Isolation and Structure Elucidation of Bioactive Secondary Metabolites from Marine Sponges and Sponge-derived Fungi

(Isolierung und strukturelle Identifizierung von biologisch aktiven Naturstoffen aus marinen Schwämme und aus Schwämmen isolierte Pilze)

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Hiermit erkläre ich ehrenwörtlich, daß ich die vorliegende Dissertation „Isolierung und strukturelle Identifizierung von biologisch aktiven Naturstoffen aus marinen Schwämme und mit Schwämmen assoziierten Pilze“ selbständig angefertigt und keine anderen als die angegebenen Quellen und hilfsmittel benutzt habe. Ich habe diese Dissertation in gleicher oder ähnlicher Form in keinem anderen Prüfungsverfahren vorgelegt. Außerdem erkäre ich, das ich bisher noch keine weiteren akademischen Grade erworben oder zu erwerben versucht habe.

Würzburg, 08. 07. 2002

Raquel C. Jadulco

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Zusammenfassung

Niedermolekulare Naturstoffe aus Bakterien, Pilzen, Pflanzen und marinen Organismen weisen eine einzigartige strukturelle Diversität auf, die für die Identifizierung neuer Leitstrukturen für die Entwicklung von Arzneistoffen und Pflanzenschutzmitteln von großer Bedeutung ist. Im Rahmen der Suche nach bioaktiven Verbindungen aus marinen Schwämmen und mit diesen Schwämmen assoziierten Pilzen wurden in dieser Arbeit insgesamt 26 Sekundärstoffe isoliert, wobei es sich bei acht Substanzen um neue Verbindungen handelt. Die Schwämme wurden im indo-pazifischen Gebiet gesammelt, insbesondere aus Indonesien und den Philippinen, so wie aus dem Mittelmeer in der Nähe der Insel Elba in Italien. Für die Entdeckung neuer bioaktiver Substanzen wurde eine Kombination von chemischen und biologischen Methoden angewendet, wodurch Extrakte mit verschiedenen Screening-Methoden auf Bioaktivität getestet worden sind. Zum Einsatz kamen dabei Versuche mit Raupen des polyphagen Nachtfalters *Spodoptera littoralis* (Noctuidae; Lepidoptera) im Hinblick auf potentielle insektizide Wirkungen, antimikrobielle Untersuchungen mit gram-negativen und gram-positiven Bakterien und dem Pilz *Candida albicans*, Zytotoxizitätstests gegenüber menschlichen Krebszellen und Toxizitätstests mit dem Krebs *Artemia salina*. Zusätzlich zur bioaktivitäts-geleiteten Isolierung von Substanzen aus aktiven Extrakten wurden daneben auch DC, UV und MS als Kriterien herangezogen, um die aus chemischer Sicht interessantesten Verbindungen zu isolieren. Damit konnten auch solche Substanzen, die nicht für die Aktivität der Extrakte im Bioscreening verantwortlich waren, weiteren Biotests unterzogen werden.

Im einzelnen wurden die folgenden Verbindungen isoliert, ihre Struktur aufgeklärt, und ihre biologische Aktivität näher charakterisiert:

1. Der antimikrobiell aktive Extrakt aus dem Pilz *Cladosporium herbarum*, der mit dem indonesischen Schwamm *Callyspongia aerizusa* assoziiert ist, ergab sieben Polyketide, die strukturell ähnlich sind, einschließlich der beiden neuen zwölf-gliedrigen Makrolide Pandangolid 3 und Pandangolid 4, sowie ein neues acetyliertes Derivat des bereits bekannten Naturstoffs 5-Hyroxymethyl-2-furancarbonsäure. Beide Furancarbonsäuren zeigten antimikrobielle Aktivität und

dürften deshalb hauptsächlich für die antimikrobielle Aktivität des Extrakts verantwortlich sein.

Daß Cladospolid B, ein bekanntes Phytotoxin, das bereits für die Arten *Cladosporium cladosporoiodes* und *C. tenuissimum* beschrieben wurde, ebenfalls aus *C. herbarum* isoliert wurde, deutet darauf hin, daß Cladospolid B als ein chemotaxonomischer Marker für bestimmte *Cladosporium*-Arten angesehen werden könnte.

2. Der antimikrobiell aktive Extrakt aus dem Pilz *Curvularia lunata*, der mit dem indonesischen Schwamm *Niphates olemda* assoziiert ist, ergab drei Substanzen, nämlich das neue antimikrobiell aktive Anthrachinon Lunatin sowie das bereits bekannte Bisanthrachinon Cytoskyrin A, und das bekannte Pflanzenhormon Abscisinsäure. Das gemeinsame Vorkommen der beiden strukturell verwandten Anthranoide könnte ein Indiz dafür sein, daß das Monomer Lunatin eine biogenetische Vorstufe des Bisanthrachinons Cytoskyrin A darstellt.

3. Ein mit dem im Mittelmeer gesammelten Schwamm *Axinella verrucosa* assoziierter Pilz der Gattung *Penicillium* ergab insgesamt sechs Substanzen, im einzelnen das bekannte Antimykotikum Griseofulvin und dessen weniger aktives Dechlor-Derivat, das bekannte Toxin Oxalin, sowie die als zytotoxisch beschriebene Verbindung Communesin B und deren neue Derivate Communesin C und Communesin D. Im Vergleich zu Communesin B erwiesen sich die neuen Communesin-Derivate als weniger aktiv gegenüber dem Krebs *A. salina.*

4. Ein bisher unidentifizierter Pilz aus dem gleichen Schwamm *Axinella verrucosa* lieferte die bekannte Substanz Monocerin, über deren phytotoxische und insektizide Eigenschaften bereits berichtet wurde.

5. Der mit dem philippinischen Schwamm *Hyrtios* aff. *reticulatus* assoziierte Pilz *Aspergillus flavus* ergab das bereits bekannte Toxin α-Cyclopiazonsäure.

6. Der indonesische Schwamm *Agelas nakamurai* lieferte vier bromierte Pyrrol-Alkaloide, nämlich die neue Substanz 4-Brompyrrol-2-carbonsäure sowie die bereits bekannten Verbindungen 4-Brompyrrol-2-carboxamid, Mukanadin B und

Mukanadin C. Alle vier Substanzen außer Mukanadin B zeigten antimikrobielle Aktivität. Bromierte Pyrrol-Alkaloide wurden in vielen Untersuchungen als typische Sekundärstoffe der Schwammgattung *Agelas* beschrieben, die bei der chemischen Verteidigung der Schwämme gegen Fische eine wichtige Rolle spielen.

7. Der indonesiche Schwamm *Jaspis splendens* ergab drei bekannte Substanzen, die für ihre antiproliferative Aktivität bekannt sind, nämlich die Depsipeptide Jaspamid (Jasplakinolid) und dessen Derivate Jaspamid B und Jaspamid C.

I. Introduction

1.1. Significance of the study

1.1.1. The need for lead compounds for drug development

Even today, after more than 100 years of research in pharmaceutical industries, there is still a great need for innovative drugs. Only one third of all diseases can be treated efficiently [Müller *et al*., 2000]. This means that there is a need for new drug entities to enable therapeutic innovations.

The economic importance of natural products is evident in a conference entitled *Profiting from biodiversity by leveraging natural product discovery*, which took place on 28-29 June 1999 in London. It was highlighted that ten of the top 20 selling medicines in 1998 were derived from natural products [Lawrence, 1999]. It was also claimed that drugs derived from natural products acquire a market share of about 80 billion US $ in the world pharmaceutical market [Müller *et al*., 2000]. Furthermore, a study using US-based prescription data from 1993 showed that over 50% of the most-prescribed drugs in the US had a natural product either as the drug, or as a ‘forebear’ in the synthesis or design of the agent [Grifo *et al*., 1997], thus demonstrating that natural products still play a major role in drug treatment.

The role of natural products in drug discovery is demonstrated by an analysis of the number and sources of anticancer and antiinfective agents, reported mainly in the Annual Reports of Medicinal Chemistry from 1984 to 1995 [Cragg, 1997]. It was observed that over 60% of the approved drugs and pre-NDA (New Drug Applications) candidates (for the period 1989-1995), excluding biologics (vaccines, monocloneals, etc. derived from mammalian sources), developed in these disease areas are of natural origin.

Drugs of natural origin have been classified as original natural products, products derived semisynthetically from natural products, or synthetic products based on natural product models [Cragg, 1997]. Bioactive natural products could thus serve as

lead structures which could then be ‘optimized’ through classical medicinal chemistry techniques and the more recent combinatorial synthesis methods to come up with new agents with improved pharmacokinetics and/or toxicology (Fig. 1.1).

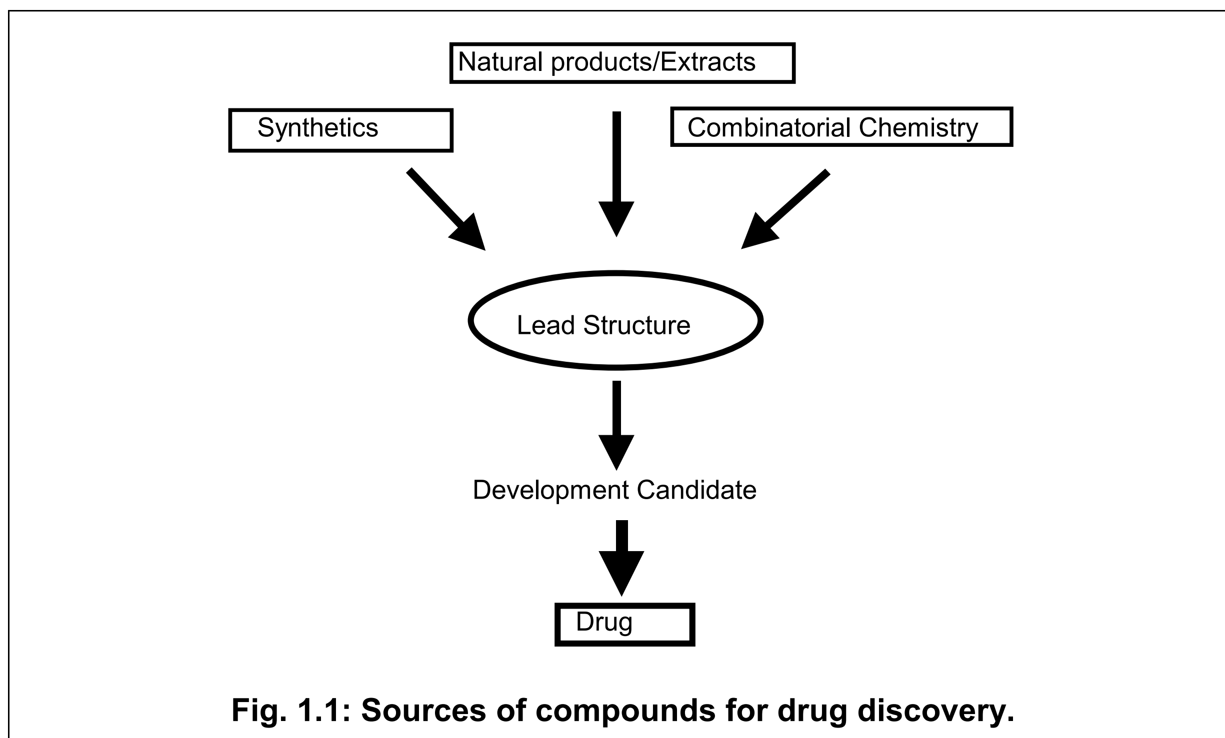


Fig. 1.1: Sources of compounds for drug discovery.

A lead compound is a compound with many of the characteristics of a desired new drug which will be used as a model for chemical modification. It must be potent, but it does not need to possess the potency at the nanomolar or picomolar levels expected of a product candidate. It must be specific for the desired target, but it does not need to possess the exquisite biochemical specificity required for a new drug. Finally, it must be available in sufficient quantities to support the early stages of development, such as biological characterization and toxicity studies, while a total synthesis of the product candidates is being completed.

Low-molecular mass natural products from bacteria, fungi, plants and marine organisms exhibit unique structural diversity, and are of maximum interest for identifying new lead structures. Thomas Henkel (Bayer AG, Wuppertal, Germany) reported that most natural products have a higher molecular weight than their synthetic counterparts, containing more rings and being generally more sterically

complex [Lawrence, 1999]. Furthermore, Henkel found that, on comparison of the compounds in a natural product datase (DNP) with a representative pool of chemical test substances (Synthetics), there was only a 60% homology. Henkel also demonstrated that by comparing the structures of natural products, synthetic drugs, and currently used drugs, there was a much higher incidence of O-containing groups in the natural product compounds. Furthermore, natural product compounds were found to contain more sp^3-hybridized bridgehead atoms. This illustrates the diverse range of compounds that can be gained from natural products that would otherwise be missed using synthetic techniques.

1.1.2. Strategies for drug development from natural products

Generally, strategies for drug discovery can be separated into three categories: chemically driven, biologically driven and a combination of both [McConnell *et al.*, 1994]. In the chemically driven or ‘traditional grind-and find’ approach, which has been pursued mainly by academic research groups, the object of the search was to find novel compounds from marine sources. Hence, extracts are ‘screened’ by TLC, ^{1}H and ^{13}C NMR for unusual and interesting patterns. The next step for this approach is then finding biological properties for purified compounds. The biologically driven strategy is the bioassay-guided approach beginning with crude extracts and has been the preferred method by modern marine natural product researchers.

The biologically driven approach which involves ‘screening’ crude extracts for biological activity, followed by the crucial work of backtracking the active compounds from the ‘hit’-extracts dominate natural products research up to the present. However, a lot of experience is required to exclude both false positive and false negative results. Considerable effort is required to get access to sufficient quantities of raw material for reproduction, isolation, structure elucidation and subsequent verification of biological activity. The complete process proved to be highly time and capacity consuming. Moreover, false positives may result when the activity shown by an extract is attributed to a synergistic effect of more than one constituent in the extract.

It seems advantageous to perform a screening with pure compounds rather than with crude extracts. For individuals, however, the problem arises of getting access to sufficient numbers of natural compounds covering a substantial structural diversity. A new approach utilized by pharmaceutical industries for the discovery of new drugs is the creation of a central natural product pool. With a natural product pool, supplied by the industry and the academic institutions, compounds are getting a more realistic chance to be discovered and highlighted in diverse target directed bioassay systems of therapeutic value. Together with high thoroughput screening (HTS), a greater number of 'hits' of lead compounds have been identified.

The development of new bioassay methods that can selectively detect biologically active molecules at very low levels as well as the advances in chemical instrumentation (e.g. high performance liquid chromatography (HPLC), high performance centrifugal countercurrent chromatography (HPCCC), capillary zone electrophoresis (CZE), high resolution mass spectrometry (HRMS), high field nuclear magnetic resonance (NMR) and X-ray crystallography which now allow the chemist to isolate submilligram quantities of the new compounds, and confidently be able to fully characterize them and identify their structures contributed to the current peak in interest in natural products.

New bioassays which target receptors and enzymes involved in pathogenesis of disease are being developed. These assays reflect new opportunities due to the recent identification of previously unrecognized biomolecular targets for therapy.

1.2. Current status of marine natural product research

Although natural products research was previously focused mainly on plants, growing interests in marine natural products have led to the discovery of an increasing number of potently active agents considered worthy for clinical application. The world's oceans cover more than 70% of the earth's surface represent our greatest resource of new natural products [McConnell *et al*., 1994]. The sea contains well over 200,000 invertebrate and algal species [Ibid]. There exist nearly 150,000 species of algae (sea weed): green (Chlorophyta), red (Rhodophyta), and brown (Phaeophyta), and some groups of marine invertebrates in which new chemical structures or

biological activities have been reported: sponges (Porifera), cnidarians or coelenterates [corals, octocorals (including sea fans), hydroids, and sea anemones], nemerteans (worms), bryozoans, ascidians (tunicates including sea squirts), molluscs (sea snails and sea slugs), and echinoderms (brittlestars, sea urchins, starfish, and sea cucumbers) [Ibid].

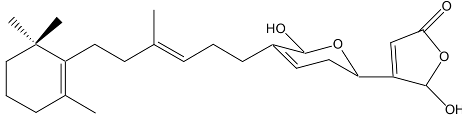
The earliest findings include the arabinose-nucleosides, known since the 1950's as constituents of the Carribean sponge *Cryptotethya crypta* (Tethydae) which served as lead compounds for the synthesis of analogues, ara-A (Vidarabin, Vidarabin Thilo®) and ara-C (Cytarabin, Alexan®, Udicil®) with improved antiviral and anticancer activity. Since then however, the systematic investigation of marine environments as sources of novel biolgically active agents only began in earnest in the mid-1970s. During the decade from 1977-1987, about 2500 new metabolites were reported from a variety of marine organisms [Newman *et al*., 2000]. Prior to 1995, a total of 6500 marine natural products had been isolated; by January of 1999, this figure had risen to approximately 10,000 [Jaspars, 1999]. These studies have clearly demonstrated that the marine environment is a rich source of bioactive compounds, many of which belong to totally novel chemical classes not found in terrestrial sources.

A recent review provided an updated list of marine natural products which are currently under clinical trials (Table 1) (Fig. 1.2) [Proksch *et al*., 2002].

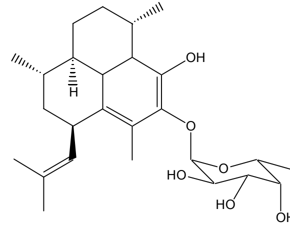
Table 1.1: Selected natural products from marine sources which are currently undergoing clinical trials.[*]

Source	Compounds	Disease area	Phase of clinical trial
Conus magnus (cone snail)	Ziconotide	Pain	III
Ecteinascidia turbinata (tunicate)	Ecteinascidin 743	Cancer	II/III
Dolabella auricularia (sea hare)	Dolastatin 10	Cancer	II
Dolabella auricularia (sea hare)	LU103793[a]	Cancer	II
Bugula neritina (bryozoan)	Bryostatin 1	Cancer	II
Trididemnum solidum (tunicate)	Didemnin B	Cancer	II
Squalus acanthias (shark)	Squalamine lactate	Cancer	II
Aplidium albicans (tunicate)	Aplidine	Cancer	I/II
Agelas mauritianus (sponge)	KRN7000[b]	Cancer	I
Petrosia contignata (sponge)	IPL 576,092[c]	Inflammation/ asthma	I
Pseudopterogorgia elisabethae (soft coral)	Methopterosin[d]	Inflammation/ wound	I
Luffariella variabilis (sponge)	Manoalide	Inflammation/ psoriasis	I
Amphiporus lactifloreus (marine worm)	GTS-21[c]	Alzheimer/ schizophrenia	I

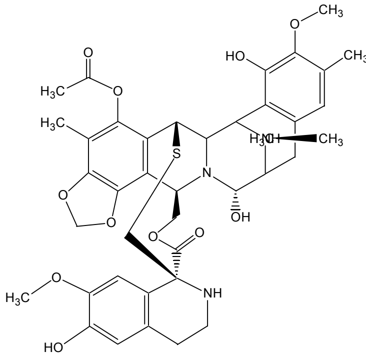
[*]reproduced from Proksch *et al* 2002.
[a]synthetic analogue of dolastatin 15
[b]Agelasphin analogue (α-galactosylceramide derivative)
[c]synthetic analogue of contignasterol (IZP-94,005)
[d]semisynthetic pseudopterosin derivative



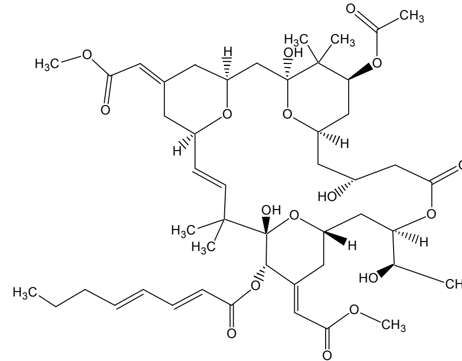
Manoalide



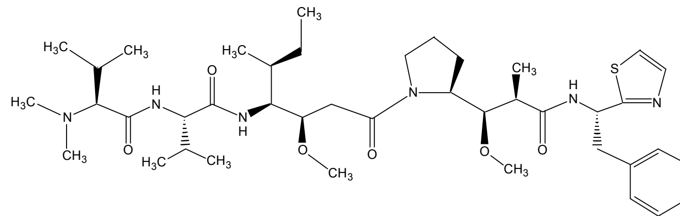
Pseudopterosin E



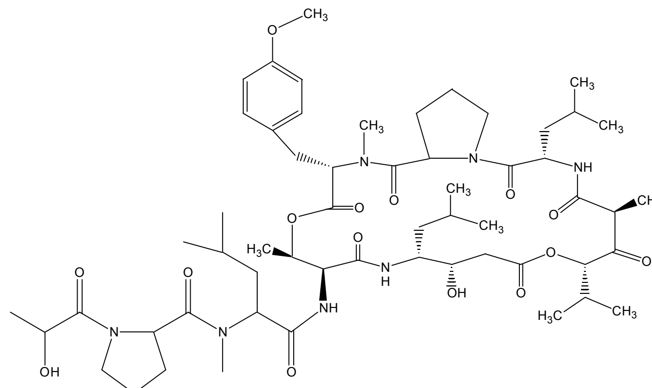
Bryostatin 1



Didemnin B



Dolastatin 10



Ecteinascidin 743

Fig. 1.2: Selected natural products currently undergoing clinical trials

1.3. Role of metabolites in host organisms

Another point of interest for marine natural product researchers is the natural function of the metabolites that they study. It has been observed that the majority of bioactive marine natural products have been isolated from soft-bodied, sessile or slow-moving marine invertebrates that are usually devoid of morphological defense structures such as spines or protective shells. This points out the ecological importance of these metabolites for the respective invertebrates and is corroborated by numerous studies which relate the accumulation of toxic natural products with their role as chemical defense to fight off potential predators (e.g. fishes) [Pawlik *et al.,* 1995; Chanas *et al*., 2996; Wilson *et al*., 1999; Lindel *et al*., 2000] or to force back neighbors competing for space [Proksch and Ebel, 1998; Proksch, 1999; McClintock, 2001]. These include studies of the feeding deterrence caused by algal metabolites and the production of metabolites by sessile organisms which inhibit settling of fouling organisms [Faulkner, 2000]. Furthermore, the observation that many nudibranchs sequestered metabolites of sponges and tunicates have led to the hypothesis that these shell-less molluscs had evolved by loss of their shell after the ancestral mollusc had acquired defense chemicals of dietary origin [Faulkner, 1983].

A study of the chemical diversity within organisms of the same species suggests that biosynthesis of secondary metabolites may be influenced by various external factors such as prevailing environmental conditions, e.g., presence of predators, infection with parasites, water temperature, depth, and nutrient levels. Internal factors, eg., stage of development/reproduction and chemical races may also play a role as well as the presence and type of symbionts within or on the tissues of a host organism.

The presence of symbionts like bacteria [Hamel *et al*., 1995], cyanobacteria [Rützler, 1990], and to a lesser extent dinophycean zooxanthellae and eucaryotic algae in sponge tissue is well documented [Ibid]. Furthermore, the result of a number of studies seems to point out that the symbionts are the true producers of the secondary metabolites isolated from marine invertebrates. Numerous examples have been cited in a review [Moore, 1999] wherein structurally related or identical compounds have been reported from taxonomically distinct invertebrates or from invertebrates and cultured microorganisms alike. A more conclusive evidence has

been given by Faulkner and co-workers when they demonstrated in several cases that secondary metabolites are cellularly located within sponge-associated microorganisms [Faulkner *et al*., 1993; Bewley *et al*., 1998]. For instance, two metabolites of the lithistid sponge *Theonella swinhoei*, swinholide A and the cyclic peptide theopalauamide, were shown to be localized in a mixed bacterial fraction and a filamentous δ-proteobacterium, respectively [Bewley *et al*., 1996]. This evidence, however, operates under the assumption that the secondary metabolites are biosynthesized within the cells in which they are localized and the possibility of an exchange of biosynthetic precursors between the partners does not exist.

Some authors consider fungi as symbionts of marine invertebrates [Müller *et al*., 1981]. However, while fungal colonies have clearly been documented on the surface of marine algae and sea grasses, there is a need to demonstrate that fungi grow within sponges and other invertebrates [Faulkner *et al*., 2000]. Because of the isolation from marine-derived fungi of secondary metabolites which have been identified from terrestrial fungi in previous examinations, there is some doubt whether some of these fungi are truly marine and whether they are epibionts, symbionts, pathogens, or merely accidentally present in or on the studied materials. In certain cases, particularly lignicolous and parasitic species, the answer is clear cut: they belong to genera unknown onland and sporulate in sea water. In other cases, typically for isolates from marine invertebrates, the answer is difficult for fungi that belong to genera well known from land, particularly when the literature fails to describe if they can sporulate in sea water. Terrestrial fungi are present in the oceans and thus, represents a major problem when trying to obtain the slow-growing marine fungi [Schaumann, 1974; Miller and Whitney, 1981].

1.4. Marine fungi as source of bioactive metabolites

The increasing number of secondary metabolites isolated from marine-derived fungi [Faulkner, 2001; Faulkner 2002] prove that they are a rich source of bioactive compounds with therapeutic potential. Natural product research using marine-derived fungi is a relatively new field. In the 1940’s, the fungus *Cephalosporium sp.* was isolated from the microbial flora of sea water collected from Cagliari, Italy.

Subsequent chemical and biological investigation of this fungus led to the discovery of the cephalosporins, now widely used in antibiotic therapy.

Marine fungi are not a taxonomically-systematically, but an ecologically-physiologically defined group of fungi (Mycophyta). They grow facultatively or obligately in oceans and ocean-associated estuarine habitats that contain brackish water including river mouths, tidal creeks and marshes, salinas, lagoons, and the like. The relatively narrow definition which is generally accepted is that obligate marine fungi are those growing and sporulating exclusively in a marine and estuarine habitat; facultative marine fungi are those isolated from marine environments but can also grow under terrestrial conditions [Kohlenmeyer and Kohlenmeyer, 1979]. Marine fungi that do not germinate in the natural marine habitat are not included in this definition. A survey showed that a total of 321 species of marine mycelial fungi have been recognized, representing 255 Ascomycetes, 60 Basidiomycetes, and 60 anamorphic ('imperfect') fungi (Deuteromycetes) [Kohlenmeyer and Volkmann-Kohlenmeyer, 1991]. This number is continuously increasing by the discovery and description of new species. Including the non-active marine fungi, the number of mycel forming higher marine fungi is estimated to be at least 6000 [Schaumann *et al*., 1993].

Many marine fungi are geographically widely distributed and appear to occur in all climates and all salinities, but are often uniquely adapted for life in the sea. They live as saprobes on algae, driftwood, decaying leaves and other dead organic material of plant and animal origin. They may also occur as parasites on mangroves, shells, crabs, sponges or in the gastrointestinal tract of fishes and are an important group of pathogens in the marine world. Other species exist as symbionts in lichenoid associations with algae on coastal cliffs. A vast number of less investigated fungal species populate the deep sea and the seafloor. Some *Endomycetes* freely float as plankton. Marine fungi represent a considerable amount of biomass in the marine habitat. They live on high energy organic substances from carbon sources that are taken up from their environment and decomposed into smaller molecules by specific ecto- and exoenzymes. Among other substrates, marine fungi decompose cellulose, lignine or alginate. They considerably contribute to the turnover of material and energy of their environment and are used themselves as source of food by other

marine organisms [Schaumann, 1974]. For ensuring their survival in their competition with other organisms, marine fungi are compellingly dependent on the production of secondary metabolites. This has been proven by the increasing number of substances isolated from marine fungi with antibacterial, antifungal, and cytotoxic activities [Faulkner 2001; Faulkner 2002]. About 70-80% of the secondary metabolites that have been isolated from marine fungi are biologically active.

It is interesting to note that fungi of the same species have been reported to yield different types of metabolites. The lignicolous fungi *Leptosphaeria oraemaris (*Pleosporaceae) yielded in one study Leptosphaerin (**1**) (Fig. 1.3) [Schiehser *et al*., 1986; Pallenberg *et al*., 1986]. A further study of *L. oraemaris*, undertaken by the group of Pietra, yielded none of the previously found metabolites, but the polyketides, leptosphaerolide (**2**), it's *o*-dihydroquinone derivative (**3**) and leptosphaerodione (**4**) (Fig. 1.3) [Guerriero *et al*., 1991]. This leads to the conclusion that the production of secondary metabolites might be highly dependent on the culture conditions and the origin of the strains. To produce these metabolites and to maximize the potential chemical diversity, they need to be grown in various nutrient-limited media. For example, media for *Penicillium spp.* that are deficient in C have been used to produce penicillins, those that are P-limited produce cephalosphorins and vancomycin, and those that are N-limited can produce carbapenems [Lawrence *et al*., 1999].

It is still questionable whether marine natural products will play a major role in drug discovery in the future as no unique secondary metabolite has yet been isolated [Grabbley *et al*., 2000]. This is due in part to the predominant isolation and cultivation of ubiquitous fungi even from samples collected in the marine environment. Today, toxic principles dominate the spectrum of biological activities in products isolated from marine sources. This maybe due to the major application of cytotoxicity-directed screening assays. However, it has to be considered that defense strategies are necessary to survive in the highly competitive marine environment, thus resulting a tremendous diversity of highly toxic compounds affecting targets that are involved in eukaryotic cell-signaling processes. The strong toxic properties of marine metabolites often preclude their application in medicine. On the other hand, a number of metabolites have proven to be valuable tools in biochemistry and cell and molecular

biology. For example, the water-soluble polyether type neurotoxin, maitotoxin, produced by the marine dinoflagellate *Gambierdiscus toxicus* heads the list of non-peptide toxins and is being used as a unique pharmaceutical tool for studying calcium transport [Murata and Yasumoto, 2000]. Currently, various other marine natural products that exhibit considerable toxic potency are hopeful candidates for clinical use, mainly in anticancer therapy.

1.5. Statement of the objective

Since marine fungi represent a rich source of bioactive metabolites which have not yet been fully exploited, this study is focused on these organisms. Organisms from the Pacific basin, mostly collected around Okinawa, account for half of all species in marine natural product patent applications; while the northwestern Atlantic accounts for about a third of the species – mostly collected in the Bahamas and the Carribean. The rest come from the Indian Ocean, the Red Sea and the Mediterranean [Bongiorni, and Pietra, 1996]. These data should not be taken as a direct indication of the most profitable areas for industrially exploitable waters but may be due to logistic and jurisdictional opportunities. The greatest biodiversity have been known to be supplied by organisms from tropical and sub-tropical waters. This study utilizes marine fungi from diverse sources: the Indo-pacific regions particularly those from Indonesian and Philippine waters, as well as those from the Mediterranean Sea near the island of Elba in Italy.

Sponges are among the well-researched marine organisms primarily because of their size and abundance. It is from sponges that 41% of discovered marine substances have been isolated and hence they present an interesting source for the isolation of marine fungi.

The metabolites isolated from marine fungi which are considered candidates for new drugs are antibacterial, antifungal, and cytototoxic. A comprehensive survey of pharmacological activity conducted by the U.S. National Cancer Institute over a period of 15 years, found that ca. 4% of the marine species (mainly animals) examined contained antitumor compound(s) [Rinehart, 2000]. Although this may be influenced by the investigation method used, it is also to be expected as these

compounds represent important chemical defense mechanisms for marine microorganisms. Hence, this study is centered on the isolation of metabolites from fungal extracts which exhibit antibacterial, antifungal, and cytotototoxic activites. For the isolation of the metabolites, a combined chemically and biologically driven approach will be used. Extracts are screened for antibacterial, antifungal, insecticidal, cytotoxic, and general toxicity activities. To supplement the bioassay-guided approach to purify the compounds responsible for the activity of the extract, TLC, UV and MS are used to isolate the chemically most interesting substances. Hence, purified compounds which are not responsible for the initial bioscreening activity may have a chance to be evaluated for other more sophisticated bioactivities.

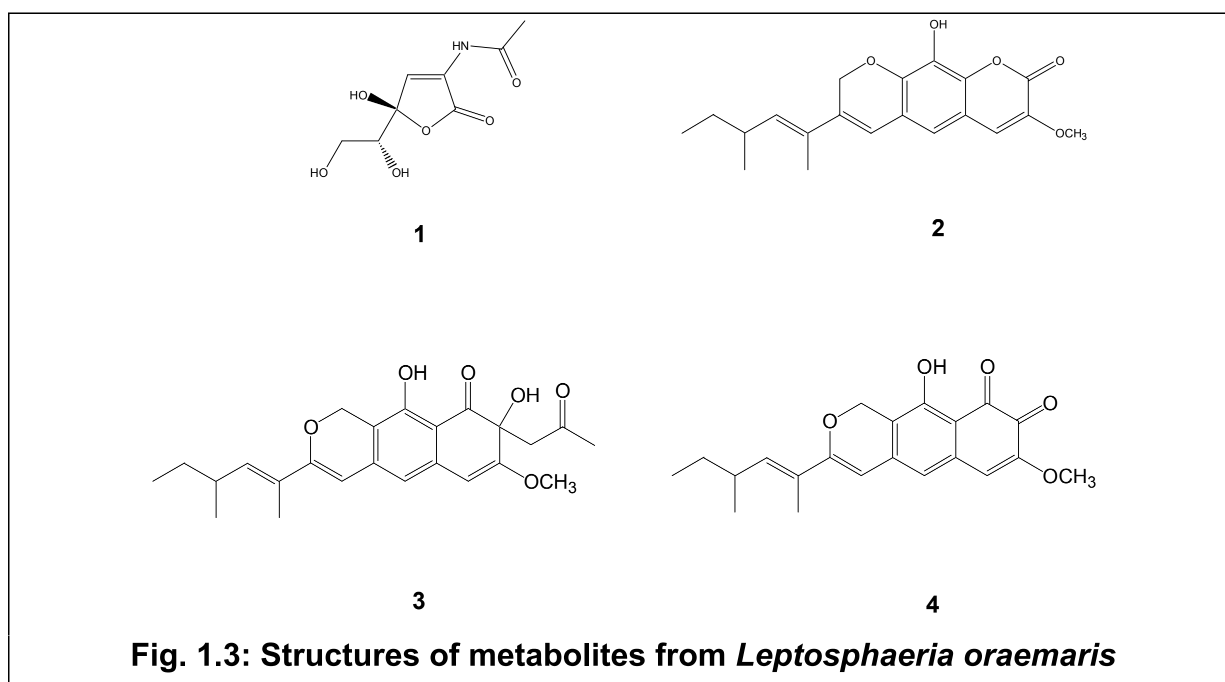


Fig. 1.3: Structures of metabolites from *Leptosphaeria oraemaris*

2. Results

The result of bioscreening of different extracts (discussed in detail later) (Table 2.1), the following materials were chosen for chemical investigations.

Table 2.1: Summary of bioscreening result showing the activities of the mterials chosen for chemical investigation.

	Brine-shrimp	**Antimicrobial**	**Insecticidal**
A. Sponges			
1. *Jaspis splendens*	+	+	+
2. *Agelas nakamurai*	-	+	-
B. Fungi			
1. *Cladosporim herbarum*	-	+	-
2. *Curvularia lunata*	-	+	-
3. *Penicillium sp.*	+	+	+
4. E00-12/11	-	+	+
5. *Aspergillus flavus*	+	+	-

2.1. Metabolites isolated from sponge-derived fungi

2.1.1. Isolated compounds from *Cladosporium herbarum* (Persoon: Fries) Link derived from *Callyspongia aerizusa*.

The ethyl acetate extract of *C. herbarum* exhibited activity against *B. subtilis* and *S. aureus* but not against *E. coli* and *C. albicans* (Table 2.2). No activity was observed in the cytotoxic, insecticidal and brine-shrimp lethality tests.

Table 2.2: Antimicrobial activity screening of the extract of C. herbarum.

Test Organism	Zone of Inhibition (mm)		
	Petroleum Ether Phase	Ethyl Acetate Phase	n-Butanol Phase
B. subtilis	n.a.	9	11
S. aureus	n.a.	13	11
E. coli	n.a.	n.a.	n.a.
HB101	n.a.	n.a.	n.a.
C. albicans	n.a.	n.a.	n.a.

*n.a. = no activity

Chemical investigation of the ethyl acetate extract yielded five structurally-related polyketides, two furanoic acid derivatives, and a phthalide. Of these eight isolated secondary metabolites, four are new compounds. The two furanoic acids: sumiki's acid and its acetyl congener were eventually found to be active against *B. subtilis and E. coli*. Since the HPLC chromatogram of the n-butanol extract showed only sumiki's acid, it was deduced that this metabolite is responsible for the antimicrobial activity of the extracts. Hence, chemical investigation was concentrated on the ethyl acetate extract.

2.1.1.1. Related macrolides found in the literature

Previously known fungal metabolites which contain twelve-membered lactone rings are shown in Fig. 2.1a. These include recifeiolide which was isolated from *Cephalosporium recifei* [Vesonder *et al*., 1971], cladospolide A, B and C from *Cladosporium cladosporioides* [Hirota *et al.*, 1985a] and *Cladosporium tenuissimum* [Fuji *et al.*, 1995], and patulolides A, B, and C from *Penicillium urticae S11r59* [Sekiguchi*, et al.* 1985*;* Rodphaya *et al.,* 1986]. A dimeric pentaketide macrolide named thiobiscephalosporolide A, the first dimeric thiomacrolide, was isolated from *Cephalosporium aphidicola* [Ackland *et al*., 1984].

A more recent report described the isolation of the polyketide metabolites *iso*-cladospolide B, pandangolide 1, pandangolide 2 and *seco*-patulolide 2 (Fig. 2.1b)

together with the previously known cladospolide B from an unidentified fungus obtained from an unidentified sponge collected in Indonesia [Smith *et al.,* 2000]. It is interesting to note that the fungal culture in this report was described as septate and having colony color of light gray to olive, which agrees with the description of *C. herbarum*. It would appear, however, that the unknown marine sponge from which their fungus was isolated, described as bright orange and encrusting, differs from *C. aerizusa*.

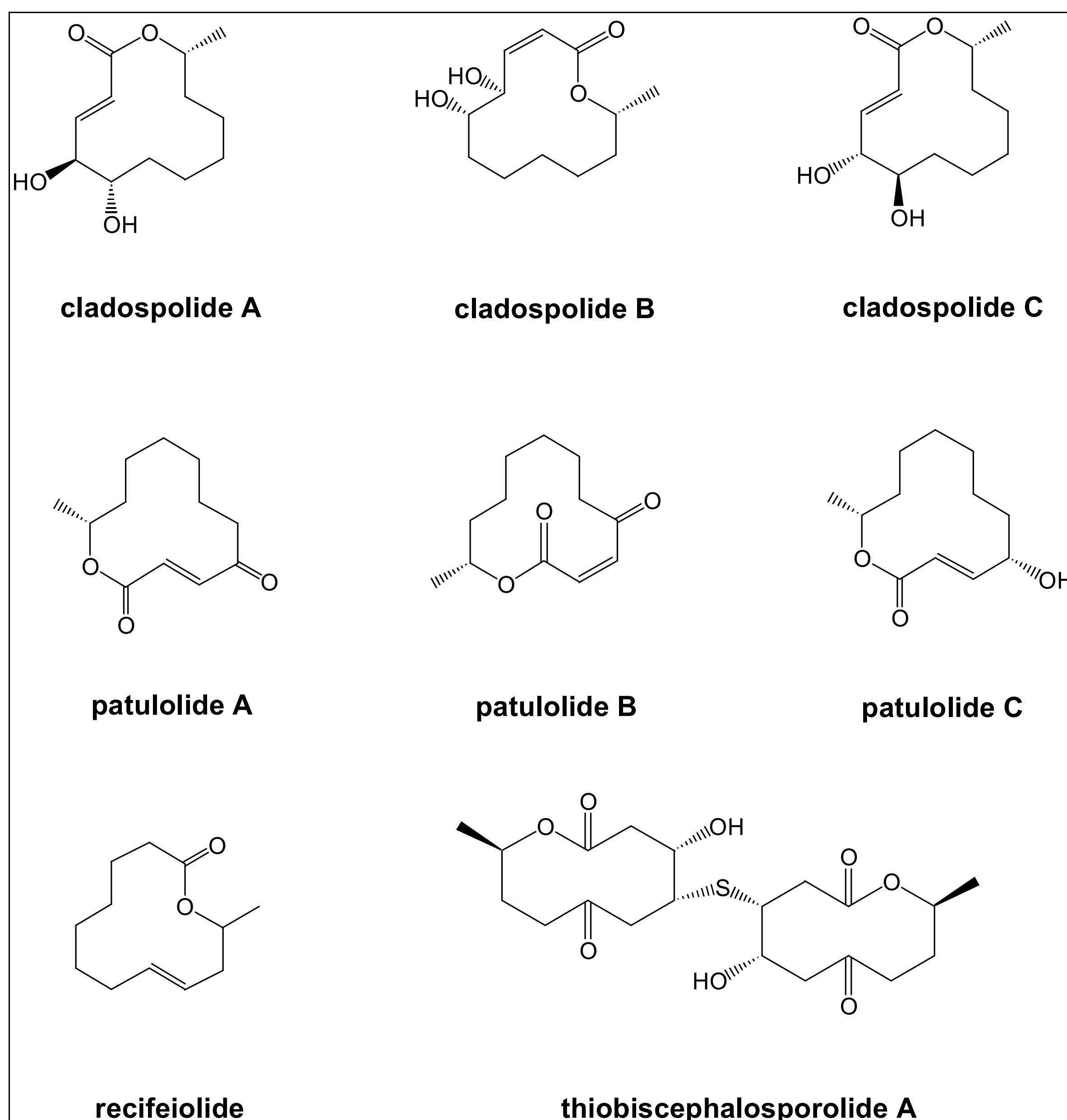


Fig. 2.1a: Some low-molecular weight macrolides isolated from fungi

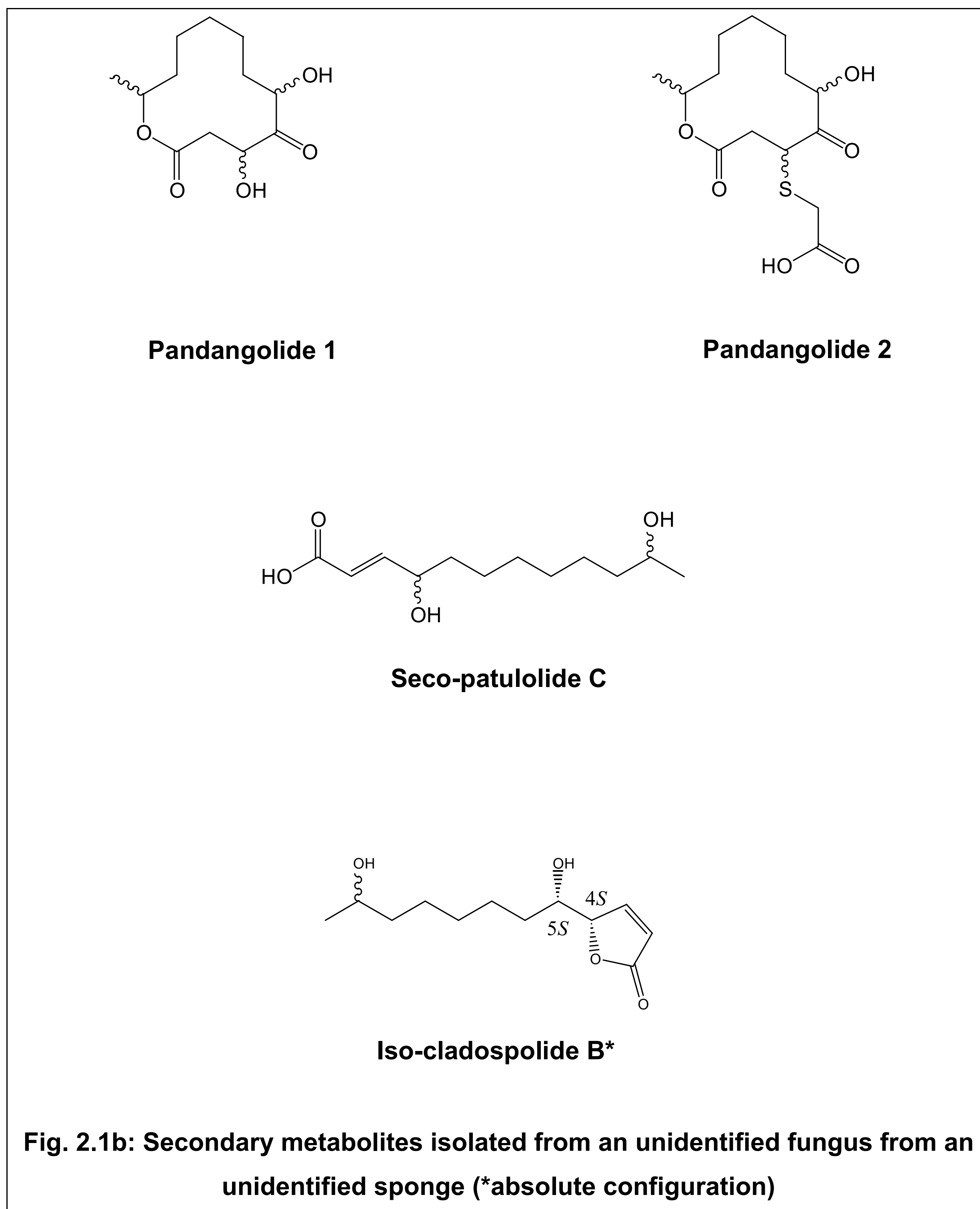


Fig. 2.1b: Secondary metabolites isolated from an unidentified fungus from an unidentified sponge (*absolute configuration)

2.1.1.2. Isolated compounds which are previously known fungal metabolites

The known compounds cladospolide B (compound **1**) (Fig. 2.1a), iso-cladospolide B (compound **2**) (Fig. 2.1b) and pandangolide 2 (compound **3**) (Fig. 2.1b) were isolated. The similarity of the UV spectra of the two isomers, compounds **1** (λ_{max} 206.3 nm) and **2** (λ_{max} 206.8 nm) indicated that the two are structurally related. Both

gave base peaks at 229 $[M+H]^+$ in the positive ion ESIMS (Fig. 2.2). The observed difference in the retention times of the two compounds in the HPLC analytical system suggested that **2** is slightly more polar than **1**. The ^1H (Fig. 2.3) and ^{13}C (Fig. 2.4) NMR spectra of the two compounds were also similar (Tables 2.3a and 2.3b).

2.1.1.2.1. Cladospolide B (1, known compound)

The ^{13}C NMR spectrum of **1** (Fig. 2.4) showed 12 carbon signals: one carboxyl, two nonsubstituted olefinic carbons, three oxygen-bound methines, five methylenes, and one methyl carbon. It gave a molecular ion peak at *m/z* 229 $[M+H]^+$ in its positive ion ESIMS spectrum (Fig. 2.2) suggesting a molecular formula of $C_{12}H_{20}O_4$. The COSY spectrum (Fig. 2.5) showed that the five methylene protons were attached to one another. Although their signals were clumped together as multiplets in the region between δ 1.40 to 1.90, it was possible to distinguish the resonances of the methylene protons H-6A and H-6B because of their correlations with H-5. Likewise, the signals corresponding to the methylene protons H-10A and H-10B were determined due to their correlations with H-11. H-11 further coupled with the methyl protons H-12 while at the other alkyl end, H-5 coupled with H-4 with a magnitude of 3.6 Hz indicating that the two hydroxyl groups at C-4 and C-5 were *cis*-oriented. H-4 was also seen to be connected to the olefinic protons H-3 and H-2. The molecular formula of the compound suggests that it must have a macrolide ring structure. The NMR data matched that of cladospolide B from the literature [Fuji *et al*., 1995].

Stereochemistry

[CD: CD: $\Delta\varepsilon_{190}$ = -2.41; $\Delta\varepsilon_{225}$ = +5.044; $\Delta\varepsilon_{282}$ = +0.123]

The coupling constant of 12.2 Hz between the two olefinic protons H-2 and H-3 of **1** indicated a *cis* configuration. Two isomers of **1**, namely, cladospolide A and C, both of which have been previously isolated with **1** from *Cladosporium tenuissimum* [Fuji *et al.,* 1995], differ from cladospolide B in having a *trans* configuration of these protons. Furthermore, a previous study [Hirota *et al*., 1985b] reported that hydrogenation of the double bond between C-2 and C-3 also produced different dihydroproducts of cladospolides A, B and C indicating different stereochemistries of the three chiral centers. The two hydroxyl groups of **1** were proven to have a *cis*

configuration due to the coupling constants of H-4 and H-5, as mentioned above. Since the $[\alpha]_D$ value (+30, *c* 0.1, MeOH) was comparable with that of cladospolide B (+45, *c* 0.4, MeOH) [Fuji *et al.,* 1995], the absolute configuration of the isolated compound is assumed to be identical to that of cladospolode B which has been established as 4*S*, 5*S* (that of C-11 was not established) by synthetic studies [Fuji *et al.*, 1995].

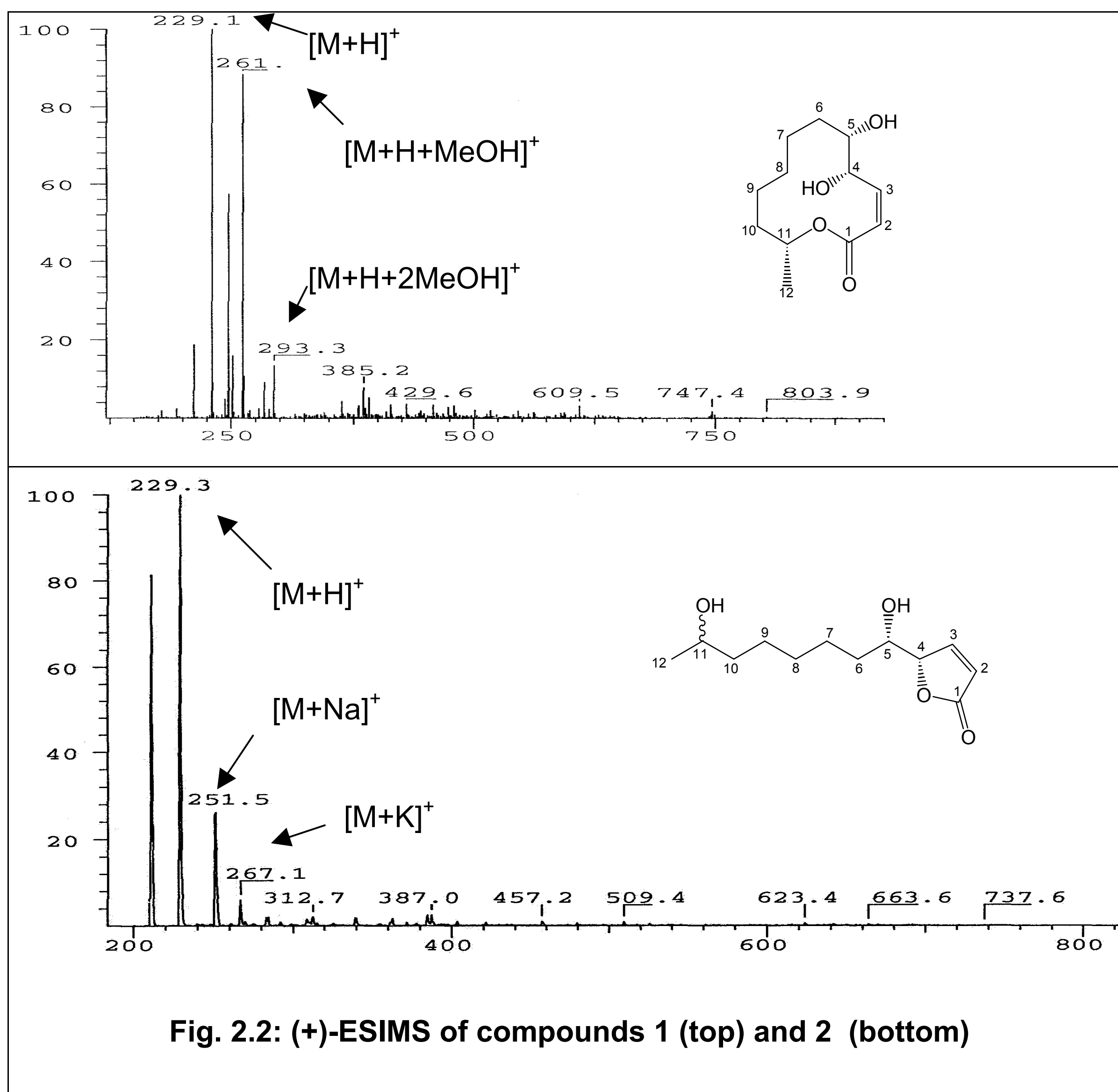


Fig. 2.2: (+)-ESIMS of compounds 1 (top) and 2 (bottom)

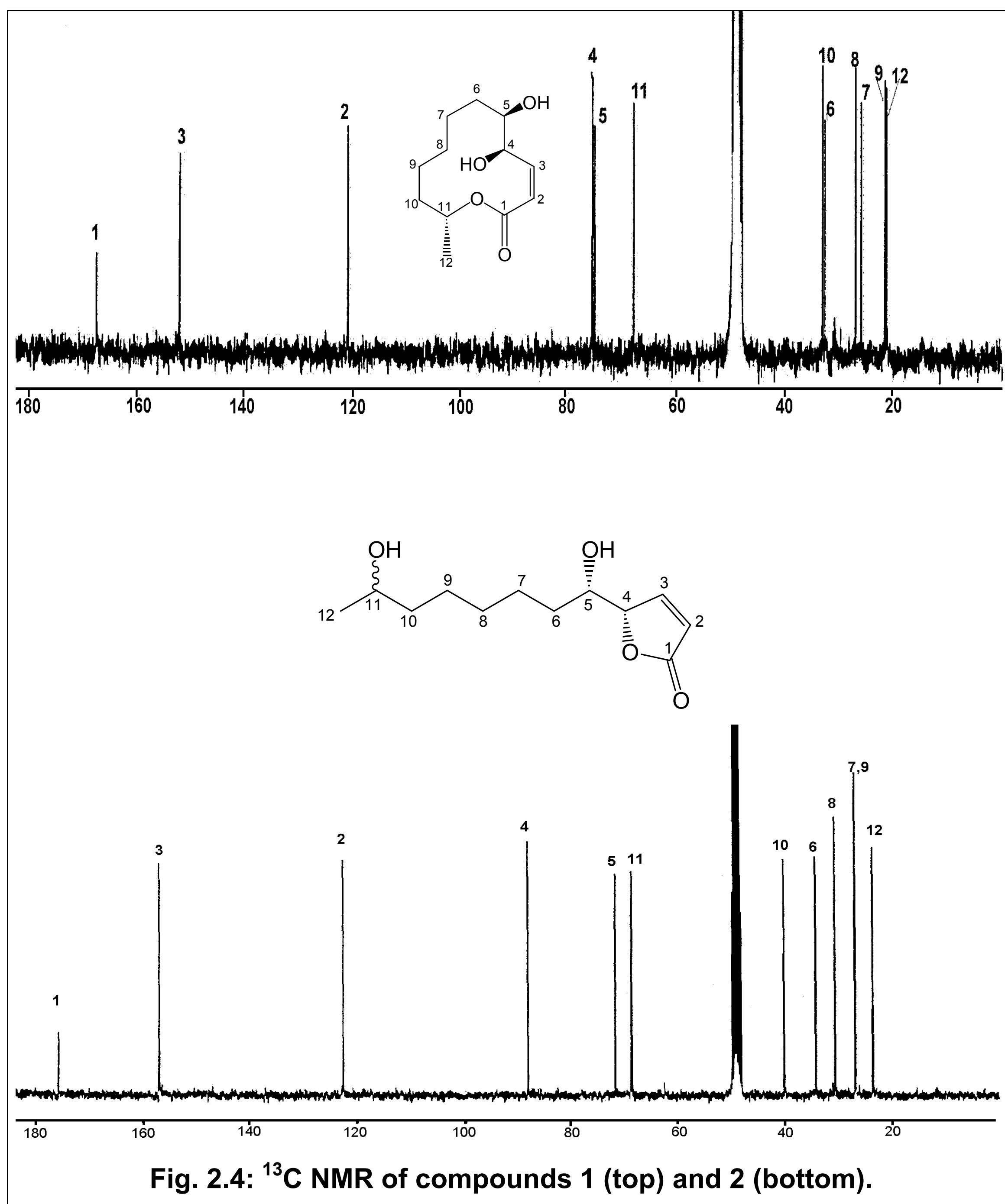


Fig. 2.4: ^{13}C NMR of compounds 1 (top) and 2 (bottom).

