# Isolation and structure elucidation of bioactive secondary metabolites of sponge-derived fungi collected from the Mediterranean sea (Italy) and Bali sea (Indonesia)

[**Isolierung und Strukturaufklärung bioaktiver Sekundärstoffe aus schwammassoziierten Pilzen aus dem Mittelmeer (Italien) und dem Balimeer (Indonesien)**]

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**Vorgelegt von**
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*To my family*

# Erklärung

Hiermit erkläre ich ehrenwörtlich, daß ich die vorliegende Dissertation „Isolierung und Strukturaufklärung bioaktiver Sekundärstoffe aus schwammassoziierten Pilzen aus dem Mittelmeer (Italien) und dem Balimeer (Indonesien)“ selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel angefertigt habe.

Diese Dissertation wurde weder in gleicher noch in ähnlicher Form in einem anderen Prüfungsverfahren vorgelegt. Außerdem erkläre ich, daß ich bisher noch keine weiteren akademischen Grade erworben oder zu erwerben versucht habe.

Düsseldorf, den 4.11.2003

Hefni Effendi

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# ZUSAMMENFASSUNG

Aus 79 schwammassoziierten Pilzstämmen, isoliert von aus Elba (Italien) und Westbali (Indonesien) stammenden Schwämmen, wurden aufgrund der Ergebnisse eines Bioaktivitätscreenings fünf Stämme zur weiteren Bearbeitung ausgewählt.

Die Proben wurden vor Ort von Tauchern gesammelt und später im Alfred Wegener Institut (AWI) für Marine und Polare Ökologie, Bremerhaven, und im Institut für Pharmazeutische Biologie, Heinrich-Heine Universität, Düsseldorf, Deutschland, kultiviert.

*Penicillium* sp. sowie *Verticillium* cf *cinnabarium* wurden von dem aus Elba stammenden Schwamm *Ircinia fasciculata* isoliert, *Fusarium* sp. von *Axinella damicornis* (ebenfalls aus Elba) und *Lecanicillium evansii* 1 und 2 aus den westbalineschen Schwämmen *Callyspongia* sp. and *Hyrtios* sp.

Das Bioaktivitätscreening, das zur Auswahl dieser fünf Stämme führte, beinhaltete die Untersuchung ihrer Wirkung auf brine shrimp (*Artemia salina*), Insekten (*Spodoptera littoralis*) und ihrer antimikrobiellen Aktivität [grampositive Bakterien (*Bacillus subtilis*, *Staphylococcus aureus*), gramnegative Bakterien (*Escherichia coli*), und drei Pilze (*Saccharomyces cerevisiae*, *Candida albicans*, *Cladosporium herbarum*)].

Bei der Auswahl dieser Stämme zur weiteren Bearbeitung wurden weiterhin die Verteilung der aus den ESI-MS Spektren zur entnehmenden Molekulargewichte und die Einzigartigkeit der UV Spektren der im jeweiligen Rohextrakt enthaltenen und in der HPLC sichtbaren Verbindungen berücksichtigt.

Die Kultivierung der Stämme 1, 2, und 3 in Form von 10 l Standkulturen in Wickerham Medium sowie deren taxonomische Einordnung wurden am AWI vorgenommen. Die Stämme 4 und 5 wurden durch das Centraalbureau voor Schimmelcultures, Baarn, die Niederlande identifiziert und erwiesen sich als neue Spezies. Diese beiden Stämme wurden in Standkulturen in Wickerham Medium am Institut für Pharmazeutische Biologie, der Heinrich-Heine Universität Düsseldorf, kultiviert.

Insgesamt 31 Verbindungen konnten nach Extraktion der Kulturen und Isolierung der gebildeten Sekundärstoffe aufgeklärt werden. Sechs dieser Verbindungen wurden von *Penicillium* sp. isoliert. Es waren Anthrachinone (Emodin, Hydroxyemodin), ein Alkaloid (Meleagrin), ein Bipeptid (Cyclo-leucylprolyl) und Triterpene (Citreohybridonol, Andrastin A).

Acht weitere Verbindungen stammen aus *Verticillium* cf *cinnabarium*, darunter einfache aromatische Verbindungen (3-Hydroxyanthranilsäure, 4-Hydroxybenzaldehyd, Tyramin), Bipeptide (Cyclo-alanyltryptophan, Cyclo-prolylvalyl, Cyclo-leucylprolyl), ein Alkaloid (Verticillin B) und ein Steroid (Lichesterol).

Drei Verbindungen (das Steroid Ergosterolperoxid, ein Triterpenacetat und ein Cerebrosid) konnten aus *Fusarium* sp. isoliert werden.

Die zwei neuen Stämme von *L. evansii* enthielten sehr verschiedenartige Metabolite. Dies fiel bereits durch das Verteilungsmuster der einzelnen Peaks in der HPLC der Rohextrakte auf und konnte später bestätigt werden.

Beide Stämme von *L. evansii* schienen in einem Medium ohne Zusatz von Meersalz zunächst viel schneller zu wachsen. Nach sieben Tagen des Wachstums waren jedoch keine merklichen Unterschiede hinsichtlich der Farbe und Dichte des Myzels mehr zu erkennen. Die Oberfläche jedes Kulturmediums war vollständig von dem weißen Myzel bedeckt.

Die *L. evansii* Kultur (Stamm 2), die ohne den Zusatz von Meersalz gezüchtet wurde, enthielt eine größere Bandbreite an Sekundärstoffen, was durch die Anzahl an Peaks im HPLC-Spektrum belegt werden konnte. Für Stamm 1 traf dies jedoch nicht zu.

Aus *L. evansii* (Stamm 1) konnten sieben Verbindungen isoliert werden, darunter phenolische Verbindungen (Terphenylin, Deoxyterphenylin, Terprenin 2, Terpreninepoxid), ein Bipeptid (Cyclo-

tyrosylprolyl) und einfache aromatische Verbindungen (Acetylhydroxybenzamid, 4-Hydroxybenzaldehyd).

Stamm 2 von *L. evansii* enthielt sieben Verbindungen: Nukleoside (Cytosinribosid, Cytosindesoxyribosid, Adenosinribosid, Adenosindesoxyribosid), Steroide (Ergosterolperoxid, Dehydroergosterolperoxid), und ein Cerebrosid (Cerebroside C).

Unter diesen Verbindungen befinden sich auch vier neue Naturstoffe (ein Triterpenacetat, Deoxyterphenylin, Terprenin 2, und Terpreninepoxid).

Bei dem Triterpenacetat handelt es sich aufgrund des Vorhandenseins einer Hydroxyl-Gruppe in Position 2 um eine neue Verbindung im Gegensatz zu ähnlichen bereits bekannten Verbindungen (Tabelle 5.1).

Bereits 100 µg Triterpenacetat zeigten eine starke Aktivität gegen *S. aureus*. Der Hemmhof zeigte eine Größe von 7 mm im Durchmesser. Außerdem konnte eine cytotoxische Wirkung auf menschliche Krebszelllinien festgestellt werden mit einer Wachstumshemmung von 91.25% (JURKAT), von 36.00% (THP-1) und von 85.67% (MM-1).

In dem neuen Deoxyterphenylin befindet sich eine Methoxygruppe in Position 4“ im Gegensatz zu der bereits bekannten Verbindung, wo sie sich in Position 2‘ befindet (Tabelle 5.1). Die ungewöhnliche Prenylseitenkette, die in Position 3 direkt an den Phenylring gebunden und nicht wie sonst über einen Sauerstoff verbunden ist, ist ein Charakteristikum für das neue Terprenin 2. In dem neuen Terpreninepoxid befindet sich eine Epoxidseitenkette (Tabelle 5.2).

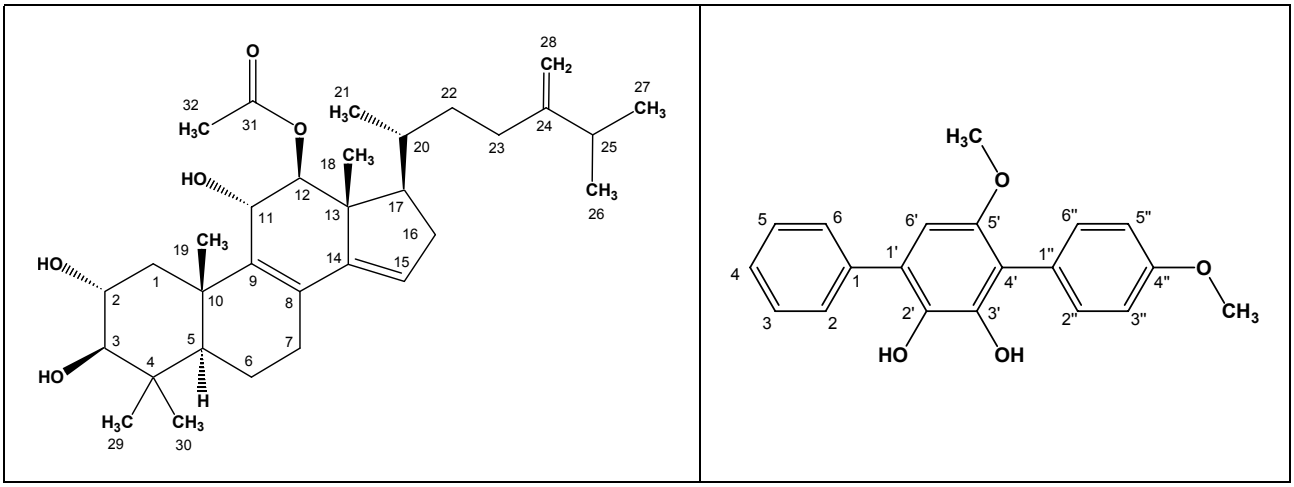


Tabelle 5.1. Triterpenacetat (Verbindung **16**) (links) und Deoxyterphenylin (Verbindung **19**) (rechts)

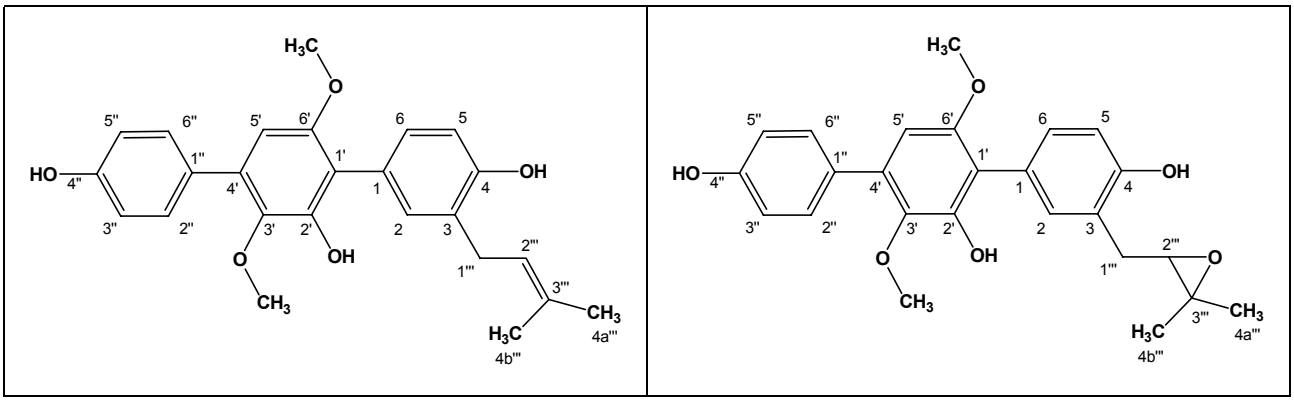


Tabelle 5.2. Terprenin 2 (Verbindung **20**) (links) und Terpreninepoxid (Verbindung **21**) (rechts)

# I. INTRODUCTION

## 1.1. Primary and secondary metabolites

The primary metabolism of an organism is the summation of an interrelated series of enzyme-catalysed chemical reactions (both degradative and synthetic) which provide the organism with its energy, its synthetic intermediates and its key macromolecules such as protein and DNA. On the other hand, secondary metabolism involves mainly synthetic processes whose end products, the secondary metabolites, play no obvious role in the economy of the organism. Whereas primary metabolism is basically the same for all living systems, secondary metabolism is restricted to the lower forms of life (e.g. species, and often strain specific) (Turner, 1971).

Other description of primary metabolism refers to all biochemical processes for the normal anabolic and catabolic pathways which result in assimilation, respiration, transport, and differentiation. Primary metabolism shared by all cells are virtually identical in most organisms. They include ubiquitous small molecule such as: sugar, amino acids, tri-carboxylic acid, or Krebs cycle intermediates, the universal building block and energy source-as well as proteins, nucleic acids, and polysaccharides, that differ in structural detail from one organism to another, but which appear to have universal function as enzyme, structural, and hereditary material. Many sugars (glucose), amino acid, low-molecular weight organic acids, many fatty acids, and some proteins are identical in animal, bacteria, fungi, plants, and other organisms (Haslam, 1986; Seigler, 1998; Torssell, 1997).

Secondary metabolisms generate diverse and seemingly less essential or non-essential by-products called secondary products. The secondary products, having no role in the basic life process, are produced by pathways derived from primary metabolic routes. Secondary metabolite products accounting for the plant colours, flavours, and smells, are sources of fine chemicals, such as: drugs, insecticides, dyes, flavours, and fragrances, and the phyto-medicines found in medicinal plants. The concept of secondary metabolism was first introduced by Kössel (1891) (Hartmann, 1985; Haslam, 1986; Seigler, 1998; Turner, 1971).

An alternative and perhaps more helpful nomenclature is to refer to metabolites as either general metabolites which are produced by a large number of organisms, or primary and secondary metabolites, which are produced by a restricted number of organisms. By this system, citric acid and ethanol can be considered to be general metabolites whereas pullulan is a special metabolite. However, citric acid and ethanol are produced in usefully high concentrations by only a few special organisms (Berry, 1988). Three following hypothesis emerged, suggesting the role of secondary metabolites.

### 1.1.1. The waste product hypothesis

The role of the secondary products has been rather ambiguous, and initially they were thought to be just waste materials. The relatively large number and amount of secondary metabolites observed in nature and the notion that these compound arose from “errors” in primary metabolisms in plants, led to the idea that secondary compounds arise and accumulate as “waste product”. However, considering their non-motile nature and the lack of sophisticated immune system, plants have to develop their own defence system against pathogens and predators, and systems to lure motile creatures, for fertilisation and dissemination (Luckner, 1990; Mothes, 1976; Seigler, 1998).

### 1.1.2. The overflow or excess primary metabolism hypothesis

In instance of unbalanced growth, secondary metabolites have been envisioned by some as shunt metabolites produced in order to reduce abnormal concentration of normal cellular constituents. The synthesis of enzymes designed to carry out secondary metabolism permits primary metabolic enzymes to continue to function until such time as circumstances are propitious for renewed metabolic

activity and growth. This could be linked to the depletion of nutrients such as phosphorus or nitrogen (Bu'Lock, 1980; Haslam, 1986).

### 1.1.3. The increased fitness hypothesis

This hypothesis is the only one that accounts for the fact that many natural products trigger very specific physiological responses in other organisms and in many cases bind to receptors with a remarkable complementary. In other words, natural products may aid an organism's survival in the absence of an immune system. This supports the hypothesis that secondary metabolites increase the fitness of individuals that possess them and that those individuals have been favoured by the process of natural selection. Secondary metabolites have an important ecological role in the interaction with the environment, and are like the communication interface between a plant and its friend and enemies in the environment (Harborne, 1986; Rosenthal and Johnson, 1979; Swain, 1977; Torssell, 1997).

The distinction amongst natural products has been recognised, because of their apparently secondary role. The substances in this group are commonly referred to as secondary metabolite. Many of the secondary products are bactericidal, repellent (by bad tastes, etc), or even poisonous to pests and herbivores. Pigments of flowers would give attractive colours for insects that help with fertilisation, or warning colours against predators. Plant pigments also provide protection against environmental harms, such as: free radicals and UV irradiation. Some of the secondary products perform signalling functions as plant hormones (Haslam, 1985).

Furthermore, many of these secondary products are originally meant for defence against herbivores such as insects which would soon come up with metabolic pathways to detoxify and even utilise these defence compounds. During evolutionary processes, animals developed a variety of dependencies to phyto-chemicals, including the secondary products that are, with or without modification, used as precursors for the synthesis of vital or beneficial molecules in the animal body.

Secondary plant products have for thousands of years played an essential role in medicine. Traditionally, they have been directly used as food and herbs. Nowadays, they are used either directly or after chemical modification. Plant, microorganism, and marine macroorganismm secondary metabolites represent a tremendous resource for scientific and clinical researches and new drug development. Overall, their pharmacological value not only remains undiminished until today, but is increasing due to constant discoveries of their potential roles in healthcare and as lead chemicals for new drug development.

## 1.2. Products of secondary metabolism

Secondary metabolic products constitute a wide array of natural products and are more complex than primary metabolic products. This is because secondary metabolites are derived from the primary products, such as amino acids or nucleotides, by modifications, such as: methylation, hydroxylation, and glycosylation (Bentley and Bennet, 1988).

The investigation of chemicals produced by plants and animals widely known as natural products chemistry has resulted in the discovery of numerous organic chemicals, many of which have found applications as pigments, fragrances, insecticides, pharmaceuticals, or biomedical tools. Previous studies, which focused on terrestrial plants and microorganisms, proved extremely fruitful, yielding many useful organic compounds, including approximately 25% of the currently used anticancer drugs, with another 25% coming from synthetic derivatives of natural products (Davidson, 1995).

Natural products will continue to be important in three areas of drug discovery: 1) as targets for production by biotechnology, 2) as a source of new lead compounds of novel chemical structure, and 3) as the active ingredients of useful treatments derived from traditional systems of medicine. Plants are not the only source of drugs. Microorganisms have been extensively screened for antibiotics. Many antibiotics such as streptomycin, neomycin, tetracycline, and chloramphenicol are produced by bacteria of the genus *Streptomyces* (Harvey, 1993).

The existence of protein compounds such as: anticancer agents (neocarzinostatin), enzyme inhibitors (tendamistat, subtilin), and lanthionine-containing antibiotics (epidermin, gallidermin) renders the border between primary and secondary metabolites to be at variance. As much as 20,000 microbial metabolites and approximately 100,000 plant products have been described so far, secondary metabolism appears as an inexhaustible source of new antimicrobials, antiviral, antitumor drugs, agricultural and pharmacological agents. Numerous secondary metabolites (benzylpenicillin, cephalosporin C, erythromycins, strobilurins, bialaphos, monacolins, polyoxins, etc.) served as lead structures for the synthetic and semi synthetic preparation of improved derivatives showing improved pharmacological properties (Dreyfuss and Chapela, 1994; Gräfe, 1999)

The major source of carbon and of energy for most heterotrophic organism is glucose, usually supplied as such in laboratory cultures and derived from carbohydrate (starch) in nature. The breakdown of glucose begins with its conversion to a triose, either by the Embden-Meyerhoft pathway or by the pentose phosphate cycle. The latter route also makes available pentoses, important in nucleotide biosynthesis, and a tetrose which can react with phosphoenolpyruvate to give shikimic acid. Shikimic acid is an intermediate for the aromatic amino acids and also for many aromatic secondary metabolites. The triose is also a precursor of serine which is converted to glycine with loss of a carbon atom which enters the C1-pool (Figure 1.2.1) (Turner, 1971).

Proceeding along the carbon pathway, the triose is converted first to pyruvate and then to acetyl coenzyme A (acetyl CoA) which is the most important single intermediate in fungal secondary metabolism. Carboxylation of acetyl CoA gives malonyl CoA, and linear condensation of acetyl CoA with several molecules of malonyl CoA leads either to the polyketides, the most numerous secondary metabolites of fungi or to the fatty acids which can in turn give rise to secondary metabolites. Alternatively, condensation of three molecules of acetyl CoA gives mevalonic acid, the key intermediate in terpene biosynthesis. Finally, by condensation of acetyl CoA with oxaloacetate, carbon from glucose enters the tricarboxylic acid (TCA) cycle which serves not only to complete the oxidation of glucose but also as a source of the carbon skeletons of several amino acids and of some secondary metabolites (Figure 1.2.1) (Turner, 1971).

Primary metabolism provides the precursors for secondary metabolism, with certain key intermediates being used more often than others. This allows a separation of secondary metabolites into four categories based on the biosynthetic precursors from which the compound is assembled and similarities to the major types of monomeric cellular constituents. Those categories include: 1) saccharides, 2) peptides, 3) acetogenins, and 4) nucleologues. However this simple classification is complicated by the mixed precursor origin of many products; substances of this kind without a dominant pathway are therefore placed in a fifth category (miscellaneous) that also includes products derived from less commonly used primary precursors (Vining, 1986).

Secondary metabolites are widely known also as natural products, relatively small and complex organic compounds. Natori (1974) classified secondary metabolites, despite their enormous diversity, on the basis of molecular skeleton as follow.
- Open-chain aliphatic or fatty compounds: fatty acids, sugars, most amino acids.
- Acyclic or cyclo aliphatic compounds: terpenoids, steroids, some alkaloids.
- Aromatic compounds : phenolic, quinones.
- Heterocyclic compounds : alkaloids, flavonoids, nucleic acid bases.

Of the top 20 best-selling pharmaceutical products in 1990, four were derived from natural products (amoxycillin, cefaclor, ceftriaxone, and lovastatin), and two others (captopril and enalapril) resulted from leads provided by a natural product (Harvey, 1993).

## 1.3. Marine natural products

Life originated in the sea and has sustained itself to the present day. The world's oceans comprise the largest part of the biosphere and contain the most ancient and diverse forms of life. The marine biotopes contain an unmatched metabolic and biodiversity. Over billions of years marine organisms have moulded the global climate and structured the atmosphere. The quantum leap of our

understanding of life during the last five years through the advent of genomics and bioinformatics at an organismal, cellular, or genetic level, has opened new perspectives (Anonymous, 2001).

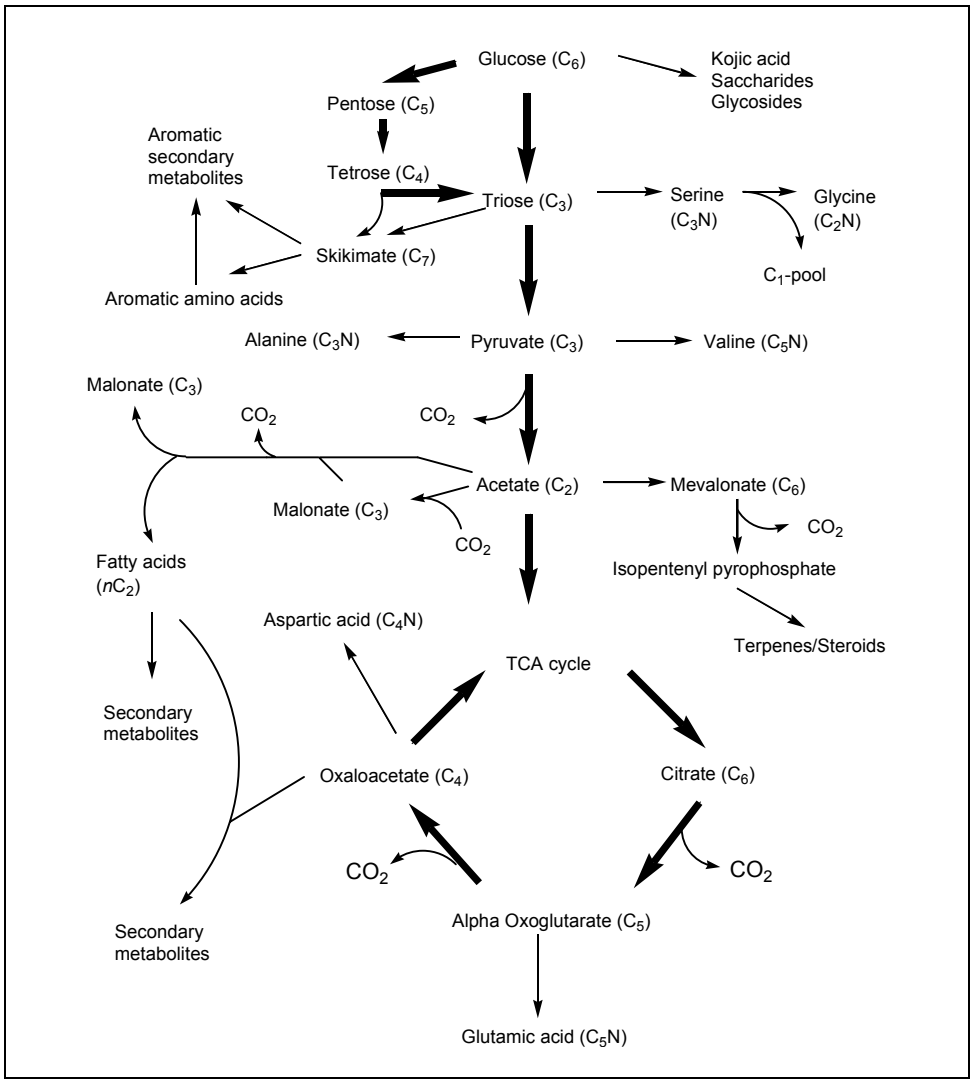



Figure 1.2.1. Scheme of the formation of secondary metabolites from the intermediates of primary metabolism (The heavy lines show the main pathways for the oxidation of glucose) (Turner, 1971)

The marine environment comprises 71 % of the earth's surface, and consists of extreme and contrasting habitats, ranging from tropical reefs to ice-shelf of the polar seas, and to black smokers in the deep sea. All life has derived from the oceans. It is estimated that 90% of all species of living organisms are to be found in the oceans. Totally different biosynthetic conditions exist in the marine environment, from those encountered on land. Many marine organisms have retained or attained a different evolutionary stage than have land-based plants and animals (Baker, 1984, Whitehead, 1999).

The biodiversity in the worlds oceans is immense, of 33 known animal phyla 15 are exclusively marine, and 32 of them have marine representatives. Some habitats are known to be particularly numerous in species. Tropical marine reefs, which represent the most diverse ecosystems encountered on earth, are comparable in diversity to tropical rain forest (de Vries and Beart, 1995; Norse, 1995).

The marine environment is literally a soup of essentially all imaginable types of microorganisms. They may occur suspended, so-called bacteria-plankton, on living or inanimate surfaces or as symbionts.

Microorganisms play important roles in all of the major element cycles in the oceans, and are intimately involved in many ecological phenomena. The marine ecosystem is unique in terms of its specific composition in organic and inorganic substances (ranging from eutrophic to oligothrophic), temperature ranges (ranging from –1,5°C in Antarctic to excess of 100°C in hot springs), pressure conditions (ranging from 1 to over 1000 atmosphere), and extensive photic and non-photic zones. Ecological niches e.g. deep-sea hydrothermal vents (temperature reaching 350°C), mangrove forest, provide habitats for the evolution of specialised microorganisms (Cowan, 1997; König and Wright, 1999).

The world oceans do indeed represent a microbial broad and microbiologically diverse resource of huge dimension about which we know relatively little. As it is estimated that less than 5% of marine bacterial and fungal species are known. Recent studies of microbial variety using analysis of RNA sequences have shown that marine microbial picoplankton (0.2 – 2.0 μm) contains a high abundance of rare species e.g. archaeal species accounting for 34%, virtually non of which have ever been isolated and chemically investigated (Biabani and Laatsch, 1998; Cowan, 1997)

It is estimated that less than 0.1% of marine microorganisms can be readily recovered by standard cultivation techniques. However, this problem may now be approached by the application of molecular phylogenetic analyses. Phylogenetically informative genes can be isolated from nucleic acids extracted from mixed microbial populations. These genes can be clonally isolated, sorted, and sequenced using standard molecular biological tools (Anonymous, 2001).

For millennia, the oceans have been a source of food, minerals, and natural products. The sea contains a wide and largely unexplored diversity of life and environment. The infrastructure and expertise are available for large scale bio-prospecting in order to identify and collect a variety of organisms or genes of potential use. Bio-screening then selects out those with the most desirable characteristics (Anonymous, 2001).

Extracted from marine mollusc, the vibrantly coloured dye tyrian purple was behind a flourishing industry for the Phoenicians about 1600 BC. Interest in this pigment declined in the middle ages and, apart from vitamins A and D from cod fish oil, it was not replaced by any other economic venture with marine natural products until quite recently (Bongiorni and Pietra, 1996). Some examples of products derived from marine organism that have long been used for the sake of human kind are presented in Table 1.3.1 (Anonymous, 2001).

Table 1.3.1. Natural products derived from marine organisms

| Products | Specific product | Sources | Uses |
|---|---|---|---|
| Algal polysaccharides | Carrageenans, Agars, Alginates | Red algae | Cosmetics, thickener, pharmacy, mucoprotector, anti coagulant, antiviral |
| Glycosamino-glycans | Chondroitin sulphate | Fish | Cosmetics, tissue replacement, anti coagulant |
| Collagen | | | Cosmetic, artificial tissue |
| Chitosan | B(1-4) *N*-acetyl glucosamine | Crustacean, shells, fungi | Cosmetics, colloids, pharmacy, microencapsulation |
| Lipids | Long chain PUFA (AA/arachidonic acid, EPA/eicosapentaenoic acid, DHA/docosohexaenoic acid) | Microalgae, seaweed, fish | Prevention of heart disease, mental development in premature children, prevention of atheosclerosis, antitumor, lipid metabolism |
| Peptides | Hormones, cyclic peptides | Fish hydrolysates | Antioxidant, immunostimulants, nutraceutical products |

The arabinose-nucleosides, known since the 1950's as constituents of the Caribbean sponge *Cryptotethya crypta* (Tethydae) which served as lead compounds for the synthesis of analogues, ara-A (Vidarabin, Thilo) and ara-C (Cytarabin, Alexan, Udicil) with improved antiviral and anticancer

activity, respectively. The discovery of sizeable quantities of prostaglandins, which had just been discovered as important mediators involved in inflammatory disease, fever and pain in the gorgonian *Plexaura homomalla* by Weinheimer and Spraggnis in 1969 is considered as the take-off point of systematic investigation of marine environments as sources of novel biologically active agents (Newman *et al*., 2000; Proksch *et al*., 2002).

By 1975 there were already three parallel tracks in marine natural product chemistry: marine toxins, marine biomedicinals and marine chemical ecology. Prior to 1995, a total 6500 marine natural products had been isolated; by January of 1999, this figure had risen to approximately 10,000. When compared with the 150,000 natural products obtained from terrestrial plants, this is a relatively small number of compounds, however an increase of 50% over a period of four years clearly represents an explosion of interest in natural products obtained from the marine environment. From 1969 – 1999 approximately 300 patents on bioactive marine natural product were issued (Faulkner, 2000; Proksch *et al*., 2002; Whitehead, 1999).

It is interesting to note that the majority of marine natural product currently in clinical trials or under preclinical evaluation is produced by invertebrates such as: sponges, tunicates, molluscs or bryozoans but not by algae. This is in sharp contrast to the terrestrial environment where plants by far exceed animals with regard to the production of bioactive natural product (Proksch *et al*., 2002). Most marine bioproduct have, as yet, been derived from relatively shallow-water organism using routine methods (i.e. scuba diving). Evaluation of the pharmaceutical, cosmetic, nutritional, and chemical potential of products derived from a deep water organisms has been limited, although at least one compound, discodermolide derived from a deep water sponge (Gunasekera *et al*., 1990; Pomponi, 1999)

Some marine organism living on a reef have neither fins, scales, fangs, claws, nor other sophisticated immune systems. In order to survive, proliferate and in response to a variety of ecological, behavioural, and physiological factors, they must produce compounds (allelo-chemical) through accumulation of toxic or distasteful natural products that are used to fight off potential predators or to force back neighbours competing for space. These metabolites also have therapeutic potential against human disease because of their very specific interaction with receptors and enzyme (McConnell *et al*., 1994; Proksch *et al*., 2002).

Sessile, soft-bodied marine invertebrates that lack obvious physical defences are therefore prime candidates to possess bioactive metabolites. Sessile marine invertebrate have a very long evolutionary history and have had ample opportunity to perfect their chemical defences. If it is assumed that secondary metabolites evolved from primary metabolites in a random manner, any newly produced secondary metabolite that offered an evolutionary advantage to the producing organism would contribute to the survival of the new strain (Faulkner, 2000).

Among the many phyla found in the oceans, the best sources of pharmacologically active compounds are bacteria (including cyanobacteria), fungi, certain algae, sponges, soft coral gorgonians, sea hare nudibranchs, bryozoans, and tunicates. Some marine organism such as: dinoflagellates, echinoderms and some fish, are well-known for their ability to produce potent toxins, but these are usually too toxic for medicinal use (Faulkner, 2000). Marine natural products are distinguished by a great chemical diversity. Halogenated compounds are particularly numerous due to the natural abundance of chlorine, bromine and to a lesser extent iodine in sea water (de Vries and Hall, 1994; Gribble, 1996; Hay and Fenical, 1996).

Compared to other marine organisms, marine fungi are poorly investigated. Due to living conditions and functions in the ecosystem, they are expected to produce a vast number of biologically active substances with new types of structure, although only a small number of potential drugs have yet been isolated from the cultivation of marine fungi (Fenical, 1997; Liberra and Lindequist, 1995; Whitehead, 1999).

The complex microbial adaptations to growing in the ocean are fundamentally different from those in land-based organisms. Nutrients are scarce in the sea, forcing many microorganisms to associate with the nutrient-rich plants and animals found nearby. Microbial symbiosis is intense. This has resulted in microorganisms that produced variety of chemical compounds for defence and competition. These compounds form the foundation for the development of marine microorganisms as a major drug resource (Fenical, 1997).

The potential applications offered by the screening of marine substances extend to pharmacology, agrochemistry, and the environment. Moreover, the use of combined approaches enhances these possibilities because marine molecules often belong to new classes without terrestrial counterparts. High-throughput screening techniques are particularly suitable for such combined approaches. In addition, marine microorganisms are a source of new genes, the exploitation of which is likely to lead to the discovery of new drugs and targets (Anonymous, 2001)

Microbial extremophiles abundant in deep hydro-thermal vents, sub-seafloor sediments, hypersaline lagoons, methane seeps, and endosymbiotic within marine animals, are ideal targets for bio-prospecting unusual and efficient enzymes and drugs. Deep-sea hydrothermal vents now offer a new source of a variety of fascinating microorganisms well adapted to these extreme environments. This new bacterial diversity includes strains able to produce new molecules such as: enzymes, polymers, and other bioactive molecules. Among these polymers, poly-β-hydroxylalkanoates (PHA) are of special interest .

In the same range of high molecular weight biological polymers, chitin, a co-polymer of *N*-acetylglucosamine and glucosamine, and chitosan, its *N*-deacetylated form, are found associated with proteins in the exoskeletons of many invertebrates species; annelids, shellfish, insect, and also in the envelope of many fungi, moulds, and yeast. Chitin polymers are natural, non-toxic and biodegradable and they have many applications in food and pharmaceuticals as well as cosmetics (Anonymous, 2001).

Nowadays, toxic principles dominate the spectrum of biological activities isolated from marine sources. This fact may partly be due to the major application of cytotoxicity directed screening assays. Nevertheless defence strategies are necessary to survive in the highly competitive marine environment, thus resulting in a tremendous diversity of highly toxic compounds affecting numerous targets involved in eukaryotic cell signalling process (Grabley and Thierckle, 1999).

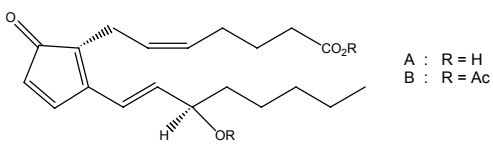
In search of marine natural products, sponges continue to dominate as a source of new compounds followed by coelenterates and the grouping of microorganism and phytoplankton (Table 1.3.2) (Blunt *et al.,* 2003).

Table 1.3.2. Distribution of marine natural products by phylum

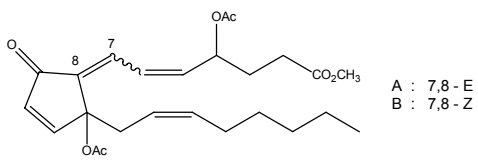
| Marine living system | Percentage of marine natural product |
|---|---|
| Sponges | 28 % |
| Coelenterates | 21 % |
| Microorganism and phytoplankton | 15 % |
| Molluscs | 7 % |
| Tunicates | 7 % |
| Red algae | 6 % |
| Echinoderms | 3 % |
| Brown algae | 2 % |
| Green algae | 1 % |

Some marine-derived compounds have generated considerable interest scientifically, commercially, and from a public and health point of view. These include prostaglandins; the potent and structurally complex toxin, palytoxin (derived from zoanthid, *Palythoa toxicus*); and one of the major causative agents of fish poisoning, ciguatoxin. Because of their unique and potent biological activities, several marine-derived compounds have already found use as biological probes or biochemical tools and sold commercially, i.e., brevetoxins B (isolated from dinoflagellate, *Gymnodinium breve*), palytoxin, okadaic acid, tetrodotoxin, saxitoxin, calyculin A, manoalide, and kainic acid (Figure 1.3.1) (McConnell *et al*., 1994).

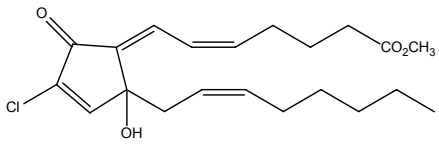
Of all the natural sources for drugs, the marine environment is clearly the last great frontier. Since the 1970s, chemists have been unravelling the fine structures of novel organic compounds produced by



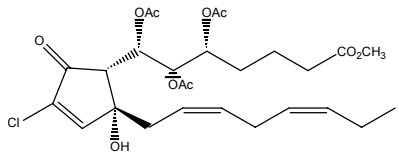

15-*epi*-$PGA_2$ (Prostaglandin)



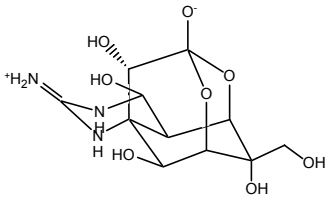

Clavulones (Prostaglandin)



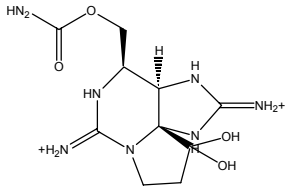
Chlorovulones (Prostaglandin)



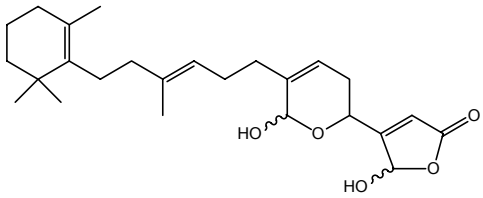
Punaglandin (Prostaglandin)



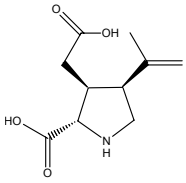
Tetrodotoxin



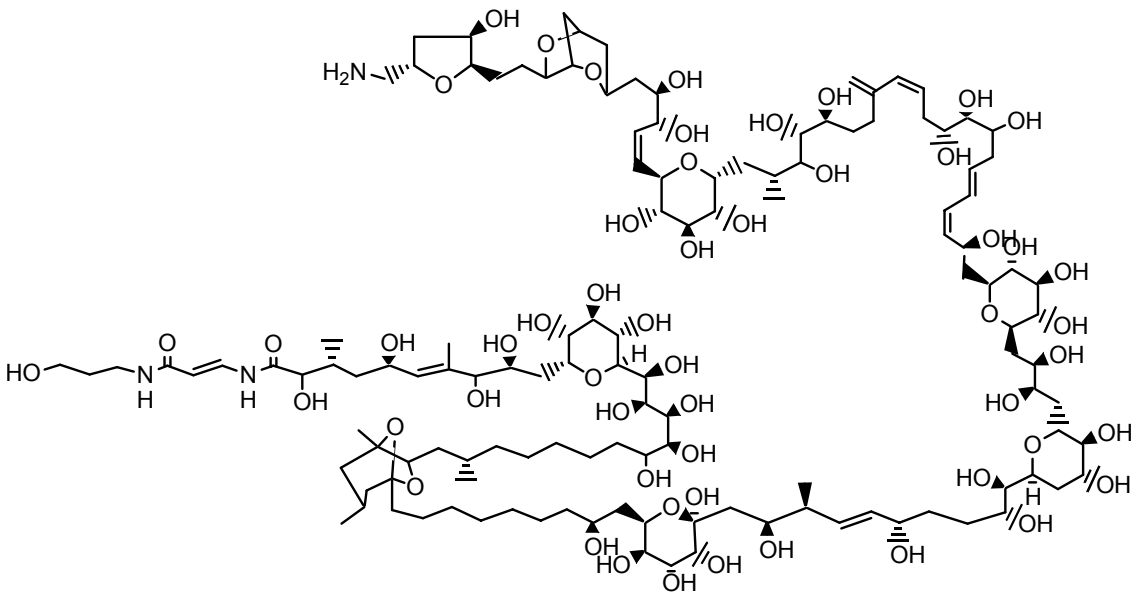
Saxitoxin



Manoalide

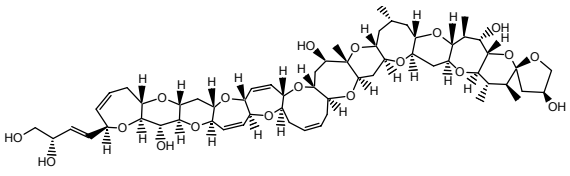


Kainic acid

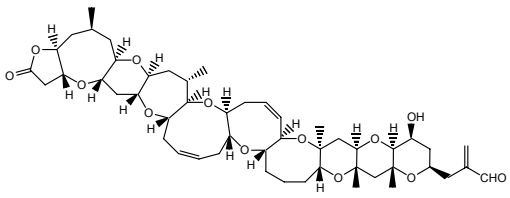


Palytoxin

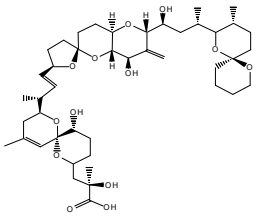
Figure 1.3.1. Several commercially available marine natural products



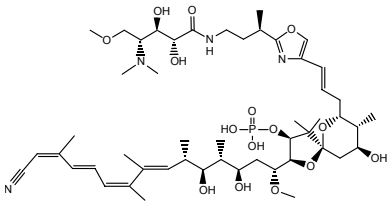
Ciguatoxin



Brevetoxin A

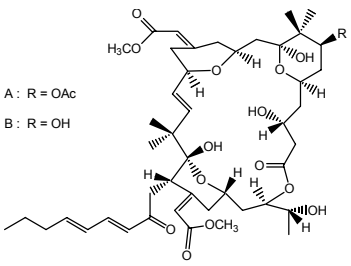


Okadaic acid

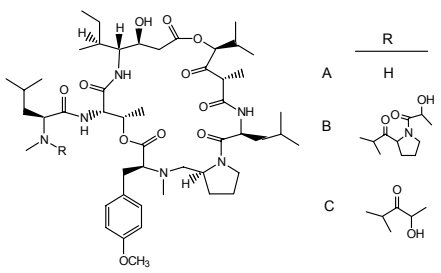


Calyculin A

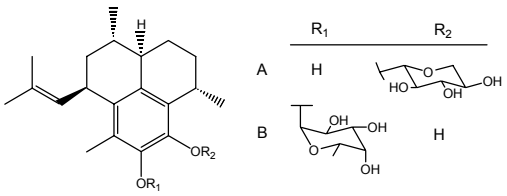
Figure 1.3.1. Several commercially available marine natural products (continued)




Bryostatin

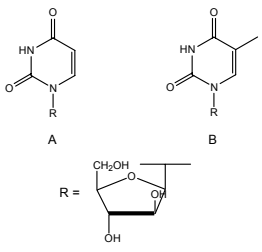



Didemnins

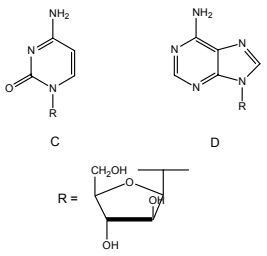



Pseudopterosins

Figure 1.3.2. Selected marine-derived compounds currently under clinical trial




Arabinosides A – B




Arabinosides C – D

Figure 1.3.3. Arabinosides A, B, C, D

marine plants and animals. The pharmaceutical industry now accepts the world's oceans as a major frontier for medical research. This emerging new field is sometimes called marine pharmacology. Although none of the discoveries has as yet led to a pharmaceutical product, there is a hope that one or more of the many marine natural products currently under investigation will eventually do so (Faulkner, 2000; Fenical, 1997).

Of all the natural sources for drugs, the marine environment is clearly the last great frontier. Since the 1970s, chemists have been unravelling the fine structures of novel organic compounds produced by marine plants and animals. The pharmaceutical industry now accepts the world's oceans as a major frontier for medical research. This emerging new field, sometimes called marine pharmacology. Although none of the discoveries has as yet led to a pharmaceutical product, there is a hope that one or more of the many marine natural products currently under investigation will eventually do so (Faulkner, 2000; Fenical, 1997).

Several examples of marine-derived compounds that have been in clinical trials include bryostatin, didemnin B, manoalide, and pseudopterosins (Figure 1.3.2). Bryostatin isolated from bryozoan, *Bugula neritina*, has antitumor activity. Didemnin B derived from tunicate, *Tridemnum solidum* possess antiviral and cytotoxicity. Manoalide which was isolated from marine sponge (*Luffariella variabilis*), demonstrated antibiotic, analgesic, and anti-inflammatory activity. Pseudopterosins derived from Caribbean gorgonian (*Pseudopterogorgia elisabethae*) was found as an analgesic and as an additive in cosmetic products (McConnell *et al.*,1994). Arabinosides (A – D) that were isolated from fermentation cultures of *Streptomyces antibioticus* were encountered to possess antiviral activity (Figure 1.3.3) (McConnell *et al.*,1994).

Faulkner (2000) reported other marine natural products under intense investigation as the potential anti cancer agents including dolastin 10, ecteinascidin 743, halichondrin B, isohomohalichondrin B, curacin A, discodermolide, eleutherobin, and sarcodictyin A (Figure 1.3.4). Anti-inflammatory agents such as: psedopterosins A and E, topsentin, debromohymenialdisine, and scytonemin are also under active investigation. In addition to those compounds being considered for medicinal use, there are an increasing number of compounds that are currently used as reagents in cellular biology. Those are the sponge metabolite, swinholide A, jaspamide, ilimaquinone, and adociasulphate. The most recent review of updated list of marine natural products which are currently under clinical trials are presented in Table 1.3.3 (Proksch *et al*., 2002).

Table 1.3.3. Several marine natural products which are currently undergoing clinical trials

| Source | Compounds | Disease | 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 |
| *D. auricularia* (sea hare) | LU103793[a] | cancer | II |
| *Bugula neritina* (bryozoan) | Bryostatin 1 | cancer | II |
| *Tridemnum 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[e] | alzheimer/ schizophrenia | I |

[a] synthetic analogue of dolastatin 15
[b] Agelasphin analogue ($\alpha$-galactosylceramide derivative)
[c] synthetic analogue of contignasterol (IZP-94,005)
[d] semisynthetic pseudopterosin derivative
[e] also known as DMXBA, 3-(2, 4-dimethoxybenzylidene)-anabaseine

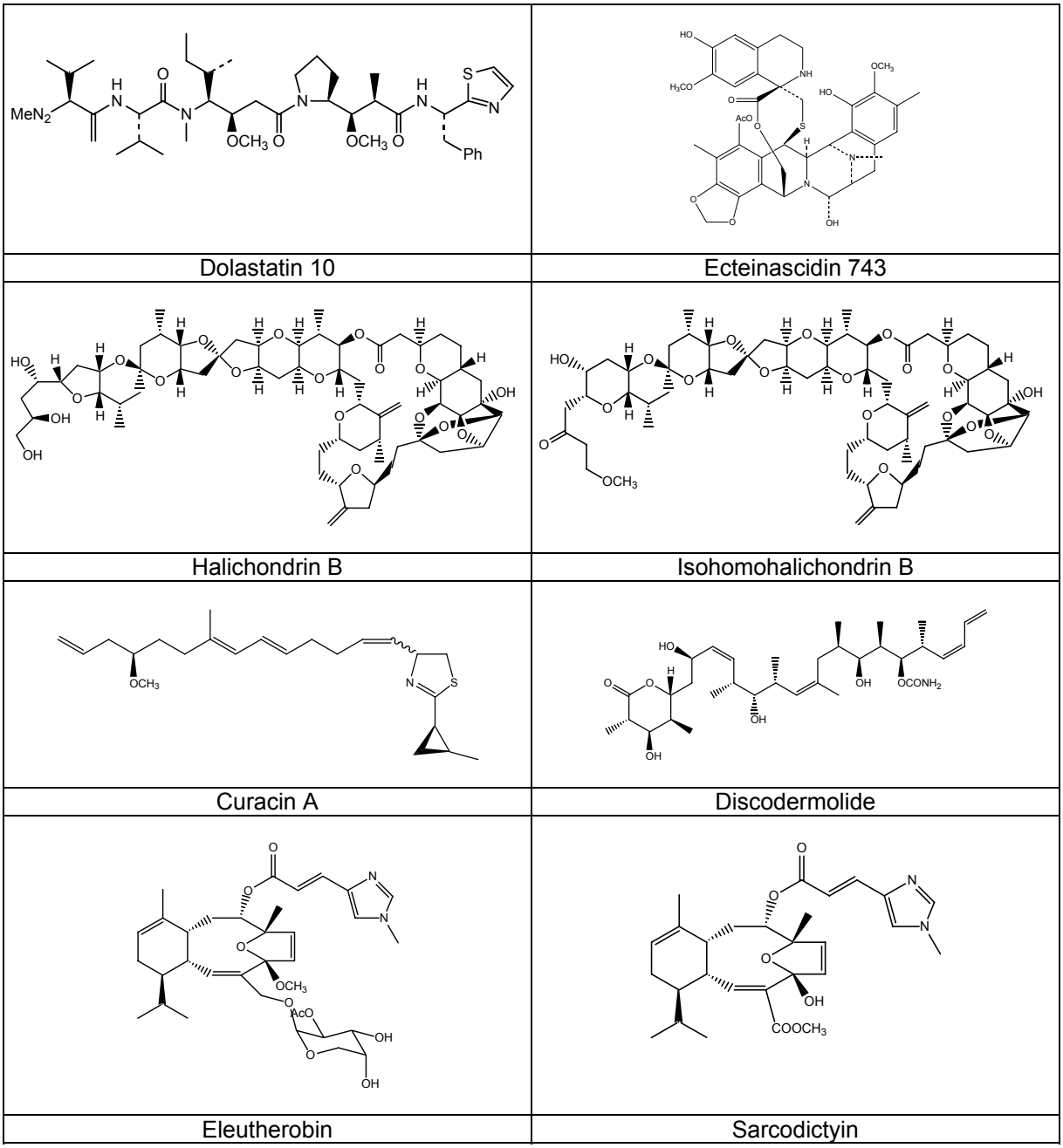



Figure 1.3.4. Marine derived compounds under intense investigation as potential anticancer agents

The pharmaceutical industry is now almost totally dependent on high-throughput screens that employ robots to perform bioassay using a 96-well plate format, This regime was designed to handle large libraries of pure compounds but, due to problems involving solubility and/or non-specific inhibition, may not provide optimal results for crude extract (Faulkner, 2000)

A serious obstacle to the ultimate development of most marine natural product that are currently under clinical trial or in preclinical evaluation is the problem of supply. Provided that the halichondrins make it to the market as new anti cancer drugs the annual need for these compounds is estimated to be in the range 1-5 kg per year, which corresponds to roughly 3,000 – 16,000 metric tonnes of sponge biomass per year. Such large amounts of biomass can never be harvested from nature without risking extinction of the respective species (Hart *et al*., 2000; Proksch *et al*., 2002).

There are a number of strategies to counteract the supply problem as follows (Battershill *et al*., 1998; Faulkner, 2000; Pomponi, 1999; Proksch *et al*., 2002).

a). Synthesis. This could be performed for relatively simple compounds. A terrestrial example of aspirin, in which the commercial product is synthesised rather than obtained from the willow bank, its natural product source. However, many bioactive marine natural products are extremely complex and require multi-step synthesis of heroic proportions. For these more complex molecules, it seems best to elucidate the mechanism of action and identify the pharmacophore so that simpler compound can be synthesised. Wender´s recent research on simplifying the bryostatin structure while retaining bioactivity is an excellent example of this approach.

b). Mariculture, bioreactor, and cell or tissue culture. Shallow water specimens may be transplanted and grown in sheltered waters or in artificial raceways but the successful culture of deep water specimens may require considerable research effort. *Bugula neritina*, the source of bryostatin has been produced under controlled conditions by CalBioMarine Technologies, while Battershill in New Zealand has reported success in growing even deep water sponges under experimental aquaculture conditions.

c). *In vitro* production. The ability to transfer genetic material from one bacterium to another, has opened up the exciting possibility of transferring segments of DNA that are responsible for the biosynthesis of secondary metabolites from uncultivable bacteria, such as symbionts, to *Escherichia coli* or other easily cultured bacteria. This technology may also be extended to transfer genetic material responsible for biosynthesis pathways from other groups of marine microorganism and eventually from marine invertebrates. This *in vitro* production are in detail explained below (Wildman, 1997).

- The production of hybrid compounds through modification of the genetic make-up of microorganisms such as what has been done with *Streptomyces* where modular gene systems occur.
- The screening of uncultivable and slow-growing microorganisms through gene transfer to host microorganisms in order to expand the diversity of microbes examined (random gene transfer).
- The horizonal transfer of genetic material between a producer and another more suitable host for compound production once a compound of interest has been detected in a producer organism (targeted gene transfer).

## 1.4. Fungi

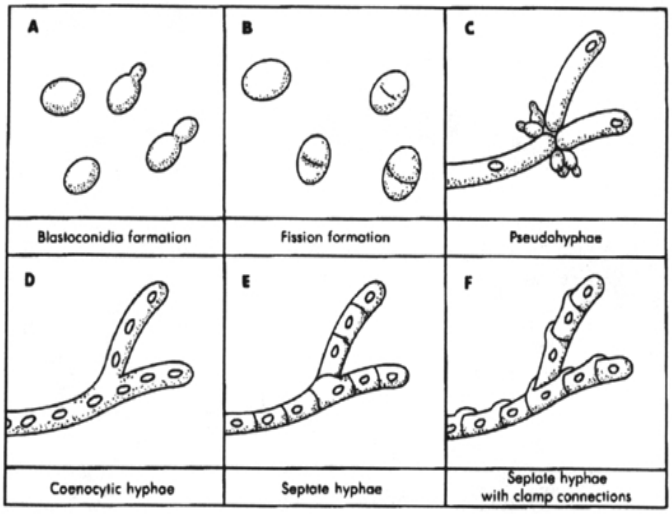
### 1.4.1. Fungal characteristics

Fungi are more generally regarded as filamentous organisms, conveniently called moulds or filamentous fungi. Moulds are important agents of decay and they are noteworthy for the ability to produce antibiotic and industrially important chemicals such as citric acid. The term fungus includes the yeasts, a group of important single-celled organisms. Many yeasts complicate the issue by being dimorphic, capable of change from a single-celled growth phase to a mycelial phase. Yeasts are widely used in the food industry, particularly in the production of alcoholic drinks such as beers and wines. It has recently become fashionable to treat yeast as if they were a group that should be considered as separate from other fungi (Dreyfuss and Chapela, 1994; Kwon-Chung and Bennett. 1992; Wainwright, 1992).

