, included two species of phylum Cnidaria, *Alcyonium antarcticum* and *Parazoanthus axinellae*; a member of phylum Chordata, *Pseudodistoma crucigaster*; the sponge *Haliclona fulva* (phylum Porifera); and three opisthobranch species (phylum Mollusca), *Placida dendritica*, *Peltodoris atromaculata*, and *Aldisa andersoni*.

The new molecules comprise two cyclic sesquiterpenes, alcyonicene (**1**) and deacetoxy-alcyonicene (**2**), two hydantoin alkaloids, parazoanthines F (**3**) and G (**4**), five linear 2-amino-alken-3-ols, crucigasterins A-E (**5-9**), thirteen high molecular weight polyacetylenes, fulvynes A-I (**12-20**) and 1-4 (**21-24**), and, finally, three chlorinated phenylpyrrolyloxazoles, phorbazoles E-G (**25-27**). The structures of these compounds were determined by an extensive use of spectroscopic techniques, mainly NMR methods. Stereochemical aspects were deeply investigated for crucigasterins by combining spectral and chemical methods.

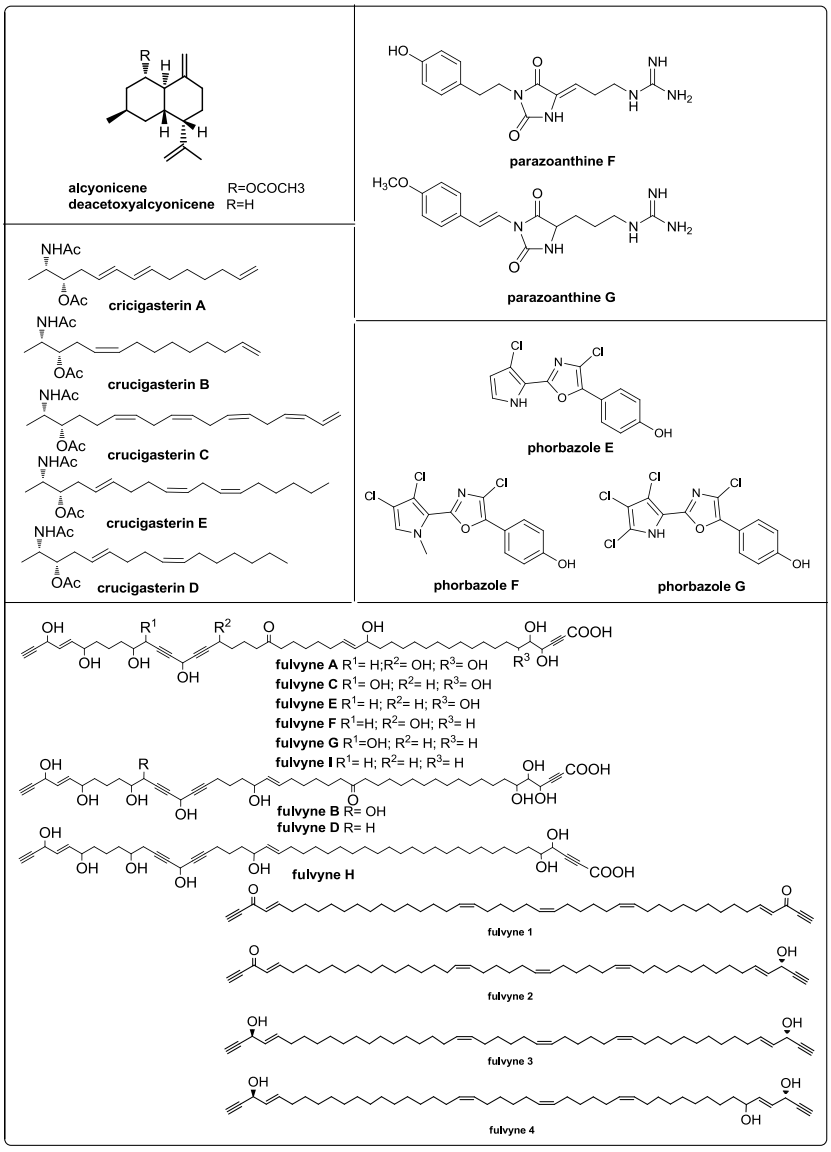


Figura 23: Summary of new natural products

From a chemical point of view, all molecules isolated in this work display interesting structural elements. Alcyonicene (**1**) and