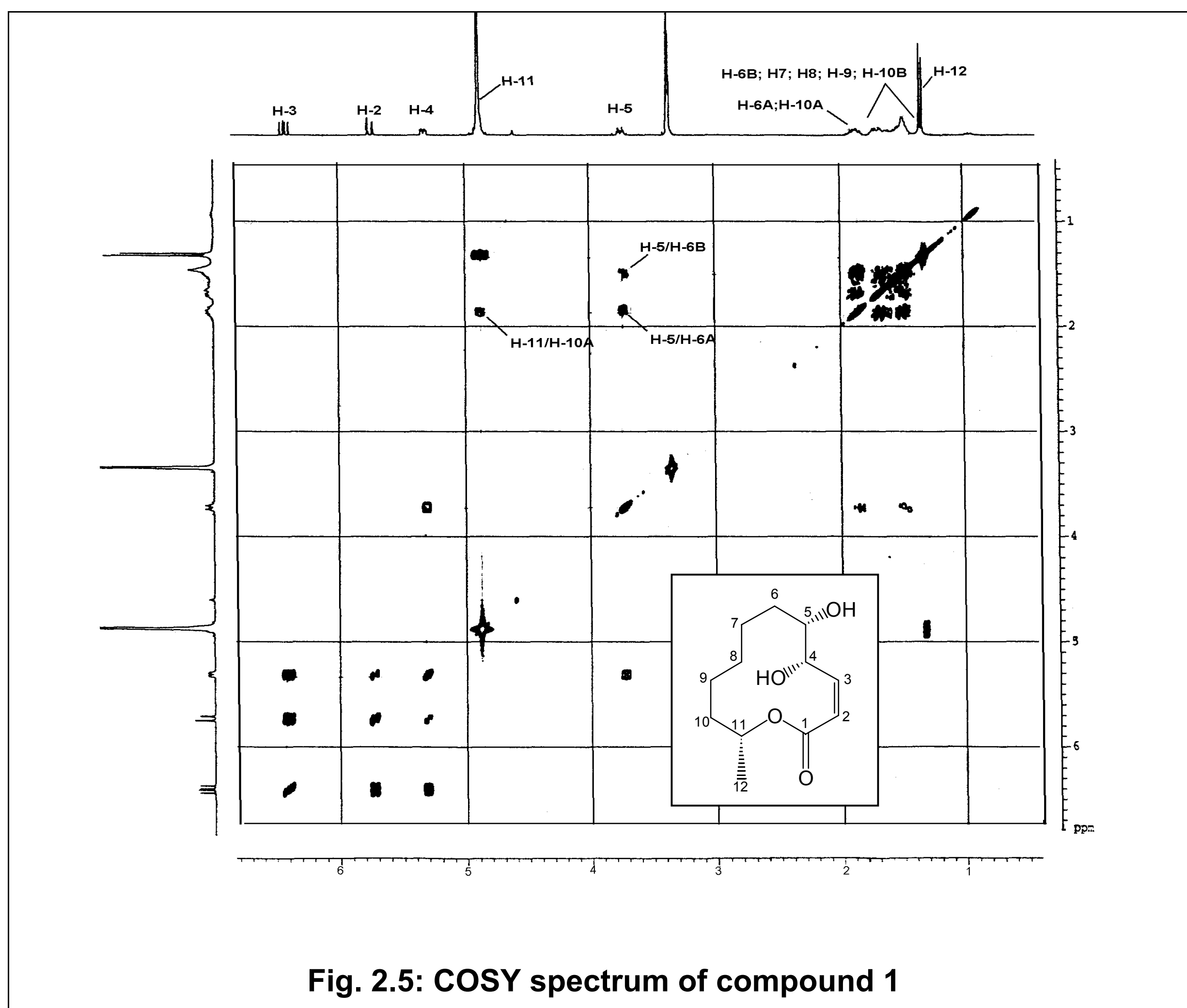


Fig. 2.5: COSY spectrum of compound 1

2.1.1.2.2. *Iso*-cladospolide B (2, known compound)

When compared with that of compound **1**, the ^{13}C spectrum (Fig. 2.3b) of **2** gave distinct differences only in the chemical shifts of C-1, C-4 and C-10. The ^{1}H NMR spectrum (Fig. 2.3) also showed upfield shifts in the signals of H-11, H_2-10 and H-6A and downfield shifts in the signals of H-2 and H-3. Furthermore, the magnitude of the coupling constant (5.8 Hz) between H-2 and H-3 suggests that the macrolide ring is replaced with a smaller ring structure. Although the HMBC spectrum (Fig. 2.6) could not distinguish whether a five- or six-membered ring is present, a literature search showed that the coupling constant between H-2 and H-3 is compatible with a five-membered lactone ring structure [Buchanan, *et al.*, 1995]. The presence of a five-membered lactone ring explained the changes in the ^{13}C chemical shifts of C-1, C-4 and C-10 as well as of H-2, H-3, H-6, H-10 and H-11. Comparison of the NMR data

of **2** with those of iso-cladospolide B isolated from an unidentified fungus derived from an unidentified sponge [Smith *et al*., 2000] confirmed that the two are identical.

Stereochemistry

[CD: CD: $\Delta\varepsilon_{190}$ = +6.43; $\Delta\varepsilon_{213}$ = -11.54; 284 = +0.093]
The protons H-4 and H-5 were seen to be *cis* oriented as deduced from the coupling constant of 3.6 Hz between the two protons. The comparable $[\alpha]_D$ values observed for **2** (–62° ,*c* 0.53, MeOH) and *iso*-cladospolide B (-90°, *c* 0.23, MeOH) [Smith *et al*., 2000] indicate that they are the same compound. The absolute configuration of C-4 and C-5 in *iso*-cladospolide B was established by synthesis in a separate study [Franck *et al.,* 2001].

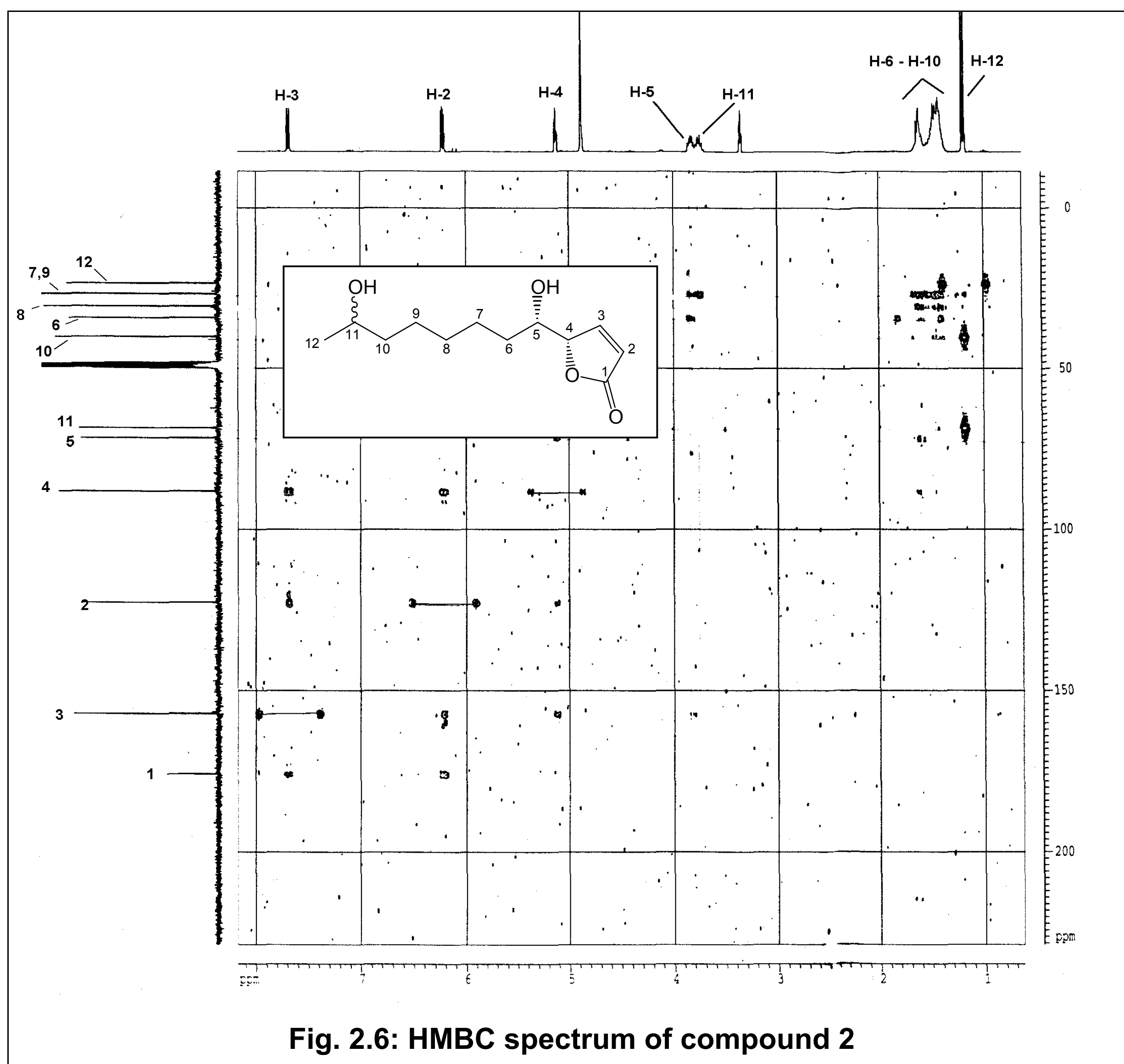


Fig. 2.6: HMBC spectrum of compound 2

2.1.1.2.3. Pandangolide 2 (3, known compound)

Compounds **3**-**5** (Fig. 2.1a,b and Fig. 2.9) exhibited highly similar ^{1}H (Table 2.3a) and ^{13}C (Fig. 2.11) (Table 2.3b) NMR spectra, from which it can be deduced that all three of them possess common structural features which distinguish them from **1** and **2**. The loss of unsaturation of C-2 and C-3 is one such feature. C-4 was also oxidized to a keto carbon and all three also have a thioalkyl side chain attached to C-3.

Compound **3** showed a molecular ion peak at *m/z* 318 $[M]^+$ with EIMS (Fig. 2.7). An intense ion peak at *m/z* 319 i$[M+H]^+$ in the positive ion ESIMS furthered verified the molecular mass which is compatible with the molecular formula $C_{14}H_{22}O_6S$. The COSY spectra (Fig. 2.8) showed the presence of three separate spin systems. The first consists of a methyl group, two methines which are geminally attached to oxygen substituents, and five methylenes. The second spin system includes a methylene H-2A and H-2B which coupled with a methine H-3. The third spin system consists of a diastereotopic methylene H-13A and H-13B which was shown by the HMBC spectrum to be attached to a carboxyl. The chemical shifts of H-3 (δ 3.67) and of C-3 (δ 41.2), as well as those of H-13A and H-13B (δ 3.36 and 3.26, respectively) and of C-13 (δ 35.0), ruled out an oxygen-containing substituent and the presence of a sulfide linking the two spin systems was proposed. The NMR data of **3** (Table 2.3a and 2.3b) was similar to published data from the previously isolated pandangolide 2 [Smith *et al.*, 2000].

Stereochemistry

[CD: CD: $\Delta\varepsilon_{214}$ = -1.196; $\Delta\varepsilon_{223}$ = -0.363; $\Delta\varepsilon_{247}$ = +1.720; $\Delta\varepsilon_{287}$ = -0.323; $\Delta\varepsilon_{342}$=+0.103]
The observed $[\alpha]_D$ value of –57° (*c* 0.72, MeOH) of **3** was similar with that of pandangolide 2 (-30, *c* 0.19, MeOH) [Smith *et al.*, 2000] suggesting that the two compounds are identical. Attempts at determining the relative stereochemistry of C-3 and C-5 of compound 3 by recording the ROESY spectrum of the compound was not successful (Fig. 2.8). No correlation was seen between H-3 and H-5 probably due to the flexibility of the bonds in the macrolide ring. Performing the Mosher reaction would only have established the stereochemistry at C-5 and possibly at C-11 after

hydrolysis of the lactone bond. However, due to low yield this reaction was not attempted.

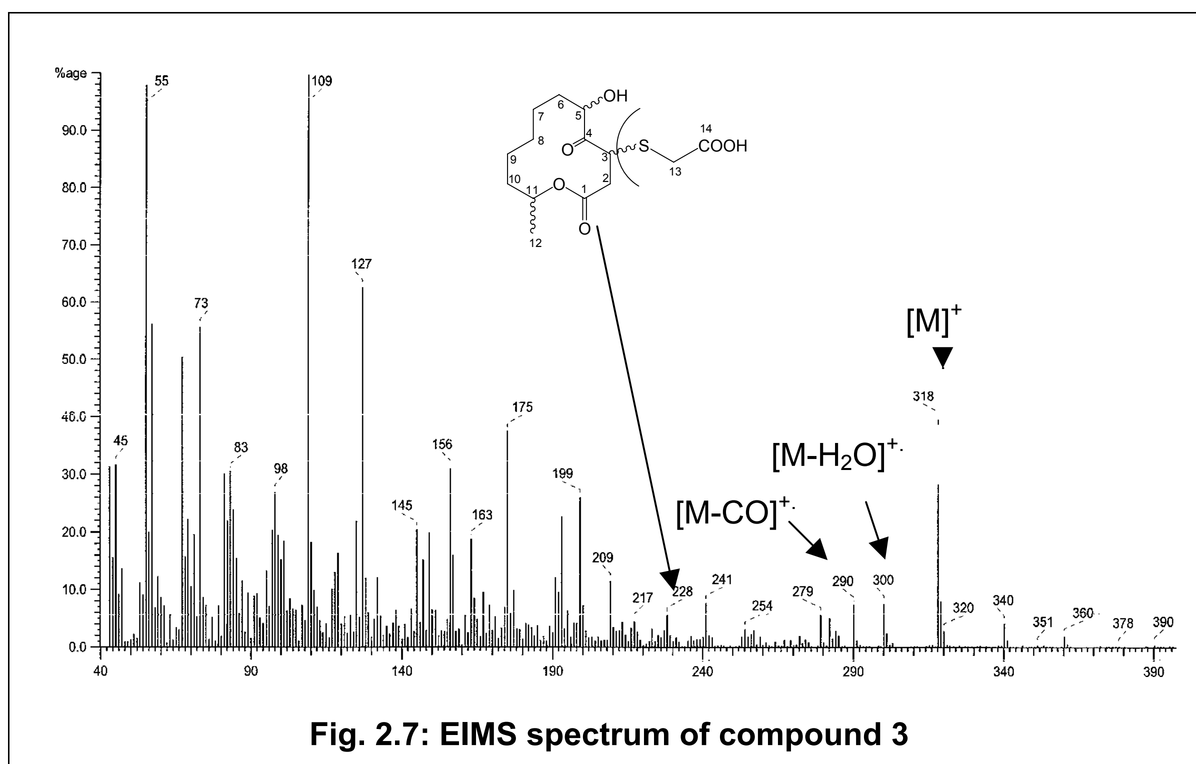


Fig. 2.7: EIMS spectrum of compound 3

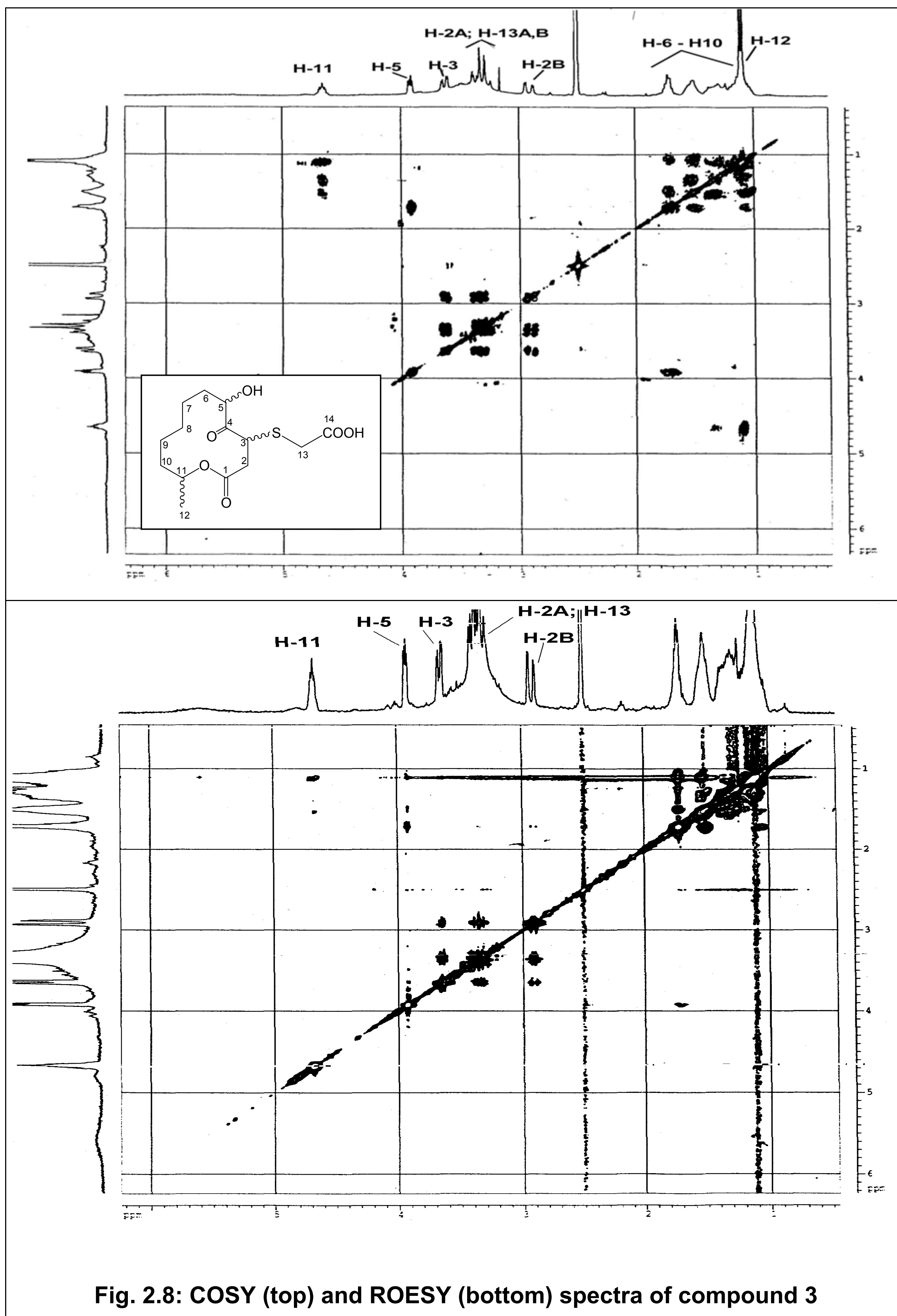


Fig. 2.8: COSY (top) and ROESY (bottom) spectra of compound 3

Table 2.3a. 1H NMR (CD3OD) data* for compounds 1-5

Atom	1	2	3	4	5
2a	5.74 (dd, 12.2, 1.5)	6.21 (dd, 5.8, 1.9)	3.34 (dd, 19.4, 12.7)	3.49 (dd, 19.4, 12.4)	3.46 (dd, 19.4, 12.7)
2b			2.91 (dd, 19.4, 3.4)	3.00 (dd, 19.4, 3.8)	2.95 (dd, 19.4, 3.8)
3	6.41 (dd, 12.2, 8.2)	7.69 (dd, 5.8, 1.6)	3.67 (dd, 12.7, 3.4)	3.72 (dd, 12.4, 3.8)	3.98 (dd, 12.7, 3.8)
4	5.31 (ddd 8.2, 3.2, 1.5)	5.12 (ddd, 3.6, 1.9, 1.6)			
5	3.72 (dd, 3.2, 10.2)	3.83 (dd, 7.6, 3.6)	3.92 (dd, 7.1, 3.7)	4.05 (dd, 8.0, 3.6)	4.06 (dd, 7.9, 3.3)
6a	1.85 (m)	1.61 (m)	1.70 (m)	1.88 (m)	1.88 (m)
6b	1.50 (m)	1.61 (m)	1.70 (m)	1.88 (m)	1.88 (m)
7a	1.40-1.80 (m)	1.45 (m)	1.05 (m)	1.64 (m)	1.67 (m)
7b	1.40-1.80 (m)	1.45 (m)	1.50 (m)	1.27 (m)	1.27 (m)
8a	1.40-1.80 (m)	1.45 (m)	1.00-1.40 (m)	1.27 (m)	1.27 (m)
8b	1.40-1.80 (m)	1.45 (m)	1.00-1.40 (m)	1.27 (m)	1.27 (m)
9a	1.40-1.80 (m)	1.45 (m)	1.00-1.40 (m)	1.47 (m)	1.47 (m)
9b	1.40-1.80 (m)	1.45 (m)	1.00-1.40 (m)	1.27 (m)	1.27 (m)
10a	1.85 (m)	1.50	1.55 (m)	1.64 (m)	1.67 (m)
10b	1.85 (m)	1.50	1.35 (m)	1.47 (m)	1.47 (m)
11	4.9 (m)	3.74 (m)	4.65 (m)	4.9 (m)	4.88 (m)
12	1.32 (d, 6.1)	1.32 (d, 6.1)	1.10 (d, 6.1)	1.23 (d, 6.2)	1.10 (d, 6.1)
13a			3.26 (d, 15.4)	3.12 (dd, 13.9, 6.5)	
13b			3.36 (d, 15.4)	3.06 (dd, 13.9, 5.5)	
14				4.41 (dd, 5.5, 6.5)	
15					
16				3.79 (s)	

* presented here as δ signal (multiplicity, *J* in Hz)

Table 2.3b. ^{13}C NMR data for compounds 1-5

Carbon Atoms	1 (CD_3OD)	2 (CD_3OD)	3 (DMSO-d_6)	4 (CD_3OD)	5 (CD_3OD)
1	167.6	175.8	169.5	172.5	174.5
2	121.1	122.7	40.5	42.2	41.9
3	152.1	157.1	41.2	43.7	43.6
4	75.7	88.2	213.9	215.0	214.5
5	75.3	71.6	75.6	77.8	77.7
6	32.5	34.2	30.9	32.4	32.4
7	25.9	26.8	20.4	21.7	21.6
8	26.9	30.6	27.5	29.0	29.0
9	21.5	26.8	22.3	23.7	23.6
10	33.0	40.1	32.5	34.1	34.0
11	68.0	68.5	72.0	74.4	74.5
12	21.3	23.5	19.6	20.0	19.9
13	-	-	35.0	36.6	-
14	-	-	broad peak	72.0	-
15	-	-	-	174.5	-
16	-	-	-	52.6	-

2.1.1.3. New isolated compounds

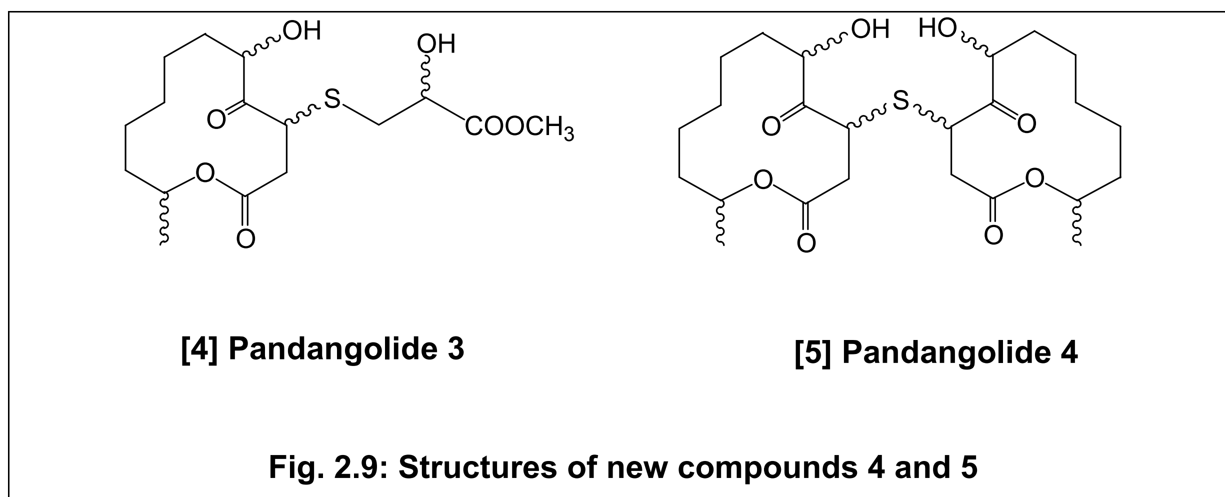


Fig. 2.9: Structures of new compounds 4 and 5

2.1.1.3.1. Pandangolide 3 (4, new compound)

HREIMS m/z 362.1391(calcd for $C_{16}H_{26}O_7S$, 362.1399)

$[\alpha]_D$ –57 (c, 0.72, MeOH)

CD: $\Delta\varepsilon_{215}$ = -1.627; $\Delta\varepsilon_{227}$ = -0.172; $\Delta\varepsilon_{240}$ = +1.726; $\Delta\varepsilon_{363}$ = 0.043

Compound **4** (Fig. 2.9) was obtained as an oil which showed a base peak in the positive ion ESIMS at *m/z* 363 $[M+H]^+$, and a molecular ion peak at *m/z* 362 $[M]^+$ with EIMS (Fig. 2.10). The exact mass of this ion, which was measured by HREIMS to be 362.1391, corresponds to the molecular formula $C_{16}H_{26}O_7S$.

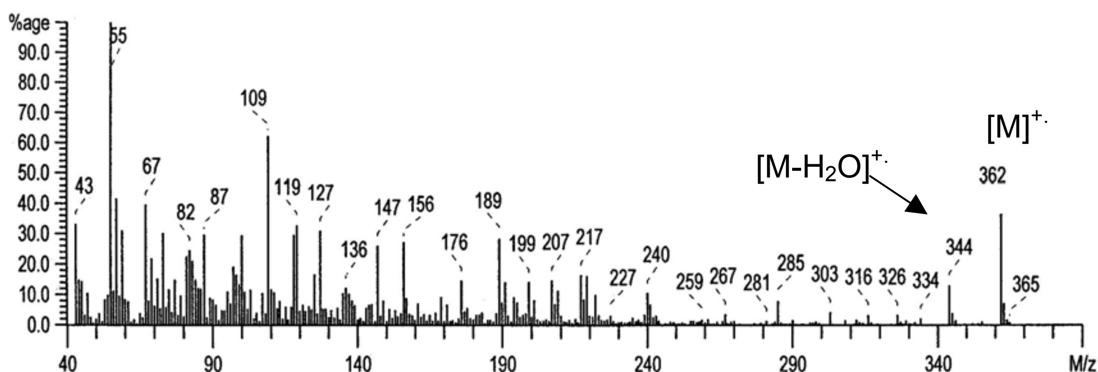


Fig. 2.10: EIMS spectrum of compound 4

The ^{13}C NMR spectrum (Fig. 2.11) of **4** was remarkably similar to that of **3**, with an additional lowfield signal from a methine carbon (C-14) at 72.0 ppm indicating an oxygen-bound carbon. Another lowfield signal at 52.6 ppm suggested a methoxyl carbon (C-16) which was attached to C-15, based on an HMBC correlation of H_3-16 (δ 3.79) with C-15 (δ 174.5) (Fig. 2.16). Correlations between H_2-13 and H-14 in the COSY spectrum (Fig. 2.12) as well as between H_2-13 with C-14 and C-15, respectively, in the HMBC spectrum, revealed the connectivities in the sulfur-containing side chain. The position of the sulfide group with respect to the macrocyclic lactone ring was evident from a long-range correlation between H-13 and C-3, and *vide infra*. The positions of C-1 to C-6 were established from correlations between H_2-2, H-3, H-5, and H_2-6 with C-4, and H_2-2 as well as H-3 with C-1, respectively. The assignments of C-7 to C-10 were based on key HMBC correlations between H-5 with C-7, H-6B with C-8, and H_3-12 with C-10, respectively.

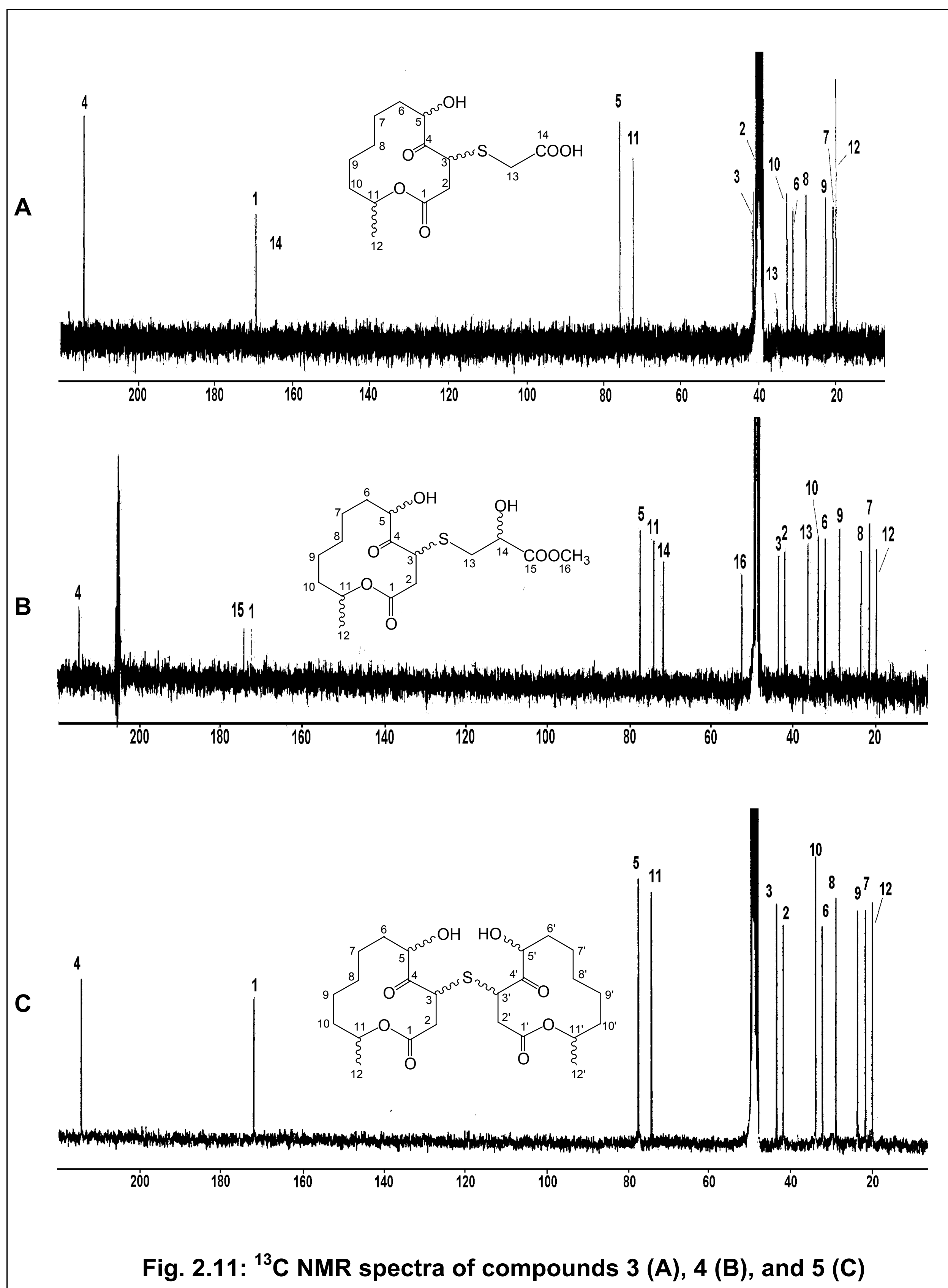


Fig. 2.11: ^{13}C NMR spectra of compounds 3 (A), 4 (B), and 5 (C)

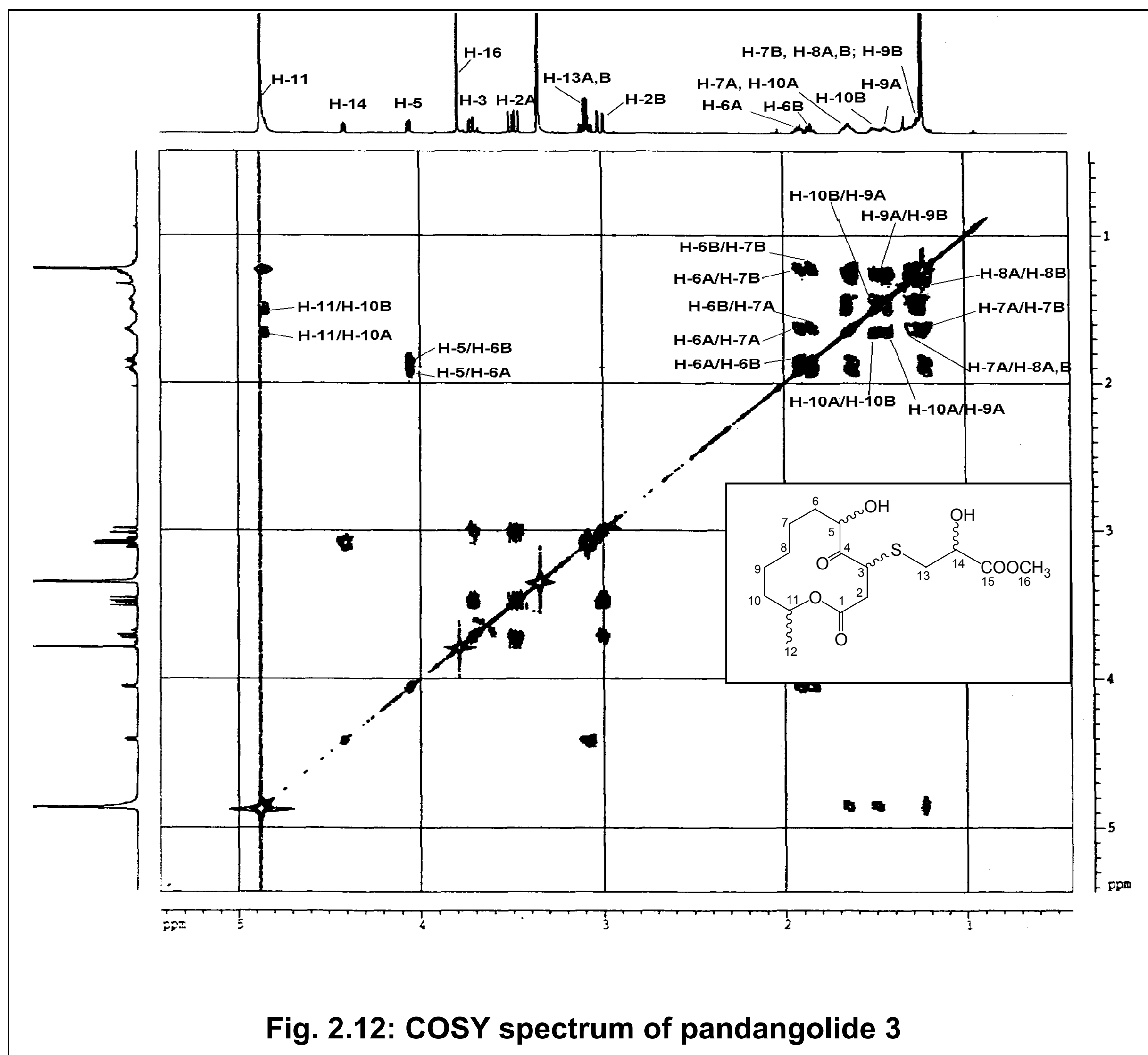


Fig. 2.12: COSY spectrum of pandangolide 3

Important correlations from the HMBC spectrum of pandangolide 3 which established the point of attachment of the sulfide side chain as well as the C-C connectivities therein are shown below (Fig. 2.13).

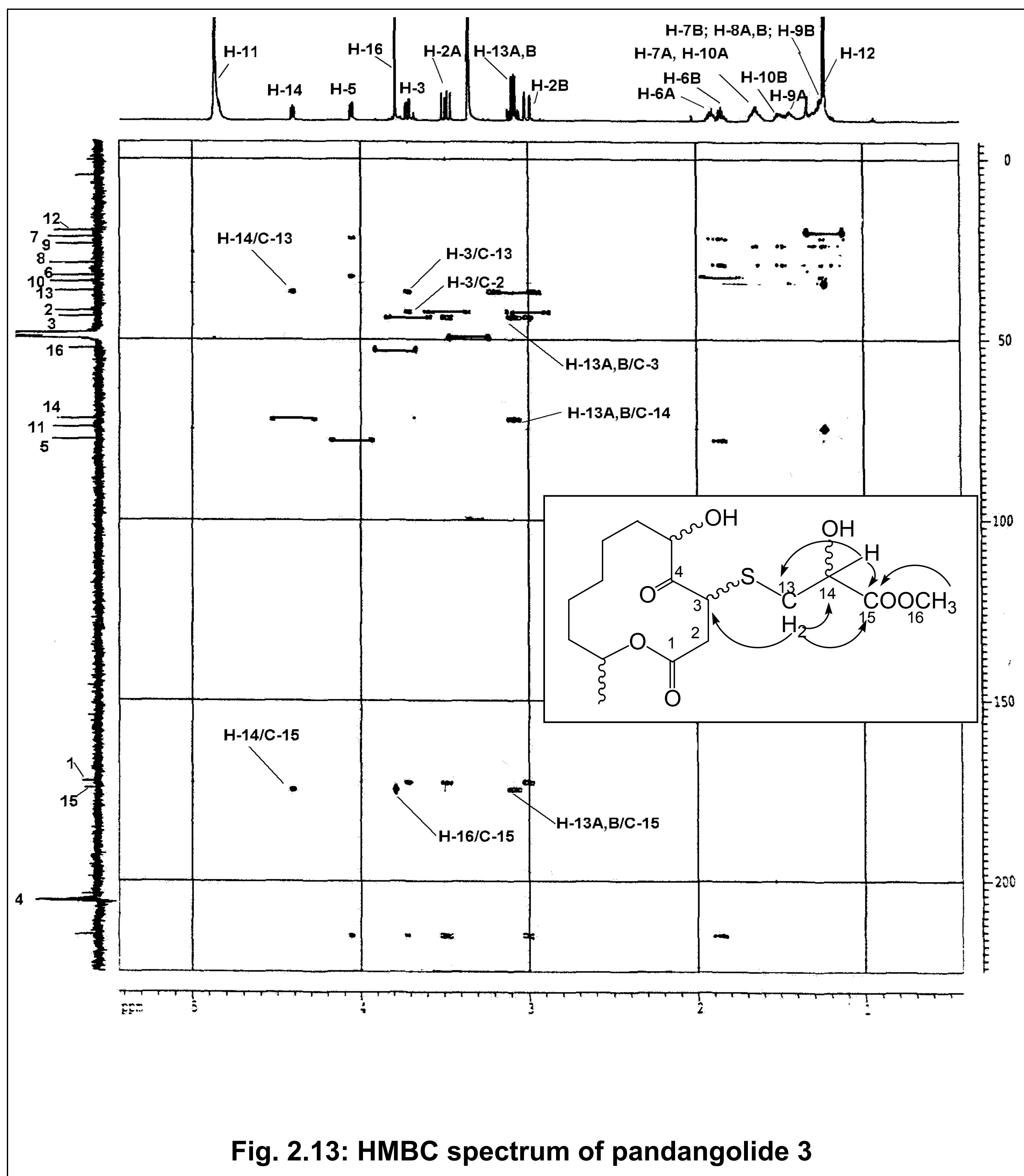


Fig. 2.13: HMBC spectrum of pandangolide 3

2.1.1.3.2. Pandangolide 4 (5, new compound)

HREIMS: *m/z* 486.2276 $[M]^+$ (calcd for $c_{24}H_{38}O_8S$, 486.2287)

$[\alpha]_D$ –55.1 (0.53, MeOH)

CD: $\Delta\varepsilon_{215}$ = 0.166; $\Delta\varepsilon_{227}$ = -1.183; $\Delta\varepsilon_{240}$ = +0.050; $\Delta\varepsilon_{363}$ =+0.0.059

Compound **5** was obtained as an oil whose positive ion ESIMS spectrum showed an intense pseudo molecular ion peak at *m/z* 487 ($[M+H]^+$). EIMS (Fig. 2.14)

measurement gave a molecular ion peak at *m/z* 486. It has a molecular weight of 486.2276 as measured by HREIMS, corresponding to the molecular formula $C_{24}H_{38}O_8S$.

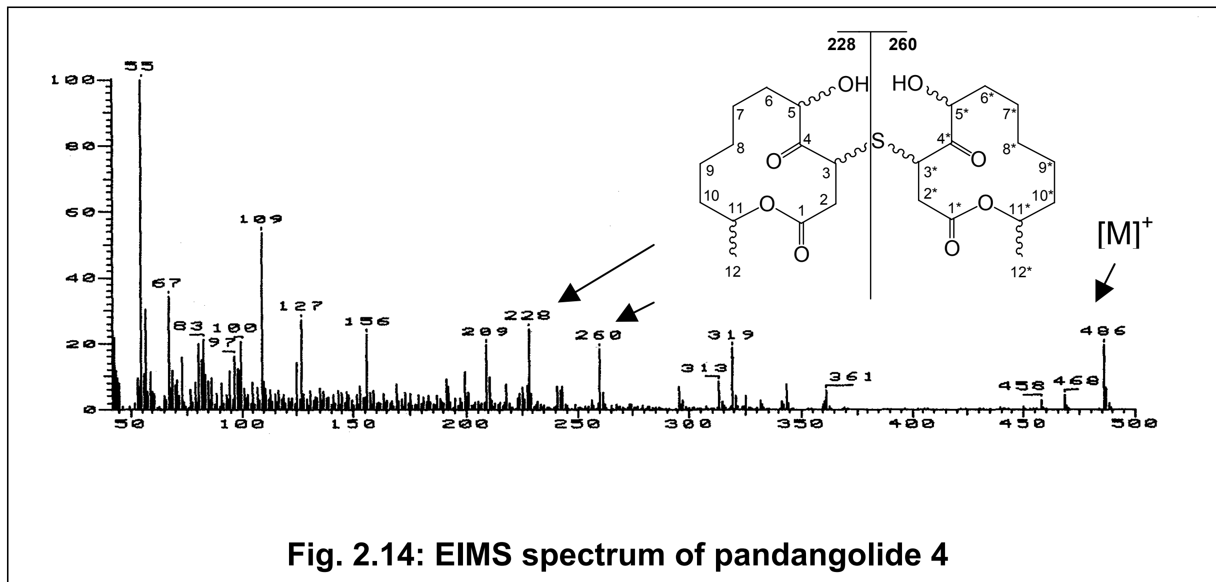


Fig. 2.14: EIMS spectrum of pandangolide 4

The ^{13}C NMR spectrum (Fig. 2.11) showed only 12 signals while integration of the proton signals in the ^1H NMR spectrum yielded only 19 protons suggesting that **5** is a symmetrical dimer. The ^{13}C NMR spectrum was similar to that of pandangolide 2 (**3**) and 3 (**4**) but with the absence of the alkyl side chain. The similarity in the chemical shifts of C-2 and C-3 in **3, 4,** and **5** indicates that in **5** the sulfide was retained, but instead of being linked to an alkyl side chain, it bridged two identical macrocyclic lactone rings to form the dimer. This was corroborated by EIMS signals at *m/z* 228 (Fig. 2.14), which corresponds to the mass of a monomer, and at *m/z* 260, corresponding to a monomer with a thiol group. Furthermore, the dimeric structure of **5** was proved by the HMBC spectrum where H-3, aside from showing a residual direct orrelation signal with C-3, also gave a long-range correlation with the respective carbon C-3' (Fig. 2.16). As in compounds **1-4** assignments of proton signals H-6 to H-10 were possible from correlations in the COSY spectrum (Fig. 2.15).

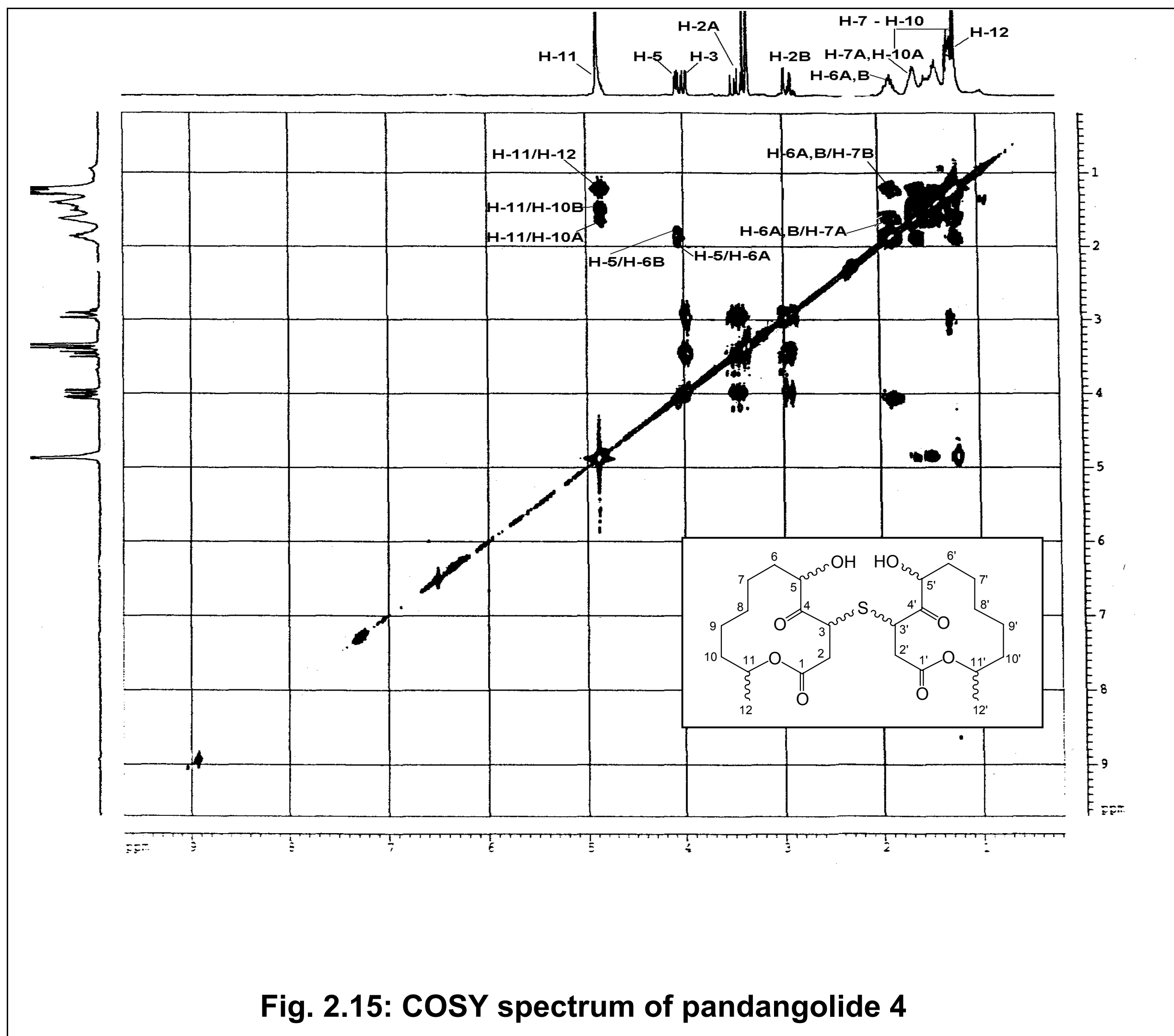


Fig. 2.15: COSY spectrum of pandangolide 4

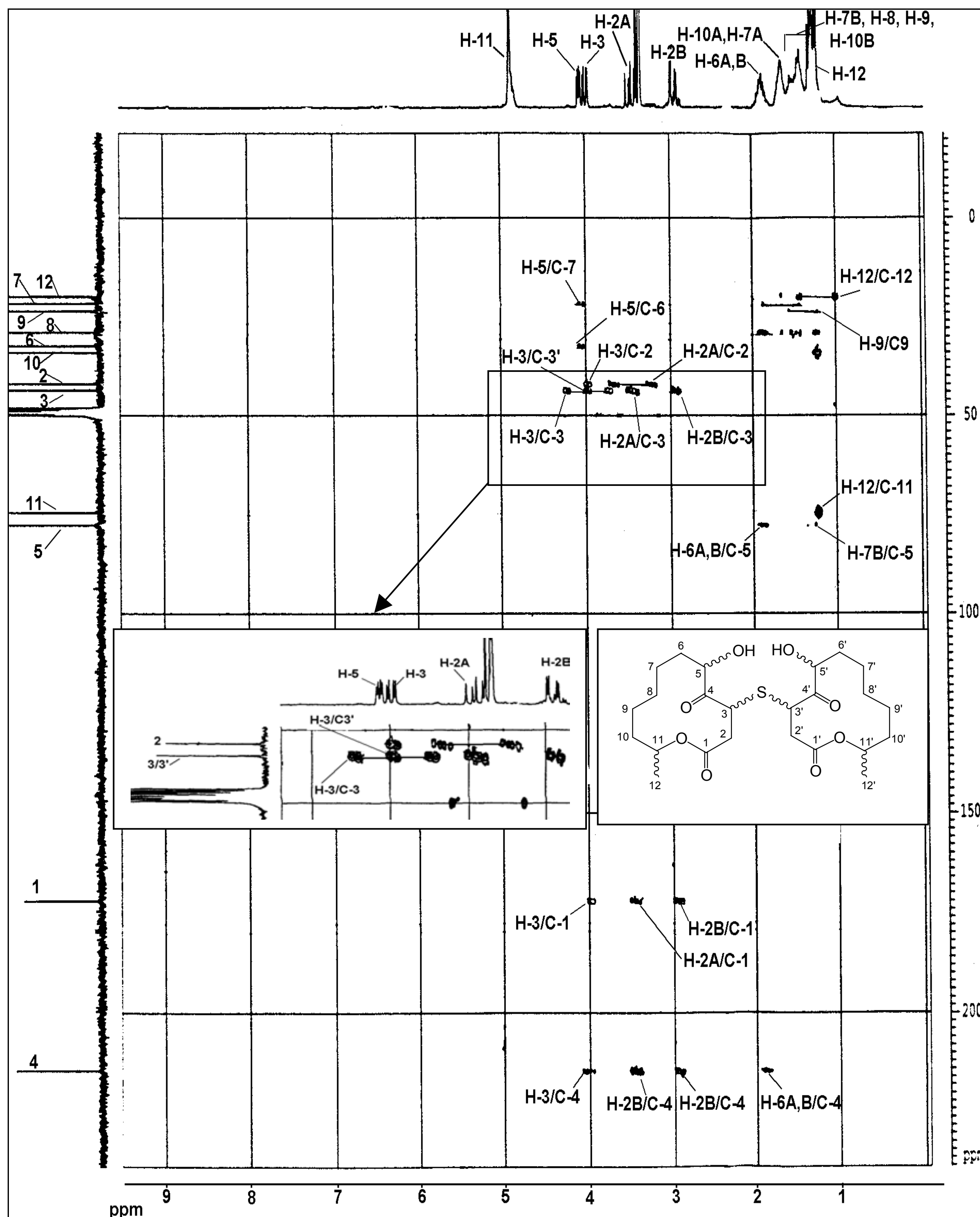


Fig. 2.16: HMBC spectrum of pandangolide 4

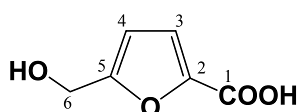
As with **4**, attempts at determining the relative stereochemistry of C-3 and C-5 in **5** proved unsuccessful when measurement of its NOESY and ROESY spectra in two different instruments exhibited no significant NOEs involving H-5, H-3 as well as H-12. This may have been due to the flexibility of the bonds in the macrolide structure. Mosher reaction, which could have established the absolute stereochemistry of C-5 and C-11 (after hydrolysis) of both compounds, as well as of C-14 of **4**, was not performed due to the limited amounts isolated.

Bioactivity

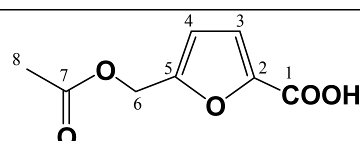
In agreement with previous reports [Smith *et al*., 2000] compounds **1-5** were inactive when tested against Gram-positive and Gram-negative bacteria. This appears to confirm previously drawn observations that antimicrobial activity of these macrolides requires the presence of a double bond flanked by two carbonyl carbons, which are common in other antimicrobial agents such as pyrenophorin, pyrenolides, and vermiculine [Rodphaya *et al*., 1986].

Likewise, the isolated macrolides also exhibited no activity against *A. salina, S. littoralis*, and human leukemia cell lines.

2.1.1.4. Furan carboxylic acid derivatives



(6) sumiki's acid



(7) acetyl sumiki's acid

Fig. 2.17: Structures of isolated furanoic acid derivatives

2.1.1.4.1. Sumiki's acid (6, known compound)

Compound **6** (Fig. 2.17), which was isolated as a brown oil, is a known fungal metabolite originally isolated from *Penicillium italicum* and *Pyricularia grisea* [Kawarada *et al.,* 1955; Munekata and Tamura, 1981]. Sumiki's acid gave an molecular ion peak at *m/z* 142 in EIMS (Fig. 2.18). This molecular mass was compatible with the molecular formula $C_6H_6O_4$. The ^{13}C (Fig. 2.21) and DEPT spectra showed five sp^2 carbons: the two at δ 119.9 and δ 110.2 were unsubstituted and of the three substituted carbons, two gave lowfield signals at δ 161.9 and δ 160.6. An oxygen-bound methylene carbon at δ 57.5 was also observed. The two olefinic protons coupled at a magnitude of 3.4 Hz compatible with protons in a furan ring. The ^{13}C and ^{1}H of **6** matched those of sumiki's acid as described in the literature [Munekata and Tamura, 1981]. Additional proof of the proposed structure was given by the fragmentation pattern in the EIMS (Fig. 2.18). Ion peaks at *m/z* 125 and 97 correponding to $[M–OH]^+$ and $[M–COOH]^+$, respectively, were observed.

2.1.1.4.2. Acetyl sumiki's acid (7, new compound)

Compound **7**, which was also isolated as a brown oil, gave a molecular ion peak at *m/z* 184 in EIMS (Fig. 2.18). An additional ion peak at 142 was also observed which could be $[M – COCH_3]^+$. It exhibited a similar fragmentation pattern as **6**, showing ion peaks at *m/z* 125 and 97 corresponding to $[M–OH]^+$ and $[M–COOH]^+$, respectively. Likewise, the UV spectra of **6** (maximum absorption at 260 nm) and **7** (maximum absorption at 256 nm) were nearly identical (Fig. 2.19).

The ^{1}H NMR spectra of both compounds (Fig. 2.20) (Table 2.4) were also very similar. The differences include the downfield shift of H-6 from 4.61 in **6** to 5.14 in **7**, together with an extra methyl group signal at 2.11 ppm in **7**. These and the MS data established the structure of **7**.

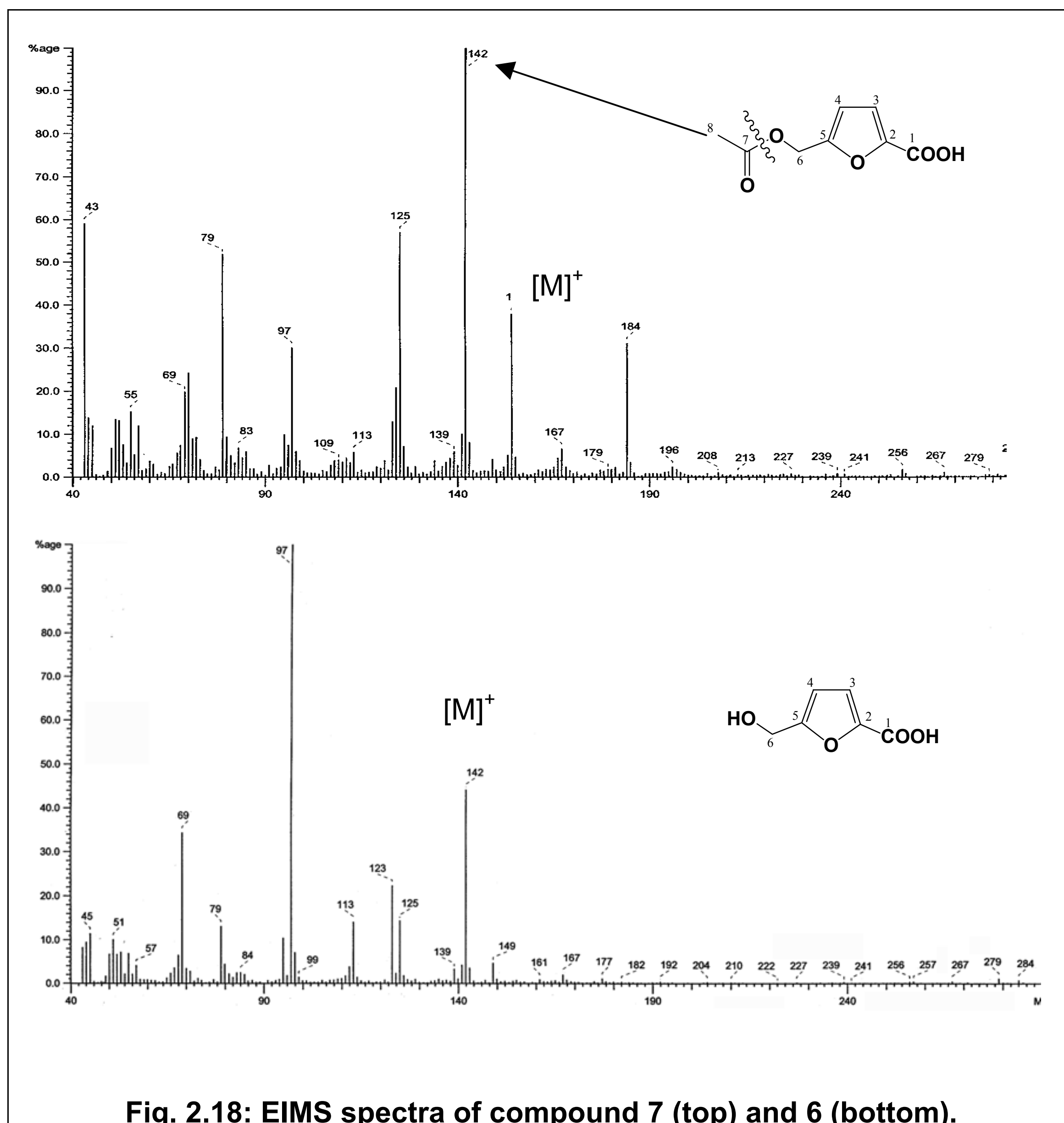


Fig. 2.18: EIMS spectra of compound 7 (top) and 6 (bottom).