The fungi are classified as eukaryotes, due to the possession of a diploid number of chromosomes and a nuclear membrane and a cell wall like plants, but they do not have chlorophyll. In most fungi, chitin (a polymer of *n*-acetyl glucosamine) is the major component of cell wall, while yeast wall is composed largely of polymers of mannose (mannans) and glucose (glucans). Fungi are not able to ingest their food like animals do, nor can they manufacture their own food the way plants do. Instead, fungi feed by absorption of nutrients from the surrounding environment. They accomplish this by growing through and within the substrate on which they are feeding (Smith, 1975; Wainwright, 1992).

There are over 100.000 species of fungi. Approximately 70,000 species of fungi have been given a valid name. Several fungi are pathogen and cause disease to man, animals, and plants. The majority of the pathogenic species are classified within the phyla zygomycota, basidiomycota, ascomycota, or the form group fungi imperfecti (Dreyfuss and Chapela, 1994; Kwon-Chung and Bennett. 1992).

Hyphae are multicellular fungi which reproduce asexually and/or sexually. Dimorphism is the condition whereby a fungus can exhibit either the yeast form or the hyphal form, depending on growth conditions. Very few fungi exhibit dimorphism. Most fungi occur in the hyphae form as branching, threadlike tubular filaments. These filamentous structure either lack cross walls (coenocytic) or have cross walls (septate). In some cases septate hyphae develop clam connections at the septa which connect the hyphal elements (Figure 1.4.1). Mushroom is a filamentous multicellular fungi which produce macroscopic fruiting bodies. Moulds, a filamentous multicellular fungi but not forming macroscopic fruiting body, are the fungi capable of producing a wide range of secondary metabolites having antibiotic properties or toxic to plants and animals (Smith, 1975; Turner, 1971).




(http://www.kcom.edu/faculty/chamberlain/Website/Lects/Fungi.htm)

Figure 1.4.1. Basic morphological structure of fungi

A mass hyphal element is termed the mycelium. Aerial hyphae often produce asexual reproduction propagules termed conidia (spores). Relatively large and complex conidia are termed macroconidia while the smaller and more simple conidia are termed microconidia. When conidia are enclosed in a sac (the sporangium), they are called endopsores. The presence and absence of conidia and their size, shape, and location are major features used in the laboratory to identify the species of fungi in clinical specimens (Smith, 1975; Turner, 1971).

All fungi are heterotrophic (saprobes, parasites, and mutualist), which means that they depend on energy-rich carbon compounds manufactured by other organisms. Most fungi are decomposers, breaking dead organisms down into detritus and returning inorganic nutrients to the ecosystem .

Fungi can grow at temperatures as low as -5$^{o}$ C and as high as 60$^{o}$ C. Certain fungi can grow under extreme acid conditions (pH 1), others can tolerate alkalinity up to pH 9. Fungi are also extremely adaptable, and can break down many substances, including some toxic pollutants. Fungi have evolved enzymes that can digest some extremely tough substrates. Chitin (insect exoskeletons), keratin (skin, hair, horn, feathers), cellulose (most plant debris) and lignin (wood), nourish many fungi, though cellulose and lignin remain completely unavailable to almost all animals (except with the collaboration of microbial symbionts).

One-third of all species of fungi are mutualists, either as mycorrhizae or lichens. Mycorrhizal fungi live on the roots of plants and provide inorganic nutrients, and often resistance to some pathogens, to the plants in exchange for organic sugars. The first colonisation of land by plants was facilitated, if not

made possible by, the ability of mycorrhizal fungi to uptake nutrients from hostile soil (Alexopoulus *et al*., 1996; Read, 1996).

A mycorrhiza is a composite structure consisting of a fungus and the root of a higher plant. Two types of mycorrhiza association can be recognised: endomycorrhizas and ectomycorrhizas. Mycorrhizal fungi render increased disease resistance to some plants (Dix and Webster, 1995; Wainwright, 1992).

The lichens, meanwhile, are fungi living in symbiotic relationships with algae or cyanobacteria. They consist of algae or bacteria trapped in the fungal hyphae. The fungus often makes up as much as 90% of the dry weight of the composite organisms. The fungus typically provides water and minerals for the algae or bacteria, in exchange for organic food from photosynthesis. The photosynthetic species in lichens are actually capable of living by themselves, but the fungal species depend on their counterparts for survival. Due to the effectiveness of the mutualist relationship in lichens, they can grow in the most inhospitable of terrestrial habitats, and often serve as key organisms in the primary succession of a habitat (Alexopoulus *et al*., 1996; Seaward, 1996; Wainwright, 1992).

The saprobic fungi are recyclers par excellence, but they are also among the world's greatest opportunists, and do not restrict their attentions to naturally occurring dead wood and leaves. Where there is a trace of moisture, their omnipresent spores will germinate, and the hyphae arising from them will attack food and fabric, paper and paint, or almost any other kind of organic matter. Some of their metabolites are extremely dangerous - even carcinogenic - if they contaminate food (mycotoxins). And parasitic fungi cause the majority of serious plant diseases (fungal plant pathology in agriculture and forestry), as well as some of animals and people (medical mycology) (Alexopoulus *et al*., 1996; Dix and Webster, 1995).

## 1.4.2. Primary and secondary metabolites of fungi

Terrestrial fungi served an enormous scope for the discovery of novel natural product in the past 60 years, many of them being potential targets for biomedical developments. The discovery of penicillin in 1929 started the era of fungal antibiotic and was followed by other important fungal metabolites like cephalosphorins, cyclosporins, and griseofulvins. Until now, fungi have only been surpassed by actinomycetales as a source for biologically active metabolites. The fungal biodiversity on land seems to be nearly exhausted. Thus, nowadays, researchers throughout the world have paid increasingly attention toward the potential of marine microorganism as an alternative source for isolation of novel metabolites (Anke and Erkel, 2002; Biabani and Laatsch, 1998; Pietra, 1997).

Although the estimated 3000 to 4000 known fungal secondary metabolites have been isolated, possibly not more than 5000 to 7000 taxonomic species have been studied in this respect. Genera such as: *Aspergillus*, *Penicillium*, *Fusarium*, and *Acremonium* are among fungi highly capable of producing a high diversity of secondary metabolite (Dreyfuss and Chapela, 1994).

Most fungal secondary metabolites are synthesised from only a few key precursors in pathways that comprise a relatively small number of reactions and which branch off from primary metabolism at a limited number of points. Acetyl-CoA is the most precursor of fungal secondary metabolites, leading to polyketides, terpenes, steroids, and metabolites derived from fatty acids. Other secondary metabolites are derived from intermediates of the shikimic acid pathway, the tricarboxylic acid cycle, and from amino acids (Dreyfuss and Chapela, 1994; Martin and Demain, 1978).

In general, secondary metabolism proceeds by pathways involving enzymes that are not used in growth and it frequently begins after active growth has ceased. In fungal batch fermentations, the growth period is often called trophophase and that during which secondary metabolites are produced is called idiophase. More fungal metabolites are derived from polyketide pathway than any other biosynthetic pathways. The term polyketide refers to an alternating sequence of $–CH_2–$ and –CO– groups, $–CH_2–CO–CH_2–CO–CH_2–CO–$; the two carbon $–CH_2–CO–$unit is related formally and biosynthetically to acetate. A complete description of a secondary metabolic pathway would require knowledge of (1) the precursor of primary metabolites from which it derives, (2) the intermediates

along the pathway, (3) the genetics and enzymology of each reaction, and (4) the regulatory process (Bentley and Bennett, 1988).
Some secondary metabolites are produced by constitutive metabolic machinery, so that specific taxa always have the potential to produce such "marker" metabolites. Pigments, toxins, and other secondary metabolites from mushroom (basidiomycetes), lichens, and other large, fleshly, or sclerotic fungi are widely accepted as constant characteristics. Data on the consistence of secondary metabolites in micro fungi are, by contrast, rather scanty (Dreyfuss and Chapela, 1994).

The mycotoxins are those mould metabolites which cause illness or death of man and domesticated animal, following consumption of a contaminated food. A very diverse group of compounds are produced by a taxonomically wide range of filamentous fungi of moulds and showing a diverse range of toxic effect (Table 1.4.1). They are of considerable interest to food industry (Smith and Moss, 1985).

Table 1.4.1. Toxic phenomena associated with mycotoxins.

| Moulds | Toxic substances | Toxic effect |
|---|---|---|
| *Aspergillus clavatus* | Patulin | Haemorrhage of lung and brain |
| *A. flavus* | Aflatoxin | Carcinogenic |
| *A. ochraceus* | Ochratoxin | Nephrotoxic |
| *A. ustus* | Austidiol | Gastro-intestinal disturbances |
| *A. versicolor* | Sterigmatocystin | Hepatic necrosis |
| *Fusarium graminearum* | Zearalenone | Oestrogenic |
| *Penicillium crustosum* | Penitrem | Tremorgenic activity |
| *P. rubrum* | Rubratoxin | Haemorrhage |
| *Rhizoctonia leguminicola* | Slaframine | Parasympathetic nervous system |

Certain filamentous fungi have also been traditionally used to improve the flavour of cheese, while others are used in Asian cultures to produce foods such as: tofu, tempeh, and miso. More recent application of fungi in the food industry include the production of flavours and colouring agents and as a protein supplement used to mimic meat. The most recent and perhaps best-known example of fungal protein is Quorn, a product that is finding favour among slimmers and health-conscious consumers because of its low energy and cholesterol content. One of the most economically important uses of fungi is in the industrial production of biochemicals such as organic acids (e.g. citric, fumaric, lactic, and itaconic acids) and gibberellins (Table 1.4.2. and 1.4.3) (Dreyfuss and Chapela 1994; Smith *et al*., 1983; Wainwright, 1992).

Moulds make 17% of all describe antibiotics. Approximately 10,000 microbial secondary metabolites have been discovered. Their unusual chemical structures include *β*-lactam rings, cyclic peptides etc. The intensity of secondary metabolism can often be increased by addition of limiting precursors. An example is the stimulation of penicillin G production by phenylacetic acid. Secondary metabolism occurs best at sub maximal growth rates. The distinction between the growth phase (trophophase) and production phase (idiophase) is sometimes very clear. A secondary metabolite is produced after growth, it is not involved in the growth of the producing culture. Nutrient limitation is the usual situation in nature resulting in very low growth rate which favour secondary metabolism. Over 40% of filamentous fungi produce antibiotics when they are freshly isolated from nature (Demain, 1996).

## 1.5. Marine fungi

The kingdom of fungi has very few organisms in the marine environment, most fungi are land based. Marine fungi are not taxonomically defined, but are classified according to their habitat and in part to their physiology, with representatives from all taxonomic groups. They are geographically widely distributed and appear to occur in all climates and salinities. Marine fungi grow facultatively or obligately in oceans and ocean-associated estuarine habitats that contain brackish-water including river mouths, tidal creeks and marshes, lagoons, and the like (Kohlmeyer and Kohlmeyer, 1979).

Obligate marine fungi are those growing and sporulating exclusively in a marine and estuarine habitat. Facultative marine fungi are those freshwater or terrestrial fungi able to grow and possibly also sporulate in the marine environment. The majority of marine fungi is isolated from intertidal zones of shores or mangroves, estuarine waters, sediments, mud, and sand dunes. Filamentous ascomycetes and fungi imperfecti are predominating the described marine fungal species. They occur as saprophytes and parasites on sea weed, drift wood and higher marine organisms in littoral and neritic environment. These fungi are not prominent in deep sea environment (Biabani and Laatsch, 1998; Liberra and Lindequist, 1995).

Table 1.4.2. Metabolites of industrial significance produced by filamentous fungi

| Fungi | Product |
|---|---|
| Antibiotics | |
| 1. *Cephalosphorium acremonium* | Cephalosporin C |
| 2. *Aspergillus fumigatus* | Fumagilin |
| 3. *Fusidium coccineum* | Fusidic acid |
| 4. *Penicillium* sp. | Griseofulvin |
| 5. *Penicillium chrysogenum* | Penicillin |
| 6. *Helminthosporium siccans* | Siccanin |
| Organic acid | |
| 7. *Aspergillus niger* | Citric acid |
| 8. *A. niger* | Gluconic acid |
| 9. *A. terreus* | Itaconic acid |
| Enzymes | |
| 10. *Aspergillus* sp. | Lipase, pentosanase, proteases |
| 11. *A. niger* | β-glucanase, cellulase, glucoamylase, glucose oxidase, lactase, pectinase |
| 12. *A. oryzae* | α-Amylase |
| 13. *A. awamori* | Glucoamylase |
| 14. *Trichoderma* sp. | Dextranase, cellulase |
| 15. *Penicillium* sp. | Dextranase |
| 16. *Mucor* sp. | Rennin |
| Traditional foods | |
| 17. Various | The softening and flavouring of cheese |
| 18. *Aspergillus oryzae* | Miso |
| 19. *Mucor, Rhizopus* | Ragi |
| 20. Mixed culture | Soya sauce |
| Miscellaneous | |
| 21. *Gibberella fujikuroi* | Gibberellins |
| 22. *G. zeae* | Zearalenone |
| 23. *Claviceps* sp. | Ergot alkaloids |
| 24. *Aurebasidium pullulans* | Pullulan |
| 25. *Sclerotium* sp. | Scleroglucan |
| 26. *Rhizopus* sp. | Steroid bioconversion |
| 27. *Fusarium* sp. | Steroid bioconversion |

The first facultative marine fungus, *Spheria typharum*, and the first obligate marine fungus, *Sphaeria posidoniae* were reported by Deamazières (1849); Durieu and Montage (1869), respectively. The publications of “Marine Mycology: The higher fungi”, a comprehensive book dealing with virtually all aspect of marine mycology, and “Illustrated key to the filamentous higher marine fungi “ marked the milestone in marine mycology (Kohlmeyer and Kohlmeyer, 1979).

A total of 321 species of marine fungi have been recognised, representing 255 ascomycetes, 60 basidiomycetes, and 60 anamorphic (imperfect/deuteromycetes). This number is continuously increasing by the discovery and description of new species. Along with the non active marine fungi, the number of marine fungi is estimated to be at least 6000. Apart from that, marine yeasts (zygomycetes), and fungi-like protists (phycomycetes) should also be taken into account. At present an estimated total of 900 species has been described, most of them belonging to ascomycetes (Biabani and Laatsch, 1998; Kohlmeyer and Kohlmeyer, 1979; Schaumann, 1993).

Table 1.4.3. Industrially exploited primary and secondary metabolites of fungi

| Metabolite | Species in Production |
|---|---|
| **Primary** | |
| Alcohol | |
| 1. Ethanol | *Saccharomyces cerevisiae* |
| Organic acid | |
| 2. Itaconic acid | *Aspergillus terreus* |
| 3. Gluconic acid | *A. niger* |
| 4. Citric acid | *A. niger, Yarrowia lipolytica* |
| Vitamin | |
| 5. Riboflavin | *Eremothecium ashbyii, Ashbya gossypii* |
| Polysaccharides | |
| 6. Scleroglucan (Polytran) | *Sclerotium rolfsii* |
| 7. Pullulan | *Aureobasisium pullulans* |
| Nucleotides | |
| 8. 5'-inosine monophosphate | Hydrolysis of *S. cerevisiae* RNA |
| 9. 5'-guanosine monophosphate | Hydrolysis of *S. cerevisiae* RNA |
| **Secondary** | |
| Antibiotic | |
| 10. Penicillins (Antibacterial: varies with specific form; in general, gram-positive bacteria and gram negative cocci) | *Penicillium chrysogenum* |
| 11. Cephalosporins (Antibacterial: broad spectrum) | *Cephalosporium acremonium* |
| 12. Griseofulvin (fungistatic) | *Penicillium griseofulvin, P. patulum* |
| 13. Fusidanes (Antibacterial: *Staphylococcus* infection) | *Fusidium coccineum* |
| Other drugs | |
| 14. Ergot alkaloids (usage: postpartum haemorrhage; induction of labour; treatment of migraine) | *Claviceps purpurea* |
| 15. Cyclosporin (immunosuppressive; prevents rejection of organ transplants) | *Trichoderma polysporum* |
| 16. Mevinolin | *Aspergillus terreus* |
| 17. Compactin | *Penicillium brevicompactum* |
| 18. Pleuromutilin | *Pleurotus mutilis* |
| Agricultural purposes | |
| 19. Gibberellins (plant growth hormone) | *Fusarium moniliforme (Gibberella fujikuroi)* |
| 20. Zearalenone (growth promoter in cattle) | *F. graminearum (Gibberella zea)* |

Marine fungi 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, shell, crabs, sponges or in the gastrointestinal tract of fishes and are an important group of pathogens in marine world. They also exist as symbionts in lichenopid associations with algae on coastal cliffs. A vast number of less investigated fungal species populate the deep sea and the seafloor. Marine fungi are concentrated mostly along the shores, the open ocean is a fungal desert where only yeast or lower fungi may be found attached to planktonic organisms or pelagic animals (Kohlmeyer and Kohlmeyer, 1979).

The marine environment does not permit the development of large, fleshly fruiting bodies, due to abrasion by waves and grains of sand impedes formation of such structures. Macromycetes growing in the leaf litter of forests need an extended nutrient-supplying mycelium and an undisturbed habitat. Similarly, soft fruiting bodies of large marine species (*Amylocarpus encephaloides*, *Eiona tunicata*, *Digitalis marine*, *Nia vibrissa*, and *Halocyphina villosa*) develop mostly in sheltered habitats, namely, on firmly anchored wood at or above the high-tide line or protected in cracks of the wood or under bark. The deep sea appears to be another environment where large fruiting bodies could develop because water currents are weak. The ascomycete *Oceanitis scuticella*, occuring at a depth of about 4000 m, has fleshy ascocarps up to 2 mm in height (Kohlmeyer and Kohlmeyer, 1979).

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. About 70-80% of the secondary metabolites that have been isolated from marine fungi are biologically active (Faulkner 2001; Faulkner 2002). The total number of characterised fungal secondary metabolites, including antibiotics, mycotoxins, pharmacologically active compounds, and those without known biological activities, are estimated around 3000 to 4000 compounds (Dreyfuss and Chapela, 1994).

The number of secondary metabolites isolated from marine-derived fungi have been increasing. This proves that they are a rich source of bioactive compounds with therapeutic potential. The success story of cephalosporin isolation, which is now widely used in antibiotic therapy, from the fungus *Cephalosporium* sp. derived from the microbial flora of sea water collected from Cagliari, Italy, in the 1940's marked the onset of interest of pursuing natural product from marine fungi (Faulkner, 2001; Faulkner 2002). Cephalosporin C becomes the only compound from a fungus isolated from a marine source that up to now has been established as a medical drug or a source for partial synthetic derivatives (Figure 1.5.1) (Biabani and Laatsch, 1998).

It remains questionable whether marine natural products derived from fungi will play a significant role in drug discovery in the future as no unique secondary metabolite has yet been isolated. This is due in part to the predominant isolation and cultivation of ubiquitous fungi even from samples collected from the marine environment (Grabbley *et al*., 2000).

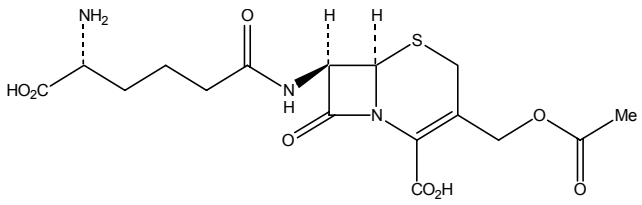



Figure 1.5.1. Cephalosphorin C

## 1.6. Drug discovery

The first step in drug discovery of natural product origin is the acquisition of samples. What comes after organisms have been procured through collection and culture can be separated broadly into drug discovery and drug development.

The drug discovery is complex and multidisciplinary. After production of a crude extract of each collected sample, primary screening with bioassays is the next step. When appropriate bioactivity is detected, various chemical techniques are employed to separate the crude extract into its components, and each component is then re-screened to identify the activity of fraction. This process of fractionation and re-screening, so called bioassay-guided fractionation, is repeated again and again until the responsible active compound has been purified.

Figure 1.6.1 depicts the magnitude of sample size and the research effort required to produce a small number of drug development candidates and the corresponding shift in costs and risks as the process progresses. Primary objective of drug discovery is to discover drug lead, or compound which may become drugs through drug development. However, in general, less than 10% of samples come through the primary screening process. At least 99% of these then drop out of the drug lead race because the compound and bioactivity combination turns out to be known, or further mechanistic studies show that the activity is not selective or not useful as a pharmaceutical. This means we are left with less than 0.1% of original samples that actually becoming drug leads and entering drug development phase, a process which itself has a high attrition rate.

It is for this reason that natural products drug discovery has been likened to "looking for a needle in a haystack". It relies on a high throughput philosophy (large numbers of biodiverse samples being put through a large number of different screens) to maximise the chance of ultimately achieving just one new drug (Illidge and Murphy, internet source).

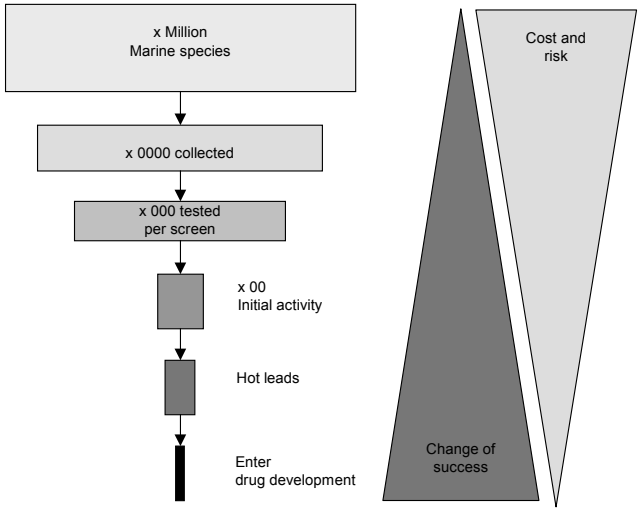



Figure 1.6.1. Orders of magnitude in drug discovery

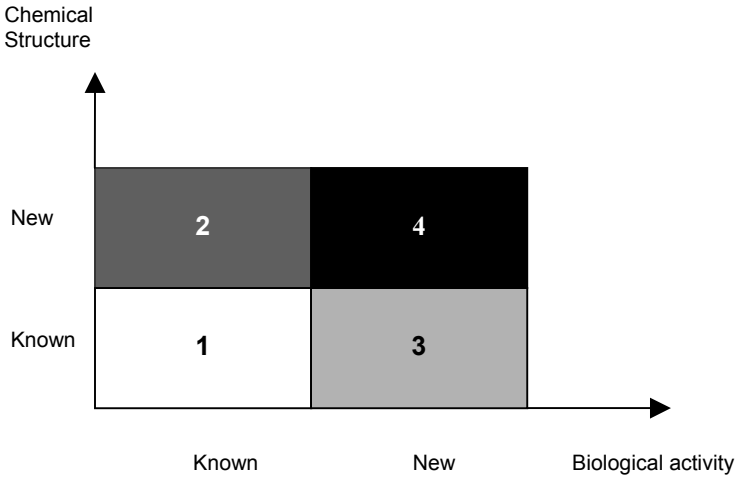



Figure 1.6.2. Relationship between chemical structure and bioactivity (Samuelsson, 1999)

The active isolated compound can then be used for development of a drug or as a model, precursor or tool in drug. The characteristic of purified active chemical compound are displayed in Figure 1.6.2 (Samuelsson, 1999).

Field 1 of Figure 1.6.2 illustrates the isolation of compounds with known structures and known activities. The only new discovery is that the compounds are found in organisms where their existence was not previously known. Field 2 describes a result which is of greater interest in the search for drugs. Compounds of new structures have been isolated but their biological activity is the same as that exerted by compounds with other related structures. Field 3 illustrates the isolation of compounds with known structures found to have hitherto not known biological activity. The fourth field is clearly of greatest interest. The work has resulted in isolation of compounds with both new structures and new biological activities.

Drug discovery process, defined as the production of drug leads, can be considered as non commercial research. On the other hand, drug development should be considered as commercial research, as it is undertaken primarily by industrial partners and is focused on compounds with a higher chance of proceeding to market. The transformation of a natural product lead into a clinically-used drug will cost upwards of 350 million dollars (Illidge and Murphy, internet source).

## 1.7. Aim and scope of the study

This study was chiefly aimed at pursuing new biologically active secondary metabolites of several sponge-derived fungi collected from Elba sea (Italy) and Bali sea (Indonesian). Research was conducted on five fungal strains including three strains of Elba sea fungi (*Penicillium* sp., *Verticillium* cf *cinnabarium*, *Fusarium* sp.), and two strains of new fungi species of Bali sea (*Lecanicillium evansii*, strain 1 and 2). The study was divided into two main parts as illustrated below.

### 1.7.1. Bioactivity screening of fungal extracts

Several pure isolates of marine fungi raw extracts, derived from 300 ml monoculture broths, were subjected to brine shrimp (*Artemia salina*), insecticidal (*Spodoptera littoralis*) assay, and antimicrobial test. Subsequently, HPLC and LC-MS measurements of raw extracts were also performed to obtain an overview of compound UV absorption patterns and the distribution of molecular weights.

The nomination of fungal strains for further investigation was based on the intensity level of bioactivity, the UV absorption characteristics displayed by the HPLC spectrum, and the uniqueness of molecular weight distribution determined by the LC-MS spectrum.

Literature searching through the available databases (Antibase, Marinlit, and Dictionary of Natural Products) of secondary metabolite having already been isolated from the prospective fungal strain for further investigation was also taken into minutely account.

Supposed that, not plenty isolated compounds reported, this nominated fungal strain was then selected for further intense secondary metabolite content investigation. Only fungi not belonging to a group of toxic species, were allowed to be selected for mass culture and followed by subsequent secondary metabolite scrutiny.

### 1.7.2. Chemical investigation of selected fungal strains

Several selected fungal strains were then cultured in large scale (10 litre media), growth in a standing culture, at constant room temperature, using artificial sea water media (Wickerham media). Fungi were harvested when rapid growth has ended, because in batch culture, the secondary metabolite often accumulates after this rapid exponential growth period.

Their secondary metabolite contents were extracted and separated on the basis of their chemical polarity, using a diverse array of chromatographic methods (VLC, column chromatography, semi preparative HPLC, TLC, etc.). The chemical investigation terminated with the structure elucidation of pure compound with the aid of MS and NMR spectra.

The pure compounds were then subjected to antibacterial assays (*Bacillus subtilis*, *Candida albicans*, *Escherichia coli*, *Staphylococcus aureus*), antifungal assays (*Cladosporium herbarum*, *Saccharomyces cerevisiae*) and cytotoxicity assays using human cancer cell lines to determine their biological activity.

# II. MATERIALS AND METHODS

## 2.1. Fungi collection and biological activity screening

Sponge-associated fungi strains investigated in this study originated from the Mediterranean Sea, Elba (Italy) and West Bali Sea (Indonesia). Sponges were collected by scuba diving. Scheme of fungi isolation from their host sponge and the subsequent biological activity screening test and mass cultivation are illustrated in Figure 2.1. A small piece of the inner part of the sponge was sliced under sterile conditions. This tiny piece was then inoculated on the surface of malt agar plates and incubated at 27$^{o}$C. In order to get a pure mono-culture of the fungi, purification through several sub-cultures onto fresh malt agar plates were repeatedly carried out.

The collected fungi were maintained under malt agar plates using the Wickerham medium (Table 2.1.1). For getting rid of bacterial contaminants, chloramphenicol (0.2 g/l), streptomycin sulphate (0.1 g/l), and penicillin G (0.1 g/l) were added to the medium. All fungal samples from Elba and Bali sea were stored in Alfred Wegener Institute (AWI) for Marine and Polar Ecology, Bremerhaven and The Institute for Pharmaceutical Biology, Heinrich-Heine University, Düsseldorf, Germany, respectively.

Table 2.1.1. Wickerham medium for marine fungi culture

| Substances | Amounts |
|---|---|
| Yeast extract (Sigma) | 3.0 g |
| Malt extract (Merck) | 3.0 g |
| Pepton (Merck) | 5.0 g |
| Glucose monohydrate (Caelo) | 20.0 g |
| Agar (Merck) | 16.0 g |
| Artificial sea water (Biomarine) | 24.4 g |
| Distilled water | 1000 ml |
| NaOH or HCl for pH adjustment (7.2 – 7.4) | Several drops |

When the pure mono-cultures of fungi in malt agar plates were accomplished, the fungi were then stored in the refrigerator at temperature of 4$^{o}$C for long term storage. In order to keep the fungi collection alive, they were transferred periodically (every 3 months) to fresh medium. For the purpose of biological activity screening test, the fungi were cultured in a liquid medium (300 ml). After a period of incubation, particularly when the rapid growth had ended, the fungi were then harvested.

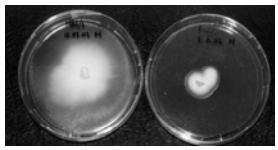
Ethyl acetate was used first to obtain the raw extract of both mycelium and broth. The raw extracts were subjected to a series of bioassays including brine shrimp assay (*Artemia salina*), insecticidal assay (*Spodoptera littoralis*), and antimicrobial assay to monitor their biological activities. Along with bioassay, a small amount of raw extract aliquot was also measured with HPLC and LC-MS for gaining the overview of chemical content through the distribution of UV absorption pattern and the distribution of compound molecular weights.

The nomination of fungi for further secondary metabolite content investigation was based on their biological activities, UV absorption patterns, and molecular weight characteristics. Before performing a mass culture (10 l), fungal samples in malt agar plate were sent to *Centraalbureau voor Schimmelcultures*, Baarn, Netherlands or to AWI, Bremerhaven for identification.

The decision of nominated fungi for further chemical investigation was taken after searching the number of secondary metabolites already isolated through the natural product database (Antibase, Marinlit, and Dictionary of Natural Product).

Those fungi, with strong biological activity, unique UV absorption pattern, specific molecular weight, relatively few reported compounds isolated from the prospective fungi, and non pathogenic were selected for further intensive secondary metabolite content investigation.

Sponge sample



Malt agar plate

Separation of fungal strain from its host sponge

Database and literature searching

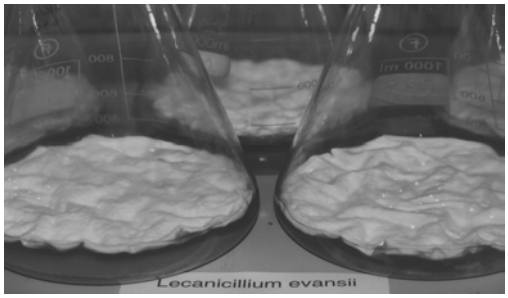
Natural product database (Antibase, Marinlit, Dictionary of Natural Product)

- Wickerham medium
- Bio-screening (brine shrimp, insecticidal, and antimicrobial assays)

Liquid culture (300 ml) for bio-screening

Fungal identification

Identification by *Centraalbureau voor Schimmelcultures*, Baarn, Netherlands or Alfred Wegener Institute of Marine and Polar Ecology, Bremerhaven, Germany




- Wickerham medium
- Stand culture
- Constant room temperature

Mass cultivation of fungi (10 l)

Figure 2.1. Fungal collection, separation, and mass cultivation scheme

## 2.2. Fungi cultivation

After conducting biological activity screening test toward 79 fungal strains, derived from several sponges, originated from Mediterranean and West Bali sea, five fungal strains were eventually selected for further scrutinising of their secondary metabolite contents (Table 2.2.1).

Table 2.2.1. Selected fungal strain for secondary metabolite isolation

| Fungal strains | Brine shrimp assay | Insecticidal assay | Antimicrobial assay |
|---|---|---|---|
| 1. *Penicillium* sp. | active | active | not active |
| 2. *Verticillium* cf *cinnabarium* | active | active | not active |
| 3. *Fusarium* sp. | active | active | active against *Bacillus subtilis* |
| 4. *Lecanicillium evansii* (strain 1) | active | not tested | not tested |
| 5. *Lecanicillium evansii* (strain 2) | active | not tested | not tested |

### 2.2.1. *Penicillium* sp., *Verticillium* cf *cinnabarium*, and *Fusarium* sp.

The sponges *Iricinia fasciculata* and *Axinella damicornis* were collected by scuba diving in Elba sea (Italy). The fungi were separated from their host sponge, and then grown on malt agar plates comprising the mixture of 15 g/l malt, 15 g/l agar, and 24.4 g/l artificial sea salt. *Penicillium* sp. derived from *Ircinia fasciculata*, *Verticillium* cf *cinnabarium* isolated from *Axinella damicornis* were mass (10 l) cultured by Dr. Schaumann.

Mass cultivation of these fungi were carried out in 30 Erlenmeyer flasks in Wickerham medium as a stand culture under constant temperature of 20$^{o}$C. This mass cultivation was carried out by Dr. Schaumann in Alfred Wegener Institute of Marine and Polar Ecology, Bremerhaven.

After 26 days incubation, into each Erlenmeyer flasks were added 300 ml ethyl acetate. The fungi were then transferred into several polyethylene bottles, and cooled under –86$^{o}$ C, and sent under dry ice to the Institute for Pharmaceutical Biology, Heinrich-Heine University, Düsseldorf.

The ethyl acetate extracts of the fungi (mycelium and culture broth) were subjected to subsequent extraction and isolation of its secondary metabolite contents presented in Figure 2.2.2 (*Penicillium* sp.), 2.2.3 (*Verticillium* cf *cinnabarium*), 2.2.4 (*Fusarium* sp.).

### 2.2.2. *Lecanicillium evansii* (strain 1 and strain 2)

The strain 1 of new fungal species of *Lecanicillium evansii* was isolated from the sponge *Callyspongia* sp., whereas the strain 2 of *L. evansii* was derived from sponge *Hyrtios* sp. Both strains were collected from West Bali Sea, Indonesia. Species identification was conducted by *the Centraalbureau voor Schimmelcultures*, Netherlands. For maintaining the culture collection, the isolated fungus was also grown on malt agar slants comprising the mixture of 15 g/l malt, 15 g/l agar, and 24.4 g/l artificial sea salt.

Mass cultivation of the fungus *L. evansii* strain 2 (10 l) carried out in 30 Erlenmeyer flasks in Wickerham medium was performed in fungal culture room, the Institute for Pharmaceutical Biology, Heinrich-Heine University, Düsseldorf. After 10 days incubation, without shaking under constant room temperature (20$^{o}$C), both fungal mycelium and the culture broth were separated. The mycelia were extracted with methanol, whereas the media were added with ethyl acetate. Both methanol-added mycelia and ethyl acetate-added media were left overnight.

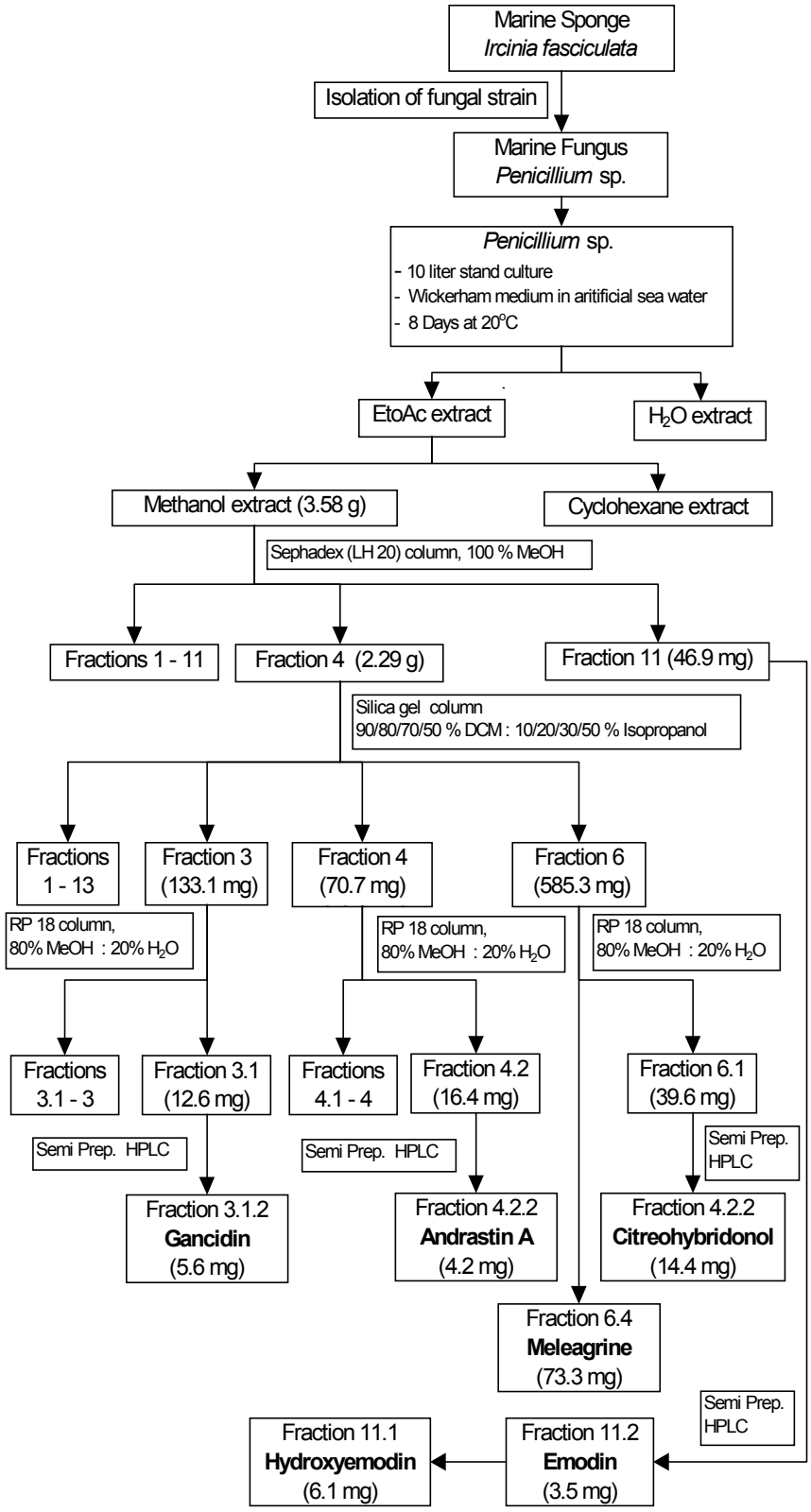



Figure 2.2.2. Secondary metabolites of *Penicillium* sp. isolation scheme

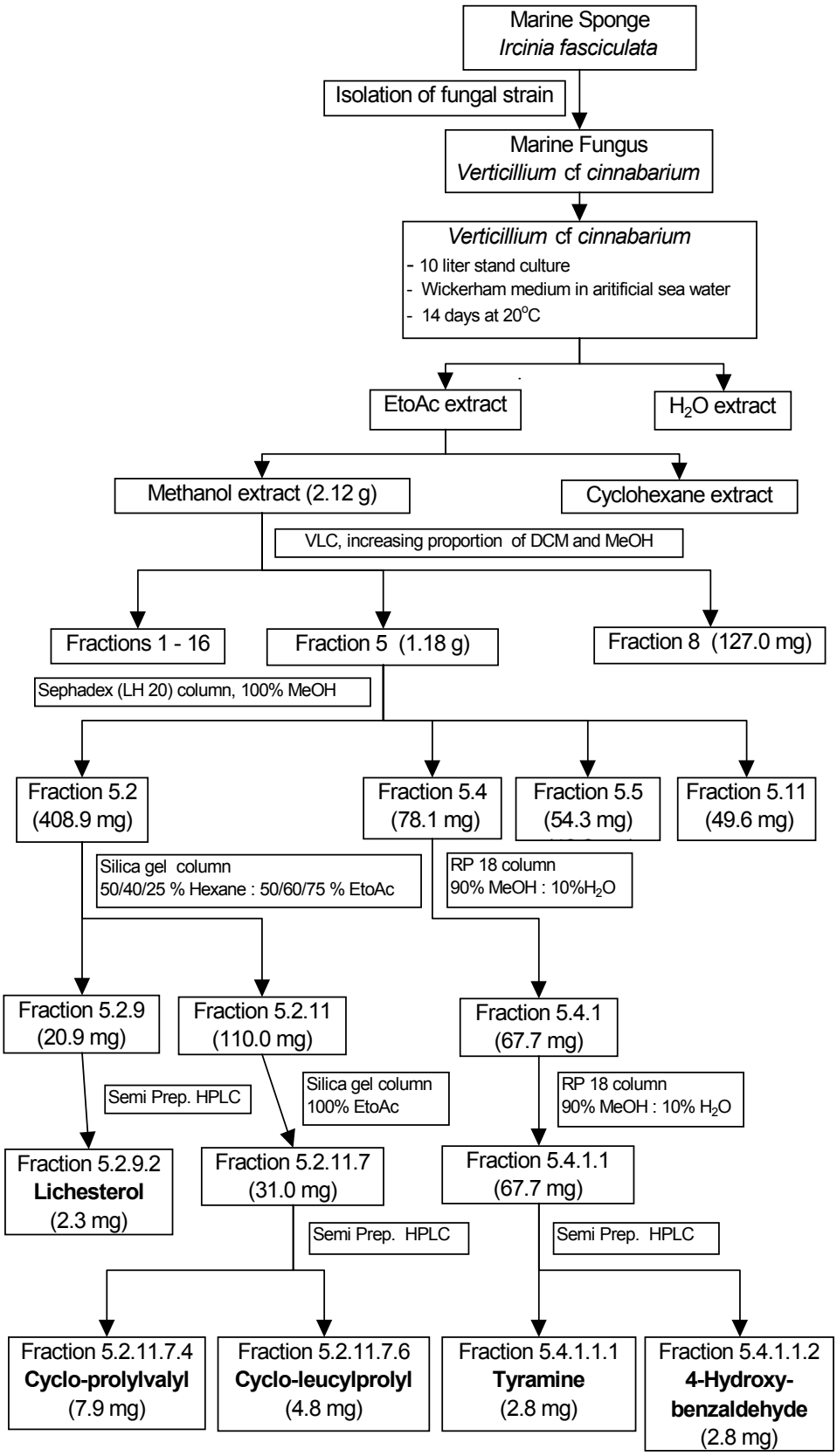



Figure 2.2.3. Secondary metabolites of *Verticillium* cf *cinnabarium* isolation scheme

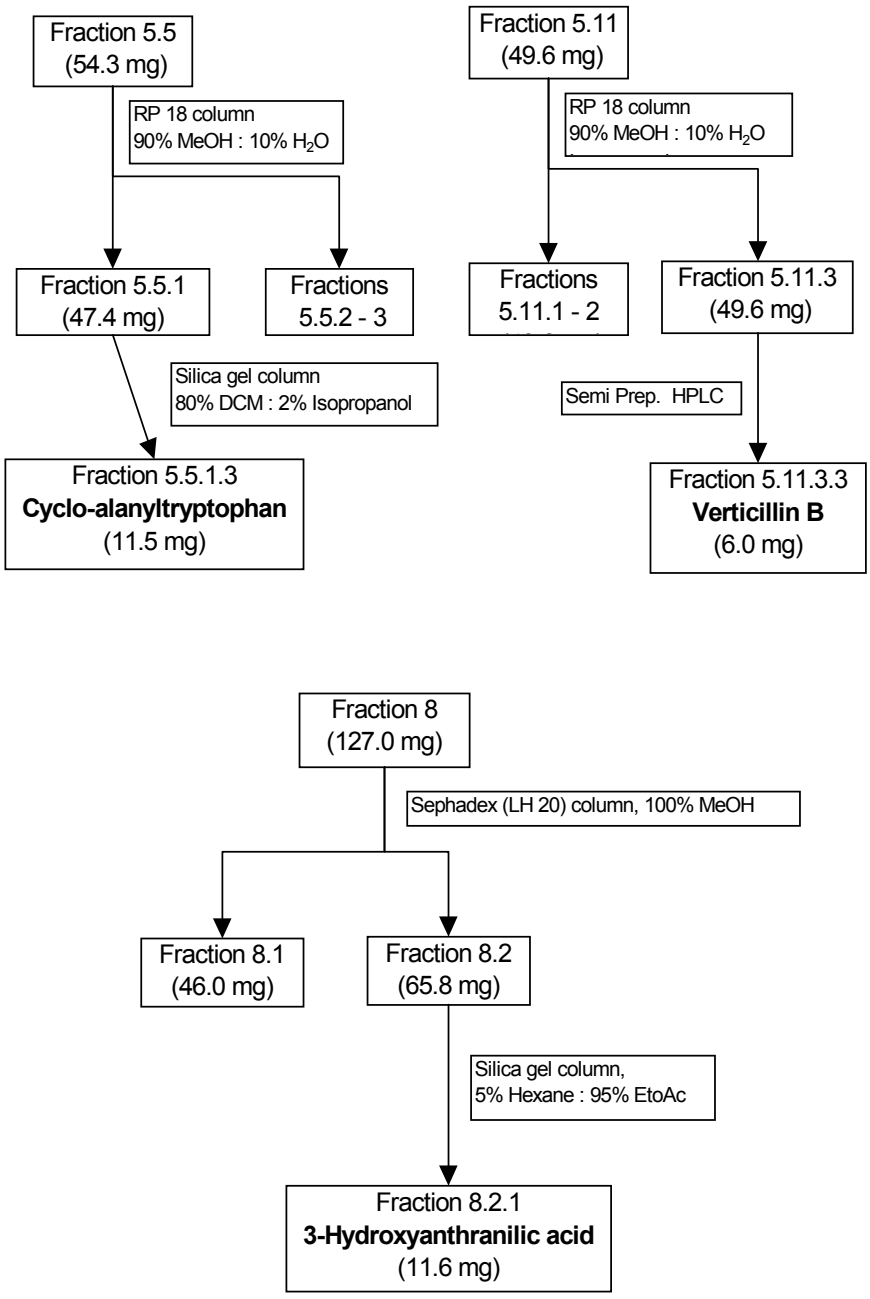



Figure 2.2.3. Secondary metabolites of *Verticillium* cf *cinnabarium* isolation scheme (continued)

Secondary metabolite content investigation of *L. evansii* strain 1 was done in 300 ml culture broth. Subsequent extraction and isolation of their secondary metabolite contents of *L. evansii* strain 1 and 2 are presented in Figure 2.2.5 and 6.

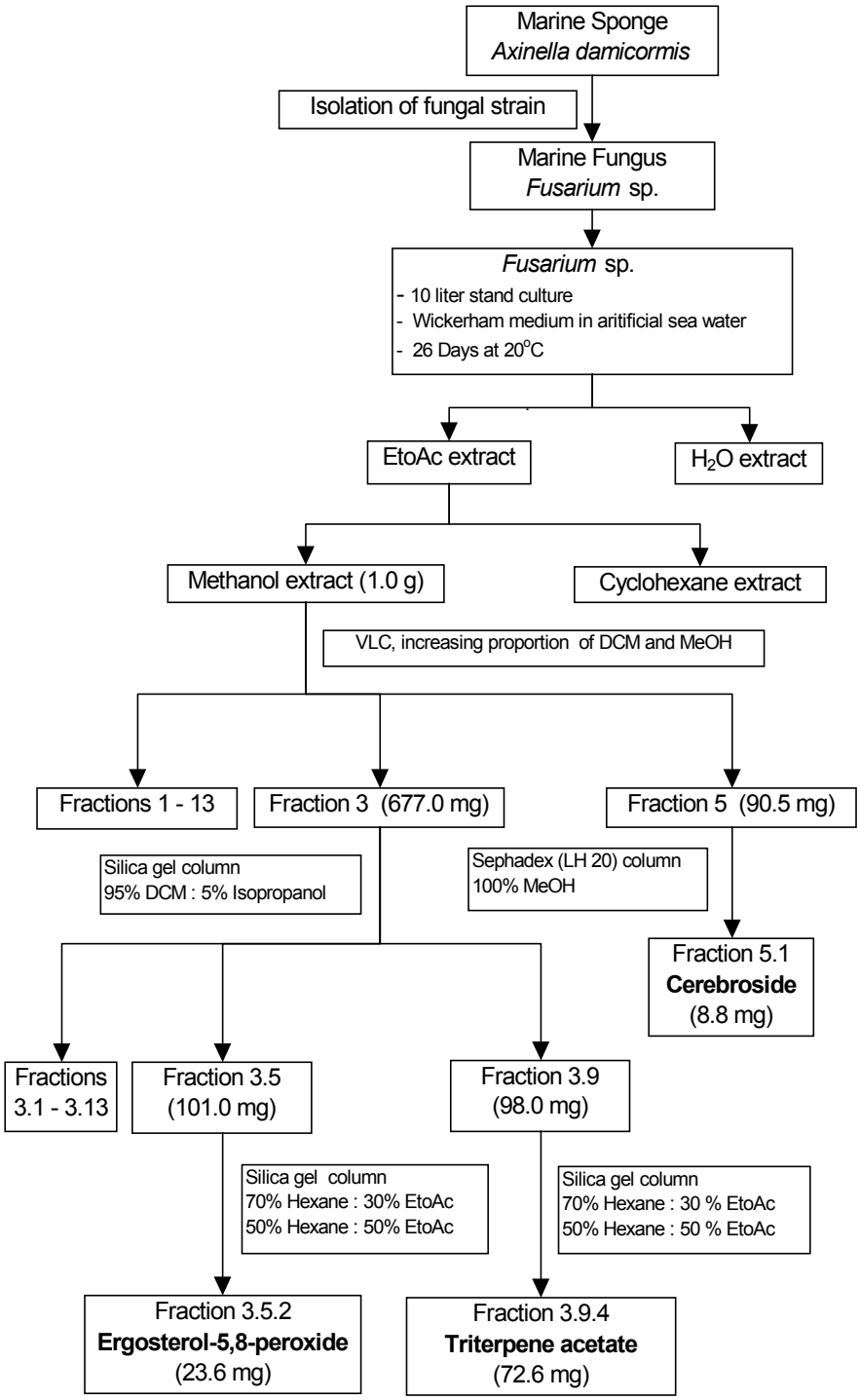



Figure 2.2.4. Secondary metabolites of *Fusarium* sp. isolation scheme

## 2.3. General chemical substances and equipments

Several chemical substances and equipments used in this study are listed in Table 2.3.1 and 2.3.2., respectively. Solvents including acetone, acetonitril, chloroform, cyclohexane, dicholoromethane, dimethylsulfoxide, ethanol, ethyl acetate, *n*-hexane, isopropanol, and methanol were purchased from the chemical storage, Heinrich-Heine University, Düsseldorf. They were all distilled prior to use and spectral grade solvents were used for spectroscopic measurements.