deacetoxy-alcyonicene (**2**) exhibited a rare bulgarane skeleton,[30] never described from the marine environment and previously described only in essential oils from *Mentha piperita*[28] and *Juniperus oxycedrus*.[29] The family of alkaloids named parazoanthines (compounds **3** and **4**) represents an example of natural 3,5-disubstituted hydantoins that do not exhibit a 3-N methyl.[47] Moreover, this rare 3,5-disubstituted hydantoin core constitutes a scaffold for the construction of bioactive molecules.[130] Crucigasterins A-E (**5**-**9**) are 2-amino-3-alcohols with an unusual *threo* relative stereochemistry being the *erythro* configuration reported for most related molecules.[68] Fulvynes (compounds **12**-**24**) belong to the interesting group of marine polyacetylenes,[78-80] high molecular weight acetogenins exhibiting several carbinol chiral centres (fulvynes A-I) or less functionalised structures (fulvynes 1-4). Finally, phorbazoles are uncommon metabolites only reported so far from a *Phorbas* sponge.[129]

A series of already reported molecules were also isolated from the studied organisms and identified by comparison of their spectroscopic data with those reported in the literature. Among them, α-pyrone propionates isolated from the sacoglossan *Placida dendritica*, cyercenes 2-4, are molecules of a certain structural interest. These compounds are able to interact with the light catalyzing the production of highly reactive singlet oxygen[131] and undergoing photorearrangement reactions that lead to the isomerisation of the double bonds in the chain.[113]

To evaluate the bioactivity of the isolated molecules, the attention has been focused towards antiproliferative and antimicrobial/antifungal activities. These studies have been carried out in collaboration with Prof. C. Irace (University of Naples) and Prof. R. Kiss (University of Bruxelles), and Prof. M. Varcamonti (University of Naples), respectively.

Due to the small amounts of pure metabolites obtained, only selected compounds were tested against different tumour and non-tumour cell lines in cytoxicity assays, against Gram+ and Gram– bacterial strains in antimicrobial tests, and against *Candida albicans*, in antifungal assays.

All parazoanthines including the known molecules were tested on different human cancer cell lines - glioma (Hs683, U373, U251), lung (A549), breast (MCF7), melanoma (SKMEL28), prostate (PC3) - but none of them exhibited significant bioactivity. However, the evaluation of the bioactivities of these molecules is still under investigation, being the next step the establishment of a possible influence of the compounds on the cell migration, element responsible of the angiogenesis.

Selected diacetyl-crucigasterins were tested for antibacterial and antifungal activities, exhibiting moderate activity. In particular, diacetyl-crucigasterins B (**6a**) and E (**9a**) were found to be active at 50 μg/mL against both *Escherichia coli* and *Candida albicans* and at 100 μg/mL against *E. coli*, respectively.

All polyoxygenated fulvynes (compounds **12**-**20**) were tested in a specific assay on a chloramphenicol resistant *B. subtilis* strain (PY79), showing a strong activity against this strain (Table 11). These molecules were also tested on a Gram+ human pathogenic strain, *Staphylococcus aureus*, exhibiting a moderate activity. In addition, compound **14**, the main metabolite of *H. fulva*, was subjected to cytotoxic assays. In particular, tested on different tumour cell lines – rat glioma (C6), rat heart (H9c2), cervical cancer (HeLa), adipose tissue (3T3-L1) - it showed selectivity for 3T3-L1 cell line being active with an IC_{50} of 55.7±11 μM (Table 12).

The chemicals isolated from opisthobranchs included metabolites probably involved in the defensive mechanisms of the molluscs. These molecules were either found to be selected from the dietary sponges, like for *P. atromaculata* and *A. andersoni*, or suggested *de novo* biosynthesised, like for *P. dendritica*.

Cyercene 4, the main metabolite of *P. dendritica*, was tested on seven human cancer cell lines (Hs683, U373, U251, A549, MCF7, SKMEL28, PC3). The biological activity of cyercene 4 was compared to that observed for aplysiopsene A,[114] a molecule isolated from the sacoglossan *A. formosa* and structurally close to cyercene 4 (Figure 16). Surprisingly, aplysiopsene A displayed significant *in vitro* growth inhibitory activity on various cancer cell lines (mean IC_{50} 5±1 μM), while cyercene 4 was weakly active (mean IC_{50} = 90 μM) (Table 13). This different activity could be due to some structural differences

including the presence of the methyl group on the pyrone moiety and in the length of the alkyl chain (Figure 16). This aspect is currently under investigation.