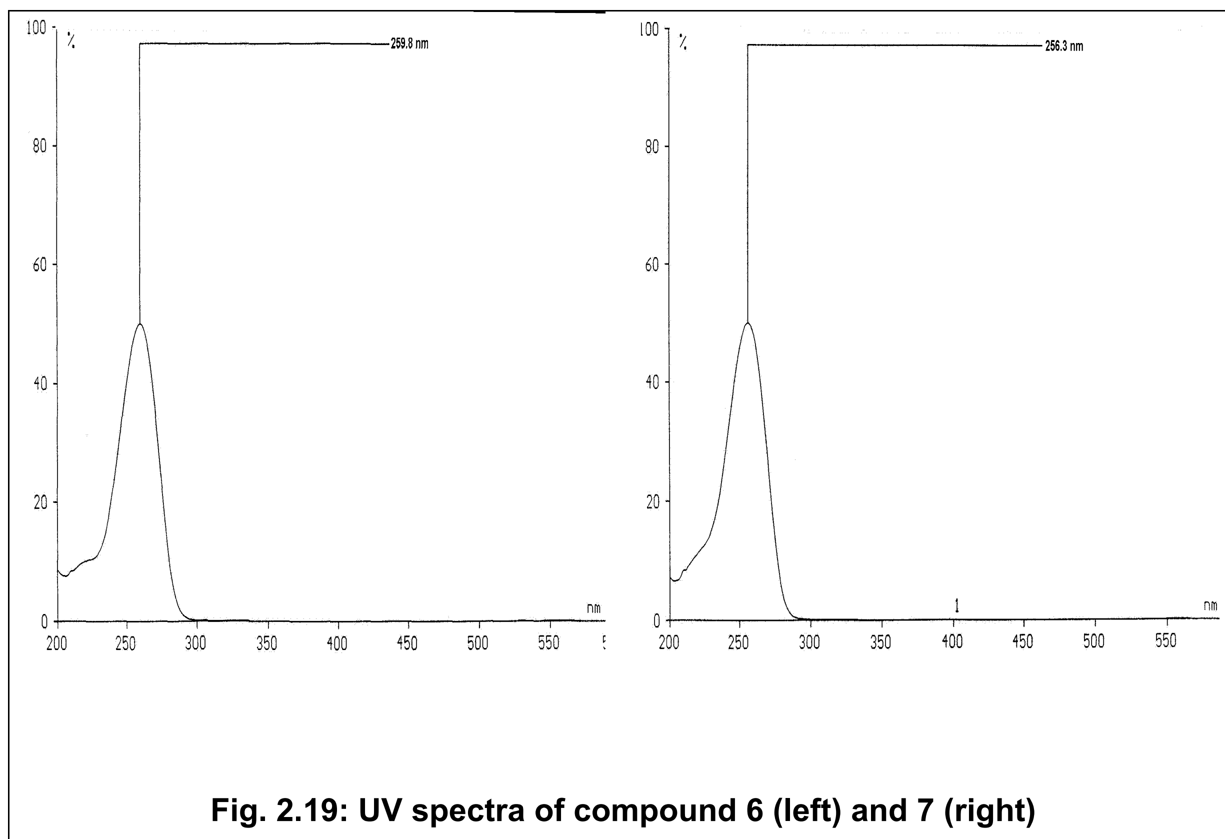


Fig. 2.19: UV spectra of compound 6 (left) and 7 (right)

Table 2.4: 1H NMR (CD_3OD) data for compounds 6 and 7

Carbon Atoms	^{13}C	^{1}H	^{1}H
	6	**6**	**7**
1	161.9		
2	145.9		
3	119.9	7.19 d, *J* = 3.5	7.16 d, *J* = 3.4
4	110.2	6.50 d, *J* = 3.5	6.61 d, *J* = 3.4
5	160.6		
6	57.5	4.60 s	5.14 s
7			
8			2.11 s

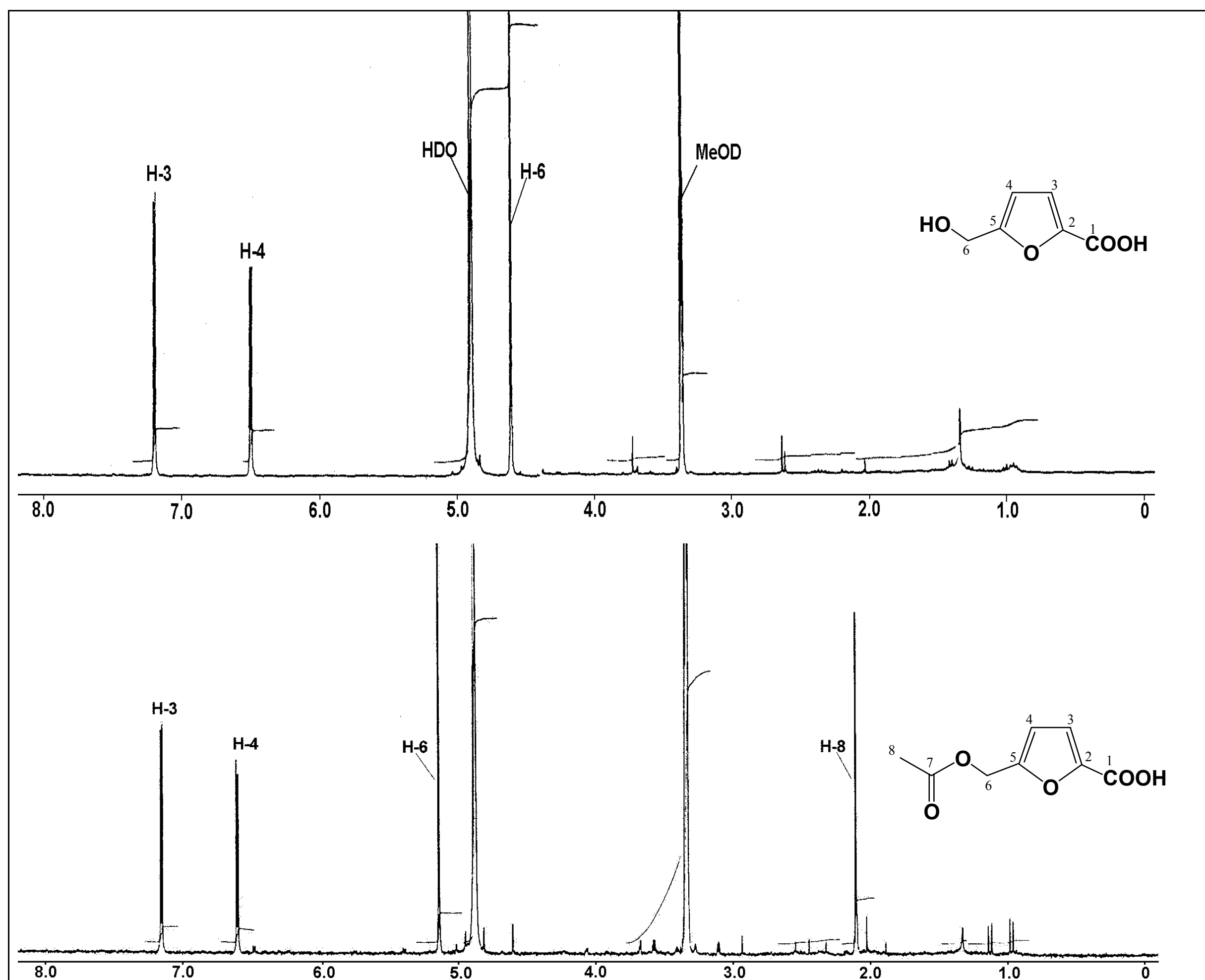


Fig. 2.20: ^{1}H NMR of compounds 6 (top) and 7

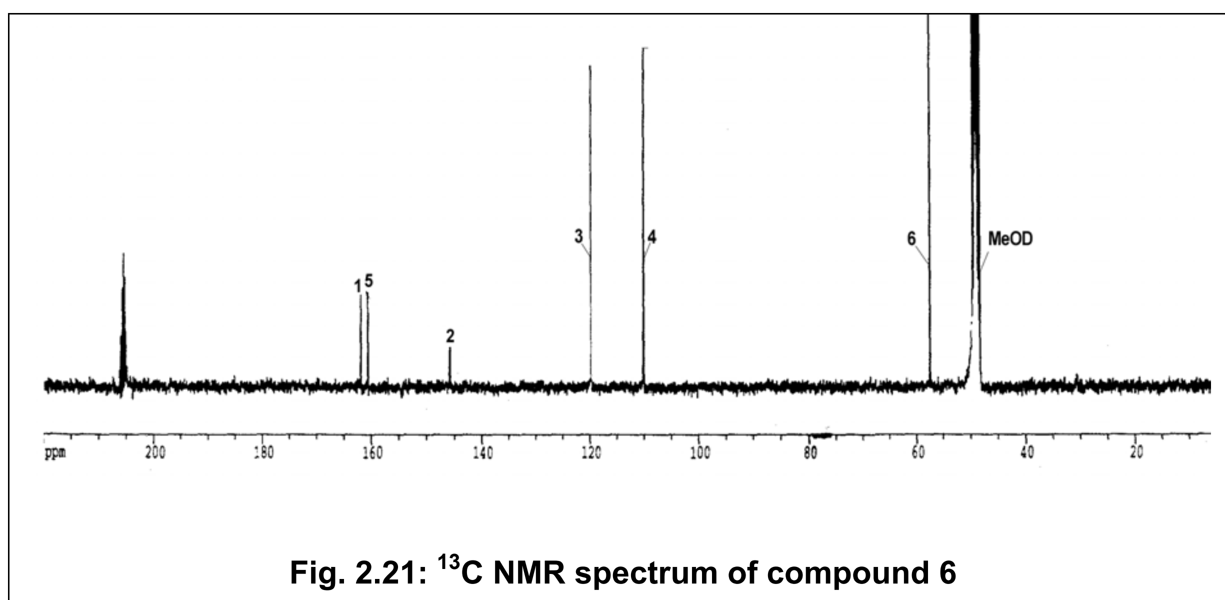


Fig. 2.21: ^{13}C NMR spectrum of compound 6

Bioactivity

Sumiki's acid was reported to exhibit some antitumor activities against SV40-transformed cels [Munekata and Tamura, 1981]. When tested agaisnt human leukemia cell lines, the isolated sumiki's acid (compound **6)**, however, was inactive. It was also inactive against *A. salina* and against the polyphagous insect, *S. littoralis*.

It was, however, found to be moderately active against *S. aureus* and *B. subtilis* (Table 2.5) and against *C. albicans* (Table 2.6).

Table 2.5: Agar plate diffusion assay data of compounds 6 and 7.

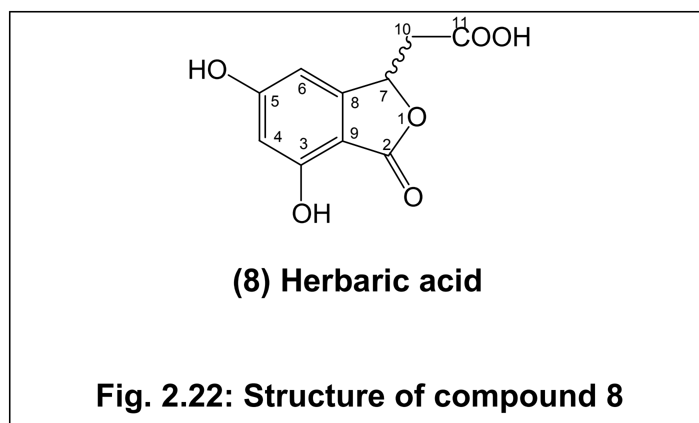
	B. subtilis	*S. auerus*	*E. coli*
EtOAc Phase (1 mg)	9 mm	13 mm	n.a.
n-BuOH Phase (1 mg)	11 mm	11 mm	n.a.
Petroleum Ether Phase (1 mg)	n.a.	n.a.	n.a.
compound **6** (5 μg)	7 mm	7 mm	n.a.
compound **7** (5 μg)	7mm	7 mm	n.a.

*Filter disk diameter is 5.5 mm

Table 2.6: Antimicrobial activity of of various concentrations of compound 6 against *C. albicans* in agar plate diffusion assay.

Loading Concentration (μg/disk)	Zone of Inhibition (mm)
1	12
5	12
10	18
50	18

2.1.1.5. Herbaric acid (8, new compound)



(8) Herbaric acid

Fig. 2.22: Structure of compound 8

Compound **8** (Fig. 2.22) was obtained as a brown, amorphous powder. The molecular formula was deduced as $C_{10}H_8O_6$ by HREIMS. Its structure was established through 1 D (^{1}H and ^{13}C) and 2 D (COSY, HMQC and HMBC) NMR (Table 2.7). The ^{13}C NMR spectrum (Fig. 2.23) showed 10 carbon atoms: two carboxyl carbons (173.0 and 171.4); six aromatic carbons, two of which are oxygen-substituted; an oxygen-substituted tertiary carbon; and one methylene group. The ^{1}H NMR (Fig. 2.24) and COSY spectra showed two *meta*-coupled aromatic protons at δ 6.42 and 6.30 (^4J = 1.6 Hz), a methylene at δ 2.91 and 2.73 with a geminal coupling constant of 16.5 Hz which further coupled to a methine at δ 5.68 (^3J = 5.2 and ^3J = 7.9 Hz, respectively). The proposed structure of herbaric acid and the assignment of NMR signals were both confirmed by HMQC and HMBC (Fig. 2.25). An apparent long range coupling between H-6 and H-7 (^4J = 0.9 Hz) was also confirmed through a homonuclear spin decoupling experiment (Fig 2.26), where irradiation at δ 5.68 (expected H-7 signal) gave an NOE effect on H-6. Furthermore, a long range correlation between H-6 and C-7 was also observed in the HMBC spectrum (Fig. 2.25). This, and a long range correlation due to W-bond coupling between H-4 and C-2 established the fused ring structure. The EIMS spectrum (Fig. 2.27) provided additional proof of the proposed structure, with strong ion peaks at *m/z* 178 and 165 corresponding to the loss of $[HCOOH]^+$ and $[CH_2COOH]^+$, respectively.

Table 2.7: NMR data for compound 8 (7) in DMSO-d6 at 600 MHz.

Position	^{13}C	^{1}H	HMBC Correlations
2	171.4 (s)	-	-
3	159.7 (s)	-	-
4	103.9 (d)	6.30 (d, 1.6)	C2, C3, C5, C9
5	167.1 (s)	-	-
6	101.7 (d)	6.42 (dd, 1.6, 0.9)	C5, C4, C7
7	78.2 (d)	5.68 (br dd, 7.9, 5.2)	C8, C10, C11
8	154.8 (s)		
9	104.5 (s)		
10 A	40.4 (t)	2.91 (dd, 5.2, 16.5)	C7, C8, C11
10 B		2.73 (dd, 7.9, 16.5)	C7, C8, C11
11	173.0 (s)		

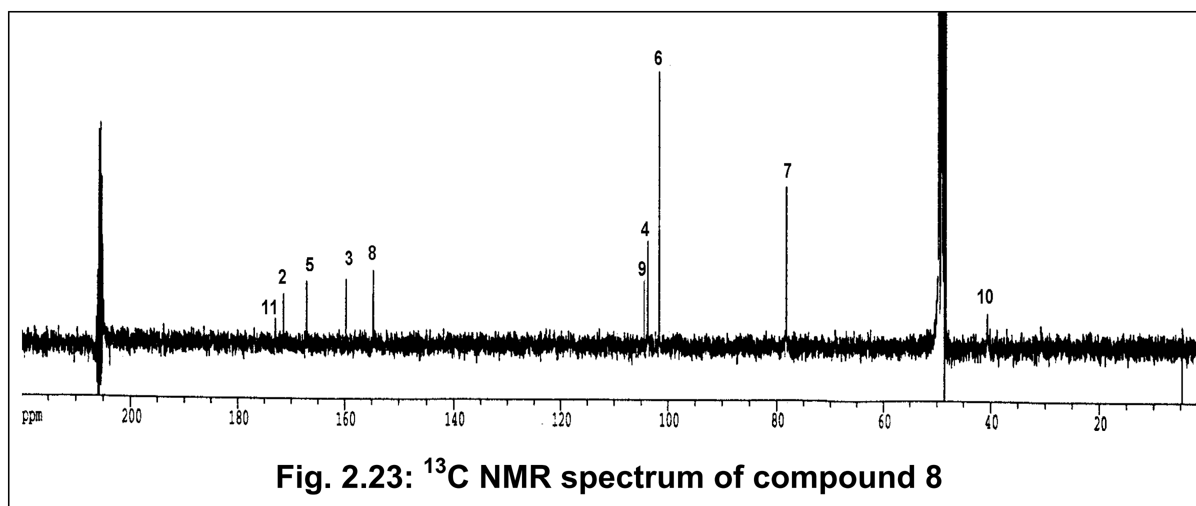


Fig. 2.23: ^{13}C NMR spectrum of compound 8

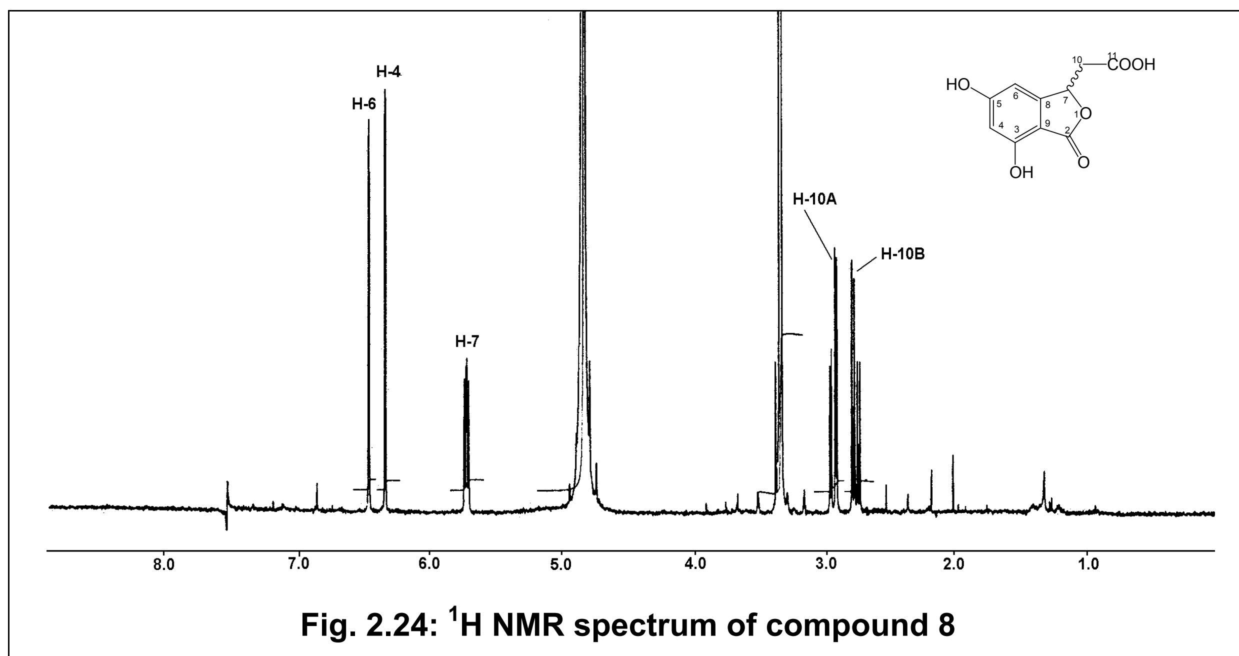


Fig. 2.24: ^{1}H NMR spectrum of compound 8

Fig. 2.25: HMBC spectrum of compound 8

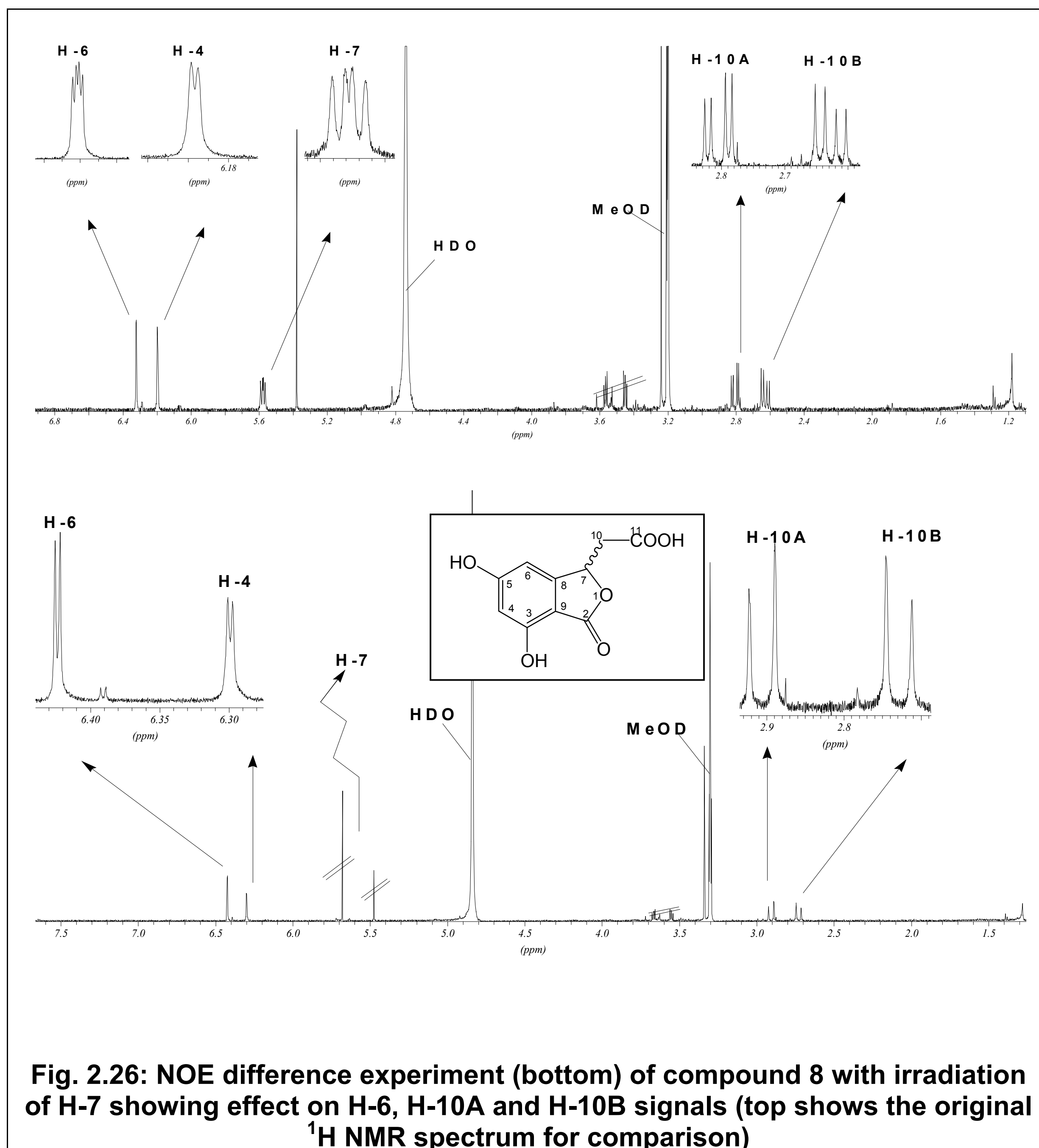


Fig. 2.26: NOE difference experiment (bottom) of compound 8 with irradiation of H-7 showing effect on H-6, H-10A and H-10B signals (top shows the original ^{1}H NMR spectrum for comparison)

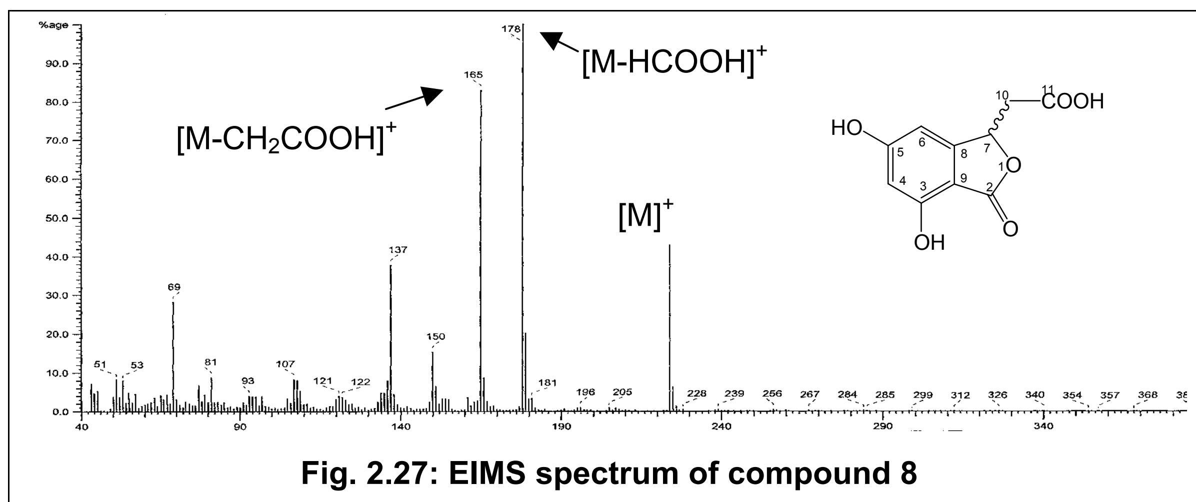


Fig. 2.27: EIMS spectrum of compound 8

Bioactivity

Herbaric acid did not show any activity against *A. salina, S. littoralis,* gram positive and gram negative bacteria, and human leukemia cell line HL-60.

2.1.2. Isolated compounds from *Curvularia Lunata* (Wakker) Boedijn derived from *Niphates Olemda*

The ethyl acetate extract of this fungus exhibited antimicrobial activities (Table 2.8). No activity was seen against the other microorganisms. Likewise, no activity was seen against *S. salina* and *S. littoralis.*

Table 2.8: Antimicrobial assay result of the extract of *Curvularia lunata*

Test Microorganism	Zone of Inhibition (mm)
B. subtilis	n. a.
S. aureus	15
E. coli	n. a.
C. albicans	n. a.

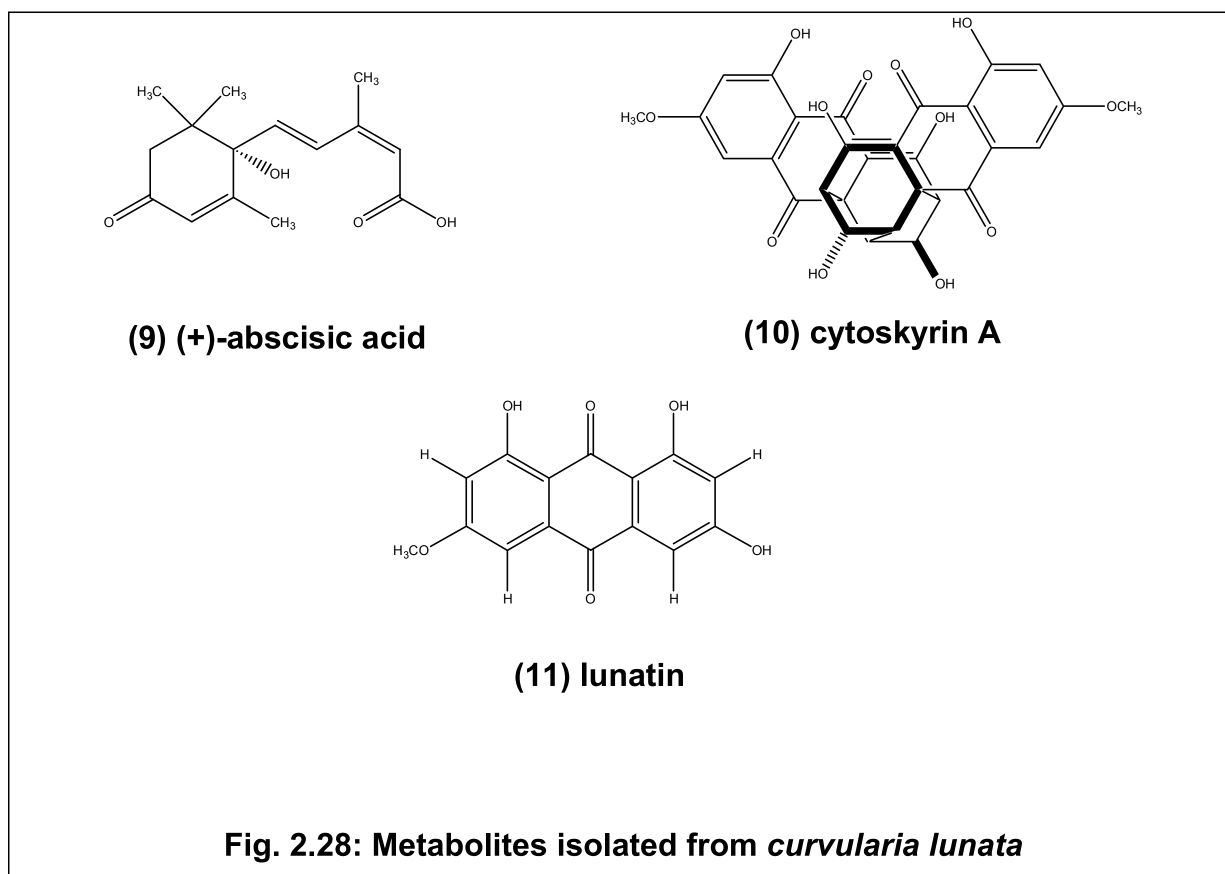


Fig. 2.28: Metabolites isolated from *curvularia lunata*

2.1.2.1. (+)-Abscisic acid (9, known compound)

Abscisin II or dormin is a monocylic sesquiterpene of universal occurrence in higher plants, such as sycamore, birch, rose, cabbage, potato, lemon, etc. It has also been isolated from phytopathogenic fungi such as *Cercospora cruenta* (fungus responsible for the leaf spot and pod blight disease complex in plants) [Oritani and Yamashita, 1985], *Macrophoma castaneicola* (fungus responsible for the black root disease in chestnuts, *Cercospora rosicola* [Assante *et al*., 1977], *Botrytis cinerea* [Marumo *et al*., 1982], and *Laminaria sp*. (Phaeophyta) [Schaffelke, 1995]. It's presence in commercial seaweed products as well as in marine algae [Crouch and Vanstaden, 1993] has also been documented, but this is the first time that abscisic acid has been isolated from a marine-derived fungus.

Structure Elucidation

Compound **9** (Fig. 2.28), which was isolated as a white amorphous powder, was the major metabolite produced by *Curvularia lunata*. It gave a molecular ion peak at *m/z*

264 in the EIMS spectra (Fig. 2.29). A fragment ion was also seen at *m/z* 246 corresponding to the loss of the hydroxyl group. The ^{13}C (Fig. 2.30) and DEPT spectra showed 15 carbons: one carbonyl; one carboxyl; six sp^2 carbons including one which is oxygen-bound; two quaternary sp^3 carbons of which one is directly bound to oxygen; one methylene and four methyl groups. The molecular formula was therefore established to be $C_{15}H_{20}O_4$. The ^1H and ^{13}C NMR spectral data (Fig. 2.30) (Table 2.9) matched those of (+)-trans abscisic acid [Ohkuma and Addicott, 1965; Constantino, *et al*., 1986]. Likewise, the EIMS spectra of compound **9** is also identical with that of (+)-abscisic acid [Constantino, *et al*., 1986; Schaffelke, 1995]. The HMQC and HMBC spectra of **9** also confirmed the proposed structure.

Stereochemistry

The coupling constant of 15.8 between H-4 and H-5 established the *trans* configuration of the double bond between C-4 and C-5, as is the case in abscisic acid. Likewise, the chemical shifts of C-2 and C-3 in compound **9**, which matched that of natural (+)-abscisic acid, also confirmed the *cis* configuration of the α, β-double bond. The stereochemistry at the chiral center C-1' of compound **9** is also identical with that (+)-abscisic acid due to the similarity in their $[\alpha]_D$ values. Compound **9** gave a $[\alpha]_D$ value of +138.35 (c. 0.47, EtOH) which is similar to the literature value of + 383° (*c* 0.23, EtOH) [Assante *et al.,* 1977]. Furthermore, the recorded CD spectra of the compound **9**: $\Delta\varepsilon_{203}$ = -5.776; $\Delta\varepsilon_{232}$ = -35.808; $\Delta\varepsilon_{264}$ = 43.718; $\Delta\varepsilon_{323}$ = -2.146 also agreed with literature data: $\Delta\varepsilon_{230}$ = -34; $\Delta\varepsilon_{262}$ = +39.5; $\Delta\varepsilon_{318}$ = -2.5) [Harada, 1973].

Bioactivity

(+)-Abscisic acid is active as a growth and development regulator [Milborrow, 1968]. Only the natural (S)-isomer is active in stomatal closure [Milborrow and Garmston 1973] whereas both enantiomers are active as germination and growth inhibitors. When tested for antimicrobial, insecticidal and brine-shrimp lethality tests, compound **9** did not show activity.

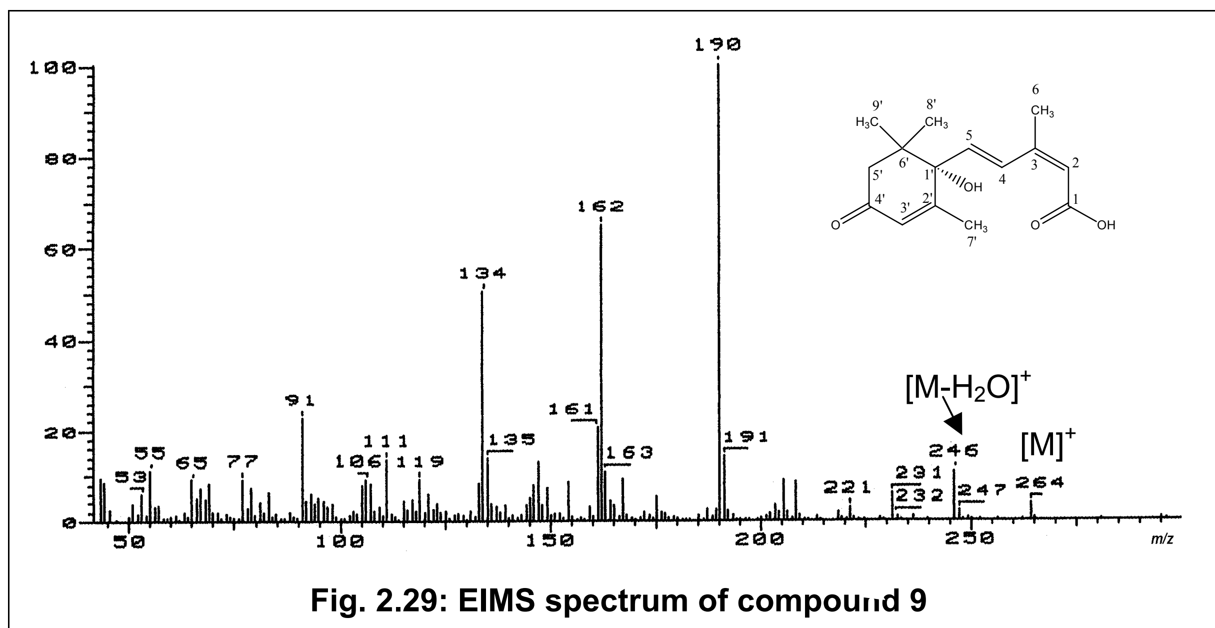


Fig. 2.29: EIMS spectrum of compound 9

Fig. 2.30: ^{13}C and ^{1}H NMR spectra of compound 9

Table 2.9: NMR data of compound 9 in DMSO-d_6 at 300 MHz

Number	^{13}C	^{1}H	HMBC
1	166.8 s		
2	118.3 d	5.67 (s)	C4, C3, C1, C6
3	149.2 s		
4	127.2 d	6.22 (d, 15.8)	C5, C2, C6, C1', C3, C2'
5	137.4 d	7.72 (dd, 15.8, 0.5)	C2, C4, C3, C2', C6', C1'
6	18.8 q	1.97 (d, 1.1)	C3, C4, C2
1'	78.3 s		
2'	163.1 s		
3'	125.9 d	5.19 (s)	C7' C5', C1'
4'	197.2 s		
5'α	49.3 t	2.53 (br d, 16.5)	C3', C1', C6', C8', C9'
5'β		2.11 (br d, 16.5)	C3', C1', C6', C8', C9'
6'	41.2 s		
7'	24.1 q	1.82 (d, 1.13)	C2', C1'', C5, C3',
8'	23.2 q	0.92 (s)	C1', C5', C6', C9'
9'	20.8 q	0.96 (s)	C1' C5', C6', C8'
C-1 O-H		12.05 (s)	
C-1'O-H		5.19 (s)	

2.1.2.2. Anthraquinones

Anthraquinones are widely distributed among microorganisms, plants, echinoderms, and insects [Thomson, 1997]. More than 500 anthraquinones have been isolated from natural sources [Ibid]. Some of these are used as purgatives (rhubarb, senna) and laxatives (emodin and danthron). Furthermore, several natural and synthetic hydroxy- and methyl- anthraquinones exhibit mutagenicity and are used as standards in the Ames/Salmonella assay [Krivobok *et al*., 1992]. In a study of the structure-mutagenicity relationships of various hydroxy- and methyl-substituted anthraquinones (emodin, danthron, physion) and of dimeric compounds (skyrin, rugulosin and rugulin), it was found out that 1- and 8-hydroxylation of the anthraquinone moiety (danthron) markedly increased the mutagenicity [Ibid]. It was also reported [Liberman *et al*., 1982] that mutagenicity decreases when hydroxylation occurs in other positions, for example with alizarin (7,8-dihydroxyanthraquinone). Other

anthraquinones with antitumor and cytotoxic activities are also found in the literature [Solis, *et al*., 1995; El-Gamal *et al*., 1995].

Some dimeric anthraquinones, also known as bisanthraquinones are shown in Fig. 2.31. Luteoskyrin and rugulosin are known to interact with DNA [Ueno, *et al*., 1967; Ueno *et al*., 1968]. Luteoskyrin has been reported to produce a BIA (Biochemical Induction Assay) response at 500 ng after extended periods of incubation [Elespuru and White, 1983]. Luteoskyrin was also reported to exhibit cytotoxicity to Reuber hepatoma H4-II-E and fibroblast Balb/3T3 cells [Akuzawa *et al.,* 1992]. Rugulosin was also reported to alter DNA [Ueno and Kubota, 1976] and to bind to DNA *in vitro* without metabolic activation [Yoshikazu *et al*., 1976].

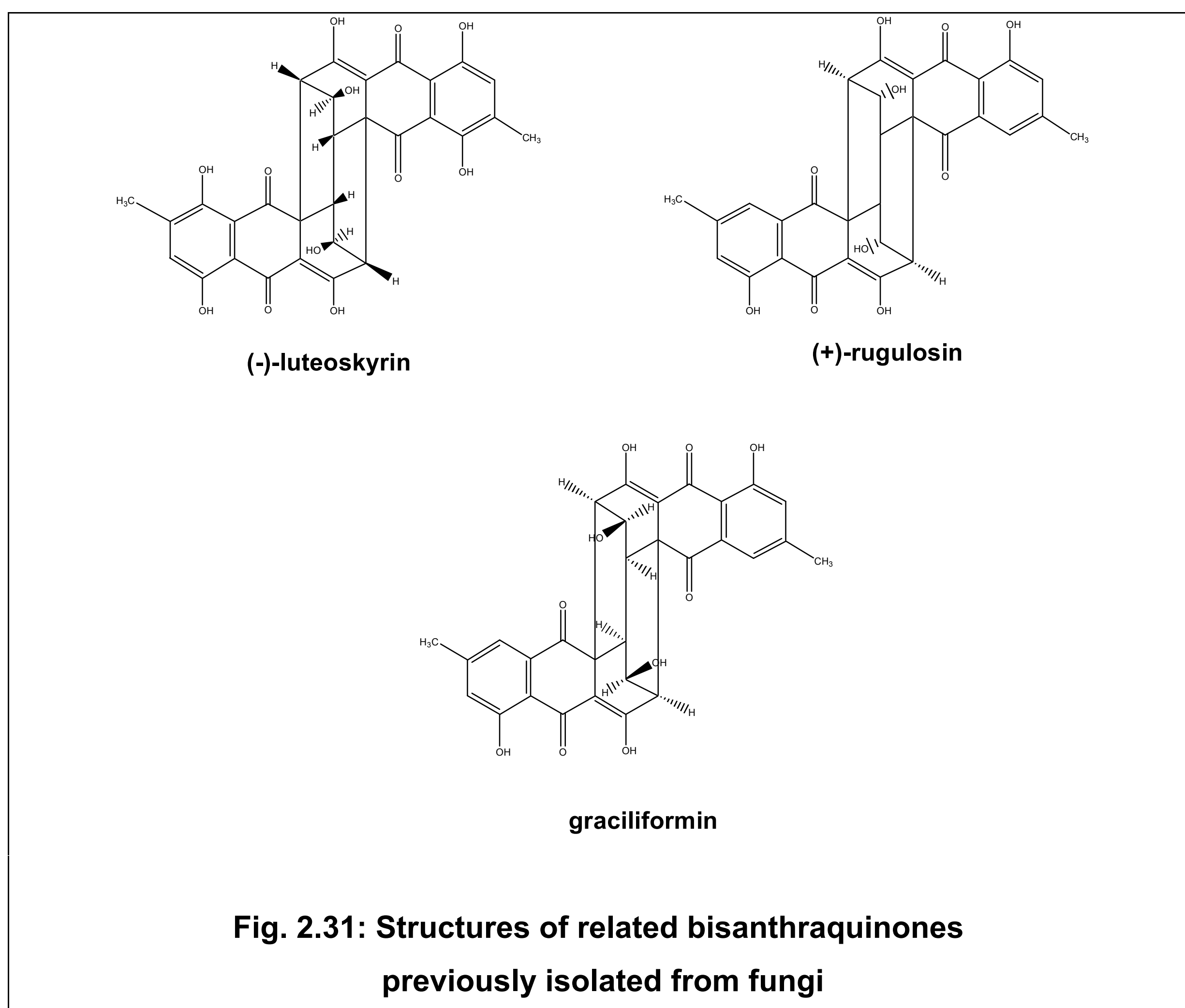


Fig. 2.31: Structures of related bisanthraquinones previously isolated from fungi

2.1.2.2.1. Cytoskyrin A (10, known compound)

Cytoskyrin A was originally reported from the endophytic fungus *Cytospora sp.* isolated from the plant *Conocarpus erecta* [Brady *et al*., 2000].

Compound **10** (Fig. 2.28) which was isolated as a yellow amorphous powder, was identified on the bases of IR, HREIMS, $[\alpha]_D$, ^1H, ^{13}C, HMBC and HMQC NMR data. It gave a molecular ion peak at 574 by EI (Fig. 2.32) and an exact mass of 574.1102 which corresponds to the molecular formula $C_{30}H_{22}O_{12}$. A fragment ion was also seen at 287 corresponding to a monomeric ion. The IR spectra (Fig. 2.33) showed a peak at 1704 and 1603 corresponding to the non-chelated C-10/C-10' and chelated C-9/C-9' carbonyls, respectively.

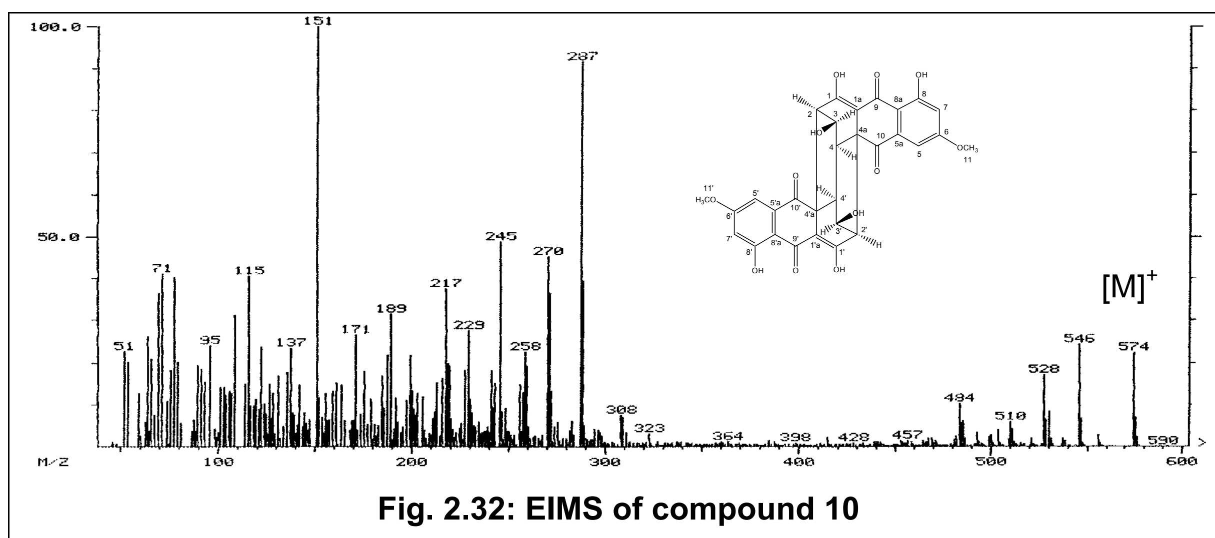


Fig. 2.32: EIMS of compound 10

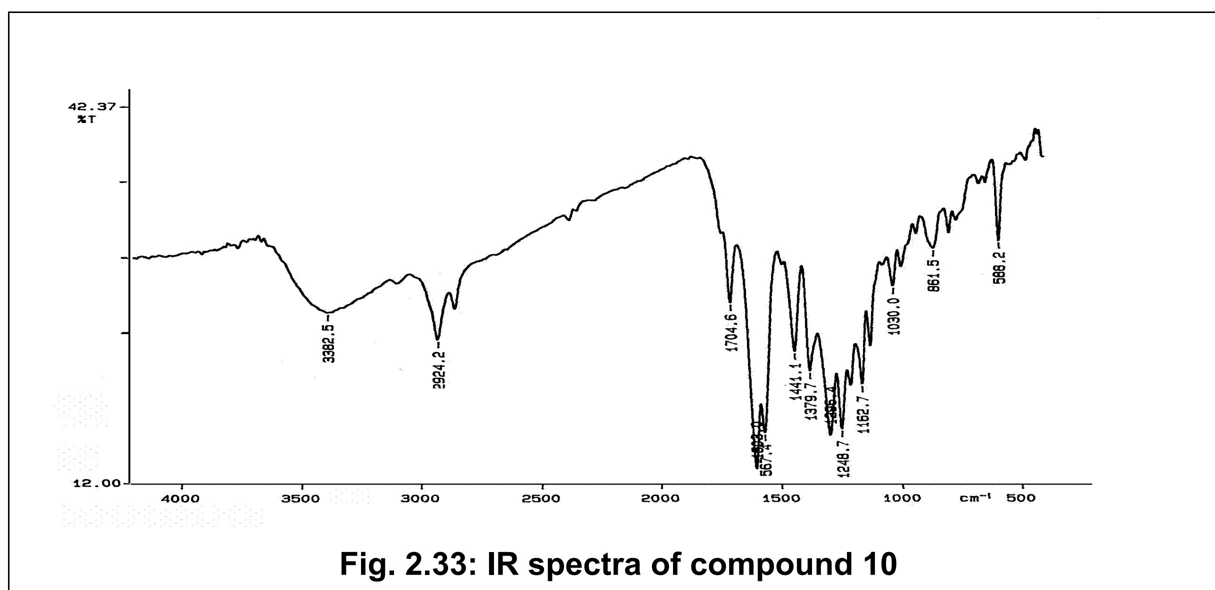


Fig. 2.33: IR spectra of compound 10

Compound **10** is a symmetrical dimer so that the ^{1}H (Fig. 2.34) and ^{13}C NMR spectra (Fig. 2.35) showed only signals corresponding to the monomer. The proton NMR showed one pair of doublets at 6.95 and 6.83 ppm with coupling constants of 2.2 Hz corresponding to the *meta*-coupled aromatic protons. A singlet at 11.69 ppm corresponds to the phenolic proton attached to C-8. A broad singlet at 14.40 ppm corresponds to the phenolic proton attached to C-1 (spectra not shown). The H-H COSY spectrum showed correlations between H-2 and H-3 and between H-3 and H-4 which attest to the vicinal positions of these three protons. The COSY spectrum also showed a long range correlation between H-2 and H-4. The hydoxyl proton (C-3—OH) which was shown as a singlet at 5.37 ppm also showed correlations with H-2 and H-4. Furthermore, long range correlations of H-2 with C-3 and C-4 and of H-4 with C-2 and C-4 were also observed. However, despite these correlations, there was no observed coupling between the signals of H-2, H-3 and H-4 in the ^{1}H NMR spectrum, with all of them appearing as singlets.

The rigid W configuration in which H-2, H-3, and H-4 are held in the central cage structure, allows for the determination, based on the Karplus equation, of the relative stereochemistry of C–2, –3, and –4 in related bisanthraquinones. CS Chem 3D Pro chemical modeling was able to predict that the experimentally observed coupling pattern will occur if the H-2, -3, and –4 protons have an all *cis* configuration within monomers of cytoskyrin A. The dihedral angles H2–C2–C3–H3 and H3–C3–C4–H4 were shown to approach 90° when a *cis, cis* configuration of each vicinal proton pair was applied on the molecular model. This also agrees with the dihedral angles measured from Dreiding models. Likewise, the dihedral angle in H4-C4-C4'-H4' was computed to be 84.9^o which explains the absence of coupling between these protons.

Meanwhile, the presence of long range correlations between H-2 and the carbons C-4a' and C-10' provided proof that the compound is a dimer (Fig. 2.36a and Fig. 2.36b). NMR data are shown in Tables 2.10a and 2.10b.

The *cis, cis* configuration for the protons H-2, -3, and –4 of cytoskyrin A in the literature was confirmed by the X-ray crystallography structure [Brady *et al*, 2000].

The $[\alpha]_D$ value observed for compound **10** (+393, c 0.184, dioxane) was in agreement with that reported for cytoskyrin A (+328, c 1.0, Acetone + 0.2% TEA) [Ibid], thus proving that the two are identical.

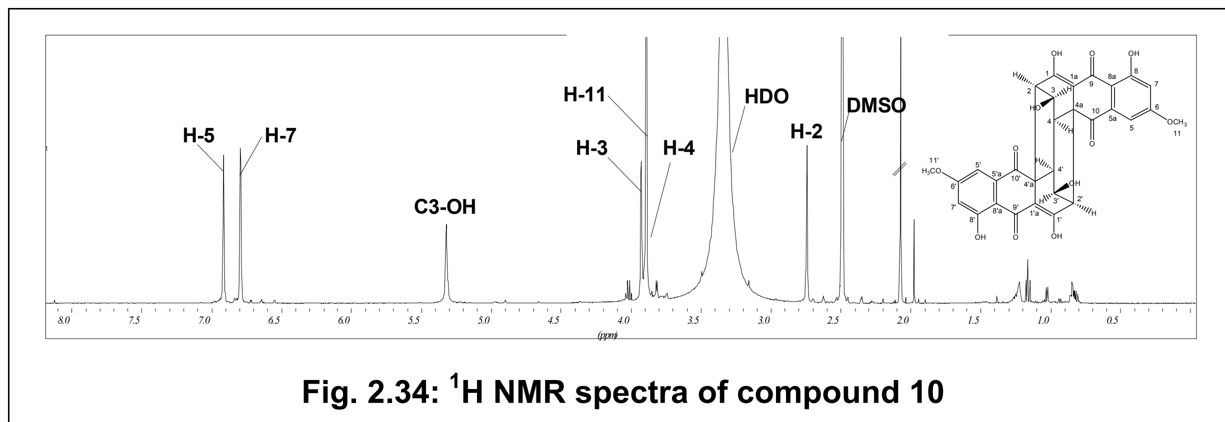


Fig. 2.34: ^{1}H NMR spectra of compound 10

Fig. 2.35: ^{13}C NMR of compounds 10 (top) and 11 (bottom).

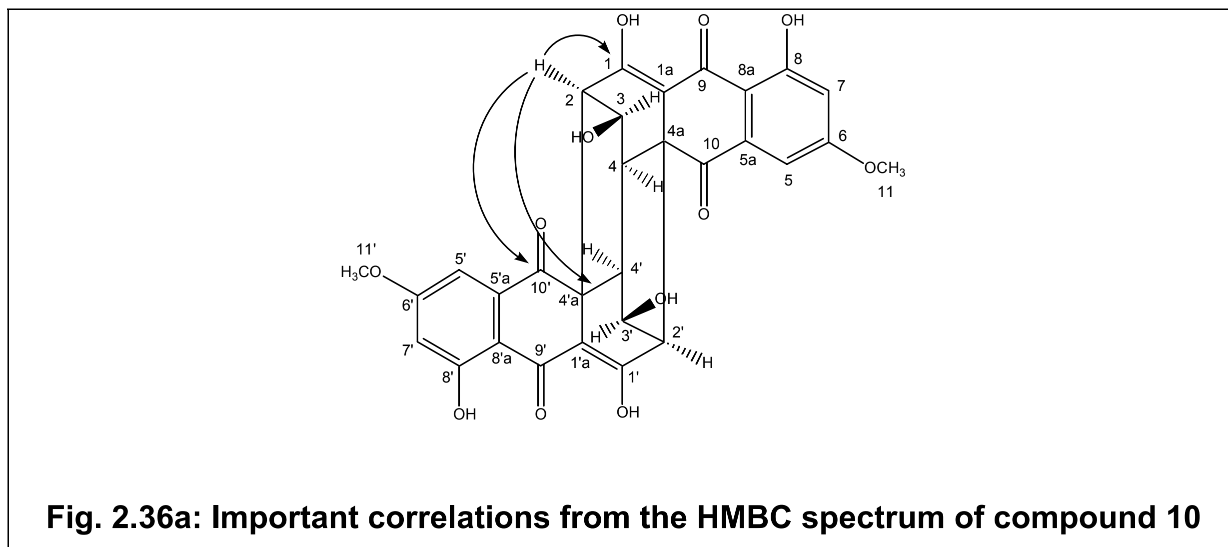


Fig. 2.36a: Important correlations from the HMBC spectrum of compound 10

H-11
H-5 H-7 C-3-OH H-3 H-4 H-2
H-3/C-4 H-2/C-4
H-2/C-4'a
H-3/C-2 H-4/C-2
H-2/C-3
H-2/C-10'
4
11
4a 2
3
1a
5,7 8a
5a
8
6
1
9
10
50
100
150
200
7.0 6.0 5.0 4.0 3.0 2.0 1.0 0
ppm

Fig. 2.36b: HMBC spectrum of compound 10

2.1.2.2.2. Lunatin (11, new compound)

Compound **11** (Fig. 2.28) was obtained as an orange-colored amorphous powder. It showed a molecular ion peak at *m/z* 286 in EIMS (Fig. 2.37). Its molecular formula was determined by HREIMS to be $C_{15}H_{10}O_6$. Its structure was established through 1 D (^{1}H, ^{13}C and DEPT) and 2 D (COSY and HMBC) NMR (Tables 2.10a and 2.10b). The ^{1}H NMR spectrum showed two phenolic protons at δ 12.31 and 12.22 (spectra not shown), two pairs of *meta*-coupled protons at δ 7.14 and 6.82 (^4J = 2.5 Hz) and at δ 7.09 and 6.56 (^4J = 2.3 Hz) and a methoxyl group at δ 3.91. The ^{13}C NMR (Fig. 2.35) and DEPT spectra revealed 15 carbon atoms: two quinone carbonyls, twelve aromatic carbons, and one methoxyl. Of the aromatic carbons, 4 were unsubstituted and of the substituted carbons, 4 were oxygen-bound. The proposed structure of compound **12** and the assignment of NMR signals were corroborated by data from the HMBC spectrum (Table 2.10b). Furthermore, the assignment of the proton signals was also made possible through an NOE experiment which showed selective enhancement of the signals for H-5 and H-7 after irradiation at δ 3.91 (H-11 signal) thereby establishing the position of the methoxy group (Fig. 2.38).

A lichen metabolite with a proposed structure identical to that of **11** was recently reported [Ivanova *et al*., 2000]. However, important differences in chemical shift data were observed such as the ^{1}H NMR data for H-4, the coupling constant between H-2 and H-4, as well as ^{13}C NMR data for C-1a, C-2 and C-4. In addition, a disparity in the chemical solution behavior of the two compounds, in which **11** was insoluble in $CHCl_3$ which was the solvent used for the measurement of the NMR spectra of the lichen metabolite, also proved that the two compounds are not identical. The lichen metabolite could thus possibly be an isomeric compound although this aspect needs further clarification.