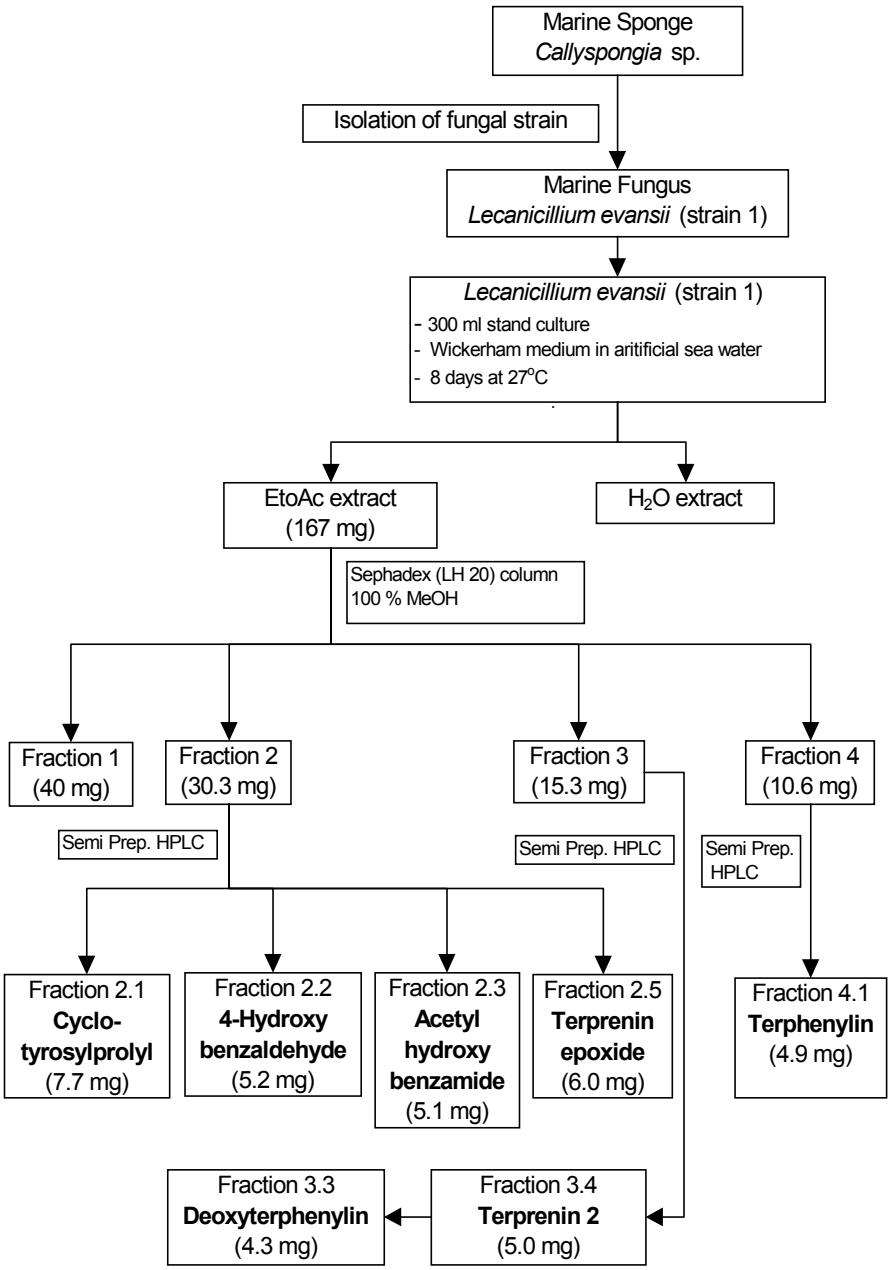



Figure 2.2.5. Secondary metabolites of *Lecanicillium evansii* (strain 1) isolation scheme

Table 2.3.1. Laboratory chemical substances

| Chemical substances | Company |
|---|---|
| 1. Anisaldehyde (4-methoxybenzaldehyde) | Merck |
| 2. Ascorbic acid (Vitamine C) | Merck |
| 3. Formaldehyde | Merck |
| 4. Gentamycin sulphate | Merck |
| 5. Glacial acetic acid | Merck |
| 6. Hydrochloric acid | Merck |
| 7. Nipagin (*p*-hydroxybenzoic acid) | Sigma |
| 8. Sodium hydroxide | Merck |
| 9. Sulphuric acid | Merck |
| 10. Trifluroacetic acid (TFA) | Merck |

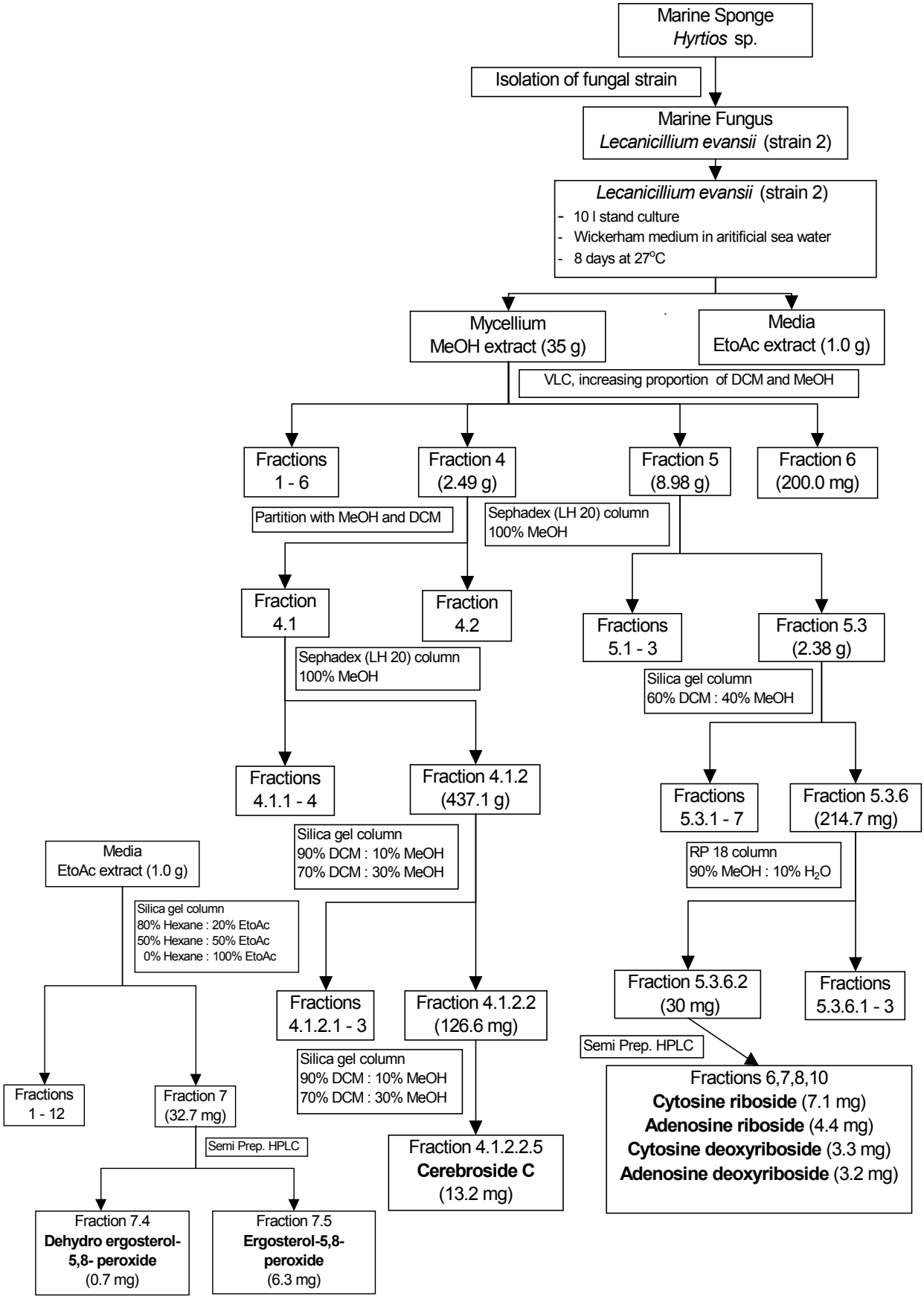



Figure 2.2.6. Secondary metabolites of *Lecanicillium evansii* (strain 2) isolation scheme

Table 2.3.2. Several equipments frequently used

| Equipments | Type and company origin |
|---|---|
| 1. Automatic pipette | Eppendropf |
| 2. Autoclave | Varioklav (Dampfsterilisator) |
| 3. Balances | Sartorius BL 1500 and, Sartorius WRC 6001 |
| 4. Centrifuge | Biofuge (pico), Heraeus |
| 5. Fraction collector | Retriever II |
| 6. Freeze dryer | Lyovac GT2 Pump Trivac D10E |
| 7. Magnetic stirrer and hot plates | IKA Combimag RCH |
| 8. Ovens | Memmert and Heraeus |
| 9. pH-meter | Inolab |
| 10. Rotary evaporator | Vacuuband |
| 11. Sonicator | Bandelin Sonorex RK 510S |
| 12. Syringe | Hamilton 1701 RSN |
| 13. Ultra thorax | Ika |
| 14. UV Lamp | Camag (254 and 366 nm) |
| 15. Shaker (mini shaker) | MS2 – IKA |
| 16. Vacuum filtration | Vacuuband (Supelco) |
| 17. Vacuum exsiccator | Savant speedvac SPD111V<br>Savant refrigerator vapour trap RVT400 Pump Savant VLP80 |

## 2.4. Chromatographic methods

### 2.4.1. Solvent extraction

An unknown natural product sample often contains a mixture of many components in a complex matrix. The components must be separated from each other so that each individual component can be identified by other analytical methods. Many organic liquids are immiscible with water. When such a liquid is added to water, two layers are formed. Whether the organic layer is in the upper or lower layer depends upon the relative density of organic liquid and water.

Suppose that an aqueous solution of two components A and B is mixed with an immiscible organic liquid, the mixture is shaken vigorously and then allowed to settle. If one of the components is more soluble in the organic layer than in the aqueous layer, then this component will be extracted into the organic layer. Assuming another component is more soluble in the aqueous layer then this component goes to the aqueous layer, resulting in two different layers. This liquid extraction is based on " a like-dislike" principle.

### 2.4.2. Thin layer chromatography (TLC)

The term chromatography, first introduced by Tswett in 1906, refers to any separation method in which the components are distributed between stationary phase and a moving (mobile) phase. The stationary phase is either a porous solid used alone or coated with a stationary liquid phase. Separation occurs because sample components have different affinities for the stationary and mobile phases and therefore move at different rates along the column. The mobile phase is called the eluent and the process by which the eluent causes a compound to move along the column is called elution.

TLC consists of a stationary phase immobilised on a glass or plastic plate, and an organic solvent. The sample, either liquid or dissolved in a volatile solvent, is deposited as a spot on the stationary phase. The bottom edge of the plate is placed in a solvent reservoir, and the solvent moves up the plate by capillary action. When the solvent front reaches the other edge of the stationary phase, the

plate is removed from the solvent reservoir. The separated spots are visualised with ultraviolet light. The different components in the mixture move up the plate at different rates due to differences in their partitioning behaviour.

TLCs were performed on pre-coated TLC plates with silica gel 60 $F_{254}$ (layer thickness 0.2 mm) with $CH_2Cl_2$:MeOH (90:10 or 95:5) for less polar compound, $CH_2Cl_2$:*iso*-$C_3H_7OH$ (75:25) or EtOAc:*n*-Hexane (70:30) for semi-polar compounds. TLC on reversed phase (RP)-C18 $F_{254}$ (layer thickness 0.25 mm) was also used for polar substances, using the solvent system of MeOH:$H_2O$ (85:15, 80:20, 70:30 or 60:40).

The bands separation on the TLC plate describing the separation of compounds were detected under UV absorbency at 254 and 366 nm, followed by spraying the TLC plates with anisaldehyde reagent with subsequent heating at 110$^{o}$C. Anisaldehyde/$H_2SO_4$ Spray Reagent (DAB 10) was prepared as follows: Anisaldehyde (5 parts), Glacial acetic acid (100 parts), Methanol (85 parts), concentrated $H_2SO_4$ (5 parts). The reagent was stored in an amber-coloured bottle and kept refrigerated until use.

TLC was always conducted to each fraction prior to further chemical work, to get the overview of the identity of each of the fraction and the qualitative purity of the fraction and the isolated compound. Band separation in TLC was also very helpful in optimising the solvent system that would be later applied for column chromatography.

## 2.4.3. Vacuum liquid chromatography (VLC)

VLC is a normal phase column using silica gel a stationary phase. In this study, prior to intense chemical investigation, the VLC was conducted toward all raw extracts. The VLC column was connected to a pump to generate vacuum condition in the column. A series of polar (e.g. methanol) and non polar (e.g. dichloromethane) solvent combinations were applied to separate the raw extract chemical content, based on their polarity difference. This technique is valuable for a great amount of raw extract. With VLC the separation of raw extract was accelerated.

## 2.4.4. Column chromatography

Fractions derived from VLC were subjected to repeated separation through column chromatography using appropriate stationary and mobile phase solvent system previously determined by TLC. Purification of fractions was later performed on semi-preparative HPLC. The mobile phase is a solvent and the stationary phase is a liquid on a solid support, a solid, or an ion-exchange resin. Liquid chromatography can be distinguished into four general classes:

a) Normal phase chromatography uses a polar stationary phase, typically silica gel in conjunction with a non-polar mobile phase (*n*-hexane, chloroform, etc). Thus hydrophobic compounds elute more quickly than do hydrophilic compounds.

b) Reverse phase (RP) chromatography uses a non-polar stationary phase and a polar mobile phase (water, methanol, acetonitril, and tetrahydrofuran). RP operates on the basis of hydrophilicity and lipophilicity. The stationary phase consists of silica packed with *n*-alkyl chains covalently bound. For instance, C-8 signifies a *n*-decyl chain and C-18 an octadecyl ligand in the matrix. The more hydrophobic the matrix on each ligand, the greater is the tendency of the column to retain hydrophobic moieties. Thus hydrophilic compounds elute more quickly than do hydrophobic compounds. RP is the most common form of liquid chromatography, primarily due to the wide range on analytes that can dissolve in the mobile phase.

c) Ion exchange chromatography involves ionic interactions. The mobile phase supports ionisation to ensure solubility of ionic solutes. The stationary phase must be partially ionic to promote some retention. Consequently, the interactions with the stationary phase are strong, and this is usually reflected in longer analysis times and broad peaks.

d) Size exclusion chromatography involves separations based on molecular size of compounds being analysed. The stationary phase consists of porous beads. The larger compounds will be excluded from the interior of the bead and thus will elute first. The smaller compounds will be allowed to enter the beads and elute according to their ability to exit from the same sized pores they were internalised through. The column can be either silica or non silica based.

Sephadex LH-20 columns with 100% methanol as eluent, silica gel columns with several eluent combinations [($CH_2Cl_2$ : MeOH), ($CH_2Cl_2$ : *iso*-$C_3H_7OH$), (*n*Hexane : EtoAc)], RP columns with methanol and nano pure water as eluent were frequently used in this study. Substances used in chromatography methods are listed in Table 2.4. 1.

Table 2.4.1. Substances for chromatography

| Chemical substances | Company |
|---|---|
| 1. Pre-coated TLC plates (AluO), Silica gel 60 F254, layer thickness 0.2 mm | Merck |
| 2. Pre-coated TLC plates (Glass), RP-18, F254 S, layer thickness 0.25 mm | Merck |
| 3. Silica gel 60, 0.04-0.063 mm mesh size | Merck |
| 4. Sephadex LH 20, 25-100 mm mesh size | Merck |
| 5. HPLC solvents (Methanol LiChroSolv HPLC) | Merck |
| 6. Phosphoric acid (85% p.a.) | Merck |
| 7. Nano pure water | Institute of Botany |

## 2.4.5. Analytical HPLC

High performance liquid chromatography (HPLC) was developed in the mid 1970's and quickly improved with the development of column packing materials and the additional convenience of on-line detectors. In the late 1970's, new methods including reverse phase liquid chromatography allowed for improved separation of very similar compounds.

HPLC utilises high pressure pumps to increase the efficiency of the separation. The use of analytical HPLC was meant to identify the distribution of peaks either from raw extracts or fractions, as well as to evaluate the purity of isolated compounds. Compounds are separated by injecting a plug of sample mixture onto the column. The different components in the mixture pass through the column at different rates due to differences in their partitioning behaviour between the mobile liquid phase and the stationary phase.

The solvent gradient applied started with 10:90 [methanol: nano pure water (adjusted to pH 2 with phosphoric acid)] to 100% methanol in 35 minutes. Auto sampler took 20 μl sample. All peaks pointing out the compounds that have UV absorption were detected by UV-VIS diode array detector. HPLC instruments consists of the reservoir of mobile phase, the pump, the injector, the separation column, and the detector. The specific analytical HPLC (Dionex) is described in Table 2.4.2.

Table 2.4.2. Analytical HPLC specification

| HPLC part | Specification |
|---|---|
| Pump | Dionex P580A LPG |
| Programme | Chromelon Ver 6.3 |
| Detector | Dionex, photo array detector UVD 340S |
| Column thermostat | STH 585 |
| Auto sampler | ASI – 100T |

## 2.4.6. Semi-preparative HPLC

Chemical separation can be accomplished using HPLC by utilising the fact that certain compounds have different migration rates in a particular column and mobile phase. Preparative HPLC refers to the process of isolation and purification of compounds. This differs from analytical HPLC, where the focus is to obtain information about the compound including identification, quantification, and resolution.

After conducting a series of column chromatographic separations, the semi-preparative HPLC equipment (Merck) was used for the isolation of pure compounds from fractions. Prior to working with semi preparative HPLC, mobile phase (eluent) system should be first pursued to find out the most appropriate mobile phase combination of either methanol and nano pure water, or acetonitril and nano pure water, with or without the addition of 0.1% TFA. The proportion of eluent combination either gradient or isocratic depends primarily on the compound UV absorption and retention time. The finding of this most suitable mobile phase solvent system was performed by using the analytical column.

Supposed that, the most appropriate eluent solvent system has already been set up, the real injection using semi-preparative column was done. Every injection carried 2 mg of the fraction dissolved in 1 ml of the solvent system. The solvent system was pumped through the column at a rate of 5 ml/min. The eluted peaks, which were clearly detected by the online UV detector, were collected separately in Erlenmeyer flasks.

## 2.5. Secondary metabolites structure elucidation

## 2.5.1. Mass spectrometry (MS)

Mass spectrometers use the difference in mass-to-charge ration (*m*/*z*) of ionised atoms or molecules to separate them from each other. Mass spectrometry is therefore useful for quantification of atoms or molecules and also for determination of chemical and structural information of molecules. In general a mass spectrometer consists of an ion source, a mass-selective analyser and an ion detector.

The output of the mass spectrometer shows a plot of relative intensity *vs* the mass-to-charge ratio (*m*/*z*). The most intense peak in the spectrum is termed the base peak and all others are reported relative to its intensity. The highest molecular weight peak observed in the spectrum will typically represent the parent molecule, minus an electron, and is termed molecular ion ($M^+$). This peak will represent the molecular weight of the compound. Its appearance depends on the stability of the compound. Double bonds, cyclic structures and aromatic rings stabilise the molecular ion and increase the probability of its appearance.

Molecules have distinctive fragmentation patterns that provide structural information to identify structural components. The process of fragmentation follows simple and predictable chemical pathways. Functional groups and overall structure determine how some portions of molecules will resist fragmenting, while other portions will fragment easily. Commonly lost fragments in mass spectrum are presented in Table 2.5.1.

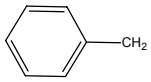
EI and FAB-MS measurements were performed by Dr. Peter Tommes of the *Institut für Anorganische Chemie und Strukturchemie*. ESI-MS measurement was conducted in the Institute for Pharmaceutical Biology, Heinrich-Heine University, Düsseldorf. The following elaborations differentiate the above mass spectra types.

#### a) EI-MS (electron impact mass spectrometry)

Vaporised sample is bombarded with a beam of electron (10 – 100 eV). A reagent gas is introduced in the source and sample is ionised by electron (500 eV). The high energy electron stream not only

ionises an organic molecule but also causes extensive fragmentation. The advantage is that fragmentation is extensive, giving rise to a pattern of fragment ions which can help to characterise the compound.

Table 2.5.1. Commonly lost fragments in mass spectrum

| Molecular weight | Commonly lost fragments |
|---|---|
| M - 15 | $CH_3$ |
| M - 17 | OH |
| M - 26 | CN |
| M - 28 | $CH_2 = CH_2$ |
| M - 29 | $CH_2 - CH_3$ |
| M - 31 | $OCH_3$ |
| M – 35 | Cl |
| M - 43 | $CH_3C = O$ |
| M - 91 |  |



**b) FAB-MS (fast atom bombardment mass spectrometry)**

In FAB a high energy beam of neutral atoms, typically Xe or Ar, strikes a solid sample causing desorption and ionisation. It is used for large biological molecules that are difficult to get into the gas phase. FAB causes little fragmentation and usually gives a large molecular ion peak, making it useful for molecular weight determination. The atomic beam is produced by accelerating ions from an ion source though a charge-exchange cell. The ions pick up an electron in collisions with neutral atoms to form a beam of high energy atoms.

**c) ESI-MS (electron spray ionisation mass spectrometry)**

A sample solution is sprayed at atmospheric pressure into the source chamber to form droplets. The droplets carry charge when they exit the capillary, and as the solvent evaporates, the droplets disappear leaving highly charged analyte molecules. ESI is particularly useful for large biological molecules that are difficult to vaporise or ionise. Comparison of different ionisation method of mass spectra are presented in Table 2.5.2.

Table 2.5.2. Comparison of different ionisation methods of mass spectra

| Ionisation methods | Typical analytes | Sample introduction | Mass range |
|---|---|---|---|
| Electron impact (EI) | Relatively small volatile | GC or liquid/solid probe | until 1,000 Dalton |
| Chemical ionisation (CI) | Relatively small volatile | GC or liquid/solid probe | until 1,000 Dalton |
| Electron spray (ESI) | Peptides, proteins, non volatile | Liquid chromatography or syringe | until 200,000 Dalton |
| Fast atom bombardment (FAB) | Carbohydrates, organometallics, peptides, non volatile | Sample mixed in viscous matrix | until 20,000 Dalton |
| Matrix assisted laser desorption (MALDI) | Peptides, proteins, nucleotides | Sample mixed in solid matrix | until 500,000 Dalton |

## 2.5.2. Nuclear magnetic resonance spectroscopy (NMR)

The first commercial NMR spectrometers for analysing $^{1}H$ content of organic molecules were introduced in the early 1950's. NMR data of protons and carbons determine chemical shift, coupling constant (multiplicity type), and peak areas (integrals).

The atomic nuclei of all elements carry a charge, which is the characteristic required to generate NMR peaks. $^{1}H$ and $^{13}C$ spin about an axis in a random fashion. However, when placed between poles of a strong magnet, the spins are aligned either parallel or anti parallel to the magnetic field, with the parallel orientation favoured since it is slightly lower in energy. The nuclei are then irradiated with electromagnetic radiation which is absorbed and places the parallel nuclei into a higher energy state; consequently, they are now in resonance with the radiation. Each H and C will produce different spectra depending on their location and adjacent molecules, or elements in the compound, because all nuclei in molecules are surrounded by electron clouds which change the encompassing magnetic field and thereby alter the absorption frequency.

The measurement of 1D and 2D NMR spectra was carried out by Dr. W. Peters at the NMR service, Heinrich-Heine University, Düsseldorf, and Dr. Victor Wray at the Institute for Biotechnology (*Gesellschaft für Biotechnologische Forschung/GBF*), Braunschweig, Germany. Proton ($^{1}H$) and carbon ($^{13}C$) NMR spectra were recorded at 300$^{o}$K on Bruker DPX 300, ARX 400 or AVANCE DMX 600 NMR spectrometers.

Several deuterated solvents (DMSO-$d_6$, $CDCl_3$, MeOD, Acetone) were used to dissolve samples for NMR measurement. The selection of which was primarily dependent on the solubility of the sample and the consideration of obtaining hydroxyl and amine group. Spectra of pure compound were processed using Bruker 1D WIN-NMR or 2D WIN-NMR software.

They were calibrated using solvent signals of carbon ($^{13}C$: $CDCl_3$ 77.00 ppm, MeOD 49.00 ppm, $CD_3COCD_3$ 30.50 ppm, DMSO-d6 39.70 ppm) and proton ($^{1}H$: $CDCl_3$ 7.26 ppm, $D_2O$ 4.79, MeOD 3.35 ppm, DMSO-d6 2.50 ppm, acetone-d6 2.05 ppm). The observed chemical shift ($\delta$) values were given in ppm and the coupling constants ($J$) in Hz.

Multiplicity for $^{13}C$ was deduced from DEPT experiments; singlet (s) = C, doublet (d) = CH, triplet (t) = $CH_2$, quartet (q) = $CH_3$. Structural assignment was based on spectra resulting from one or more of the following NMR spectra measurement: $^{1}H$, $^{13}C$, DEPT, $^{1}H \rightarrow {}^{1}H$ COSY, $^{1}H \rightarrow {}^{13}C$ direct correlation (HMQC), $^{1}H \rightarrow {}^{13}C$ long range correlation (HMBC), ROESY and NOE.

## 2.5.3. Optical activity

Optically active compounds contain at least one chiral centre. Optical activity is a macroscopic property of a collection of these molecules that arises from the way they interact with light. The instrument with which optically compounds are studied is a polarimeter. This equipment consists of a light source, two polarising filters and a cell that contains a solution of an optically active compound.

A method of differentiating enantiomers is based on the following differences: the *d* or (+) optical isomer rotates the light plane clockwise (dextro-rotary) and the *l* or (-) optical isomers rotates the light plane counter-clockwise (levo-rotary)

Amino acids have the formula $^{+}H_3NCH(R)CO_2^{-}$. The central C atom is surrounded by four different groups (except in the case of glycine, where R = H), so that amino acids are chiral. All naturally-occurring amino acids exist as only one of the two possible enantiomers, and so by extension, all proteins and enzymes are also chiral.

A Perkin-Elmer-241 MC Polarimeter was used for analysing optical rotation. The substance was stored in a 0.5 ml cuvette with 0.1 dm length. The angle of rotation was measured at the wavelength of 546 and 579 nm of a mercury vapour lamp at room temperature (25$^{o}$C). The following expression was used for calculating specific optical rotation.

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

Where: $[\alpha]_D^{20}$ = The specific rotation at 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, calculated by the following formula.

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

where: [α] = The angle of rotation (°)
*l* = The length of polarimeter tube (dm),
*c* = The concentration of the substance (g/100 ml of the solution)

## 2.6. Bioassays

Extraction and isolation of secondary metabolite in this research was a bioassay-guided study. Prior to conducting the chemical investigation, a number of fungal raw extracts underwent a series of biological screening tests including brine shrimp assay, insecticidal assay, and cytotoxicity test.

Only fungi having a high toxicity were selected for further chemical investigation. Pure isolated compounds were also subjected to antimicrobial assay to obtain more specific biological activity. Four types of bioassay applied in this study are elaborated below.

### 2.6.1. Brine shrimp assay

Brine shrimp assay is an *in vivo* lethality test involving the whole body of a tiny crustacean, brine shrimp (*Artemia salina* Leach). The brine shrimp lethality assay is considered as a useful tool for preliminary assessment of toxicity. From a pharmacological point of view, a good relationship has been found with the brine shrimp lethality test to detect antitumoral compounds in terrestrial plant extract (Carballo *et al*., 2002).

Newly hatched brine shrimps were used as test organisms. It has been shown that *A. salina* is highly vulnerable to toxins at the early development stage. Acute effect which is a quick short term response, signified by the death of brine shrimp, instead of chronic effect which has a long term effect, was selected as a parameter of classifying toxicity level of test substance.

#### 2.6.1.1. Sample preparation

Substances to be tested were diluted in an organic solvent and the appropriate amount was poured into a 10 ml test vial. Bioassay was done on 0.5 mg crude extract and various concentrations of the pure compound.

The test substance in test vial was then dried under nitrogen. To achieve better mixture between test substance and artificial sea water, 20 μl DMSO was added. Control vials containing DMSO only were also prepared.

### 2.6.1.2. Hatching the eggs

As much as 33 g salt mixture (Biomarine, Erkrath, Germany) was dissolved in 1000 ml distilled water, thoroughly mixed with the help of magnetic stirrer. A small amount of dried brine shrimp eggs (Dohse, Bonn, Germany) were hatched in a small tank filled with 1000 ml artificial sea water. After 48 hours, the eggs would hatch.

Twenty nauplii were taken from the hatching tank, and transferred into each test vial (test substance + artificial sea water + DMSO). If the transfer of 20 nauplii finished, artificial sea water was then added to make the end volume of 5 ml. The experimental vials were left overnight, under illumination, in constant room temperature.

After 24 hours, survivors and dead brine shrimp were counted. The more the death of brine shrimp, the more toxic of the test substance. Test substance resulting in 50% death of test animals was regarded as having bioactivity.

## 2.6.2. Insecticidal bioassay

### 2.6.2.1. Culture maintenance

*Spodoptera littoralis* has a relatively short life cycle (4 weeks). The relatively ease to maintain them in the laboratory with an artificial diet composed mainly of leguminous beans in agar makes a wide range application of this insect for insecticidal assay for a number of natural products. This insect is one of the most hazardous pest found in the Mediterranean region and Africa.

In order to keep *S. littoralis* continuously alive in the insect room, the maintenance of egg, larvae, pupae, and adult was done in a regular basis. Fresh diet was supplied to the larvae, pupae, and adults every other days. Larvae were reared in different plastic box according to their age level. Larvae boxes were kept in a chamber at 28$^{o}$C.

The number of larvae at their pre-pupal stage were reduced to prevent cannibalism. Pre-pupal stage were transferred into a plastic box containing no diet until they reached pupal stage. The pupal stage were then stored in a dark humid chamber at 28$^{o}$C until they developed to their adult stage.

A 10 litre plastic pail, lined with filter paper on which the female can lay their eggs, was used for rearing the adults. The adults feed on sugar solution incorporated in a cotton which was placed onto a petri disc. The plastic pail was maintained at a constant temperature of 28$^{o}$C.

Along with the replacement of diet, the removal of dead adults, the laid eggs sticking on the paper were also collected every two days and transferred to the dark chamber until the neonate larvae hatch. This culture maintenance was done all year around.

### 2.6.2.2. Artificial diet

Table 2.6.1 presents the substance used for preparing 5 litre artificial diet. White beans (600 g) were mixed with 1600 ml water and left overnight. This mixture was then brought into suspension and homogenised with the help of mixer. Substances 2 – 6 were added into the bean suspension.

Table 2.6.1. Substances for insect artificial diet

| Substances | Amounts |
|---|---|
| 1. White beans (Müller's Mühle GmbH) | 600 g |
| 2. Hefe/yeast extract (Bella back ) | 100 g (4 packs) |
| 3. Ascorbic acid / Vitamin C (Caelo) | 12 g |
| 4. Nipagin/*p*-hydroxybenzoic acid ethyl ester (Sigma) | 12 g |
| 5. Gentamycin sulphate (Serva) | 720 mg |
| 6. Formaldehyde (Merck) | 4 ml |
| 7. Agar (Caelo) | 40 g |

As much as 40 g agar was boiled in 1000 ml water and cooled down to ca 50$^{o}$C, then added into the bean suspension, and thoroughly mixed. The agar bean mixture was poured into a several plastic box and cooled in room temperature to solidify. The ready to use agar bean diet was then stored in the refrigerator.

## 2.6.2.3. Chronic feeding assay

Insecticidal assay for crude extract and fraction needed concentrations of 5.0 mg and 1.0 mg, respectively. Test substances were evenly mixed with 0.735 g freeze-dried diet (artificial diet without agar). Then 2 ml methanol was added to homogenise the mixture. The homogenised mixture was left overnight in the fume hood to allow evaporation of the methanol.

Gentamycin sulphate (53.2 mg) was dissolved in 100 ml water. Into the already dried mixture was incorporated 1.41 ml gentamycin sulphate to prevent the growth of fungi in the diet. Agar (7.2 g) dissolved into 200 ml water was boiled, and cooled until ca 50$^{o}$C. Around 2.2 ml agar was added into the dried diet, thoroughly mixed, and allowed to solidify. The solidified agar and diet were transferred into a small plastic experimental box.

Into each plastic box were introduced 20 neonate larvae of *S. littoralis* . Those boxes were covered with a small size net, and maintained in the incubation chamber with constant temperature of 28$^{o}$C for one week. To keep the agar mixture always with enough moisture, a wet towel which covered the experiment boxes was soaked with water daily. Biological activity of test substance was determined as the survival or growth rate in percent relative to controls.

## 2.6.3. Antimicrobial assay

Four bacterial strains: gram positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, gram negative bacterium *Escherichia coli* and three fungal strains: *Saccharomyces cerevisiae*, *Candida albicans*, and *Cladosporium herbarum* were applied as test microbes in this study.

## 2.6.3.1. Culture preparation

Bauer Kirby Test developed by Bauer *et al*. (1966) was performed in this study. A few colonies of the selected test microorganisms were sub-cultured in 4 ml of tryptose-soy broth (Sigma) and allowed to grow for 2 to 5 hours to reach a microbial suspension of moderate cloudiness.

The suspension was then diluted with sterile saline solution to a density visually equivalent to that of a barium sulphate standard solution. This standard was prepared by mixing 0.5 ml (1% $BaCl_2$) with 99.5 ml (1% 0.36 N $H_2SO_4$).

The previously prepared microbial broth was inoculated onto a surface of petri dish containing a mixture of medium and agar plates, and dispersed evenly by means of sterile beads and allowed them to settle for a while. Media used for this antimicrobial assay are presented in Table 2.6.2, 3 and 4. This 1000 ml media were divided into 32 plates. Each plate could hold 8 test substances.

Table 2.6.2. Agar plate medium (Luria Bertoni) for *B. subtilis* and *E. coli*

| Substances | Amounts |
|---|---|
| Sodium chloride (Aldrich) | 10.0 g |
| Tryptone (Sigma) | 10.0 g |
| Yeast extract (Sigma) | 5.0 g |
| Distilled water | 1000.0 ml |
| NaOH and HCl for pH adjustment to 7 | |
| Agar (Caelo) | 15.0 g |

Table 2.6.3. Universal broth medium (medium 186) for yeast for *S. cerevisiae*

| Substances | Amounts |
|---|---|
| Glucose (Caelo) | 10.0 g |
| Peptone (Merck) | 5.0 g |
| Malt extract (Merck) | 3.0 g |
| Yeast extract (Sigma) | 3.0 g |
| Distilled water | 1000.0 ml |
| Agar (Caelo) | 15.0 g |

Table 2.6.4. Potato dextrose agar (medium 129) for *C. herbarum*

| Substances | Amounts |
|---|---|
| Potatoes infusion (200 g scrubbed and sliced potatoes was boiled in 1000 ml water for 1 hour, and then passed through fine sieve) | 1000.0 ml |
| Glucose (Caelo) | 20.0 g |
| Agar (Caelo) | 15.0 g |

## 2.6.3.2. Agar plate diffusion assay

Aliquots of the test substance were incorporated into a sterile filter paper discs (5 mm diameter) to obtain a dish loading concentration of 500 μg for crude extracts and two various concentrations (100 and 50 μg) for pure compounds.

The test substance-impregnated paper discs were allowed to dry for a while, and placed on the surface of agar media previously seeded with the appropriate test microorganisms. The experimental petri disc along with petri disc containing no test substance were incubated at 37$^{o}$C for 24 hours for antibacterial assay and under room temperature for around two or three days for antifungal assay.

During the assay, the test substance which was previously dispersed in filter paper disc diffused through the agar medium. The size of the clear zone (measured in mm diameter) around the filter paper disc denoted the growth inhibition which related to the level of antimicrobial activity of the test substance. The wider the growth zone inhibition the greater bioactivity of the test substance.

## 2.6.4. Cytotoxicity test

Cytotoxicity relates to the degree to which an agent possesses a specific destructive action on certain cells. Cytotoxicity testing is a rapid, standardised, sensitive, and inexpensive means to determine whether a material contains significant quantities of biologically harmful extractable agents. The high sensitivity of the test is due to the isolation of the test cells in cultures and the absence of the protective mechanisms that assist cells within the body.

A mammalian cell culture medium is the preferred extractant because it is a physiological solution capable of extracting a wide range of chemical structures, not just those soluble in water.

Cytotocixity test by using human cancer cell lines JURKAT, THP-1, and MM-1 (Schneider *et al*., 1977; Tsuchiya *et al*., 1980) was performed by Dr. Klaus Steube of *Deutsche Sammlung von Mikroorganismen und Zellkulturen GmBH*, Braunschweig, Germany. Cells grown in plastic flasks were grown under a media supplemented with 10-20% heat inactivated fetal bovine serum and cultivated at 37$^{o}$C in a humidified atmosphere containing 5% $CO_2$.

Ethylene glycol monomethyl ether (EGMME) or DMSO was used for dissolving test substances, test substances were then diluted in RPM1-1640 culture medium and stored at –20$^{o}$C. For the experiment the test substance concentration was set to be 0.1%.

Exponentially growing cells, detected by more than 90% viability as measured by trypan blue exclusion, were harvested. Those cells were re-suspended in fresh medium to give a concentration (2 to 4 x $10^5$ cells/ml). The number of cell was counted by a cell counting chamber after staining the cells with trypan blue.

As much as 90 μl cell cultures were seeded out into 96-well flat-bottom culture plates. Into this culture, the mixture of 10 μl medium and 0.1% test substance was added. Cytotoxicity was evaluated by the MTT assay after 48 hours incubation. Cells were harvested by a multiple automatic sample harvester, and glass fibre filters was used for collecting the cells (Steube *et al.*, 1998).

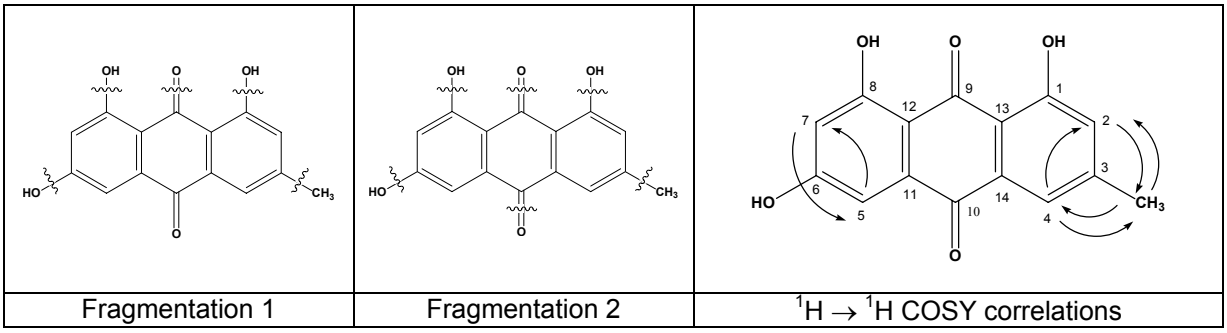
# III. RESULTS

## 3.1. Isolated secondary metabolites of fungus *Penicillium* sp.

Six secondary metabolites were isolated from fungus *Penicillium* sp that had been obtained from the sponge *Ircinia fasciculata*. They included emodin (compound **1**), hydroxyemodin (compound **2**), gancidin (compound **3**), meleagrin (compound **4**), citerohybridonol (compound **5**), and andrastin A (compound **6**). The following description elaborates more detailed on the above compounds.

### 3.1.1. Compound 1 (emodin)

The EI-MS spectrum of compound 1 presented ion peaks at *m*/*z* 270 $[M]^+$, 186 $[M\text{-}3OH\text{-}CH_3\text{-}H_2O]^+$ (fragment 1), 140 $[M\text{-}3OH\text{-}CH_3\text{-}O_2]^+$ (fragment 2) (Figure C1.1). Both fragments indicated that this compound contained three hydroxyl groups and one methyl group. NMR data of compound 1 are presented in Table C1.1.




| Fragmentation 1 | Fragmentation 2 | $^1H \rightarrow {}^1H$ COSY correlations |
|---|---|---|

Figure C1.1. Hypothetical fragmentation in the EI-MS spectrum and COSY correlations of compound **1** (emodin)

The $^1H$ NMR spectrum of compound **1** (Table C1.1, Figure C1.2 and C1.3) revealed four methine protons in the aromatic region [$\delta$ 7.45 (H2), 6.98 (H4), 7.04 (H5), 6.37 (H7)], one singlet (methyl proton) [$\delta$ 2.32 (H3)] attaching to phenyl ring. Furthermore, meta correlation was observed between H5 and H7 as pointed out by their doublet multiplicity and coupling constant of 2.0 Hz for H5 and 2.0 Hz for H7. Correlation between H5 and H7 was also readily observed in the COSY spectrum (Table C1.1 MeOD, Figure C1.1 and C1.4).

Meta position between H2 and H4 was explained by three bonds correlation between H3 ($CH_3$) and H2, H4 in the COSY correlation. Both H2 and H4 also connected to methyl at position 3 (Table C1.1 MeOD, Figure C1.1 and C1.4). The existence of methyl protons attaching to phenyl ring was indicated by a singlet proton located at downfield $\delta$ 2.32 (H3/$CH_3$).

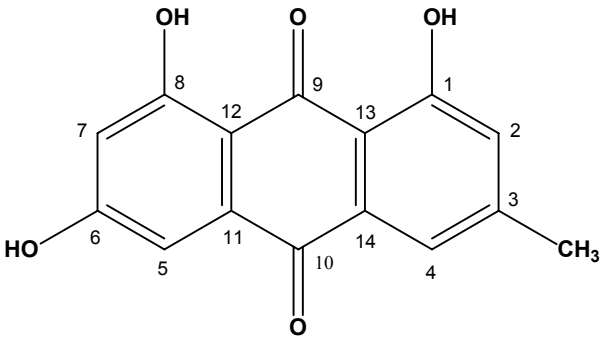
Comparison of $^1H$ NMR spectrum (MeOD and DMSO) of compound **1** with that of emodin (standard) exhibited similar chemical shifts, leading to the conclusion that the compound **1** was emodin (1,6,8-trihydroxy-3-methyl-anthraquinone). Antimicrobial assay results of compound **1** are displayed in Table C1.2

Table C1.2. Antimicrobial activity of compound **1** (emodin)

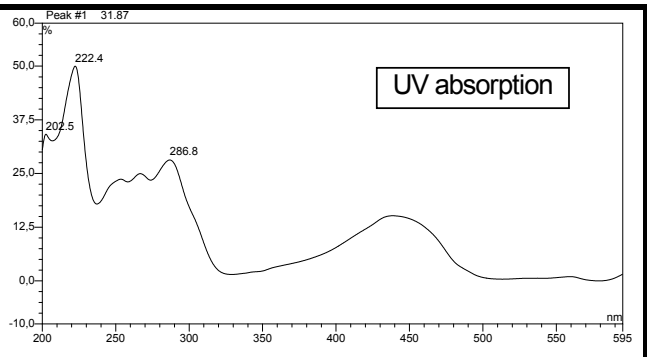
| Compound **1** | *Staphylococcus aureus* | *Bacillus subtilis* | *Escherichia coli* | *Candida albicans* | *Saccharomyces cerevisiae* | *Cladosporium herbarum* |
|---|---|---|---|---|---|---|
| 50 μg | not active | not active | not active | not active | not active | not active |
| 100 μg | not active | 7 mm | 9 mm | 10 mm | not active | not active |

## Emodin (compound 1)

| | |
|---|---|
| CAS Registry Number | : 518-82-1 |
| Characteristic | : orange powder |
| Formula | : $C_{15}H_{10}O_5$ |
| Molecular Weight | : 270 g/mol |
| Amount | : 3.5 mg |
| Source | : *Penicillium* sp. derived from *Ircinia fasciculata* |




| | |
|---|---|
| Retention Time | : 31.74 min |
| $R_f$ | : 0.71 |
| Fluorescence, 254 nm | : orange |
| 366 nm | : orange |
| Anisaldehyde/$H_2SO_4$ | : orange |




EI-MS (*m*/*z*, rel. int.) : 270 $[M]^+$ $_{(100)}$, $242_{(8.8)}$, $214_{(10.9)}$, $186_{(5.3)}$, $169_{(4.0)}$, $140_{(11.5)}$, $129_{(6.5)}$, $32_{(43.2)}$

Table C1.1. NMR data of compound **1** (emodin)

| Position | $\delta$ $^{13}$C (ppm), multiplicity ($J$ in Hz) (Cohen and Towers, 1995 in DMSO) | $\delta$ $^{1}$H (ppm), multiplicity ($J$ in Hz) (standard in DMSO) | $\delta$ $^{1}$H (ppm), multiplicity ($J$ in Hz) (standard in MeOD | $\delta$ $^{1}$H (ppm), multiplicity ($J$ in Hz) (in DMSO) | $\delta$ $^{1}$H (ppm), multiplicity ($J$ in Hz) (in MeOD) | COSY (H→ H) (in MeOD) |
|---|---|---|---|---|---|---|
| 1 (OH) | 161.90 (s) | 11.30 (s) | | 12.23 (s) | | |
| 2 | 124.80 (d) | 7.32 (s) | 7.35 (s) | 7.45 (s) | 7.54 (s) | H3 ($CH_3$), H4 |
| 3 | 149.30 (s) | | | | | |
| 3 ($CH_3$) | | 2.33 (s) | 2.24 (s) | 2.38 (s) | 2.42 (s) | H2, H4 |
| 4 | 121.20 (d) | 7.01 (s) | 6.88 (s) | 7.10 (s) | 7.06 (s) | H2, H3 ($CH_3$) |
| 5 | 109.70 (d) | 6.98 (d, 2.2) | 6.97 (d, 2.2) | 6.88 (s) | 7.30 (d, 2.0) | H7 |
| 6 (OH) | 167.00 (s) | 11.87 (s) | | | | |
| 7 | 108.60 (d) | 6.48 (d, 2.2) | 6.36 (d, 2.2) | 6.19 (s) | 6.47 (d, 2.0) | H5 |
| 8 (OH) | 165.90 (s) | 11.95 (s) | | 12.49 (s) | | |
| 9 | 191.90 (s) | | | | | |
| 10 | 182.20 (s) | | | | | |
| 11 | 136.60 (s) | | | | | |
| 12 | 110.00 (s) | | | | | |
| 13 | 114.40 (s) | | | | | |
| 14 | 134.00 (s) | | | | | |

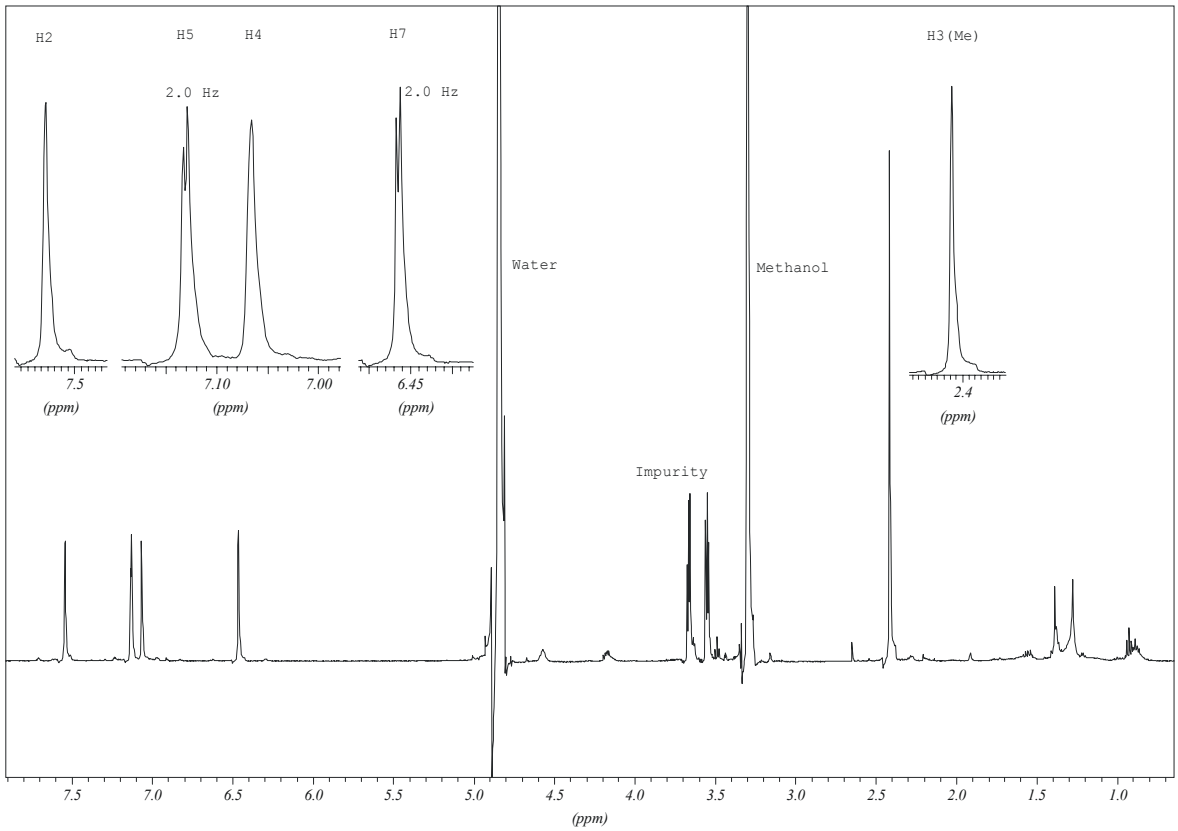



Figure C1.2. $^{1}$H NMR spectrum of compound **1** (emodin) in MeOD

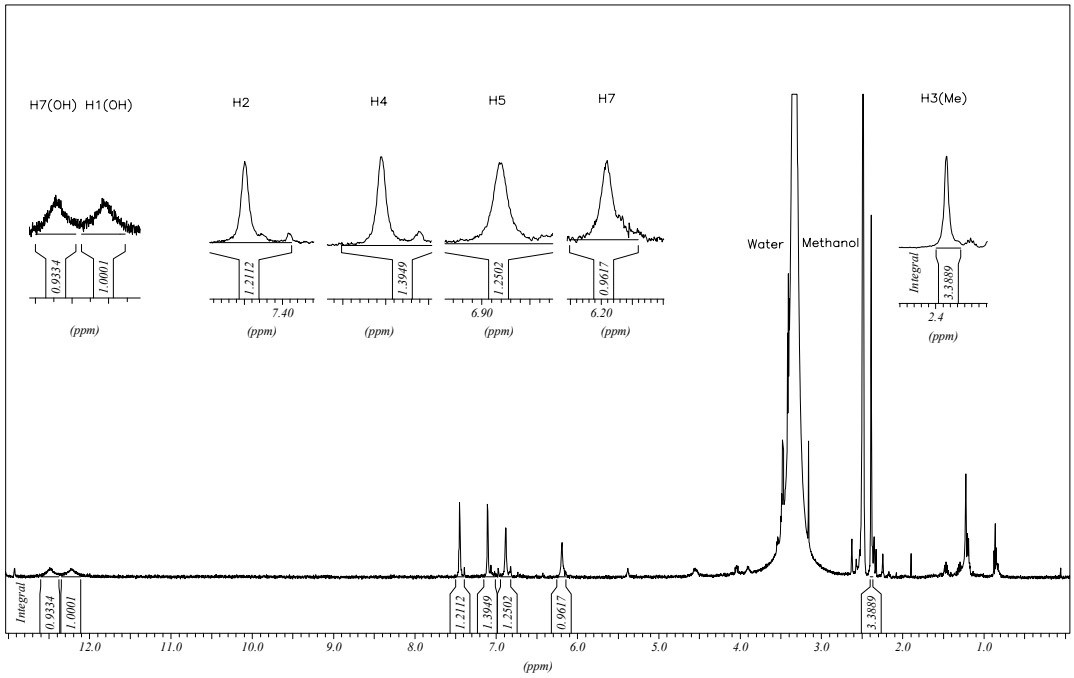



Figure C1.3. $^{1}$H NMR spectrum of compound **1** (emodin) in DMSO

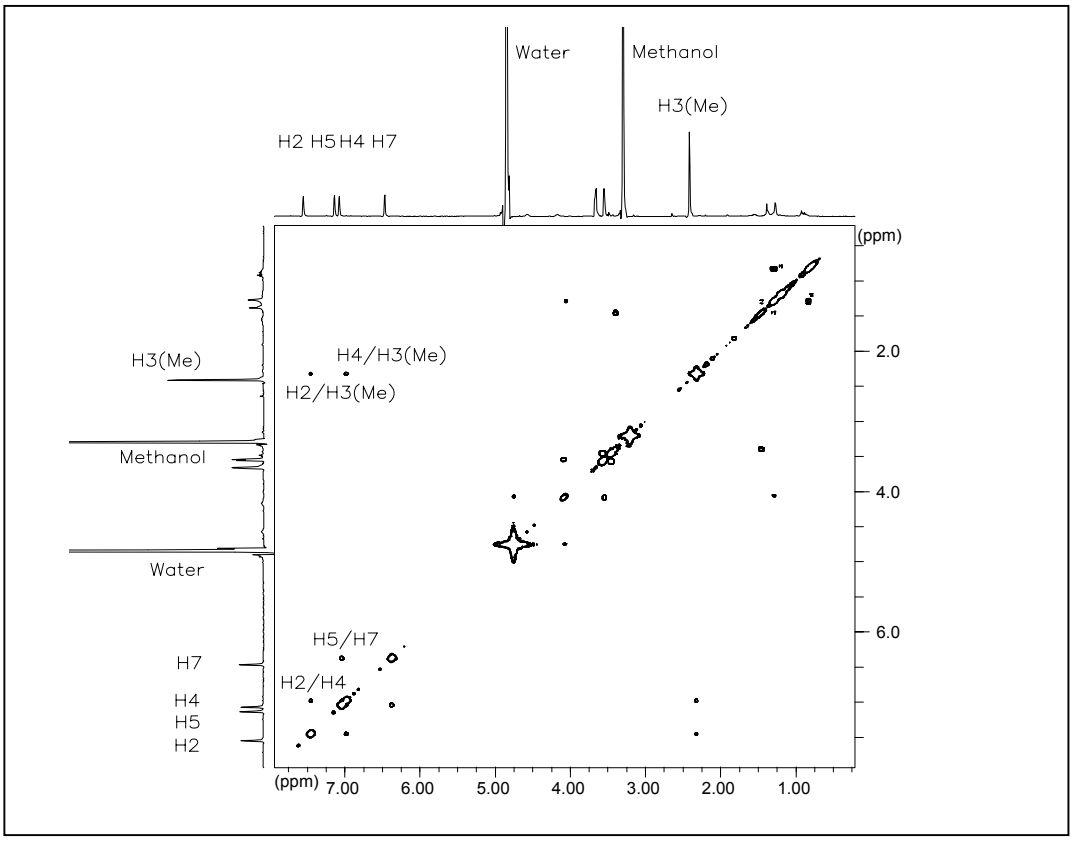
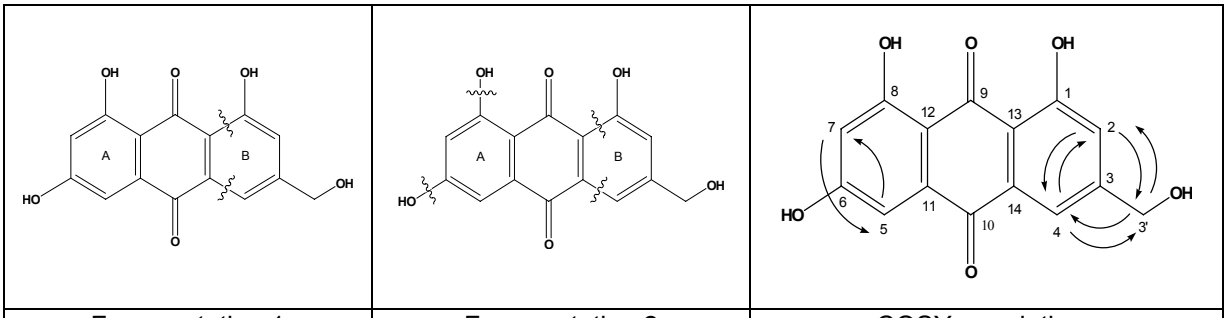



Figure C1.4. COSY spectrum of compound **1** (emodin) in MeOD

## 3.1.2. Compound 2 (hydroxyemodin)

Compound **2** when subjected to mass spectrometry (EI-MS) showed major ions at *m*/*z* 286 $[M]^+$, 188 $[M\text{-ring B}]^+$ (fragment 1) and 154 $[M\text{-ring B-2OH}]^+$ (fragment 2) (Figure C2.1), giving a molecular weight of 286 g/mol and suggesting a molecular formula of $C_{15}H_{10}O_6$.




| Fragmentation 1 | Fragmentation 2 | COSY correlations |
|---|---|---|

Figure C2.1. Hypothetical fragmentation in the ESI-MS spectrum and COSY correlations of compound **2** (hydroxyemodin)

The $^1$H NMR spectrum of compound **2** (Table C2.1, Figure C2.2) exhibited four aromatic methine protons [*δ* 7.24 (H2), 7.71 (H4), 7,16 (H5), 6.50 (H7)], two aliphatic methylene protons [*δ* 3.34 ($CH_2OH$)]. Two hydroxyl groups [*δ* 12.25 (H1), 12.16 (H8) ] attaching to aromatic ring were clearly observed in the $^1$H NMR data in DMSO (Table C2.1). Multiplicity (doublet) with coupling constant (2.5 Hz) of H5 and H7 indicated that both protons had meta correlation. This was also proved by the correlation between H5 and H7 and vice versa in the COSY spectrum. The second spin system existed at H2, H3' ($CH_2OH$) and H4 in which each proton had prominent correlation to each other in the COSY spectrum (Figure C2.1 and C2.3).