Phorbazoles were considered with regards with their cytotoxic activity. The main metabolites of *A. andersoni*, phorbazole E (**25**) and F (**26**), were assayed using a rat glioma C6 cell line. Preliminary results indicated a weak cytotoxicity for both molecules.

Part of the results obtained under this Ph.D. project have been published on peer-reviewed international journals.[24,68,88] Chemical data regarding mollusc species have to be integrated with the results of the biological activity evaluation - work still in progress - and will be submitted for publication (papers in preparation). The studies that have been here described represent a little but significant contribution to the exploring of marine environment and to the identification of new bioactive molecules.

List of publications and poster communications to international symposium

Pubblications:

1. E. Manzo, M. L. Ciavatta, **G. Nuzzo** and M. Gavagnin. Terpenoid Content of the Antarctic Soft Coral *Alcyonium antarcticum*. *Natural Product Communications* **2009**, *4*, 1615-1619;

Abstract: Chemical investigation of the soft coral *Alcyonium antarcticum*, collected off Terra Nova Bay, resulted in the isolation of twoclosely related sesquiterpenes, alcyonicene (**1**) and deacetoxy-alcyonicene (**2**), along with known terpenoid compounds. The structure elucidation of the new molecules, possessing a rare bulgarane skeleton, has been made mainly by NMR techniques.

2. M. L. Ciavatta, E. Manzo, **G. Nuzzo**, G. Villani, M. Varcamonti and M. Gavagnin. Cricigasterins A-E, antimicrobial amino alchohols from the Mediterranean colonial ascidian *Pseudodistoma crucigaster*. *Tetrahedron* **2010**, *66*, 7533-7538;

Abstract: Five new unsaturated 2-amino-3-alcohols, crucigasterins A-E (**2-6**), were isolated together with known related compound **7** from the Mediterranean ascidian *Pseudodistoma crucigaster* and characterised as diacetyl derivatives (**2a-6a**) by spectroscopic methods. The *threo*-relative configuration of the amino alcohol portionwas inferred by NOE analysis of the oxazolidinone derivative of crucigasterin A (**2**) as well as by ^{13}C NMR comparison with synthetic *threo* and *erythro* model compounds. The co-occurring metabolites were assumed to have the same relative configuration as **2** by comparison of the diagnostic carbon value of C-1. The absolute stereochemistry of compound **7** that had not been previously reported was determined by applying the modified Mosher's method on the corresponding N-acetyl derivative. The same absolute configuration was suggested for the other co-occurring crucigasterins by biogenetic considerations. Antibacterial and antifungal activities of selected crucigasterins were also evaluated.

3. **G. Nuzzo**, M.L. Ciavatta, G. Villani, E. Manzo, A. Zanfardino, M. Varcamonti and M. Gavagnin. Fulvynes, antimicrobial polyoxygenated acetylenes from the Mediterranean sponge *Haliclona fulva*. *Tetrahedron* **2012**, *68*, 754-760.

Abstract: Nine linear polyoxygenated acetylenes, fulvynes A-I (**1**-**9**), exhibiting the uncommon 2-yne carboxylic acid functionality, have been isolated from the butanolic extract of the Mediterranean sponge *Haliclona fulva*. Their structures possess a long alkyl chain with several oxygenated carbons, and were established by detailed spectroscopic analysis. All fulvynes were found to be active against a chloramphenicol-resistant strain of *Bacillus subtilis*.

Poster communications:

1. **19-23/07/09** Oporto (Portogallo), VI European Conference of Marine Natural Products: "New antimicrobial 2-amino-alken-3-ols from the Mediterranean colonial ascidian *Pseudodistoma crucigaster";* M. L. Ciavatta, G. Nuzzo, E. Manzo, G. Villani, A. Zanfardino, M. Varcamonti and M. Gavagnin;.

2. **5-9/06/11** Napoli NatPharma: Nature Aided Drug Discovery (NADD) 2011: "Fulvynes A-I, Selective Antimicrobial Oxygenated Polyacetylenes from the Mediterranean Sponge *Reniera fulva*"; G. Nuzzo, M. L. Ciavatta, G. Villani, E. Manzo, A. Zanfardino, M. Varcamonti and M. Gavagnin;

3. **12-15/06/2011** Kolymvari – Crete, Greece Trends in Natural Products Research: A PSE Young Scientists' Meeting: "Fulvynes A-I, Selective Antimicrobial Oxygenated Polyacetylenes from the Mediterranean Sponge *Reniera fulva*"; G. Nuzzo, M. L. Ciavatta, G. Villani, E. Manzo, A. Zanfardino, M. Varcamonti and M. Gavagnin.

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