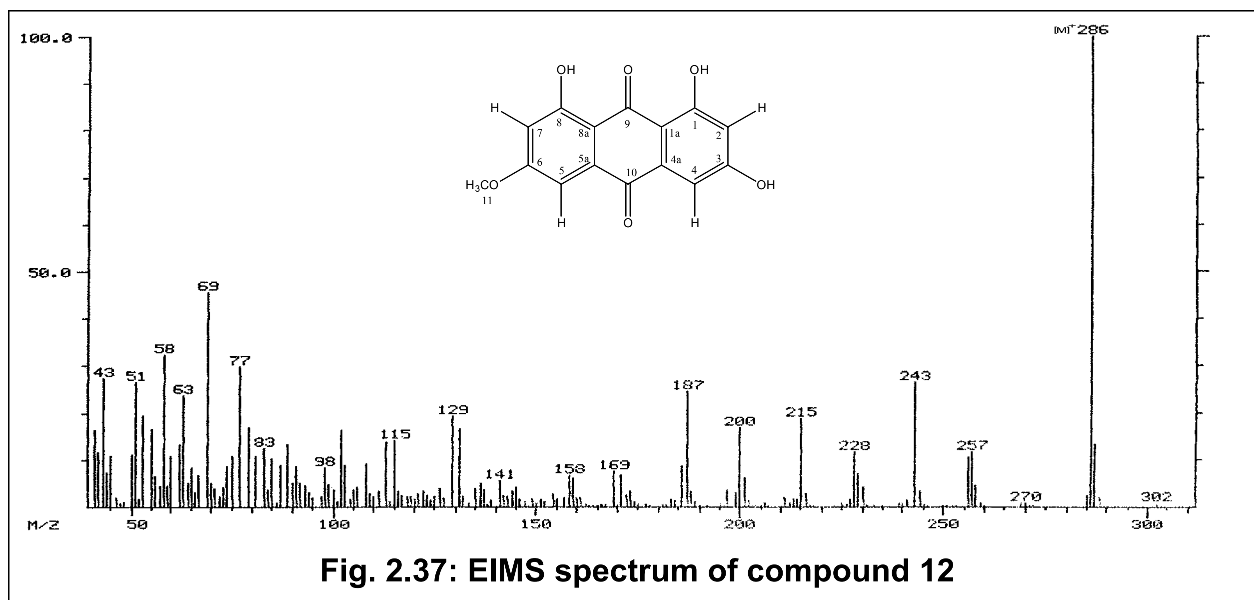


Fig. 2.37: EIMS spectrum of compound 12

H-11
H-5
H-4
H-7
H-2

Fig. 2.38: NOE difference spectrum of compound 11 (bottom) showing effects on H-4 and H-2 after irradiation of H-11 [original ^{1}H NMR spectrum is shown (top) for comparison]

Table 2.10a: ^{13}C NMR data for compounds 10 and 11 in DMSO-d_6 at 400 MHz and 300 MHz, respectively.

Atom Number	**10**	**11**
1	184.0 (s)	164.4 (s)
1a	104.2 (s)	108.3 (s)
2	60.6 (d)	108.1 (d)
3	72.4 (d)	165.9 (s)
4	49.5 (d)	109.3 (d)
4a	58.9 (s)	134.6 (s)
5	106.3 (d)	107.4 (d)
5a	135.8 (s)	134.8 (s)
6	165.5 (s)	165.5 (s)
7	106.3 (d)	106.6 (d)
8	162.6 (s)	164.1 (s)
8a	110.9 (s)	109.6 (s)
9	184.1 (s)	188.4 (s)
10	193.3 (s)	181.1 (s)
11	56.2 (s)	56.2 (q)

Table 2.10b: ^{1}H and HMBC NMR data of compounds 10 and 11 in DMSO-d_6 at 400 MHz and 300 MHz, respectively

H	**^{1}H (m, *J* in Hz) 10**	**HMBC Correlations**	**^{1}H (m, *J* in Hz) 11**	**HMBC Correlations**
1				
1a				
2	2.77 (s)	C2 (direct), C1, C1a, C3, C4, C4a, C10	6.56 (d, 2.3)	C1, C1a, C3
3	3.95 (s)	C3 (direct), C1, C2, C4, C4a		
4	3.91 (s)	C1a, C2, C4, C4a’, C10’	7.09 (d, 2.3)	C1a, C3, C10
4a				
5	6.95 (d, 2.2)	C5 (direct), C6, C8a, C10	7.14 (d, 2.5)	C7, C8a, C10
5a				
6				
7	6.83 (d, 2.2)	C7 (direct), C6, C8, C8a,	6.82 (d, 2.5)	C5, C6, C8, C8a
8				
8a				
9				
10				
11	3.91 (s)	C11 (direct), C11	3.91 (s)	C6
C3-OH	5.37 (s)			
C8-OH	11.69 (s)		12.31 (s)	
C1-OH	14.40 (s)		12.22 (s)	

Bioactivity

Cytoskyrin A (**10**) was reported to show strong biochemical induction assay (BIA) activity down to 12.5 ng in the standard assay [Brady *et al*., 2000]. BIA is a rapid (colorimetric) bacterial assay which measures the induction of the SOS response in bacteria and is used to identify compounds that inhibit DNA synthesis either directly by inhibiting the DNA replication machinery or more often indirectly by modifying DNA. Preliminary studies was also reported to show that cytoskyrin A inhibits both in vitro DNA synthesis and transcription to a greater extent than either luteoskyrin or cytoskyrin B [Ibid].

In this study, cytoskyrin A (**10)** was tested for activity against human leukemia HL-60 cell line. The result showed inhibition of cell growth by 35% when applied in concentration of 5 μg/ml. Lunatin (**11)** on the other hand, exhibited no activity.

Antimicrobial tests showed that both anthraquinones, **10** and **11,** were active against *S. aureus, E. coli, and B. subtilis* but had no activity against *C. albicans* (Table 2.11). A comparative test on their activities against *S. aureus* indicated that compound **11** was slightly more potent than compound **10** (Table 2.12).

Table 2.11: Zones of inhibition in agar-plate diffusion assay.

Compound	*S. aureus*	*E. coli*	*E. coli HBI-101*	*B. subtilis*	*C. albicans*
11					
(5 μg)	8.5 cm	9.0 cm	8.0 cm	7.5 cm	n.a.
(10 μg)	10.0 cm	11.0 cm	10.5 cm	9.0 cm	n.a.
10					
(5 μg)	8.5 cm	9.0 cm	8.0 cm	8.0 cm	n.a.
(10 μg)	10.0 cm	11.0 cm	9.0 cm	12.0 cm	n.a.

Table 2.12: Agar plate diffusion assay for compounds 10 and 11

Amount (μg)	Zone of inhibition (mm) against *S. aureus*	
	10	**11**
1	15	n. a.
5	15	12
10	15	14

2.1.3. Isolated compounds from fungi derived from *Axinella verrucosa*

Twenty-two fungi species were isolated from the sponge *Axinella verrucosa* which was collected in Secca di Corallo, Elba, Italy. Bioscreening of the ethyl acetate extracts of these fungi showed the extract E00-12/3 which later identified as *Penicillium sp.* as exhibiting strong activities (Fig. 2.39; Table 2.13). An unidentified fungus E00-12/11 which exhibited antifeedant activity (30% growth rate) against *Spodoptera littoralis* was also chemically investigated when its HPLC chromatogram showed only one major metabolite.

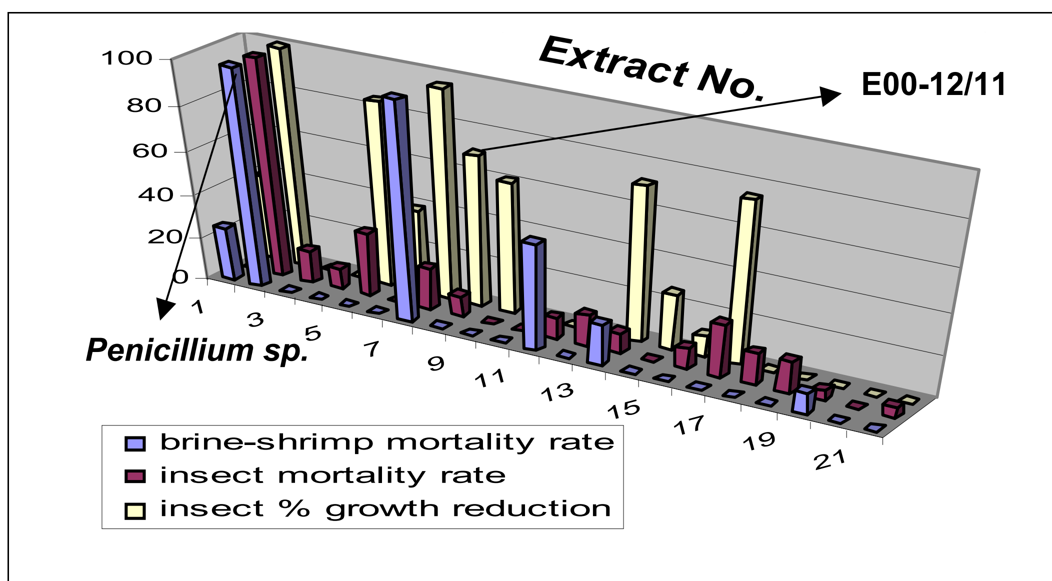


Fig. 2.39: Bioscreening result of 21 extracts of various fungal strains isolated from the Mediterranean sponge *Axinella verrucosa.*

Table 2.13: Bioscreening result of the extracts of 22 fungal strains isolated from Mediterranean sponges.

Sample from 300 mL Fungal Culture	Brine-shrimp Mortality Rate		Insect Mortality	Insect Growth Reduction
	500 µg	50 µg		
E00-12/1	25	0	0	40
E00-12/3	100	100	100	100
E00-12/4	0	0	15	0
E00-12/5	0	0	10	0
E00-12/6	0	0	30	85
E00-12/7	0	0	0	39
E00-12/8	100	85	20	96
E00-12/11	0	0	10	70
E00-12/13	0	0	0	61
E00-12/14	0	0	0	0
E00-12/16	50	0	10	0
E00-12/21	0	0	15	0
E00-12/22	20	0	10	72
E00-12/24a	0	0	0	26
E00-12/25	0	0	10	10
E00-12/27	0	0	25	75
E00-12/31	0	0	15	0
E00-12/32	0	0	15	0
E00-12/36	10	0	5	0
E00-12/46	0	0	0	0
E00-12/51	0	0	5	0

2.1.3.1. Isolated compounds from the fungus *Penicillium spp.*

2.1.3.1.1. Oxaline (12, known compound)

Oxaline [Nagel *et al*., 1976] belongs to a group of mycotoxins which includes meleagrin [Kawai *et al*., 1984], the glandicolins [Dictionary of Natural Products, 2000] and the structurally-related roquefortines [Ohmomo *et al*., 1975] which are all metabolites of *Penicillium spp*. (Fig. 2.40a). Mycotoxins are secondary metabolites of fungi which are ubiquitous in a broad range of commodities and feeds, and are toxic to mammals, poultry and fish. These compounds are characterized by having interesting structural features, namely, an N-OH group, a dehydrohistidine unit, an isoprenoid side chain, and a single carbon atom having three nitrogen functionalities.

Structure Elucidation

Compound **12** (Fig. 2.40b) was isolated as white scaly powder. It gave a molecular ion peak at *m/z* 446 in (-)-ESIMS (Fig. 2.41). Its ^{1}H NMR spectrum (Fig. 2.42) is similar to that of oxaline isolated from cultures of the toxicogenic fungus *Penicillium oxalicum* [Nagel *et al*., 1976]. Furthermore, a fragment ion at *m/z* 377 in the (-)-ESIMS spectrum which represents the loss of the isoprenoid side chain is also typical of oxaline and its derivatives [Ibid]. From the COSY spectra, an ABCD spin system was observed from the aromatic resonances at δ 7.61(H-4), δ 7.29 (H-6), δ 7.09 (H-5) and δ 7.00 (H-7). An exomethylene moeity also comprised an ABX system at δ 5.97(H-22), δ 5.00 (H-23A), and δ 4.94 (H-23B). Two singlets at δ 3.65 and at δ 3.54 were assigned to two methoxyl groups H-27 and H-26, respectively. Two geminal methyl groups gave a 6-proton singlet at δ 1.24. Two singlets were also observed corresponding to signals from H-8 (δ 5.39) and H-15 (δ 8.10). To the imidazole protons H-18 and H-20 were assigned the signals at δ 8.96 and 8.19.

The proton and carbon signals (Table 2.14) were assigned based on comparison with literature data [Nagel *et al*., 1976; Konda *et al*., 1980] and confirmed by HMBC. Observed C-H correlations are shown below (Fig. 2.43).

The relative stereochemistry of compound **12** at the stereogenic centers C-2 and C-3 was also assumed to be identical with that reported in the literature, based on similarities of the observed $[\alpha]_D$ value of -24° (*c* 0.168, MeOH) with literature value of –45° (*c* 0.3, MeOH) [Nagel *et al*., 1976; Konda *et al*., 1980].

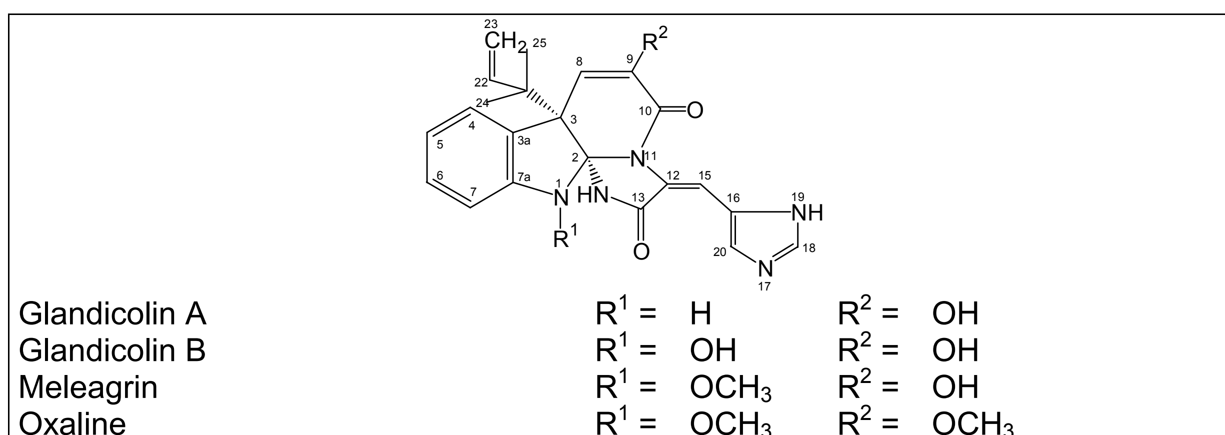
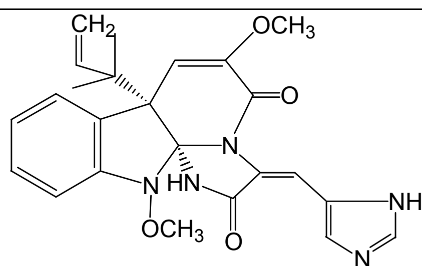
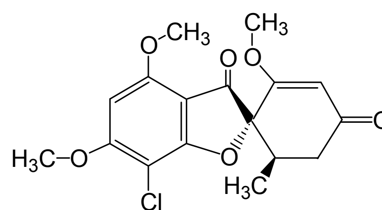


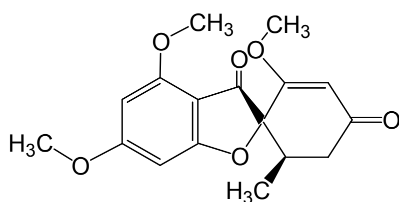
Fig. 2.40a: Related mycotoxins isolated from fungi



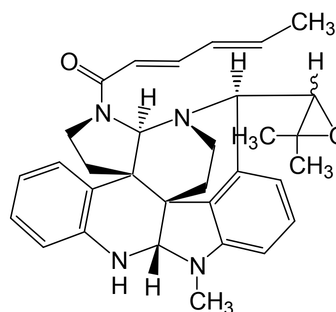
(12) oxaline



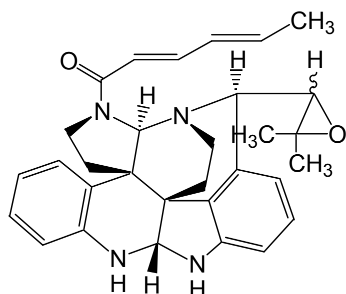
(13) griseofulvin



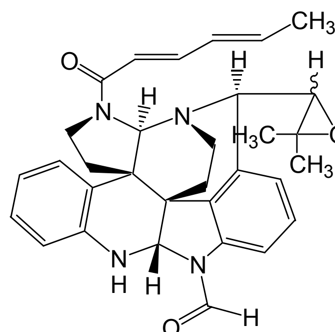
(14) dechlorogriseofulvin



(15) communesin B*



(16) communesin C*



(17) communesin D*

Fig. 2.40b: Compounds isolated from *Penicillium spp.*
(*relative configuration showed)

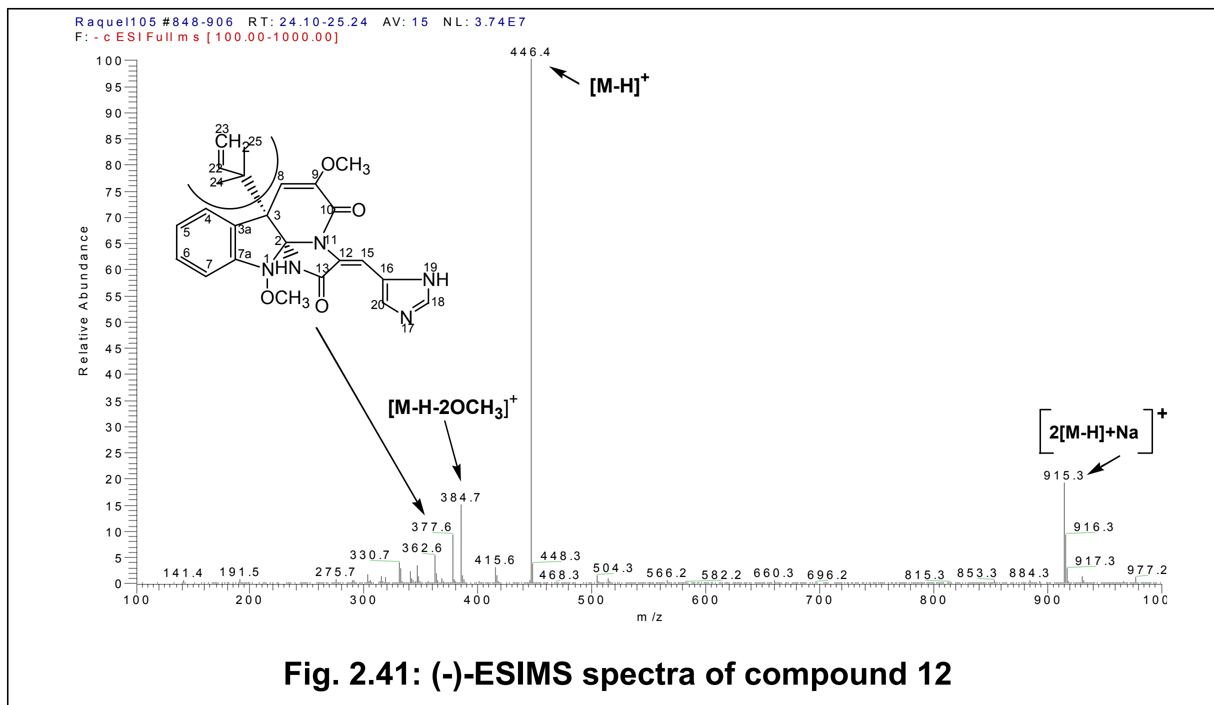


Fig. 2.41: (-)-ESIMS spectra of compound 12

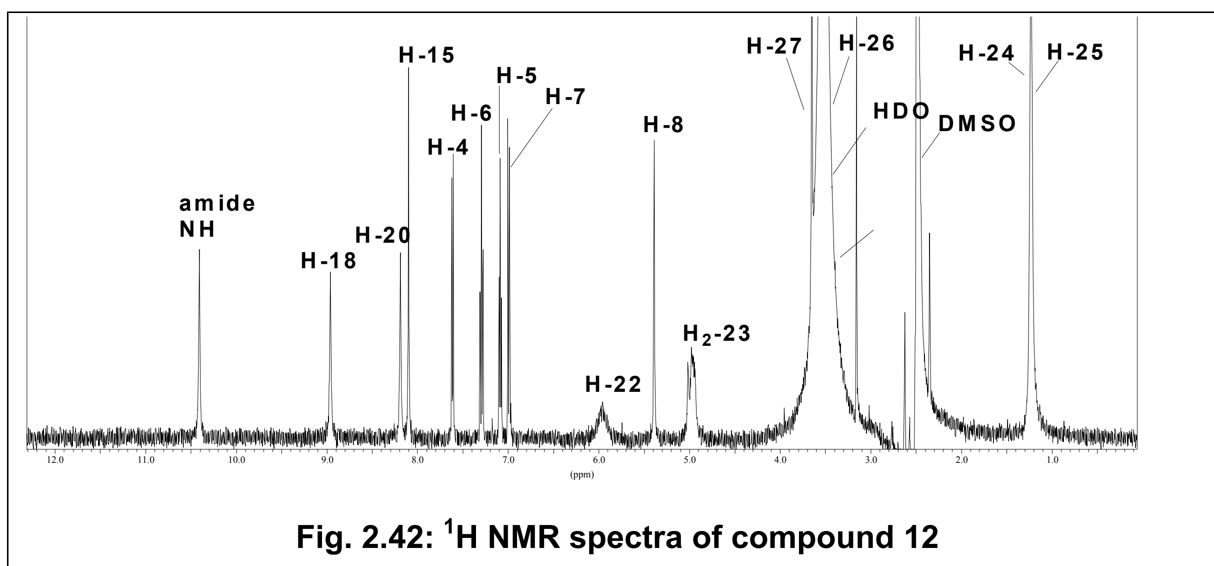


Fig. 2.42: ^{1}H NMR spectra of compound 12

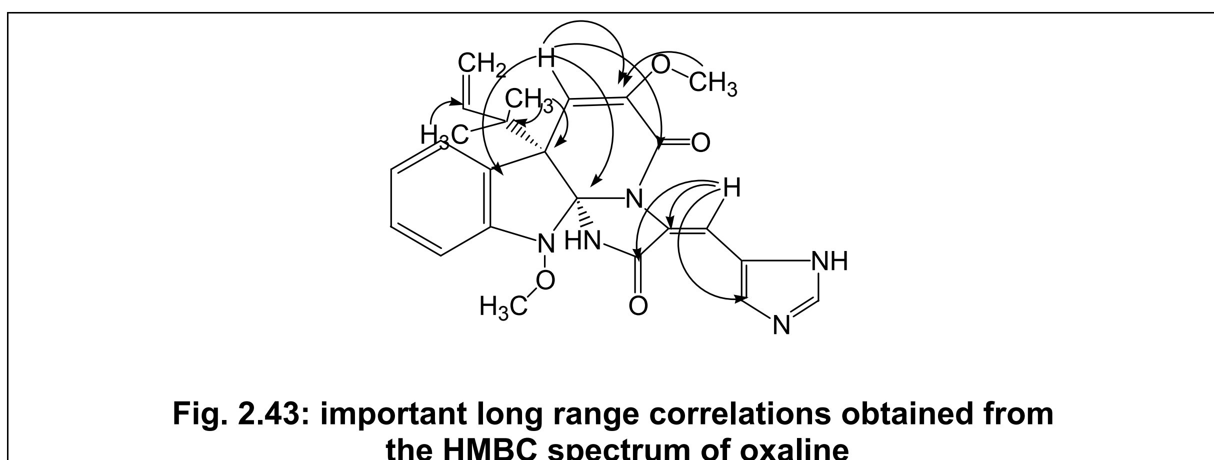


Fig. 2.43: important long range correlations obtained from the HMBC spectrum of oxaline

Bioactivity

Oxaline has been considered as a mycotoxin [Lund and Frisvad, 1994; Steyn 1995; Kozlovskii *et al*., 2000]. Roquefortine, a toxin which has been reported to affect the central nervous system, has also been found to be the precursor in the biosynthesis of oxaline [Reshetilova *et al.,* 1995].

In bioassay screenings performed in the laboratory, oxaline has been found to be active against Gram positive and Gram negative bacteria. At a loading concentration of 50 μg, it inhibited growth of *S. aureus, E. coli, B. subtilis and C. albicans* by 7, 10, 10 and 15 mm, respectively.

No activity was observed against *A. salina and P. littoralis.*

Table 2.14: NMR data of compound 12

C	δ (lit)	δ (Actual)*	^{1}H
2	101.6 s	101.4 s	
3	52.6 s	53.0 s	
3a	146.5 s	146.4 s	
4	124.7 d	124.4 d	7.61 (d, 7.88)
5	123.3 d	122.8 d	7.09 (t, 7.57)
6	128.4 d	130.0 d	7.29 (t, 7.57)
7	112.0 d	111.8 d	7.00 (d, 7.88)
7a	146.6 s	147.3 s	
8	107.0 d	109.0 d	5.39 (s)
9	126.0 s	125.7 s	
10	157.3 s	156.8 s	
12	123.1 s	121.8 s	
13	166.1 s	163.9 s	
15	109.7 d		8.10 (s)
16	126.2 s		
18	136.4 d		8.96 (s)
20	133.8 d	130.9 d	8.19 (s)
21	42.5 s	42.0 s	
22	142.8 d	144.0 d	5.97 (m)
23	113.9 d		4.94 (d, 10.41)
			5.00 (d, 17.02)
24	24.1 q		1.24 (s)
25	23.7 q		1.24 (s)
26	55.7 q	55.0 q	3.54 (s)
27	65.2 q	64.1 q	3.65 (s)
N-H			10.41 (s)

* obtained from HMBC spectra

2.1.3.1.2. Griseofulvin (13, known compound)

Griseofulvin was first reported in 1939 *from Penicillium griseofulvum* [Oxford and Simonart, 1939]. Since then, it has also been isolated from cultures of *P. janczewskii, P. patulum, P. albidum, P. raciborskii, P. melinii, P. urticae, P. raistrickii, P. brefeldianum, P. viridi-cyclopium, P. brunneo-stoloniferum; P. janthinellum, P. martensii, P. nigricans, P. urticae, Carpenteles, P. hordei brefeldianum, Nigrospora oryzae, Nigrospora saccharii, Aspergillus versicolor* [Antibase, 2002].

Structure Elucidation

The (+)-ESIMS spectrum of **13** (Fig. 2.44) showed a molecular ion peak at 353-355 $[M+H]^+$, corresponding to the molecular formula $C_{17}H_{17}{}^{35}ClO_6$. The ^1H (Fig. 2.45) and ^{13}C NMR spectra (Fig. 2.46) was identical with that of griseofulvin in the literature. Two keto groups were evident, as well as four unsaturated carbons which resonated in the low field region, indicating direct bonding with oxygens. Five signals were also observed in the region between 90 to 104 ppm corresponding to four unsaturated carbons and an sp^3 quaternary carbon with a particularly deshielded nucleus from direct bonding with an oxygen and a keto group. In the aliphatic region, three signals corresponding to the three methoxyl carbons were also observed between 56 to 57 ppm. Only two remaining ^{13}C signals at δ 36.8 and 14.3 were seen. However, the ^1H NMR showed the presence of a methyl group and three aliphatic protons which exhibited a AMX three spin system, suggesting the presence of yet another carbon overlapping with the DMSO peak. The assignment of cabon and proton signals (Table 2.15) were based on comparison with literature data [Sato, 1976; Levine *et al*, 1971].

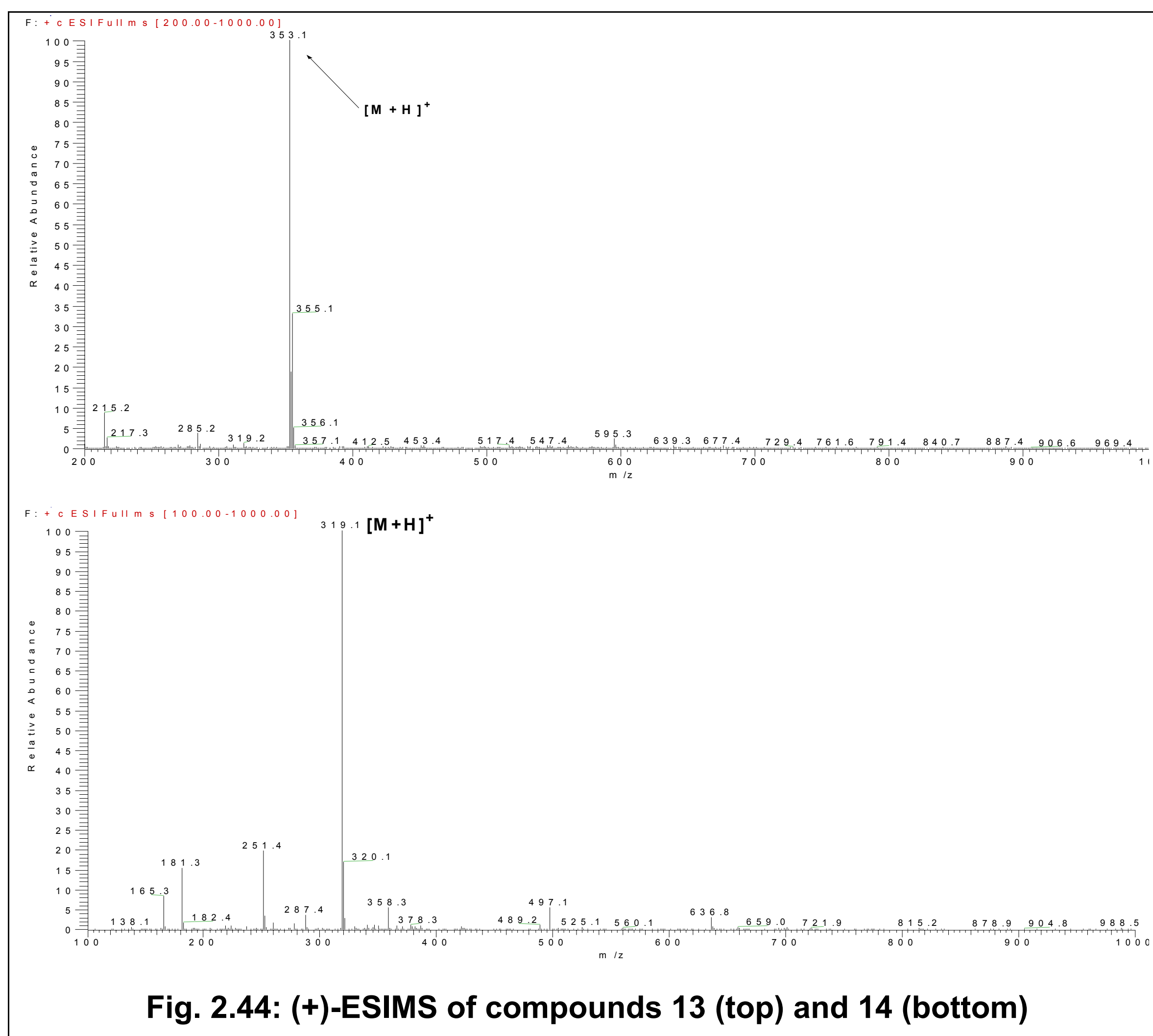


Fig. 2.44: (+)-ESIMS of compounds 13 (top) and 14 (bottom)

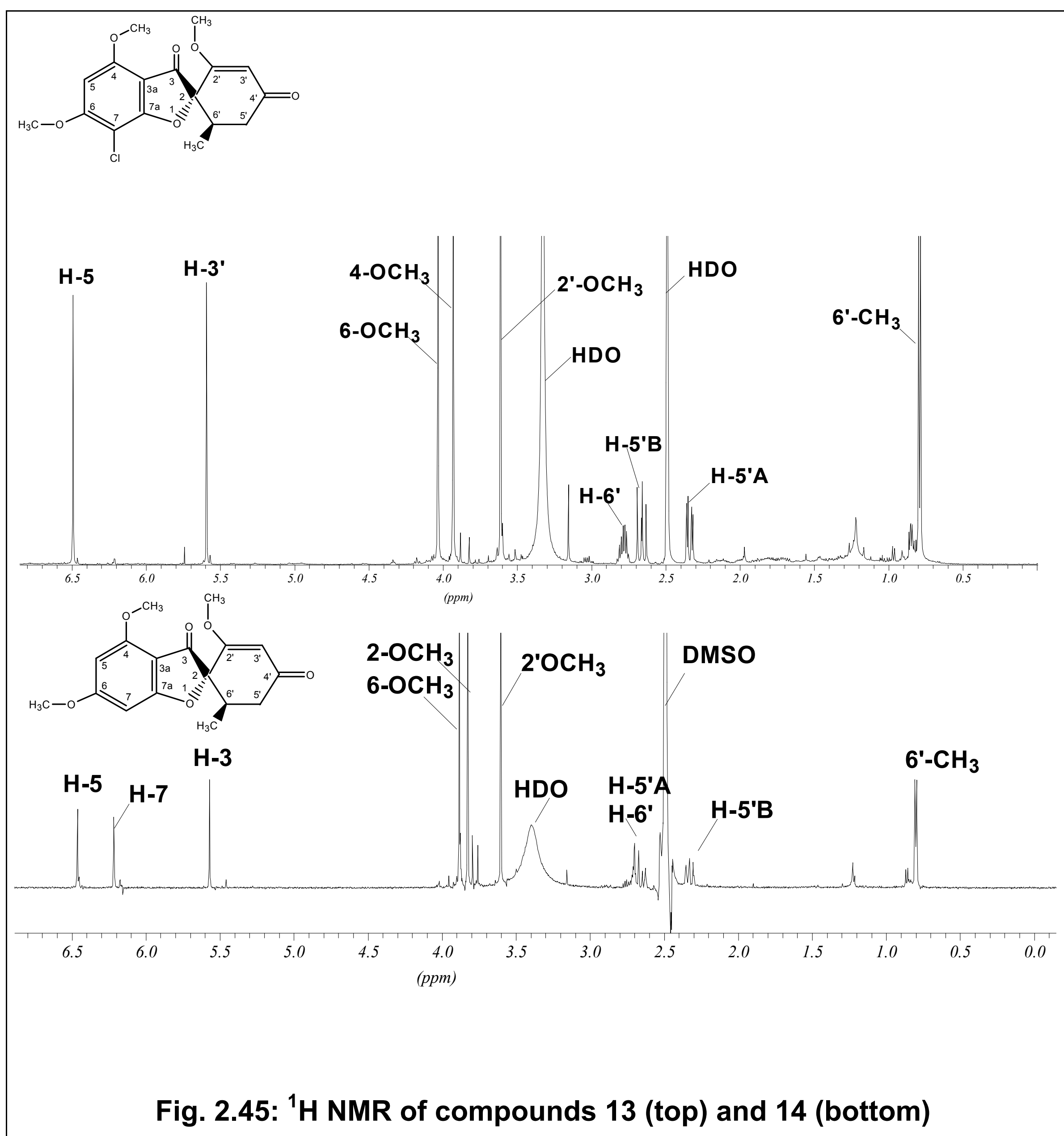


Fig. 2.45: ^{1}H NMR of compounds 13 (top) and 14 (bottom)

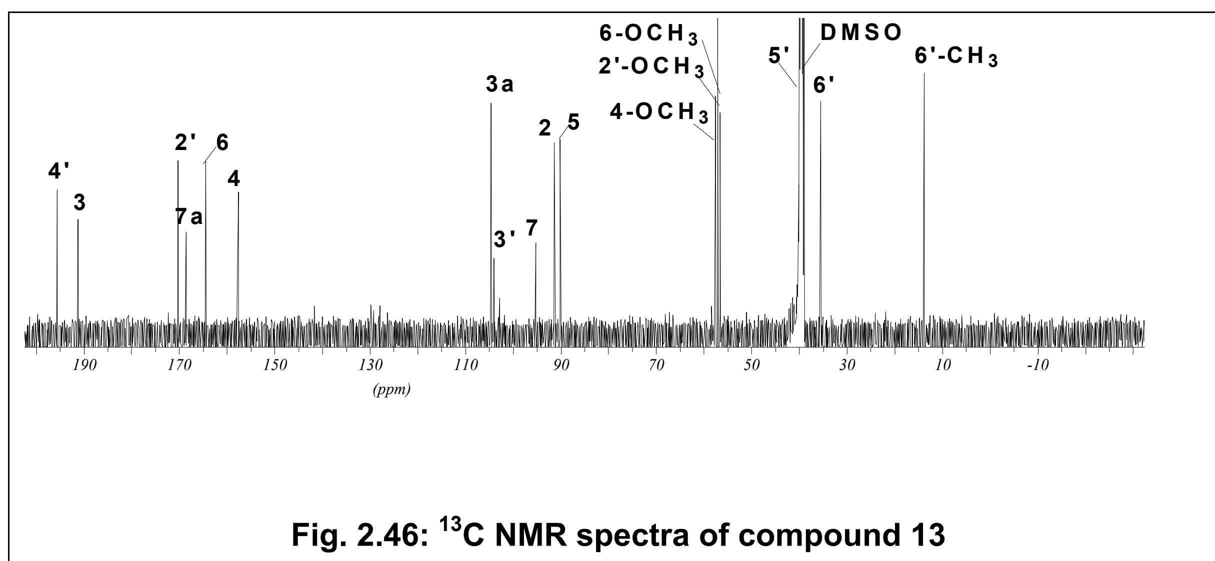


Fig. 2.46: ^{13}C NMR spectra of compound 13

Furthermore, an x-ray analysis of a single crystal of compound **13** (Fig. 2.47) found it to be identical with griseofulvin as reported in the literature [Brown and Sim, 1963]. Optical rotation data obtained for compound **13** (α_D=+315, *c* 0.410, $CHCl_3$) was similar to literature data (α_D=+370, *c* 0.410, $CHCl_3$) [Grove *et al.*, 1952].

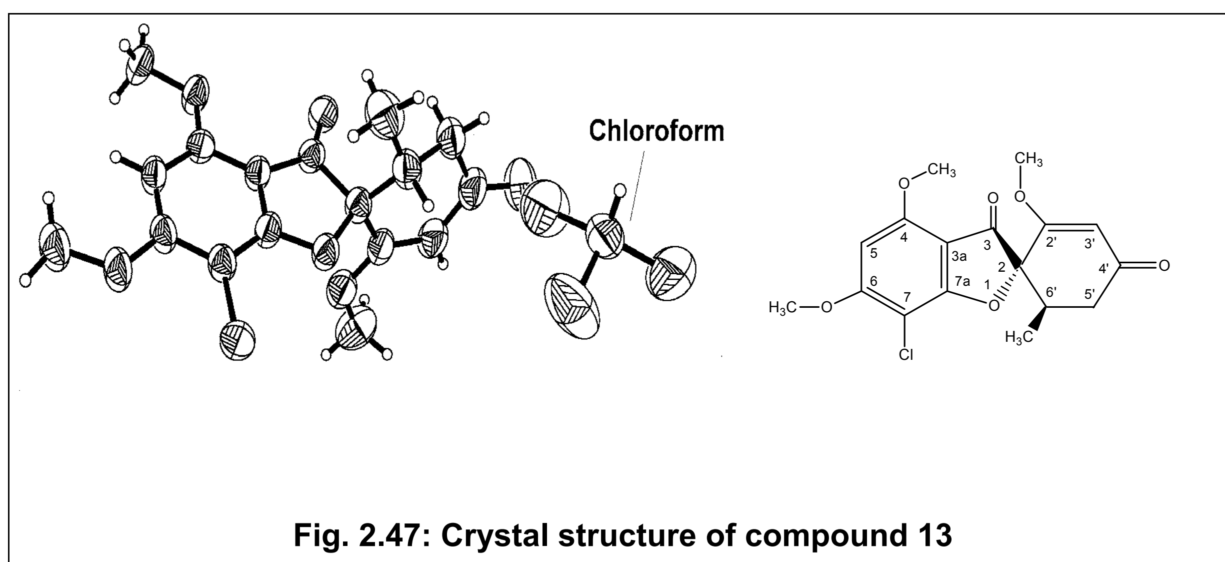


Fig. 2.47: Crystal structure of compound 13

2.1.3.1.3. 7-Dechlorogriseofulvin (14, known compound)

Dechlorogriseofulvin was first isolated from *Penicillium griseofulvum and P. janczewskii* in 1951 [MacMillan, 1951]. It has also been reported to be a product of the cultures of *P. urticae, P. nigricans, Aspergillus versicolor, Coriolus vernocipes* [Antibase, 2002].

Structure Elucidation

The UV and (+)-ESIMS (Fig. 2.44) spectra of compound **14** was similar with that of **13**. Eluting 1.5 minutes earlier than griseofulvin in the RP-18 HPLC analytical system, **14** also exhibited a shift in its UV absorption maxima from 292 to 288 nm when compared with **13**. It showed a molecular ion peak at *m/z* 319 corresponding to $[M+H]^+$ in (+)-ESIMS (Fig. 2.44) which was compatible with the molecular formula $C_{17}H_{18}O_6$ suggesting the loss a chloro group from griseofulvin. This was confirmed by the ^1H NMR (Fig. 2.45) which was similar with that of **13** except for an additional aromatic proton which was *meta*-coupled with another aromatic proton (*J* =1.7) (Table 2.15).

The absolute configuration of compound **14** is also deduced to be identical with that of dechlorogriseofulvin due to the similarity in their $[\alpha]_D$ values [observed $[\alpha]_D$ +191 (*c* 0.081, MeOH); lit. $[\alpha]_D$ +390 (*c* 1.0, Me_2CO) [Macmillan, 1951].

Table 2.15: NMR data of compounds 13 and 14

	13		**14**
	^{13}C	^{1}H	^{1}H
1			
2	91.0		
3	191.2		
3a	104.6		
4	157.6		
5	90.1	6.49 (s)	6.46 (d, 1.7)
6	164.5		
7	95.2		6.21 (d, 1.7)
7a	168.6		
2'	170.3		
3'	104.0	5.59 (s)	5.57 (s)
4'	195.6		
5'	40.5	2.66 (dd, 16.7,13.2)	2.66 (d, 14.1, 12.9)
		2.34 (dd, 16.7, 4.7)	2.32 (dd, 14.1, 2.8)
6'	36.8	2.79 (ddd, 6.6, 13.2, 4.7)	2.79 (m)
4-OCH_3	57.1	3.93 (s)	3.83 (s)
6-OCH_3	56.4	4.03 (s)	3.88 (s)
2'-OCH_3	56.6	3.61 (s)	3.60 (s)
6'-CH_3	14.3	0.79 (d, 6.6)	0.80 (d, 6.3)

Bioactivity

Griseofulvin is a widely used antifungal agent with affinity to keratin. It is also a possible human carcinogen with LD_{50} of 1200 mg/kg. Dechlorogriseofulvin, on the other hand, was reported to possess a much weaker antifungal activity against the fungus *Botrytis allii* (6.25 μg/mL least concentration compared to griseofulvin's 0.05 μg/mL) [MacMillan, 1951].

Compound **13** was found active against *B. subtilis* and *C. albicans* down to a loading concentration of 1 μg, but not with *S. aureus* and *E. coli* (Table 2.16). When tested against *C. albicans* at a concentration of 10 μg/disk in the agar diffusion test, no

activity was exhibited by **14**. Both compounds **13** and **14** were inactive in the brine-shrimp lethality and insecticidal tests.

Table 2.16: Agar diffusion assay data of compound 13

Concentration (μg)	Zone of inhibition (mm)			
	S. aureus	*E. coli*	*B. subtilis*	*C. albicans*
1	no activity	no activity	7	15
5	no activity	no activity	10	18
10	no activity	no activity	10	15

2.1.3.1.4. Communesin B (15, known compound)

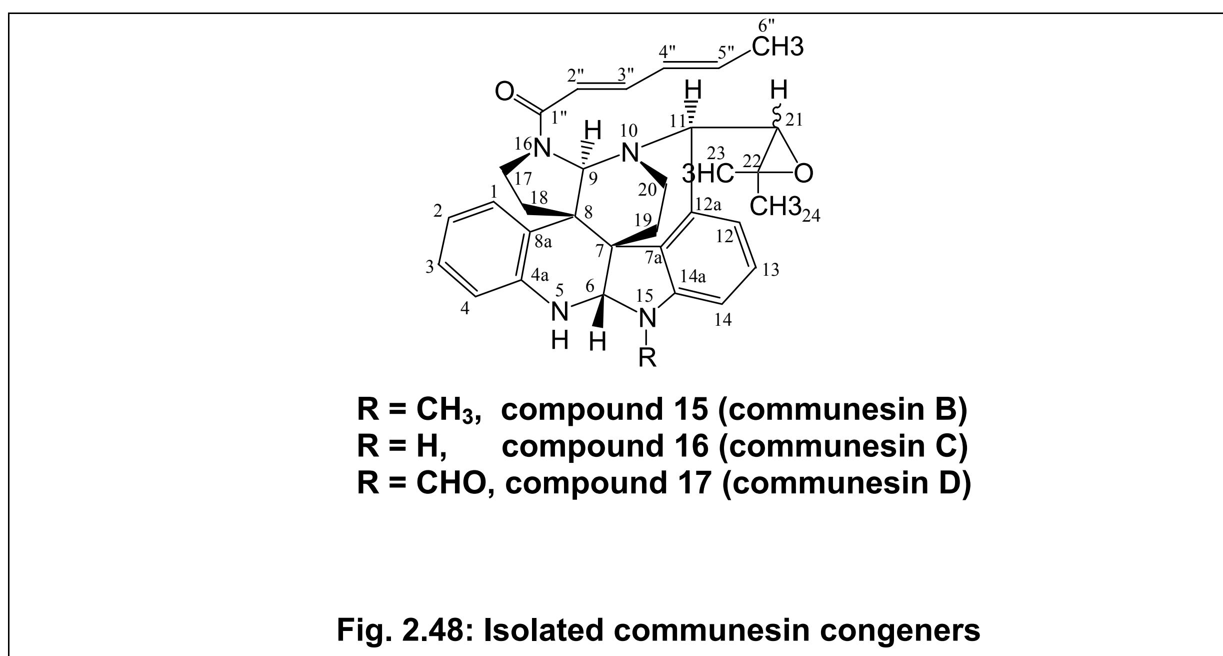


Fig. 2.48: Isolated communesin congeners

Communesin B (Fig. 2.48) is a metabolite previously isolated from a strain of *Penicillium spp.* which was associated with the marine alga *Enteromorpha intestinalis* [Numata *et al*., 1993]. It was isolated with a congener, communesin A, possesssing an acetyl group attached to N-16. Both compounds were reported to exhibit moderate to potent cytotoxic activity in the P-388 lymphocytic leukemia test system in cell culture (ED_{50} 3.5 and 0.45 μg/ml for communesin A and B, respectively) [Ibid].

Structure Elucidation

Compound **15** (Fig. 2.48) showed a molecular ion peak at *m/z* 509 in the (+)-in ESIMS (Fig. 2.52A) which is compatible with the molecular formula $C_{32}H_{36}N_4O_2$. The fragmentation pattern of the molecular ion of **15** showed the presence of an ion corresponding to $[M+H-71]^+$ which could be due to the loss of the dimethylethoxide moeity. This fragment ion were later found to be present in the ESIMS spectra of the other communesin congeners, compounds **16** and **17** (Fig. 2.52b and 2.52c). The ^{13}C (Fig. 2.49) and ^{1}H (Fig. 2.52) of **15** agreed with the NMR data of communesin B as reported in the literature [Ibid].

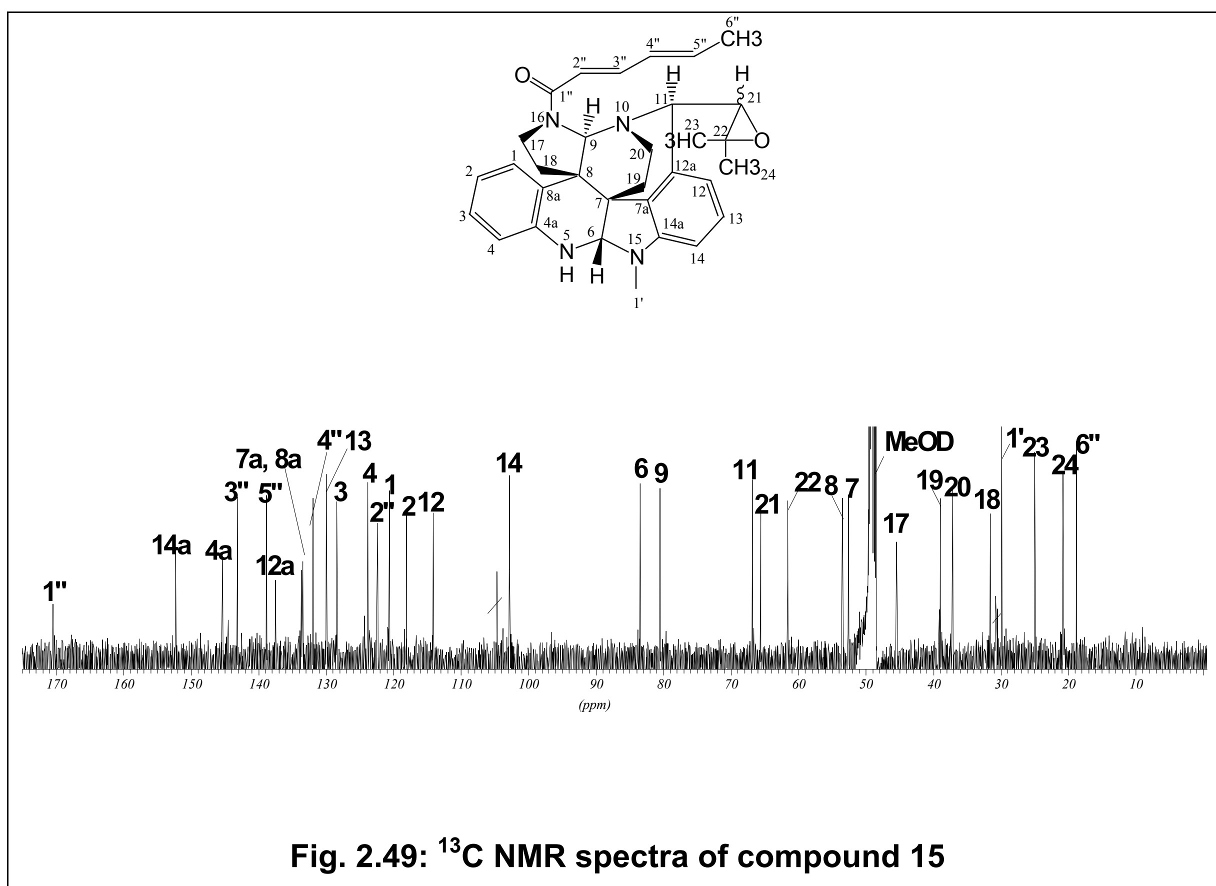


Fig. 2.49: ^{13}C NMR spectra of compound 15

The assignment of ^{13}C and ^{1}H NMR signals was established by HMQC and the C-H connectivities were confirmed by HMBC (Table 2.17). Some of the important correlations are shown below (Fig. 2.50a).

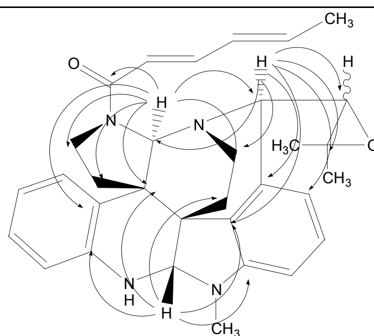


Fig. 2.50a: Important HMBC correlations in the spectra of compound 15

It is interesting to point out that the observed $[\alpha]_D$ value of **15** which was –58° (*c*, 0.1, MeOH) is identical with that of the reported value of communesin A (-58° *c* 0.14, $CHCl_3$), the N16-acetyl congener of communesin B, but deviates from the reported value of communesin B (+8, *c* 0.23, $CHCl_3$) [Numata *et al*., 1993]. Although the absolute configurations of the stereogenic centers of communesins A and B in the literature were not determined [Ibid], the identical relative stereochemistry and $[\alpha]_D$ values of **15** and communesin A attest to their identical absolute configuration. However, no conclusion can be drawn on the relationships of **15** and communesin B except that the two have the same relative stereochemistry at all their stereogenic centers except at C-21 as gathered from a ROESY experiment on **15.** The stereochemistry at C-21 could not be deduced from the ROESY spectra and could be the differing point in the stereochemistries of **15** and communesin B. Observed NOE's are shown below (Fig. 2.50b).

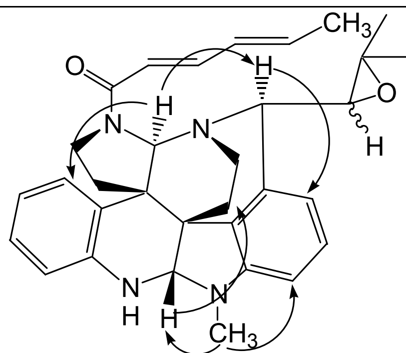


Fig. 2.50b: Important NOEs found in the ROESY spectra of compound 15

2.1.3.1.5. Communesin C (16, new compound)

HREIMS *m/z* 494.2668 (calcd for $C_{31}H_{34}N_4O_2$, 494.2682)
$[\alpha]_D$ -30 (*c* 0.038, MeOH)

Compound **16** and **17** (Fig. 2.40) exhibited similar UV spectra as **15** (Fig. 2.51). Compound **16** gave a base ion peak at m/z 495 in (+)-ESIMS (Fig. 2.52B) which is 14 amu less than **15**. Its molecular formula was established by HREIMS to be $C_{31}H_{34}N_4O_2$. Moreover, as with **15,** the mass spectra also showed a strong ion peak at m/z 424 corresponding to $[M+H-71]^+$ possibly due to the loss of the dimethylethoxide moeity. The ^{1}H NMR spectra of **16** (Fig. 2.53) also was almost identical with that of **15** (Table 2.17). The difference lies only in the absence of the N-methyl group which is seen at δ 2.78 in **16**. Slight shifts of the H-6 and of the aromatic H-14 signals to lower fields were also observed in **16**. The COSY spectrum (Fig. 2.54) showed that all the spin systems found in **15** were retained in **16**. Hence it is postulated that the new derivative **16**, to which the name communesin C is assigned, is the N-demethyl derivative of communesin B.

The stereochemistry of compound **16** is assumed to be identical with that of **15** due to the similarity in their observed $[\alpha]_D$ values [-30 (*c* 0.038, MeOH) for **16** and –58 (*c* 0.1, MeOH) for **15**].