Table C2.1. NMR data of compound **2** (hydroxyemodin)

| Position | δ $^1$H (ppm), multiplicity (*J* in Hz) (Kalidhar, 1989 in $CDCl_3$) | δ $^1$H (ppm), multiplicity (*J* in Hz) (in DMSO) | δ $^1$H (ppm), multiplicity (*J* in Hz) (in MeOD) | COSY (H→ H) (in MeOD) |
|---|---|---|---|---|
| 1 (OH) | | 12.25 (br s) | | |
| 2 | 7.42 | 7.23 (s) | 7.24 (s) | H3' ($CH_2$), H4 |
| 3 | | | | |
| 4 | 8.19 | 7.63 (s) | 7.71 (s) | H2, H3' ($CH_2$) |
| 5 | 7.99 | 7.05 (s) | 7.16 (d, 2.5) | H7 |
| 6 (OH) | | | | |
| 7 | 7.29 | 6.45 (s) | 6.50 (d, 2.5) | H5 |
| 8 (OH) | | 12.16 (br s) | | |
| 15 ($CH_2$) | | 4.59 (s) | 4.67 (s) | H2, H4 |

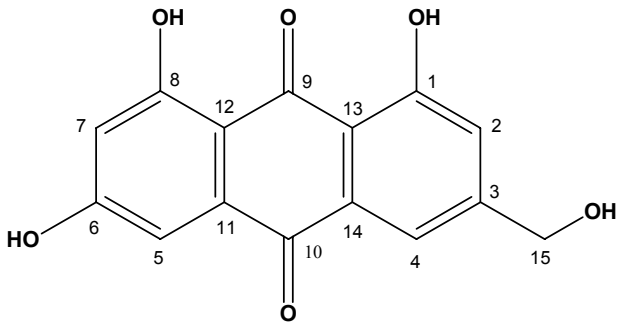
Comparison of the $^1$H NMR spectrum of compound **2** with that reported by Kalidhar (1989) indicated similar chemical shifts, bringing to the conclusion that compound **2** was hydroxyemodin/citreorosein (1,6,8-trihydroxy-3-hydroxymethyl-anthraquinone). Antimicrobial activity of compound **2** is presented in Table C2.2.

Table C2.2. Antimicrobial activity of compound **2** (hydroxyemodin)

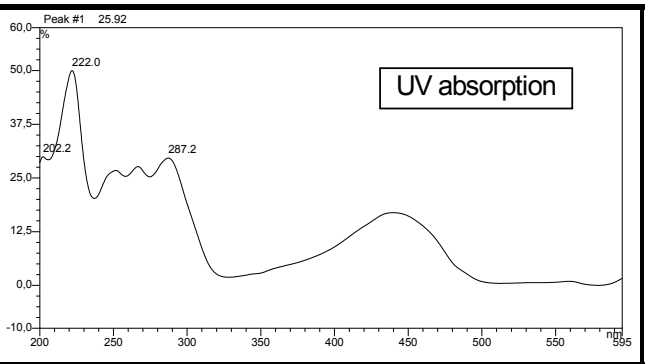
| Compound **2** | *Staphylococcus aureus* | *Bacillus subtilis* | *Escherichia coli* | *Candida albicans* | *Saccharomyces cerevisiae* | *Cladosporium herbarum* |
|---|---|---|---|---|---|---|
| 50 μg | not active | not active | not active | 9 mm | not active | not active |
| 100 μg | not active | not active | 9 mm | 12 mm | not active | not active |

## Hydroxyemodin (compound 2)

| | |
|---|---|
| CAS Registry Number | : 481-73-2 |
| Characteristic | : orange powder |
| Formula | : $C_{15}H_{10}O_6$ |
| Molecular Weight | : 286 g/mol |
| Amount | : 6.1 mg |
| Source | : *Penicillium* sp. derived from *Ircinia fasciculata* |




| | |
|---|---|
| Retention Time | : 26.05 min |
| $R_f$ | : 0.56 |
| Fluorescence, 254 nm | : orange |
| 366 nm | : orange |
| Anisaldehyde/$H_2SO_4$ | : orange |




EI-MS (*m/z*, rel. int.) : 286 $[M]^+_{(100)}$, $258_{(42.4)}$, $216_{(7.6)}$, 188 $[M\text{-ring B}]^+_{(4.1)}$, 154$[M\text{-ring B-2OH}]^+_{(5.3)}$, $138_{(6.6)}$, $116_{(11.0)}$, $92_{(3.6)}$, $70_{(13.2)}$

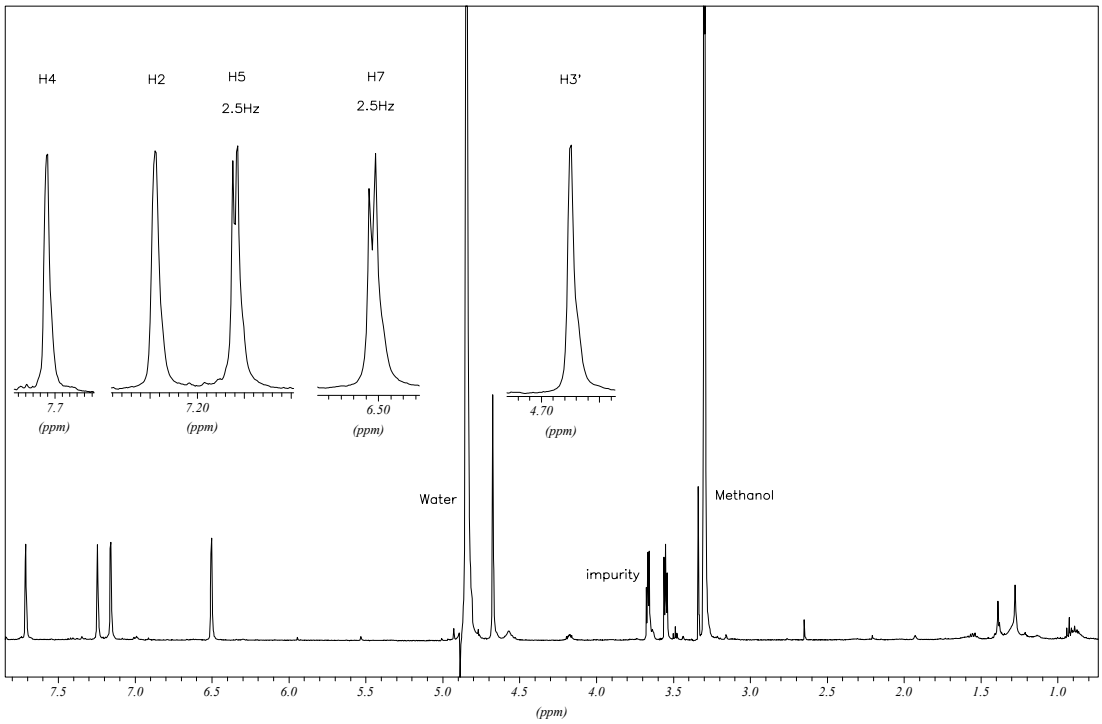



Figure C2.2. $^{1}$H NMR spectrum of compound **2** (hydroxyemodin) in MeOD

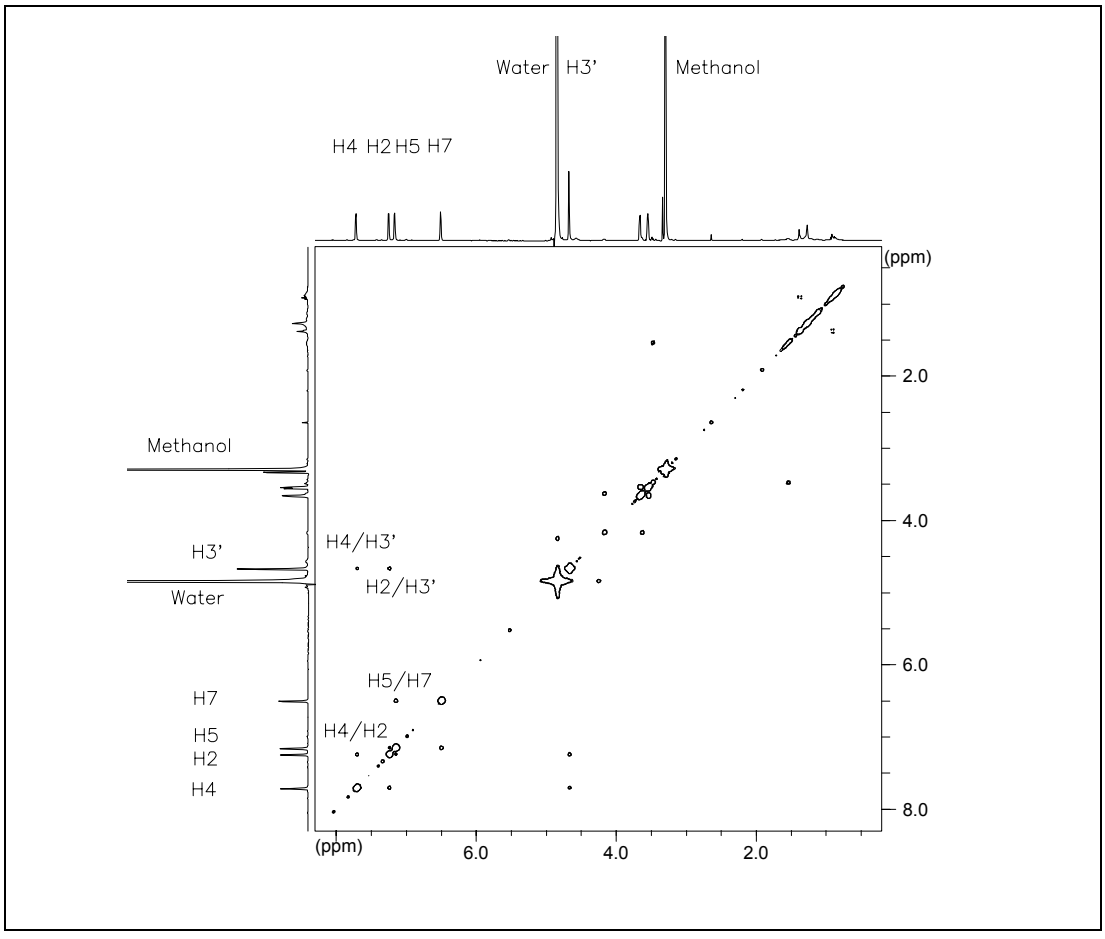
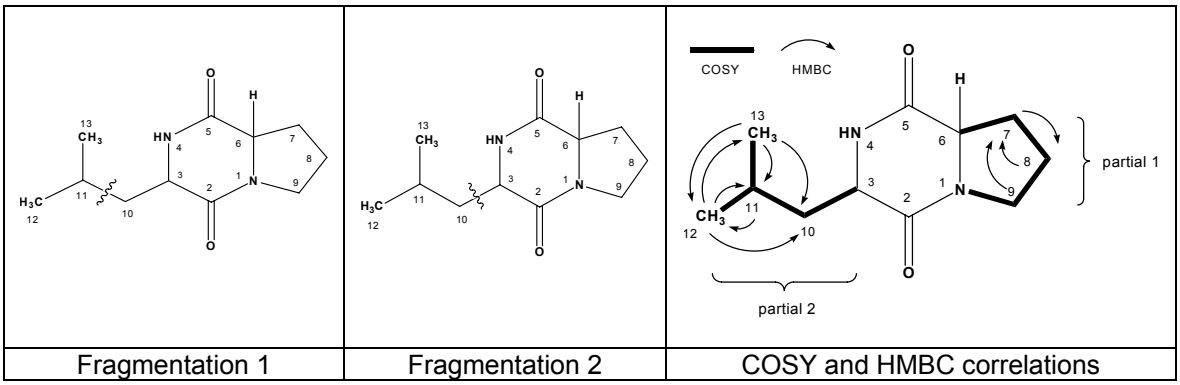



Figure C2.3. COSY spectrum of compound **2** (hydroxyemodin) in MeOD

## 3.1.3. Compound 3 (gancidin/cyclo-leucylprolyl)

The EI-MS spectrum of compound **3** gave major ions at *m*/*z* 195 $[M\text{-}CH_3]^+$, 167 $[M\text{-}CH\text{-}2CH_3]^+$ (fragment 1), 154 $[M\text{-}2CH\text{-}CH_3]^+$ (fragment 2) (Figure C3.1), proposing a molecular weight of 210 g/mol and a molecular formula of $C_{11}H_{18}N_2O_2$.




Fragmentation 1 | Fragmentation 2 | COSY and HMBC correlations

Figure C3.1. Hypothetical fragmentation of compound **3** (gancidin) in the EI-MS spectrum, COSY and HMBC correlations

The $^1H$ NMR spectrum (Table C3.1, Figure C3.2) exhibited two aliphatic methyl protons [$\delta$ 0.95 (H12), 1.0 (H13)], four methylene protons (H7, H8, H9, H10), and two methine protons [$\delta$ 4.11 (H3), 1.73 (H11)].

The $^{13}C$ NMR spectrum indicated two singlet carbonyl carbons [$\delta$ 170.00 (C2), 166.00 (C5)], three doublet carbons [$\delta$ 54.95 (C3), 60.59 (C6), 26.08 (C11)], four triplet carbons [$\delta$ 29.38 (C7), 23.86 (C8), 46.75 (C9), 39.71 (C10)], and two quartet carbons [$\delta$ 23.60 (C12), 21.19 (C13)]. Furthermore, the existence of methylene carbons at positions 7, 8, 9, and 10 was also proved by the DEPT spectrum (Table C3.1, Figure C3.3).

The COSY spectrum revealed two partial structures (Figure 3.1). The first partial structure connected protons at position 6, 7, 8 and 9, while the second partial structure correlated protons at positions 3, 10, 11, 12 and 13. Proton and carbon position assignments were established by connecting these partial structures with the aid of long range correlations of the HMBC spectrum (Figure C3.1).

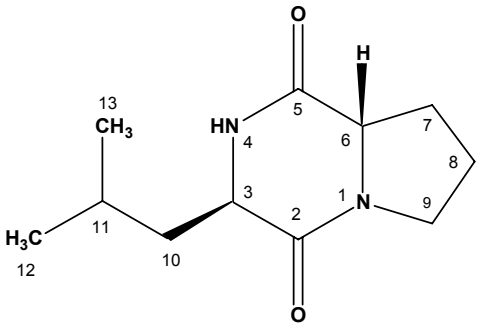
All direct correlations among protons and their corresponding carbons were readily detected in the HMQC spectrum (Table C3.1). Three downfield carbons [$\delta$ 54.95 (C3), 58.93 (C6), 45.42 (C9)] and three downfield protons [$\delta$ 4.11 (H3), 4.24 (H6), 3.61 (H9)] due to the existence of nitrogen and carbonyl atoms nearby distinctly appeared in the $^1H$ and $^{13}C$ NMR spectra (Figure C3 and C3.3).

Optical rotation of compound **3** was $-82.7^o$, whereas optical rotation of cyclo(*D*-leucyl-*L*-prolyl) previously isolated from the sponge *Calyx* cf *podatypa* was $-91.2^o$ (Adamczeski *et al.,* 1995), suggesting both compounds are the same.

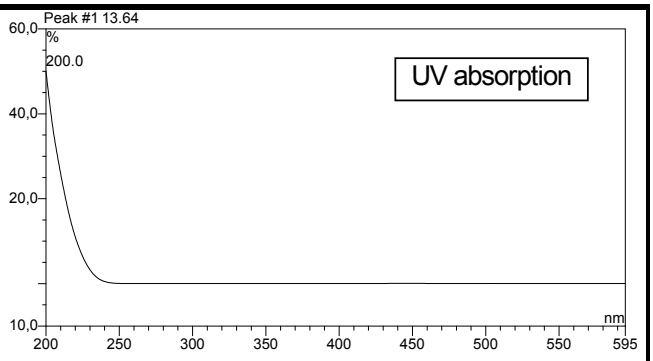
As much as 50 µg and 100 µg of compound **3** inhibited 7 mm growth zone of *Staphylococcus aureus* and 7 mm growth zone of *Bacillus subtilis*, respectively.

## Gancidin / Cyclo(leucylprolyl) (compound 3)

| | | |
|---|---|---|
| CAS Registry Number | : | 36238-67-2 |
| Characteristic | : | white powder |
| Formula | : | $C_{11}H_{18}N_2O_2$ |
| Molecular Weight | : | 210 g/mol |
| Amount | : | 5.6 mg |
| Source | : | *Penicillium* sp. derived from *Ircinia fasciculata* |




| | | |
|---|---|---|
| Retention Time | : | 13.57 min |
| $R_f$ | : | 0.81 |
| Fluorescence, 254 nm | : | + |
| 366 nm | : | - |
| Anisaldehyde/$H_2SO_4$ | : | - |




| | | |
|---|---|---|
| Optical Rotation $[\alpha]_D^{20}$ | : | experiment = -82.7° (c=0.1 MeOH)<br>literature = -91.2° (MeOH) |
| EI-MS (*m*/*z*, rel. int.) | : | 195 $[M\text{-}CH_3]^+_{(1.1)}$, 167$[M\text{-}CH\text{-}2CH_3]^+_{(2.9)}$, 154 $[M\text{-}2CH\text{-}CH_3]^+_{(74.7)}$ |

Table C3.1. NMR data of compound **3** (gancidin)

| Position | $\delta$ $^{13}$C (ppm) (Antibase, 2002 in $CDCl_3$) | $\delta$ $^{13}$C (ppm) (in $CDCl_3$) | DEPT | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) (Antibase, 2002 in $CDCl_3$) | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) ( in $CDCl_3$) | COSY (H → H) | HMQC (H → C) direct | HMBC (H → C) |
|---|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | |
| 2 | 170.40 (s) | 170.06 (s) | | | | | | |
| 3 | 53.34 (s) | 53.48 (d) | | 4.12 (t, 4.0) | 4.11 (t, 4.2) | H10, H11 | C3 | |
| 4 | | | | 6.01 (s) | | | | |
| 5 | 166.24 (s) | 166.16 (s) | | | | | | |
| 6 | 58.93 (d) | 59.06 (d) | | 4.04 (dd, 9.5, 3,5) | 4.00 (dd, 9.4, 3,7) | H7, H8 | C6 | |
| 7 | 28.00 (t) | 29.38 (t) | 29.38 (t) | 2.14 (m), 1.53 (m) | 2.10 (m), 1.70 (m) | H8, H9 | C7 | C8 |
| 8 | 22.68 (t) | 23.96 (t) | 23.96 (t) | 2.00 (m), 1.92 (m) | 2.00 (m), 1.91 (m) | H6, H7, H9 | C8 | C7 |
| 9 | 45.42 (t) | 45.57 (t) | 45.57 (t) | 3.62 (m), 3.52 (m) | 3.61 (m), 3.54 (m) | H7, H8 | C9 | C7 |
| 10 | 38.42 (t) | 38.77 (t) | 39.77 (t) | 2.36 (m), 2.14 (m) | 2.35 (m), 2.13 (m) | H3, H11 | C10 | |
| 11 | 24.52 (d) | 24.84 (d) | | 1.74 (m) | 1.73 (m) | H3, H13 | C11 | C12 |
| 12 | 23.21 (q) | 22.80 (q) | | 0.96 (d, 6.5) | 0.95 (d, 6.0) | H11, H13 | C12 | C10, C11, C13 |
| 13 | 21.19 (q) | 21.27 (q) | | 1.00 (d, 6.5) | 1.00 (d, 6.0) | H11, H12 | C13 | C10, C11, C12 |

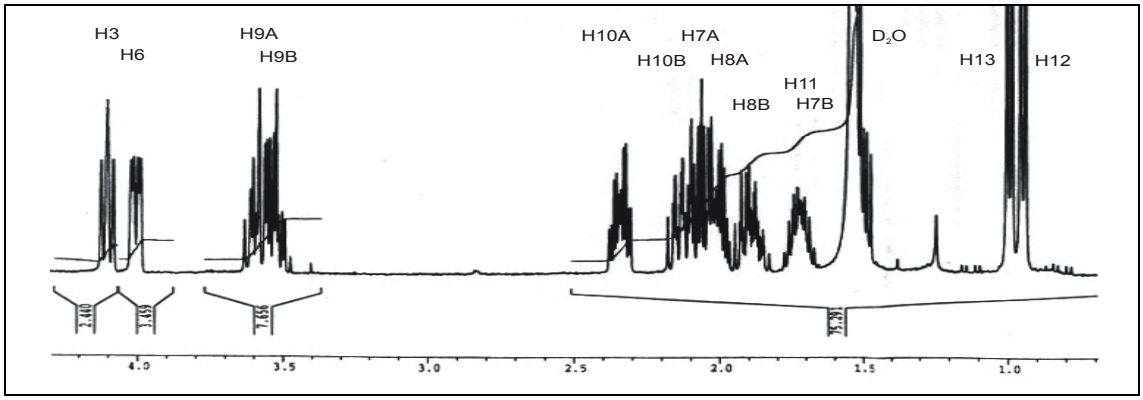



Figure C3.2. $^{1}$H NMR spectrum of compound **3** (gancidin)

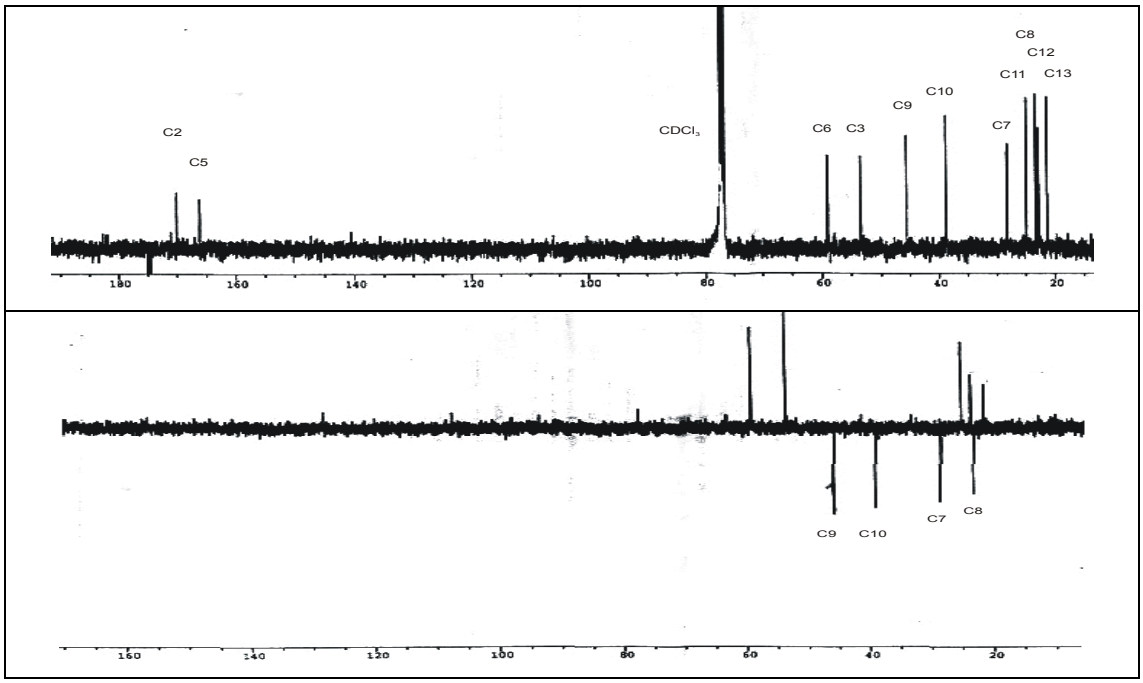
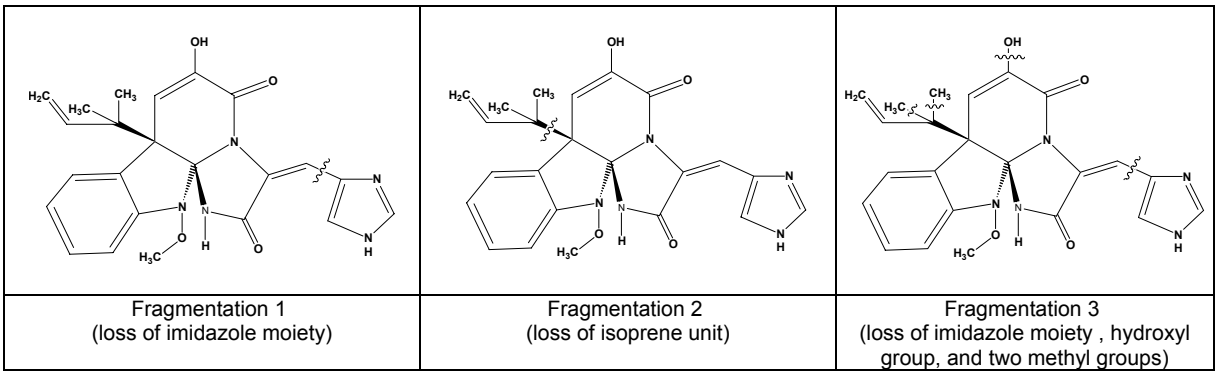



Figure C3.3. $^{13}$C NMR and DEPT spectrum of compound **3** (gancidin)

## 3.1.4. Compound 4 (meleagrine)

The ESI-MS spectrum of compound **4** showed an intense ion peak at *m*/*z* 434 $[M+H]^+$ (positive), suggesting a molecular weight of 433 g/mol which contained an odd number of nitrogen atoms, and suggesting a molecular formula of $C_{23}H_{23}N_5O_4$.

EI-MS analysis confirmed the molecular weight with ions at *m*/*z* 433 $[M]^+$, 365$[M\text{-}C_3H_4N_2]^+$ (fragment 1), 364 [M-isoprene group]$^+$ (fragment 2), 318 $[M\text{-}C_3H_4N_2\text{-}2CH_3\text{-}OH]^+$ (fragment 3). The presence of an imidazole moiety, an isoprenoid side chain, and a hydroxyl group were clearly evident from the fragments observed (Figure C4.1).



| Fragmentation 1 (loss of imidazole moiety) | Fragmentation 2 (loss of isoprene unit) | Fragmentation 3 (loss of imidazole moiety , hydroxyl group, and two methyl groups) |
|---|---|---|

Figure C4.1. Hypothetical fragmentation of compound **4** (meleagrine) in the EI-MS spectrum

Four aromatic proton signals of the $^1$H NMR spectrum (Table C4.1, Figure C4.3) in meleagrine appeared at *δ* 7.52 (H4), 7.02 (H5), 7.25 (H6), 6.96 (H7). The COSY spectrum distinctly exhibited an ABCD spin system in the area of aromatic resonances. This proposed that the tryptophan moiety of meleagrine has no substituent on the aromatic ring. The methoxy group, hence, should be on the nitrogen (N1). In addition, no long range correlation in the HMBC spectrum of methoxy group to its adjacent carbons verified the position of this methoxy bound to nitrogen atoms at position 1 (Table C4.1, Figure C4.6)

Furthermore, two doublet exo-methylene protons at *δ* 4.98, 5.00 (H23), two geminal methyl protons at *δ* 1.18 (H24, H25), and one singlet methoxy protons at *δ* 3.65 (H1) were visible. These geminal methyls suggested the presence of one isoprenyl unit in the molecule. The existence of isoprenyl group was confirmed by the HMBC correlation (Figure C4.6).

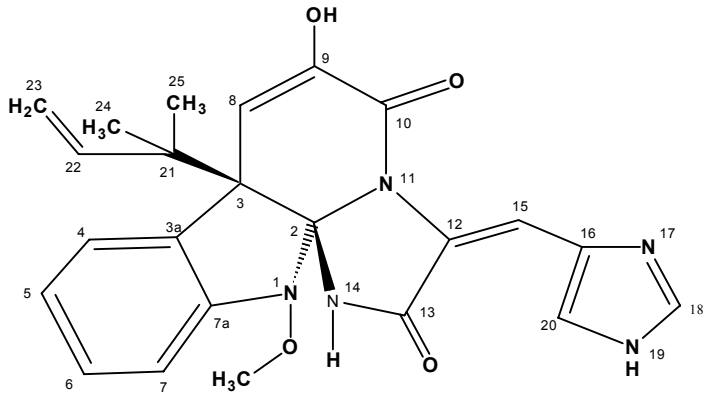
Two olefinic singlet protons [*δ* 5.24 (H8), 817 (H15)] and two other downfield singlet protons assigned to the signal of imidazole protons [*δ* 7.75 (H18), 7.52 (H20)] appeared. Correlation of olefinic protons (H8, H15) and their neighbouring carbons was also determined by the HMBC spectrum (Figure C4.6).

Due to the non existence of $^{13}$C data of reported meleagrin, $^{13}$C NMR data of oxaline previously isolated from *Penicillium* (Konda *et al*., 1980) was taken as comparison. Meleagrine has only one methoxy group. Methoxy group on position nine at oxaline was replaced by hydroxyl group. Resonance of C9 of meleagrine shifted to higher field at *δ* 142.8 compared to that of C9 oxaline (146.4 ppm) (Table C4.1, Figure C4.5).

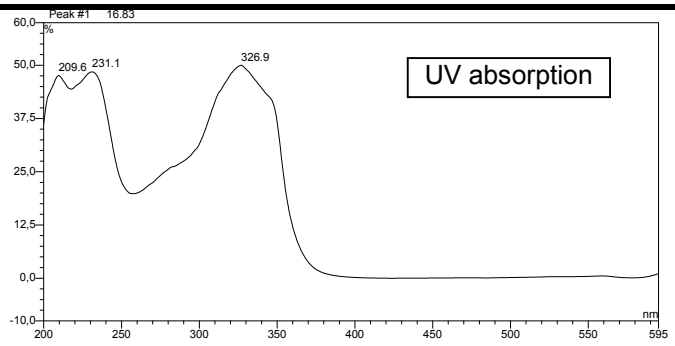
Two carbonyls [*δ* 158.2 (C10), 164.4 (C13)] and one doublet carbon [*δ* 142.5 (C22)] carrying an exo-methylene group (C23) were observed. The presence of exo-methylene group was proven also by triplet carbon signal [*δ* 112.4 (C23)] in the DEPT spectrum. One quarternary methoxy carbon [*δ* 64.2 (C1)] and two methyl carbons [*δ* 23.7 (C24), 23.1 (C25)] also appeared (Table C4.1, Figure C4.5).

## Meleagrine (compound 4)

| | | |
|---|---|---|
| CAS Registry Number | : | 71751-77-4 |
| Characteristic | : | pale yellow powder |
| Formula | : | $C_{23}H_{23}N_5O_4$ |
| Molecular Weight | : | 433 g/mol |
| Amount | : | 78.3 mg |
| Source | : | *Penicillium* sp. derived from *Ircinia fasciculata* |



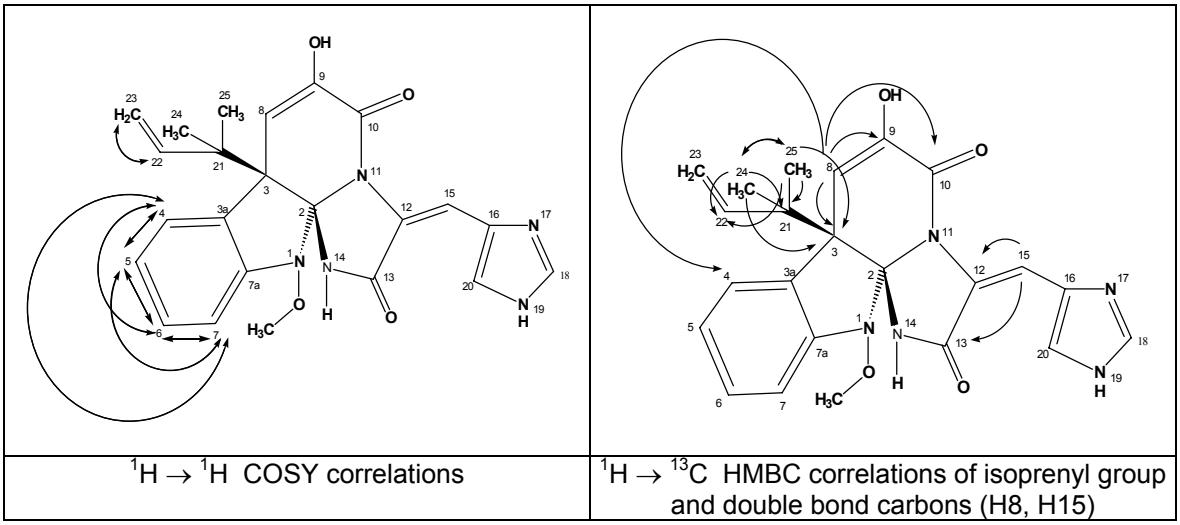

| | | |
|---|---|---|
| Retention Time | : | 16.90 min |
| $R_f$ | : | 0.81 |
| Fluorescence, 254 nm | : | + |
| 366 nm | : | orange |
| Anisaldehyde/$H_2SO_4$ | : | brown |




| | | |
|---|---|---|
| Optical Rotation $[\alpha]_D^{20}$ | : | experiment = -125° (c=0.1, $CHCl_3$) |
| | | literature = -116° (c=0.088, $CHCl_3$) (Nozawa and Nakajima, 1979) |
| ESI-MS (*m/z*) | : | 434 $[M+H]^+$ (positive) |
| EI-MS (*m/z*, rel. int.) | : | 433 $[M]^+_{(42.5)}$, 365$[M\text{-}C_3H_4N_2]^+_{(100)}$, 364 [M-isoprene unit]$^+_{(75.9)}$, 318 $[M\text{-}C_5H_{11}N_2O]^+_{(36.6)}$, $277_{(45.0)}$, $261_{(13.2)}$, $154_{(86.5)}$, $125_{(28.9)}$, $107_{(37.0)}$, 69 $[C_5H_9$/isoprene unit$]^+_{(83.7)}$ |

Table C4.1. NMR data of compound **4** (meleagrine)

| Position | $\delta$ $^{13}$C (ppm) ( in DMSO) | DEPT | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) (Kawai *et al.*, 1984 in $CDCl_3$) | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) (in DMSO) | COSY (H → H) | HMQC (H → C) direct | HMBC (H → C) |
|---|---|---|---|---|---|---|---|
| 1 (N) | | | | | | | |
| 2 | 101.2 (s) | | | | | | |
| 3 | 52.6 (s) | | | | | | |
| 3a | 126.0 (s) | | | | | | |
| 4 | 124.6 (d) | | 7.58 (br d, 7.5) | 7.52 (d, 7.0) | H5, H6, H7 | C4 | C3, C6, C7,C7a |
| 5 | 123.1 (d) | | 7.07 (br t, 7.5) | 7.02 (br t, 7.6) | H4, H6, H7 | C5 | C3a, C6, C7,C7a |
| 6 | 127.9 (d) | | 7.30 (br t, 7.5) | 7.25 (br t, 7.6) | H5, H7 | C6 | C4, C5, C7,C7a |
| 7 | 111.5 (d) | | 6.95 (br d, 7.5) | 6.96 (d, 7.0) | H4, H5, H6 | C7 | C3a, C5, C6,C7a |
| 7a | 146.2 (s) | | | | | | |
| 8 | 107.0 (d) | | 5.50 (s) | 5.24 (s) | | C8 | C2, C3, C3a,C4, C5, C9, C10, C22 |
| 9 (OH) | 142.8(s) | | | 10.0 (br s) | | | |
| 10 | 158.5 (s) | | | | | | |
| 11 | | | | | | | |
| 12 | 124.6 (s) | | | | | | |
| 13 | 165.0 (s) | | | | | | |
| 14 (NH) | | | | 9.20 (s) | | | |
| 15 | 109.1 (d) | | 8.27 (s) | 8.17 (s) | | C15 | C12, C13 |
| 16 | 127.9 (s) | | | | | | |
| 17 | | | | | | | |
| 18 | 136.5 (d) | | 7.61 (s) | 7.75 (s) | | C18 | |
| 19 (NH) | | | 12.72 (br s) | 12.76 (br s) | | | |
| 20 | 133.5 (d) | | 7.25 (s) | 7.52 (s) | | | |
| 21 | 41.7 (d) | | | | | | |
| 22 | 142.8 (d) | | 6.12 (dd, 17.2, 10.6) | 6.00 (br s) | H23 | | |
| 23 | 111.5 (t) | 111.5 (t) | 5.10 (d, 17.2) H23A; 5.06 (d, 10.6) H23B | 5.00 (br d, 18.3) H23A; 4.98 (br d, 18.3) H23B | H22 | C23 | |
| 24 | 23.7 (q) | | 1.35 (s) | 1.18 (s) | | C24 | C3, C21, C22, C25 |
| 25 | 23.1 (q) | | 1.24 (s) | 1.18 (s) | | C25 | C3, C21, C22, C24 |
| 1'-$OCH_3$ | 64.7 (q) | | 3.73 (s) | 3.65 (s) | | C1' | |




| $^{1}H \rightarrow {}^{1}H$ COSY correlations | $^{1}H \rightarrow {}^{13}C$ HMBC correlations of isoprenyl group and double bond carbons (H8, H15) |
|---|---|

Figure C4.2. COSY and HMBC correlations of compound **4** (meleagrine)

Nitrogen carrying carbon was seen at $\delta$ 101.1 (C2) positioned far more downfield than proton-binding cyclic carbon position due to the presence of nitrogen at positions 1 and 14. Similar phenomenon occurred at C12, C16, C18, C20 locating more downfield than proton-carrying double bond carbon location because also of the existence of nitrogen nearby. Likewise, oxygen-carrying double bond carbon was also evident at position 9 ($\delta$ 143.2) which also located more downfield than proton-carrying double bond carbon (Table C4.1).

Comparison of $^{1}H$ NMR spectrum of compound **4** with that of meleagrine isolated from *Penicillium meleagrinum* quoted by Kawai *et al*. (1984) proved quite similar. The proton chemical shifts difference of both compounds varied only from 0.01 until 0.27 ppm.

The assignment of stereogenic centres at C2 and C3 of compound **4**, therefore, was assumed also the same with that of meleagrine. This was also supported by almost the same optical rotation of both compounds.

As much as 100 μg compound **4** caused 9 mm growth zone inhibition of *Candida albicans* in the antimicrobial assay.

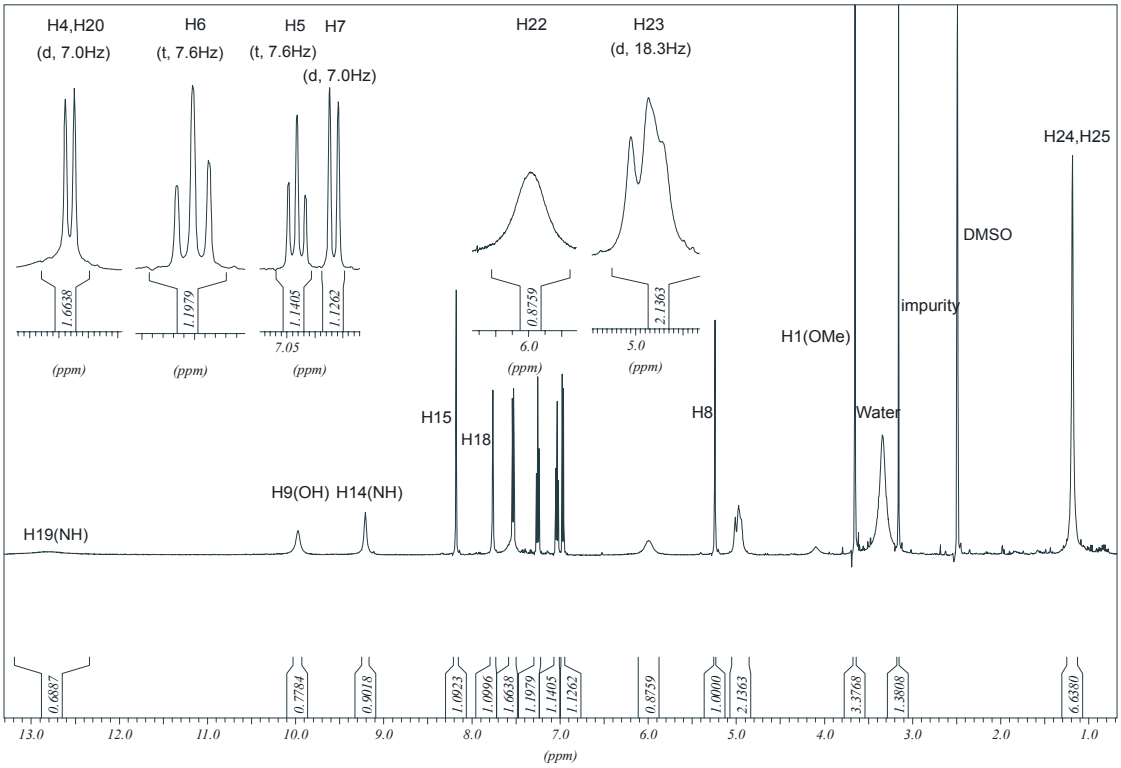



Figure C4.3. $^{1}$H NMR spectrum of compound **4** (meleagrine)

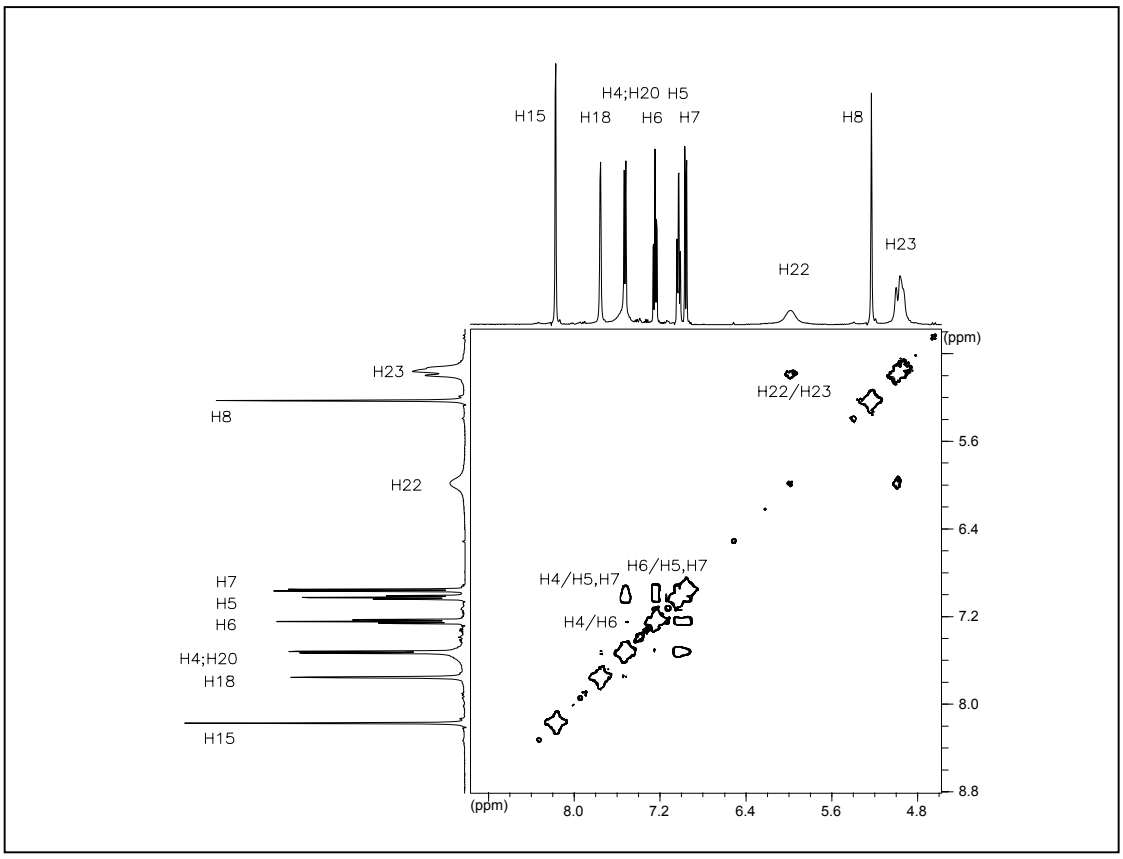



Figure C4.4. COSY spectrum of compound **4** (meleagrine)

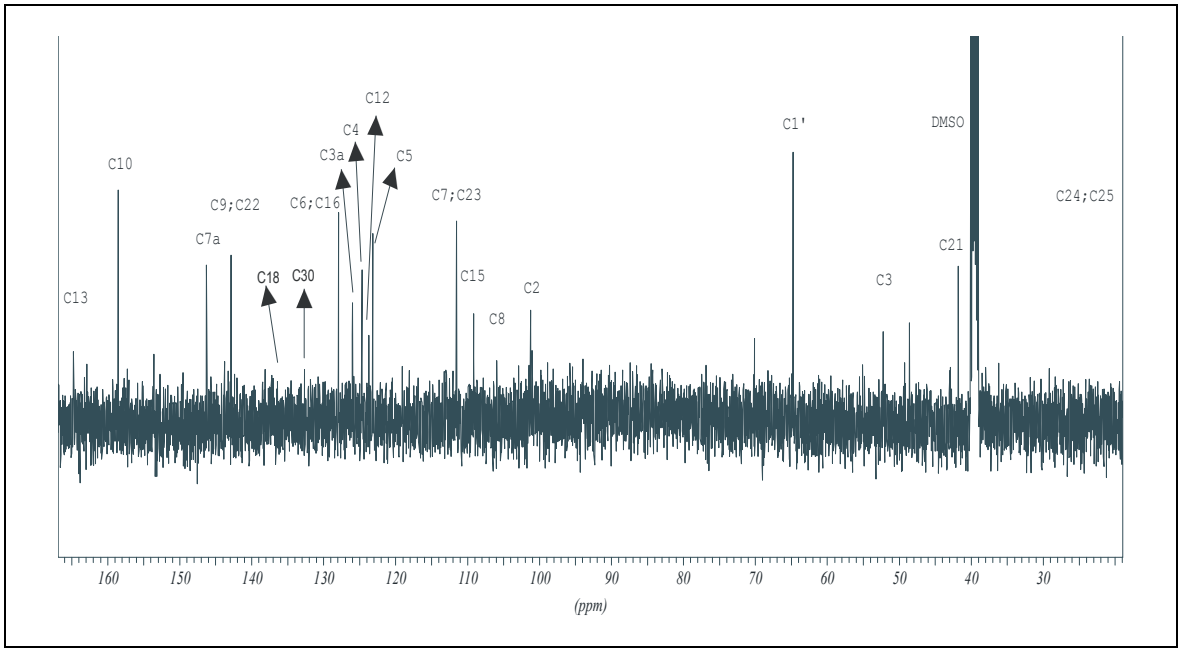



Figure C4.5. $^{13}$C NMR spectrum of compound **4** (meleagrine)

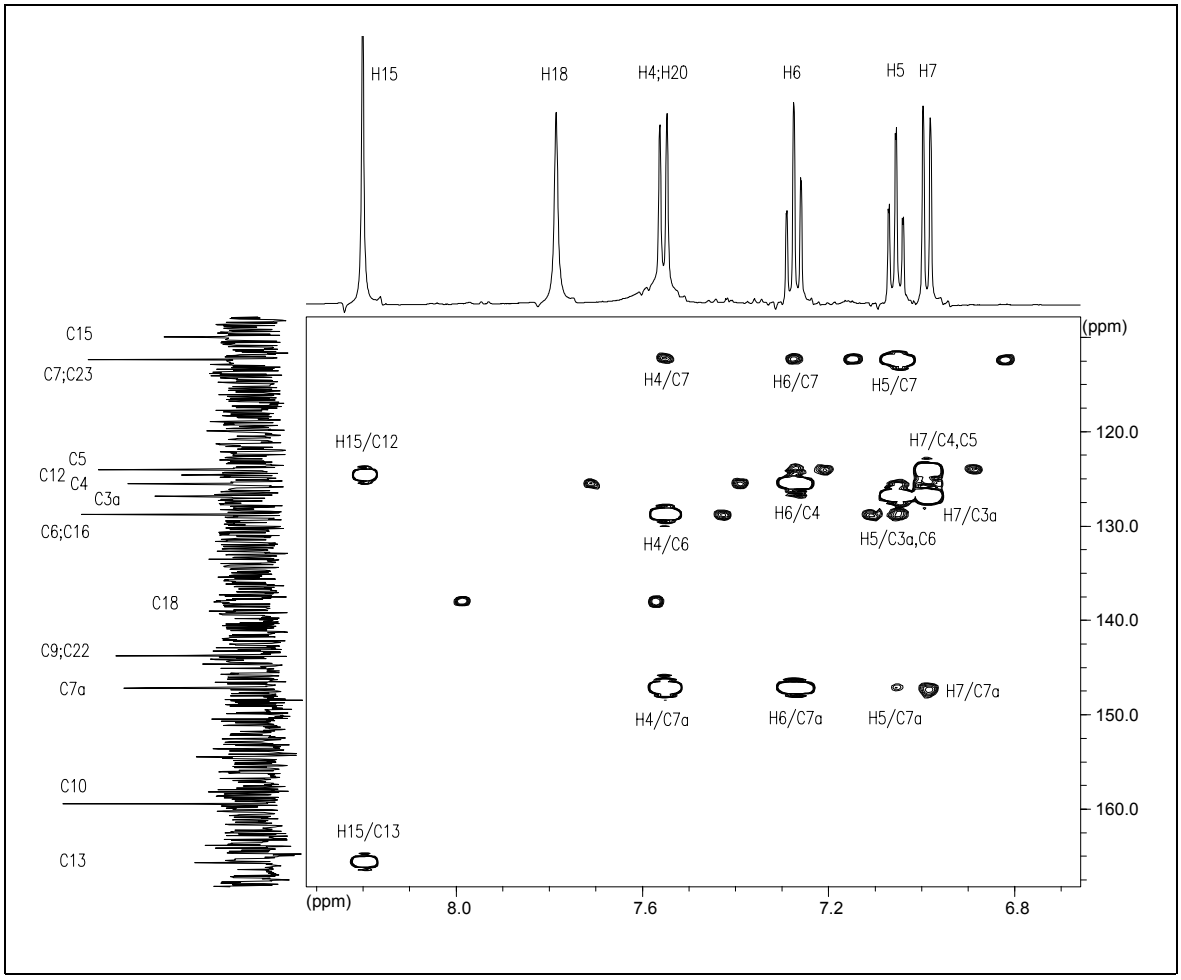



Figure C4.6. HMBC spectrum of compound **4** (meleagrine)

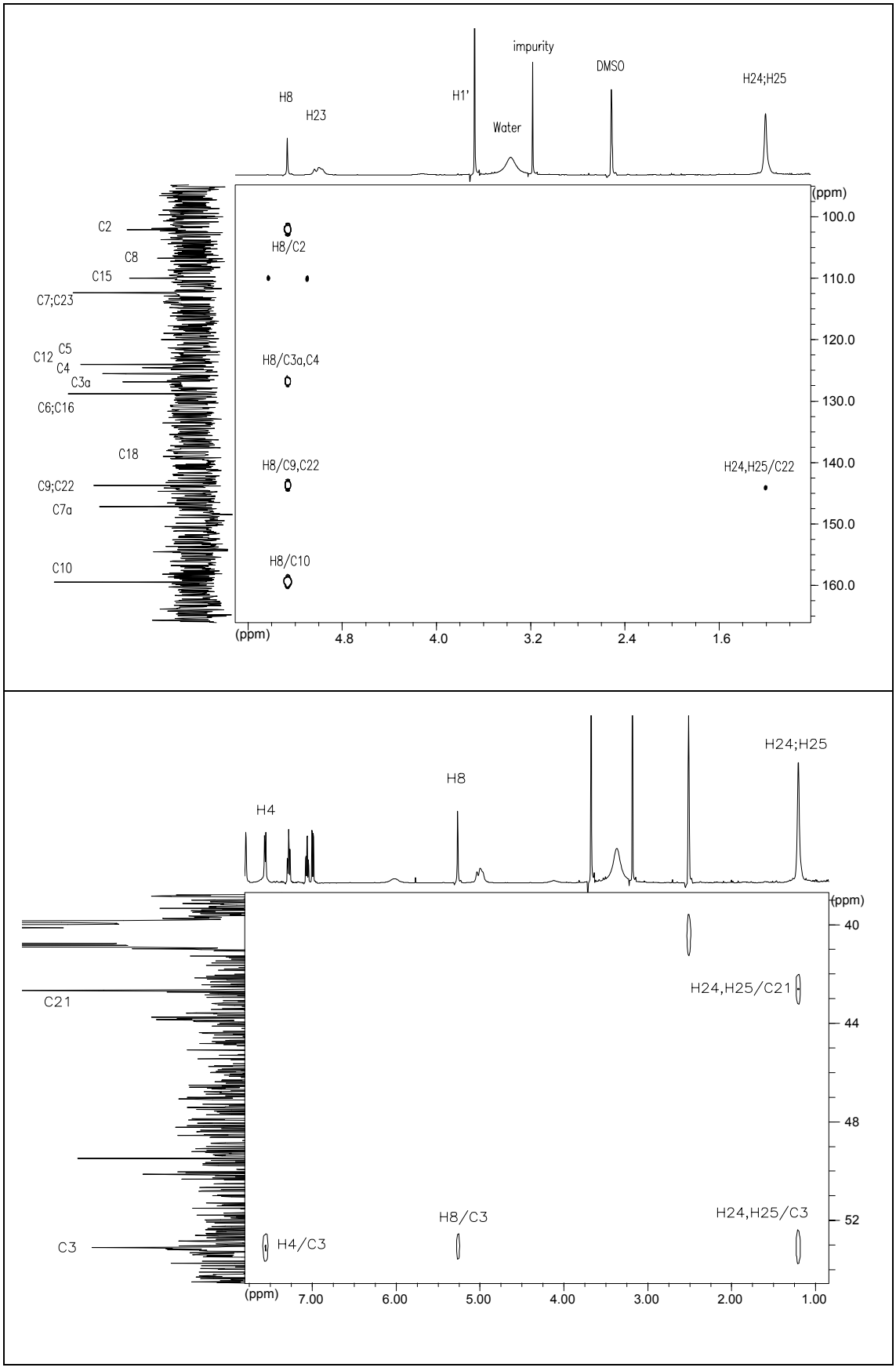



Figure C4.6. HMBC spectrum of compound **4** (meleagrine) (continued)

### 3.1.5 Compound 5 (citreohybridonol)

The structure of compound **5** was derived on the basis of spectroscopic analysis. Assignments of the $^{1}$H NMR (Figure C5.2), $^{13}$C NMR (Figure C5.3), COSY (Figure C5.4), HMQC (Figure C5.6), and HMBC (Figure C5.7) spectra are depicted in Table C5.1.

Molecular formula and molecular weight of compound **5** were $C_{28}H_{36}O_8$, and 500 g/mol, respectively. These were confirmed by signals found in the ESI-MS spectrum at *m*/*z* 501$[M+H]^+$ (positive), 499$[M-H]^-$ (negative), and in the EI-MS spectrum at *m*/*z* 523 $[M+Na]^+$.

The solubility of this compound in chloroform was not as good as in DMSO. Signals with their multiplicity appearing in the $^{1}$H NMR spectrum of chloroform-dissolved compound **5**, consequently, were also not as straightforward as those in the DMSO-dissolved compound **5**. It is in accordance with the reported data of $^{1}$H NMR of citreohybridonol dissolved in chloroform that showed several overlapping peaks.