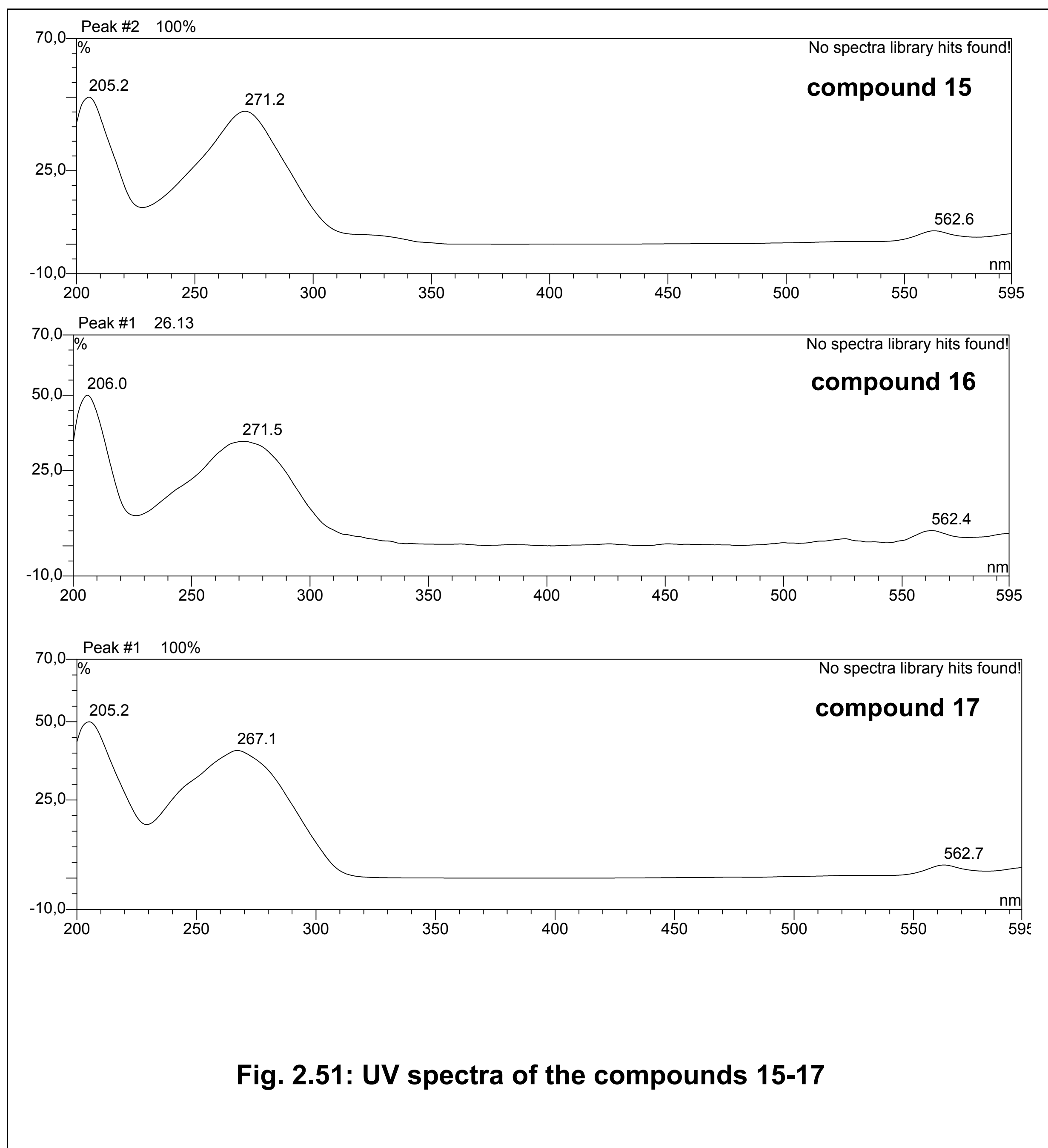


Fig. 2.51: UV spectra of the compounds 15-17

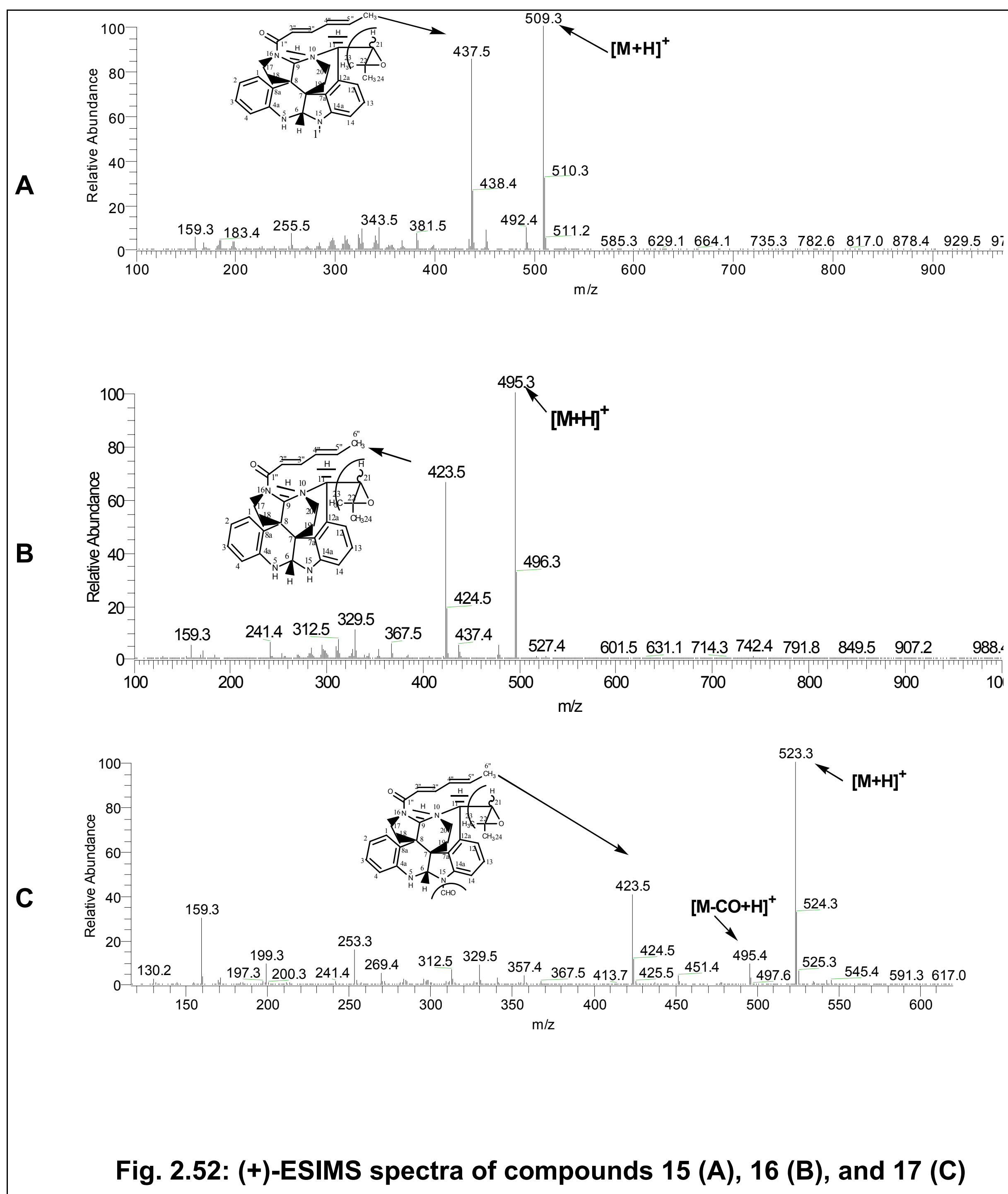


Fig. 2.52: (+)-ESIMS spectra of compounds 15 (A), 16 (B), and 17 (C)

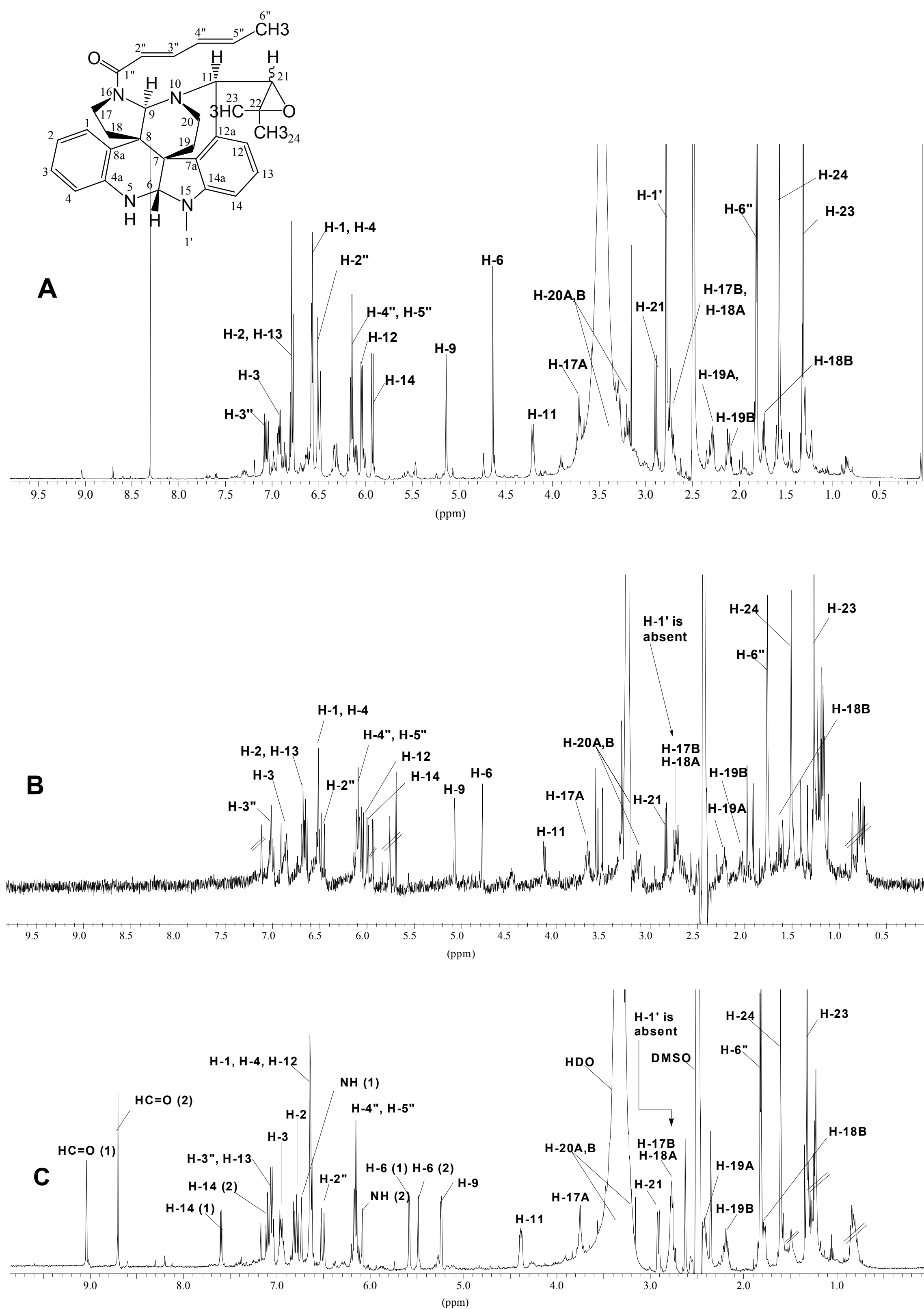


Fig. 2.53: H NMR spectra of compounds 15 (A), 16 (B), and 17 (C)

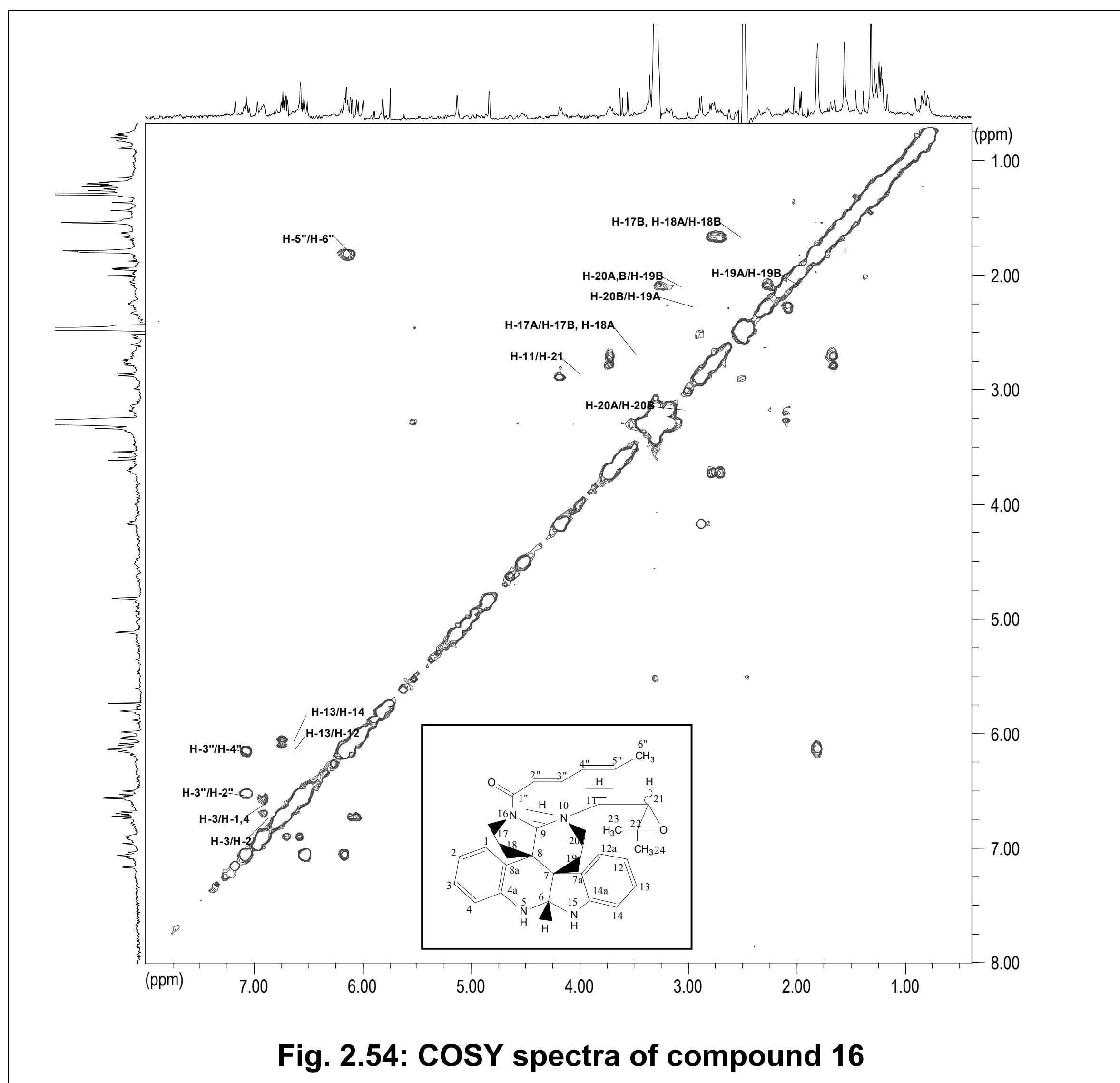


Fig. 2.54: COSY spectra of compound 16

2.1.3.1.6. Communesin D (17, new compound)

HREIMS m/z 522.2621 (calcd for $C_{32}H_{34}N_4O_3$, 522.2631)

$[\alpha]_D$ +23.3^o (*c* 0.039, MeOH)

The UV (Fig. 2.51) and (+)-ESIMS spectra (Fig. 2.52) of compound **17**, to which the name communesin D is assigned, is also similar to that of **15** and **16**. The (+)-ESIMS spectra (Fig. 2.52) showed a base peak at *m/z* 523, which is 14 amu more than **15**. The molecular formula was established by HREIMS to be $C_{32}H_{34}N_4O_3$. The fragmentation pattern of the molecular ion in (+)-ESIMS showed the presence of an ion peak at *m/z* 495 corresponding to $[M+H-28]^+$, which could be ascribed to the loss of a carbonyl group. As in both communesins B and C, there is also the presence of an ion peak at *m/z* 423 corresponding to a loss of the dimethylethoxide group and the

formyl group. The EIMS spectra of **17** (Fig. 2.56) also showed an ion peak at *m/z* 451 corresponding to $[M-71]^{+\cdot}$ which repeated this observation that the loss of the dimethylepoxide moiety is characteristic in the fragmentation patterns of these compounds.

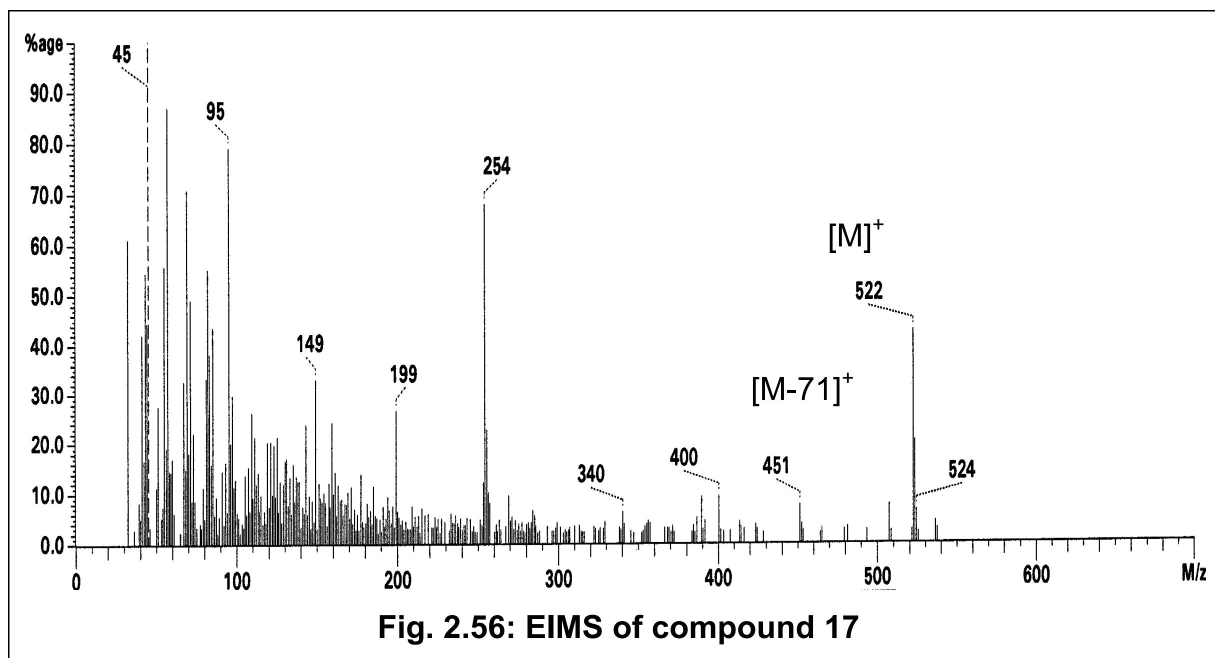


Fig. 2.56: EIMS of compound 17

The ^1H and the 2D NMR (COSY, ROESY and HMBC) spectra of the compound established its structure as the formamide derivative of communesin B (Table 2.17).

The ^1H NMR spectrum of **17** was also highly similar to both **15** and **16** (Fig. 2.53). The most prominent difference is the loss of the H-1' signal at δ 2.78 which was present in **15**. Furthermore an additional proton signal at δ 8.70 was observed in **17**, corresponding to a formyl proton. The H-6 signal was also seen at a lower field at δ 5.55 compared to that of **15** which resonated at δ 4.65, presumably due to the greater deshielding effect of the amide moeity in **17**. The COSY spectrum (Fig. 2.55) showed that all the spin systems found in **15** and **16** are also retained: there were no changes in the resonances of the olefinic protons from the conjugated side chain, the two separate N-ethylene moeities, the alkyl epoxide moeity and the aromatic protons H-1 to H-4. However, it was observed that the doublet signals from the aromatic protons H-12 and H-14 from the other aromatic ring were shifted to lower fields (Table 2.17), probably due to the resonance-enhanced electron-withdrawing effect of the formamide group.

An interesting feature in the NMR spectra of **17** in DMSO-d_6 (Fig. 2.53c) showed the presence of two stereoisomers of this compound due to the differences in the orientation of the N-formyl group. DMSO-d_6 allowed the slow interconversion of the two isomers which caused the appearance of two signals of the protons of the formyl group as well as those of NH, and H–14 which resonated at different chemical shifts due to the deshielding effect of the formyl carbonyl. This was again evident in the COSY spectra of **17** which showed correlations between NH-A with H-6B and between NH-B with H-6B. The ROESY spectra of **17** also showed correlations between the CHO-A with H-14B and between CHO-B with NH-A and NH-A. The two stereoisomers are shown in Fig. 2.57 with important NOE's from the ROESY spectra.

The relative stereochemistry of **17** was found to be identical with **15**, as deduced from its ROESY spectrum. Important correlations are shown below (Fig. 2.57). Its $[\alpha]_D$ value of +23.3^o *c* 0.039, MeOH) however deviates from **15** and is more related to that of communesin B in the literature (+8, *c* 0.23, $CHCl_3$) [Numata *et al.*, 1993]. The HMBC spectra confirmed the proposed structure which showed correlation between both the formyl proton and H-13 with C-14a. Important correlations are shown below (Fig. 2.58).

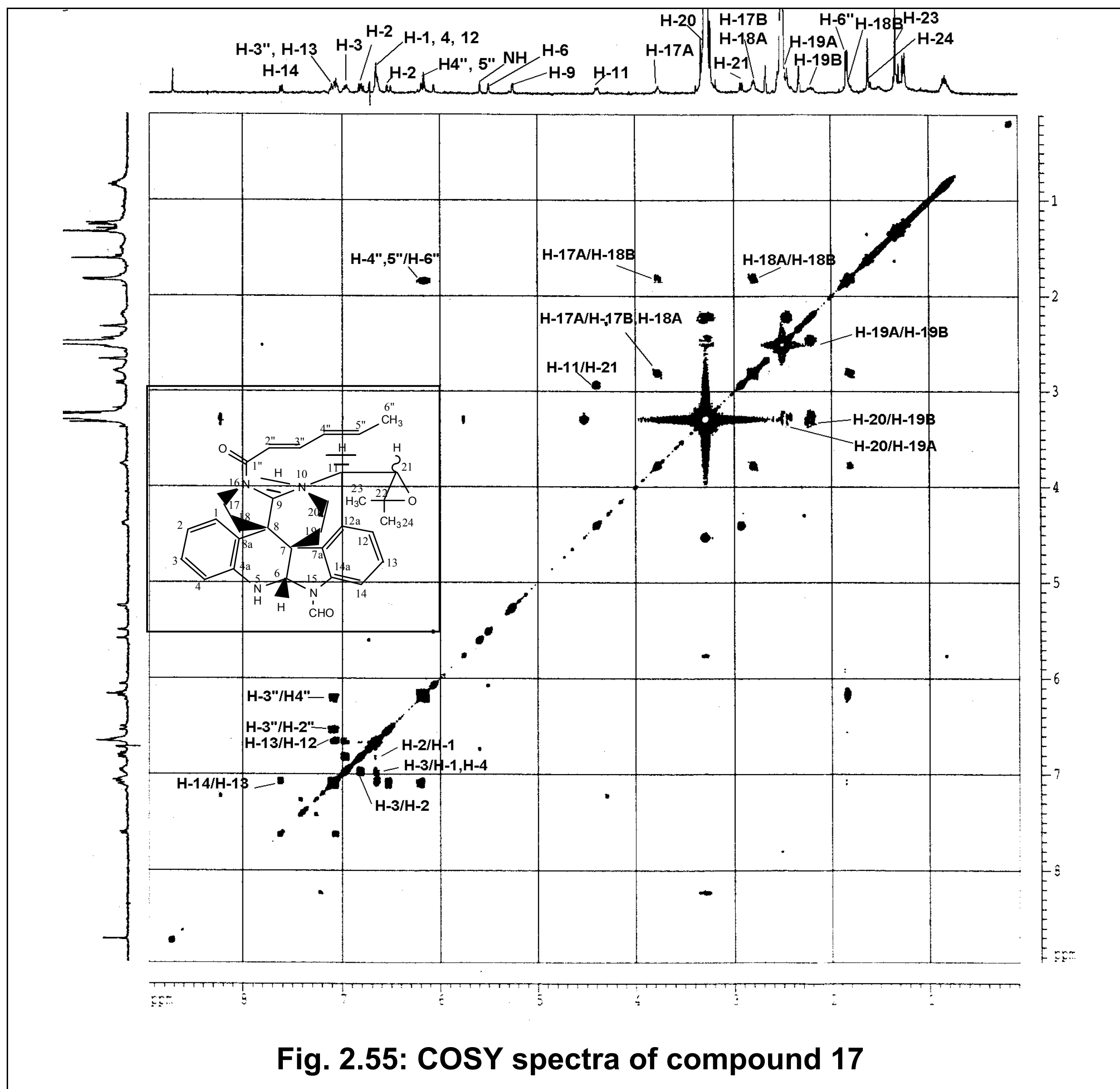


Fig. 2.55: COSY spectra of compound 17

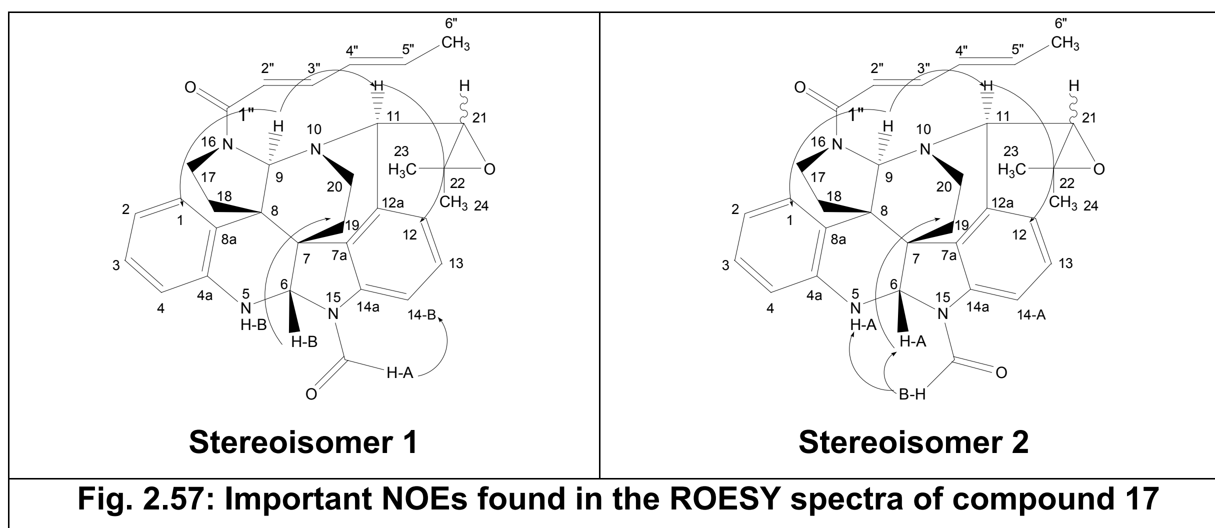


Fig. 2.57: Important NOEs found in the ROESY spectra of compound 17

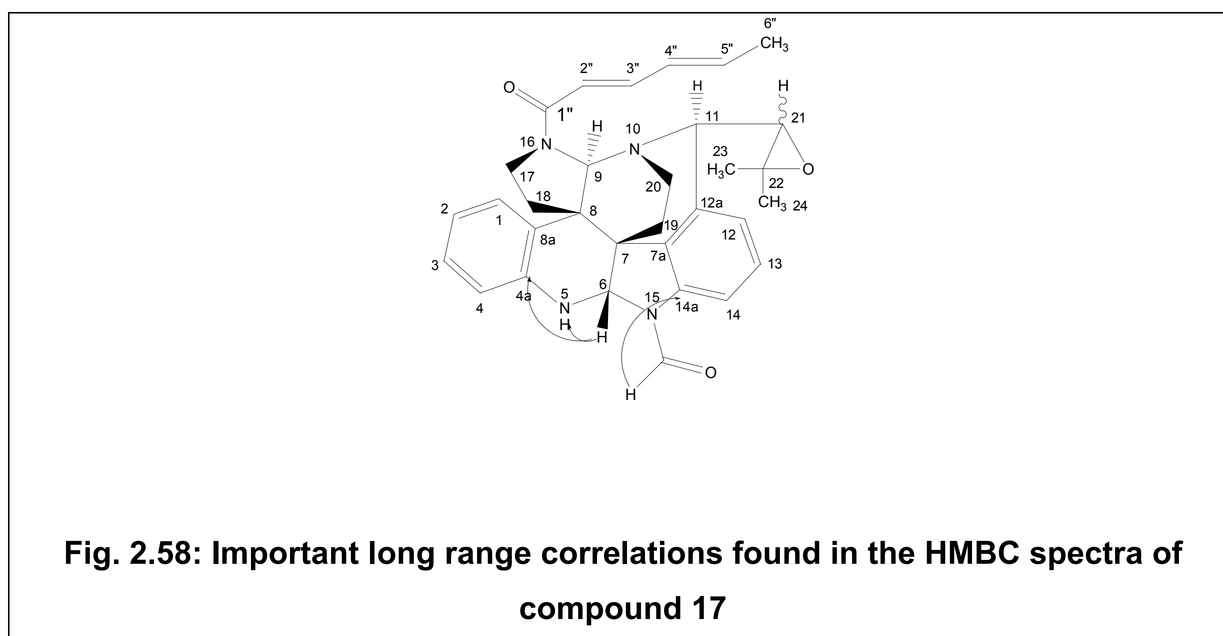


Fig. 2.58: Important long range correlations found in the HMBC spectra of compound 17

Table 2.17: NMR data of the compounds 15-17 measured at 500 Mhz

C#	**15** ^{1}H DMSO-d_6	**15** ^{13}C MeOD	**16** ^{1}H DMSO-d_6	**17** ^{1}H DMSO-d_6	**17** ^{13}C* DMSO-d_6
1	6.57	120.62	6.57	6.64	120.0
2	6.79	118.06	6.7	6.79	120.0
3	6.92	128.41	6.9	6.95	129.0
4	6.57	123.81	6.57	6.67	120.1
4a		145.31			142.3
6	4.65	83.45	4.83	5.50	76.4
7		52.56			51.1
7a		133.63			135.5
8		53.45			53.6
8a		133.42			131.5
9	5.14	80.50	5.12	5.24	80.0
11	4.20	66.80	4.18	4.39	64.6
12	6.04	114.10	6.1	6.64	139.5
12a		137.50			138.8
13	6.79	129.93	6.74	7.04	132.0
14	5.92	102.77	6.03	7.60	107.4
14a		152.30			141.0
17	2.73	45.44	2.78	2.79	
	3.72		3.73	3.76	
18	1.74	31.52	1.67	1.78	
	2.73		2.78	2.79	
19	2.12	38.94	2.09	2.19	
	2.31		2.27	2.43	
20	3.20	37.14	3.2	3.20	35.8
	3.30		3.3	3.30	
21	2.90	65.59	2.88	2.92	63.5
22		61.59			60.0
23	1.32	25.00	1.32	1.33	24.9
24	1.56	20.79	1.55	1.61	20.5
1'	2.78	29.88			
1"		170.47			167.2
2"	6.50	122.36	6.53	6.52	122.0
3"	7.06	143.12	7.07	7.08	144.7
4"	6.14	131.91	6.16	6.17	131.1
5"	6.14	138.81	6.16	6.17	137.0
6"	1.82	18.80	1.81	1.83	50.7
NH		4.32	br s	5.59	
HC=O				8.70	

*obtained from HMBC spectrum

Bioactivity:

Compound **15** showed strong activity against *A. salina, S. littoralis*, and the human leukemia cell lines. It was also active against *S. aureus, B. sub*tilis and *C. albicans* with a minimum loading concentration of 0.5 μg, but not against *E. coli.* (Table 2.18-2.21).

Table 2.18: Agar plate diffusion assay of compound 15.

Concentration (μg/disk)	Zone of inhibition (mm)			
	S. aureus	*E. coli*	*B. subtilis*	*C. albicans*
0.5	10	n.a	9	12
2.5	10	n.a	9	12
5	10	n.a	10	15

Table 2.19: Brine-shrimp lethality test for compounds 15-17

Concentration	Mortality (%)		
(μg/mL)	**15**	**16**	**17**
	LC_{50} = 0.300	LC_{50} = 1.933	LC_{50} = 0.567
0.0	0	0	0
0.2	10	0	0
0.4	50	0	0
0.6	100	0	0
0.8	100	10	40
1.0	100	25	100
2.0	100	50	100

Table 2.20: Insecticidal test for compound 15 (LC_{50} = 6.9 ppm)

Concentration (ppm)	Mortality (%)
0	0
5	50
15	80
20	80
50	100

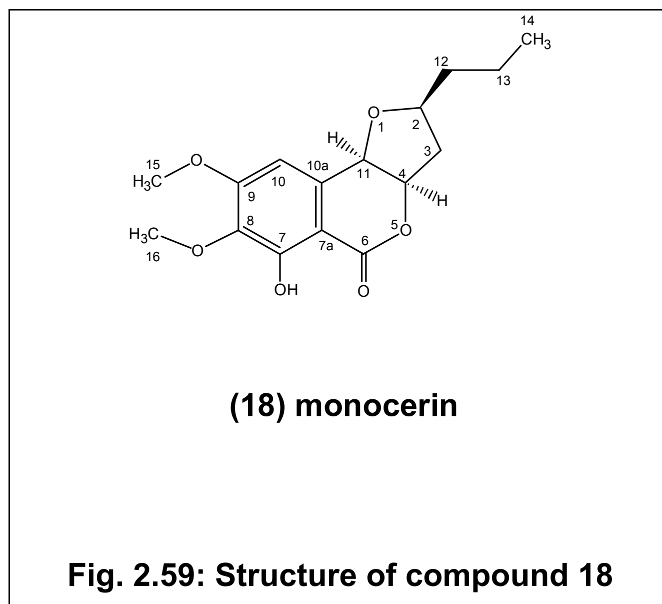
Table 2.21: Cytotoxicity tests for compound 15

Concentration (μg/mL)	Growth rate (%) in different cell lines		
	JURKAT[+] EC_{50} = 6.159	THP-1 * EC_{50} = 14.23	MM-1[§] EC_{50} = 7.918
0	100.0	100.0	100
1	89.3	86.0	93.0
2.5	92.0	-	92.3
5.0	67.0	90.0	87.0
10	31.8	86.0	43.7
20	25.0	-	-
25	1.7	10.0	5.3
50	2.0	-	-

[+]values taken from two separate MTT assays and two separateThy assays.
* values taken from MTT assay
[§] values taken from both MTT and Thy assays

2.1.3.2. Isolated compounds from an unidentified fungus derived from the sponge *Axinella verrucosa*

Monocerin (18, known compound)



(18) monocerin

Fig. 2.59: Structure of compound 18

Monocerin was originally isolated from *Helminthosporium monoceras* [Aldridge, 1970]. It has since been reported to be a product of cultures from the fungi *Fusarium larvarum, Drechslera ravenelli, Drechslera turcicum* and *Readeriella mirabilis* [Dictionary of Natural Products on CD-ROM, 2002].

Structure Elucidation

Compound **18** (Fig. 2.59) gave a molecular ion peak at *m/z* 308 by EIMS (Fig. 2.60). This molecular mass was compatible with the molecular formula $C_{16}H_{20}O_6$. The ^{13}C (Fig. 2.61) and DEPT showed sixteen carbons: one carboxyl, five substituted aromatic carbons, one unsubstituted aromatic carbon, two methoxyls, three oxagen-bound methines, three methylenes and one methyl. Assignment of one bond C-H connectivities was established by HMQC. The COSY showed that H-11 coupled with H-4, which was connected to a diastereotopic methylene H-3A and H-3B. These two geminal protons were also seen to correlate with the methine proton H-2, which was further connected to the methylene protons H-12. H-12 was was seen to be attached to H-13 and H-14 in a side chain. Two-bonds and three-bonds C-H connectivities were established by HMBC. NMR data of **18** are presented in Table 2.22).

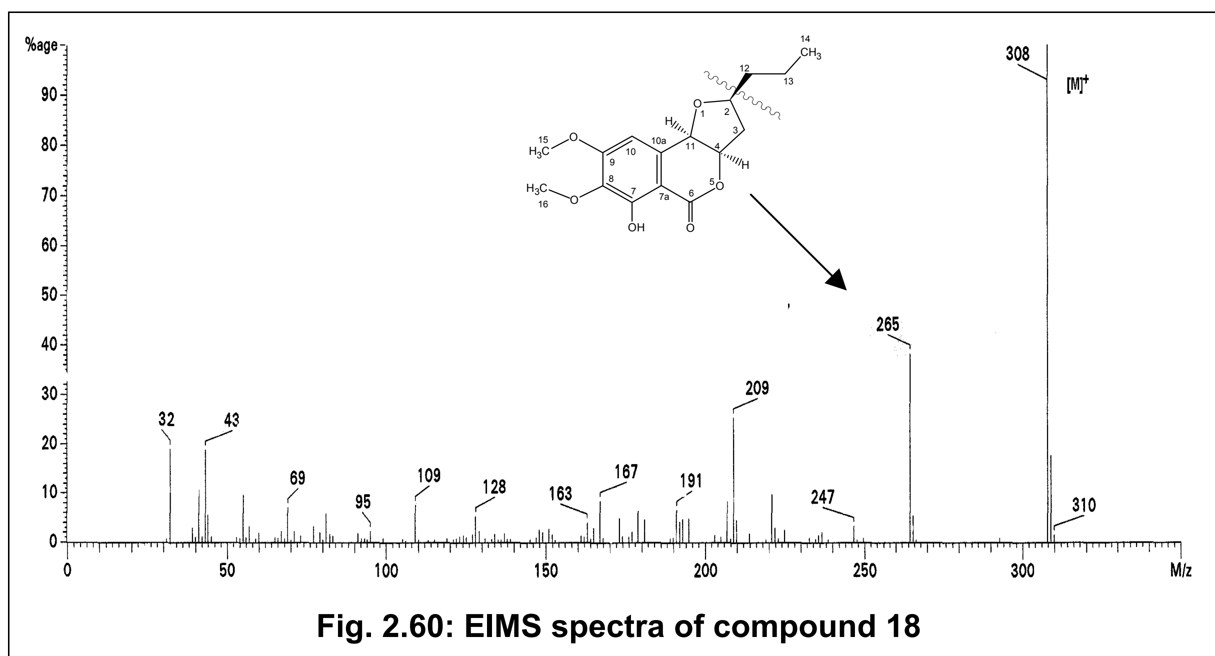


Fig. 2.60: EIMS spectra of compound 18

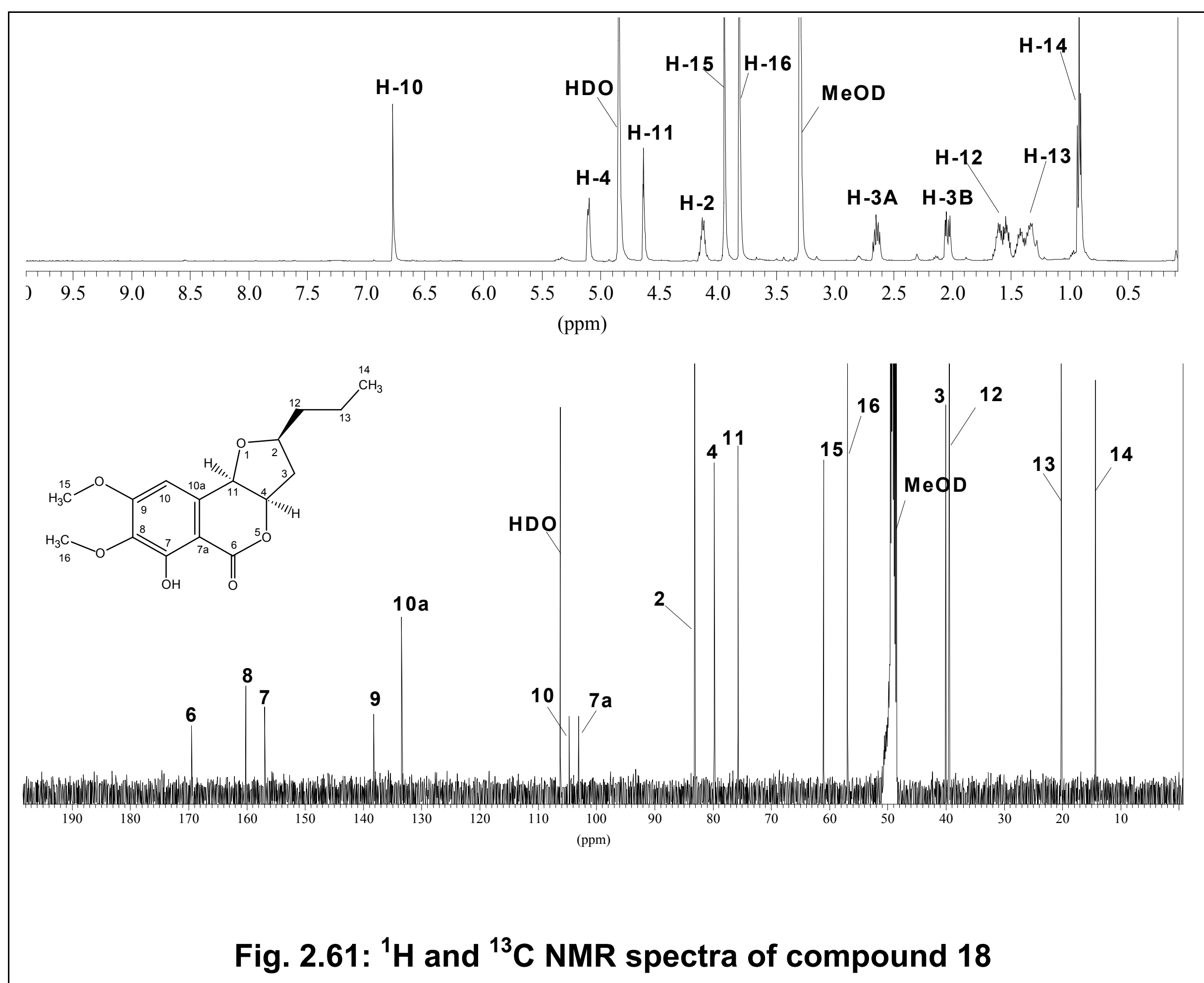


Fig. 2.61: ^{1}H and ^{13}C NMR spectra of compound 18

Further proof of the proposed structure was supplied by the fragmentation pattern in EIMS. A strong ion peak at *m/z* 265 (40%) due to the loss of the n-propyl side chain was observed. Further fragment ion at *m/z* 247 represents a further loss of a hydroxyl group.

The stereochemistry at the three stereogenic centers are believed to be identical with monocerin. The coupling constant of 2.81 Hz between H-4 and H-11 indicate a *cis* configuration between these protons, mirroring that of monocerin. Measured $[\alpha]_D$ values of monocerin + 51 (*c* 0.144, MeOH) was almost identical with literature data of $[\alpha]_D$ +53 (*c* 0.85, MeOH) [Aldridge and Turner, 1970]. Monocerin has been reported as having an absolute stereochemistry 2*S*, 4*R* 11*R*, as established through chiroptical and ^{1}H NMR analysis, and confirmed by synthesis [Grove and Pople, 1979; Mori and Takaishi, 1989].

Table 2.22: NMR data of compound 18

C	^{13}C	^{1}H	HMBC
2	83.5	4.12 (m)	
3A	40.5	2.64 (ddd, 14.5, 8.83, 6.31)	C12, C4,
3B		2.02 (br dd, 14.5, 5.68)	C12, C4, C2, C11
4	80.2	5.10 (br dd, 5.68, 2.81)	C2, C11
6	169.9		
7	157.3		
7a	105.0		
8	160.6		
9	138.6		
10	106.6	6.77 (s)	C11, C7a, C10a, C8, C7, C9, C6
10a	133.8		
11	76.1	4.63 (d, 2.81)	C4, C7a, C10, C10a
12	39.8	1.60 (m)	C14, C13, C3, C4
13	20.6	1.40 (m)	C14, C12
14	14.7	0.92 (t, 7.36)	C13, C12
15	61.4	3.94s)	C9
16	57.3	3.82 (s)	C8

Bioactivity

Monocerin was first reported as a toxin isolated from *Helminthosporium monoceras* [Aldridge and Turner, 1970]. It was found to be active against powdery mildew on wheat. It has also been found to be a metabolite of the entomogenous fungus *Fusarium larvarum* Fuckel and was found to possess insecticidal activities. Furthermore, monocerin isolated from an American *Exserohilum turcicum* derived from *Sorghum hapelense,* exhibited phytotoxicity. A French strain of the fungus *Exserohilum turcicum* isolated from maize, also yielded monocerin, which gave phytotoxic properties [Cuq *et al.,* 1993]. It caused necrotic lesions on punctured leaves, the size of which is in direct relation with the dose applied. It also inhibits the root growth of pregerminated seeds: the median dose (ID_{50}) being around 10^{-3} M. It decreased the viability of maize root cap cells and mesophyll protoplast suspensions: the ID_{50} was 2.5 – 5 x 10^{-4} M for cells and around 8 x 10^{-5} M for protoplasts [Ibid].

Compound **18** was found to be active against *S. aureus, B. subtilis and C. albicans* with zones of inhibition of 10, 10 and 15 mm, respectively, using a concentration of 50 µg/mL. It was also found to be an potent antifeedant of the polyphagous insect *S. littoralis* exhibiting ED_{50} of 14 ppm. It was inactive in the brine-shrimp lethality test.

2.1.4. Isolated compound from *Aspergillus flavus*, Link: Fries derived from *Hyrtios aff. reticulatus*

The extracts obtained from 14 various cultures of fungal strains collected from different species of Philippine sponges were initially screened for biological activity. Results of the screening (Fig. 2.62a) led to the selection of the extract of *Aspergillus flavus* associated with the sponge *Hyrtios* aff. *reticulatus,* which showed 95% mortality rate in the brine-shrimp lethality test, 25% growth rate in the insecticidal test. It also showed activities in the animtimicrobial assay (Table 2.23a).

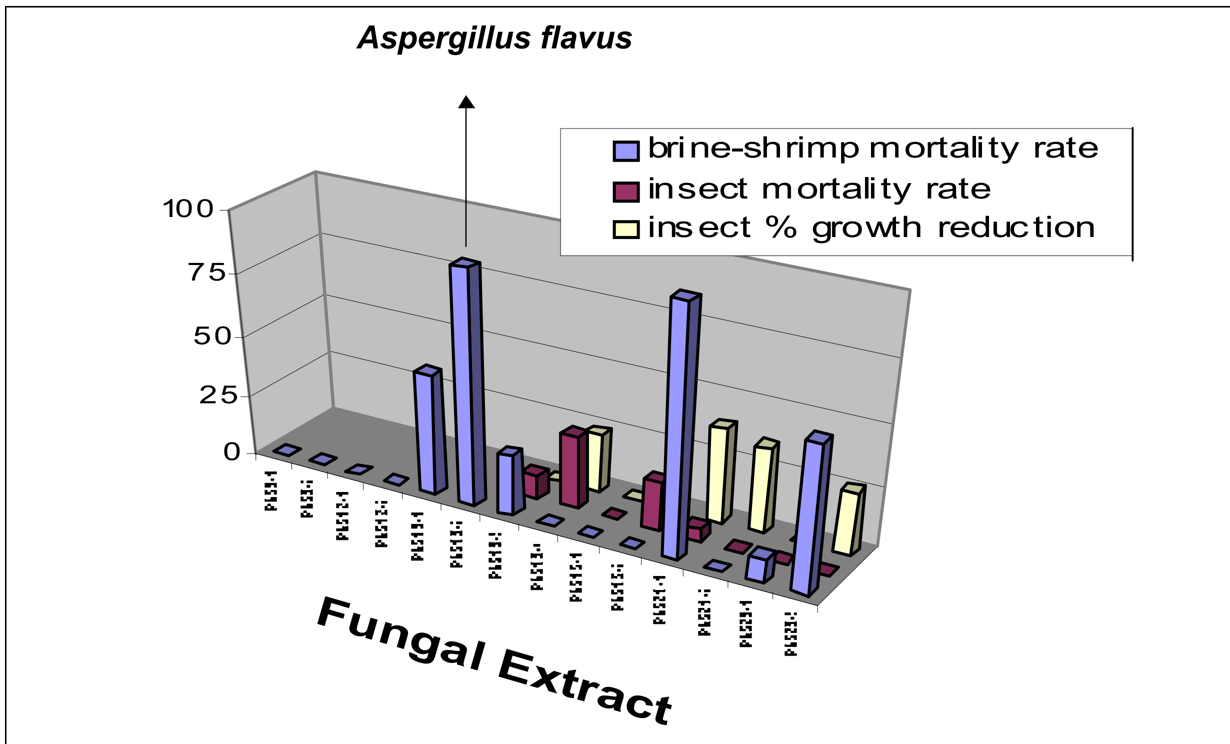
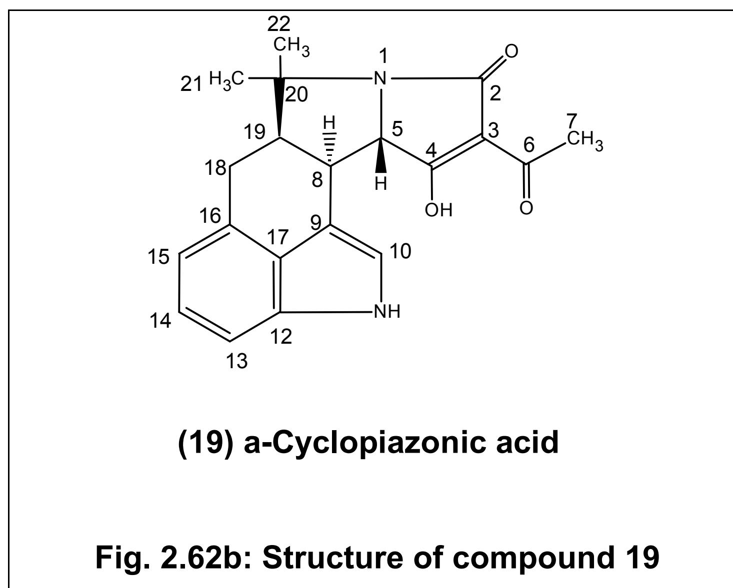


Fig. 2.62a: Result of the bioscreening of Philippine-collected fungal strains

Table 2.23a: Antimicrobial assay result of the extract of *Aspergillus flavus*

Test Microorganism	Zone of Inhibition (mm)
B. subtilis	10
S. aureus	11
E. coli	n. a.
C. albicans	10

α-Cyclopiazonic acid (19, known compound)



(19) a-Cyclopiazonic acid

Fig. 2.62b: Structure of compound 19

This toxic alkaloid was first isolated from *Penicillium cyclopium* Westling in 1968 [Holzapfel, 1968]. This known mycotoxin appears to be produced mainly by *Penicillium camemberti, Penicillium commune, Penicillium griseofulvum, Aspergillus versicolor, Aspergillus flavus and Aspergillus oryzae* [Dictionary of Natural Products on CD-ROM, 2002].

Compound **19** (Fig. 2.62b) gave a molecular ion peak at *m/z* 337 $[M+H]^+$ in the positive ion ESIMS spectrum which is compatible with the molecular formula $C_{20}H_{20}N_2O_3$. The ^1H and ^{13}C NMR (Fig. 2.63) matched that of α-cyclopiazonic acid in the literature [Nolte *et al.*, 1979].

The COSY spectra showed three separate spin systems. The indole proton at δ 10.7 was seen to couple to its neighboring proton H-10. The three aromatic protons at δ 6.76, δ 6.98, and δ 7.13 exhibited an ABC system. H-15 at δ 6.76 was *ortho*-coupled (J = 8.2) to proton H-14 at δ 6.98, which is furthered *ortho*-coupled to H-13 at δ 7.13 (J = 6.9). The third spin system presented an XMA_2B system [Holzapfel, 1968]. H-5 at δ 3.70 coupled with H-8 at δ 3.41 with a magnitude of 11.03 Hz indicating an *anti*-configuration. H-8 further coupled with H-19 at δ 2.38 with a magnitude of 5.65 Hz which suggests a *cis*-configuration. H-19 also showed coupling with geminal protons (H-18A and B) centered at δ 2.90 and 2.96. The relative configuration of these three

stereogenic centers C-5, C-8 and C-19 as derived from the coupling constants of the attached protons are identical the reported relative configuration of α-cyclopiazonic acid [Holzapfel, 1968]. The similarities in the $[\alpha]_D$ values of compound **19** (-122°, *c* 0.0214, MeOH) and α-cyclopiazonic acid (-109.4°, c 0.885, $CHCl_3$) [Pohland *et al.,* 1982]] proved that the two compounds are identical.

The assignments of the ^{13}C and ^{1}H signals (Table 2.23b) were based on comparison with literature data. The proposed structure was corroborated by HMBC.

Bioactivity

α-Cyclopiazonic acid is a toxin, the oral administration of which to male and female rats proved fatal after one to five days, and gave LD_{50} values of 36 and 63 mg kg^{-1}, respectively [Purchase, 1971]. In rats, cyclopiazonic acid produces degenerative changes and necrosis in the liver, pancreas, spleen, kidney, salivary glands, myocardium, and skeletal muscle. Although the teratogenic potential proved to be low in rats [Morrissey, *et al*., 1984], significant retardation in embryonic skeletal development was evident after administration of 5-10 mg of cyclopropiaqzonic acid during pregnancy. When chicken were fed rations containing 100 mg kg^{-1} cyclopropiazonic acid for seven weeks, the test group experienced decreased weight gain, poor feed conversion, and a sixfold increase in mortality compared to chickens receiving toxin-free feed [Dorner, 1983]. Postmortem examination revealed proventricular lesions characterized by mucosal erosion and hyperemia, and yellow foci in the livers and spleens. The birds also experienced mucosal necrosis in the gizzard, and hepatic and splenetic necrosis and inflammation.

It also inhibits sarcoplasmic reticulum Ca^{2+}-ATPase and ATP-dependent Ca^{2+} transport and is used as a pharmacologic tool for studying intracellular Ca^{2+} homeostasis [Dictionary of Natural Products on CD-ROM, 2002].

Compound **19** showed antimicrobial activity in the agar plate diffusion assay with 50 µg/disk causing zones of inhibition of 10 mm against each of the organisms *S. aureus, B. subtilis and C. albicans*. It also showed antifeedant activity against *S. littoralis* with ED_{50} of 10.08 ppm.

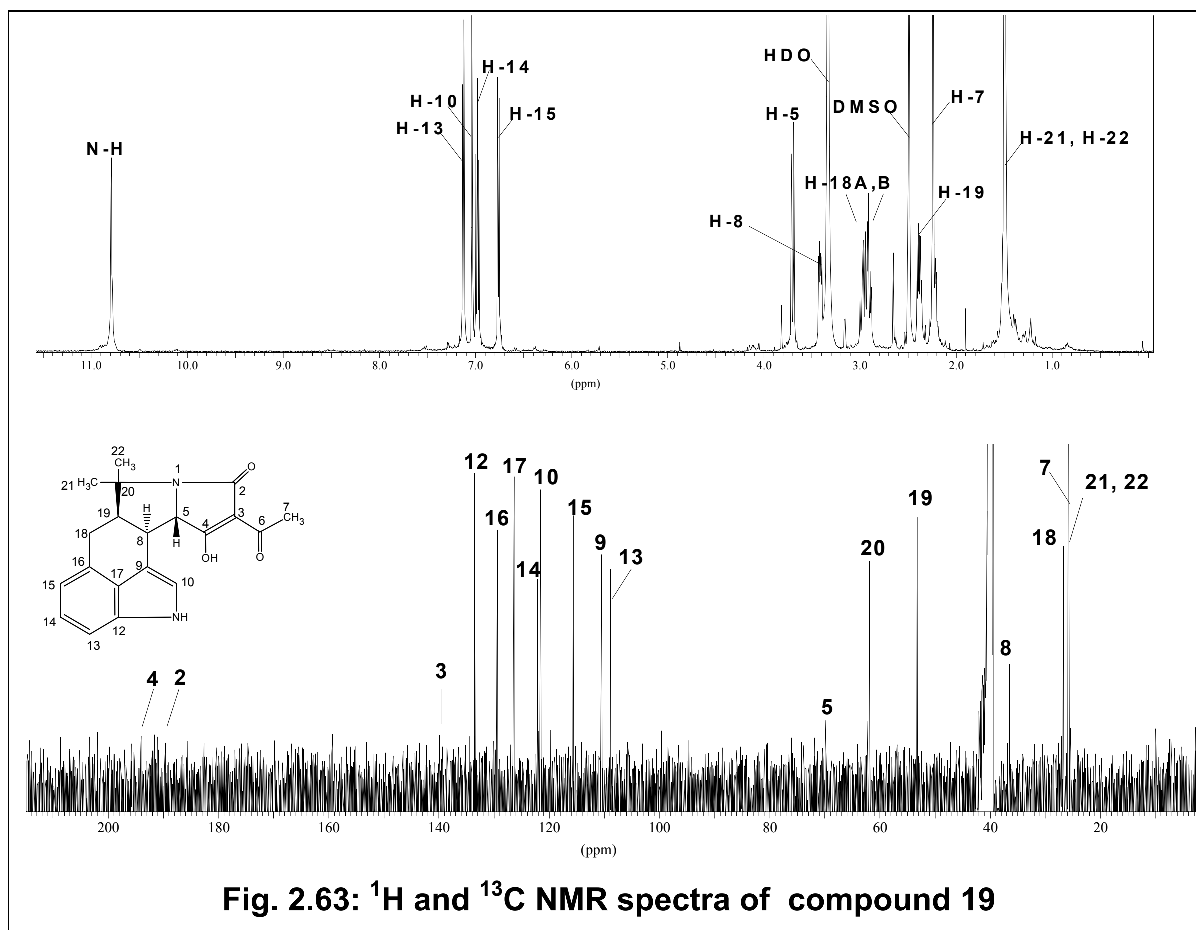


Fig. 2.63: ^{1}H and ^{13}C NMR spectra of compound 19

Table 2.23b: NMR data for compound 19 (DMSO-d_6 in 500MHz)

	^{13}C	^{1}H	HMBC
2			
3	140.0		
4	195.2		
5	69.0	3.70 (d, 11.03)	C4, C8, C9
6	192.0		
7	25.4	2.24 (s)	C3, C6
8	36.1	3.41 (dd, 10.72, 5.65)	C4, C5, C9, C10, C17, C18, C19
9	110.1		
10	121.2	7.04 (d, 1.89)	
N-H		10.7 (s)	C9, C10, C12, C17
12	133.1		
13	108.5	7.13 (d, 8.2)	C14, C15, C17
14	121.7	6.98 (dd, 8.2, 6.9)	C13, C15, C16, C12
15	115.3	6.76 (d, 6.9)	C9, C12, C17
16	129.0		
17	126.0		
18	26.3	2.90 (dd, 15.77, 11.98)	C8, C15, C16, C17, C19
		2.96 (dd, 15.77, 5.65)	
19	52.9	2.38 (ddd, 11. 98, 5.65, 5.65)	C5, C8, C9, C18
20	61.5		
21	25.3	1.50 (s)	C19, C20, C22
22	25.3	1.49 (s)	C19, C20, C21

2.2. Secondary metabolites isolated from sponges

2.2.1. Isolated compounds from *Agelas Nakamurai*

Sponges belonging to the genus *Agelas* have been found to be rich sources of bromopyrrole alkaloids. This group of alkaloids is represented by oroidin (Fig. 2.64a) which was first isolated in 1971 from *Agelas oroides* [Forenza *et al*., 1971]. Subsequently, many other species of *Agelas, Axinella, Acanthella, Hymeniacidon, Phakellia, and Pseudaxinyssa* and *Halichondria* [Walker *et al*., 1981; Nakamura *et al*., 1984; Gunasekera *et al*., 1989; Cafieri *et al*., 1997] have been reported to contain high levels of oroidin and/or its cyclic analogues. Therefore, the oroidin alkaloids are

useful chemotaxonomic markers for axinellid sponges that were once allied with the *Agelasida* [Hao *et al.,* 2001]. Oroidin and 4,5-dibromopyrrole-2-carboxylic acid were discovered to be responsible for the chemical defense of *Agelas clathrodes* and five other species of *Agelas* from tropical Caribbean coral reefs [Chanas *et al*., 1996).

This group of compounds exhibits interesting bioactivities such as insecticidal, antimicrobial, Na, K-ATPase inhibitory, and antiserotonergic activities. Sceptrin (Fig. 2.64a) and its analogues exhibited potent antibacterial/antifungal [Bernan *et al.,* 1993] activities, anti-muscarinic activity [Rosa *et al.,* 1992], and anti-histaminic activity [Cafieri *et al.,* 1997]. Furthermore, oxysceptrin is also a potent actomyosin ATPase activator [Kobayashi *et al.,* 1991].

The total extract from the sponge *Agelas nakamurai* showed antimicrobial activity (Table 2.24a). Upon chemical investigation of the extract, compounds **20-24** (Fig. 2.64b) were isolated, with 4-bromopyrrole 2-carboxamide (**20**) and 4-bromopyrrole 2-carboxylic acid (**21**) as the major compounds. This was the first time that compound **21** was isolated from a natural source.

Table 2.24a: Antimicrobial assay result of the extract of *Agelas nakamurai*

Test Microorganism	*B. subtilis*	*S. aureus*	***E. coli***	*C. albicans*
Zone of Inhibition (mm)	25	22	15	25

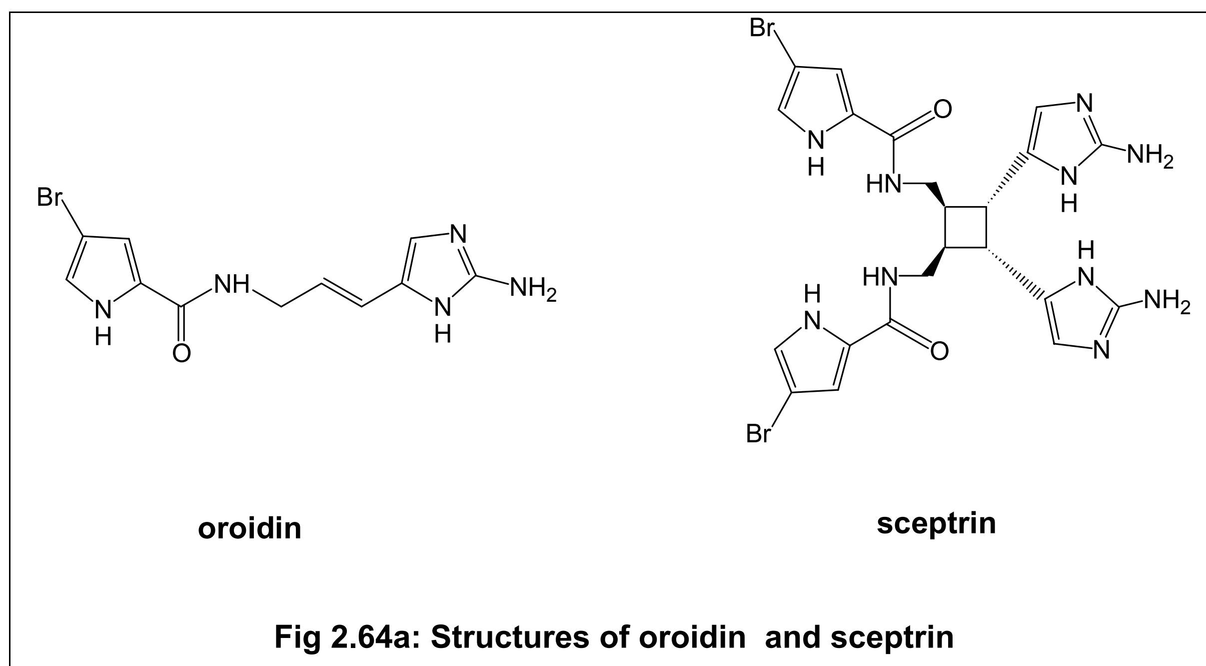


Fig 2.64a: Structures of oroidin and sceptrin

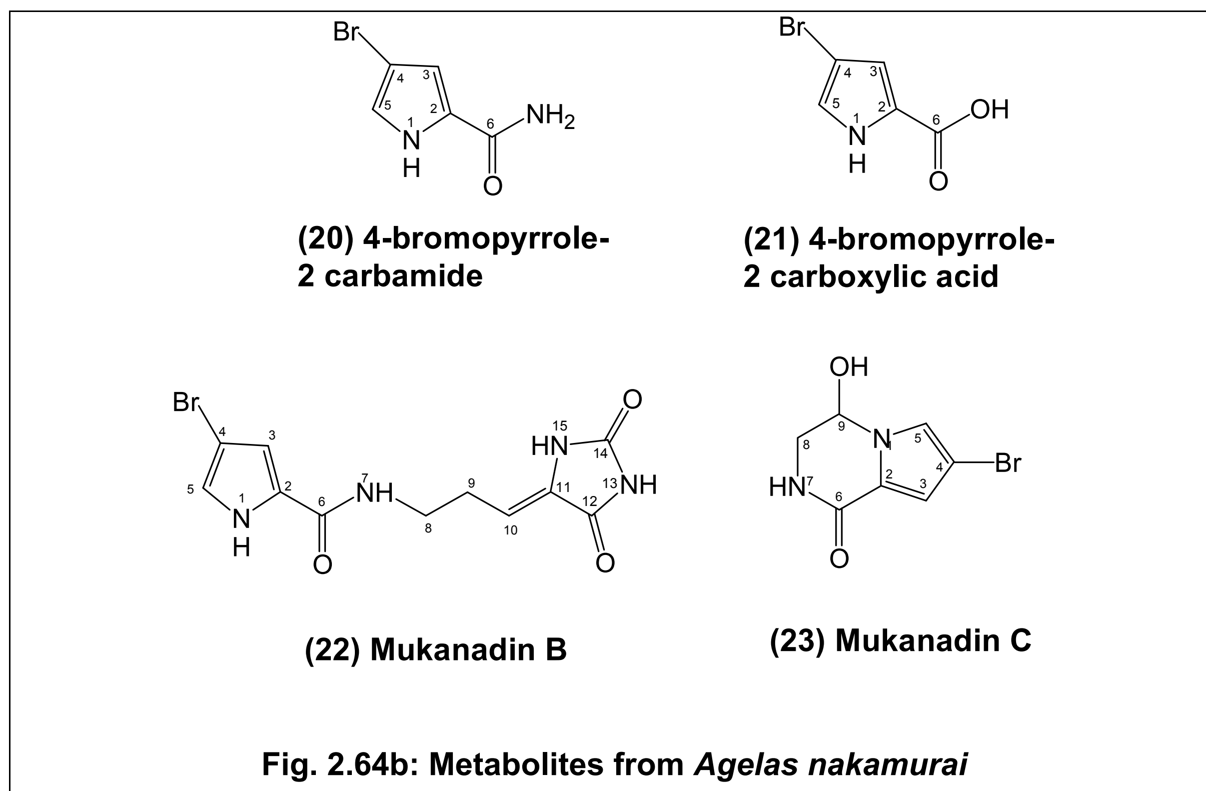


Fig. 2.64b: Metabolites from *Agelas nakamurai*

2.2.1.1. 4-bromopyrrole 2-carboxamide (20, known compound)

4-bromopyrrole 2-carboxamide has been previously isolated from the sponge *Acanthella carteri* Dendy, 1889 (*Acanthella aurantiaca* Keller, 1889) [Mancini *et al.,* 1997] and was reported to exhibit activity against NSCLC-N6 human non-small-cell-lung carcinoma, recording IC_{50} of 9.4 μg/ml [Ibid]. Its isomer, 5-bromopyrrole carboxamide, which showed antimicrobial activity against Gram-positive bacteria and fungi [Iwagawa *et al*., 1998] has been isolated from *Agelas nakamurai* [bid]. 4,5-Dibromopyrrole 2-carboxamide has been originally isolated from *Agelas oroides* [Forenza *et al*., 1971] and subsequently in *Agelas mauritiana* [Tsukamoto *et al*., 1996b] and showed antifouling activity against the ascidian *Ciona savignyi* at a concentration of 2.5 μg/mL [Ibid].

The structure of compound **20** (Fig. 2.64b) was established through EIMS (Fig. 2.65), ^{1}H (Fig. 2.66), and ^{13}C NMR data (Fig. 2.67) and by comparison with literature data [Mancini *et al.,* 1997]. NMR data are presented in Table 2.24b.

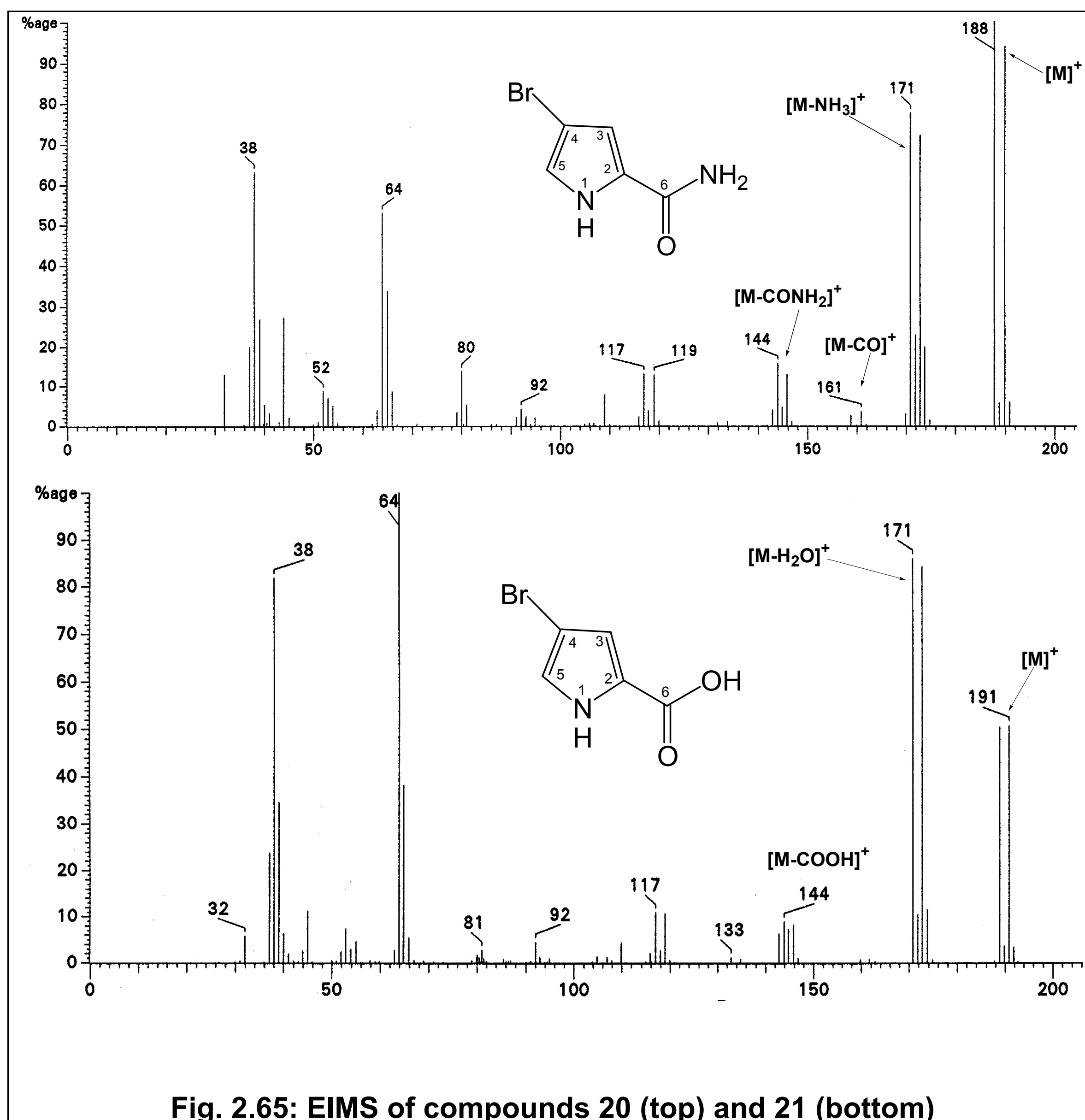


Fig. 2.65: EIMS of compounds 20 (top) and 21 (bottom)

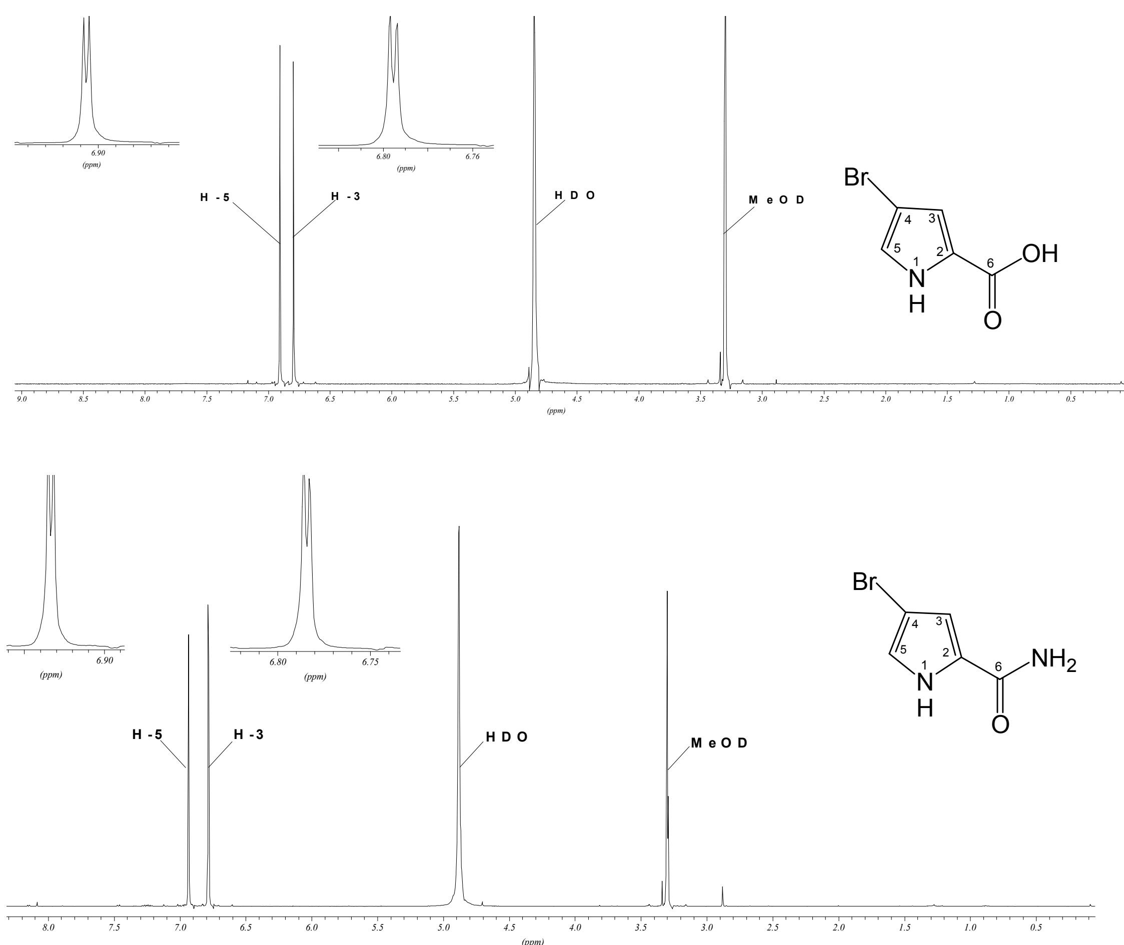


Fig. 2.66: ^{1}H NMR of compounds 21 (top) and 20 (bottom)

2.2.1.2. 4-bromopyrrole 2-carboxylic acid (21, new compound)

Although this alkaloid has been produced in the past through synthesis [Anderson *et al*., 1965; Fringuelle *et al*., 1969], this is the first time that it has been isolated from sponge. A related alkaloid, 4,5-dibromopyrrole carboxylic acid, has been originally isolated from *Agelas oroides* [Forenza *et al.,* 1971] and was subsequently found *in Agelas sventres*. It has been reported to exhibit antifeeding deterrent activity against *Thalassoma bifasciatum* with a minimum concentration of 0.8 mg/mL [Assman *et al.,* 2001].

Compound **21** was isolated as a white powder. It gave a molecular ion peak at *m/z* 189-191 in EIMS (Fig. 2.65). Its molecular formula was established by HREIMS to be

$C_5H_4{}^{79}BrNO_2$ (188.94232; expected 188.9425 calc for $C_5H_4{}^{79}BrNO_2$).The ^1H NMR spectrum was similar to that of the amide except for a broad signal at 11.5 ppm due to the carboxyl proton (spectra not shown). The ^{13}C NMR spectrum also showed that the pronounced difference was the upfield shift of the C-2 signal due to the greater shielding effect of the carboxylic group, whereas a downfield shift was observed for C-5 which might be due to the resonance-stabilized electron-withdrawing effect of the carboxylic substituent.

Table 2.24b: ^{13}C and ^1H NMR data of compounds 20 and 21 measured with DMSO-d_6 at 500 MHz

	^{13}C		^1H	
	20	**21**	**20**	**21**
1				11.5 (br s)
2	127.2	125.0		
3	123.1	124.2	6.84 (d, 1.6)	6.83 (d, 1.6)
4	97.5	97.8		
5	114.2	117.6	6.95 (d, 1.6)	6.97 (d, 1.6)
6	164.6	163.4		

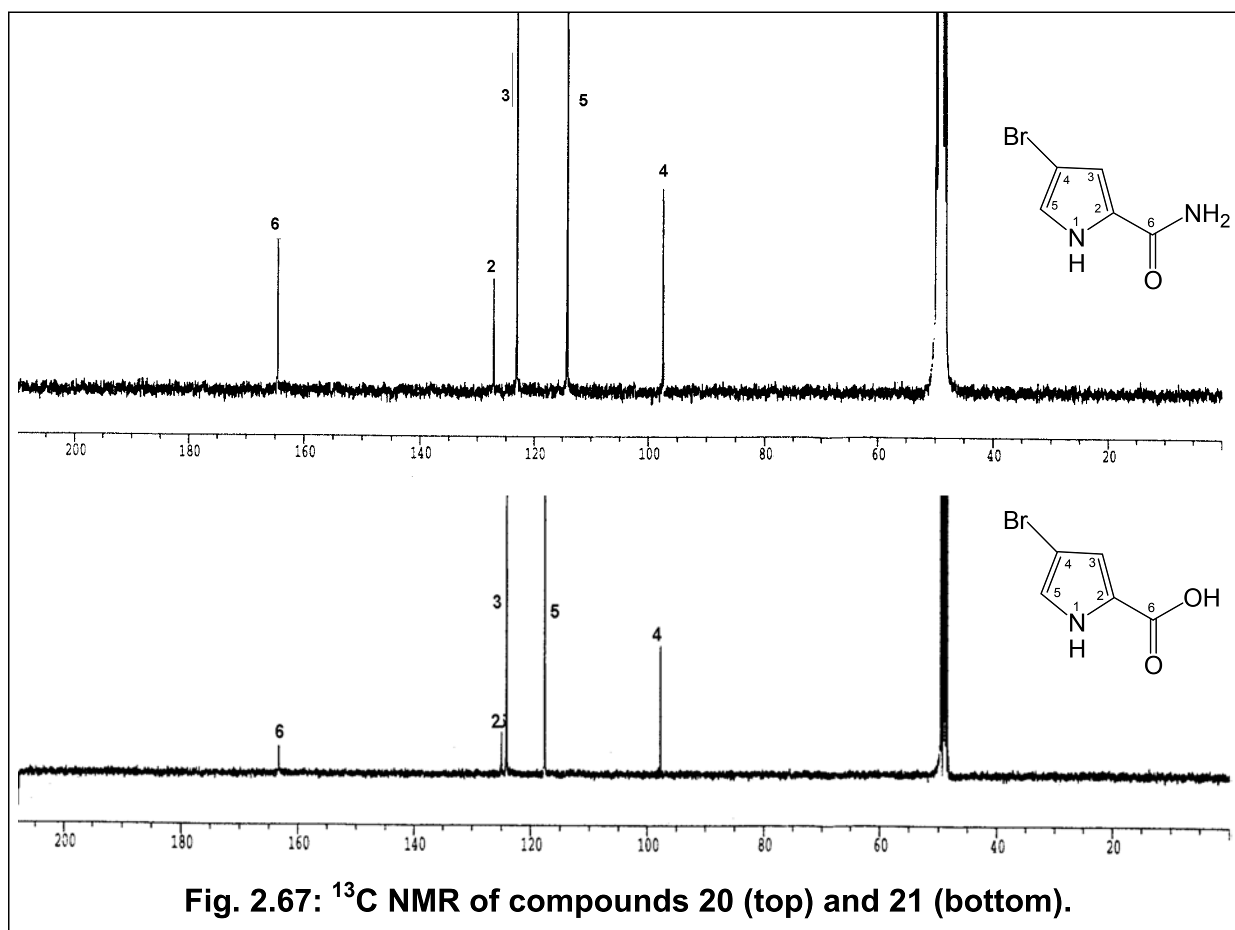


Fig. 2.67: ^{13}C NMR of compounds 20 (top) and 21 (bottom).

2.2.1.4. Mukanadin B (22, known compound)

The (+)-ESIMS (Fig. 2.68) showed pseudomolecular ion peaks at *m/z* 327 and 329 $[M+H]^+$ which is compatible with the molecular formula $C_{11}H_{12}BrN_4O_3$. Its ^1H and ^{13}C NMR spectra (Fig 6.69) are superimposable with that of mukanadin B which was also isolated from the same sponge species [Uemoto *et al.,* 1999]. NMR data are presented in Table 2.25.

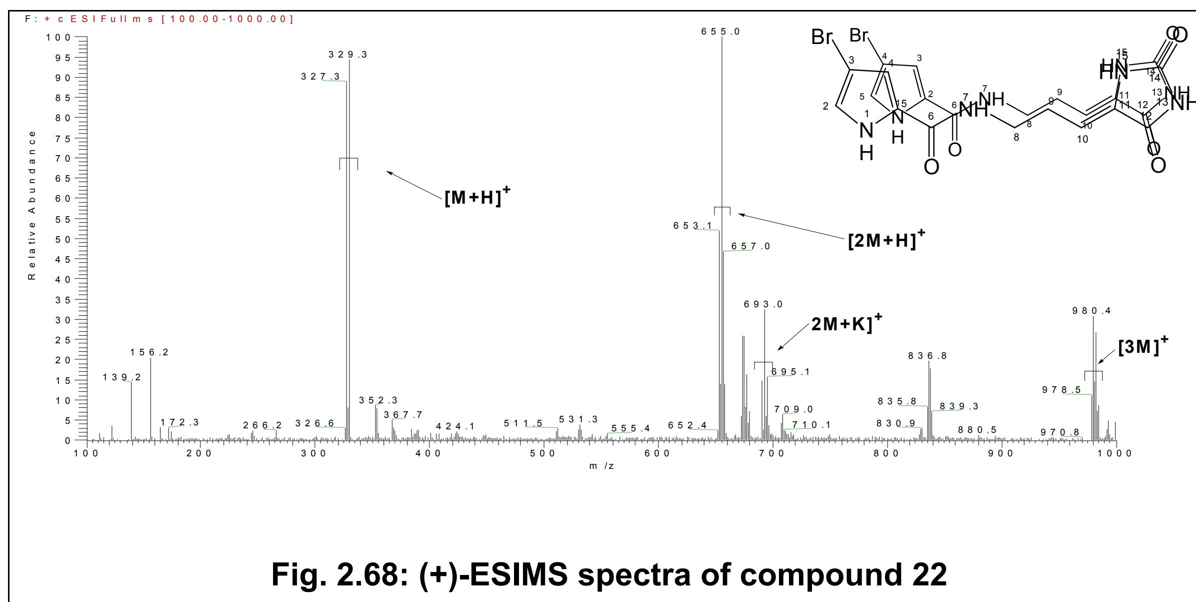


Fig. 2.68: (+)-ESIMS spectra of compound 22

Table 2.25: NMR data of compound 22

	^{13}C	H
1		11.80 (s)
2	126.8	
3	111.3	6.81 (dd, 2.52, 1.89)
4	94.8	
5	121.1	6.96 (dd, 2.8, 1.6)
6	159.6	
7		8.17 (t, 5.8)
8	37.6	3.23 (dt, 6.6, 5.8)
9	26.8	2.36 (dt, 7.6, 6.6)
10	108.8	5.53 (t, 7.6)
11	131.2	
12	164.2	
13		10.15 (s)
14	154.8	
15		10.95 (s)

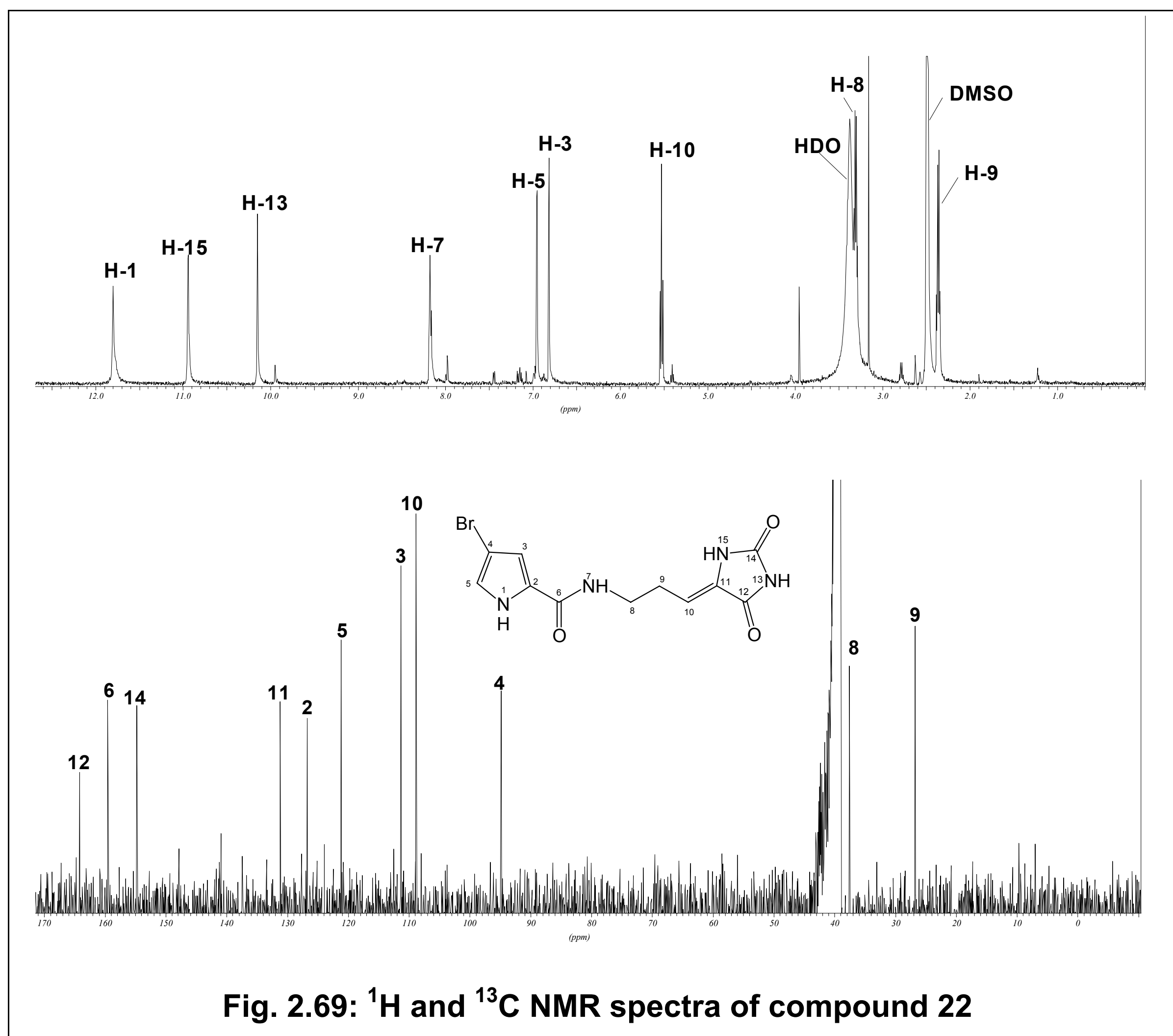


Fig. 2.69: ^{1}H and ^{13}C NMR spectra of compound 22

2.2.1.3. Mukanadin C (23, known compound)

Mukanadin C was previously isolated from the Okinawan sponge *Agelas nakamurai* [Uemoto *et al*., 1999] and from *Axinella carteri* [Cong-Jun *et al*., 1998]. It is the 2-debromo analogue of longamide which showed moderate antimicrobial activity [Cafieri *et al*., 1995].

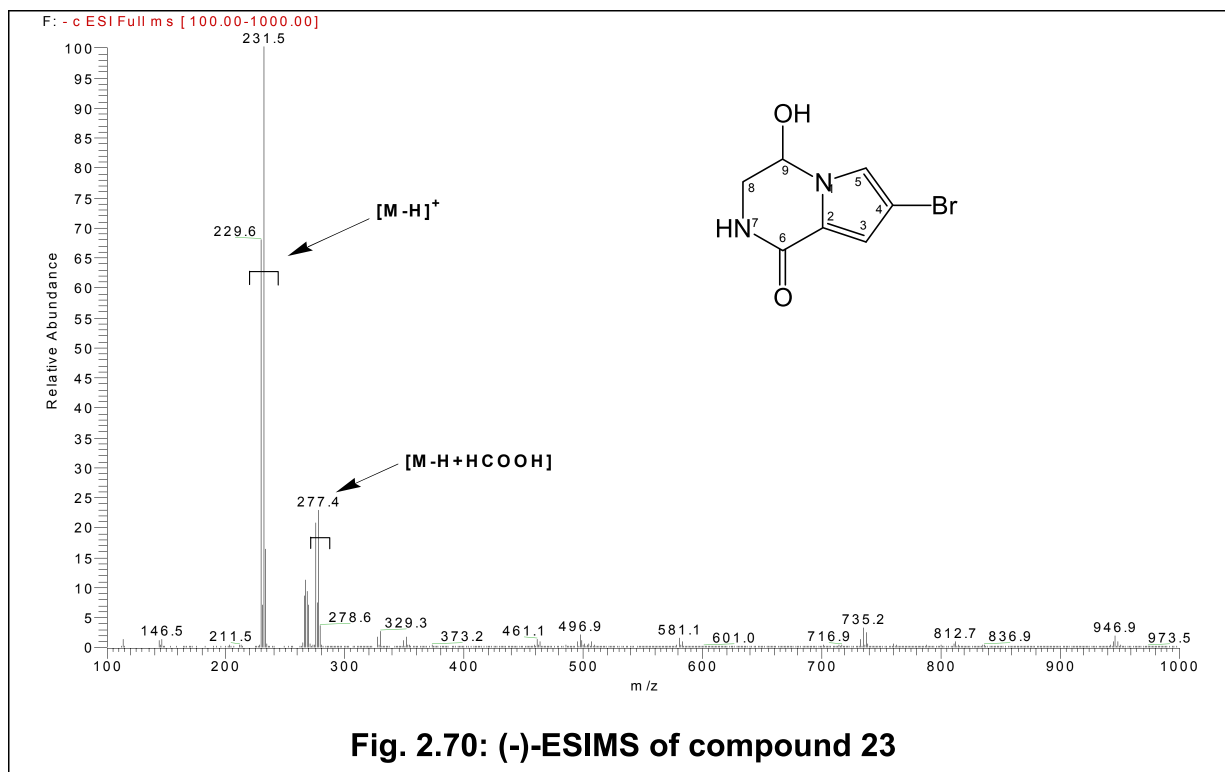


Fig. 2.70: (-)-ESIMS of compound 23

Structure Elucidation

Compound **23** was isolated as a white amorphous powder. It gave pseudomolecular ion peaks at m/z 230 and 231 $[M]^+$ in the EIMS. Likewise the (-)-ESIMS (Fig. 2.70) showed pseudomolecular ion peaks at m/z 229 and 231 $[M-H]^+$. Hence, the molecular formula was deduced to be $C_7H_7O_2N_2{}^{79}Br$. The ^1H and ^{13}C NMR data of **23** (Fig. 2.71; Table 2.26) agree with that of (±) mukanadin C [Uemoto *et al.*, 1999; Cong-Jun *et al.*, 1998].

The $[\alpha]_D$ which was measured at 24 ^{o} C gave a value of 0° (*c* 0.5, MeOH) which agreed with literature data (0°, *c* 1.0, MeOH) [Uemoto *et al.*, 1999] indicating that the isolate consists of a racemic mixture of two enantiomers differing in stereochemistry at C-9.

Table: 2.26: NMR data of compound 23 in DMSO-d_6 measured at 500 MHz

	^{13}C	^{1}H
1		6.75 (d, 5.68)
2	124.5	
3	113.1	6.63 (d, 1.90)
4	96.0	
5	121.6	7.16 (d, 1.90)
6	157.9	
7		7.73 (br s)
8A	46.0	3.59 (ddd, 13.2, 3.3, 1.7)
8B		3.30 (under HDO signal)
9	74.3	5.57 (br d, 3.79)

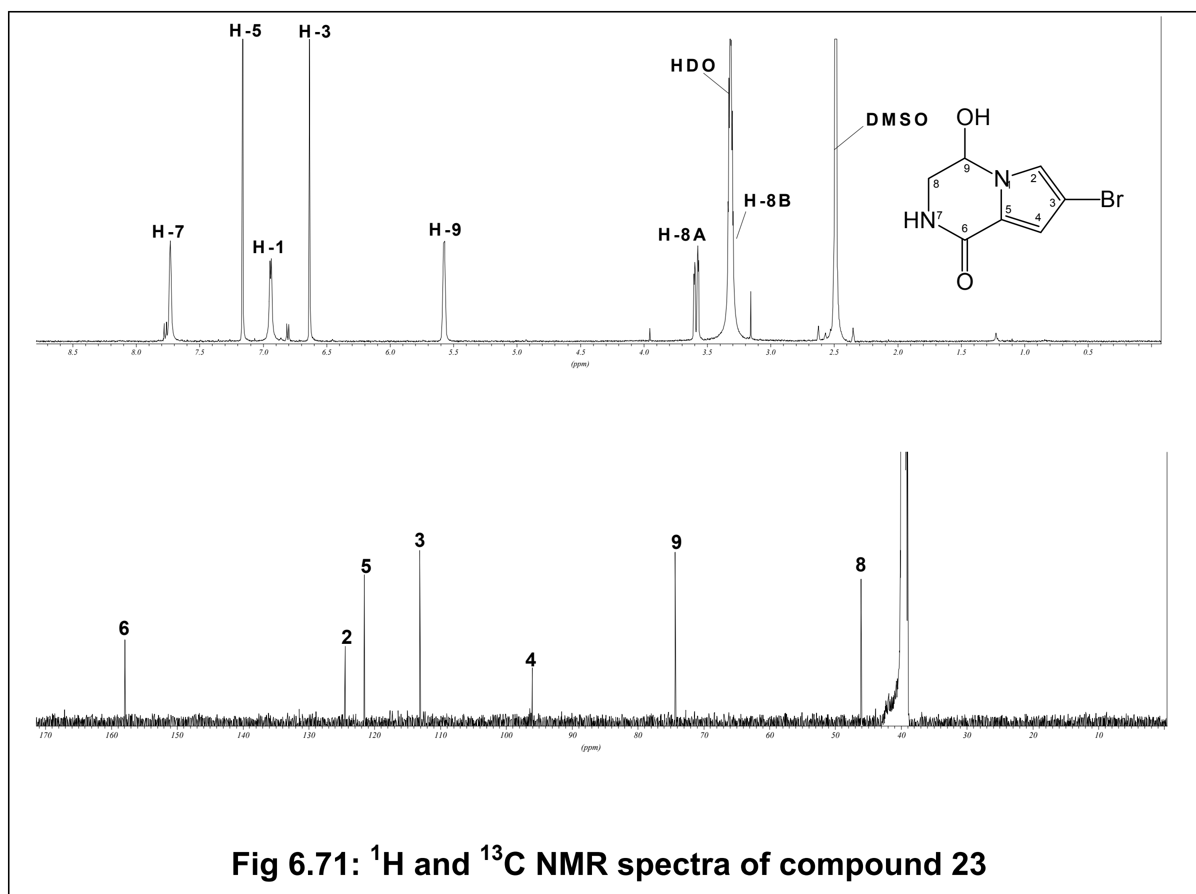


Fig 6.71: ^{1}H and ^{13}C NMR spectra of compound 23

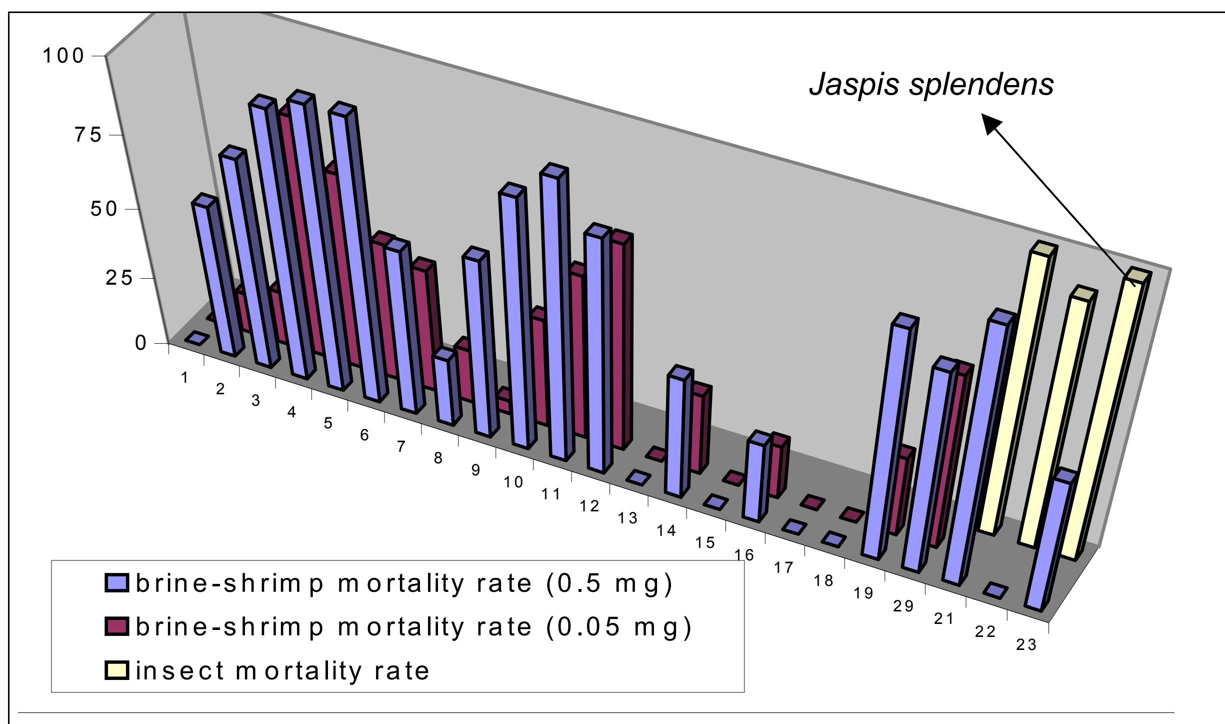
Bioactivity

Compound **20, 21** and **23** showed moderate antimicrobial activities while compound **22** was inactive (Table 2.27). Compounds **20-24** exhibited no activity in the insecticidal, brine-shrimp lethality and cytotoxicity tests.

Table 2.27: Agar plate diffusion assay of compounds 20, 21, and 23.

Concentration (μg/disk)	Zone of inhibition (mm)			
Compound **20**	*S.A*	*E.C.*	*B.S.*	*C.A*
5	n.a.	n.a.	n.a	12
10	8	12	10	12
50	10	10	12	15
Compound **21**				
5	n.a.	n.a.	10	15
10	8	10	10	15
50	10	12	10	15
Compound **23**				
5	n.a.	n.a.	10	10
10	n.a.	n.a.	11	12

2.2.2. Isolated compounds from *Jaspis splendens*



From 23 sponge extracts screened mainly for activity against brine-shrimp, the ethyl acetate extract of the sponge *Jaspis splendens* exhibited strong activities in the brine-shrimp lethality tests with mortality rates of 100% (0.5 mg extract) and 100% (0.05 mg extract) and 50% mortality rate in the insecticidal test (Fig. . Upon chemical investigation, the main component, jaspamide (jasplakinolide) (compound **24**) was isolated, together with trace amounts of its known derivatives, jaspamide B (compound **25**) and jaspamide C (compound **26**) (Fig. 2.72). All three compounds were observed to possess nearly identical UV spectra (Fig. 2.73). The HPLC chromatogram of the extract containing the three compounds also showed that jaspamide B and C were more polar than jaspamide (Fig. 2.73). Compounds **25** and **26** differ from **24** only in the polypropionate moeity, with the tripeptide portion of jaspamide retained unaltered in both derivatives.

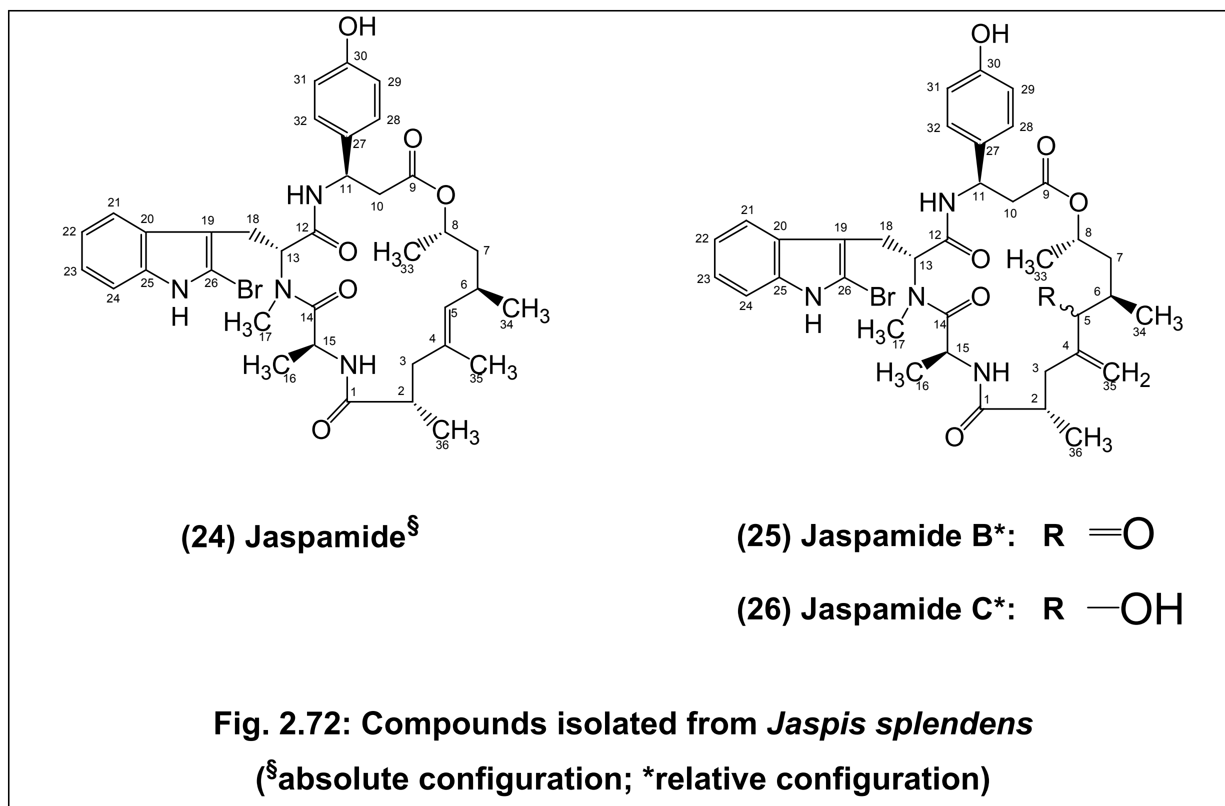
2.2.2.1. Jaspamide / Jasplakinolide (24, known compound)

This bioactive depsipeptide was first isolated by two independent groups from the sponge *Jaspis* cf. *Johnstoni* in 1986 [Zabriskie *et al*., 1986; Crews *et al*., 1986]. Since then, the presence of jaspamide in other sponge genera, including *Auletta* cf.

Constricta [Crews *et al*., 1994] and *Hemiasterella minor* [Talpir *et al*., 1994] has also been reported.

Jaspamide represents a new class of cyclic depsipeptides. It contains a propionate unit and two rare amino acids, β-tyrosine previously reported in the edeine peptides and 2-bromoabrine which is apparently a new amino acid [Zabriskie *et al*., 1986].

The data (Table 2.29) from the (+)-ESIMS (Fig. 2.74), ^{1}H (Fig. 2.75) and ^{13}C (Fig. 2.76) NMR spectra of **24** matched that of jaspamide [Zabriskie *et al*., 1986; Crews *et al*., 1986]. Its $[\alpha]_D$ value of + 65.8 (*c* 0.496, $CHCl_3$) matched that of jaspamide (+65, *c* 1.535, $CHCl_3$) [Zabriskie, *et al.,* 1986] which has an absolute configuration of 2*S*, 6*R*, 8*S*, 11*R*, 13*R*, 15*S* as determined hydrolysis, chemical derivatization and chiral HPLC of the alanine moeity and by X-ray crystal analysis of the acetate derivative of jaspamide.



(24) Jaspamide§

(25) Jaspamide B*: R =O

(26) Jaspamide C*: R —OH

Fig. 2.72: Compounds isolated from *Jaspis splendens*
(§absolute configuration; *relative configuration)

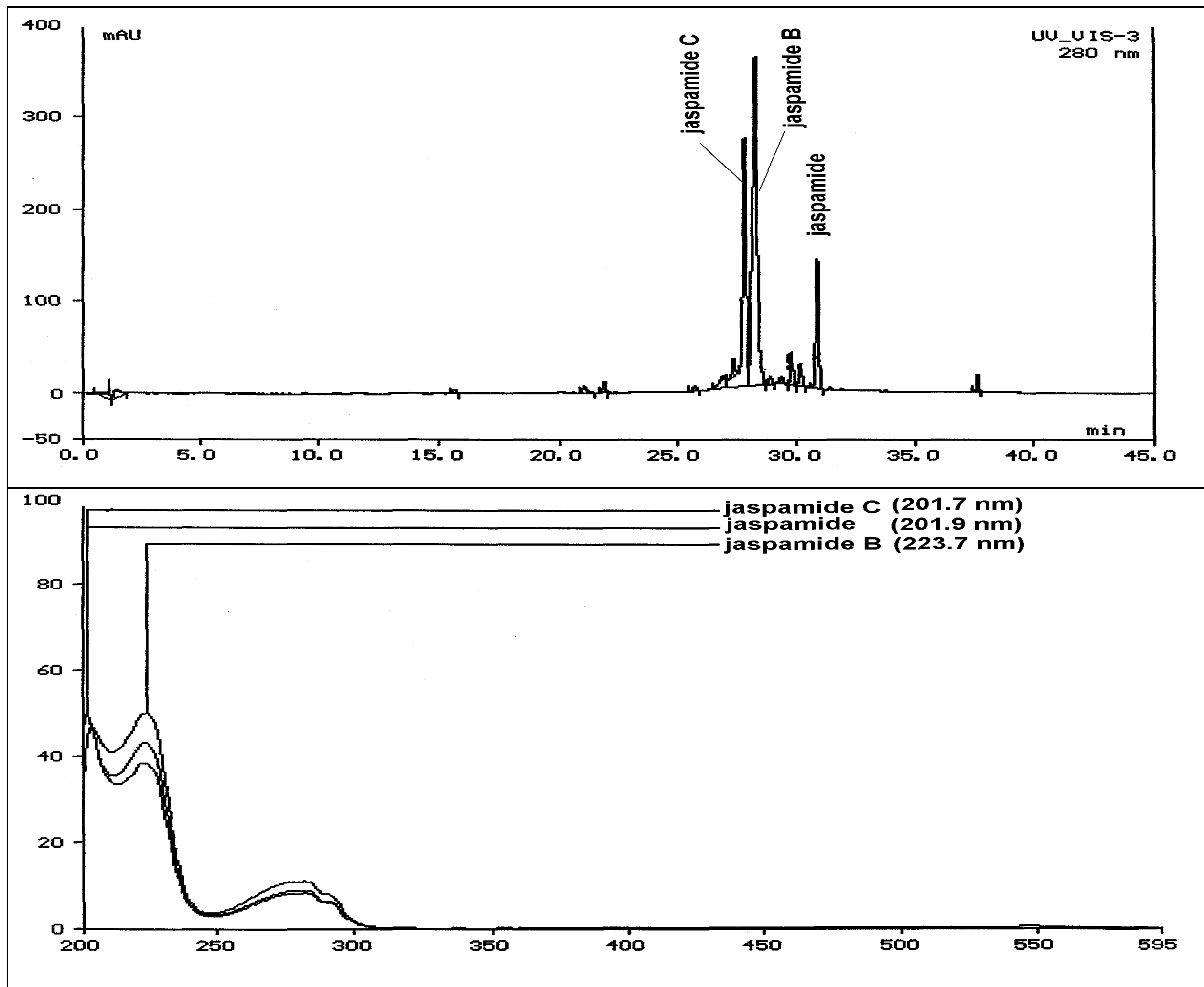


Fig. 2.73: HPLC chromatogram with corresponding UV spectra of compounds 24-26

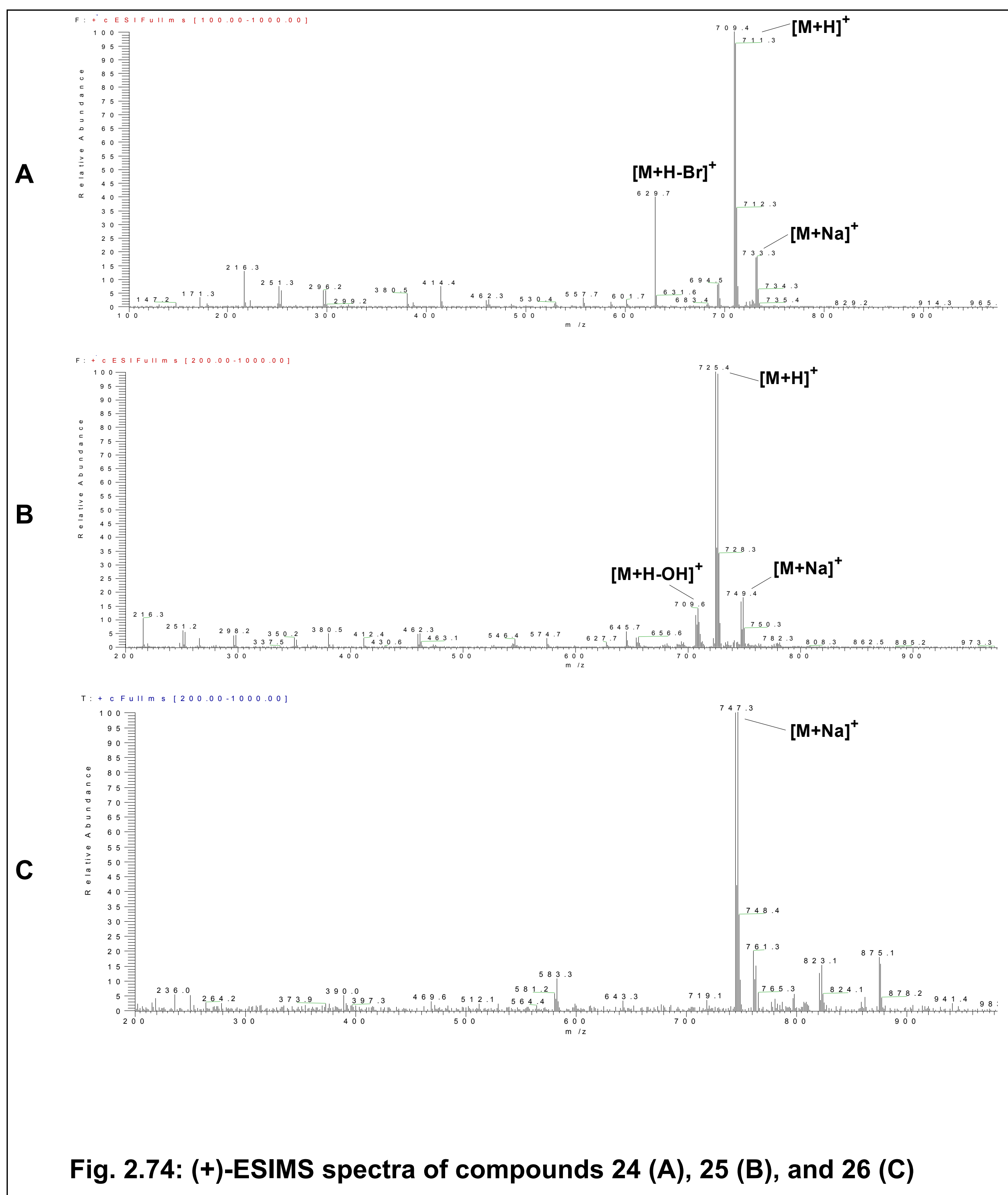


Fig. 2.74: (+)-ESIMS spectra of compounds 24 (A), 25 (B), and 26 (C)

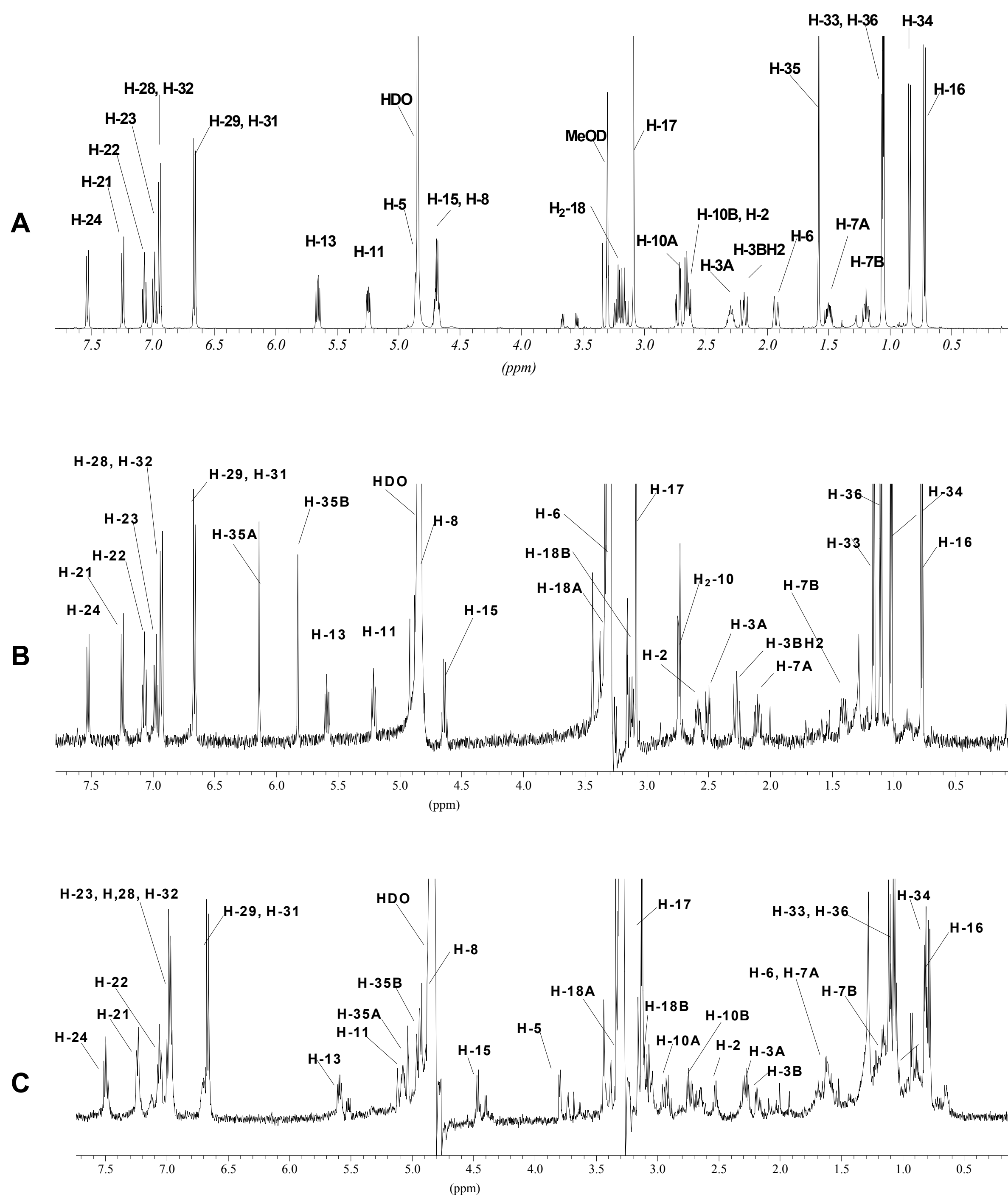


Fig. 2.75: ^{1}H NMR spectra of compounds 24 (A), 25 (B), and 26 (C)

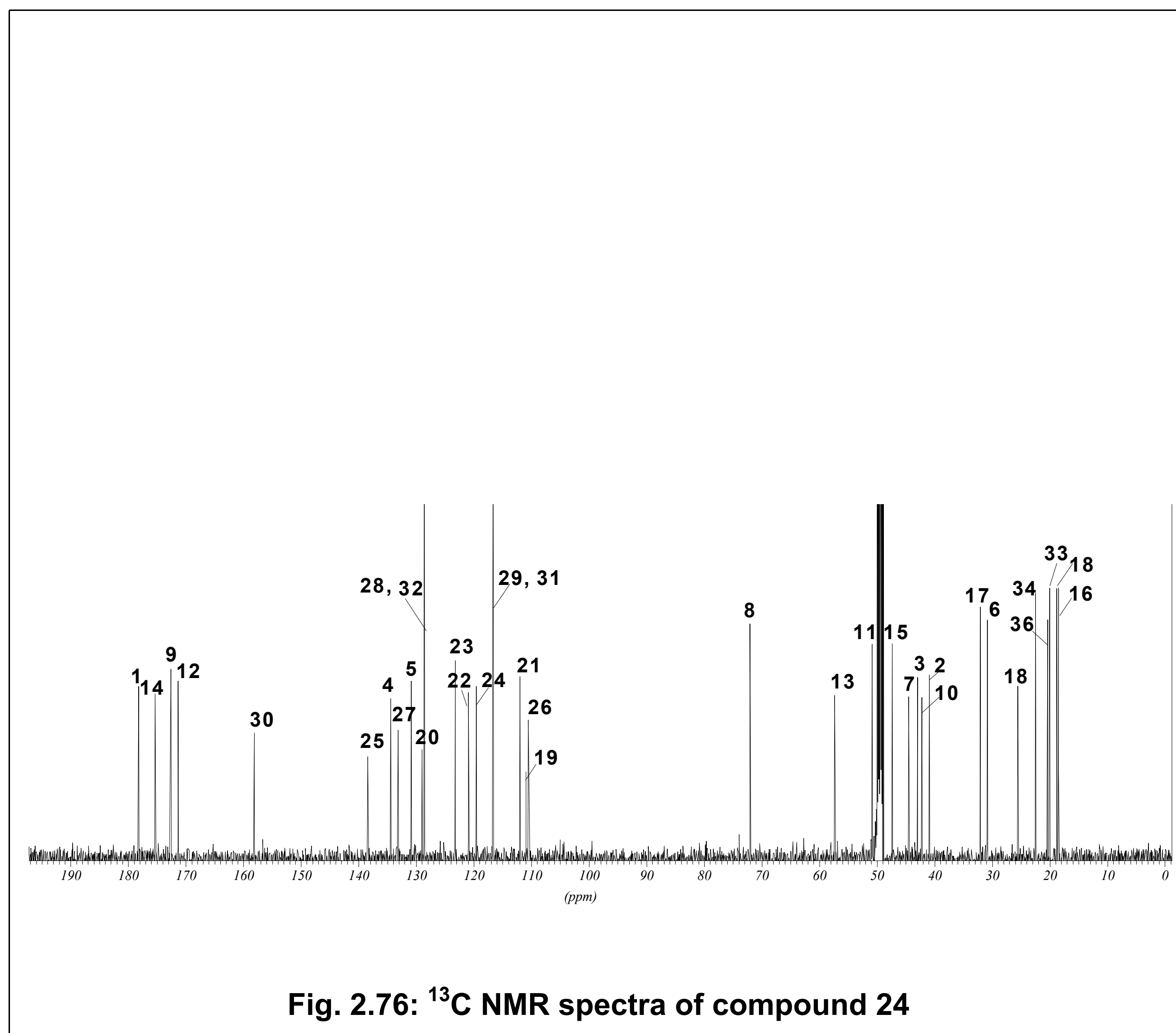


Fig. 2.76: ^{13}C NMR spectra of compound 24

2.2.2.2. Japamide B (25, known compound)

Jaspamide B and C have been isolated from the sponge *Jaspis splendas* collected in Vanuatu [Zampella *et al*., 1999]). Both compounds were reported to inhibit the in vitro growth of th NSCLC-N6 human tumor cel lines with EC_{50} values in the μg/mL range [Ibid].