In the $^{1}$H NMR spectrum, one methyl of acetoxyl group [$\delta$ 2.00 (H27)], one methoxy group [$\delta$ 3.42 (H28)], and one olefinic proton [$\delta$ 5.26 (H11)] clearly appeared. Six other singlet methyl signals [$\delta$ 1.30 (H18), 1.04 (H20), 1.76 (H21), 1.13 (H22), 0.90 (H24), 0.79 (H25)] attaching to nonprotonated carbon were also indicative. Among these six methyls, two of them (H18, H21) were vinyl methyls bound to double bond carbon, two others (H24, H25) were assigned as geminal methyls (Table C5.1, Figure C5.2).

Furthermore, the presence of two typical low field proton signals bound to oxygen-carrying carbons was evident at $\delta$ 4.50 (H3) and 4.78 (H6). The direct correlation of each proton to their neighbouring protons was clearly presented in the COSY spectrum in which there were three partial COSY correlations namely H1, H2, H3 (part 1), H6, H7 (part 2), and H9, H11, H12, H21 (part 3) (Figure C5.1 and C5.4).

The carbon types, revealed by the HMQC experiment, included one methoxy group (C28) characterised by low field chemical shift (50.37 ppm), seven methyl groups (C18, C20, C21, C22, C24, C25, C27), three methylene groups (C1, C2, C7), five methine groups (C3, C5, C6, C9, C11), twelve non-protonated carbon atoms, four of which were in carbonyl grouping: $\delta$ 194.55 (ketone, C17), 173.18 (methyl ester, C19), 179.31 (lactone, C23), 169.75 (acetoxy, C26) (Figure C5.5).

Further $^{13}$C spectrum analysis indicated the presence of two pairs double bond resonance at $\delta$ 118.57 (C11), 140.35 (C12), 165.24 (C15), 112.26 (C16). Two methine carbon signals [$\delta$ 75.53 (C3), 77.98 (C6)], characteristic of bearing an oxygen atom, were present (Table C5.1, Figure C5.3).

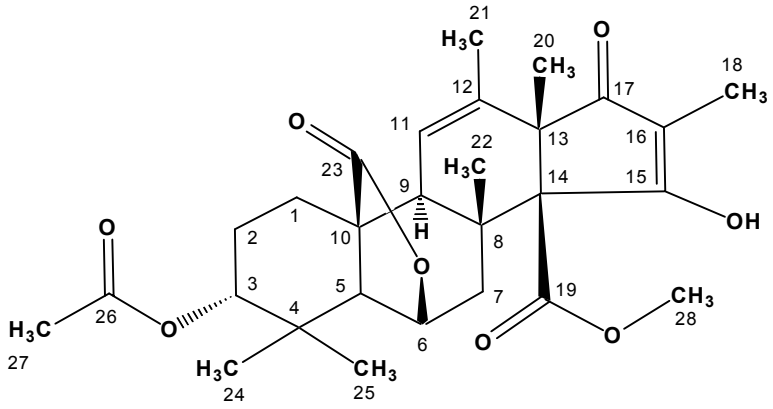
Long range correlation of seven methyl and one methoxy groups with their adjacent carbons was readily shown in the HMBC spectrum (Figure C5.1, C5.6). This HMBC correlation aided also the assignment of methyl and methoxy groups position in the molecule.

The $^{1}$H NMR spectrum of compound **5** in comparison with that of citerohybridonol isolated from *Penicillium citreo-viride* reported by Kosemura *et al*. (1992) indicated similar chemical shifts, leading to the conclusion that both compounds were identical.

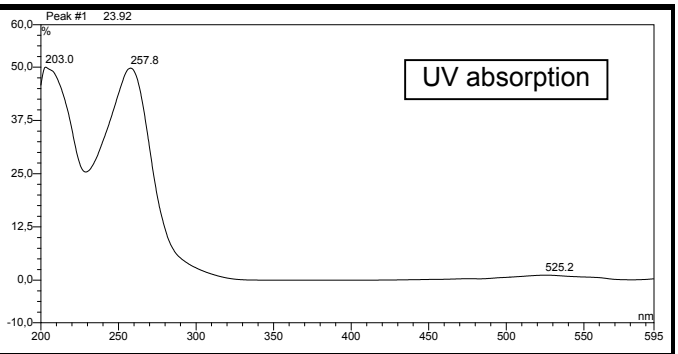
As much as 50 and 100 μg of compound **5** inhibited 7 and 10 mm growth zone of *Candida albicans*, respectively.

## Citreohybridonol (compound 5)

| | |
|---|---|
| Chemical Abstract | : 117:251584 |
| Characteristic | : pale yellow powder |
| Formula | : $C_{28}H_{36}O_8$ |
| Molecular Weight | : 500 g/mol |
| Amount | : 14.4 mg |
| Source | : *Penicillium* sp. derived from *Ircinia fasciculata* |




| | |
|---|---|
| Retention Time | : 23.81 min |
| $R_f$ | : 0.85 |
| Fluorescence, 254 nm | : + |
| 366 nm | : pale yellow |
| Anisaldehyde/$H_2SO_4$ | : - |




| | |
|---|---|
| Optical Rotation $[\alpha]_D^{20}$ | : experiment = +49.8° (c=0.1, $CHCl_3$) |
| | literature = +67.3° (c=0.066, $CHCl_3$) (Kosemura *et al*., 1992) |
| ESI-MS (*m/z*) | : 501$[M+H]^+$ (positive), 523$[M+Na]^+$ (positive), 499[M-H]- (negative) |
| FAB-MS (*m/z*, rel. int.) | : 523 $[M+Na]^+_{(3.5)}$, $329_{(4.0)}$, $289_{(2.7)}$, $307_{(3.0)}$, $277_{(45.0)}$, $197_{(3.8)}$, $176_{(26.7)}$, $154_{(43.2)}$, $136_{(40.4)}$, $107_{(28.7)}$, $89_{(60.8)}$ |

Table C5.1. NMR data of compound **5** (citreohybridonol)

| Position | $\delta$ $^{13}$C (ppm) (in DMSO) | DEPT | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) (Kosemura *et al*., 1992 in $CDCl_3$) | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) (in DMSO) | COSY (H → H) | HMQC (H → C) direct | HMBC (H → C) |
|---|---|---|---|---|---|---|---|
| 1 | 20.84 (t) | 20.84 (t) | 1.25 – 2.25 (m) overlapped | 1.95 (d, 3.5) H1A;<br>1.19 (ddd, 13.6, 13.6, 5.4) H1B | H2, H1B | | H1A → C3, C5, C10, C23<br>H1B → C2, C9, C10, C23 |
| 2 | 22.08 (t) | 22.08 (t) | 1.25 – 2.25 (m) overlapped | 1.49 – 1.61 (m) | H1B, H3 | C2 | C1 |
| 3 | 75.53 (d) | | 4.65 (dd, 2.5, 2.5) | 4.50 (br s) | H1, H2 | C3 | C1, C4, C5, C26 |
| 4 | 33.86 (s) | | | | | | |
| 5 | 53.90 (d) | | 1.25 – 2.25 (m) overlapped | 1.89 (s) | | C5 | C1, C4, C7, C9, C10, C23, C24 |
| 6 | 77.98 (d) | | 4.72 (d, 3.9) | 4.78 (d, 4.1) | H7B | C6 | C4, C5, C8, C10, C23 |
| 7 | 36.44 (t) | 36.44 (t) | 3.63 (d, 14.2) H7A;<br>2.51 (dd, 14.2, 4.4) H7B | 3.89 (d, 14.2) H7A;<br>2.23 (dd,14.2, 4.4) H7B | H6 | C7 | H7A →C5, C6, C8, C14, C22<br>H7B →C5,C6, C8, C9, C22 |
| 8 | 41.19 (s) | | | | | | |
| 9 | 50.67 (d) | | 1.25 – 2.25 (m) overlapped | 2.48 (d) | H11, H21 | C9 | C5, C8, C10, C11, C12, C14, C22, C23 |
| 10 | 43.24 (s) | | | | | | |
| 11 | 118.57 (d) | | 5.67 (br s) | 5.26 (br s) | H9, H21 | C11 | C8, C9, C10, C13, C21 |
| 12 | 140.35 (s) | | | | | | |
| 13 | 60.37 (s) | | | | | | |
| 14 | 69.96 (s) | | | | | | |
| 15 | 165.24 (s) | | | | | | |
| 16 | 112.26 (s) | | | | | | |
| 17 | 194.55 (s) | | | | | | |
| 18 | 8.03 (q) | | 1.33 (s) | 1.30 (s) | | C18 | C16, C17 |
| 19 | 173.18 (s) | | | | | | |
| 20 | 17.78 (q) | | 0.94 (s) | 1.04 (s) | | C20 | C12, C13, C14, C17 |
| 21 | 20.70 (q) | | 1.87 (s) | 1.76 (s) | H9, H11 | C21 | C11, C12, C13 |
| 22 | 24.44 (q) | | 1.32 (s) | 1.13 (s) | | C22 | C7, C8, C9, C14 |
| 23 | 179.31 (s) | | | | | | |
| 24 | 25.86 (q) | | 0.94 (s) | 0.90 (s) | | C24 | C3, C4, C5, C10, C25 |
| 25 | 22.16 (q) | | 0.89 (s) | 0.79 (s) | | C25 | C3, C4, C5, C24 |
| 26 | 169.75 (s) | | | | | | |
| 27 | 21.12 (q) | | 2.02 (s) | 2.00 (s) | | C27 | C3, C26 |
| 28 | 50.37 (q) | | 3.67 (s) | 3.42 (s) | | C28 | C14, C19 |

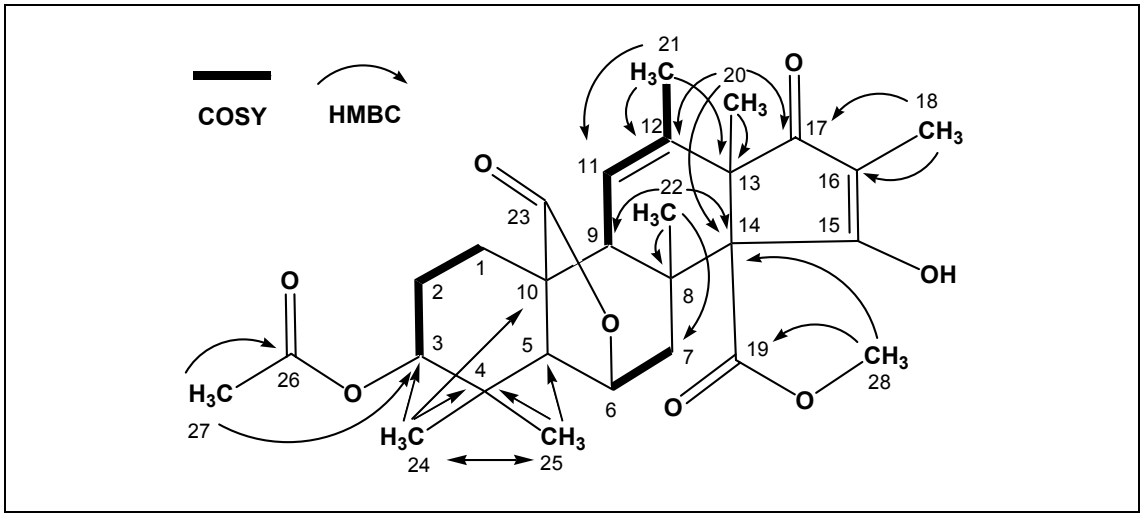



Figure C5.1. COSY and methyl HMBC correlations of compound **5** (citreohybridonol)

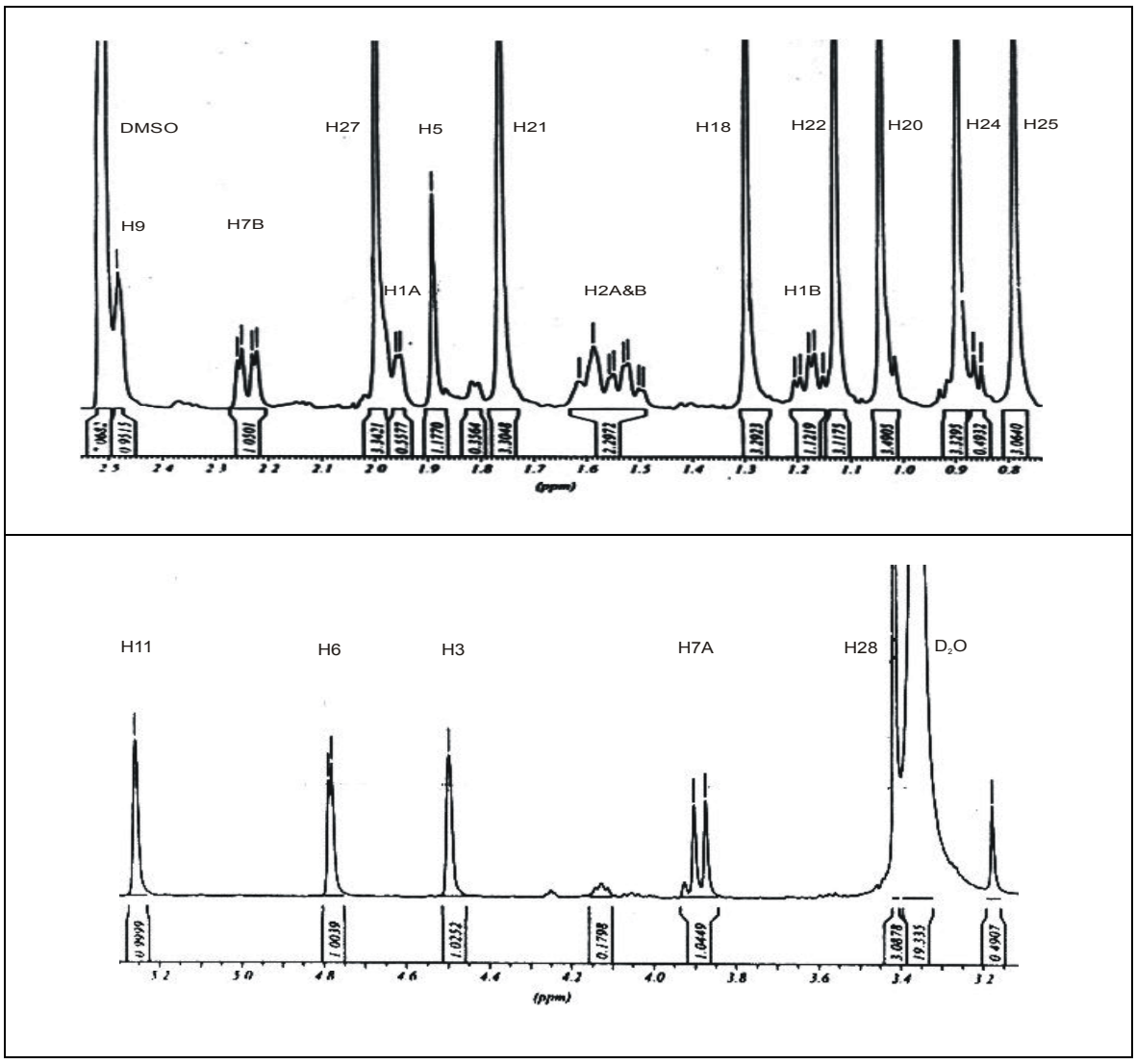



Figure C5.2. $^{1}$H NMR spectrum of compound **5** (citreohybridonol)

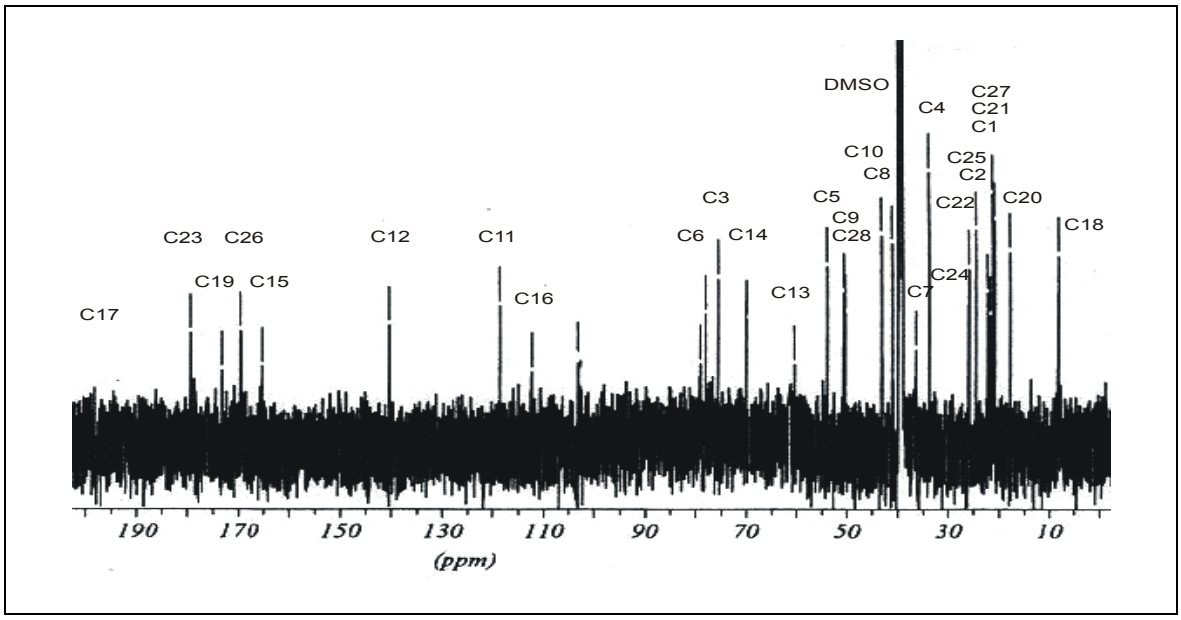



Figure C5.3. $^{13}C$ NMR spectrum of compound **5** (citreohybridonol)

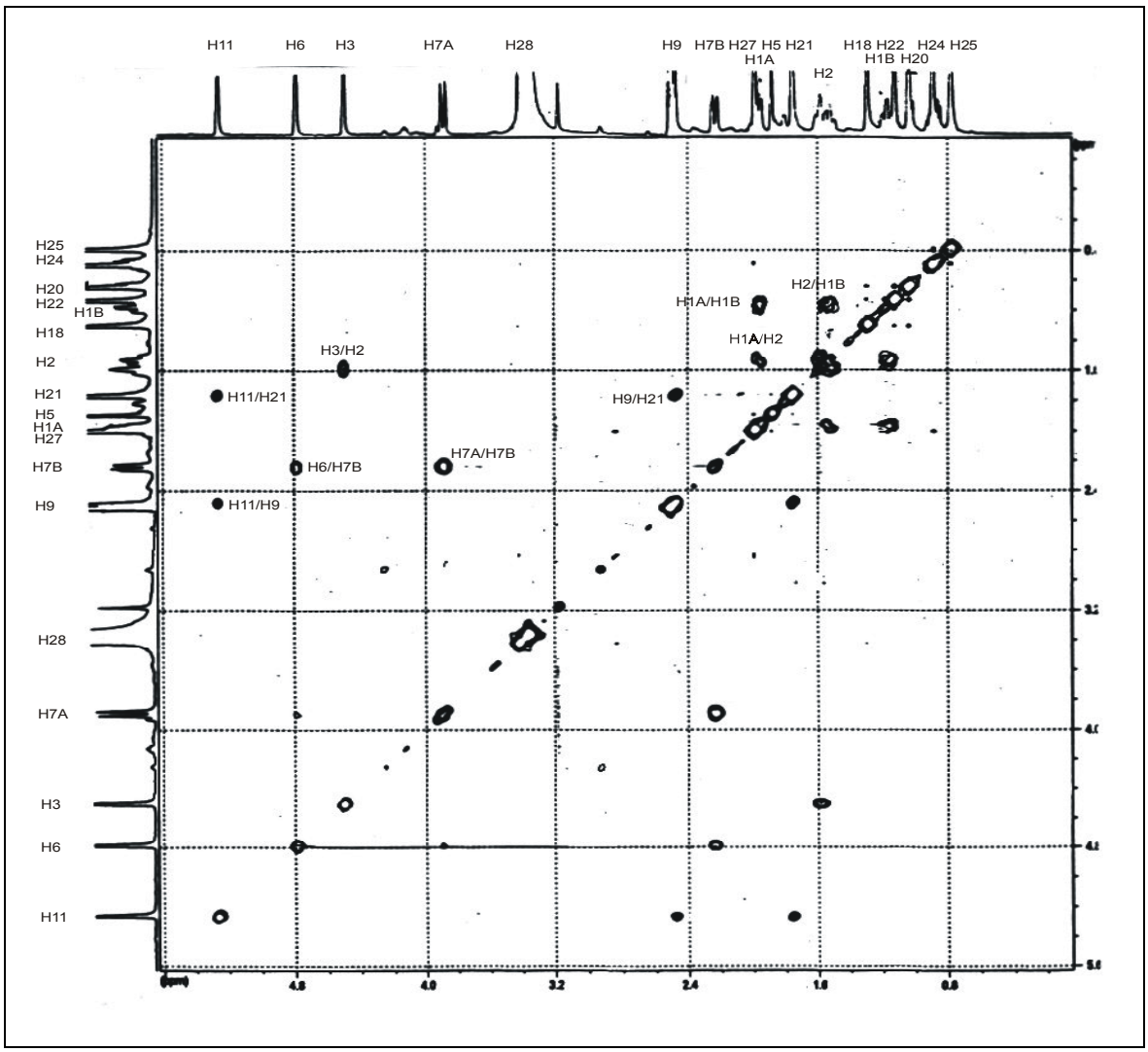



Figure C5.4. COSY spectrum of compound **5** (citerohybridonol)

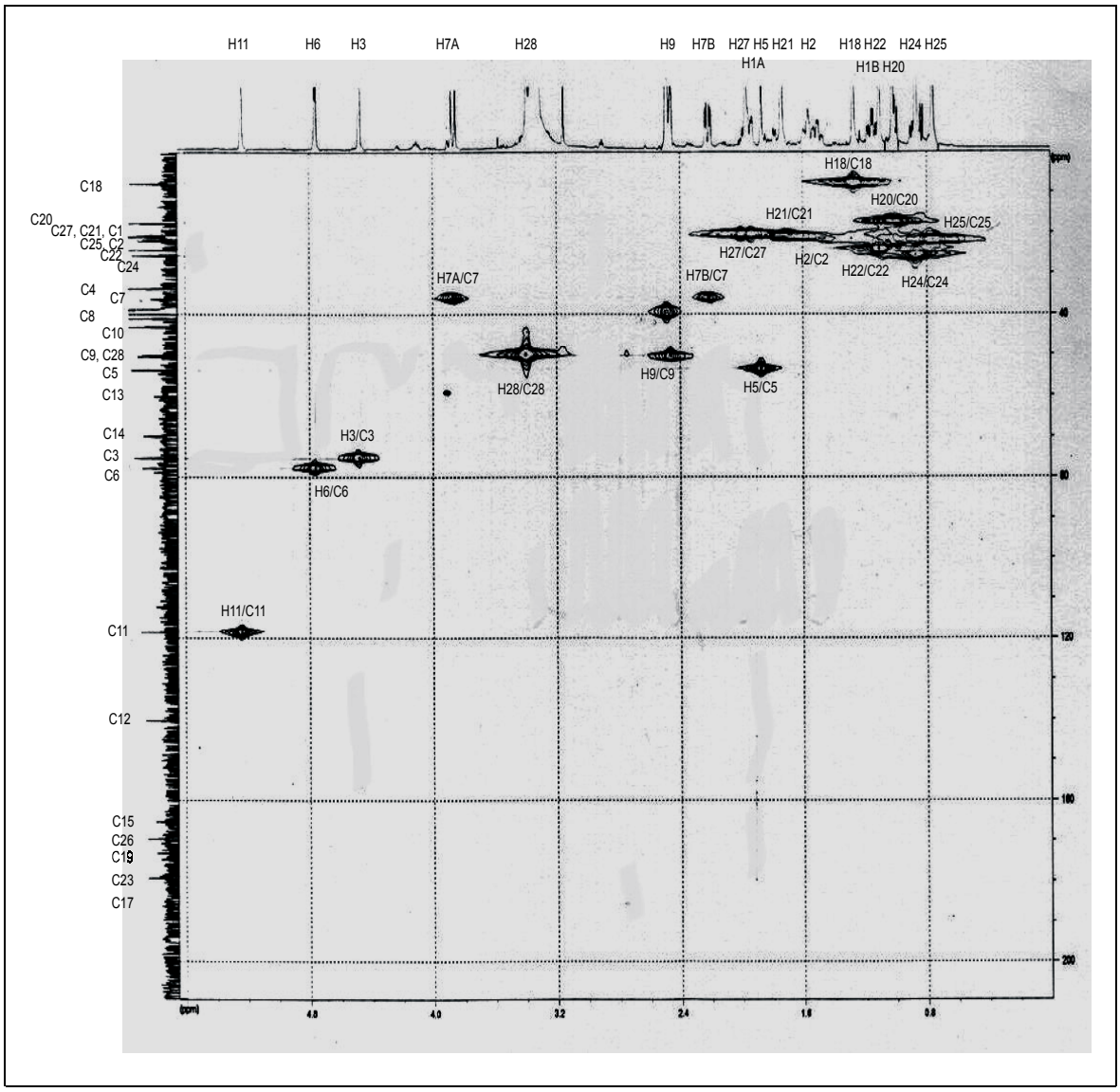



Figure C5.5. HMQC spectrum of compound **5** (citerohybridonol)

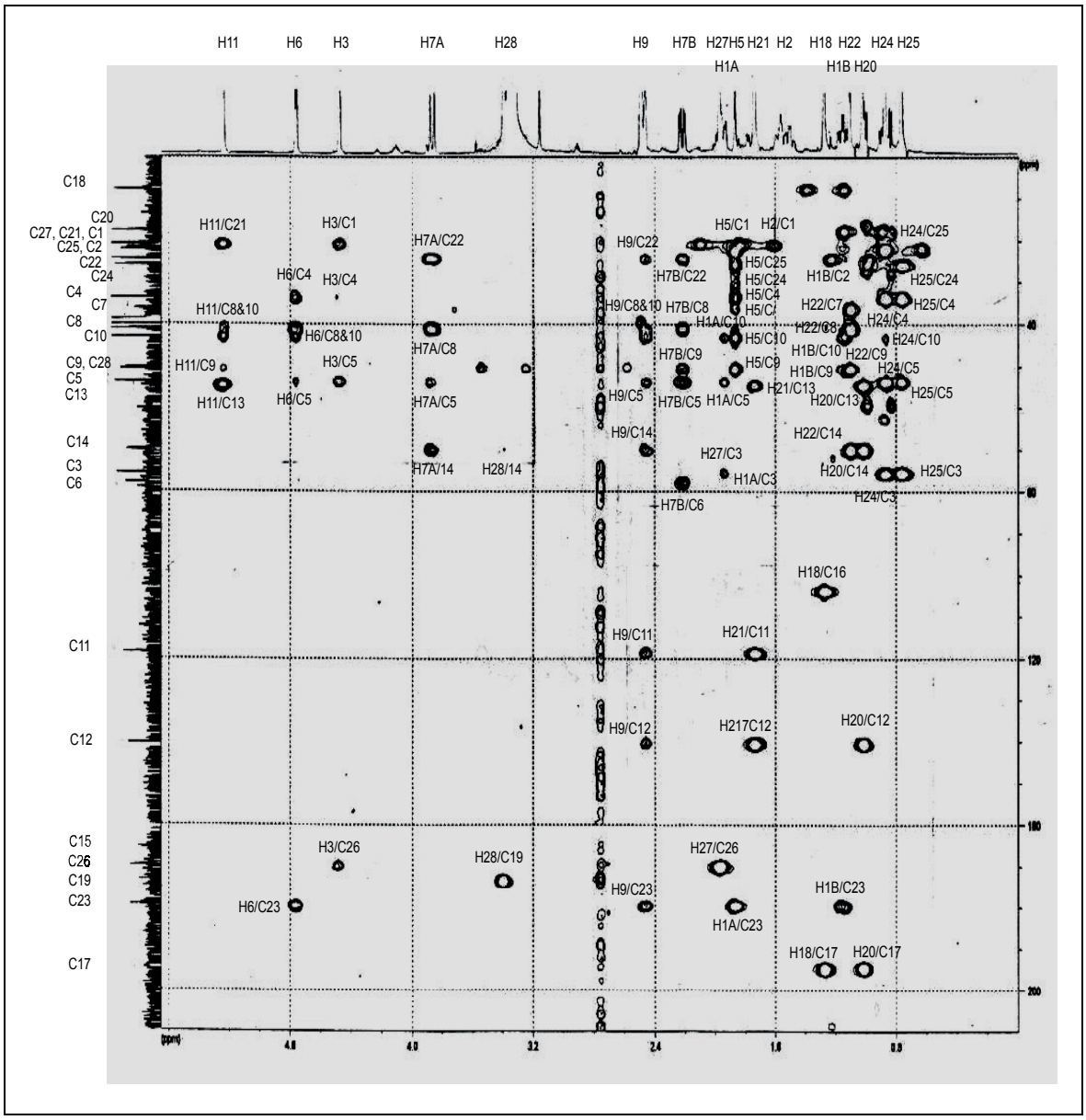



Figure C5.6. HMBC spectrum of compound **5** (citerohybridonol)

## 3.1.6. Compound 6 (andrastin A)

The ESI-MS spectrum of compound **6** revealed the molecular weight of 486 g/mol and suggested the molecular formula of $C_{28}H_{38}O_7$. The ESI-MS spectrum gave ion peaks at *m*/*z* 487.2 [ $M+H]^+$ (positive), 485.6 $[M-H]^-$ (negative).

Furthermore, in the EI-MS appeared ions at *m*/*z* 486$[M]^+$, 457 $[M-2CH_3]^+$ (fragment 1), 397 [M-acetoxy-$2CH_3]^+$ (fragment 2), 365 [M-acetoxy-$3CH_3$-$OH]^+$ (fragment 3) and 135 [acetoxy + methyl ester + $OH]^+$ (fragment 4) (Figure C6.1).

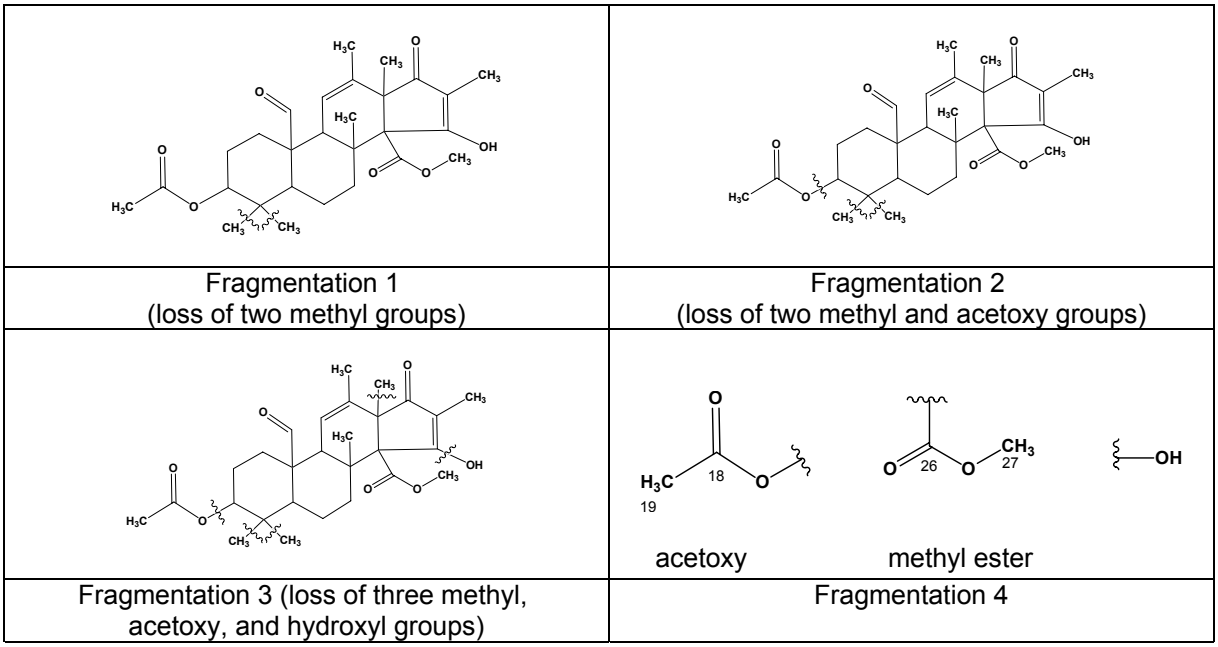



Figure C6.1. Hypothetical fragmentation of compound **6** (andrastin A) in the EI-MS spectrum

The $^1$H NMR spectrum revealed eight methyl signals (H19, H20, H21, H22, H24, H25, H27, H28) consisting of one acetoxy methyl (*δ* 2.04, H19), one methyl ester (*δ* 3.56, H27), two methyls attaching to double bond carbons [*δ* 1.70 (H24), 1.57 (H28)], four methyls bound to fully substituted carbons [*δ* 0.94 (H20), 0.86 (H21), 1.22 (H22), 1.14 (H25)] (Table C6.1, Figure C6.3).

The presence of an aldehyde proton [*δ* 10.16 (H23)] embedded in the deshielding zone of the C = O, and one isolated olefinic proton [*δ* 5.34 (H11)] were also evident (Table C6.1, Figure C6.3).

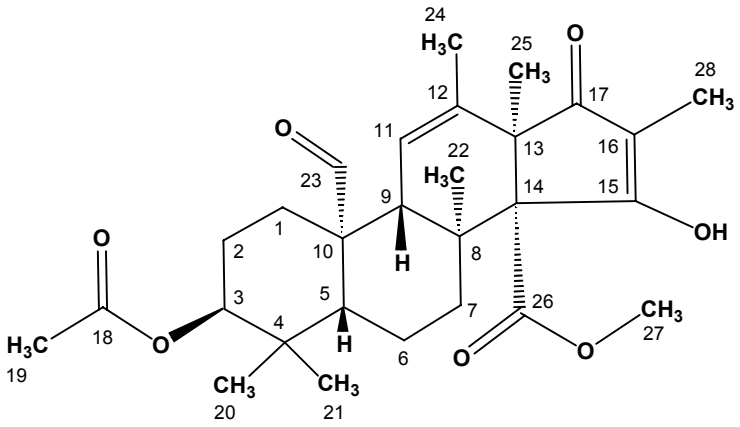
The appearance of one typical low field aldehyde carbon [ *δ* 206.80 (C23)], one isolated ketone [δ 200.40 (C17)], and two esters [*δ* 172.20 (C18), 171.80 (C26)] in the $^{13}$C NMR spectrum validated the difference of this compound with citreohybridonol (Table C6.1).

Four typical signals of double bond carbons namely *δ* 123.60 (C11), 137.00 (C12), 187.40 (C15, oxygen-carrying double bond carbon), 114.50 (H16) also supported the structure assignment. Eight methyl carbon signals classified into one acetoxy methyl (C19), one ester methyl (C27), two geminal methyls (C20, C21), two vinyl methyls (C24, C28), and two methyls bound to fully substituted carbons (C22, C25) clearly appeared in the $^{13}$C NMR spectrum (Table C6.1).

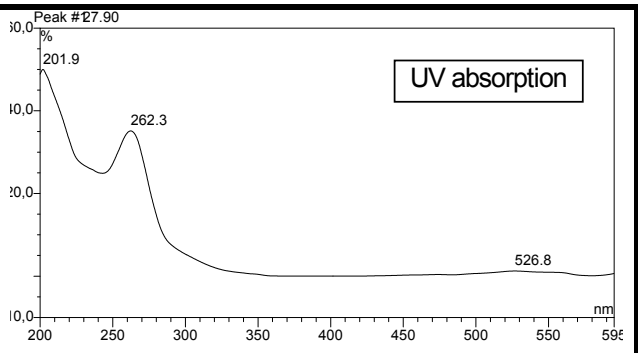
By looking at the COSY spectrum (Figure C6.2 and C6.4), three partial structures namely : $–CH_2–CH_2–CH–O–$ (within ring A, positions H1, H2, H3); $–CH–CH_2–CH_2–$ (within ring B, positions H5, H6, H7) and –CH–CH= (within ring C, positions H9, H11, H12) were deduced. Direct correlation of protons to their corresponding carbons were showed in the HMQC spectrum (Figure C6.5).

## Andrastin A (compound 6)

| | |
|---|---|
| CAS Registry Number | : 174232-42-9 |
| Characteristic | : white powder |
| Formula | : $C_{28}H_{38}O_7$ |
| Molecular Weight | : 486 g/mol |
| Amount | : 4.2 mg |
| Source | : *Penicillium* sp. derived from *Ircinia fasciculata* |




| | |
|---|---|
| Retention Time | : 27.98 min |
| $R_f$ | : 0.90 |
| Fluorescence, 254 nm | : + |
| 366 nm | : light blue |
| Anisaldehyde/$H_2SO_4$ | : - |




| | |
|---|---|
| Optical Rotation $[\alpha]_D^{20}$ | : experiment = -57.7° (c=0.1, MeOH) |
| | literature = -46.4° (c=0.6, MeOH) (Omura *et al*., 1996) |
| ESI-MS (*m*/*z*) | : 487.2$[M+H]^+$ (positive), 485.6$[M-H]^-$ (negative) |
| EI-MS (*m*/*z*, rel. int.) | : 486$[M]^+$ $_{(5.9)}$, 457$_{(9.6)}$, 397$_{(21.4)}$, 365$_{(26.9)}$, 309$_{(7.2)}$, 215$_{(10.8)}$, 183$_{(12.3)}$, 147$_{(8.6)}$, 135$_{(10.9)}$, 83$_{(12.1)}$ |

Table C6.1. NMR data of compound **6** (andrastin A)

| Position | $\delta$ $^1$H (ppm), multiplicity (*J* in Hz) (Shiomi *et al*., 1996 in MeOD) | $\delta$ $^1$H (ppm), multiplicity (*J* in Hz) (in MeOD) | COSY (H → H) (in $CDCl_3$) | HMQC (H→ C) direct | HMBC (H→ C) |
|---|---|---|---|---|---|
| 1 | 2.30 (ddd, 12.4, 3.3, 3.3) H1A; 0.98 (ddd, 5.0, 12.4, 13.0) H1B | (ddd,14.2, 3.2, 3.2) H1A; 0.95 (br s) H1B | H2, H23 | C1 | C3, C4, C5 |
| 2 | 2.05 (m); 1.59 (m) | 2.06 (m); 1.58 (m) | H1 | C2 | |
| 3 | 4.62 (dd, 2.4, 2.4) | 4.60 (dd, 2.5, 2.5) | H2, H5 | C3 | |
| 4 | | | | | |
| 5 | 1.84 (dd, 15.7, 2.4) | 1.84 (dd, 13.2, 2.5) | H3, H6, H7 | C5 | |
| 6 | 2.08 (m); 1.70 (m) | 2.06 (m); 1.70 (m) | H5 | | |
| 7 | 3.00 (ddd, 13.0, 12.9, 4.0) H7A; 2.25 (ddd, 12.9, 3.1, 3.1) H7B | 3.08 (ddd, 13.9, 9.5, 4.0) H7A; 2.25 (ddd, 14.2, 3.2, 3.2) H7B | H5, H6 | C7 | C5 |
| 8 | | | | | |
| 9 | 2.13 (s) | 2.15 (s) | | C9 | C24 |
| 10 | | | | | |
| 11 | 5.39 (br s) | 5.34 (br s) | H9 | | |
| 12 – 18 | | | | | |
| 19 ($CH_3$) | 2.05 (s) | 2.04 (s) | | C19 | C18 |
| 20 ($CH_3$) | 0.95 (s) | 0.94 (s) | | C20 | C3, C4, C5, C21 |
| 21 ($CH_3$) | 0.88 (s) | 0.86 (s) | | C21 | C3, C4, C5, C20 |
| 22 ($CH_3$) | 1.24 (s) | 1.22 (s) | | C22 | C7, C8, C9, C14 |
| 23 | 10.18 (s) | 10.16 (s) | | | |
| 24 ($CH_3$) | 1.75 (br s) | 1.74 (s) | | C24 | C11, C12 |
| 25 ($CH_3$) | 1.16 (s) | 1.14 (s) | | C25 | C12, C13, C14 |
| 26 | | | | | |
| 27 (O-$CH_3$) | 3.58 (s) | 3.56 (s) | | C27 | C26 |
| 28 ($CH_3$) | 1.59 (s) | 1.57 (s) | | C28 | C16 |

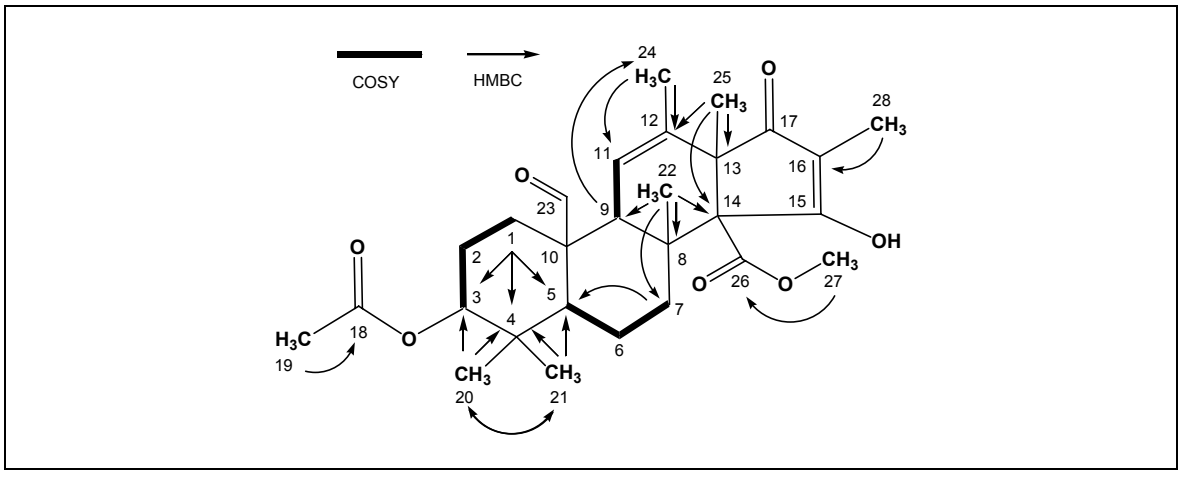



Figure C6.2. COSY and HMBC correlations of compound **6** (andrastin A)

The HMBC spectrum connected partial structures established by $^1H \rightarrow {}^1H$ correlation in the COSY spectrum to form rings A, B, C, and D (Figure C6.2 and C6.6). Andrastin A has a similar structure as citreohybridonol. However, the lactone in citreohybridonol at position 10 is replaced by an aldehyde group in andrastin A.

Similarity of chemical shifts, multiplicities, and coupling constants in the $^1H$ NMR spectrum of compound **6** with those of andrastin A derived from *Penicillium* sp., along with the similarity of compound **6** optical rotation (57.7°) and literature optical rotation (46.4°) (Shiomi et al., 1996) resulted in the conclusion that both compounds and their stereochemistry were identical. Antimicrobial assay of compound **6** did not demonstrate any activity.

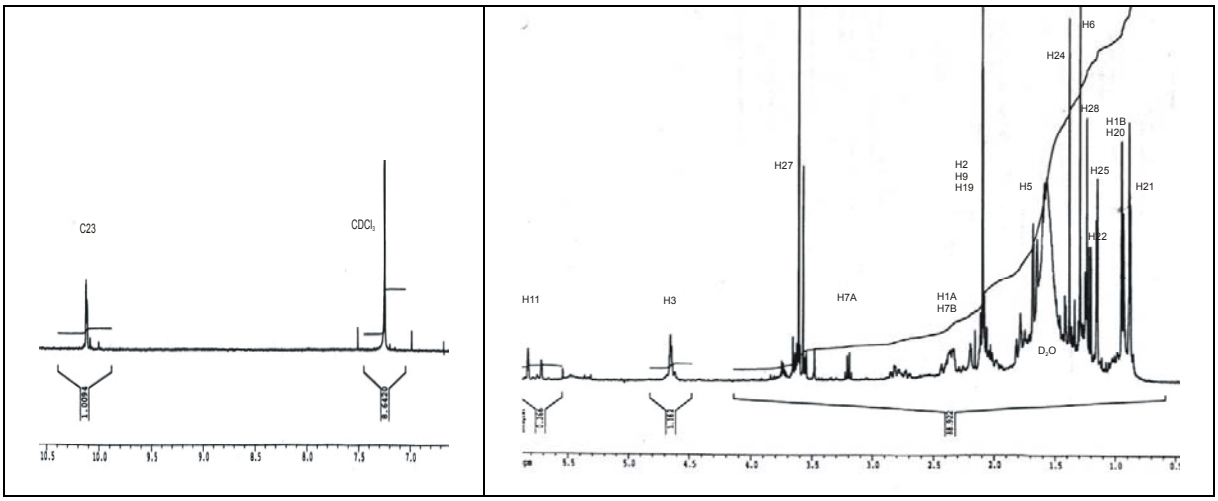



Figure C6.3. $^1H$ NMR spectrum of compound **6** (andrastin A) in $CDCl_3$

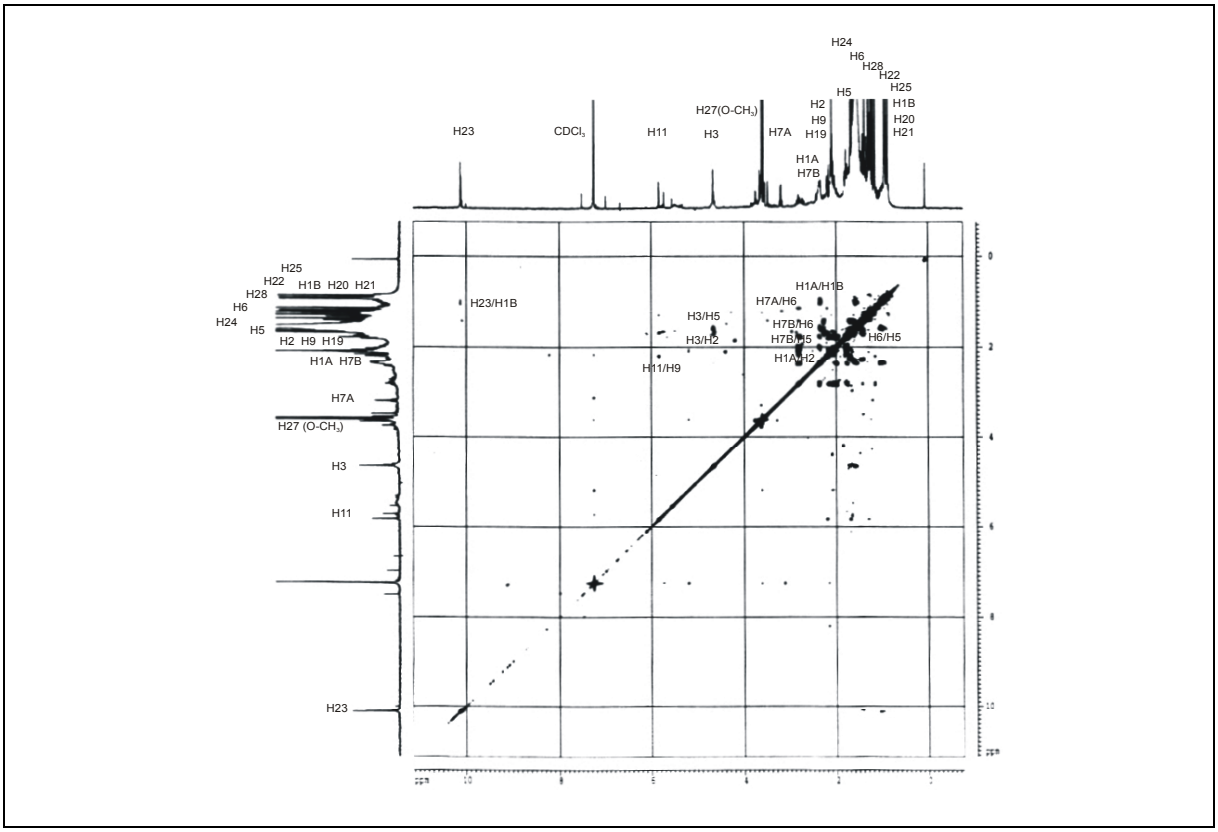



Figure C6.4. COSY spectrum of compound **6** (andrastin A) in $CDCl_3$

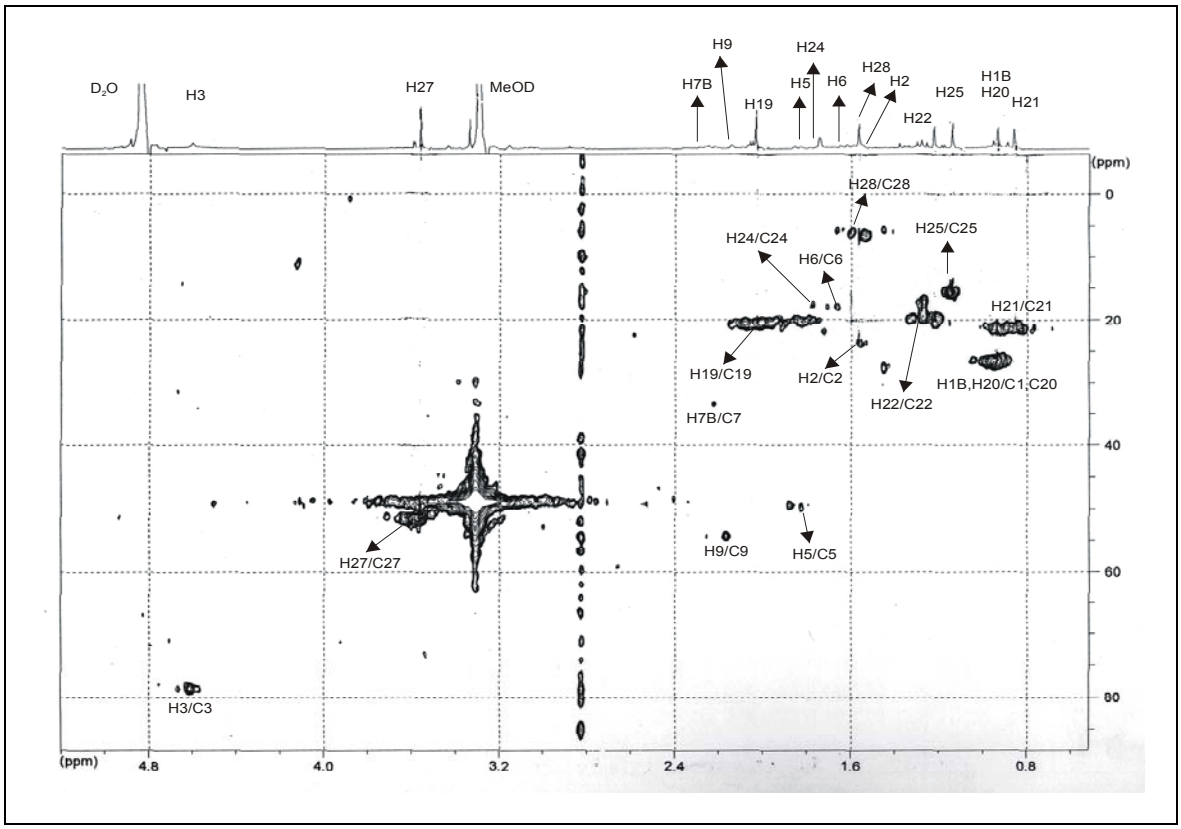



Figure C6.5. HMQC spectrum of compound **6** (andrastin A) in MeOD

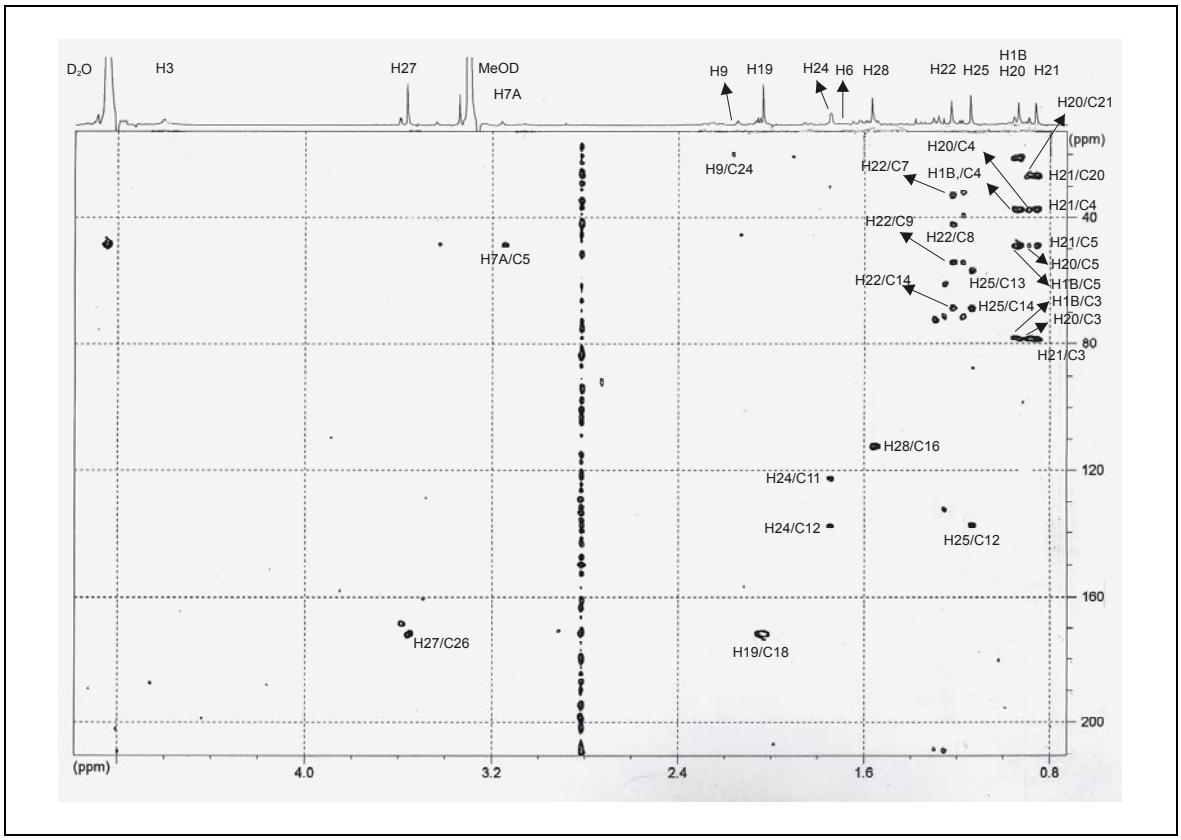



Figure C6.6. HMBC spectrum of compound **6** (andrastin A) in MeOD

## 3.2. Isolated secondary metabolites of fungus *Verticillium* cf *cinnabarium*

Eight compounds were successfully isolated from the fungus *Verticillium* cf *cinnabarium*, that had been derived from the sponge *Ircinia fasciculata*. They included 3-hydroxyanthranilic acid (compound **7**), 4-hydroxybenzaldehyde (compound **8**), tyramine (compound **9**), cyclo(alanyltryptophan) (compound **10**), cyclo(prolylvalyl) (compound **11**), cyclo(leucylprolyl) (compound **12**), verticillin B (compound **13**), and lichesterol (compound **14**).

### 3.2.1. Compound 7 (3-hydroxyanthranilic acid)

The molecular weight and molecular formula of compound **7** (3-hydroxyanthranilic acid) were determined 153 g/mol and $C_7H_7NO_3$, respectively. These were confirmed by the EI-MS spectrum showing a peak at *m*/*z* 153 $[M]^+$ with a distinctive fragmentation as follows: *m*/*z* 135 $[M\text{-}H_2O]^+$ (fragment 1), 107 $[M\text{-}COOH]^+$ (fragment 2), 91 $[M\text{-}COOHNH_2]^+$ (fragment 3), 79 [M of $CH_4O_3N_1]^+$ (fragment 4) (Figure C7.1).

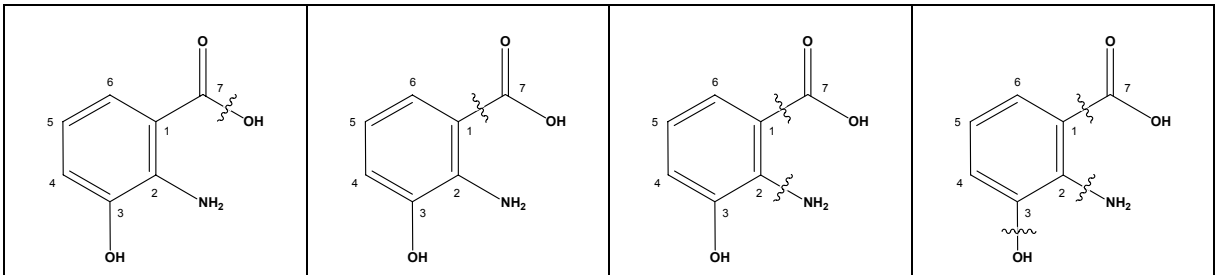



Figure C7.1. Hypothetical fragmentation of compound **7** (3-hydroxyanthranilic acid) in the EI-MS spectrum

There were only three proton signals in the aromatic region in the $^1H$ NMR spectrum. Those protons were $\delta$ 6.85 (H4), $\delta$ 6.49 (H5), $\delta$ 7.38 (H6). From their multiplicity and coupling constant, ortho position of each proton to their neighbouring proton was indicative (Table C7.1, Figure C7.2).

One carbonyl [$\delta$ 171.87 (C7)] in the low field of the $^{13}C$ NMR spectrum was observed. In addition to that, there were three singlet carbons and three doublet carbons (Table C7.1, Figure C7.3).

Comparison of compound **7** NMR spectrum with that of 2-amino-3-hydroxybenzoic acid from Aldrich (1992) confirmed that both compounds were identical. Bioactivity of compound **7** is presented in Table C7.2.

Table C7.1. NMR data of compound **7** (3-hydroxyanthranilic acid)

| Position | $\delta$ $^{13}C$ (ppm) (in DMSO) | $\delta$ $^1H$ (ppm), multiplicity (*J* in Hz) (Aldrich, 1992 in $CDCl_3$ + DMSO) | $\delta$ $^1H$ (ppm), multiplicity (*J* in Hz) (in DMSO) |
|---|---|---|---|
| 1 | 112.65 (s) | | |
| 2 | 142.01 (s) | | |
| 3 | 146.19 (s) | | |
| 4 | 118.02 (d) | 6.80 (d) | 6.85 (d, 7.8) |
| 5 | 116.13 (d) | 6.40 (t) | 6.49 (t, 8.0, 7.8) |
| 6 | 123.26 (d) | 7.30 (d) | 7.38 (d, 8.3) |
| 7 (COOH) | 171.87 (s) | | |

## 3-Hydroxyanthranilic acid (compound 7)

CAS Registry Number : 548-93-6

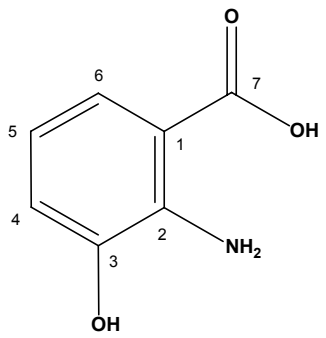
Characteristic : Yellow powder

Formula : $C_7H_7NO_3$

Molecular Weight : 153 g/mol

Amount : 11.6 mg

Source *Verticillium* cf *cinnabarium* derived from *Ircinia fasciculata*

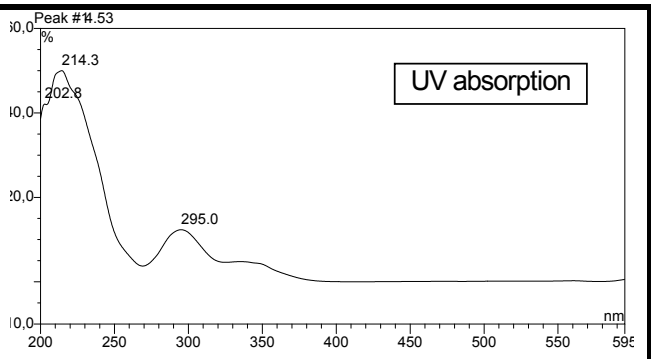



Retention Time : 4.36 min.

$R_f$ : 0.45

Fluorescence, 254 nm : +
366 nm : blue

Anisaldehyde/$H_2SO_4$ : +




EI-MS (*m/z*, rel. int.) : 153 $[M]^+$ (100), 135 $[M-H_2O]^+$(57.4), 107 $[M-COOH]^+$(97.1), 91$[M-COOHNH_2]^+$(2.0), 79 [M of $CH_4O_3N]^+$(24.7)

Table C7.2. Antimicrobial activity of compound **7** (3-hydroxyanthranilic acid)

| Compound **7** | *Staphylococcus aureus* | *Bacillus subtilis* | *Escherichia coli* | *Candida albicans* | *Saccharomyces cerevisiae* | *Cladosporium herbarum* |
|---|---|---|---|---|---|---|
| 50 μg | not active | 7 mm | not active | not active | not active | not active |
| 100 μg | 7 mm | 10 mm | not active | not active | not active | not active |

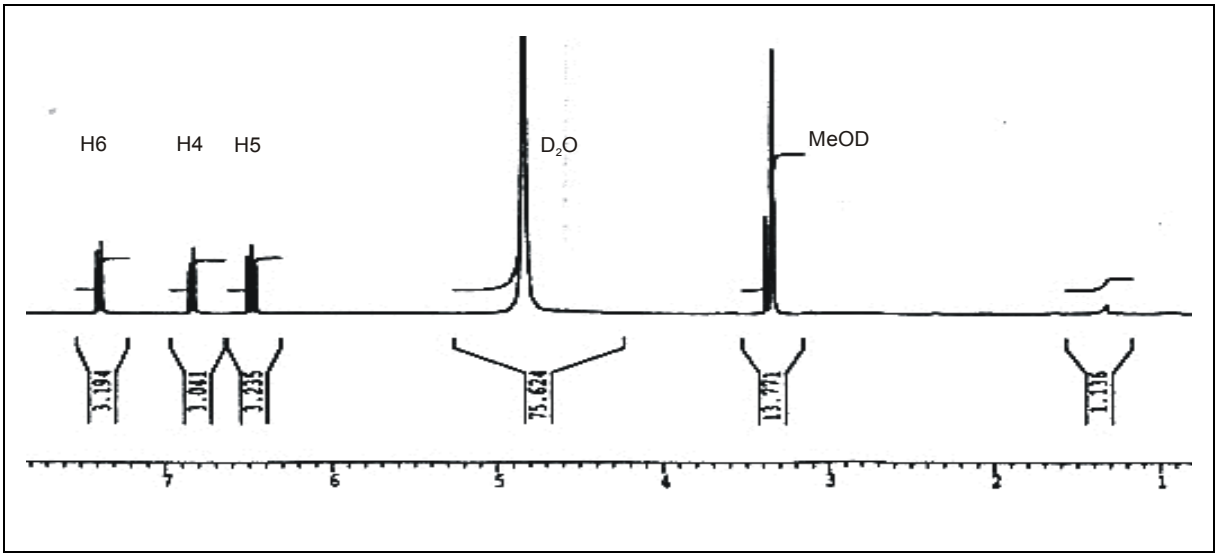



Figure C7.2. $^{1}$H NMR spectrum of compound **7** (3-hydroxyanthranilic acid)

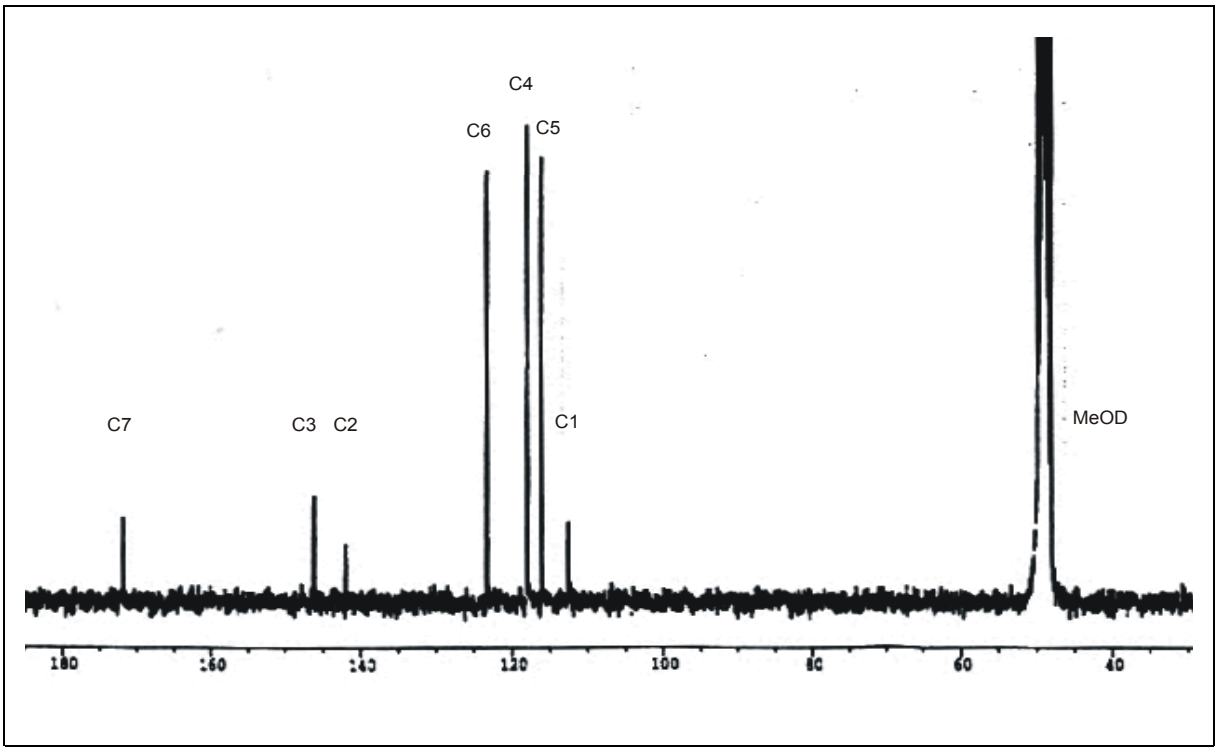



Figure C7.3. $^{13}$C NMR spectrum of compound **7** (3-hydroxyanthranilic acid)

## 3.2.2. Compound 8 (4-hydroxybenzaldehyde)

Molecular weight of this compound was 122 g/mol, determined by the ESI-MS spectrum, exhibiting a peak at *m*/*z* 121 $[M-1]^-$ and suggesting a molecular formula of $C_7H_6O_2$.

Para-substituted phenyl ring of compound **8** was evident from proton signals at $\delta$ 7.78 (H2, H6) and $\delta$ 6.91 (H3, H5). This AA'BB' spin system was apparently displayed by doublet multiplicty and ortho coupling with the magnitude of 8.8 Hz of two pair protons. Carbonyl proton found at low field chemical shift $\delta$ 9.75 (s, H7) also distinctly appeared in the $^1$H NMR spectrum (Table C8.1).

Comparison of chemical shift of the $^1$H NMR data of compound **8** with that of a standard compound (para-hydroxybenzaldehyde) in the same NMR solvent (MeOD) was absolutely identical (Table C8.1). Antimicrobial assay compound **8** did not exhibit any bioactivity.

Table C8.1. NMR data of compound **8** (4-hydroxybenzaldehyde)

| Position | $\delta$ $^{13}$C (ppm), (Standard in MeOD) | $\delta$ $^1$H (ppm), multiplicity (*J* in Hz) (Standard in MeOD) | $\delta$ $^1$H (ppm), multiplicity (*J* in Hz) (in MeOD) |
|---|---|---|---|
| 1 | 129.43 (s) | | |
| 2 | 133.84 (d) | 7.76 (d, 8.5) | 7.78 (d, 8.8) |
| 3 | 117.16 (d) | 6.91 (d, 8.5) | 6.91 (d, 8.8) |
| 4 | 165.57 (s) | | |
| 5 | 117.16 (d) | 6.91 (d, 8.5) | 6.91 (d, 8.8) |
| 6 | 133.84 (d) | 7.76 (d, 8.5) | 7.78 (d, 8.8) |
| 7 | 193.22 (d) | 9.75 (s) | 9.75 (s) |

## 4-Hydroxybenzaldehyde (compound 8)

CAS Registry Number : 1233-08-0

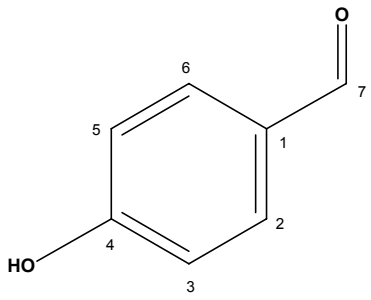
Characteristic : white powder

Formula : $C_7H_6O_2$

Molecular Weight : 122 g/mol

Amount : 5.2 mg

Source : *Verticillium* cf *cinnabarium* derived from *Ircinia fasciculata*

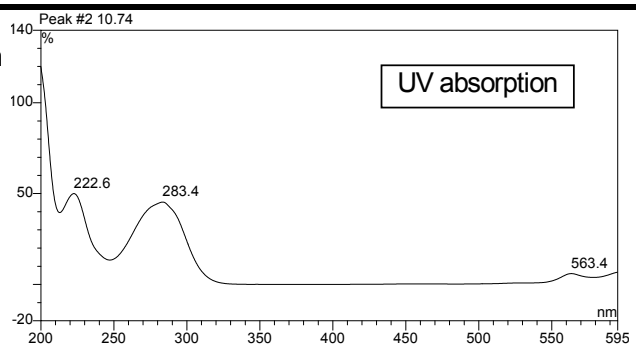



Retention Time : 10.69 min

$R_f$ : 0.91

Fluorescence, 254 nm : +
366 nm : +

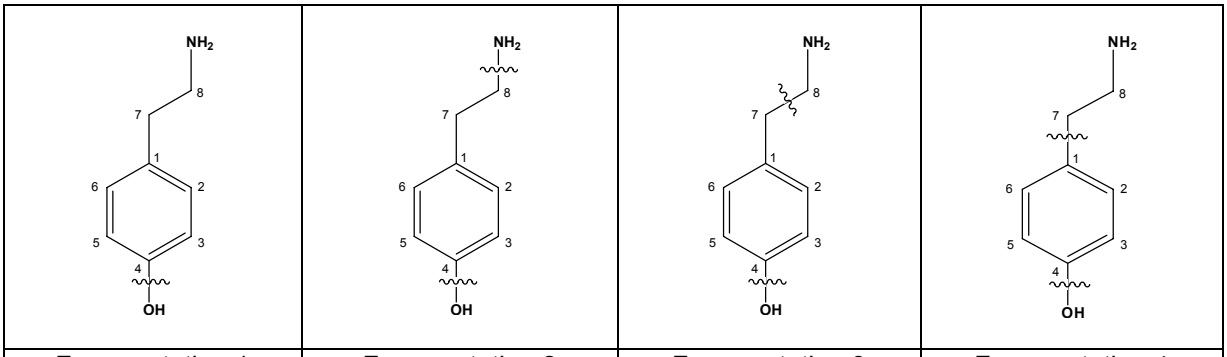
Anisaldehyde/$H_2SO_4$ : -




ESI-MS (*m/z*) : 121.6 $[M-H]^-$ (negative)

## 3.2.3. Compound 9 (tyramine)

The EI-MS spectrum of compound **9** showed clear-cut fragmentation namely: *m/z* 137 $[M]^+$, 120 $[M\text{-}OH]^+$ (fragment 1), 107 $[M\text{-}NH_2OH]^+$ (fragment 2), 91 $[M\text{-}CH_2NH_2OH]^+$ (fragment 3), 77 $[M\text{-}CH_2CH_2NH_2OH]^+$ (fragment 4) (Figure C9.1). This fragmentation confirmed that the molecular weight and molecular formula were 137 g/mol and $C_8H_{11}NO$, respectively.




| Fragmentation 1 | Fragmentation 2 | Fragmentation 3 | Fragmentation 4 |
|---|---|---|---|

Figure C9.1. Hypothetical fragmentation of compound **9** (tyramine) in the EI-MS spectrum

Two triplet protons [$\delta$ 2.70 (H7) and 3.60 (H8)] at aliphatic region were observed in the $^1H$ NMR spectrum. An AA'BB' spin system representing para-substituted phenyl ring was also clearly found through analysis of proton coupling constant and $^1H \rightarrow {}^1H$ COSY correlation. Another spin system (A2B2) was depicted on protons (H7 and H8) with their coupling constants of 6.5 – 7.6 Hz at the aliphatic region (Table C9.1, Figure C9.5).

Four carbons of phenyl ring were connected to their corresponding protons as conspicuously seen in the HMQC spectrum (Figure C9.6). Two carbons [$\delta$ 39.43 (C7), $\delta$ 64.99 (C8)] at low field representing aliphatic chain of the compound were also distinctly observed in the $^{13}C$ NMR spectrum (Figure C9.3 and C9.4).

Correlation among protons with their neighbouring carbons was indicated clearly in the HMBC spectrum (Figure C9.7). Shielding effect of hydroxyl group toward C3, C5, and C1 caused these three carbons to appear more upfield than the adjacent carbons (Table C9.1).

Table C9.1. NMR data of compound **9** (tyramine)

| Position | $\delta$ $^{13}C$ (ppm) | $\delta\,^1H$ (ppm), multiplicity (*J* in Hz) in MeOD | COSY (H → H) | HMQC (H → C) direct | HMBC (H → C) |
|---|---|---|---|---|---|
| 1 | 129.31 (s) | | | | |
| 2 | 130.58 (d) | 7.10 (dd, 8.2, 1.9) | H3 | C2 | C6, C4 |
| 3 | 115.83 (d) | 6.80 (dd, 8.2, 1.9) | H2 | C3 | C2, C6 |
| 4 | 156.49 (s) | | | | |
| 5 | 115.83 (d) | 6.80 (dd, 8.2, 1.9) | H6 | C5 | C2, C6 |
| 6 | 130.58 (d) | 7.10 (dd, 8.2, 1.9) | H5 | C6 | C2, C4 |
| 7 | 39.43 (t) | 2.70 (t, 7.3) | H8 | | C2, C6, C8 |
| 8 | 64.99 (t) | 3.60 (t, 7.3) | H7 | | C2, C6 |

Antimicrobial assay performed with compound **9** as a test substance did not reveal any biological activity.

## Tyramine (compound 9)

CAS Registry Number : 51-67-2

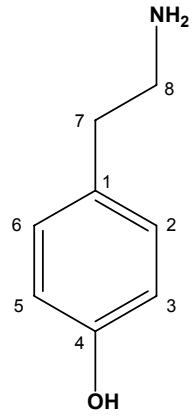
Characteristic : white powder

Formula : $C_8H_{11}NO$

Molecular Weight : 137 g/mol

Amount : 2.8 mg

Source : *Verticillium* cf *cinnabarium* derived from *Ircinia fasciculata*



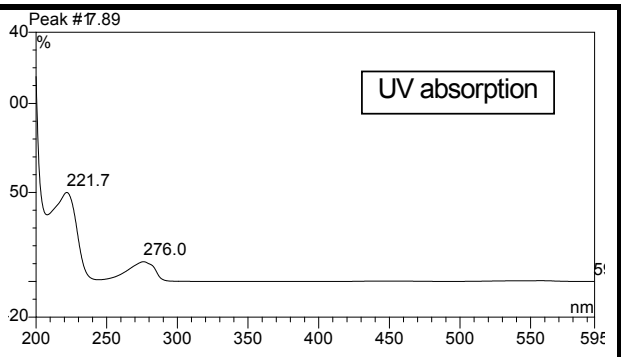

Retention Time : 8.02 min

$R_f$ : not measured

Fluorescence, 254 nm : not measured

366 nm : not measured

Anisaldehyde/$H_2SO_4$ : not measured




EI-MS (*m/z*, rel. int.) : 137 $[M]^+_{(24.3)}$, 120 $[M\text{-}OH]^+_{(0.7)}$, 107 $[M\text{-}NH_2OH]^+_{(100)}$, 91$[M\text{-}CH_2NH_2OH]^+_{(1.7)}$, 77$[M\text{-}CH_2CH_2NH_2OH]^+_{(11.9)}$

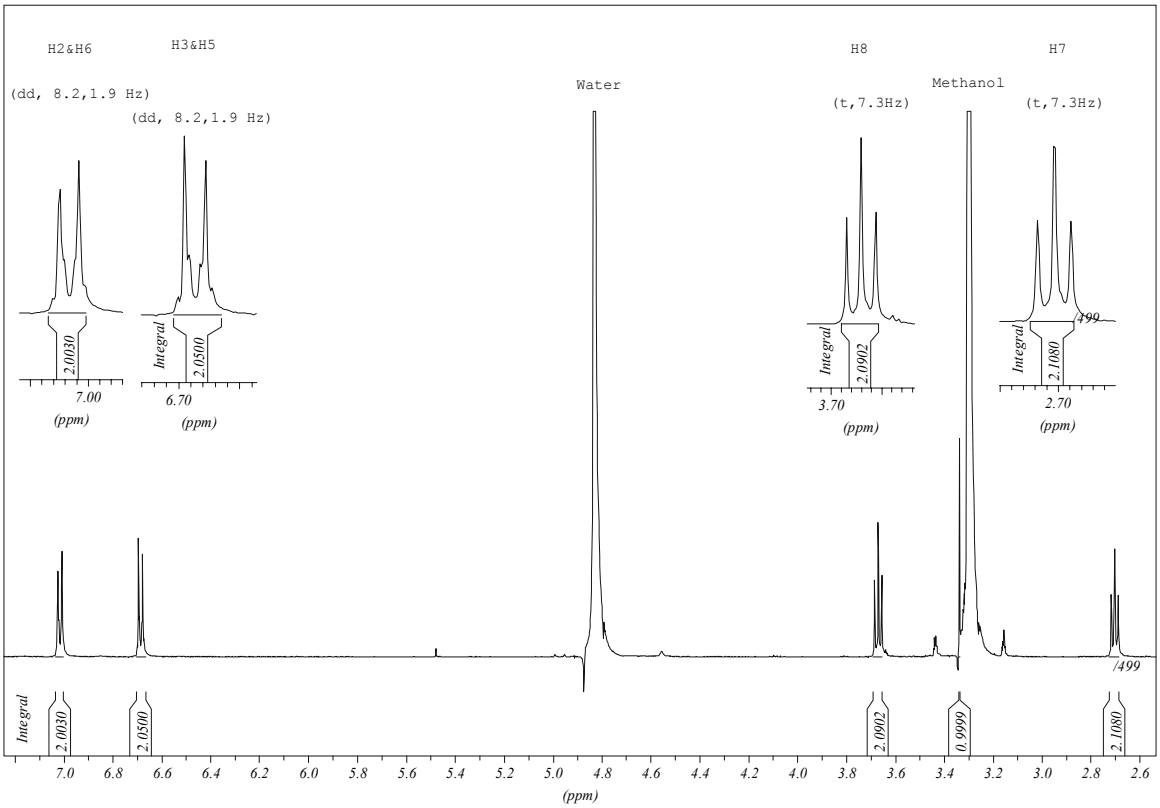



Figure C9.2. $^{1}$H NMR spectrum of compound **9** (tyramine)

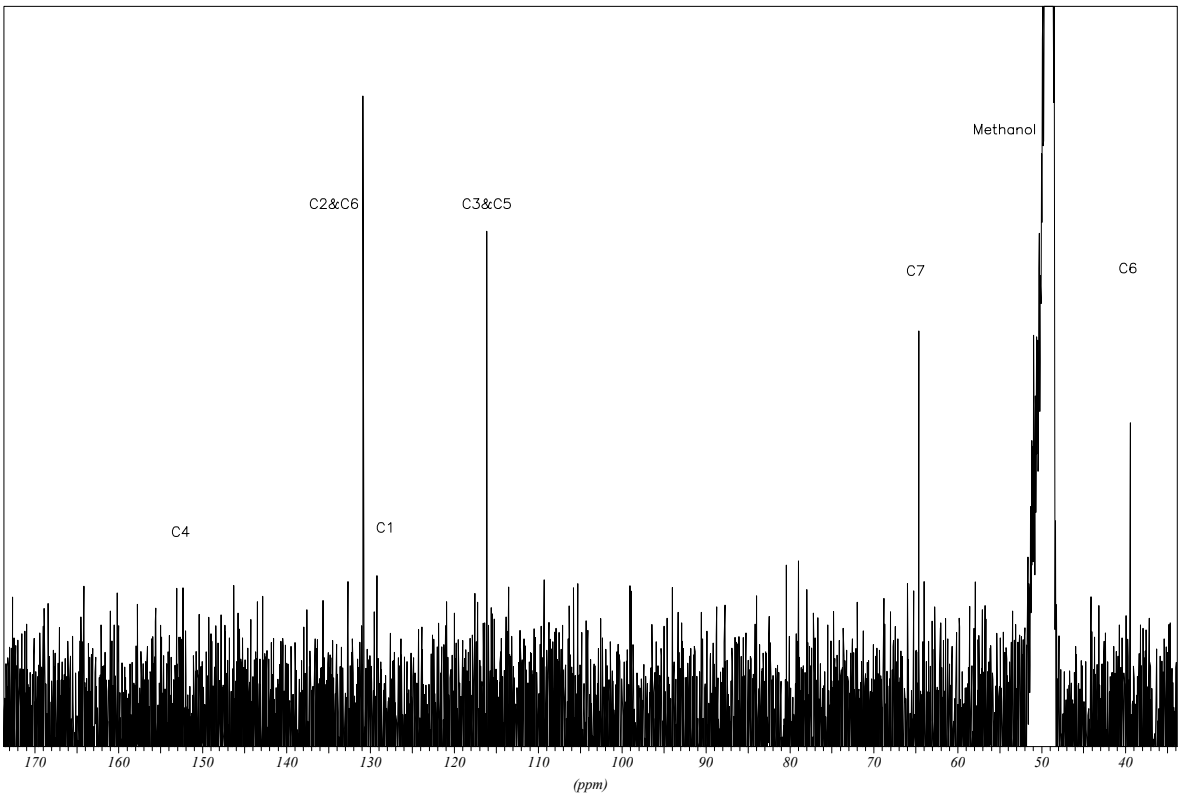



Figure C9.3. $^{13}$C NMR spectrum of compound **9** (tyramine)

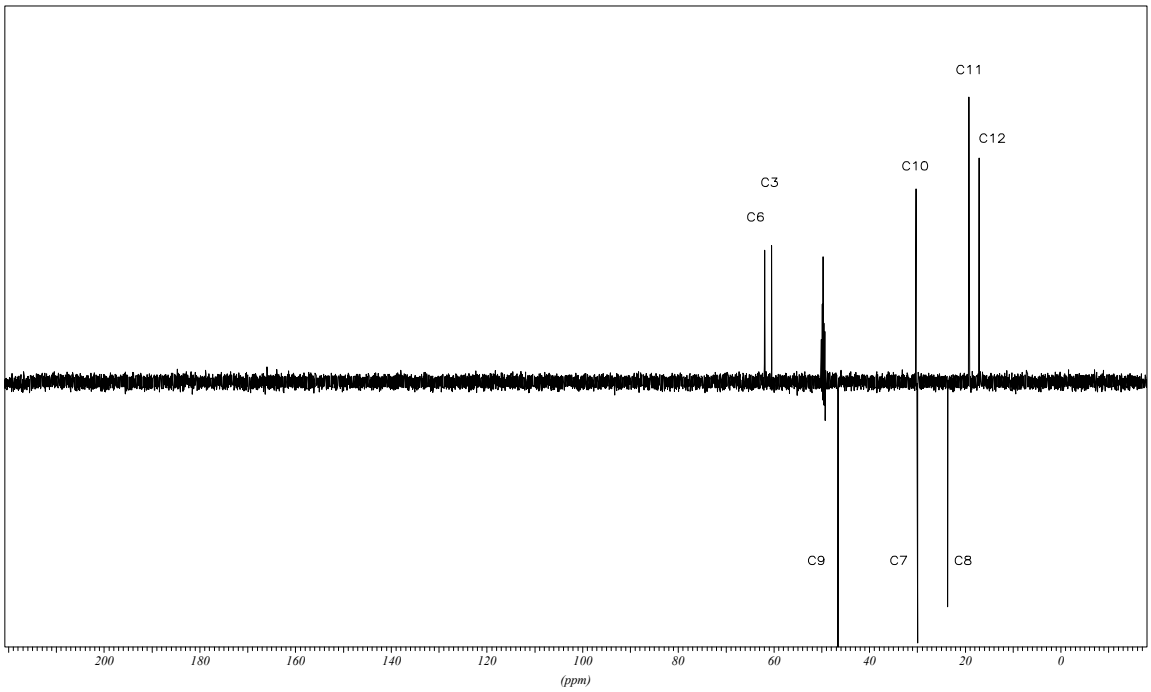



Figure C9.4. DEPT spectrum of compound **9** (tyramine)

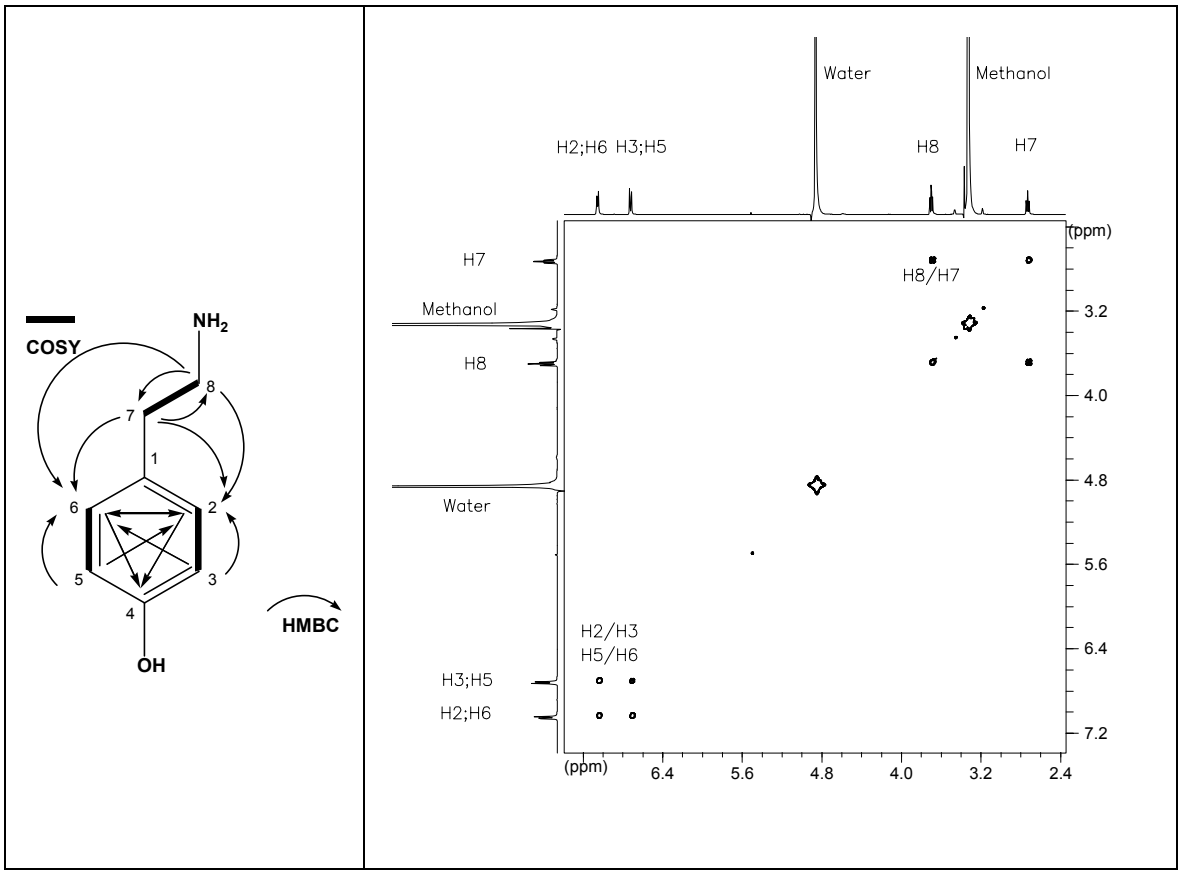



Figure C9.5. COSY spectrum of compound **9** (tyramine)

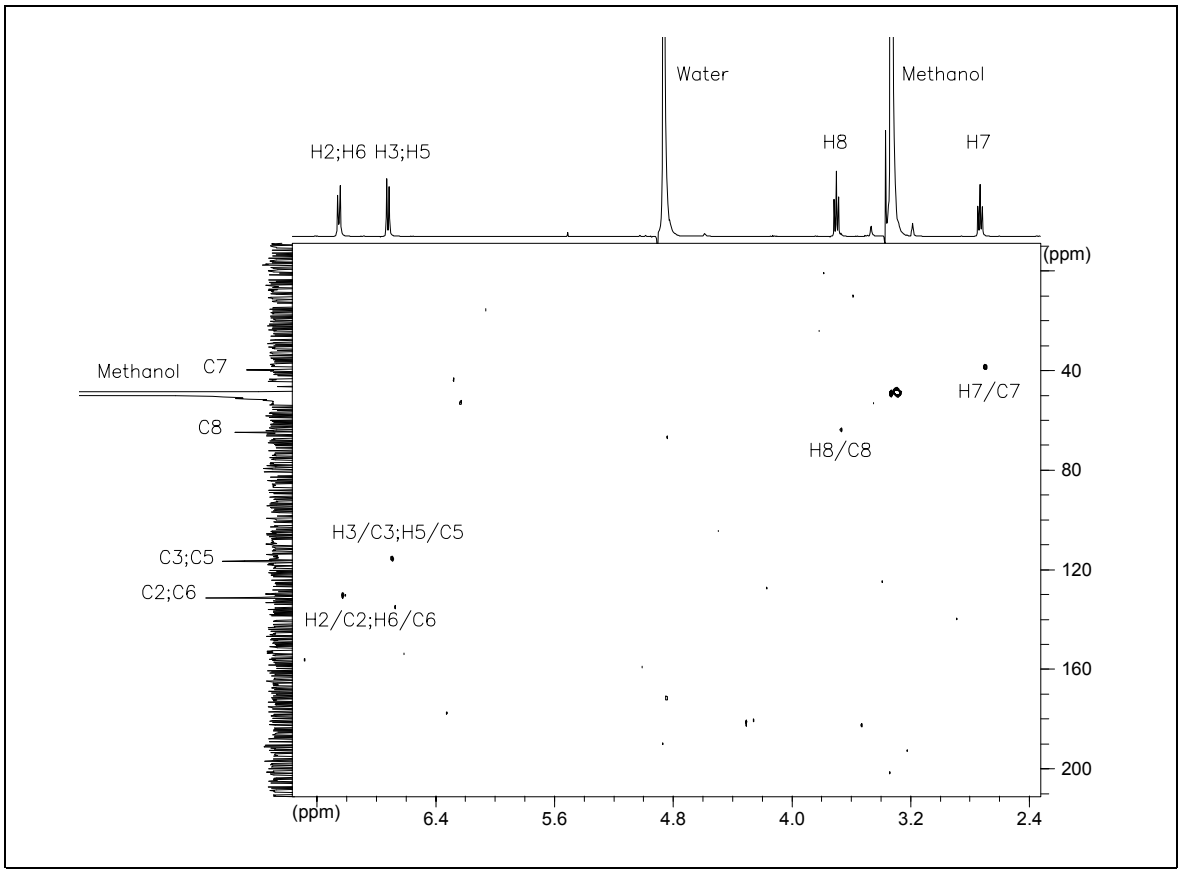



Figure C9.6. HMQC spectrum of compound **9** (tyramine)

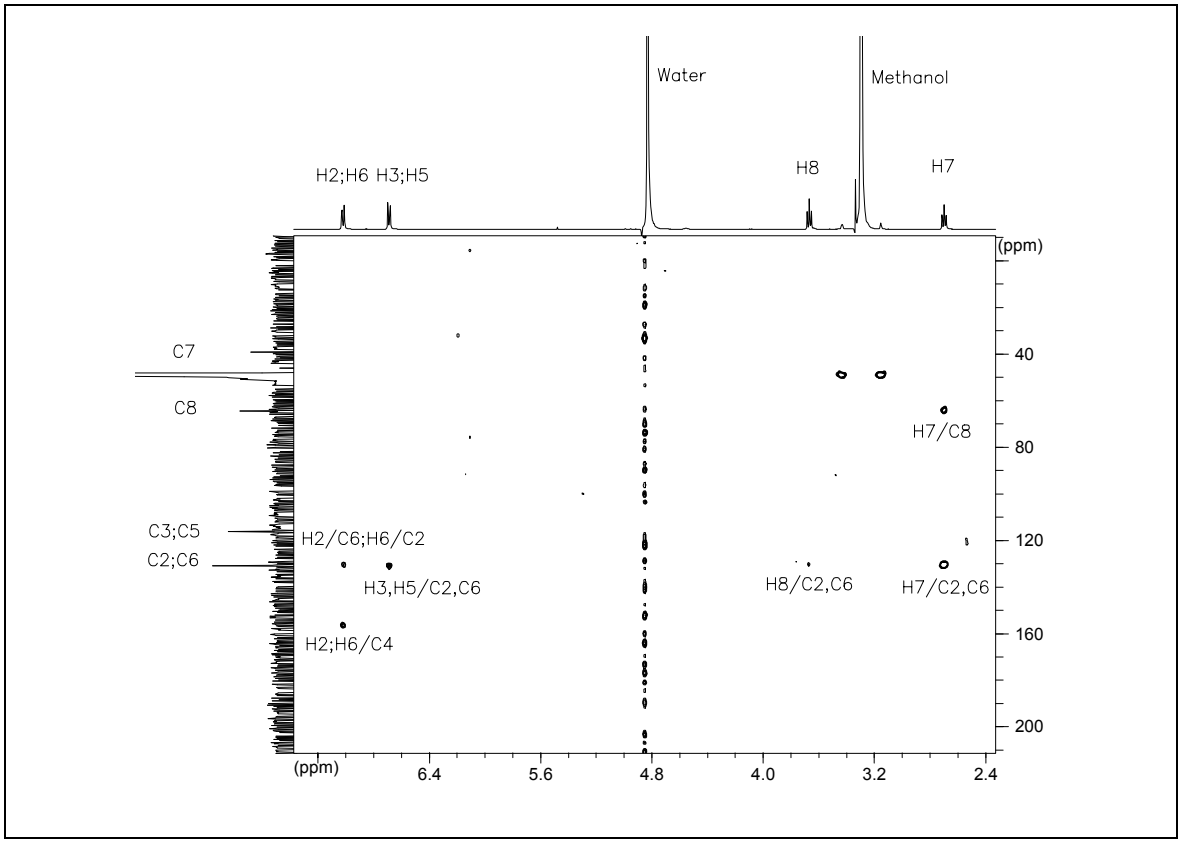
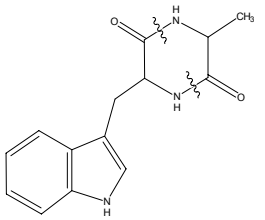


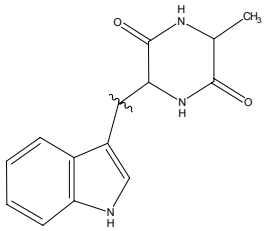

Figure C9.7. HMBC spectrum of compound **9** (tyramine)

## 3.2.4. Compound 10 (cyclo-alanyltryptophan)

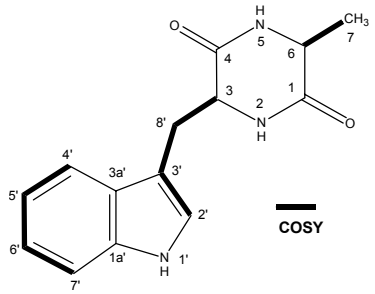
The ESI-MS spectrum of compound **10** showed a peak at *m*/*z* 258 $[M+H]^+$ (positive). In addition, EI-MS spectrum analysis revealed intense peaks at *m*/*z* 257 $[M]^+$, 185 $[M-C_3H_5O_1N_1]^+$ (fragment 1), 130 $[M-C_3H_5O_1N_1]^+$ (fragment 2) corresponding to the loss of diketopiperazine (Figure C10.1). These MS spectra gave a molecular weight of 257 g/mol and indicated a molecular formula of $C_{14}H_{15}N_3O_2$.



Fragmentation 1



Fragmentation 2




$^1H \rightarrow {}^1H$ COSY correlations

Figure C10.1. Hypothetical fragmentation in the EI-MS spectrum and COSY correlations of compound **10** (cyclo-alanyltryptophan)

In the $^1H$ NMR spectrum of compound **10**, typical downfield proton of *δ* 7.10 (H2'), characteristic of indole ring proton was observed. Through COSY analysis, a spin system of ABCD was observed at aromatic region, containing protons varying from *δ* 7.03 to *δ* 7.64. This ABCD spin system was also readily verified by the multiplicity and coupling constant of protons at position H4' [*δ* 7.35 (ddd, 7.2 Hz/ortho, 1.9 Hz/meta, 0.9 Hz/para)], H5' [7.03 (m)], H6' [7.11 (m)], H7' [7.64 (ddd, 7.2 Hz/ortho, 1.9 Hz/meta, 0.9 Hz/para)]. At the high field region, singlet proton [*δ* 0.42 (H7)], representing methyl was identified (Table C10.1, Figure C10.2).

Two carbonyl singlet carbons at downfield [*δ* 170.65 (C1), 169.52 (C4)] of the $^{13}C$ NMR spectrum appeared. In addition, there were also observed three more singlet carbons [C1a'(nitrogen-carrying double bond carbon), C3', C3a'], seven doublet carbons [C3, C6, C2'(nitrogen-binding double bond carbon), C4', C5', C6', C7'], one triplet carbon (C8'), and one quartet carbon (C7). The DEPT spectrum confirmed the existence of methylene carbon at position 8' (Figure C10.4).

The optical rotation of compound **10** was –13.3°, it was an accordance with the optical rotation of synthetic cyclo(*D*-alanyl-*D*-tryptophan) which was –10.2° (Nakashima and Slater, 1969) (Table C10.2). Bioactivity of compound **10** are presented in Table C10.3.

Table C10.2. Optical rotation of cyclo-alanyltryptophan (Nakashima and Slater, 1969)

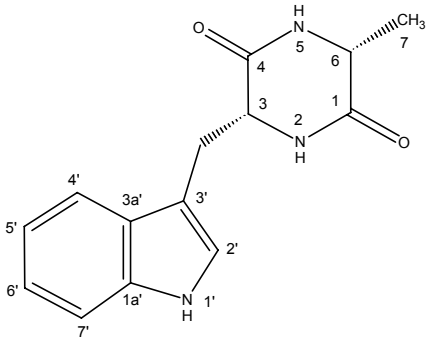
| Cyclo(alanyltryptophan) | $[\alpha]_D$ (°) |
|---|---|
| Cyclo(*L*-alanyl-*L*-tryptophan) | +10.4 [c=0.480 EtOH] |
| Cyclo(*D*-alanyl-*D*-tryptophan) | -10.2 [c=0.048 EtOH] |
| Cyclo(*D*-alanyl-*L*-tryptophan) | +75.6 [c= 0.049 EtOH] |
| Cyclo(*L*-alanyl-*D*-tryptophan) | -74.2 [c= 0.051 EtOH] |

Table C10.3. Antimicrobial activity of compound **10** (cyclo-alanyltryptophan)

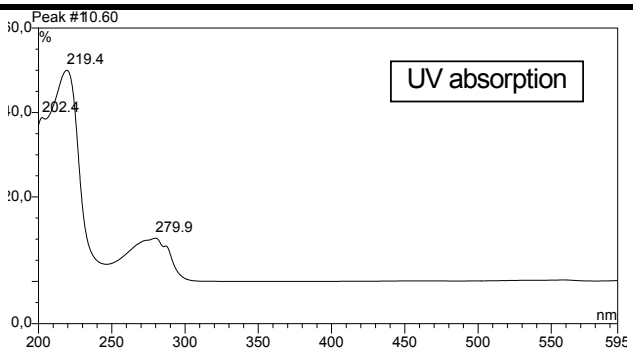
| Compound **10** | *Staphylococcus aureus* | *Bacillus subtilis* | *Escherichia coli* | *Candida albicans* | *Saccharomyces cerevisiae* | *Cladosporium herbarum* |
|---|---|---|---|---|---|---|
| 50 μg | 7 mm | not active | not active | 7 mm | not active | not active |
| 100 μg | 7 mm | not active | not active | 10 mm | not active | not active |

## Cyclo-alanyltryptophan (compound 10)

| | |
|---|---|
| Chemical abstract | : DA:C-299 |
| Characteristic | : pale yellow powder |
| Formula | : $C_{14}H_{15}N_3O_2$ |
| Molecular Weight | : 257 g/mol |
| Amount | : 11.5 mg |
| Source | : *Verticillium* cf *cinnabarium* derived from *Ircinia fasciculata* |




| | |
|---|---|
| Retention Time | : 10.70 min |
| $R_f$ | : 0.48 |
| Fluorescence, 254 nm | : + |
| 366 nm | : blue |
| Anisaldehyde/$H_2SO_4$ | : lila |




| | |
|---|---|
| Optical Rotation $[\alpha]_D^{20}$ | : experiment = -13.3° (c=0.1, EtOH) |
| | literature = -10.2° (EtOH) (Nakashima and Slater, 1969) |
| ESI-MS (*m/z*) | : 258.2 $[M+H]^+$ (positive) |
| EI-MS (*m/z*, rel. int.) | : 257 $[M]^+$ (7.2), 185 $[M-C_3H_5O_1N_1]^+$(0.5), 130 $[M-C_3H_5O_1N_1]^+$(100) |

Table C10.1. NMR data of compound **10** (cyclo-alanyltryptophan)

| Position | $\delta$ $^{13}$C (ppm) (in MeOD) | DEPT | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) (in MeOD) | COSY (H→ H) |
|---|---|---|---|---|
| 1 | 170.65 (s) | | | |
| 2 | | | | |
| 3 | 57.50 (d) | | 4.31 (m) | H8A, H8B |
| 4 | 169.52 (s) | | | |
| 5 | | | | |
| 6 | 51.76 (d) | | 3.74 (q, 7.0) | H7 |
| 7 | 20.03 (d) | | 0.42 (d, 7.0) | H6 |
| 1‘ | | | | |
| 1a‘ | 137.83 (s) | | | |
| 2‘ | 125.82 (d) | | 7.05 (m) | H8‘A, H8‘B |
| 3‘ | 112.16 (s) | | | |
| 3a‘ | 129.21 (s) | | | |
| 4‘ | 120.16 (d) | | 7.35 (ddd, 7.2, 1.9, 0.9) | H5‘, H6‘, H7‘ |
| 5‘ | 122.49 (d) | | 7.03 (m) | H4‘, H6‘, H7‘ |
| 6‘ | 119.98 (d) | | 7.11 (m) | H4‘, H5‘, H7‘ |
| 7‘ | 109.35 (d) | | 7.64 (ddd, 7.2, 1.9, 0.9) | H4‘, H5‘, H6‘ |
| 8’A | 30.84 (t) | 30.84 (t) | 3.49 (dd, 14.1, 4.0) | H3, H2‘, H8’B |
| 8’B | | | 3.19 (dd, 14.1, 4.0) | H3, H2‘, H8’A |

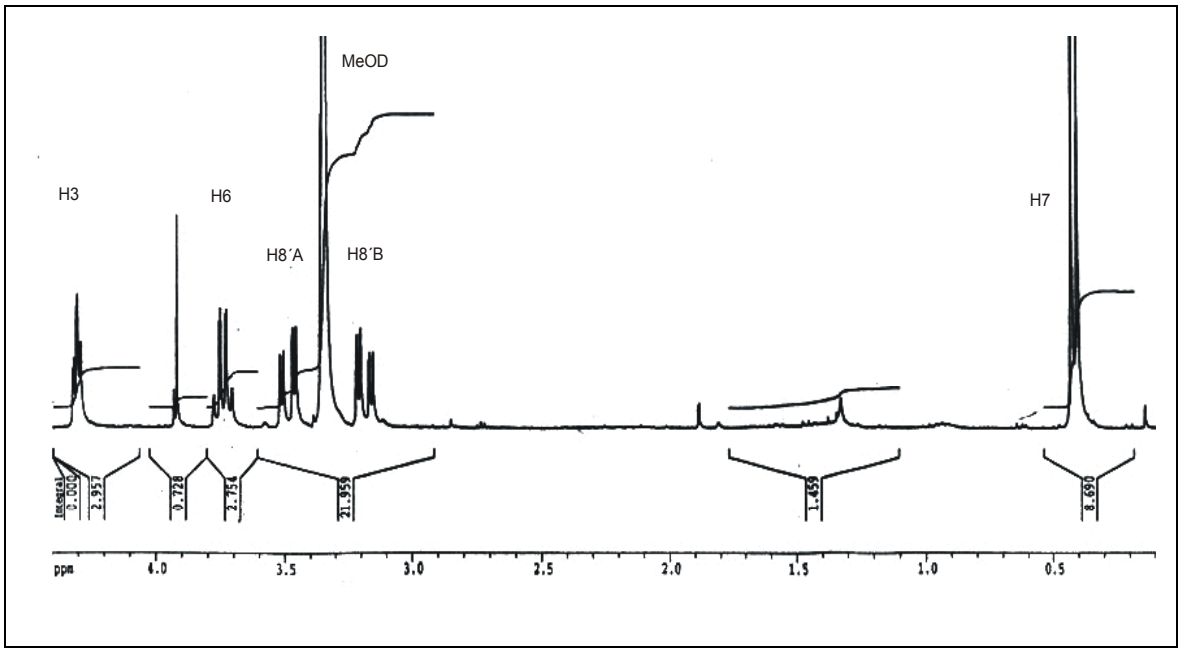



Figure C10.2. $^{1}$H NMR spectrum of compound **10** (cyclo-alanyltryptophan)

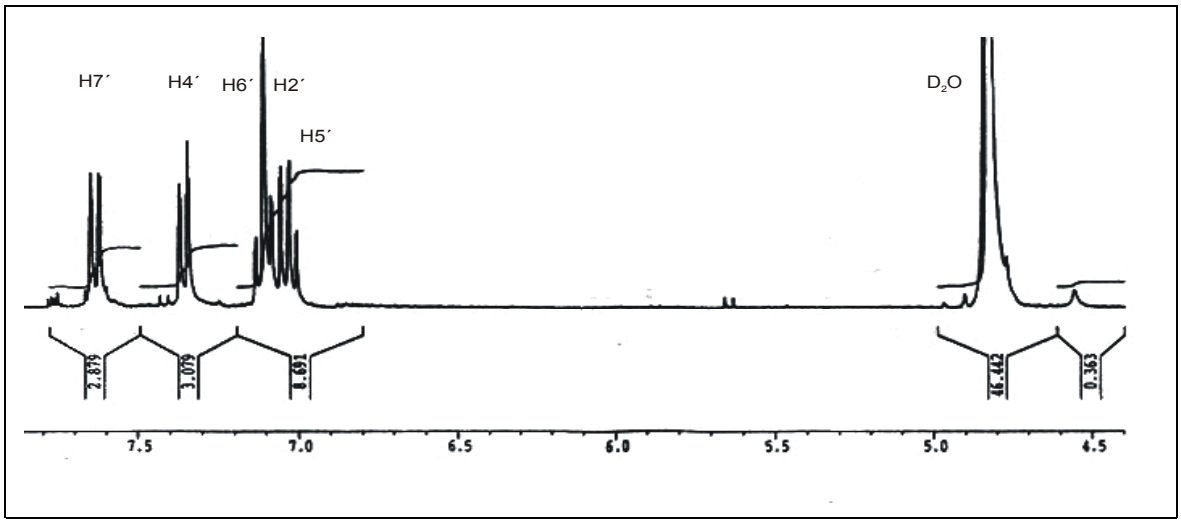



Figure C10.2. $^{1}$H NMR spectrum of compound **10** (cyclo-alanyltryptophan) (continued)

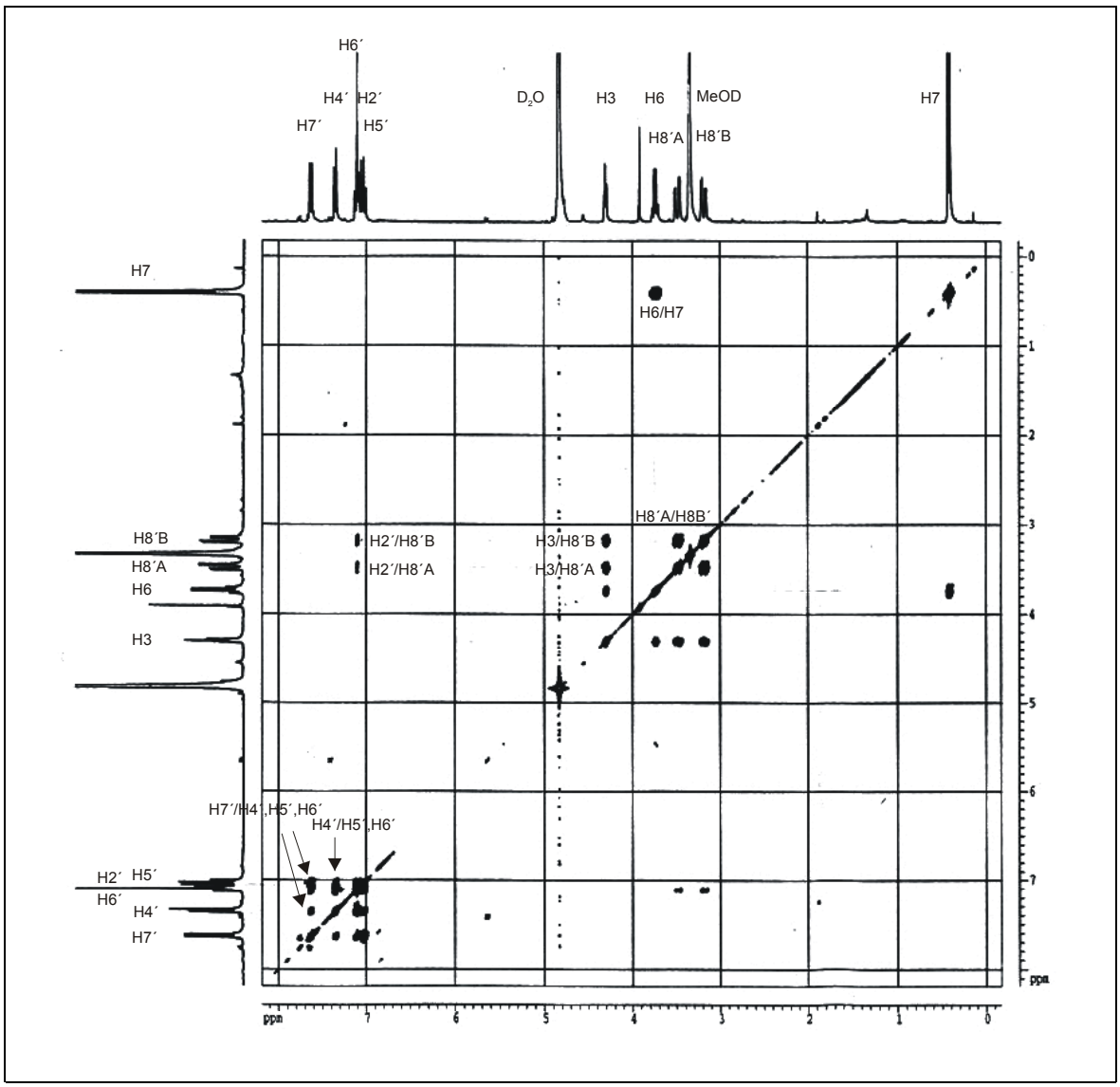



Figure C10.3. COSY spectrum of compound **10** (cyclo-alanyltryptophan)

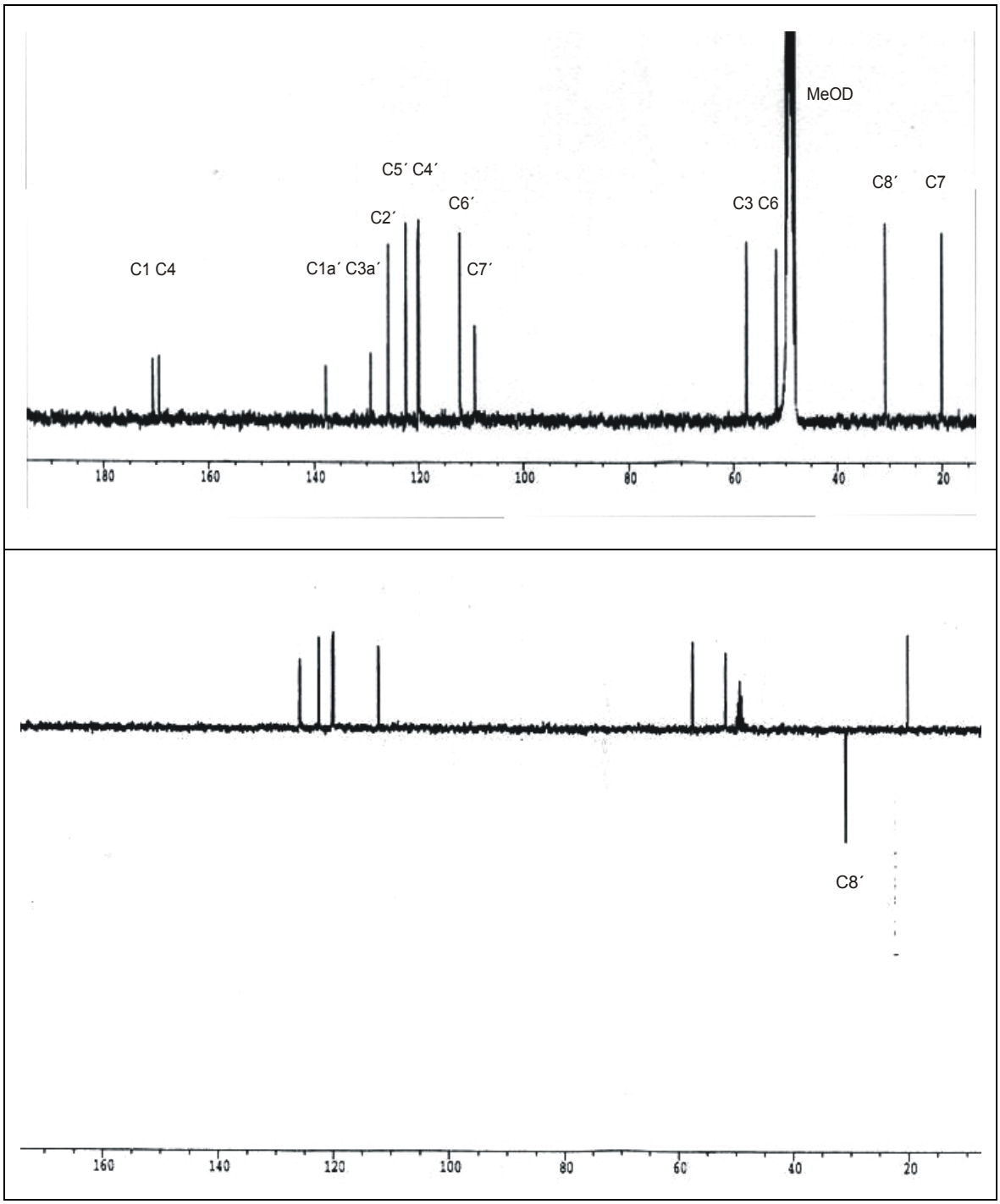



Figure C10.4. $^{13}C$ NMR and DEPT spectra of compound **10** (cyclo-alanyltryptophan)

## 3.2.5. Compound 11 (cyclo-prolylvalyl)

The ESI-MS spectrum of compound **11** presented an ion peak at *m*/*z* 197 $[M+H]^+$ (positive). This ESI-MS spectrum gave molecular weight and molecular formula of 196 g/mol and $C_{10}H_{16}N_2O_2$, respectively.