Structure elucidation

Compound **25** was isolated as a white amorphous powder. It showed pseudomolecular ion peaks at *m/z* 745-747 $[M+Na]^+$ in (+)-ESIMS (Fig. 2.74), indicating a molecular weight which is 14 amu more than that of **24**. The ^{1}H and COSY (Fig. 2.77) spectra matched that of Jaspamide B as reported in the literature [Zampella *et al*., 1999]. When compared with that of **24**, the ^{1}H NMR spectrum (Fig.

2.75) of compound **25** showed the absence of the olefinic carbon-bound methyl H-35 which had appeared as a singlet at δ 1.58 in **24**. Instead, two olefinic protons which both appeared as singlets were observed at δ 5.83 and 6.14. Furthermore, the COSY spectrum of **25** (Fig. 2.77) also indicated that the signal corresponding to the olefinic proton H-5 in **24** was also missing. Thus, compared to **24**, compound **25** showed a shortened spin system encompassing the polyproprionate moeity: H-33 correlated with H-8, which correlated with H-7A and H-7B. These geminal protons further coupled with H-6, which in turn coupled with the methyl H-34.

Moreover, the resonances of H-3A, H-3B, H-6, H-7, and H-8 were also shifted to lower fields (Table 2.28). These features in the NMR data of the **25** are compatible with the structure of jaspamide B.

Stereochemistry

The absolute stereochemistry of jaspamide B in the literature was not determined whereas its relative stereochemistry was assumed to be similar to that of jaspamide due to the similarities in the ^{1}H and ^{13}C NMR chemical shifts observed for the tripeptide portion of jaspamide and jaspamide B [Zampella *et al.,* 1999]. Compound **25** showed similar $[\alpha]_D$ value (+20, *c* 0.05, $CHCl_3$) with that of jaspamide B (+11.4, *c* 0.0014, $CHCl_3$) indicating that the two are probably the same compound.

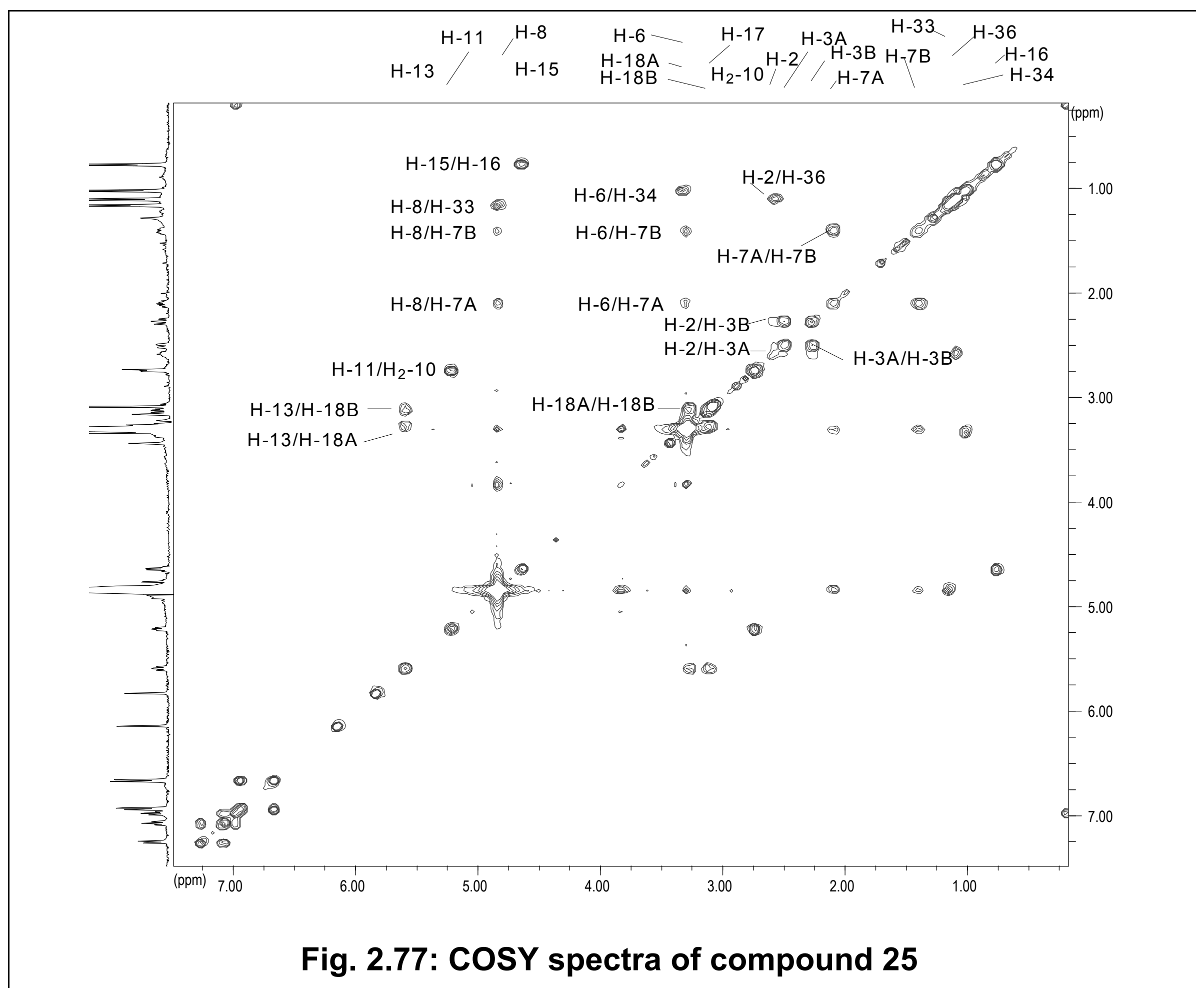


Fig. 2.77: COSY spectra of compound 25

2.2.2.3. Jaspamide C (26, known compound)

Compound **26** showed pseudomolecular ion peaks at 725 and 727 in (+)-ESIMS (Fig. 2.74), two mass units more than **25**. The presence of an ion peak at *m/z* 708 and 709 in the (+)-ESIMS spectra, which could be due to an $[M+H-OH]^+$ ion, was not apparent in the fragmentation patterns of **24** and **25**, suggesting the possible presence of a hydroxyl group in **26**. Its ^1H NMR spectrum (Fig. 2.75) gave proofs to this claim. The presence of two singlets at 5.09 and at 4.99 ppm suggested that the exomethylene protons (C-35) found in **25** were retained. Furthermore, a signal at δ 3.88 confirmed the presence of the expected methine H-5. The hydroxyl group at C-5 was confirmed by the ^{13}C spectra which showed an additional oxygen bound sp^3 carbon at 78.9 ppm (Table 2.30). The ^1H NMR data of compounds **24-26** are presented in Table 2.28. The COSY spectrum of **26** (Fig. 2.78) showed that all spin systems in **25** are retained. The HMBC spectrum showed correlations between the methyl protons H-34, as well as the exomethylene protons H-35A and H-35B, with this carbon. It was therefore assigned as C-5. Further key correlations found in the HMBC spectra are shown below (Fig. 2.79).

Stereochemistry

Like jaspamide B, the stereochemistry of jaspamide C in the literature was not established. Compound **26** exhibited an $[\alpha]_D$ value of +9.0168 (*c* 0.168, MeOH) which was related to that of jaspamide C [+25.4 (*c* 0.0013, $CHCl_3$)] [Zampella *et al.*, 1999] which indicate that the two may have the same stereochemistry.

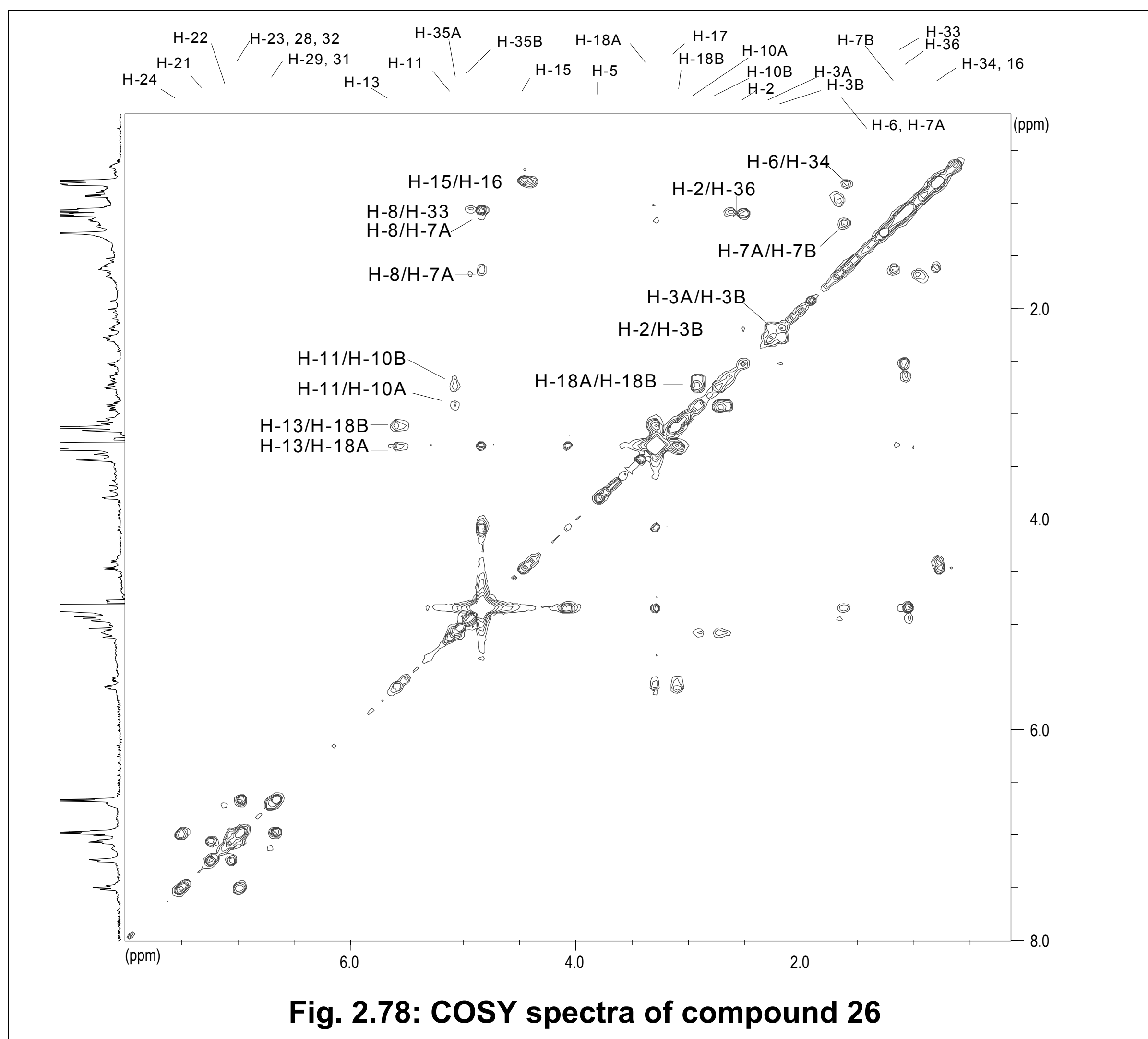


Fig. 2.78: COSY spectra of compound 26

Table 2.28: ^{1}H NMR data of jaspamide and derivatives.

	Jaspamide	Jaspamide B	Jaspamide C
1			
2	2.65 (m)	2.58 (m)	2.52 (q, 6.9)
3	2.30 (m)	2.51 (dd, 12.5, 3.3)	2.28 (dd, 15.3, 6.9)
	2.19 (dd, 15.2, 11.7)	2.27(dd, 12.5, 11.7)	2.18 (dd, 15.3, 6.9)
4			
5	4.85 (HDO peak)		3.86 (d, 5.3)
6	1.93 (m)	3.30 (MeOD peak)	1.62 (m)
7	1.50 (ddd, 13.3, 9.8, 6.0)	2.10 (ddd, 14.5, 8.5, 6.9)	1.62 (m)
	1.19 (ddd, 13.3, 8.2, 5.4)	1.41 (ddd, 14.5, 6.9, 5.1)	1.17 (m)
8	4.69 (m)	4.85 (HDO peak)	4.85 (HDO peak)
9			
10	2.73 (dd, 15.5, 4.0)	2.74 (d, 8.2)	2.94 (dd, 16.1, 7.9)
	2.65 (dd, 15.5, 8.5)	2.74 (d, 8.2)	2.74 (dd, 16.1, 6.3)
11	5.25 (dd, 8.5, 4.0)	5.21 (dd, 7.3, 5.9)	5.09 (dd, 12.0, 5.7)
12			
13	5.66 (dd, 9.8, 6.9)	5.59 (dd, 9.5, 6.9)	5.59 (dd, 10.0, 6.3)
14			
15	4.69 (q, 6.6)	4.64 (q, 6.6)	4.40 (q, 6.6)
16	0.72 (d, 6.6)	0.77 (d, 7.0)	0.79 (d, 6.9)
17	3.09 (s)	3.09 (s)	3.12 (s)
18	3.22 (dd, 14.8, 6.9)	3.30 (MeOD peak)	3.30 (MeOD peak)
	3.16 (dd, 14.8, 9.8)	3.11 (dd, 14.8, 9.5)	3.10 (m)
19			
20			
21	7.24 (d, 7.9)	7.25 (d, 7.9)	7.50 (d, 8.2)
22	7.07 (t, 7.9)	7.07 (t, 7.9)	7.06 (t, 8.2)
23	6.99 (ddd, 8.2, 7.9, 1.0)	6.97 (dd, 8.2, 7.9)	6.98 (m)
24	7.53 (d, 8.2)	7.52 (d, 8.2)	7.50 (d, 8.2)
25			
26			
27			
28	6.94 (br d, 8.5)	6.93 (d, 8.5)	6.98 (d, 8.5)
29	6.66 (d, 8.5)	6.66 (d, 8.5)	6.67 (d, 8.5)
30			
31	6.66 (d, 8.5)	6.66 (d, 8.5)	6.67 (d, 8.5)
32	6.94 (br d, 8.5)	6.93 (d, 8.5)	6.98 (d, 8.5)
33	1.06 (d, 6.3)	1.16 (d, 6.3)	1.06 (d, 7.9)
34	0.84 (d, 6.6)	1.02 (d, 6.7)	0.82 (d, 6.6)
35	1.58 (s)	6.14 (s)	5.04 (s)
		5.83 (s)	4.94 (s)
36	1.06 (d, 6.9)	1.10 (d, 7.0)	1.11 (d, 6.9)

Table. 2.29: NMR data of compound 24

	^{13}C	^{1}H	HMBC
1	177.8		
2	40.5	2.52 (q, 6.9)	
3	42.6	2.28 (dd, 15.3, 6.9)	C1, C2, C4, C5, C35
		2.18 (dd, 15.3, 6.9)	C4
4	134.0		
5	130.5	3.86 (d, 5.3)	C5 (direct)C3, C8, C35
6	30.5	1.62 (m)	
7	44.1	1.62 (m)	C5, C6, C8, C33, C34
		1.17 (m)	C5, C6, C8, C33, C34
8	71.6	4.85 (HDO peak)	C9
9	172.2		
10	41.9	2.94 (dd, 16.1, 7.9)	C10 (direct), C9, C11, C26
		2.74 (dd, 16.1, 6.3)	C10 (direct), C9, C11, C26
11	50.4	5.09 (dd, 12.0, 5.7)	C10, C26
12	170.9		
13	56.9	5.59 (dd, 10.0, 6.3)	C12, C14, C16, C18, C19
14	174.9		
15	46.9	4.40 (q, 6.6)	C1, C14, C17
16	18.1	0.79 (d, 6.9)	C16 (direct), C13, C14,
17	31.7	3.12 (s)	C17, C14, C15
18	25.1	3.30 (MeOD peak)	C12, C13, C21, C26
		3.10 (m)	C12, C13, C21, C26
19	110.5		
20	129.0		
21	111.6	7.50 (d, 8.2)	C19, C20, C23, C25
22	120.5	7.06 (t, 8.2)	C22 (direct), C20, C21
23	122.9	6.98 (m)	C23 (direct), C21, C25
24	119.2	7.50 (d, 8.2)	C24 (direct), C20, C22
25	138.0		
26	110.1		
27	132.7		
28	128.6	6.98 (d, 8.5)	C28 (direct), C11, C30, C32
29	116.2	6.67 (d, 8.5)	C29 (direct), C27, C30, C31
30	157.7		
31	116.2	6.67 (d, 8.5)	C31 (direct), C27, C29, C30, C
32	128.2	6.98 (d, 8.5)	C32 (direct), C11, C28, C30
33	19.6	1.06 (d, 7.9)	C7, C8
34	22.0	0.82 (d, 6.6)	C5, C6, C7
35	18.4	5.04 (s)	C4, C5
		4.94 (s)	
36	20.0	1.11 (d, 6.9)	C1, C2

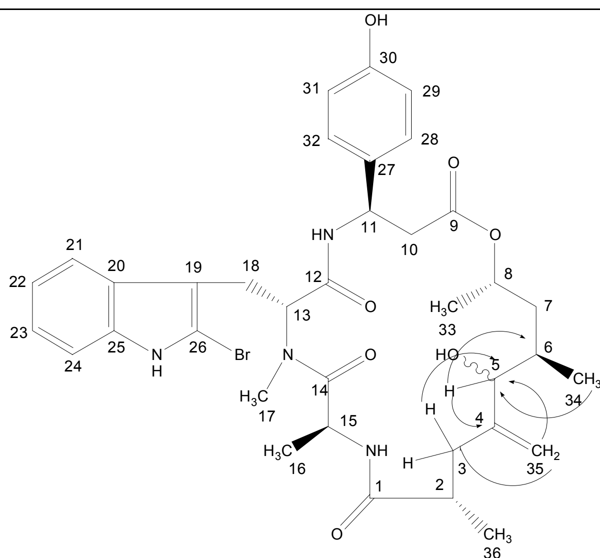


Fig. 2.79: Key HMBC correlation found for compound 26

Table 2.30: NMR data of compound 26

	^{13}C	^{1}H	HMBC
1	178.9		
2	40.5	2.52 (dd, 14.1, 6.9)	C1, C36
3	37.4	2.28 (dd, 14.5, 6.8)	C1, C2, C4, C35, C36
		2.18 (dd, 15.3, 6.8)	C1, C2, C4, C5, C35
4	150.0		
5	79.0	3.86 (d, 5.33)	C5 (direct), C4, C6, C35
6	33.5	1.62 (m)	
7	42.5	1.62 (m)	
		1.17 (m)	
8	71.5	4.85 (HDO peak)	
9	172.3		
10	41.5	2.94 (dd, 7.9, 16.1)	C9, C11
		2.74 (dd, 16.2, 6.3)	C9, C11, C27
11	51.1	5.0 (m)	C9, C10, C27, C28, C32
12	171.4		
13	57.7	5.65 (dd, 10.0, 6.3)	C13 (direct), C12, C17
14	176.5		
15	47.0	4.40 (dd, 13.7, 7.0)	C15 (direct), C14
16	16.6	0.79 (d, 6.9)	C16 (direct), C13, C14, C15
17	32.7	3.12 (s)	C17(direct), C13, C14, C15
18	24.7	3.30 (MeOD peak)	C12, C13, C20, C26
		3.10 (m)	C12, C13, C20, C26
19	-		
20	128.7		
21	119.1	7.50 (d, 8.2)	C21 (direct), C23, C25,
22	121.1	6.96 (t, 8.0)	C22 (direct), C20, C24
23	122.9	7.06 (t, 8.0)	C23 (direct), C21, C25
24	111.7	7.24 (dd, 8.1)	C24 (direct), C20, C22
25	138.1		
26	110.6		
27	133.0		
28	128.7	6.98 (d, 8.5)	C28 (direct), C11, C30, C32
29	116.3	6.67 (d, 8.5)	C29 (direct), C27,C31, C30
30	156.8		
31	116.3	6.67 (d, 8.5)	C31 (direct), C27, C29, C30
32	128.7	6.98 (d, 8.5)	C32 (direct), C11, C28, C29
33	21.3	1.06 (d, 7.9)	C33 (direct), C8, C7
34	14.7	0.82 (d, 6.6)	C34 (direct), C5, C6, C7
35	113.3	5.05 (s)	C35 (direct), C3, C5
		4.95 (s)	C35 (direct), C3, C5
36	18.5	1.11 (d, 6.9)	C36 (direct), C1, C2, C3

Bioactivity

Jaspamide has been reported to possess remarkable biological activities such as antifungal and antiproliferative activities [Zabriskie *et al*., 1986]. In particular, it showed anthelminthic, cytotoxic, selective antimicrobial, insecticidal, and ichthytotoxic activities [Zabriskie *et al*., 1986; Crews *et al*., 1986; Braekman *et al*., 1987].

Jaspamide B and C were also reported to exhibit cytotoxicity against the human NSCLC-N6 cancer cell line with IC_{50} values of 3.3 and 1.1 μg/mL, respectively [Zampella *et al*., 1999].

Compound **24** exhibited activities in all biotests. In the brine-shrimp lethality test, it gave a 100% mortality rate at 100 ppm. When tested against *S.littoralis*, jaspamide showed strong insecticidal activity causing 100% mortality rate at 50 ppm. When tested for antimicrobial activity, jaspamide showed strong activity against *Candida albicans* but exhibited no activity against gram-positive and gram-negative test organisms (Table 2.31). Compound **24** also showed activities against human leukemia cell lines (Table 2.32).

Table 2.31: Agar plate diffusion assay of compound 24

Concentration (μg/disk)	Zone of Inhibition (mm) *S. aureus*	*E. coli*	*B. subtilis*	*C. albicans*
1	n.a.	n.a.	n.a.	7
5	n.a.	n.a.	n.a.	20
10	n.a.	n.a.	n.a.	20
50	n.a.	n.a.	n.a.	28

Table 2.32: Cytotoxicity test for compound 24

Concentration (μg/mL)	Growth rates (%) of different cell lines	
	JURKAT	THP-1
0.1	56	56
1	22	11
2.5	-	8
5	-	5
10	10	2
20	4	-
25	-	2

3. Materials and Methods

3.1. Biological materials

Samples include sponges and sponge-associated fungi collected in Indonesia, the Philippines and Italy. The sponges were collected by scuba diving and voucher specimen were identified by Dr. Rob W. M. van Soest of the Zoological Museum, Amsterdam. For the isolation of fungi, the scheme illustrated in Fig.3.1 was used, a piece of the inner part of the sponge was cut under sterile conditions, and inoculated on agar slants and incubated at 27°C. A pure culture of the fungi was prepared through repeated inoculation on fresh malt agar plates. The fungi were then grown in 300 mL cultures and after a period of incubation, both the mycelium and broth were extracted. The extracts were screened for biological activity and those exhibiting significant bioactivity were mass cultivated under the same growth conditions. A sample culture of the fungi were sent to the Centraalbureau voor Schimmelcultures (CBS), Baarn, Netherlands or to the Alfred-Wegener-Institut für Polar- und Meeresforschung for identification.

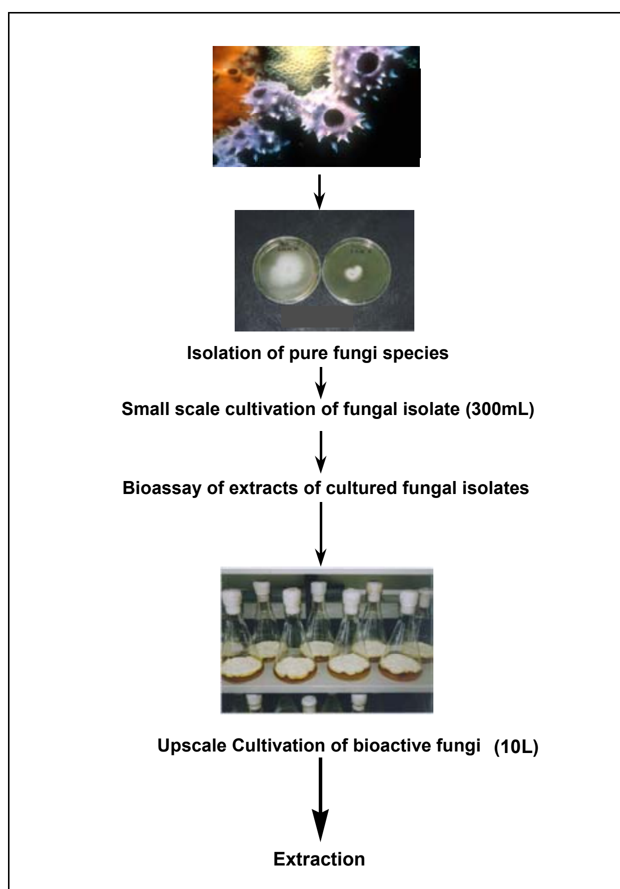


Fig. 3.1: Fungal collection, isolation, and cultivation scheme

3.1.1. Sponge-derived fungi

The isolated fungi were grown on agar plates using the GPNS medium developed by Schaumann. For eliminating bacterial contaminants Chloramphenicol – 0.2 g/l; Streptomycine Sulfate – 0.1 g/l; Penicillin G- 0.1 g/l were added.

Glucose-Monohydrate	1.0 g
Peptone (aus Casein/merck)	0.5 g
Yeast Extract (Merck)	1.0 g
Ammonium nitrate	1.0 g
Agar (Merck)	16.0 g
Sea water (ca. 30% sea salt)	1000 ml
pH (NaOH/HCl)	7.2-7.4

After purification, the fungi is grown on 300 mL medium in a 1 L flask and grown for approximately one month. The fungi is then screened for bioactivity (brine-shrimp, insecticidal, antimicrobial, cytotoxic). After the optimum growth time, the culture is added with Ethylacetate and allowed to stand overnight. The broth and mycelium are then extracted exhaustively with ethyl acetate.

Bioactive fungi are then re-grown in 10 L medium under the same culture condition as the bioscreening culture.

3.1.1.1. *Cladosporium herbarum*

Samples of the sponge *Callyspongia aerizusa* were collected near Bali Barat National Park in Indonesia. The fungus was grown on malt agar slants consisting of 15 g/l agar, and 24.4 g/l artificial sea-salt mixture. The isolated fungus was identified as *Cladosporium herbarum* by the Centraalbureau voor Schimmelcultures (CBS), Baarn, Netherlands. Mass cultivation of the fungus was carried out in erlenmeyer flasks in malt extract broth consisting of 1.5% malt extract in distilled water supplemented with 2.44% of SERA artificial sea salt mixture at pH 7.6 – 7.8. After 41 days incubation at room temperature without shaking, the fungal biomass was

separated from the culture broth, and both the mycelium and the broth were extracted with ethyl acetate.

3.1.1.2. *Curvularia lunata*

Samples of the sponge *Niphates olemda* (De Laubenfels, 1954) were collected by scuba diving in Bali Barat National Park in Indonesia. A voucher fragment is kept in 70% methanol under the registration number ZMA POR. 13438 in the Zoological Museum, Amsterdam. The isolated fungus was identified as *curvularia lunata* by the Centraalbureau voor Schimmelcultures (CBS), Baarn, Netherlands. Mass cultivation of the fungus was carried out in Erlenmeyer flasks in malt extract broth consisting of 1.5% malt extract in distilled water supplemented with 2.44% of SERA artificial sea salt mixture at pH 7.6 – 7.8. After 32 days of incubation at room temperature without shaking, the fungal biomass was separated from the culture broth, and both the mycelium and the broth were extracted with ethyl acetate.

3.1.1.4. *Pennicillium spp.* and an unidentified fungus

From the sponge *Axinella verrucosa* collected in Secca di Corallo, Elba, Italy was isolated twenty-two fungi species which have been grown in 1L-Erlenmeyer flasks containing 300 mL culture broths of the Wickerham medium which consists of yeast extract (3 g/L), maltextract (3 g/L), peptone (5 g/L), glucose (10 g/L), sea salt (24.4 g/L).The pH of the medium was adjusted to 7.2 – 7.4 using NaOH and HCl. After 21 days at room temperature, the culture broths were added with 300 mL EtOAc, transferred in polyethylene and cooled under –86° C until they are transported per courier under dry ice to the University of Düsseldorf. The ethyl acetate extracts of the fungi were subjected to preliminary bioactivity screening.

3.1.1.5. *Aspergillus flavus*

Samples of the demosponge *Hyrtios aff. reticulatus* (Thiele, 1898) were collected near the island of Cabilao, Loon, Bohol in the central part of the Philippines. A voucher sample of the sponge is kept at the Zoological Museum, Amsterdam, with the registration number ZMA POR.16869. The fungus isolated from this sponge was

cultivated in Wickerham medium as described for *Penicillium spp.* After 30 days at room temperature, the culture broths were added with 300 mL EtOAc and left overnight at room temperature. The ethyl acetate extracts of the fungi were subjected to preliminary bioactivity screening.

3.1.2. Marine sponges

3.1.2.1. *Agelas nakamurai*

The sponge (Fig. 3.2) was collected from a depth of 44 ft. near Pulau Kapoposang in Indonesia. It forms an irregular thick-walled flabellate mass with an irregular surface. The skeleton is a fine-meshed reticulation of sponge fibers of which the primary ones are cored with one to four spicules in cross section, and the connecting fivers are uncored. All fibers are lightly echinated. The spicules are the usual verticillated acanthostyles 180-285 x 12-20 μm with 17-26 whorls of spines. A voucher specimen has been deposited in the Zoological Museum Amsterdam, reg. no. ZMA POR. 16735.

Fig.3.2: *Agelas nakamurai*

3.1.2.2. *Jaspis splendens*

The sponge was collected in 1996 in Ujung Pandang, Indonesia. It was identified by Dr. Van Soest of the Zoological Museum Amsterdam.

3.2. Chemicals used

3.2.1. General laboratory chemicals

Anisaldehyde (4-methoxybenzaldehyde)	Merck
L-(+)-Ascorbic acid	Merck
Glacial acetic acid	Merck
Formaldehyde	Merck
Gentamycin sulfate	Merck
Hydrochloric acid	Merck
Nipagin A (*p*-hydroxybenzoic acid)	Sigma
Sodium hydroxide	Merck
Concentrated sulfuric acid	Merck
Trifluroacetic acid (TFA)	Merck

3.2.2. Culture nutrient media

Agar-agar	Merck
Ammonium nitrate	Merck
Artificial sea salt	SERA
Chloramphenicol	Merck
Glucose-monohydrate	Merck
Yeast Extract	Merck
Penicillin G	Merck
Peptone (aus casein)	Merck
Streptomycine Sulfate	Merck

3.2.3. Solvents

Acetone
Acetonitrile
Chloroform
Dichloromethane
Diethylether
Dimethylsulfoxide
Ethanol
Ethyl Acetate
Hexane
Methanol

Solvents were purchased from the Institute of Chemistry, University of Wuerzburg. They were distilled prior to use and spectral grade solvents were used for spectroscopic measurements.

3.2.4. Chromatography:

Item	Supplier
Precoated TLC plates (AluO, Silica Gel 60 F254, layer thickness 0.2 mm	Merck
Pre-coatred TLC plates (Glass), RP-18, F254 S, layer thickness 0.25 mm	Merck
Silica Gel 60, 0.04-0.063 mm mesh size	Merck
Sephadex LH 20, 25-100 mm mesh size	Merck
Pre-packed LOBAR Columns	Sigma
Silica Gel 60, 40-63 m, 25 mm i.d. x 310 mm	
RP C18, 40-63 m, 25 mm i.d. x 310	
HPLC Solvents:	
Methanol LiChroSolv HPLC	Merck
ortho-Phosphoric Acid 0.15%, pH 2.0 (prepared from *ortho*-Phosphoric Acid 85% p.a.)	Merck
Nanopure Water	Barnstead

3.3 Equipments used

Balances	Mettler AT 200
	Mettler AT 250
	Mettler PE 1600
	Sartorius RC210P
Centrifuge	TGA-Ultracentrifuge, Rotor 65.13; Konton
Fraction collector	ISCO Cygnet
Freeze Dryer	a) Christ Alpha 2-4 with Equipment Control; LDC-IM and Vacuum Pump DVO 004B
	b) LYOVAC GT2
	Pump TRIVAC D10E
Hot plates	Camag
Syringe	Hamilton 1701 RSN
Mill	Moulinex 354
Magnetic Stirrer	Variomag Multipoint HP
Mixer	Braun
PH-Electrode	WTW pH 350
Rotary Evaporator	Buchi Rotavap RE111;
	Buchi Rotavap R-200
Drying Ovens	Heraeus T 5050
Sonicator	Bandelin Sonorex RK 102
UV Lamp	Camag (254 and 366 m,)
UV/Vis Spectrophotometer	Perkin-Elmer Lambda 2
Vibrofix Shaker	Janke & Kunkel, VF 1
Vacuum Filtration	Supelco
Vacuum Exsicator	Savant SpeedVac SPD111V
	Savant Refrigerator Vapor Trap RVT400
	Pump Savant VLP80

HPLC equipment:

I. Gynkotek

Pump:	Gynkotek, M40
HPLC Program:	Gynkosoft (v.5.4)
Detector:	Gynkotek, Photodiode Array Detector UVD 340
Autosampler:	Gynkotek Autosampler GINA 50
Printer:	NEC P60

II. Dionex

Pump:	Dionex P580A LPG
HPLC Program:	Chromeleon Ver 6.3
Detector:	Dionex, Photodiode Array Detector UVD 340S
Column thermostat:	STH 585
Autosampler:	ASI-100T

3.4. Chromatographic methods

3.4.1. Thin layer chromatography

TLC were performed on precoated TLC plates with Si gel 60 F_{254} (layer thickness 0.2 mm, E. Merck. Darmstadt, Germany) with CH_2Cl_2:MeOH (90:10 or 95:5) or CH_2Cl_2:*iso*-C_3H_7OH (75:25) for semi-polar compounds. TLC on Diol plates were also used for other semi-polar compound using EtOAc:n-Hexane (70:30) or EtOAc:Acetone (9:1). TLC on reversed phase (RP)-C18 F_{254} (layer thickness 0.25 mm, E. Merck, Darmstadt, Germany) was sometimes used for polar substances and using the solvent system MeOH:H_2O (85:15, 80:20, 70:30 or 60:40).

The compounds were detected from their UV absorbance at 254 and 366 nm or by spraying the TLC plates with anisaldehyde reagent follwed by heating at 110°C.
Anisaldehyde/H_2SO_4 Spray Reagent (DAB 10)

Anisaldehyde:	5 parts
Glacial Acetic acid:	100 parts
Methanol:	85 parts

Were mixed, to which 5 parts of concentrated H_2SO_4 were added slowly. The reagent was stored in an amber-colored bottle and kept refrigerated until use.

TLC was used to monitor the identity of each of the fractions and the qualitative purity of the isolated compound. It was also utilized to optimize the solvent system that would be applied for column chromatography.

3.4.2. Column chromatography

Crude extracts were subjected to repeated separation through column chromatography using appropriate stationary phase and solvent system previously determined by TLC. Further purification of fractions were later performed on prepacked LOBAR columns.

The following separation systems were used:

a) Stationary Phase: Sephadex LH-20 (25-100 m diameter)
   Solvent system: MeOH or acetone
b) Stationary Phase: RP-18 (LOBAR, size A and B)
   Solvent system: MeOH : Nanopure water
c) Stationary Phase: Diol (LOBAR, size A and B)
   Solvent system: EtOAc : Hexane or EtOAc : Acetone
d) Stationary Phase: Silica gel (LOBAR, size A and B)
   Solvent system: CH2Cl2 : MeOH or CH2Cl2 : *iso*-C_3H_7OH

3.4.3. Semipreparative HPLC

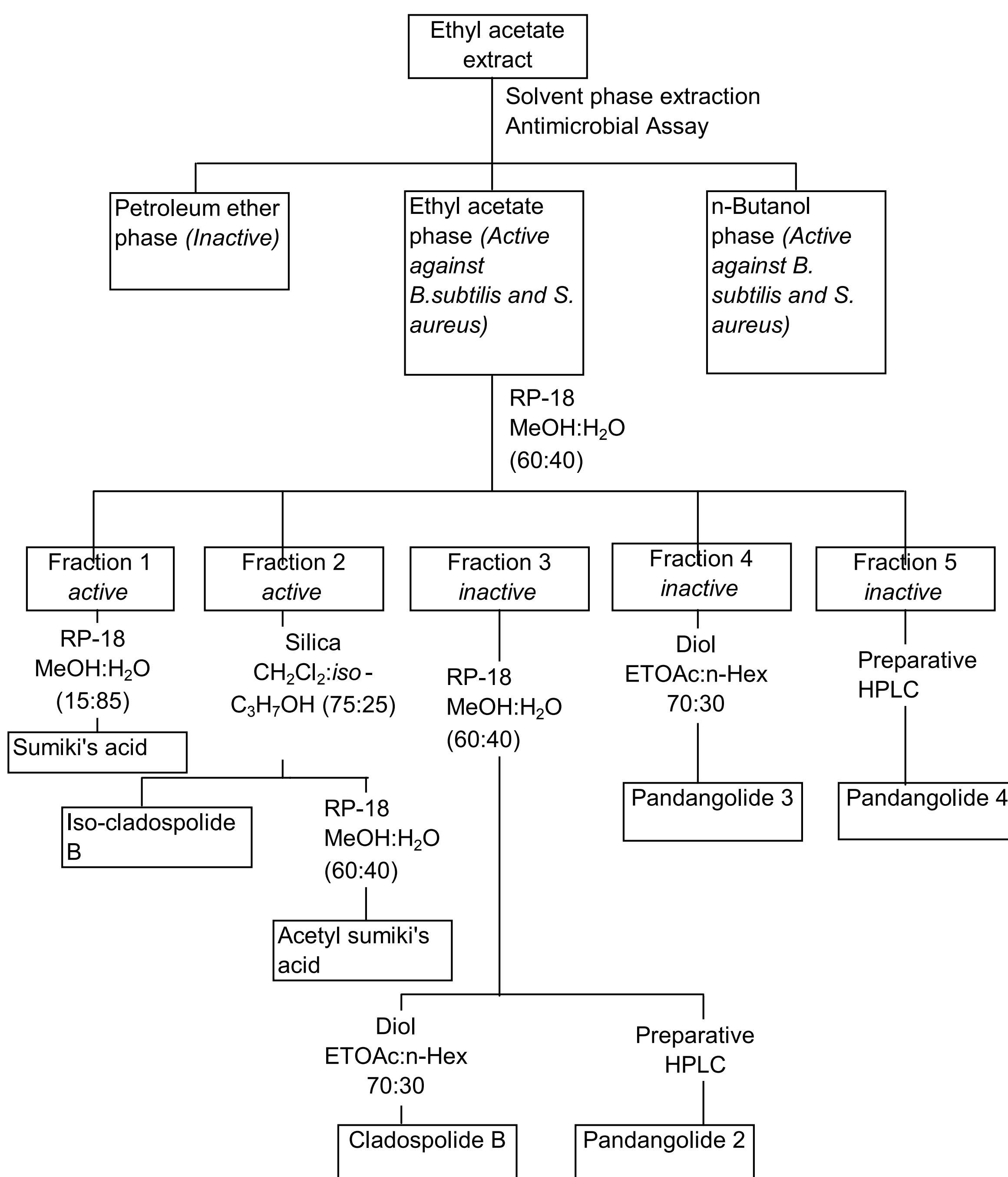
The semipreparative HPLC was used for the isolation of pure compounds from fractions previously separated using column chromatography. Each injection consists of 2 mg of the fraction dissolved in 1 mL of the solvent system. The solvent system, which is composed of MeOH and Nanopure water with 0.1% TFA, was pumped through the column at a rate of 5 mL/min. The eluted peaks which were detected by the online UV detector were collected separately in Erlenmeyer flasks.

3.4.4. Analytical HPLC

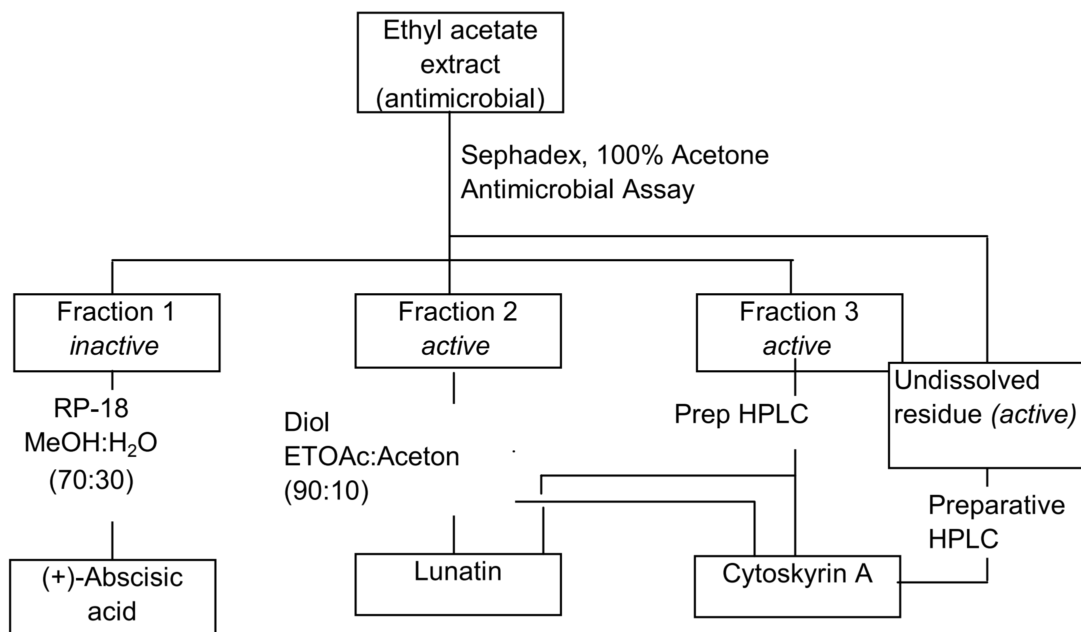
Analytical HPLC was used to identify interesting peaks from extracts and fractions as well as to evaluate the purity of isolated compounds. The gradient used started with 10:90 [MeOH: nanopure H_2O (0.1% *o*-phosphoric acid)] to 100% MeOH in 35 minutes. Peaks are detected by UV-VIS diode array detector.

3.5. Procedure for the isolation of the secondary metabolites

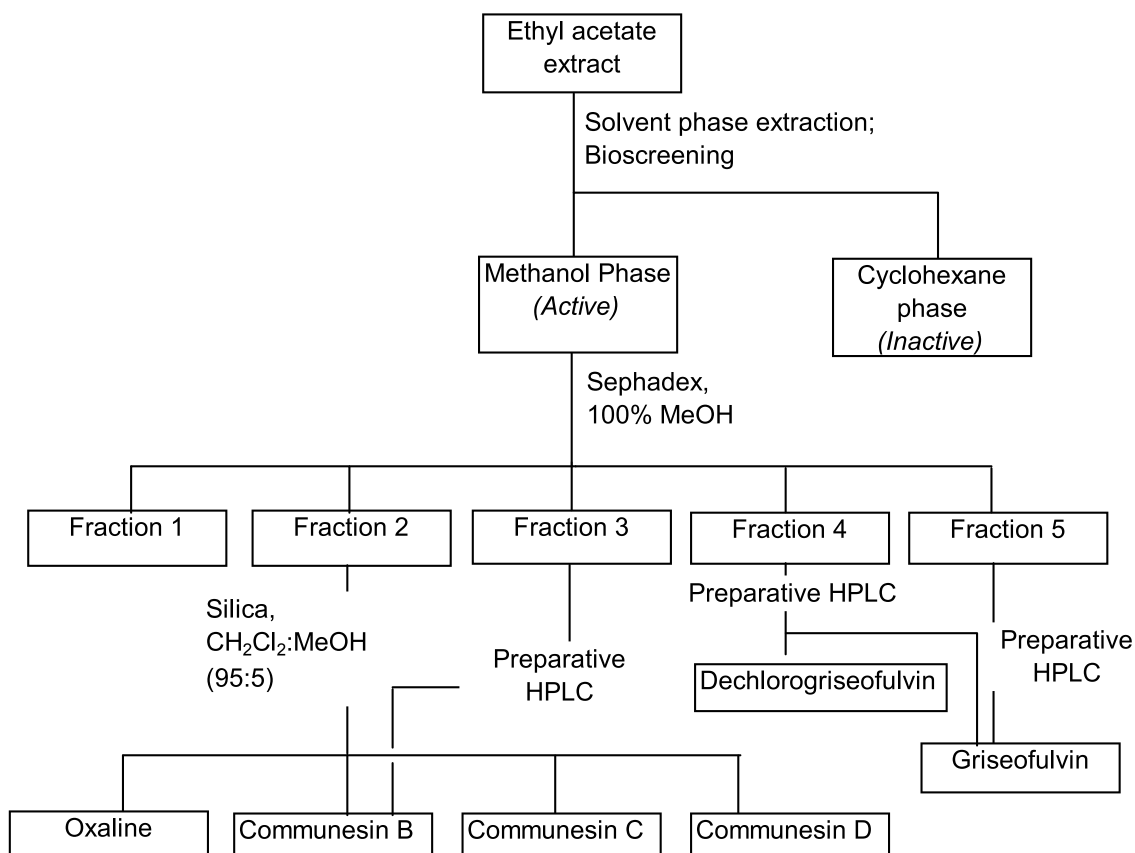
3.5.1. Isolation of the secondary metabolites from *Cladosporium herbarum*



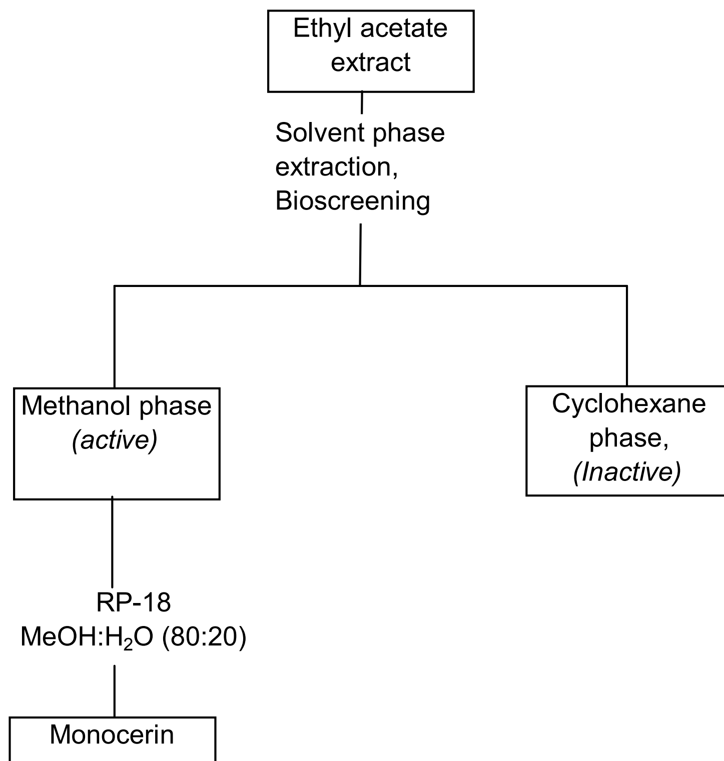
3.5.2. Isolation of metabolites from *Curvularia lunata*



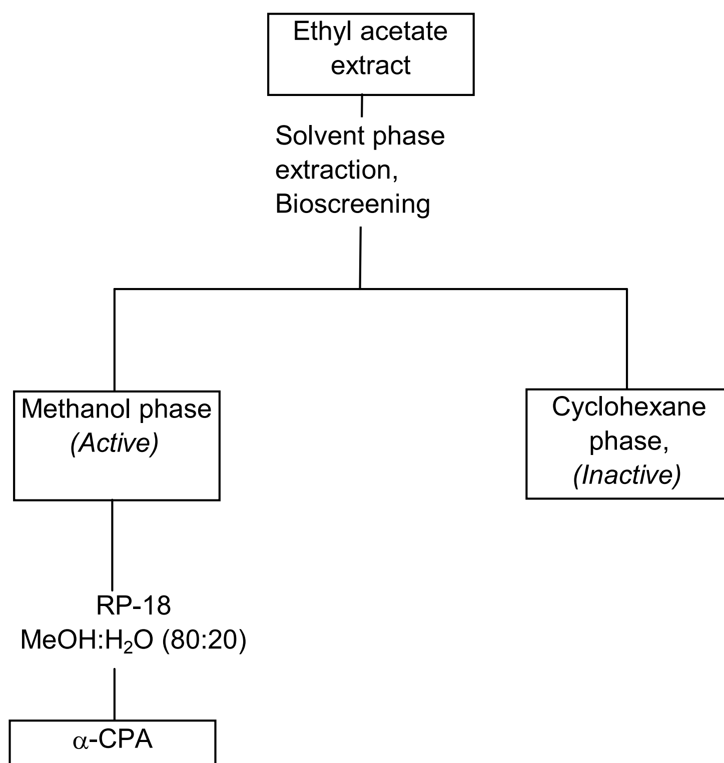
3.5.3. Isolation of metabolites from *Penicillium spp.*



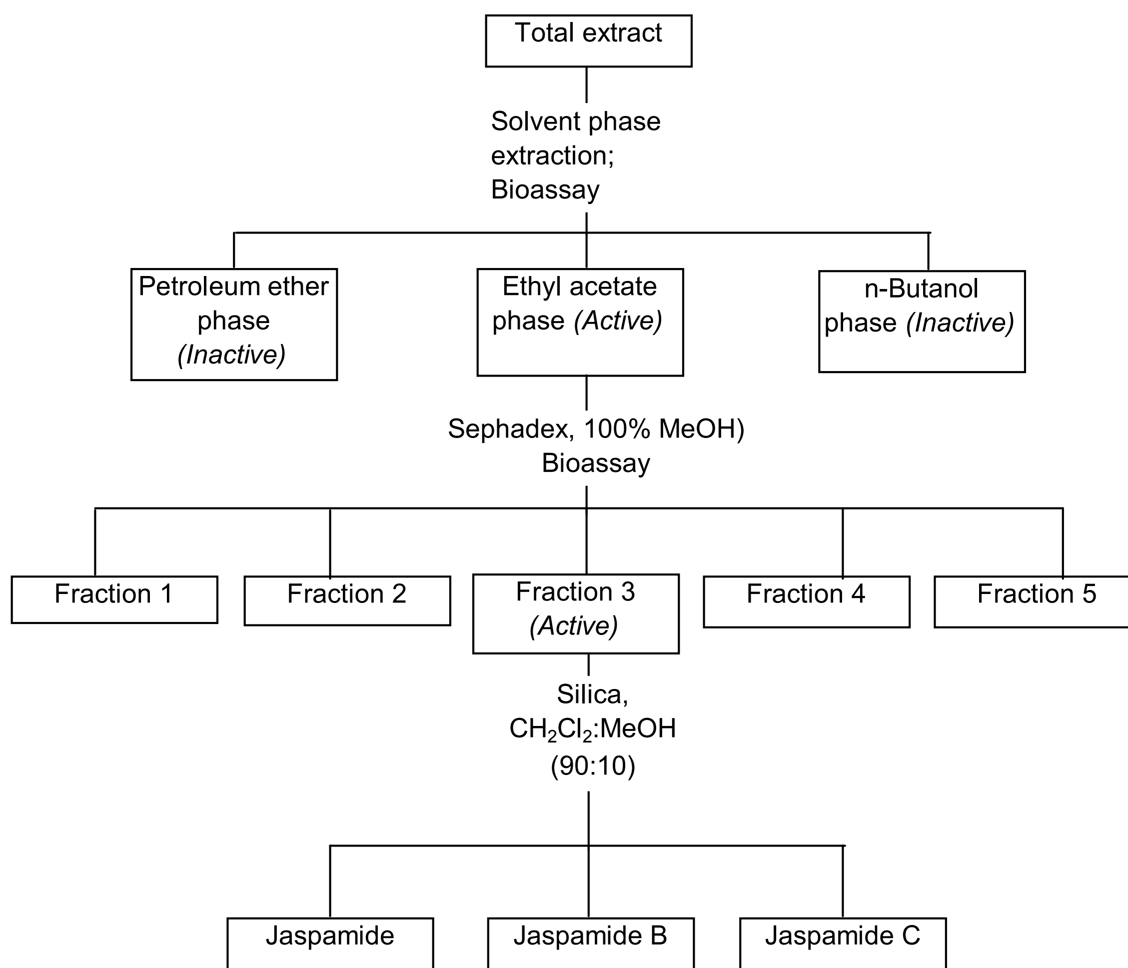
3.5.4. Isolation of monocerin from an unidentified fungus



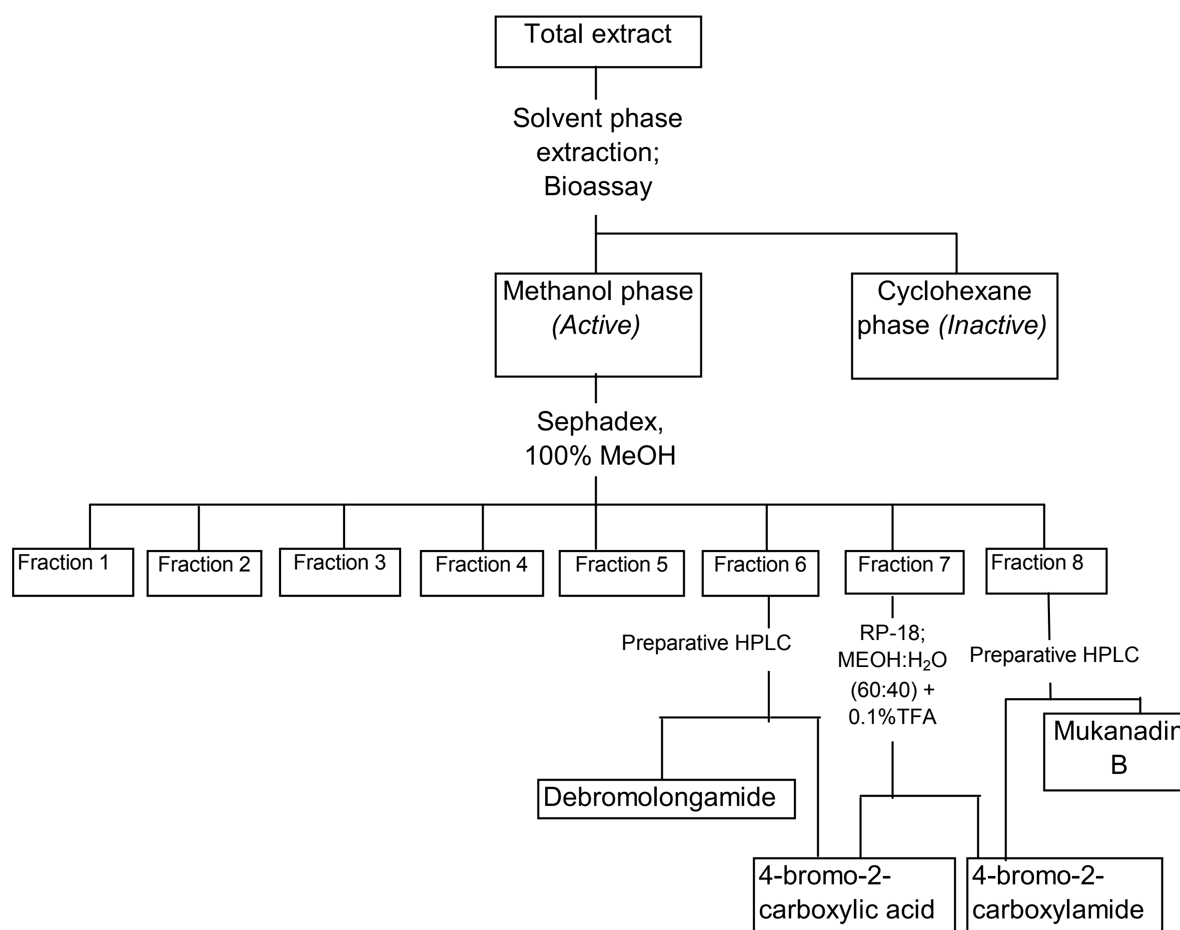
3.5.5. Isolation of α-cyclopiazonic acid from *Aspergillus flavus*



3.5.6. Isolation of metabolites from *Jaspis splendens*



3.5.7. Isolation of metabolites from *Agelas nakamurai*



3.6. Structure elucidation of the isolated secondary metabolites

3.6.1. Mass spectrometry (MS)

Low resolution MS. Low resolution mass spectra were measured by ESI-, EI-, CI- and FAB-MS on a Finnigan MAT 8430 mass spectrometer. Measurements were done by Dr. Ludger Witte at the Institute of Organic Chemistry, University of Braunschweig, Dr. Albrecht Berg of Hans Knoll Institut für Naturstofforschung, Jena, and Dr. Peter Tommes of HHU Düsseldorf.