The $^1H$ NMR spectrum of compound **11** (Table C11.1, Figure C11.2) showed two doublet protons of methyl groups [$\delta$ 0.93 (H11), 1.08 (H12)]. Four downfield proton signals [$\delta$ 4.02 (H3), 4.19 (H6), 3.49 (H9A), 3.54 (H9B)] were also observed indicating that these protons were bound to nitrogen-carrying carbons.

Protons at $\delta$ 4.02 (H3), 2.47 (H10), 0.93 (H11), and 1.08 (H12) were positioned close to each other, as indicated by the correlation in the COSY spectrum, which was also assured by long range correlations in the HMBC spectrum (Figure C11.1, C11.3, and C11.6).

The presence of three nitrogen-binding carbons was visible due to the following signals [$\delta$ 61.24 (C3), 59.75 (C6), 46.57 (C9)] in the $^{13}C$ NMR spectrum. In addition, two carbonyls [$\delta$ 170.75 (C2), 165.26 (C5)], one doublet carbon [$\delta$ 61.24 (C3)], three triplet carbons [$\delta$ 29.92 (C7), 23.65 (C8), 46.57 (C9)], and two quartet carbons [$\delta$ 16.36 (C11), 18.55 (C12)] were encountered in the $^{13}C$ NMR spectrum (Figure C11.4). The DEPT spectrum proved the existence of three methylene carbons (C7, C8, C9) (Table C11.1, Figure C11.5).

All protons directly connected to their corresponding carbons in the HMQC spectrum. The HMBC spectrum confirmed that the side chain (C10, C11, C12) of this bipeptide was attached to position three (C3) of diketopiperazine. This was clearly seen by the presence of two bond couplings between H10 and C3 and three bond couplings between H11, H12 and C3 (Table C11.1, Figure C11.1, C11.5, and C11.6).

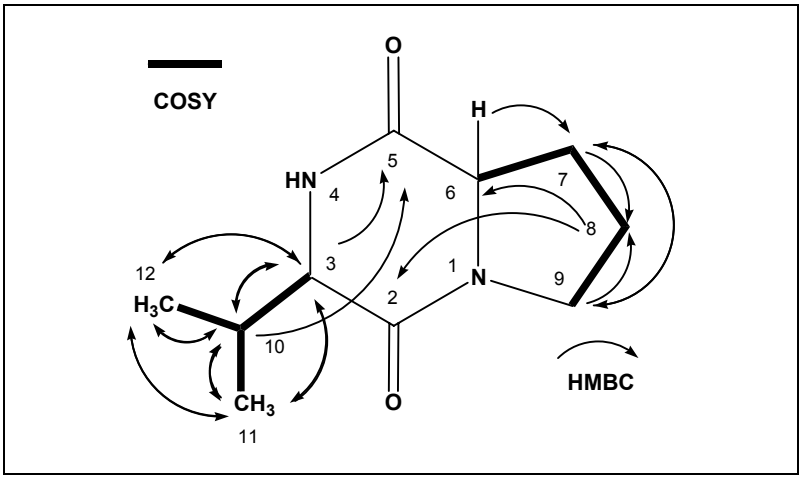
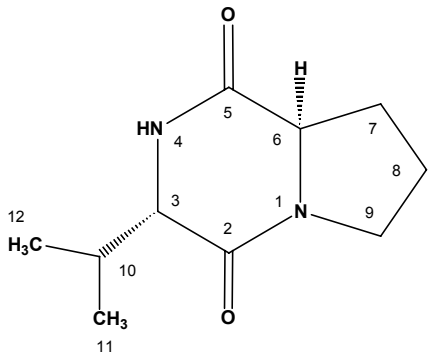



Figure C11.1. COSY and HMBC correlations of compound **11** (cylylo-prolylvalyl)

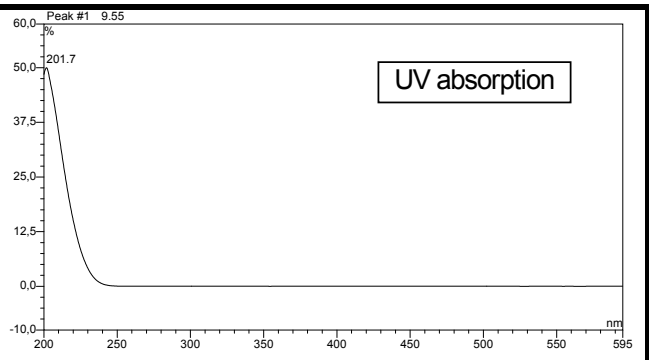
Optical rotation (-102.7°) of compound **11** and optical rotation (-103°) of cyclo(*L*-prolyl-*D*-valyl) (Adamczeski *et al.,* 1995) were almost identical, suggesting that both compounds were the same. Antimicrobial assay of compound **11** did not present any bioactivity.

## Cyclo-prolylvalyl (compound 11)

| | |
|---|---|
| Chemical abstract | : DA:C-316 |
| Characteristic | : white powder |
| Formula | : $C_{10}H_{16}N_2O_2$ |
| Molecular Weight | : 196 g/mol |
| Amount | : 7.9 mg |
| Source | : *Verticillium* cf *cinnabarium* derived from *Ircinia fasciculata* |




| | |
|---|---|
| Retention Time | : 9.63 min |
| $R_f$ | : 0.64 |
| Fluorescence, 254 nm | : + |
| 366 nm | : - |
| Anisaldehyde/$H_2SO_4$ | : - |




| | |
|---|---|
| Optical Rotation $[\alpha]_D^{20}$ | : experiment = -102.7° (c=0.1, MeOH)<br>literature = -103° (MeOH) (Adamczeski *et al*., 1995) |
| ESI-MS (*m*/*z*) | : 197.2 $[M+H]^+$ (positive) |

Table C11.1. NMR data of compound **11** (cyclo-prolylvalyl)

| Position | $\delta$ $^{13}$C (ppm) (in MeOD) | DEPT | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) (in MeOD) | COSY (H → H) | HMQC (H → C) direct | HMBC (H → C) |
|---|---|---|---|---|---|---|
| 1 | | | | | | |
| 2 | 172.57 (s) | | | | | |
| 3 | 61.24 (d) | | 4.02 (d, 2.5) | H10 | C3 | C5, C10, C11, C12 |
| 4 | | | | | | |
| 5 | 167.57 (s) | | | | | |
| 6 | 59.75 (d) | | 4.19 (dd, 6.9, 1.3) | H7A,B; H8A,B | C6 | C7 |
| 7 | 29.92 (t) | 29.92 (t) | | | | |
| 7A | | | 2.31 (ddd, 11.9, 6.9, 1.3) | H8A,B; H7B | C7 | C9 |
| 7B | | | 2.00 (m) | H8A,B | C7 | C6, C8 |
| 8 | 23.65 (t) | 23.65 (t) | | | | |
| 8A | | | 1.92 (m) | H7A,B | C8 | C2, C6 |
| 8B | | | 1.92 (m) | H7A,B | C8 | C2, C6 |
| 9 | 46.57 (t) | 46.57 (t) | | | | |
| 9A | | | 3.49 (m) | H8A,B; H7B | C9 | C7, C8 |
| 9B | | | 3.54 (m) | H8A,B; H7B | C8 | C7, C8 |
| 10 | 29.60 (d) | | 2.47 (q, 6.9, 6.9, 2.5) | H11, H12 | C10 | C3, C5, C11, C12 |
| 11 | 16.36 (q) | | 0.93 (d, 6.9) | H12, H10 | C11 | C3, C10, C12 |
| 12 | 18.55 (q) | | 1.08 (d, 6.9) | H11, H10 | C12 | C3, C10, C11 |

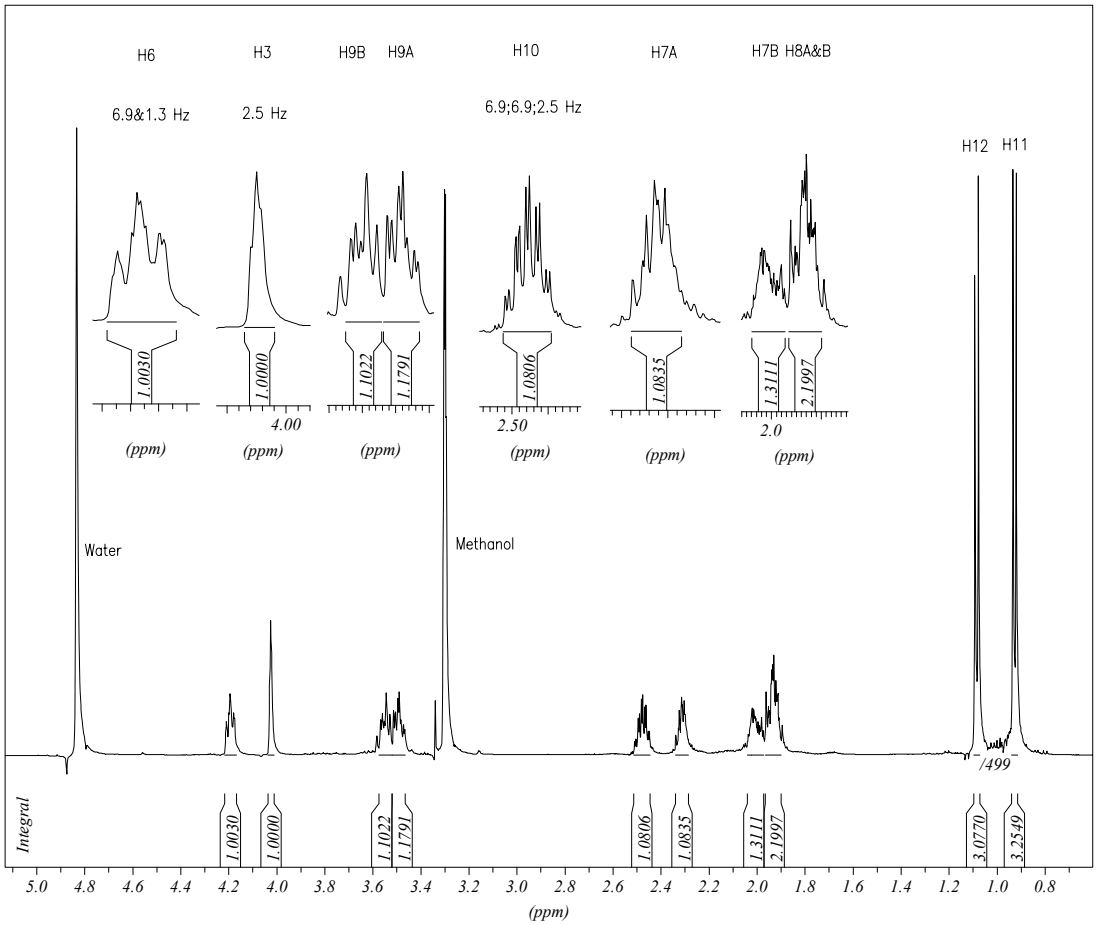



Figure C11.2. $^{1}$H NMR spectrum of compound **11** (cyclo-prolylvalyl)

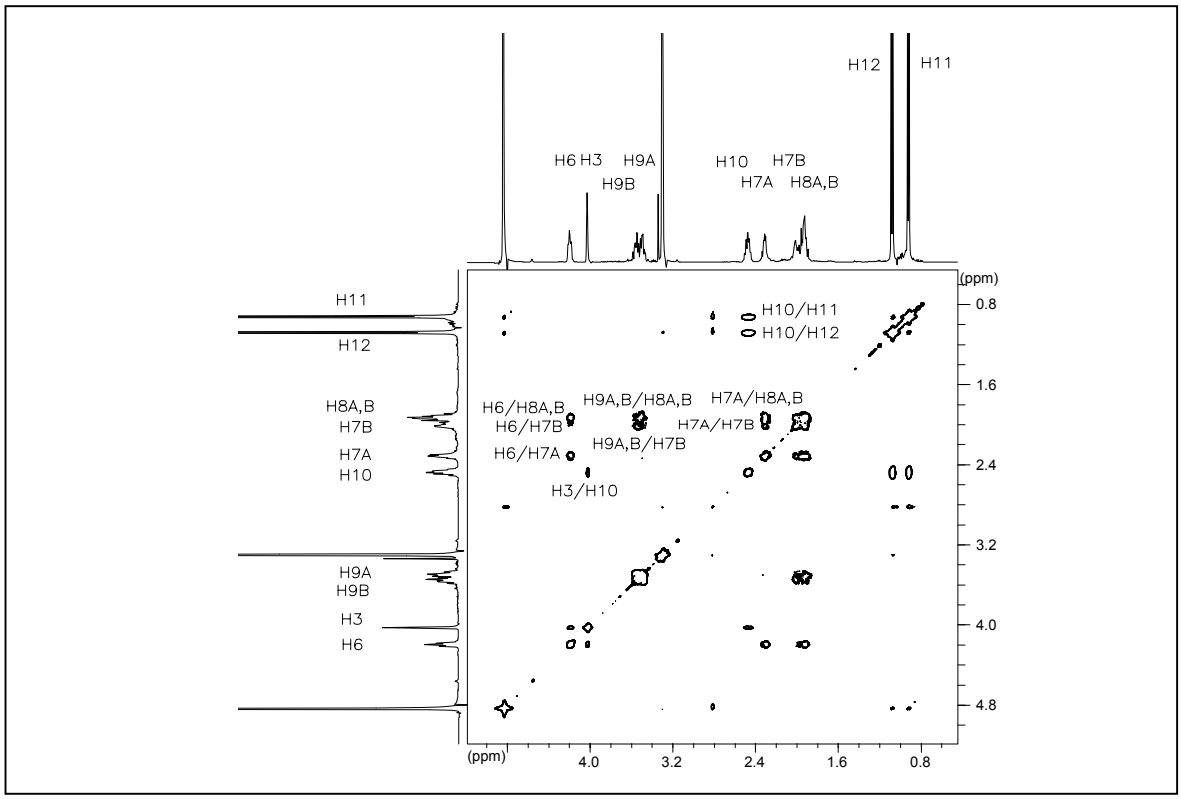



Figure C11.3. COSY spectrum of compound **11** (cyclo-prolylvalyl)

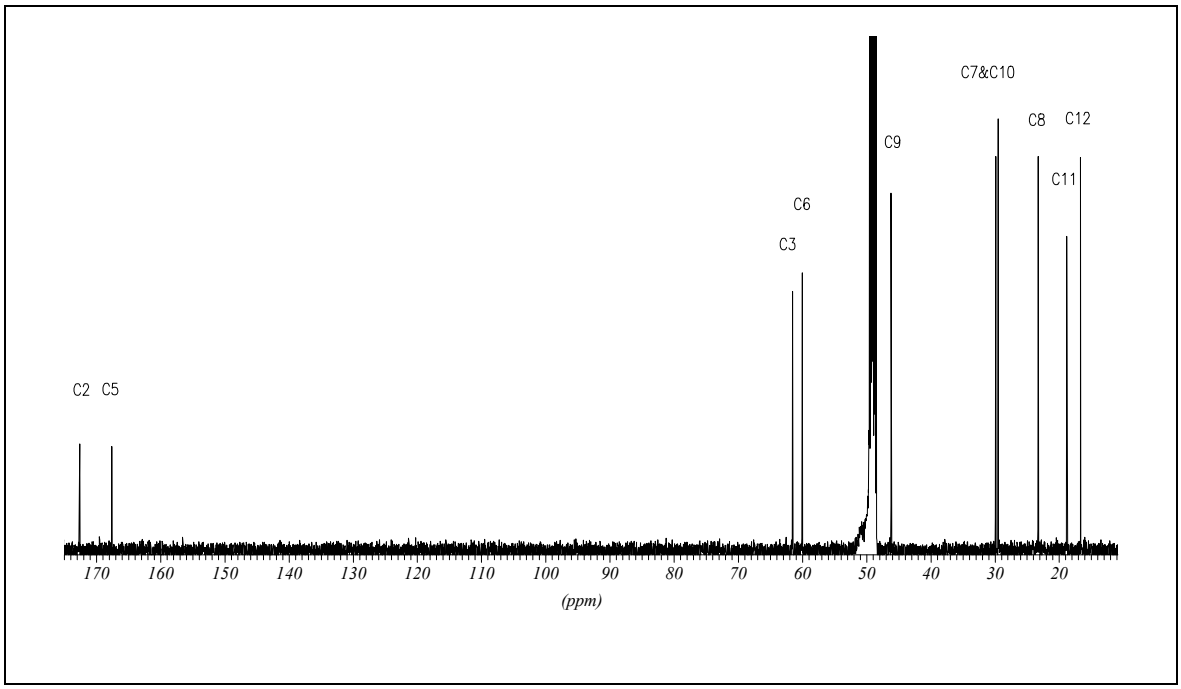



Figure C11.4. $^{13}$C NMR spectrum of compound **11** (cyclo-prolylvalyl)

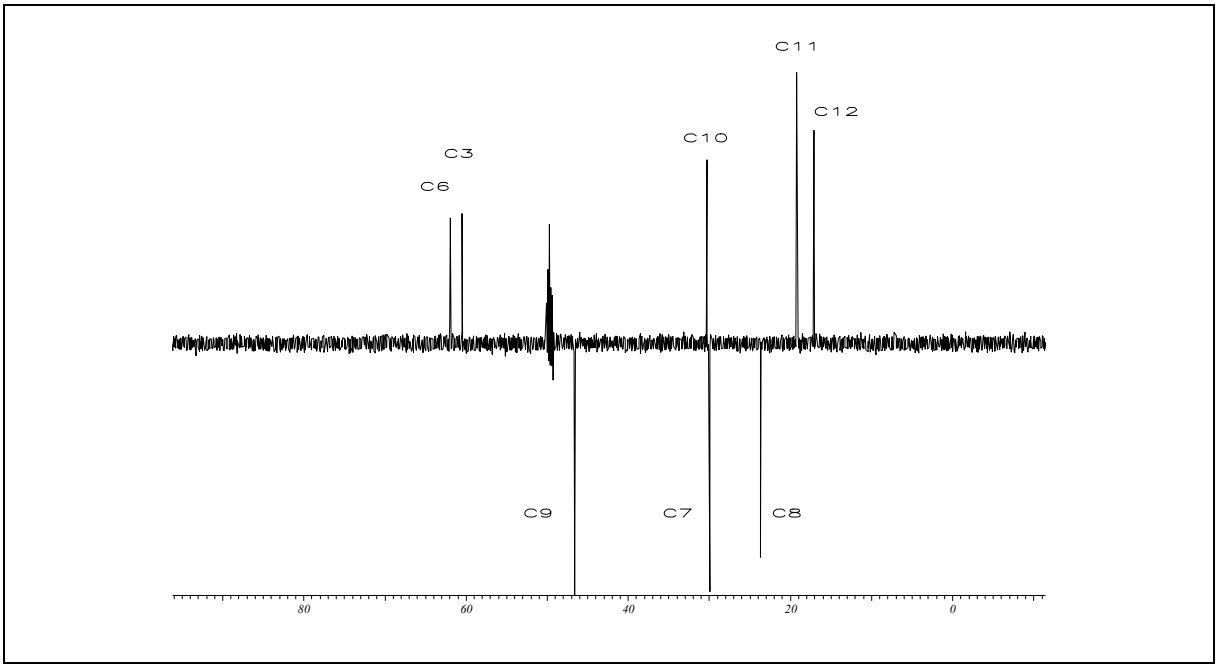



Figure C11.5. DEPT spectrum of compound **11** (cyclo-prolylvalyl)

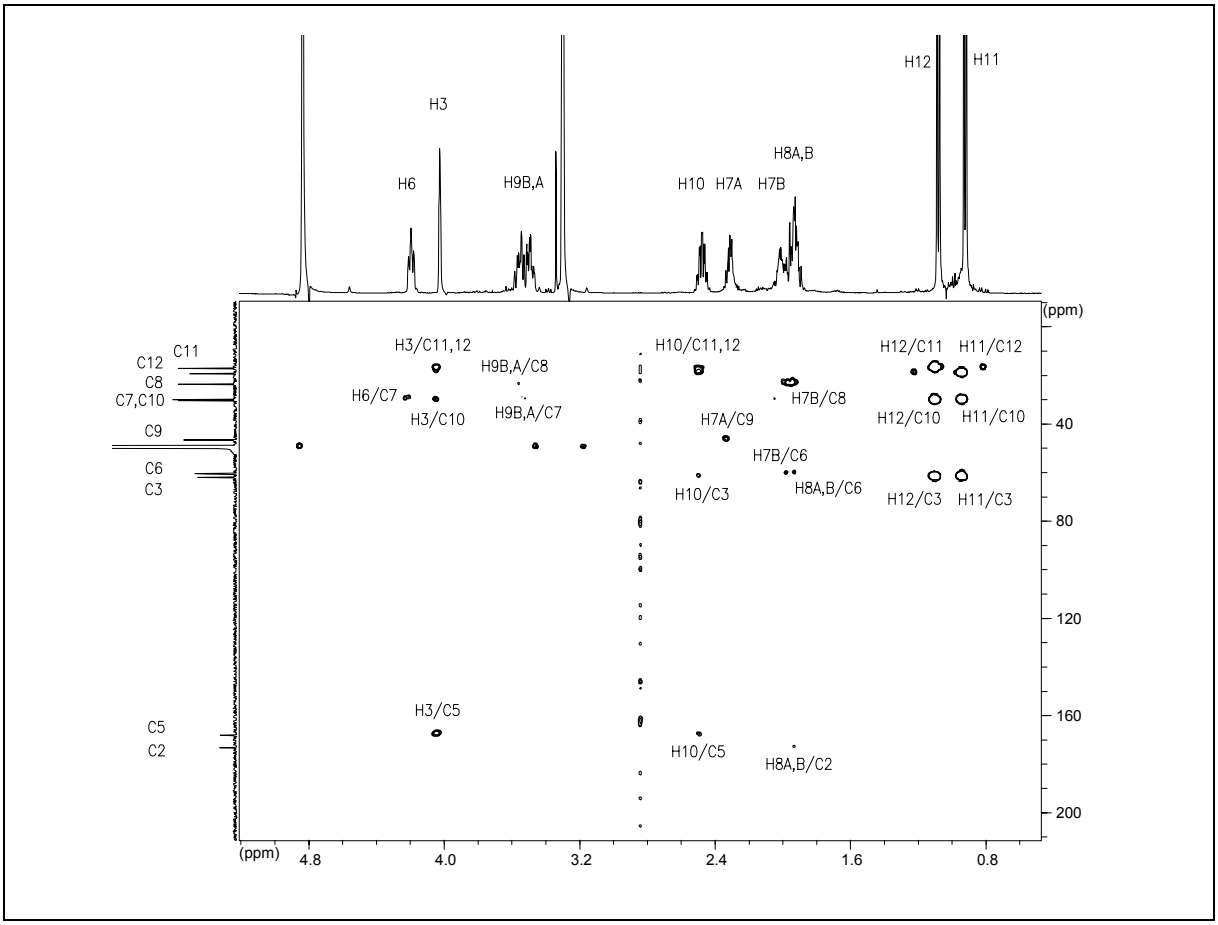



Figure C11.6. HMBC spectrum of compound **11** (cyclo-prolylvalyl)

## 3.2.6. Compound 12 (cyclo-leucylprolyl)

The ESI-MS spectrum of compound **12** exhibited an ion peak at *m*/*z* 211.2 $[M+H]^+$, implying a molecular weight of 210 g/mol and a molecular formula of $C_{11}H_{18}N_2O_2$.

The presence of two quarternary methyl groups [$\delta$ 0.95 (H12 and H13)] was evident in the $^1$H NMR spectrum. Furthermore, three protons at downfield [$\delta$ 4.11 (H3), 4.24 (H6), 3.50 (H9)] and their downfield respective carbons [$\delta$ 54.36 (C3), 59.99 (C6), 46.15 (C9)], characteristic of nitrogen-carrying carbons, were readily observed (Table C12.1, Figure C12.1).

The COSY and HMBC spectra clearly pinpointed correlations between the methyl protons, proving geminal position of these two methyls at C11. Side chain of this compound was connected to its parent molecule at position C3. This connection was explained by the HMBC spectrum in which there were three bond couplings between H11 and C3 as well as two bond couplings between H3 and C10 (Table C12.1, Figure C12.1, C12.3 and C12.7).

The diketopiperazine skeleton was apparent from the characteristic two singlet amides at low field in the $^{13}$C NMR spectrum [$\delta$ 172.79 (C2), 168.91 (C5)] and the nitrogen-carbon residues at $\delta$ 54.36 (C3), 59.99 (C6), 46.15 (C9) (Figure C12.4). Four methylene carbons at $\delta$ 28.78 (C7), 23.36 (C8), 46.57 (C9), and 39.12 (C10) were assured by the DEPT spectrum (Figure C12.5).

In addition, three methine carbons at $\delta$ 54.36 (C3), 59.99 (C6), 25.49 (C11), two quaternary carbons at $\delta$ 21.91 (C12), and 22.99 (C13) were also encountered in the $^{13}$C NMR spectrum (Table C12.1). Direct correlations of protons to their respective carbons were revealed in the HMQC spectrum (Figure C12.6).

Table C12.1. NMR data of compound **12** (cyclo-leucylprolyl)

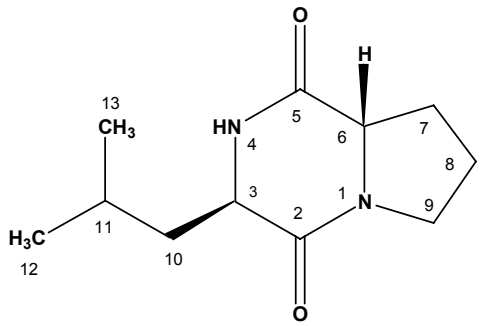
| Position | $\delta$ $^{13}$C (ppm) (in MeOD) | DEPT | $\delta$ $^1$H (ppm), multiplicity (*J* in Hz) (in MeOD) | COSY (H → H) | HMQC (H → C) direct | HMBC (H → C) |
|---|---|---|---|---|---|---|
| 1 | | | | | | |
| 2 | 172.79 (s) | | | | | |
| 3 | 54.36 (d) | | 4.11 (t, 5.0) | H10, H11 | C3 | C10 |
| 4 | | | | | | |
| 5 | 168.91 (s) | | | | | |
| 6 | 59.99 (d) | | 4.24 (dd, 6.9, 1.9) | H7, H8 | C6 | |
| 7 | 28.78 (t) | 28.78 (t) | 2.28 (m) | H8 | | C8, C9 |
| 8 | 23.36 (t) | 23.36 (t) | 2.00 (m) | H7, H9 | | C5, C7 |
| 9 | 46.15 (t) | 46.57 (t) | 3.50 (m) | H8 | C9 | C7, C8 |
| 10 | 39.12 (t) | 39.12 (t) | 1.90 (m) | H11, H12, H13 | C10 | C3, C5, C11, C12, C13 |
| 11 | 25.49 (d) | | 1.50 (m) | H10, H3 | | C3, C12, C13 |
| 12 | 21.91 (q) | | 0.95 (d, 6.3) | H13, H11, H10 | C12 | C10, C11, C13 |
| 13 | 22.99 (q) | | 0.95 (d, 6.3) | H12, H11, H10 | C13 | C10, C11, C12 |

Comparison of proton chemical shifts of compound **12** and of the one isolated from the sponge, *Tedania ignis*, reported by Schmitz *et al*. (1983) showed that both spectra were very similar, suggesting that the two compounds are identical.

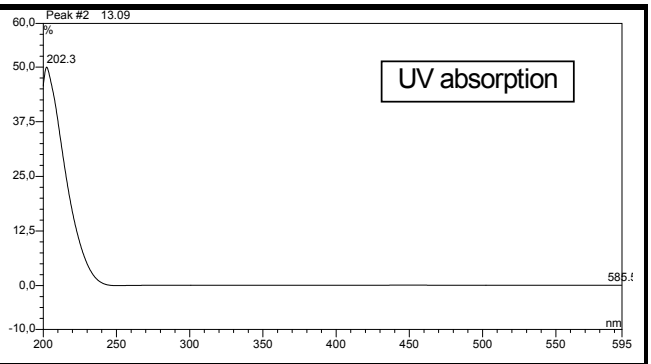
Comparison of optical rotation of compound **12** (–60$^o$) and the optical rotation of cyclo(*D*-leucly-*L*-prolyl) (-78$^o$) (Table C12.2) ended with the conclusion that both compounds had the same stereochemistry. Antimicrobial assay of compound **12** demonstrated no bioactivity.

## Cyclo-leucylprolyl (compound 12)

| | |
|---|---|
| Cemical Abstract | : 123:222507 |
| Characteristic | : white powder |
| Formula | : $C_{11}H_{18}N_2O_2$ |
| Molecular Weight | : 210 g/mol |
| Amount | : 4.8 mg |
| Source | : *Verticillium cf cinnabarium* derived from *Ircinia fasciculata* |




| | |
|---|---|
| Retention Time | : 12.97 min |
| $R_f$ | : 0.65 |
| Fluorescence, 254 nm | : + |
| 366 nm | : - |
| Anisaldehyde/$H_2SO_4$ | : - |




| | |
|---|---|
| Optical Rotation $[\alpha]_D^{20}$ | : experiment = -60° (c=0.1, EtOH)<br>literature = -78° (EtOH) |
| ESI-MS (*m/z*) | : 211.2 $[M+H]^+$ (positive) |

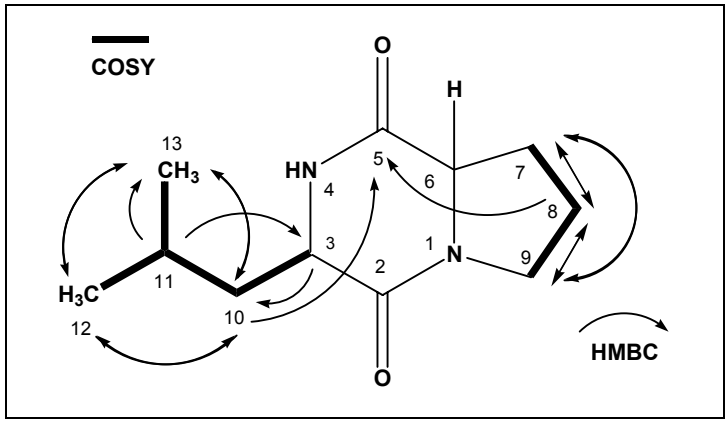



Figure C12.1. COSY and HMBC correlations of compound **12** (cyclo-leucylprolyl)

Table C12.2. Optical rotations for diketopiperazine of the formula cyclo(*L*-Pro-*D*/*L*-Xxx) (Adamczeski *et al.,* 1995)

| Xxx | $[\alpha]_D$ ($^o$) |
|---|---|
| *L*-Leu | -156 [a] |
| | -143 [EtOH] |
| | -142 [EtOH] |
| | -91.3 [$H_2O$][b] |
| *D*-Leu | -91.2 [a] |
| | -78 [EtOH] |
| *L*-Val | -185 [a] |
| | -180 [EtOH] |
| | -161 [EtOH] |
| | -134 [EtOH] |
| *D*-Val | -120 [EtOH] |
| | -103 [a] |

[a] = 0.01 N NaOH in aqueous MeOH (1:1, v/v)
[b] This value is inconsistent with other reported data

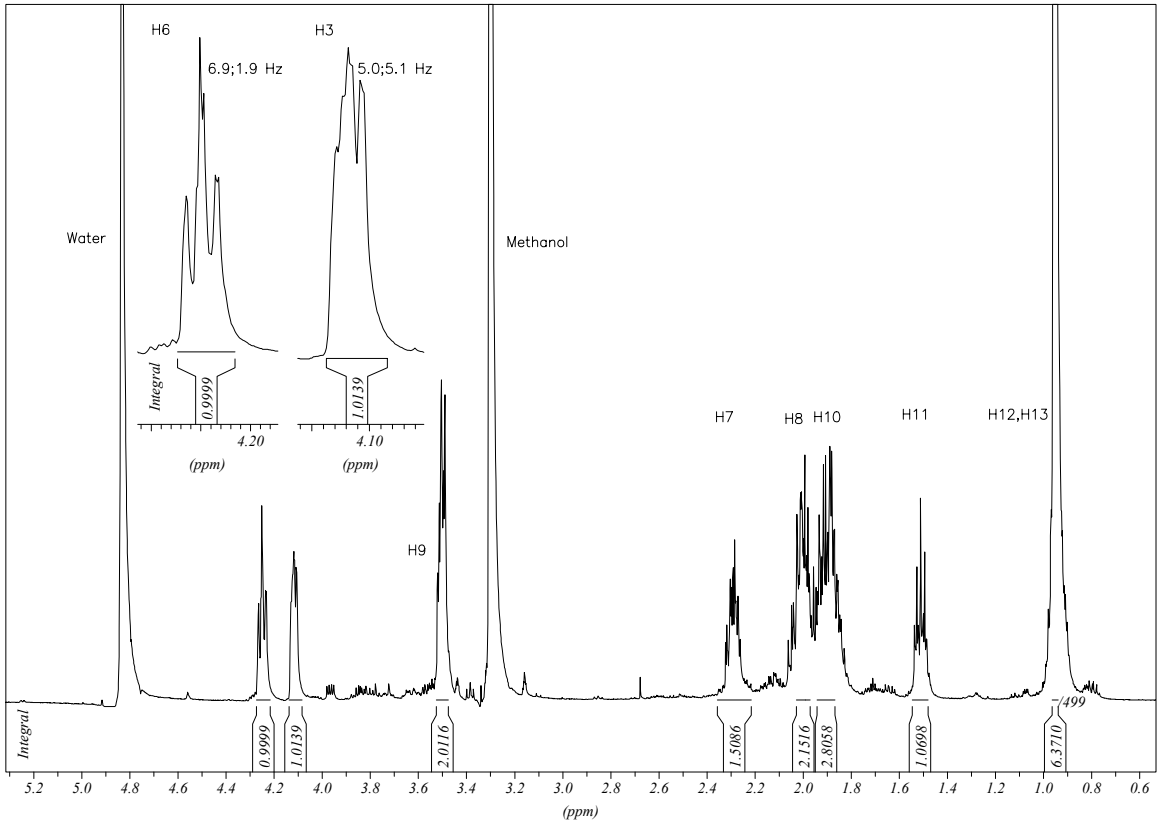



Figure C12.2. $^{1}$H NMR spectrum of compound **12** (cyclo-leucylprolyl)

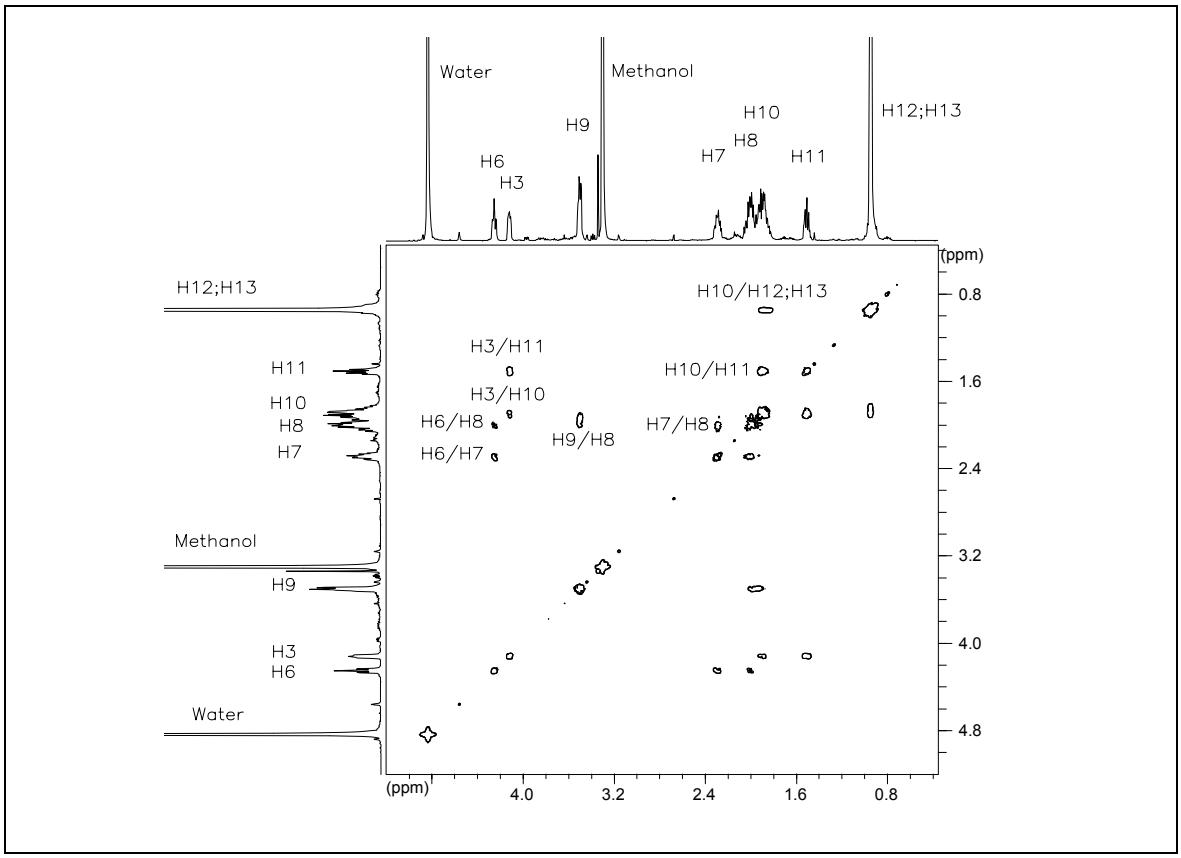



Figure C12.3. COSY spectrum of compound **12** (cyclo-leucylprolyl)

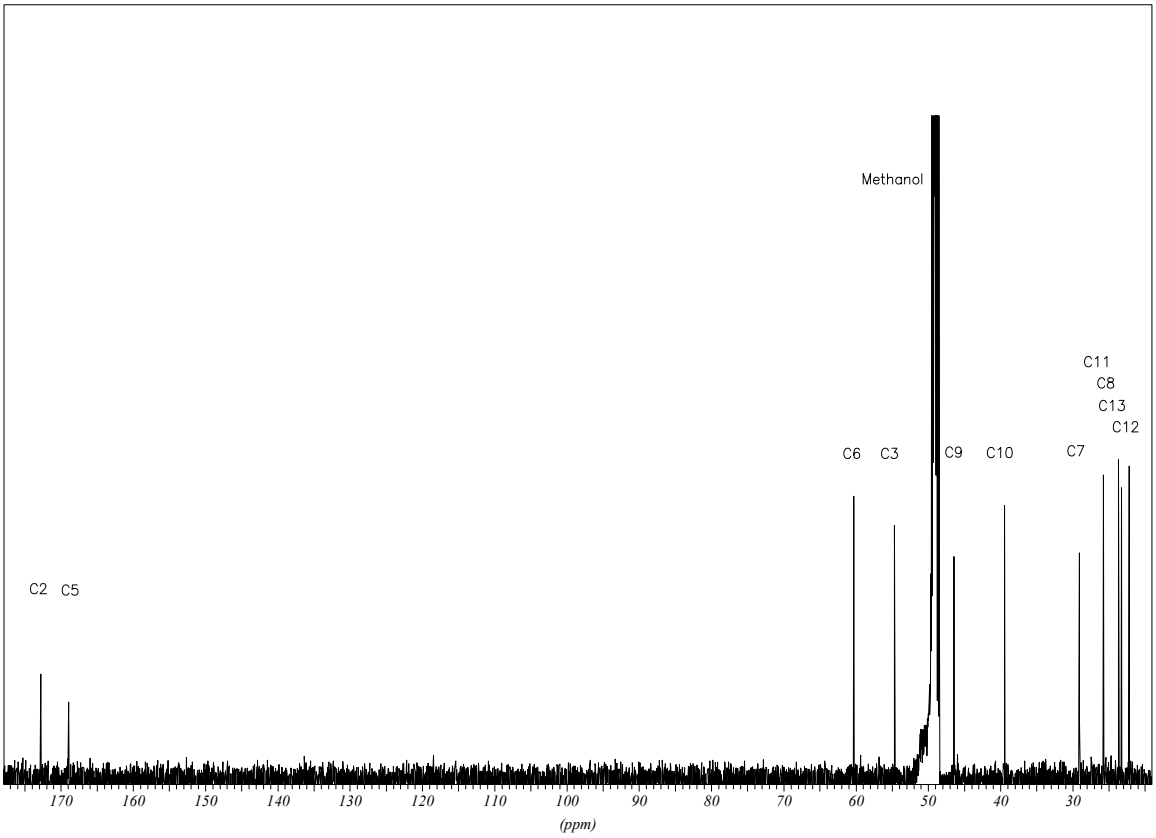



Figure C12.4. $^{13}C$ NMR spectrum of compound **12** (cyclo-leucylprolyl)

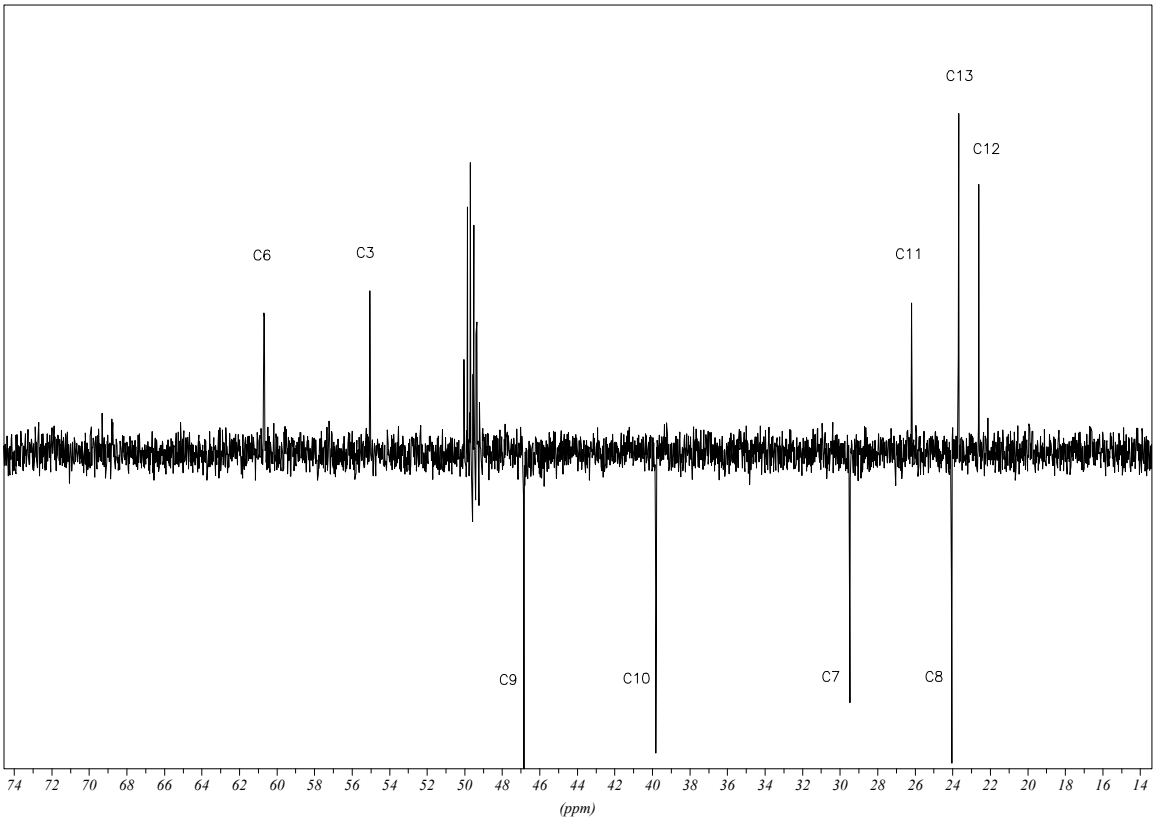



Figure C12.5. DEPT spectrum of compound **12** (cyclo-leucylprolyl)

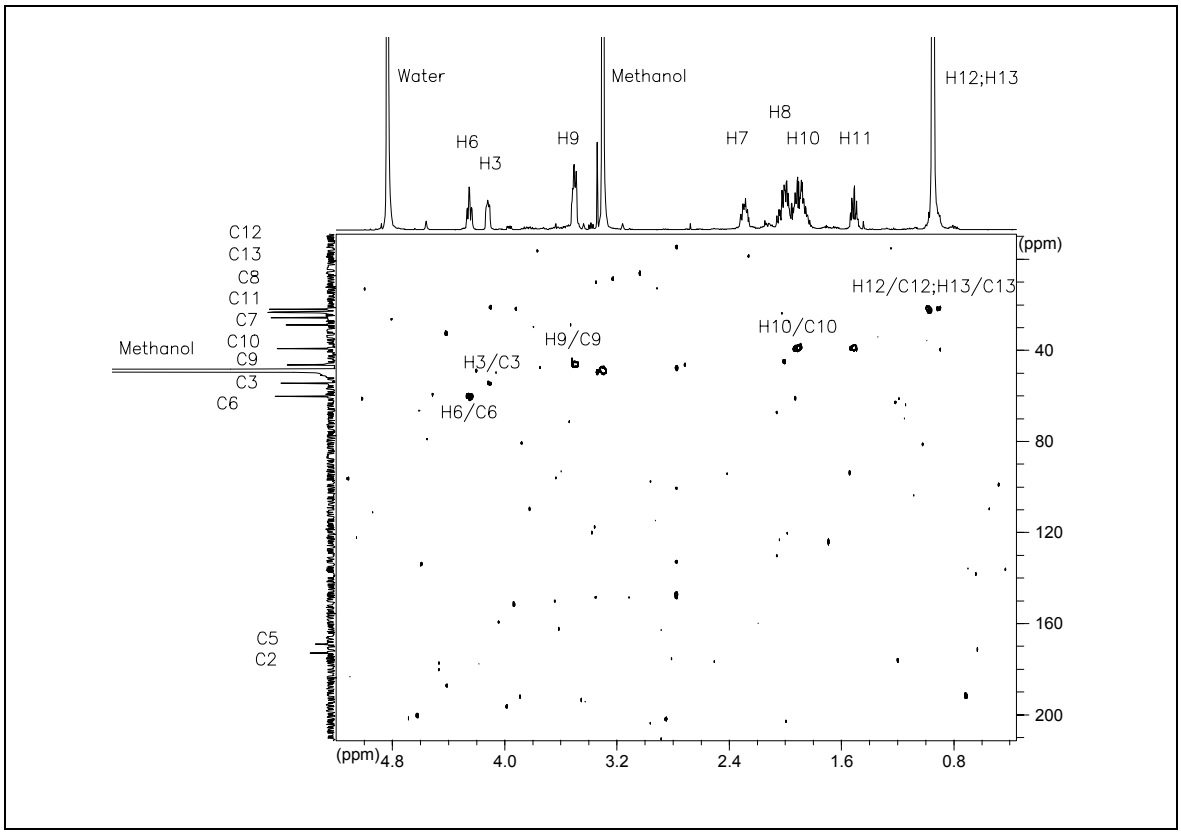



Figure C12.6. HMQC spectrum of compound **12** (cyclo-leucylprolyl)

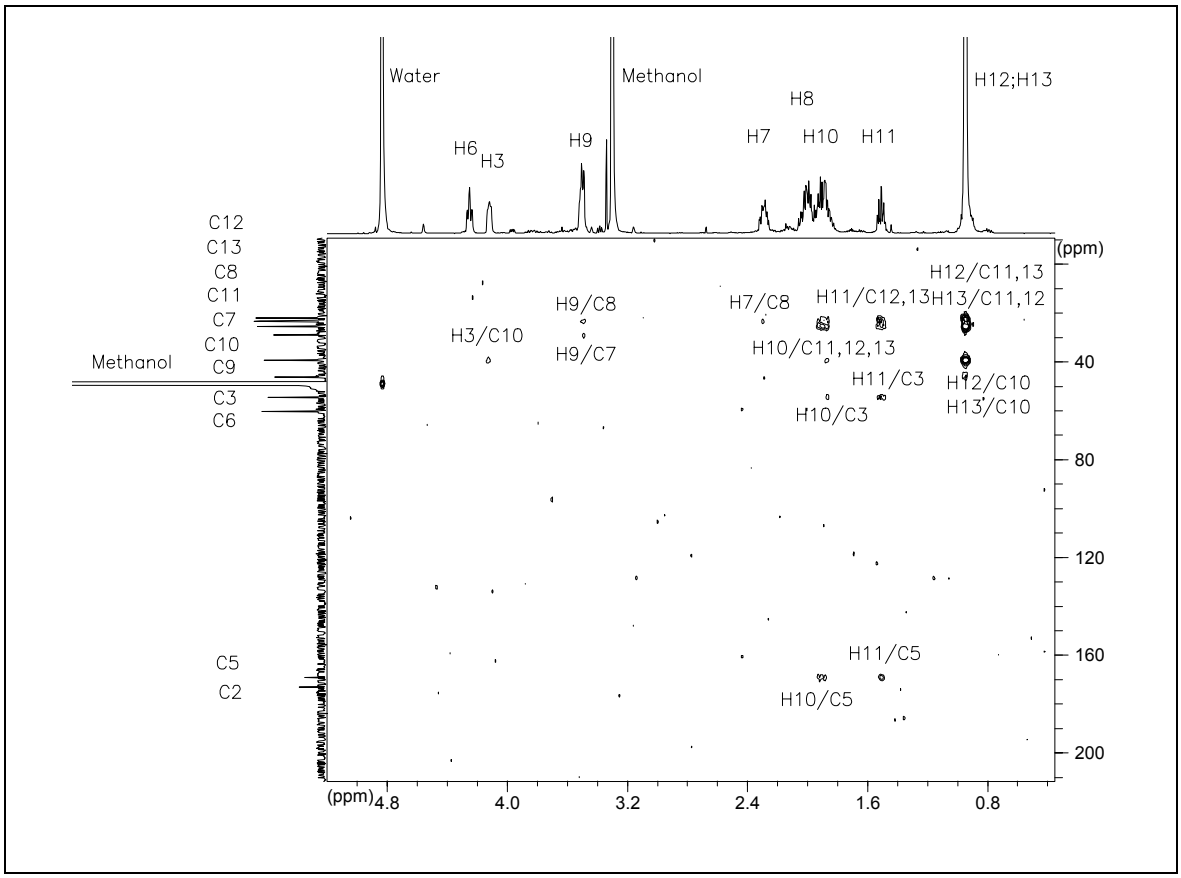



Figure C12.7. HMBC spectrum of compound **12** (cyclo-leucylprolyl)

## 3.2.7. Compound 13 (verticillin B)

Molecular weight and molecular formula of this compound were 712 g/mol and $C_{30}H_{28}N_6O_7S_4$, respectively. It was suggested by the ESI-MS spectrum prevailing ion peaks at *m*/*z* 713.1 $[M+H]^+$ (positive) and 711.6 $[M-H]^-$ (negative).