EIMS (electron impact mass spectroscopy) analysis involves vaporizing a compound in an evacuated chamber and then bombarding it with electrons having 25-80 eV (2.4-7.6 MJ/mol) of energy. The high energy electron stream not only ionize an organic molecule (requiring about 7-10 eV) but also cause extensive fragmentation (the strongest single bonds in organic molecules have strengths of about 4 eV). The

advantage is that fragmentation is extensive, giving rise to a pattern of fragment ions which can help to characterize the compound. The disadvantage is the frequent absence of a molecular ion. For standard MS/MS measurements a gradient of 10:90 [ACN : nanopure H_2O (0.1% HCOOH)] to 100% Acetonitrile in 35 min was used.

ESIMS (electron spray ionization) is a method for ejecting ionized molecules from a solution by creating a fine spray of highly charged droplets in the presence of a strong electric field. This type of ionizaiton is highly conducive to the formation of multiply charged molecules.

High resolution MS. High resolution is achieved by passing the ion beam through an electrostatic analyser before it enters the magnetic sector. In such a double-focusing mass spectrometer, ion masses can be measured with an accuracy of about 1 ppm. With measurement of this accuracy, the atomic composition of the molecular ions can be determined.

LCMS. High-pressure liquid chromatography (HPLC) is a powerful method for the separation of complex mixtures, especially when many of the components may have similar polarities. If a mass spectrum of each component can be recorded as it elutes from the LC column, quick characterization of the components is greatly facilitated. Usually, ESIMS is interfaced with LC to make an effective on-line LC/MS. HPLC/ESI-MS was carried out using a Finnigan MAT TSQ-7000 mass spectrometry connected to a UV detector. The samples were dissolved in water/MeOH mixtures and injected to HPLC/ESI-MS set-up. HPLC was run on a Nucleosil C-18 reversed-phase column. Measurements were done at the Institute of Food Chemistry, University of Würzburg and at HHU Düsseldorf.

3.6.2. Nuclear magnetic resonance spectroscopy (NMR)

NMR measurements were done by Dr. Victor Wray at the Gesellschaft für Biotechnologiesche Forschung (GBF) in Braunschweig and by Prof. Dr. Peters at the Institut für Organische Chemie und Makromolekulare Chemie of the Heinrich-Heine Universität Düsseldorf. ^{1}H and ^{13}C NMR spectra were recorded at $300^{o}K$ pm Bruker DPX 300, ARX 400 or AVANCE DMX 600 NMR spectrometers. All 1D and 2D

spectra were obtained using the standard Bruker software. This sample is dissolved in a deuterated solvent (i.e. DMSO-d_6, $CDCl_3$, MeOD), the choice of which is dependent on the solubility of the sample. Solvent signals at 3.30 ppm and 49.0 ppm (CD_3OD) and at 2.49 ppm and 39.5 ppm (DMSO-d_6) were considered as internal standard (reference signal). The observed chemical shift (δ) values were given in ppm and die coupling constants (J) in Hz.

3.6.3. Infrared spectroscopy (IR)

Infrared spectra were recorded at the Institut of Organic Chemistry, University of Wuerzburg on an FTIR Spektrometer Perkin-Elmer 1605 using KBr disks as beamsplitter. About 1.0 mg of the solid compound is finely ground in a small agate mortar with a drop of liquid hydrocarbon, Nujol. The solid is ground with 10 to 100 times its bulk of pure KBr and the mixture was pressed into a disc using a mould and a hydraulic press.

3.6.4. CD

CD spectrum were recorded Jobin Yvon CD 6 using methanol as solvent. CD is a measurement of the difference in the absorbance by a chiral molecule of left and right circularly polarized light. CD curves are plots of $\Delta\varepsilon$ against the wavelength. The samples were done at the Institute of Organic Chemistry, University of Würzburg.

3.6.5. Optical activity

Optical rotation was determined on a Perkin-Elmer-241 MC Polarimeter by measuring the angle of rotation at the wavelength of 546 and 579 nm of a mercury vapor lamp at room temperature (25°C) in a 0.5 mL cuvette with 0.1 dm length. The specific optical rotation was calculated using the expression:

$$[\alpha]_D^{20} = \frac{[\alpha]_{579} \times 3.199}{4.199 - \frac{[\alpha]_{579}}{[\alpha]_{546}}}$$

where $[\alpha]_D^{20}$ = the specific rotation at the wavelength of the sodium D-line, 589 nm, at a temperature of 20°C.

$[\alpha]_{579}$ and $[\alpha]_{546}$ = the optical rotation at wavelength 579 and 546 nm respectively, calculated using the formula:

$$[\alpha]_\lambda = \frac{100 \times \alpha}{l \times c}$$

where α = the measured angle of rotation in degrees,

l = the length in dm of the polarimeter tube,

c = the concentration of the substance expressed in g/100 mL of the solution

3.7. Bioassay

The methods for the detection of biological activity of natural proudct mixtures can best be divided into two groups for screening purposes: general screening bioassays and specilized screening bioassays. The search for specific pharmacologic activites often overlooks other useful activities which are not detected or are ignored, in the screening process. Furthermore specific test methods are often cumbersome and expensive. Hence, for bioassay-guided isolation of actives from natural product mixtures single, inexpensive 'bench-top' bioassays for the rapid screening of extracts and fractions have to be employed. Since most active plant principles are toxic at elevated doses, a possible approach to developing an effective general bioassay might be simply to screen for substances that are toxic to zoologic systems. Once such substances have isolated, a battery of specific and more sophisticated bioassays could then be employed.

3.7.1. Brine-shrimp assay

This technique is an *in vivo* lethality test in a tiny crustacean, the brine shrip (*Artemia salina* Leach). It has been previously utilized in various bioassay systems including in the analysis of pesticide residues, mycotoxins, stream pollutants, anesthetics, dinoflagellate toxins, morphine-like compounds, toxicity of oil dispersants, cocarcinogenicity of phorbol esters and toxicants in marine environments [Meyer *et*

al., 1982]. This test takes into account the basic premise that pharmacology is simply toxicology at a lower dose, and that toxic substances might indeed elicit, at a lower non-toxic dose, interesting pharmacologic effects [McLaughlin, 1991]. The procedure determines LC_{50} values in μg/ml of active compounds and extracts in the brine medium.

Sample preparation. The test samples were dissolved in an organic solvent and the appropriate amount is trasferred to a 10-ml sample vial. For crude extracts, 0.5 μg of the compound were used and various concentrations of the pure compound.The samples were then dried under nitrogen and the dried samples were reconstituted with 20 μL DMSO. Control vials containing DMSO were also prepared.

Hatching the eggs. Brine shrimp eggs (Dohse, Aquristik GmbH, Bonn, Germany) were hatched in a small tank filled with artificial sea water which was prepared with a commercial salt mixture (Sera Sea-Salt, Aquaristik GmbH, Bonn, Germany) and distilled water. After 48 hours, 20 nauplii were collected by pipette (counted macroscopically in the stem of the pipette against a lighted background) and transferred into each test sample vial. Artificial sea water was then added to make 5 mL. The vials were maintained under illumination. Survivors were counted, with the aid of a magnifying glass after 24 hours and the percent deaths at each dose and control were determined. The LC_{50}'s were determined using the probit analysis method.

3.7.2. Insecticidal bioassay

The polyphagous pest insect *Spodoptera littoralis* Boisd. (Noctuidae, Lepidoptera), commonly known as Egyptian cotton worm, has proved to be a good test model for an insecticidal bioassay on a broad spectrum of chemical classes of natural products. The larvae of *S. littoralis* feed on a wide variety of plant species and are one of the most robust and hazardous pest insects in the Mediterraneian as well as in Africa. *S. littoralis* has a short life cycle of 4 weeks and is easy to maintain in the laboratory by utilizing an artificial diet consisting mainly of leguminous beans in agar.

Culture conditions. Larvae of S. littoralis were from a laboratory colony reared on artificial diet under controlled conditions [Srivastava *et al.,* 1991]. Optimum growth was achieved under standardized conditions at a temperature of 28°C and a controlled day and night phase of 16 and 8 h, respectively.

Larvae at their pre-pupal stage were regularly separated (to prevent from being cannibalized) and set on Vermiculit, a kind of silicated mineral, until they reach the pupal stage. The pupae were then stored to a dark humid chamber at 28°C until they develop to their final adult stage. The adults were then transferred to a 10-liter plastic pail, lined with filter paper on which the female can lay their eggs. The adults feed on saccharose solution at this stage, they were maintained at a controlled day and night phase of 16 and 8 h, respectively, and at a temperature of 28°C. The laid eggs were collected every two days and again transferred to the dark chamber until the neonate larvae hatch.

Artificial diet. The artificial diet consisting mainly of leguminous beans in agar were first left to stand overnight in 460 mL water and then homogenized in a mixer. To the bean suspension, the following substances were added:

3.0 g	L-(+)- Ascorbic acid
3.0 g	Nipagin
30.0 g	Baker's Yeast
0.180 g	Gentamycin Sulfate
1.0 mL	Formaldehyde Solution

To this bean homogenate, warm agar suspension (10 g agar cooked in 315 mL water and cooled down to 50°C) was added and thoroughly mixed. The agar bean mixture was then poured into a 3-liter GERDA box and cooled to room temperature to solidify. The solidified agar-bean diet was then stored in the refrigertor until use.

Chronic feeding assay. The neonate larvae of *S. littoralis* are forced on a diet treated with a known concentration of a test sample, then the larvae are monitored after 6 days. The diet consist of a freeze-dried form of the above-described bean homogenate mixture, this time consisting only of nipagin, baker's yeast and formaldehyde solution. The test sample, dissolved in 2 mL carrier solvent, was

incorporated to 0.735 g of the freeze-dried homogenate, and the mixture is allowed to stand overnight to allow evaporation of the carrier solvent. For biological screening studies, concentrations of 5.0 and 1.0 mg were used for crude extracts and pure compounds, respectively. To the treated homogenate, 2.2 mL of warm agar suspension was added and then left to solidify at room temperature. Twenty neonate larvae of *S. littoralis* were used per assay whose growth are then monitored after 6 days of incubation at 28^oC in a dark humid chamber. Activity is measured as the survivial rate or growth rate of the surviving larvae when compared to controls.

$$\% \text{ survival rate} = \frac{\text{number of surviving larvae (sample)}}{\text{number of surviving larvae (control)}} \times 100$$

$$\% \text{ growth rate} = \frac{\text{Average larval weight (sample)}}{\text{Average larval weight (control)}} \times 100$$

A subsequent experiment on the active compound was analyzed for activity at a range of doses. Potency was determined as the effective concentration (EC_{50}) of the test substance added to that diet necessary to cause a 50% reduction in weight. Lethal concentration that cause 50% death (LC_{50}) were also determined. The ED_{50} and LC_{50} were calculated by probit analysis from the dose response curve.

3.7.3. Antibacterial activity

Microorganisms. Crude extracts and pure compounds were tested for activity against the following standard strains: gram positive bacteria *Bacillus subtilis* 168 and *Staphylococcus aureus* ATCC 25923, gram negative bacteria *Escherichia coli* ATC 25922 and *Escherichia coli* HB 101. The bacterial strains were from the laboratory cultures of the Institut für Infektionsbiologie, University of Würzburg.

Culture preparation. The agar diffusion assay was performed according to the Bauer-Kirby-Test (DIN 58940, Bauer et al. 1966). Prior to testing, a few colonies (3 to 10) of the organism to be tested were subcultured in 4 ml of tryptose-soy broth (Sigma, FRG) and incubated for 2 to 5 h to produce a bacterial suspension of moderate cloudiness. The suspension was diluted with sterile saline solution to a

density visually equivalent to that of a $BaSO_4$ standards, prepared by adding 0.5 ml of 1% $BaCL_2$ to 99.5 ml of 1% H_2SO_4 (0.36 N). The prepared bacterial broth is inoculated onto Müller-Hinton-Agar plates (Difco, USA) and dispersed by means of sterile beads.

Agar diffusion assay. For screening, aliquots of the test solution were applied to sterile filter-paper discs (5 mm diameter, Oxoid Ltd.) to give a final disc loading concentration of 500 μg for crude extracts and various concentrations (50, 25, 10, 5 and 1μg/disk) for pure compounds. The impregnated discs were placed on agar plates previously seeded with the selected test organisms, along with discs containing solvent blanks. The plates were incubated at 37°C for 24 h then antimicrobial activity was recorded as the clear zone of inhibition surrounding the disc at which the diameter was measured in mm.

3.7.4. Cytotoxicity test

Cytotoxicity tests were carried out by Dr. Klaus Steube of DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmBH, Braunschweig, Germany). The tests used human leukemia cell lines U-937 [Sundstrom *et al*., 1976], MONO-Mac-6 [Ziegler-Heitbrock *et al.,* 1988], JURKAT [Schneider *et al*., 1977], THP-1 [Tsuchiya *et al*., 1980], and MM-1.

The cells were grown in plastic flasks (Nunc, Wiesbaden, Germany). All cultures were propagated using standardized media supplemented with 10-20% heat-inactivated fetal bovine serum and were cultivated at 37°C in a humidified atmosphere containing 5% CO_2.

The freeze-dried compound were dissovled in EGMME (ethylene glycol monomethyl ether) or DMSO, diluted in RPM1-1640 culture medium and stored frozen at –20°C. At the final dilution used in the experiments, the EGMME/DMSO concentration was 0.1%.

For the experiments, exponentially growing cells (viability of >90% as measured by trypan blue exclusion) were harvested, washed and resuspended in fresh medium at

a final concentration ranging between 2 and 4 x 10^5 cells/mL (depending on the cell line). Total cell number and viability were determined in a cell counting chamber after staining the cells with trypan blue. Aliquots of 90 µL were seeded out into 96-well flat-bottom culture plates (Nunc, Wiesbaden, Germany). Ten microliters of medium with the solvent EGMME/DMSO or the different concentration of the test compound were added. After a 48 h incubation/cultivation period the extent of cytotoxicity was evaluated by the MTT assay [Steube *et al*, 1998] or by the Thy assay. In the latter, cytotoxicity was determined by incorporaion of [^{3}H]thymidine. Radioactive incorporation was carried out for the last 3 h of the 48 h incubation period. One µCi of [methyl-^{3}H]-thymidine (Amersham-Buchler, Braunschweig, Germany; specific activity 0.25 mCi/µmol) was added in a 20 µL volume to each well. Cells were harvested on glass fiber filters with a multiple automatic sample harvester, and radioactivity was determined in a liquid scintillation counter (1209 Rackbeta, LKB, Freiburg, Germany).

All experiments were carried out in triplicates and repeated three times. As controls, media with 0.1% EGMME/DMSO were included in the experiments.

4. Discussion

4.1. The isolation of known compounds and dereplication

The search for new bioactive compounds from nature using bioassay-guided isolation has the disadvantage of having a high probability of isolating previously known secondary metabolites. This can be minimized by a system known as ‘dereplication’ which can be performed by subjecting the extract to an early form of detection. The taxonomy, biological and chemical profiles of the extract are investigated. The chemical profiles are usually determined by TLC and the more modern online HPLC-NMR or HPLC-MS analyses followed by consultation of several databases. This, however, involves interdisciplinary cooperation (i.e., identification of the marine organism may have to be tasked to taxonomists). This requires time, which may not always be at the disposal of every graduate research student. Hence, chemical investigation of an unidentified marine organism may be undertaken which then precludes this dereplication step. It is nevertheless important to point out that the method for choosing extracts for study utilized by our research group is not without the application of modern instrumentation such as HPLC with diode array detection and online HPLC-ESIMS and of up-to-date database such as Antibase® 2002, Dictionary of Natural Products on CD-ROM 2002, and Marinlit.

This research work made possible the isolation of five structurally-related polyketides, including two new twelve-membered macrolides; two furoic acid compounds including one which has been isolated for the first time; one new phthalide; two anthraquinones one of which is a new metabolite; the known plant hormone abscisic acid; the known phytotoxin monocerin; the known mycotoxins oxaline and α-cyclopiazonic acid; the antifungal griseofulvin and its dechloro derivative; the cytotoxic alkaloid communesin B and its two new derivatives; the bromo-pyrrole alkaloids from sponge which include the new 4-bromo-pyrrole-2-carboxylic acid, and the known compounds: 4-bromo-pyrrole-2-carboxamide, mukanadin B and mukanadin C; and the known depsipeptide jasplakinolide (jaspamide) and its two known derivatives jaspamide B and C from sponge.

4.2. Fungal metabolites as antibiotics

Antibiotics, the most important type of microbial metabolites, represent one of the largest and most diverse groups of natural products. The inhibition of one organism by another has been observed by early microbiologists and the term antibiotic was then used to mean 'a chemical substance which has the capacity for inhibiting growth and even destroying other microorganisms in dilute solutions' [Vandamme, 1984]. The first antibiotics isolated were detected using simple screens against common bacteria and it was in the selection of antibacterial antibiotics that the greatest success was achieved, although screens were also carried out for antifungal, antimycoplasma, antispiroketal, antiprotozoal, antitumor and antiviral compounds. The agricultural antibiotics are used as feed additives or plant protectants against bacterial, fungal and viral infections. Others are applied as preservatives or biochemical reagents.

To date, several tens of thousands of metabolites have been isolated from microorganisms. Only a very small percentage of these have antibiotic activity, and it is probably true to say that the majority of them have no known biological activity [Berry *et al.,* 1993]. This does not mean that they do not have any natural role since it has been hypothesized that such compounds may have a variety of roles such as acting as chelating agents, hormones, pheromones, as well as being competitive agents in natural environments [Ibid]. It was also observed that many of them have biological activities which are not directly related to their natural environment [Nisbet and Porter, 1989].

4.3. Metabolites from *Curvularia lunata*

4.2.1. Antimicrobially-active anthraquinones

The ethyl acetate extract of the fungus *Curvularia lunata* exhibited strong antimicrobial activities against *B. subtilis* and *S. aureus* during the preliminary screening. Chemical investigation of the extract yielded the antibacterial anthraquinones: lunatin (compound **11**) and cytoskyrin A (compound **10**) (Fig. 2.28). Both anthraquinones showed selective antibacterial activity against the gram positive

bacteria *B. subtilis* and *S. aureus.* It also appeared that cytoskyrin A, a bisanthraquinone, is more potent than the monomeric anthraquinone lunatin, with the former exhibiting activity with as low as 1 μg loading amount (1.74 nmol) compared to 5 μg (17.5 nmol) of the latter. Cytoskyrin A also showed proliferative activity against LH-60 cell line upon prolonged incubation whereas lunatin was inactive. This seems to point out to the importance of the dimeric structure in bisanthraquinones for cytotoxic activity, especially when literature reports of cytotoxic activities of similar bisanthraquinones such as luteoskyrin and rugulosin (Fig. 2.31) [Akuzawa *et al*., 1992] are also considered.

4.2.2. Biosynthesis of bisanthraquinones

The co-isolation of the monomeric lunatin and the bisanthraquinone, cytoskyrin A, indicates the role of monomeric anthraquinones as precursors in the biosynthesis of bisanthraquinones. Although the exact biosynthetic pathway is still not clear, this seem to favor the biosynthetic pathway proposed by Takeda [Takeda *et al*., 1973]. The co-isolation from *Penicillium islandicum* of a series of anthraquinonoids, which include monomeric and dimeric anthraquinones as well as modified bisanthraquinones, have led to their theory that bisanthraquinones have been formed biogenetically by the base-catalyzed Michael-type intramolecular condensation of a hypothetical intermediate, a partially hydrogenated bianthraquinone. A schematic diagram of this proposed biosythetic pathway is shown below (Fig. 4.1) incorporating the co-isolated anthraquinonoids catenarin, (+)-iridoskyrin, (-)-rubroskyrin and (-)-luteoskyrin.

However, the isolation of the partially hydrolyzed monomeric anthraquinone, 5,6-dihyrocatenarin and 5,6,7,8-tetrahydrocatenarin from a strain of *Penicillium islandicum* [Bu'Lock and Smith, 1968] provides a possible alternative pathway, which is the dimerization of partially hydrolyzed 1,3,6,8-tetrahydroxyanthraquinone monomers by a one-electron oxidation and condensation process to form the bisanthraquinone [Brady *et al*., 2000].

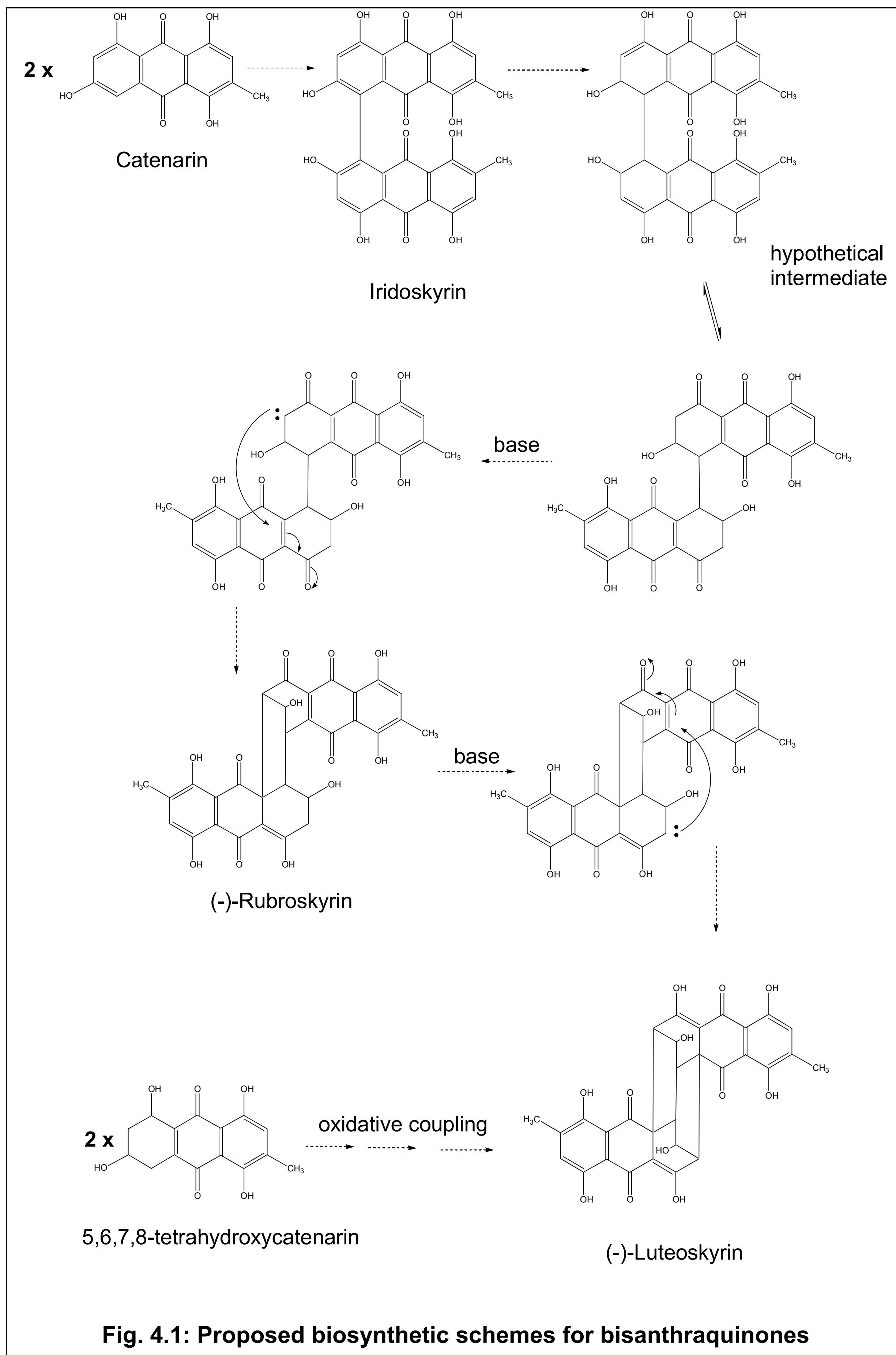


Fig. 4.1: Proposed biosynthetic schemes for bisanthraquinones

4.2.2. Abscisic acid

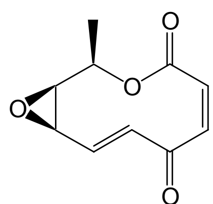
The isolation of (+)-abscisic acid (compound **9,** Fig. 2.28) as the major metabolite of *C. lunata* raises the question of the role of this plant hormone in marine biology. Its presence in marine algae [Crouch and Vanstaden, 1993] suggest that abscisic acid's role as growth-regulating hormone in higher plants may also apply in marine flora. However, this is the first time that abscisic acid is isolated from a marine-derived fungus which suggests a possible similar relationship between abscisic acid-producing plant-pathogenic fungi like *Cercospora cruenta* [Oritani and Yamashita, 1985]*, Macrophoma castaneicola* [Arai et a., 1989]*, Cercospora rosicola* [Assante *et al*., 1977]*, Botrytis cinerea* [Marumo *et al*., 1982]*,* and *Laminaria spp.* [Schaffelke, 1995] with higher plants and between marine fungi and marine algae.

4.4. *Cladosporium herbarum*

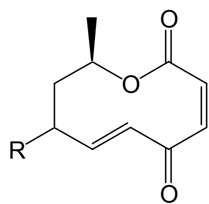
4.4.1. Antimicrobial metabolites

The second fungal extract, that of *Cladosporium herbarum* also exhibited selective activity against gram positive bacteria *B. subtilis and S. aureus*, with the activity limited to the ethyl acetate and the *n*-butanol solvent phases. Comparison of the HPLC chromatograms of the two solvent phases showed the presence of a common polar metabolite which was later identified as sumiki's acid (compound **6**, Fig. 2.17), a furoic acid derivative. Also isolated in trace amounts was the acetyl congener of sumiki's acid (compound **7**, Fig. 2.17). Both compounds showed selective antimicrobial activities against *B. subtilis and S. aureus* down to a loading amount of 5 μg in the agar diffusion assay.

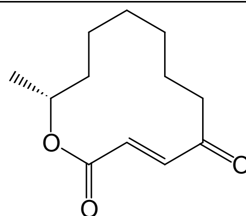
The four isolated macrolides and the structurally-related γ-lactone (compounds **1-5**) (Fig. 2.1 and Fig. 2.9) exhibited no antimicrobial activities. This appears to confirm previously drawn observations that antimicrobial activity of macrolides requires the presence of a double bond flanked by two carbonyl carbons, which are common in other antimicrobial agents such as patulolide A and B, pyrenophorin, pyrenolides, vermiculine, among others (Fig. 4.2) [Rodphaya *et al*., 1986; Antibase, 2002].



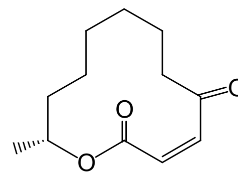
pyrenolide A



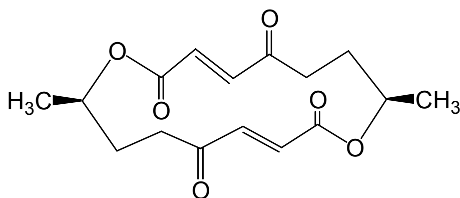
R = H, pyrenolide B
R = OH, pyrenolide C



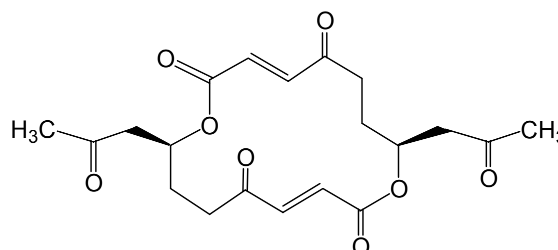
patulolide A



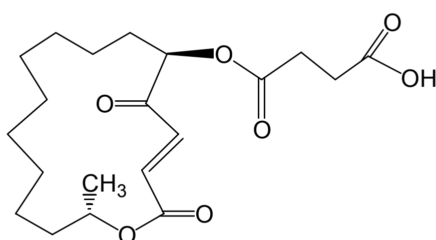
patulolide B



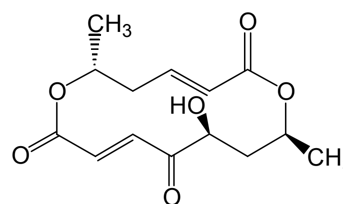
pyrenophorin



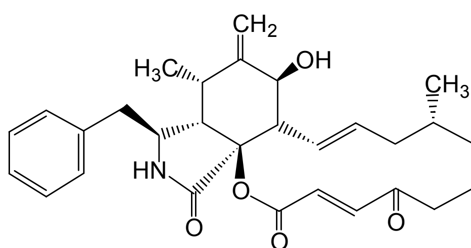
vermiculin



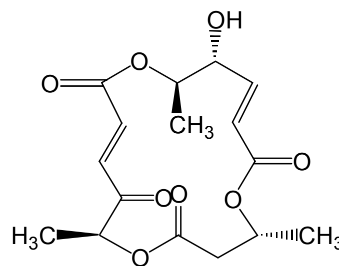
(-)-A-26771B



grahamimycin A



cytochalasin A



macrosphelide-B

Fig. 4.2: Macrolides with antimicrobial activities [Antibase, 2002]

4.4.2. Compounds with potential phytotoxic activities

4.4.2.1. Macrolides

Cladospolide A has been reported to inhibit root growth of lettuce seedlings and cladospolide B was found to inhibit shoot elongation of rice seedlings without causing necrosis to the plant.

Phytotoxins and phytotoxic fungi are of importance in natural product research because of their potential use in the production of agrochemicals. Cladospolide B (compound **1**, Fig. 2.1), for instance, and other metabolites exhibiting plant growth-inhibiting activities, may be used to induce dwarfism, a very important biological phenomenon in agriculture, because it prevents crops from lying on the ground and produces a better harvest. Conversion of a normal plant into a dwarf is usually brought about by chemical plant growth retardants. In many cases, dwarfing agents act by blocking gibberellin biosynthesis; therefore these compounds are sometimes used in biosynthetic studies of gibberellins.

Phytotoxic fungi, on the other hand have been used as herbicides. In recent years, spores of several fungal pathogens have been sucessfully formulated as weed control agents known as mycoherbicides, which can be sprayed onto the target weed in the manner of a conventional herbicide [Burge, 1993]. The advantages include low persistence, high specificity, environmental safety and relatively low production costs.

4.4.2.2. Herbaric Acid

Also co-isolated was the new phthalide herbaric acid (compound **8**, Fig. 2.22) which was found to posses no biological activities in the screenings performed. Herbaric acid is the 5-hydroxyl derivative of the known toxin iso-ochracinic acid previously isolated from *Alternaria kikuchiana*, a parasite responsible for the black spot disease on Japanese pears [Kameda and Namiki, 1974]. Other related phthalides possessing phytotoxic activities include convolvulol, convolvulanic acid A and B, 4-methyl-7-hydroxyphthalide [Antibase, 2002]. Here is another case of a marine-derived fungi producing secondary metabolites with potential phytotoxic activities. There is, of

course, the possiblity that these fungi are of terrestrial origin and not ‘true’ or obligate marine fungi. However, as in *Curvularia lunata*, there is also the probability that this fungus is not a sponge symbiont but could be originally associated with marine algae with which it interacts through the production of these secondary metabolites.

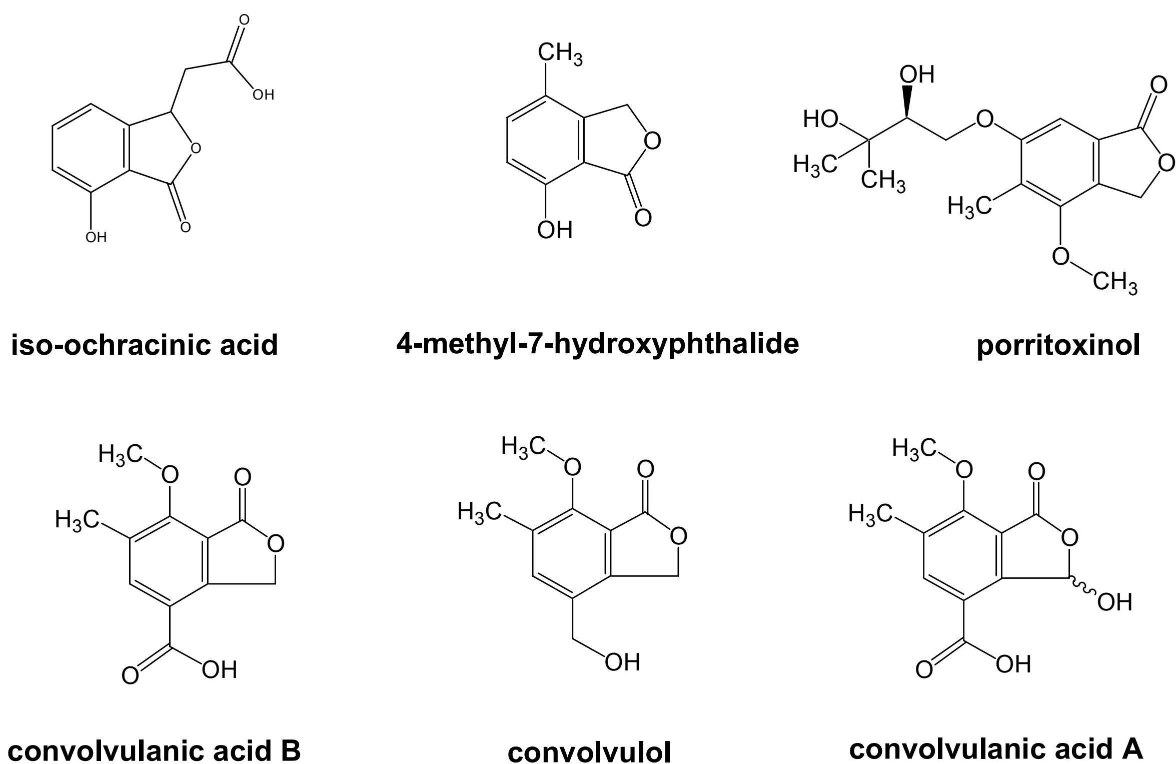


Fig.4.3: Phytotoxic phthalides previously isolated from fungi [Antibase, 2002]

4.4.5. Biosynthetic pathways for polyketides (compounds 1-7)

The similarity in the structures of the macrolides, compounds **1, 3, 4** and **5**, clearly suggests that all of them are synthesized through the same biosynthetic pathway. A scheme for the possible synthetic pathway of cladospolide B (compound **1**), iso-cladospolide B (compound **2**), is proposed below (Fig. 4.4).

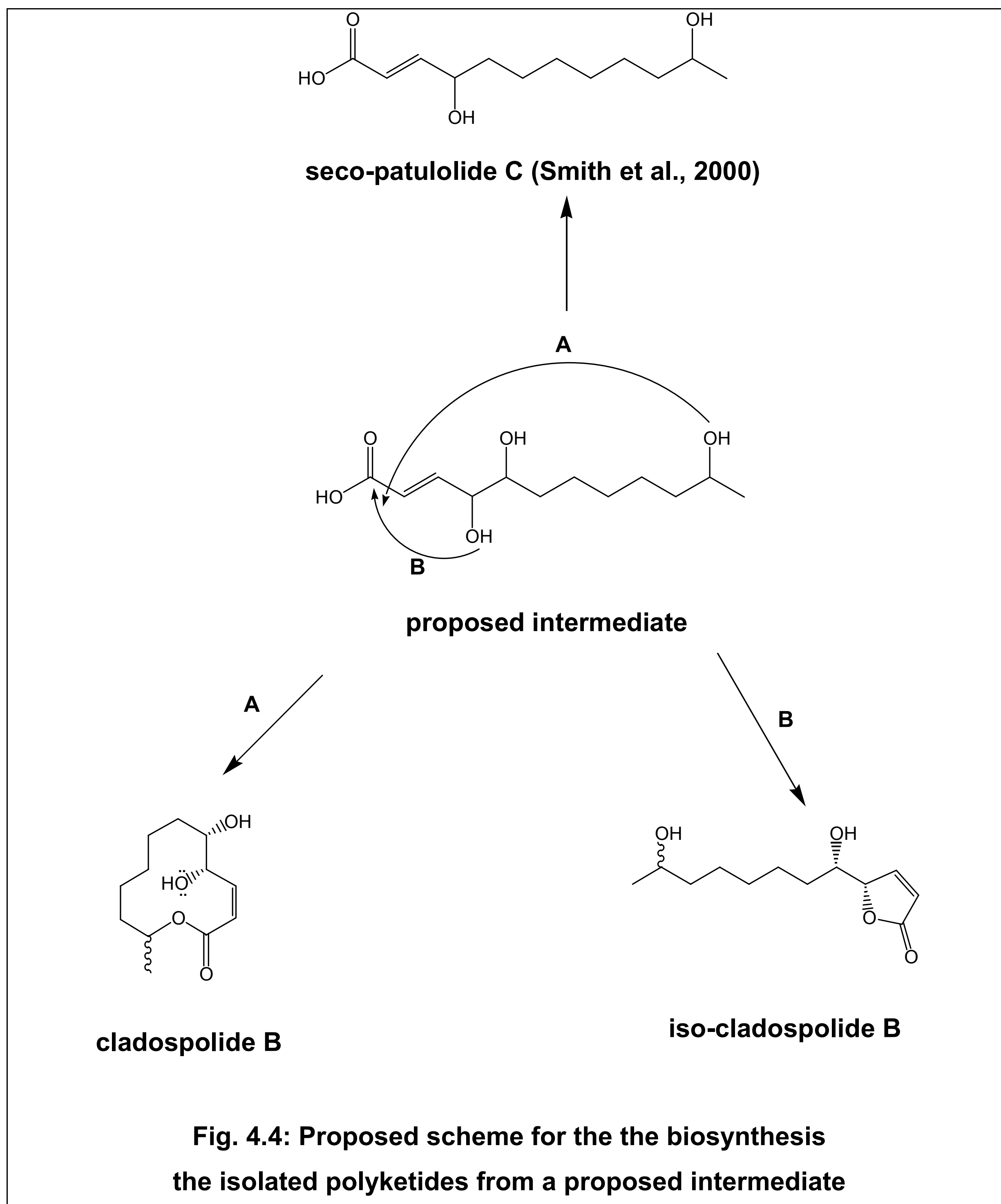


Fig. 4.4: Proposed scheme for the the biosynthesis the isolated polyketides from a proposed intermediate

4.4.6. Different set of metabolites isolated from two *C. herbarum* strains

It is interesting to note that a separate investigation of another strain of *C. herbarum* which was isolated from the sponge *A. aerophoba* performed by another researcher in our research team yielded mainly pyrone derivatives (Fig. 4.5) [Jadulco *et al.*, 2002]. Since both fungal strains were cultivated under the same conditions the chemical differences found are likely to be caused by different fungal genotypes.

However, the isolation of cladospolide A, B and C from two *Cladosporium* species, namely, *Cladosporium cladosporioides* [Hirota *et al.*, 1985a] and *Cladosporium tenuissimum* [Fuji *et al.*, 1995], suggests that these macrolides maybe metabolic markers of this genus.

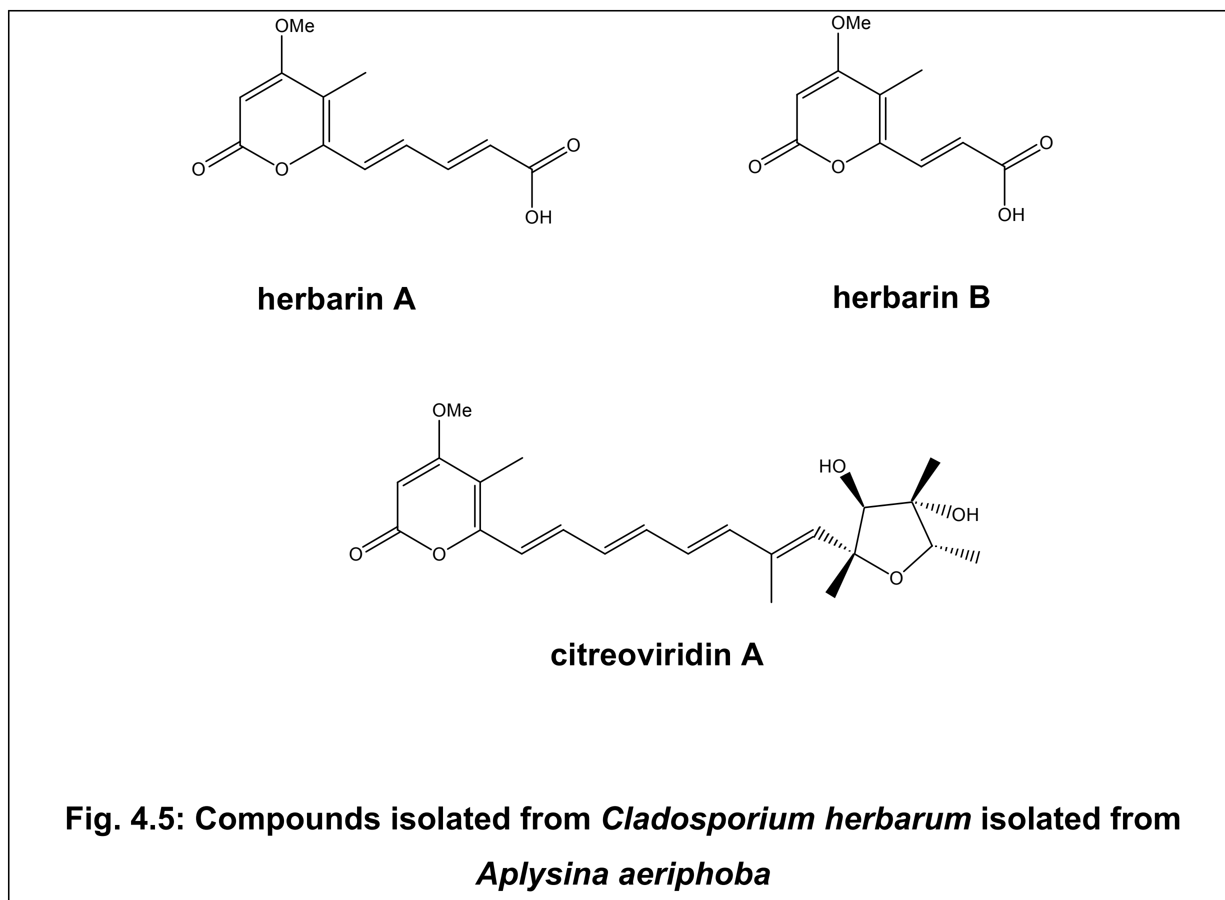


Fig. 4.5: Compounds isolated from *Cladosporium herbarum* isolated from *Aplysina aeriphoba*

4.5. Fungal metabolites from the extract of *Penicillium spp.*

4.5.1. Metabolites as taxonomic markers

The ethyl acetate extract of a *Penicillium* species yielded griseofulvin (compound **13**, Fig. 2.40) as the main metabolite together with trace amounts of dechlorogriseofulvin (compound **14,** Fig. 2.40) and oxaline (compound **12,** Fig. 2.40). Griseofulvin is a well-known antifungal. In the laboratory, it showed activity against *C. albicans* with a zone of inhibiton of 15 mm by 1 μg/disk loading concentration. Dechlorogiseofulvin did not exhibit activity against *C. albicans* in the screening using loading concentration of 10 μg/disk. It has been reported has a much weaker antifungal

activity – 125 times less active than griseofulvin [MacMillan, 1951], indicating the importance of the chloro substituent for this action.

The pattern of metabolites produced by a fungi can also be used as a taxonomic marker [Dreyfuss and Chapela, 1994]. Mycotoxins produced by different strains of *Penicillium* species isolated from different sources were studied. It was found out that individual strains can be characterized by their specific profiles of N-containing secondary metabolites and produced mycotoxins of different structural types. The alkaloids oxaline, alpha-cyclopiazonic acid, and griseofulvin are among a series of metabolites (along with toxins such as meleagrin, ochratoxin A, citreoviridin, roquefortin, among others) which are produced in peculiar combinations, the production of which can identify a certain fungal taxon or a series of taxa along with morphological and physiological data. *Penicillium coprophilum*, for instance is known to produce griseofulvin, dechlorogriseofulvin, roquefortine C, meleagrin, and oxaline [Frisvad and Filtenborg, 1990]. Thus, the taxonomic identity of a strain can indicate the presence of the corresponding marker metabolite; alternatively, a single metabolite or metabolite pattern frequently can point to a specific fungal taxon or series of taxa.

4.5.2. Communesins

Also isolated was communesin B (compound **15**, Fig. 2.40) and trace amounts of two derivatives, communesins C (compound **16,** Fig. 2.40) and D (compound **17**, Fig. 2.40). Communesin B exhibited potent toxicities in the brine-shrimp lethality test, insecticidal, antimicrobial and cytotoxicity tests. When the brine-shrimp lethality activities of the three derivatives were compared, it was found out that communesin C was the least active with an LC_{50} of 1.93 μg/mL, suggesting the importance of the N-15-substituent. Communesin B, which has a methyl group at N-15, was the most active with an LC_{50} of 0.3 μg/mL, whereas communesin D which a formyl group at N-15 has an LC_{50} of 0.57 μg/mL.

4.6. Metabolite from *Aspergillus flavus* and an unidentified fungus

Monocerin (compound **18**, Fig. 2.59) which was isolated from an unidentified fungus has been reported to possess phytotoxic activities and insecticidal activities. Against *S. littoralis*, it exhibited strong antifeedant activity ED_{50} of 14.78 ppm. It also showed antimicrobial activities against Gram-positive and Gram-negative bacteria. However, it did not exhibit toxicity against *A. salina*. Likewise, $\alpha-$cyclopiazonic acid (compound **19**, Fig. 2.62), a well-known toxin, which was isolated from *Aspergillus flavus* also exhibited antimicrobial activity and strong antifeedant activity against *S. littoralis*, but had no activity against *A. salina*. Since both are expected to exhibit general toxicities, their inactivity against brine-shrimp may have something to do with their poor solubility in polar solvents where the assay is based.

The identification of cyclopiazonic acid is of particular importance because of its effect in human health. Normally produced by *Aspergillus flavus, Penicillium cyclopium, P. camemberti, P. commune,* and *P. griseofulvum* and a common contaminant of agricultural products, it has been associated with human toxicosis commonly known as 'kodua poisoning,' a nonlethal illness characterized in humans by fatigue, tremors, slurring of speech, and nausea [Abramson, 1997]. It has been shown to be carried over from chicken feed into the muscle tissue of chicken, thus posing a risk of contaminating human diet by chicken meat containing this toxin.

4.7. Metabolites from sponges

4.7.1. Relationship between structure and antimicrobial activities of isolated bromopyrrole derivatives from *Agelas nakamurai*

The ethyl acetate of A. nakamurai showed strong antimicrobial activities against *B. subtilis, S. aureus, E. coli and C. albicans*. Chemical investigation of the extract yielded the antimicrobially active bromo-pyrrole alkaloids: 4-bromopyrrole-2-carboxamide (compound **20**, Fig. 2.64b) and its new acid derivative 4-bromopyrrole-2-carboxylic acid (compound **21**, Fig. 2.64b), and the known metabolites mukanadin B (compound **22**, Fig. 2.64b) and C (compound **23**, Fig. 2.64b). It has been established that these brominated compounds which are produced by sponges in

Agelas [Urban *et al*., 1999], *Axinella* [Walker *et al*., 1981], *Hymedinacidon* [Kobayashi *et al.,* 1997], *Stylotella* [Kato *et al.,* 1995], and *Pseudoceratina* [Tsukamoto *et al.,* 1996a] serve as chemical defense for the sponges against the attack of fishes [Wilson *et al*., 1999; Lindel *et al*., 2000]. Bromopyrrole derivatives have also shown a variety of biological activities including antimicrobial (Urban *et al*, 1999; Walker *et al.*, 1981), tyrosine kinase inhibitory (Kobayashi *et al.,* 1997), and antifouling (Kato, *et al.*, 1995; Tsukamoto *et al.*, 1996b) activities.

Compounds **20, 21,** and **23** showed antimicrobial activities against Gram-positive and Gram-negative bacteria. This observation, when considered with a literature survey of bromopyrrole alkaloids isolated from sponge (Table 4.1), appears to prove that a bromo substituent at C-4 and an amide, carboxyl or a cyano group are required for antimicrobial activity. When the amide is substituted, the antimicrobial activity seem to disappear as in compound **22** (entry 3 in Table 4.1), or to became selective as in compound **23** (entry 2 in Table 4.1). When a second bromo group is present at C-5, activity seems to be retained when an amide group is present at C-2 as in longamide (entry 8 in Table 4.1) and the antimicrobially active compound sceptrin (Fig. 2.64a). Entries 8 and 9 in Table 4.1 which both possess two bromine atoms and a carboxyl and cyano groups at C-2, respectively, were antimicrobially inactive. When the bromo substituent is only at C-5, activity was seen to disappear or to be more selective, as exemplified by compounds 7 and 6 in Table 4.1, respectively.

4.7.2. Relationship between structure and cytotoxic activities of jaspamide and its derivatives

The sponge *Jaspis splendens* yielded the peptides jaspamide (jasplakinolide), jaspamide B and jaspamide C. Jaspamide is well known for its antiproliferative activity. A literature report comparing the cytotoxic activities of the three jaspamide derivatives against human NSCLC-N6 cancer cell line found jaspamide B and C to possess slightly lower activities with IC_{50} values of 3.3 and 1.1 μg/mL, respectively, compared to an IC_{50} value of 0.36 μg/mL for jaspamide (Zampella, *et al*, 1999). Since there is no drastic change in the core structure of the molecule, this difference might be due to the decreased polarity of jaspamide which might allow it greater permeability through cell membranes as its more polar congeners.

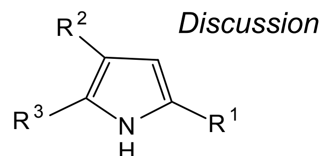


Table 4.1. Biological activites of known bromopyrrole derivatives.

R^1	R^2	R^3	Source	Activity	Reference
1. COOH	Br	H	*A. nakamurai*	Antimicrobial activity against Gram positive and Gram negative bacteria	present work
2. CONH-R^4	Br	H	*A. nakamurai*	Antimicrobial activity against Gram positive bacteria	present work
3. CONH-R^5	Br	H	*A. nakamurai*	No antimicrobial activity	present work
4. $CONH_2$	Br	H	*A. nakamurai*	Antimicrobial activity against Gram positive bacteria and Gram negative bacteria	present work
5. $CONH_2$	Br	H	*Acanthella carteri*	Cytotoxic against NSCLC-N6 human non-small-cell-lung carcinoma (IC_{50} 4.8 μg/mL)	Mancini *et al*., 1997
6. $CONH_2$	H	Br	*A. nakamurai*	Antimicrobial activity against Gram positive bacteria and fungi	Iwagawa, *et al*., 1998
7. CONH(CH_2OMe)	H	Br	*A. nakamurai*	no antimicrobial and cytotoxic activities	Iwagawa, *et al*., 1998
8. CONH-R^4	Br	Br	*Agelas longissima*	Moderate antimicrobial activity	Cafieri *et al*., 1995]
9. CN	Br	Br	*Agelas oroides*	Moderate cytotoxicity; no antimicrobial, antifungal, algicidal, antimalarial activities	König, *et al*., 1995]
10. COOH	Br	Br	*A.oroides* *A. sventres*	No cytotoxic, antimicrobial, antifungal, algicidal, antimalarial activites Antifeedant against *Thalassoma bifasciatum* at a min conc of 0.8 mg/ml	[König, *et al*., 1995]; [Assman, *et al*., 2001]
11. $COOCH_3$	Br	Br	*Agelas oroides*	No antimicrobial and cytotoxic activities	König, *et al*., 1995
12. $CONH_2$	Br	Br	*A.oroides* *A.mauritiana*	Antifouling activity against the ascidian *Ciona savignyi* at a conc of 2.5 μg/mL) Cytotoxic against NSCLC-N6 human non-small-cell-lung carcinoma (IC_{50} 9.4 μg/mL)	[Tsukamoto *et al*., 1996b]; [Mancini *et al*., 1997]

R4 = fused ring of mukanadin C (compound 23, Fig. 2.64b); R5 = aliphatic chain of mukanakin B (compound 22, Fig. 2.64b)

5. Summary

Low-molecular mass natural products from bacteria, fungi, plants and marine organisms exhibit unique structural diversity which are of interest for the identification of new lead structures for medicinals and agrochemicals. In the search for bioactive compounds from marine sponges and sponge-associated fungi, this research work resulted to the isolation of twenty-six compounds, eight of which are new metabolites. The sponges were collected from the Indo-pacific regions, particularly those from Indonesian and Philippine waters, as well as those from the Mediterranean Sea near the island of Elba in Italy. A combination of the chemically- and biologically-driven approach for drug discovery was employed, wherein extracts were screened for antibacterial, antifungal and cytotoxic activities. In addition to the bioassay-guided approach to purify the compounds responsible for the activity of the extract, TLC, UV and MS were also used to isolate the chemically most interesting substances. Hence, purified compounds which are not responsible for the initial bioscreening activity may have a chance to be evaluated for other bioactivities.

Enumerated below are the compounds which have been isolated and structurally elucidated and whose bioactivities have been further characterized.

1. The extract of the fungus *Cladosporium herbarum* associated with the sponge *Callyspongia aerizusa* afforded seven structurally related polyketides, including two new twelve-membered macrolides: pandangolide 3 and 4, and a new acetyl congener of the previously isolated 5-hydroxymethyl-2-furoic acid. The two furoic acid analogues isolated were found to be responsible for the antimicrobial activity of the extract. The isolation of the known phytotoxin Cladospolide B from *Cladosporium herbarum*, which was originally known from *Cladosporium cladosporioides* and *C. tenuissimum,* indicates the possibility that Cladospolide B may be a chemotaxonomic marker of particular *Cladosporium* species.

2. The extract of the fungus *Curvularia lunata* associated with the Indonesian sponge *Niphates olemda* yielded three compounds, namely the new antimicrobially-active anthraquinone lunatin, the known bisanthraquinone cytoskyrin A, and the known plant hormone abscisic acid. The co-occurrence of

the two structurally-related anthraquinones suggests that the monomeric lunatin may be a precursor in the biosynthesis of the bisanthraquinone cytoskyrin A.

3. The fungus *Penicillium spp.* associated with the Mediterranean sponge *Axinella verrucosa* yielded six compounds, namely the known antifungal griseofulvin and its less active dechloro analogue; the known toxin oxaline; and the known cytotoxic metabolite communesin B and its two new congeners communesin C and D. The new communesins were less active than communesin B in the brine-shrimp lethality test.

4. An unidentified fungus which was also isolated from the same Mediterranean sponge *Axinella verrucosa* as *Penicillium spp.* yielded the known compound monocerin which has been reported to possess phytotoxic and insecticidal activities.

5. The fungus *Aspergillus flavus* associated with the Philippine sponge *Hyrtios aff. reticulatus* yielded the known toxin α-cyclopiazonic acid.

6. The Indonesian sponge *Agelas nakamurai* yielded four bromopyrrole alkaloids namely the new compound 4-bromo-pyrrole-2-carboxylic acid, and the known compounds: 4-bromo-pyrrole-2-carboxamide, mukanadin B and mukanadin C. All of the four compounds except mukanadin B were found to be antimicrobially-active. Bromopyrrole alkaloids are well-known metabolites of the genus *Agelas* and are proven to play an important role in the chemical defense of the sponge against predation from fishes.

7. The Indonesian sponge *Jaspis splendens* yielded three known substances which are known for their antiproliferative activities, namely the depsipeptides jaspamide (jasplakinolide), and its derivatives jaspamide B and jaspamide C.

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List of Abbreviations

$[\alpha]_D$	: specific rotation at the soldium D-line
BuOH	: butanol
br	: broad signal
CD	: circular dichroism
CI	: chemical ionization
COSY	: correlation spectroscopy
d	: doublett
dd	: double doublett
ddd	: double double doublett
DEPT	: distortionless enhancement by polarization transfer
DMSO	: dimethylsulfoxide
DNA	: deoxyribonucleic acid
ED	: effective dose
EI	: electron impact
ESI	: electron spray ionization
EtOAc	: ethyl acetate
eV	: electronvolt
Fig	: Figure
g	: gram
h	: hour
HMBC	: heteronuclear multiple bond connectivity
HMQC	: heteronuclear multiple quantum coherence
HPLC	: high performace liquid chromatography
Hz	: herz
IR	: infrared spectroscopy
LC	: lethal concentration
lit.	: literature
m	: multiplett
MeOD	: deuterated methanol
MeOH	: methanol
mg	: milligram
mL	: milliliter

μg	: microgram
μL	: microliter
mRNA	: messenger-RNA
MS	: mass spectrometry
m/z	: mass per charge
n.a	: no activity
ng	: nanogram
nm	: nanometer
NMR	: nuclear magnetic resonance
NOE	: nuclear Overhauser effect
ppm	: parts per million
q	: quartett
RNA	: ribonucleic acid
RP-18	: reversed phase C-18
s	: singlett
t	: triplett
TFA	: trifluoroacetic acid
TLC	: thin layedr chromatography
UV	: ultra-violet

Publikationsliste

Jadulco R, Proksch P, Wray V, Sudarsono, Berg A, and Gräfe U (2001): New macrolides and furan carboxylic acid derivative from the sponge-derived *Cladosporium herbarum*, *J Nat Prod* **64**, 527-530.

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