One typical singlet methyl proton, $\delta$ 3.00 (H13, H13‘), attached to nitrogen was observed in the $^1$H NMR spectrum (Table C13.1, Figure C13.1 and C13.2). In addition, another singlet proton at downfield, $\delta$ 4.55 (H11, H11‘), bound to oxygen-carrying carbon, was also encountered.

Double doublet multiplicity of H7 with coupling constant of 7.6 Hz (ortho coupling) and 2.5 Hz (meta coupling), triplet multiplicity of H8 and H9 with coupling constant of 7.6 Hz (ortho coupling), and doublet multiplicity of H10 with coupling constant of 7.6 Hz, along with COSY correlations of these protons (H7, H8, H9, and H10), suggested the presence of an ABCD spin system in the aromatic ring (Table C13.1, Figure C13.1).

The integration of the $^1$H NMR spectrum revealed only half of the actual proton number, implying a dimeric structure. However, since there were only half (1,5) methyl singlet protons [$\delta$ 1.87 (H14’)] and one doublet methylene ($CH_2$) proton [$\delta$ 5.65 (H14)], it was concluded that this compound had a non-symmetrical dimeric structure (Table C13.1, Figure C13.1 and C13.2).

Table C13.1. NMR data of compound **13** (verticillin B)

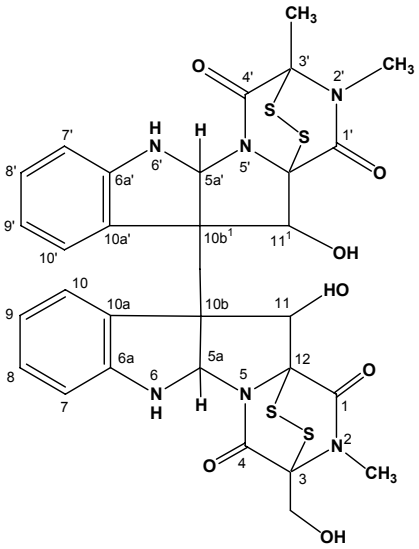
| Position | $\delta$ $^1$H (ppm), multiplicity (*J* in Hz) (Saito *et al*., 1988 in DMSO) | $\delta$ $^1$H (ppm), multiplicity (*J* in Hz) (in MeOD) | COSY (H→ H) |
|---|---|---|---|
| 5a | 5.48 (d, 4.1) | 5.07 (d, 1.9) | |
| 6 | 6.49 (d, 4.1) (NH) | | |
| 7 | 6.54 - 7.71 | 6.73 (dd, 7.6, 2.5) | H9 |
| 8 | 6.54 - 7.71 | 7.81 (t, 7.6) | H7, H9 |
| 9 | 6.54 - 7.71 | 7.08 (t, 7.6) | H7, H10 |
| 10 | 6.54 - 7.71 | 6.58 (d, 7.6) | H9, H7 |
| 11 | 4.89 | 4.55 (s) | |
| 12 | | | |
| 13 | 3.01 | 3.00 (s) | |
| 14 | 5.82 | 5.65 (d, 3.2) | |
| 14‘ | | 1.87 (s) (1.5 H) | |

Comparison of $^1$H NMR spectrum of compound **13** with that of verticillin B derived from *Chaetomium* spp. and *Verticillin* sp. isolated by Saito *et al*. (1988) and Minato *et al*. (1973), respectively, proved similar chemical shifts with the difference varying from 0.1 until 0.63 ppm.

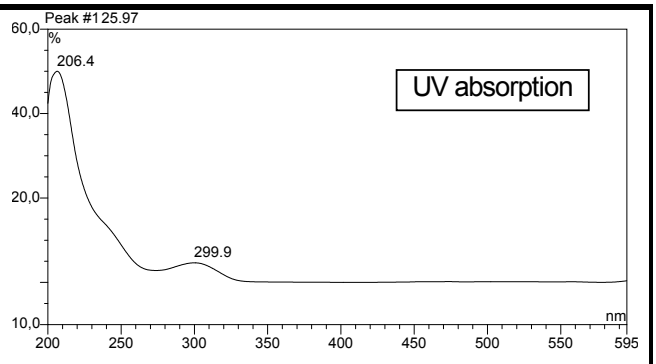
As much as 100 μg compound **13** (verticillin B) caused a 10 mm zone of growth inhibition of *Bacillus subtilis* in the antimicrobial assay.

## Verticillin B (compound 13)

| | |
|---|---|
| CAS registry number | : 52212-86-9 |
| Characteristic | : pale yellow powder |
| Formula | : $C_{30}H_{28}N_6O_7S_4$ |
| Molecular Weight | : 712 g/mol |
| Amount | : 6.0 mg |
| Source | : *Verticillium* cf *cinnabarium* derived from *Ircinia fasciculata* |



| | |
|---|---|
| Retention Time | : 25.86 min |
| $R_f$ | : 0.81 |
| Fluorescence, 254 nm | : + |
| 366 nm | : - |
| Anisaldehyde/$H_2SO_4$ | : - |




| | |
|---|---|
| Optical Rotation $[\alpha]_D^{20}$ | : +115° (c=0.1, DMSO) |
| ESI-MS (m/z) | : 713 $[M+H]^+$(positive), 711.6 $[M-H]^+$(negative) |

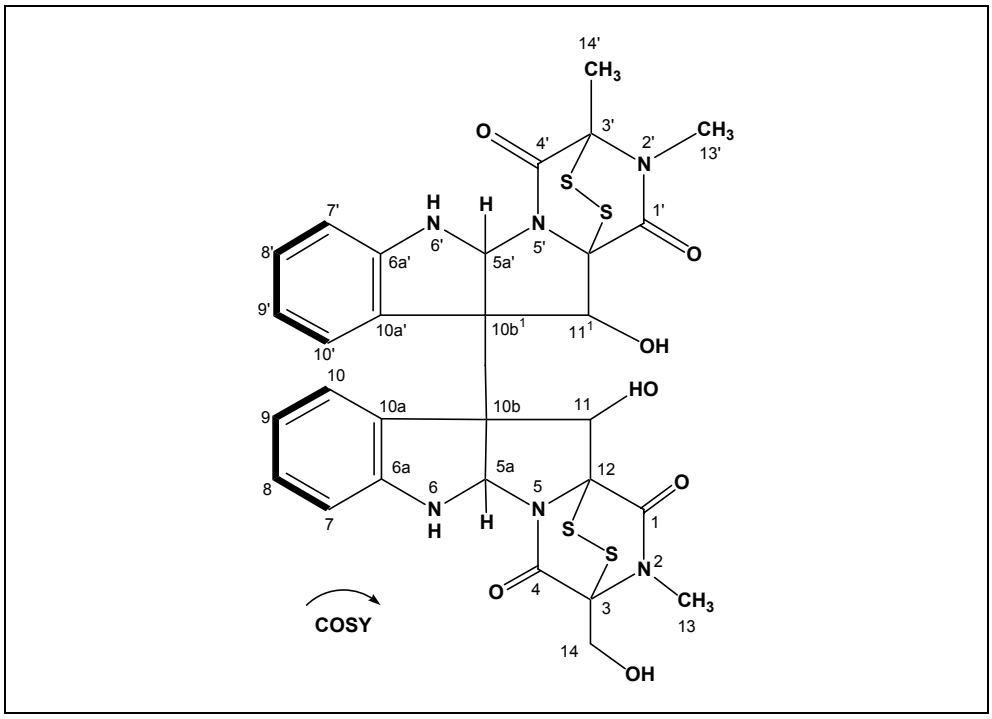



Figure C13.1. COSY correlations of compound **13** (verticillin B)

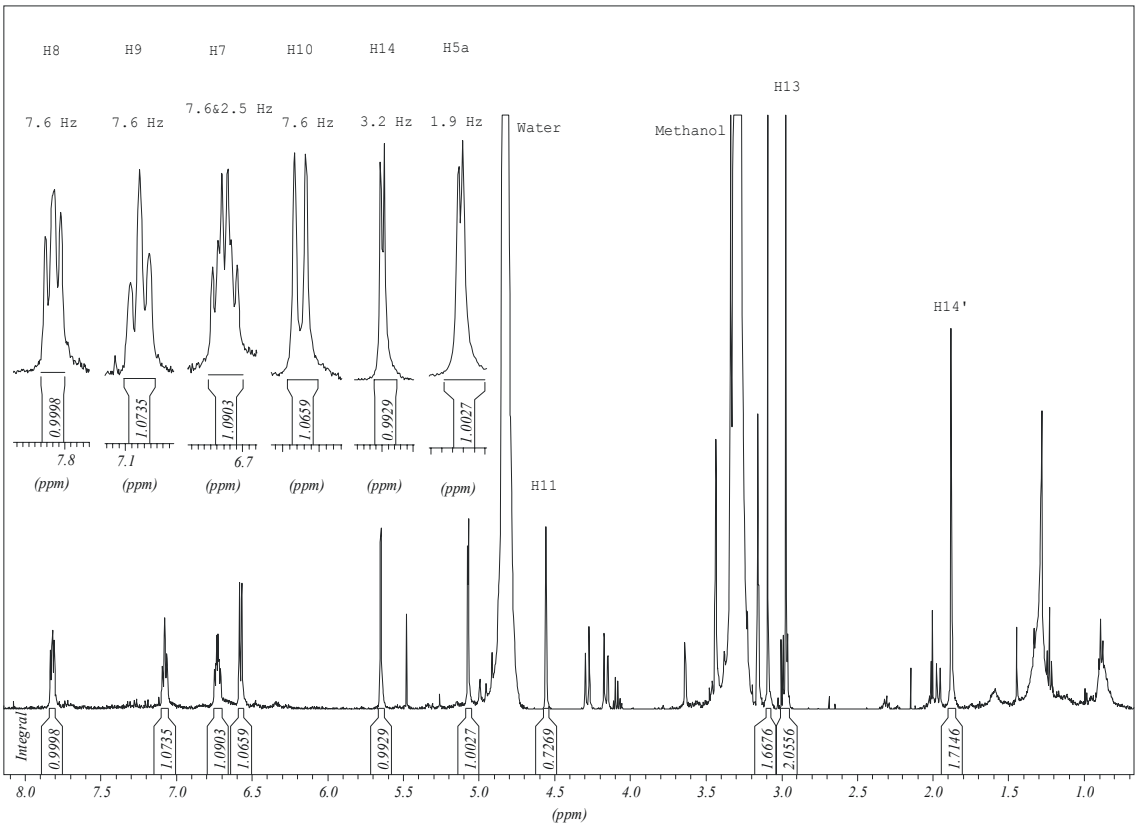



Figure C13.2. $^{1}$H NMR spectrum of compound **13** (verticillin B)

## 3.2.8. Compound 14 (lichesterol)

The EI-MS spectrum of compound **14** gave peaks at *m/z* 410 $[M]^+$, 427 $[M+OH]^+$, 396 $[M-CH_3]^+$ (fragment 1), 378 $[M+H-HO_2]^+$ (fragment 2), 298 [M-side chain]$^+$ (fragment 3), 267 [M-side chain-$CH_3$-OH]$^+$ (fragment 4) (Figure C14.1). Fragment 2 and 4 indicated that the compound had one hydroxyl group. The molecular weight and molecular formula were determined as 410 g/mol and $C_{28}H_{42}O_2$, respectively.

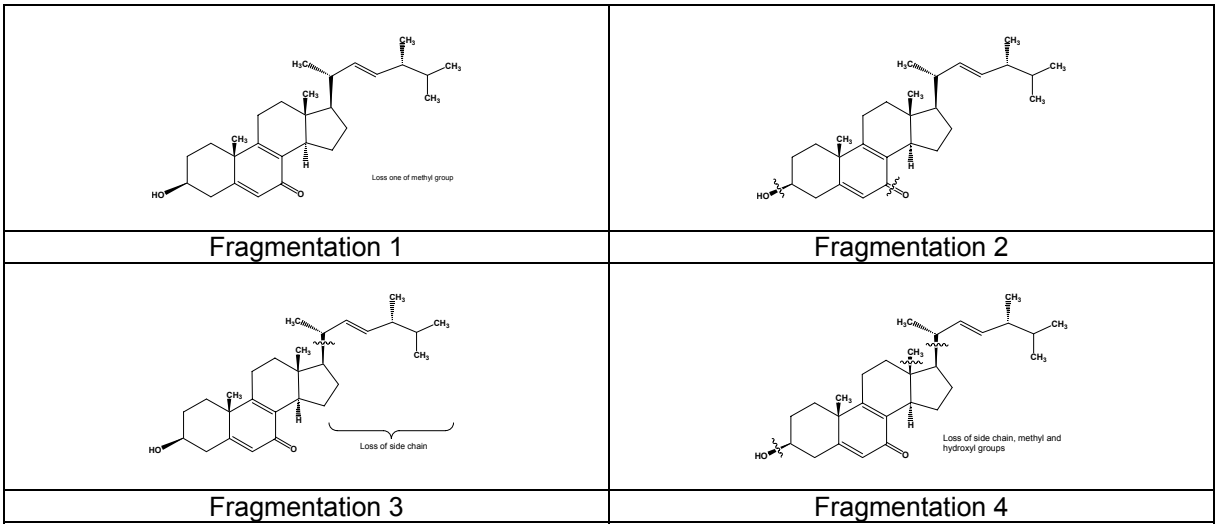



Figure C14.1. Hypothetical fragmentation of compound **14** (lichesterol) in the EI-MS spectrum

The $^1H$ and $^{13}C$ NMR spectra indicated a typical steroidal pattern. The $^1H$ NMR spectrum showed signals due to two tertiary methyl groups [$\delta$ 0.73 (H18), 1.42 (H19)], four methyl groups of sterol side chain [$\delta$ 1.05 (H21), 0.89 (H26), 0.91 (H27), 0.98 (H28)], seven methylene protons [$\delta$ 1.30 (H1), 1.95 (H2), 2.60 (H4), 1.45 (H11), 2.20 (H12), 1.45 (H15), 1.33 (H16)], five methine protons [$\delta$ 2.30 (H14), 1.33 (H17), 2.15 (H20), 1.90 (H24), 1.52 (H25)], one methine proton on carbon bearing hydroxyl group [$\delta$ 3.59 (H3)], and three olefinic protons [$\delta$ 6.05 (H6), 5.25 (H22), 5.28 (H23)]. The presence of 7-ketone function caused the olefinic proton (H6) signal to resonate further downfield at $\delta$ 6.05 (Table C14.1, Figure C14.3).

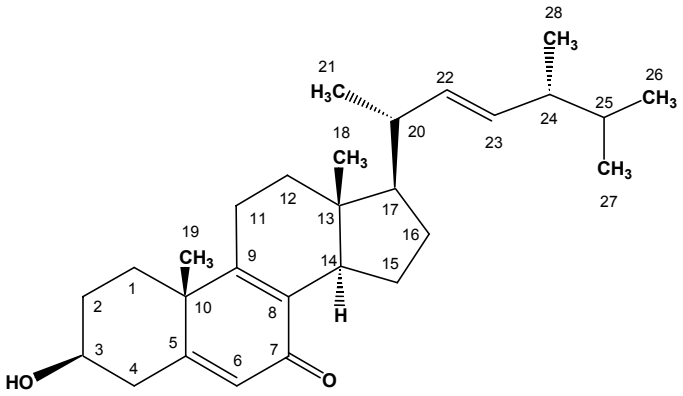
The $^{13}C$ NMR spectrum contained twenty eight signals that included three pairs double bonds [$\delta$ 166.20 (C5), 126.60 (C6), 134.80 (C8), 165.20 (C9), 136.90 (C22), 133.30 (C23)], one carbonyl [$\delta$ 188.00 (C7)], two singlet carbons [$\delta$ 38.70 (C10), 44.00 (C13)], six doublet carbons [$\delta$ 72.70 (C3), 49.80 (C14), 54.70 (C17), 41.70 (C20), 44.30 (C24), 34.40 (C25)], seven triplet carbons [$\delta$ 35.80 (C1), 31.30 (C2), 42.80 (C4), 25.80 (C11), 36.70 (C12), 25.90 (C15), 30.50 (C16)], and six quartet carbons [$\delta$ 12.30 (C18), 24.30 (C19), 21.60 (C21), 20.40 (C26), 20.00 (C27), 18.20 (C28)] (Table C14.1, Figure C14.4).

The existence of seven triplet carbons (methylene carbon) was supported by the DEPT spectrum. The resonances of the corresponding protons were assigned by the HMQC spectrum. The presence of six degrees of unsaturation was straightforwardly explained by the downfield carbon resonances of C5, C6, C8, C9, C22, and C23. Likewise, the carbon signal at $\delta$ 188.00 (C7) clearly denoted the existence of a ketone group in the steroid skeleton (Table C14.1, Figure C14.4).

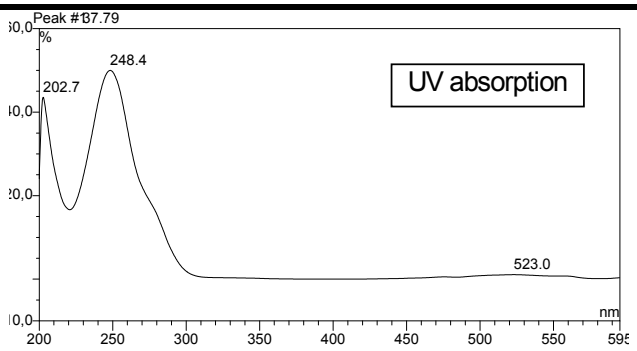
Furthermore, the occurrence of six methyl groups was also evident in the $^{13}C$ NMR spectrum [C18 ($\delta$ 12.30), C19 ($\delta$ 24.30), C21 ($\delta$ 21.60), C26 ($\delta$ 20.40), C27 ($\delta$ 20.00), C28 ($\delta$ 18.20)]. Long range correlation of these methyl groups with their neighbouring carbons was apparently displayed in the HMBC spectrum which also assisted the assignment of these methyls position in the sterol skeleton. Similarly, the position assignment of olefinic proton was also aided by the long range correlation in the HMBC spectrum (Figure C14.2 and C14.5).

## Lichesterol (compound 14)

| | |
|---|---|
| CAS Registry Number | : 2000942-18-3 |
| Characteristic | : white powder |
| Formula | : $C_{28}H_{42}O_2$ |
| Molecular Weight | : 410 g/mol |
| Amount | : 2.3 mg |
| Source | : *Verticillium* cf *cinnabarium* derived from *Ircinia fasciculata* |




| | |
|---|---|
| Retention Time | : 37.70 min |
| $R_f$ | : 0.57 |
| Fluorescence, 254 nm | : + |
| 366 nm | : - |
| Anisaldehyde/$H_2SO_4$ | : + |




| | |
|---|---|
| Optical Rotation $[\alpha]_D^{20}$ | : experiment = -15.9° (c=0.1, $CHCl_3$)<br>literature = -28.3° ($CHCl_3$) (Ishizuka *et al*., 1997) |
| EI-MS (*m/z*, rel. int.) | : 411 $[M+H]^+$ (60.4), 427 $[M+O]^+$ (51.2), 396 $[M+H-CH_3]^+$ (26.1),<br>378 $[M+H-HO_2]^+$ (15.1), 285 [M-side chain]$^+$ (15.7),<br>253 [M-side chain-$CH_3OH$]$^+$ (22.8) |

Table C14.1. NMR data of compound **14** (lichesterol)

| Position | $\delta$ $^{13}$C (ppm) (in MeOD) | DEPT | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) (in MeOD) | COSY (H → H) | HMQC (H → C) direct | HMBC (H → C) |
|---|---|---|---|---|---|---|
| 1 | 35.8 (t) | 35.8 (t) | 1.30 (m) | | C1 | C2, C4, C19 |
| 2 | 31.3 (t) | 31.3 (t) | 1.95 (m) | | C2 | |
| 3 | 72.7 (d) | | 3.59 (m) | | | |
| 4 | 42.8 (t) | 42.8 (t) | 2.60 (m) | H2 | C4 | C2, C3, C5, C6, C9 |
| 5 | 166.2 (s) | | | | | |
| 6 | 126.6 (s) | | 6.05 (d) | H4 | C6 | C4, C8 |
| 7 | 188.0 (s) | | | | | |
| 8 | 134.8 (s) | | | | | |
| 9 | 165.2 (s) | | | | | |
| 10 | 38.7 (s) | | | | | |
| 11 | 25.8 (t) | 25.8 (t) | 1.45 (m) | | C11 | C5, C8, C9, C12, C13, C14 |
| 12 | 36.7 (t) | 36.7 (t) | 2.20 (m) | H9 | C12 | C9, C11, C13, C14 |
| 13 | 44.0 (s) | | | | | |
| 14 | 49.8 (d) | | 2.30 (m) | H15 | C14 | C13 |
| 15 | 25.9 (t) | 25.9 (t) | 1.45 (m) | H14, H16, H17 | C15 | C8, C9, C13, C14 |
| 16 | 30.5 (t) | 30.5 (t) | 1.33 (m) | | C16 | C18, C22 |
| 17 | 54.7 (d) | | 1.33 (m) | | C17 | C16, C18, C22 |
| 18 | 12.3 (q) | | 0.73 (s) | | C18 | C12, C13, C14, C17 |
| 19 | 24.3 (q) | | 1.42 (s) | | C19 | C1, C2, C5, C8, C9, C10 |
| 20 | 41.7 (d) | | 2.15 (m) | H16, H17, H22, | C20 | C13, C14, C22, C23 |
| 21 | 21.6 (q) | | 1.12 (d, 6.6) | H20 | C21 | C17, C20, C22 |
| 22 | 136.9 (d) | | 5.25 (m) | H20 | C22 | C20, C24 |
| 23 | 133.3 (d) | | 5.28 (m) | H20 | C23 | C20, C24 |
| 24 | 44.3 (d) | | 1.90 (m) | H23, H25, H28 | C24 | C22, C23, C25, C26, C27, C28 |
| 25 | 34.4 (d) | | 1.52 (m) | H24, H26, H27 | C25 | C23, C24, C26, C27, C28 |
| 26 | 20.4 (q) | | 0.89 (d, 6.7) | H25, H27 | C26 | C24, C25, C27, C28 |
| 27 | 20.0 (q) | | 0.91 (d, 6.7) | H25, H26 | C27 | C24, C25, C26, C28 |
| 28 | 18.2 (q) | | 0.98 (d, 6.8) | H24 | C28 | C23, C24, C25, C26, C27 |

The side chain of the sterol was connected to the core molecule at position C17. This was confirmed by the COSY spectrum through the existence of correlation between H20 and three neighbouring protons (H17, H16, H22). The presence of correlation in the HMBC spectrum between H20 and a number of neighbouring carbons (C13, C24, C22, C23) as well as correlation between H17 and several carbons (C22, C18, C16) located nearby also confirmed this side chain connection to the sterol main skeleton. Another proof of side chain correlation was deducted from the three bond couplings of H21 toward C17 (Figure C14.2 and C14.5).

Comparison of the $^{1}$H NMR spectrum of compound **14** with that of lichesterol derived from *Grifolia frondosa*, isolated by Ishizuka *et al*. (1997) showed similarity of chemical shifts with differences varying from 0.04 until 0.08 ppm. This led to the conclusion that both compounds are identical.

The optical rotation of lichesterol and compound **14** were -28.3$^{o}$ and -15.9$^{o}$, respectively. This optical rotation, thus, supported the conclusion that both compounds had the same stereochemistry. Antimicrobial assay of compound **14** presented no bioactivity.

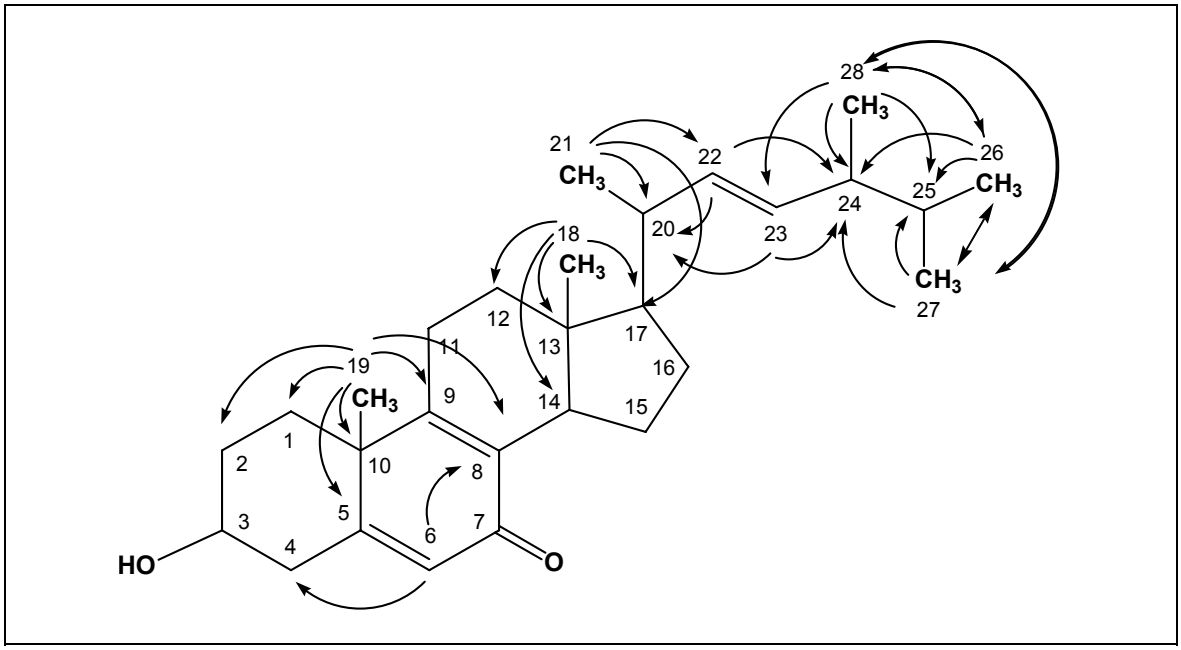



Figure C14.2. Methyl and olefinic proton HMBC correlations of compound **14** (lichesterol)

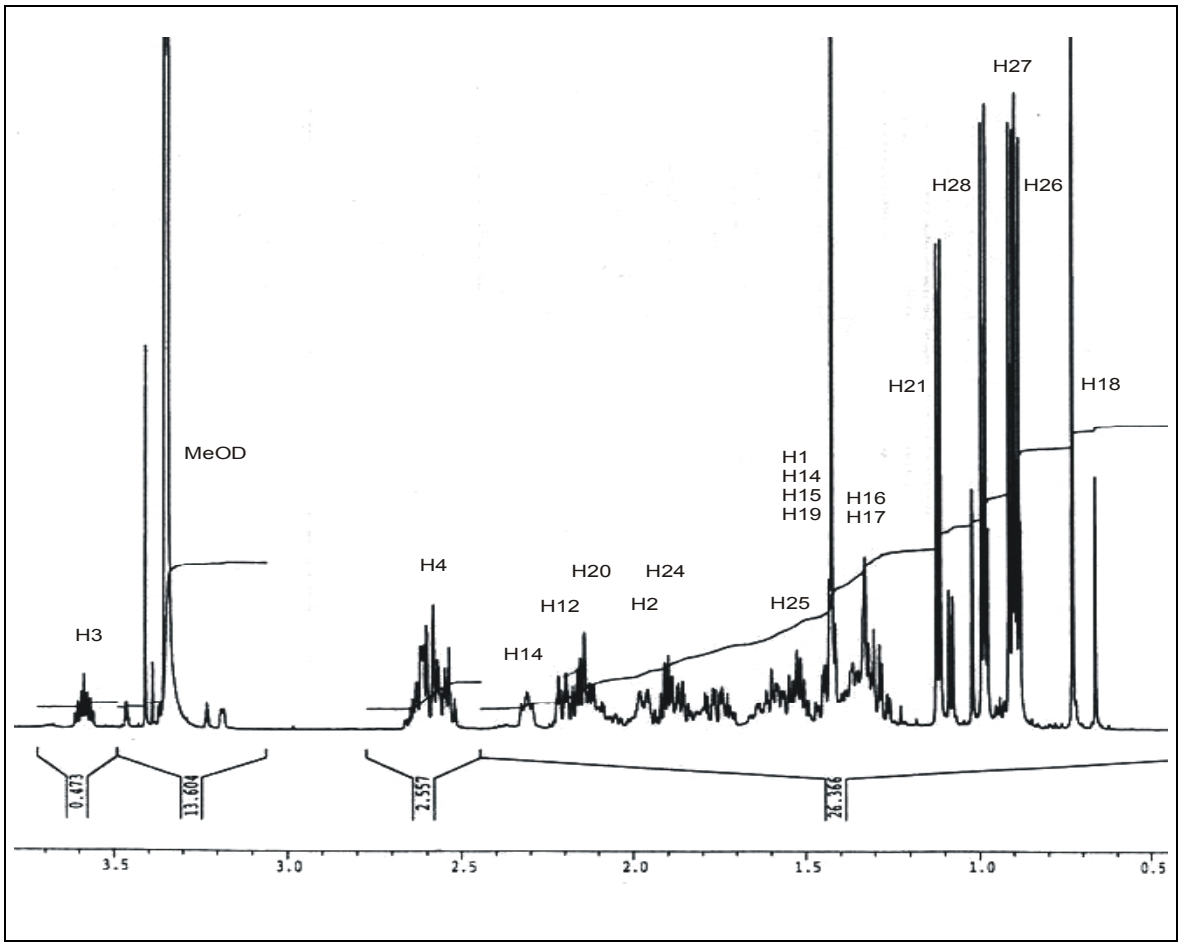



Figure C14.3. $^{1}$H NMR spectrum of compound **14** (lichesterol)

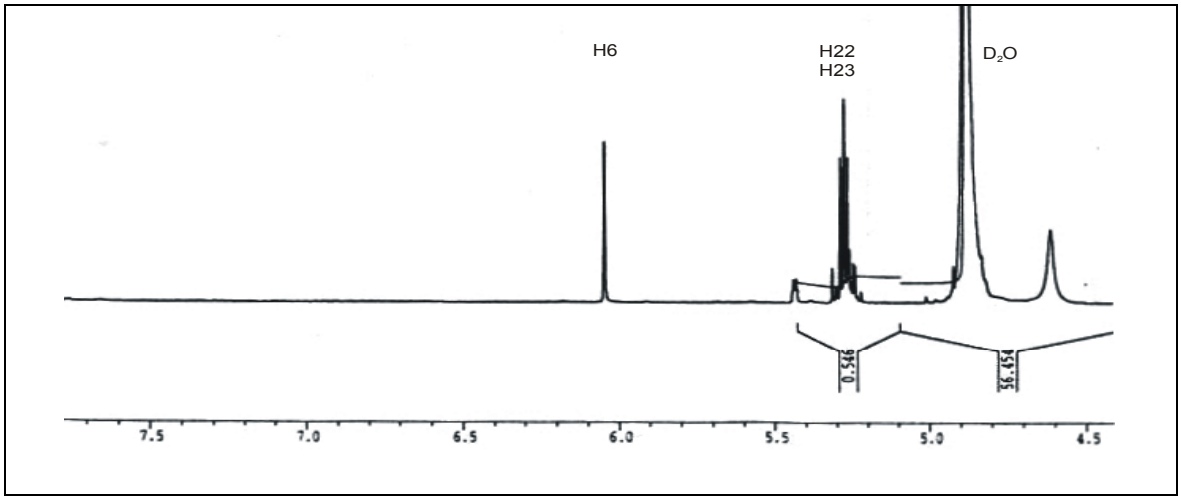



Figure C14.3. $^{1}$H NMR spectrum of compound **14** (lichesterol) (continued)

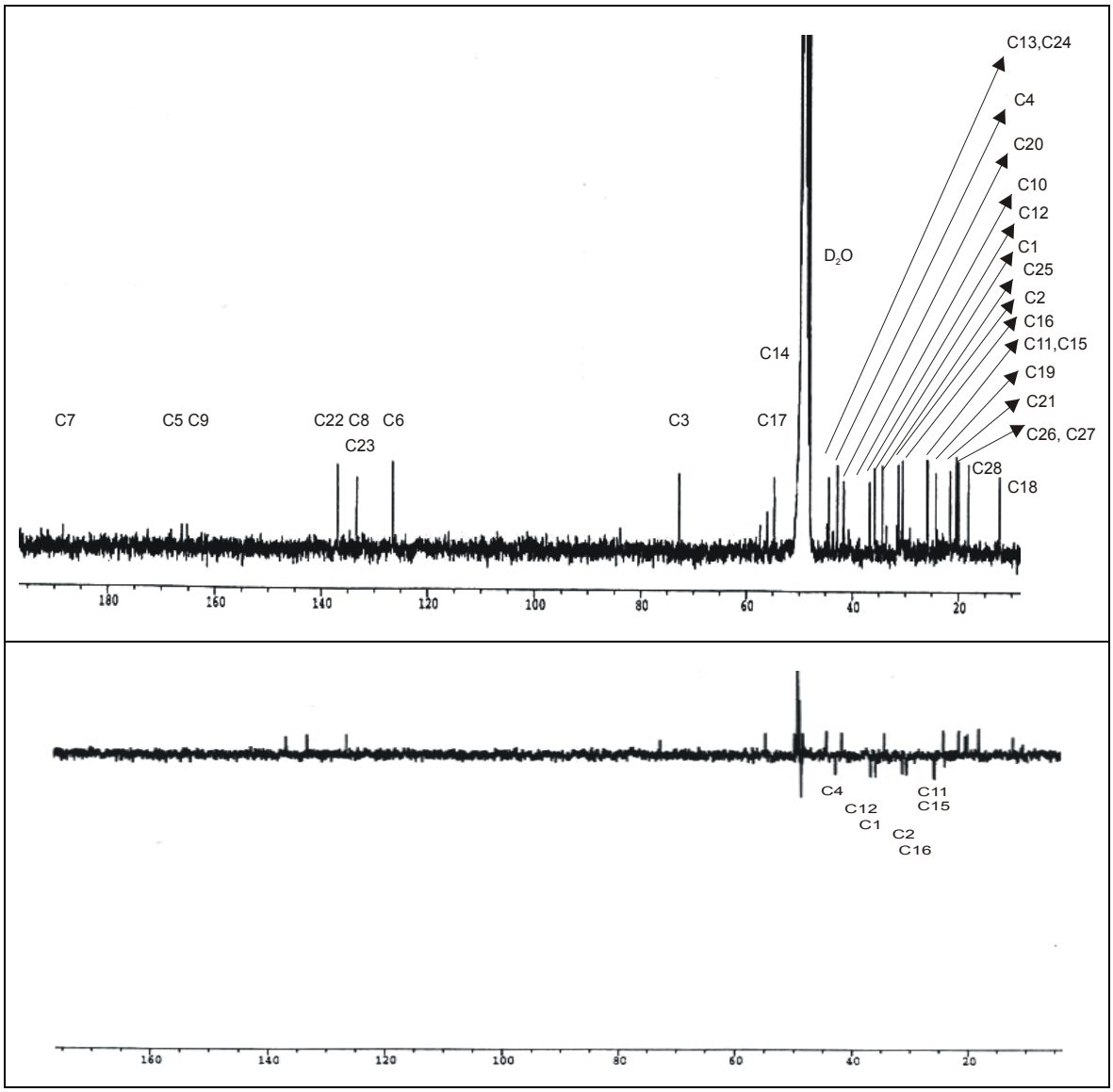



Figure C14.4. $^{13}$C NMR and DEPT spectra of compound **14** (lichesterol)

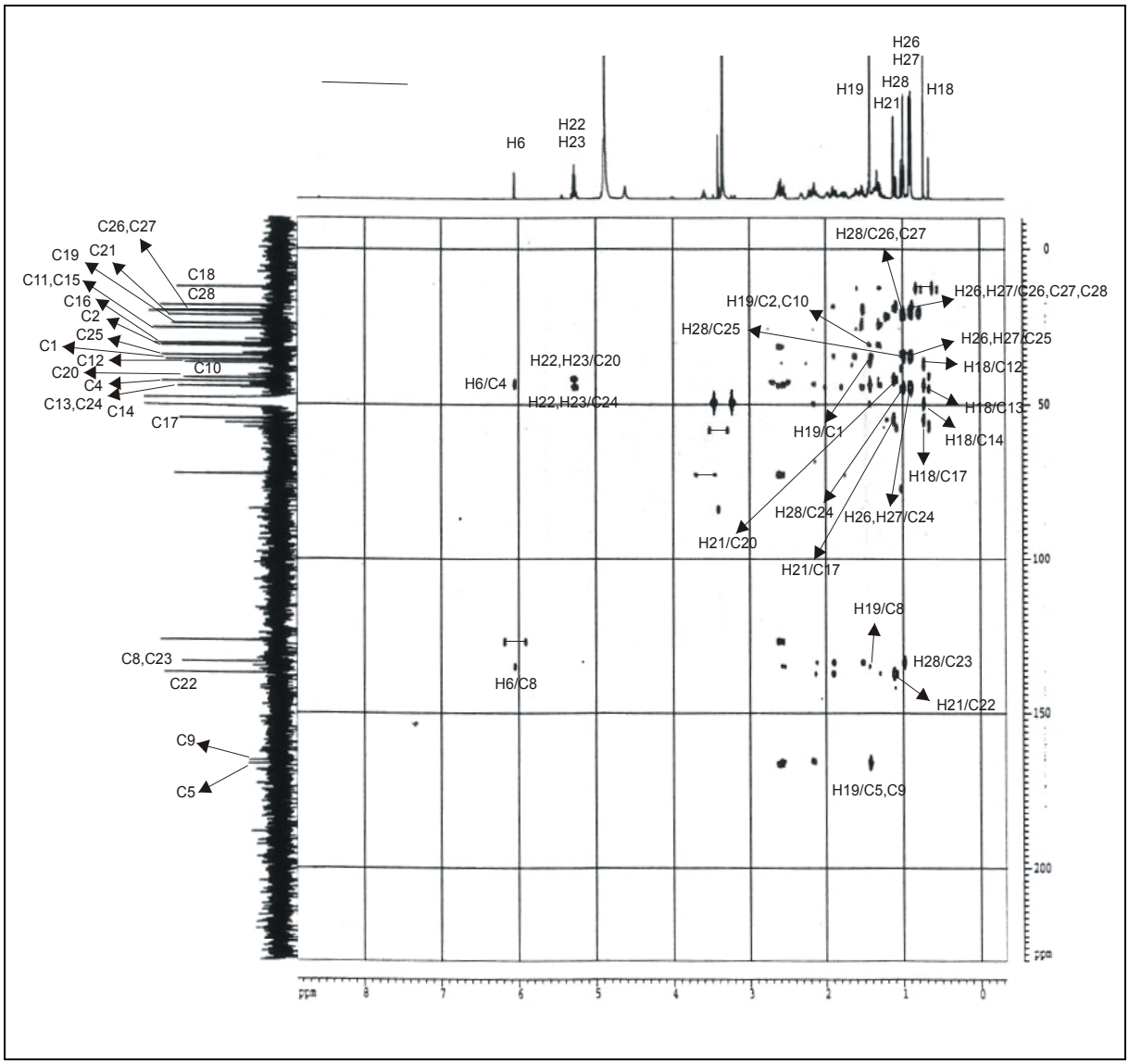


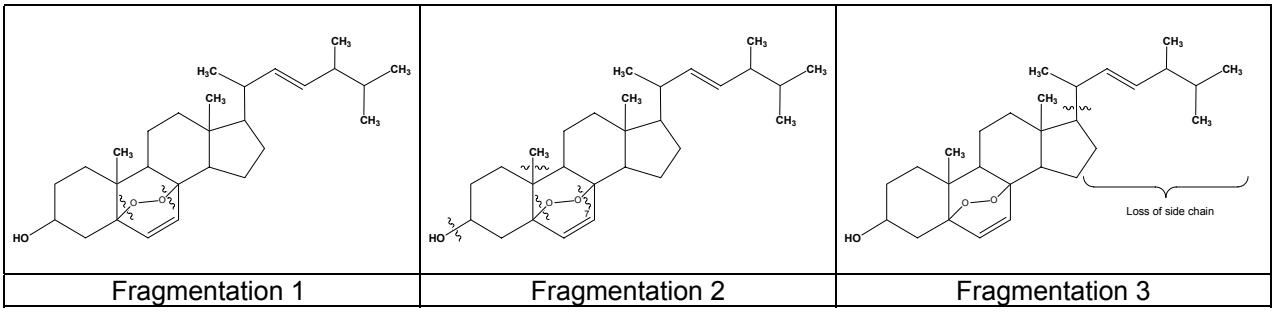

Figure C14.5. Methyl and olefinic proton HMBC spectra of compound **14** (lichesterol)

## 3.3. Isolated secondary metabolites of fungus *Fusarium* sp.

Three compounds were isolated from the fungus *Fusarium* sp. that had been derived from the sponge *Axinella damicornis*. They were an ergosterol peroxide (compound **15**), a triterpene acetate (compound **16**), and a cerebroside (compound **17**).

### 3.3.1. Compound 15 (ergosterol-5,8-peroxide)

In the FAB-MS spectrum of compound **15**, intense peaks were detected at *m*/*z* 451 $[M+Na]^+$, 396, 363, and 303, corresponding to the following hypothetical fragmentation $[M-O_2]^+$ (fragment 1), $[M-H_2O-O_2-CH_3]^+$ (fragment 2), $[M-O_2-C_3H_7O]^+$, and [M-side chain]$^+$ (fragment 3), respectively (Figure C15.1). This FAB-MS spectrum gave a molecular weight of 428 g/mol and an empirical formula of $C_{28}H_{44}O_3$.




| Fragmentation 1 | Fragmentation 2 | Fragmentation 3 |
|---|---|---|

Figure C15.1. Hypothetical fragmentation of compound **15** (ergosterol-5,8-peroxide) in the FAB-MS spectrum

Two downfield doublet signals [$\delta$ 6.22 (H6) and 6.49 (H7)] in the $^1$H NMR spectrum revealed the presence of a disubstituted double bond in the sterol skeleton. Other characteristic signals were two olefinic protons [$\delta$ 5.19 (H22, H23)] representing a double bond in the sterol side chain, one proton attached to oxygen-bearing carbon [$\delta$ 3.95 (H3)], and six methyl groups [$\delta$ 0.80 (H18), 0.87 (H19), 0.99 (H21), 0.80 (H26), 0.82 (H27), 0.90 (H28)] (Table C15.1, Figure C15.4).

At high field in the $^1$H NMR spectrum, two singlet signals [$\delta$ 0.80 (H18), 0.87 (H19)] were observed, representing methyl groups in the sterol nucleus. Four other methyl signals (H21, H26, H27, H28) belonged to the side chain (Table C15.1, Figure C15.4).

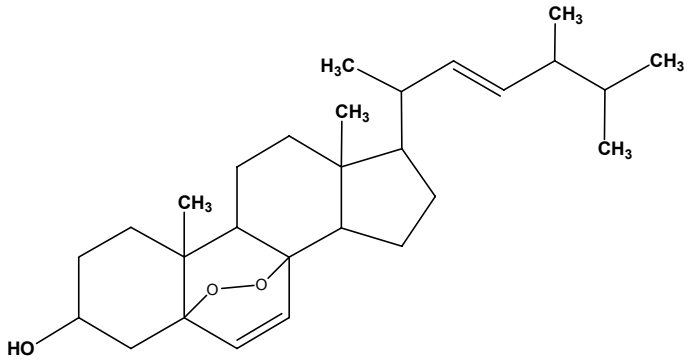
The $^{13}$C NMR spectrum analysis corroborated the presence of 28 carbons. The DEPT spectrum verified the existence of 24 protonated carbons, They consisted of six quartet carbons (methyl), seven triplet carbons (methylene), and eleven doublet carbons (methine).

Four non-protonated carbons (quarternary) [$\delta$ 79.5 (C5 ), 82.2 (C8), 36.9 (C10), 44.6 (C13)] were readily assigned due to the lack of these signals in the DEPT spectrum (Table C15.1, Figure C15.5).

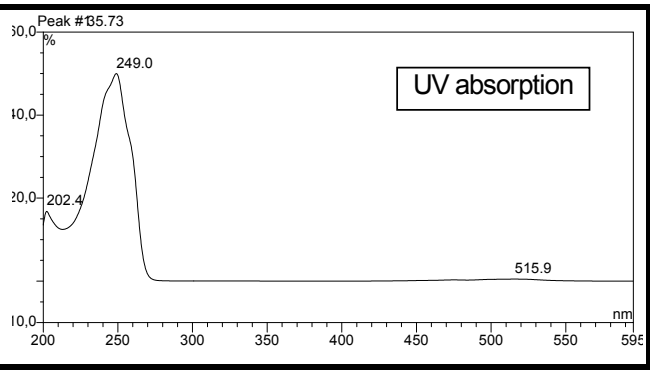
Two pairs of olefinic carbon signals [$\delta$ 130.7 (C6), 135.4 (C7), 132,3 (C22), 135.2 (C23)] were distinctly observed. The HMBC correlations (Figure C15.2 and C15.6) differentiated clearly the position of these two double bond pairs, namely in the sterol nucleus (first pair, C6 and C7) and in the side chain (second pair, C22 and C23). These double bond assignments were proved by the following connections: H6 to C1, C5, C8; and H7 to C4, C5, C8, C14; as well as both H22 and H23 to C20 and C24 (Table C15.1).

## Ergosterol-5,8-peroxide (compound 15)

| | |
|---|---|
| CAS Registry Number | : 2061-64-5 |
| Characteristic | : white crystals |
| Formula | : $C_{28}H_{44}O_3$ |
| Molecular Weight | : 428 g/mol |
| Amount | : 23.6 mg |
| Source | : *Fusarium* sp. derived from *Axinella damicornis* |




| | |
|---|---|
| Retention Time | : 35.69 min |
| $R_f$ | : 0.41 |
| Fluorescence, 254 nm | : + |
| 366 nm | : + |
| Anisaldehyde/$H_2SO_4$ | : brown |




Optical Rotation $[\alpha]_D^{20}$ : experiment = +16.5° (c=0.1, $CHCl_3$)
literature = -25° ($CHCl_3$) (Gunatilaka *et al*., 1981)

FAB-MS (*m/z*, rel. int.) : 451$[M+Na]^+_{(6.0)}$, 396 $[M-O_2]^+_{(42.7)}$, 377$_{(23.3)}$, 363$[M-H_2O-O_2-CH_3]^+_{(10.1)}$, 337$[M-O_2-C_3H_7O]^+_{(5.3)}$, 303[M-side chain]$^+_{(2.1)}$

Table C15.1. NMR data of compound **15** (ergosterol-5,8-peroxide)

| Position | $\delta$ $^{13}$C (ppm) (in $CDCl_3$) | DEPT | $\delta$ $^{1}$H (ppm) (Bok *et al*., 1999 in $CDCl_3$) | $\delta$ $^{1}$H (ppm) (in $CDCl_3$ ) | HMBC (H→ C) |
|---|---|---|---|---|---|
| 1 | 37.0 (t) | 37.0 (t) | | 2.10 (m) | C2, C3, C5 |
| 2 | 30.1(t) | 30.1 (t) | | 1.94 (m) | C4 |
| 3 | 66.5 (d) | | 3.96 (m) | 3.95 (m) | |
| 4 | 51.2 (t) | 51.2 (t) | | | |
| 5 | 79.5 (s) | | | | |
| 6 | 130.7 (d) | | 5.95 (d, 8.4) | 6.22 (d, 8.5) | C1, C5, C8 |
| 7 | 135.5 (d) | | 6.29 (d, 8.5) | 6.49 (d, 8.5) | C4, C5, C8, C14 |
| 8 | 82.2 (s) | | | | |
| 9 | 34.8 (d) | | | | |
| 10 | 36.9 (s) | | | | |
| 11 | 20.7 (t) | 20.7 (t) | | | |
| 12 | 39.4 (t) | 39.4 (t) | | | |
| 13 | 44.6 (s) | | | | |
| 14 | 51.8 (d) | | | | |
| 15 | 28.6 (t) | 28.6 (t) | | | |
| 16 | 23.4 (t) | 23.4 (t) | | | |
| 17 | 56.3 (d) | | | 1.20 (m) | C12, C13, C14, C17, C20 |
| 18 | 12.9 (q) | | 0.82 (s) | 0.80 (s) | C11, C12, C13, C14, C16, C17, C20 |
| 19 | 18.2 (q) | | 0.88 (s) | 0.87 (s) | C1, C2, C4, C5, C6, C8, C9, C10 |
| 20 | 39.7 (d) | | | | |
| 21 | 20.9 (q) | | 1.00 (d, 6.0) | 0.99 (d, 6.5) | C17, C20, C22, C23 |
| 22 | 132.3 (d) | | 5.18 (m) | 5.19 (m) | C20, C24 |
| 23 | 135.2 (d) | | 5.18 (m) | 5.19 (m) | C20, C24 |
| 24 | 42.8 (d) | | | 1.85 (m) | C22, C23 |
| 25 | 33.1 (d) | | | 1.47 (m) | C24, C26, C27, C28 |
| 26 | 19.9 (q) | | 0.82 (d, 8.5) | 0.80 (d, 6.6) | C24, C25, C27, C28 |
| 27 | 19.7 (q) | | 0.83 (d, 8.0) | 0.82 (d, 6.6) | C24, C25, C26, C28 |
| 28 | 17.5 (q) | | 0.91 (d, 7.0) | 0.90 (d, 6.7) | C23, C24, C25, C26, C27 |

Likewise, through HMBC spectrum analysis, assignment of two methyl signals located in the sterol nucleus and four methyl signals in the side chain was proven. These methyl groups in relation to their neighbouring carbons were presented as follows: H18 had several cross peaks with C11, C12, C13, C14, C16, C17, C20; H19 connected to C1, C2, C4, C5, C6, C8, C9, C10, confirming that these two methyl signals were attached to the sterol nucleus.

The other four methyl signals, H21 related to C17, C20, C22, C23; H26 connected to C24, C25, C27, C28; H27 had cross peaks with C24, C25, C26, C28; and the last methyl signal (H28) had connections with C23, C24, C25, C27. These long range correlations assigned the position of these methyl groups in the sterol side chain (Figure C15.2 and C15.6).

The singlet carbon signals in the lower field [$\delta$ 79.5 (C5), 82.2 (C8)] were bound to oxygen, forming the peroxide. Another typical oxygen-carrying carbon was also observed at $\delta$ 66.5 (C3). This oxygen-carrying C3 was confirmed by the presence of a particular proton [$\delta$ 3.95 (H3)] attached to the oxygen-carrying carbon.

Comparison of the $^{1}$H NMR data of compound **15** with those reported by Bok *et al*. (1999) showed identical chemical shifts with differences varying from 0.01 to 0.27 ppm, leading to the conclusion that both compounds are the same.

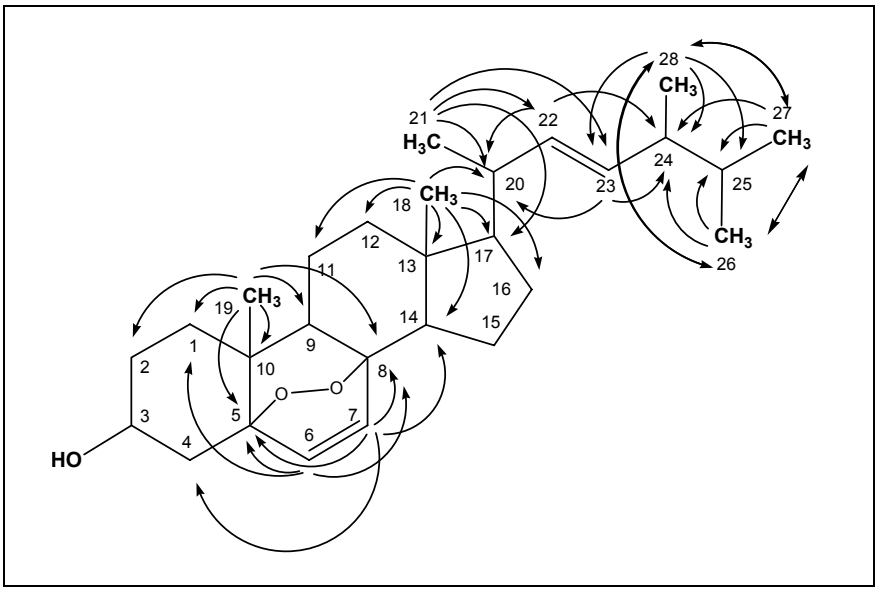



Figure C15.2. HMBC of methyl groups and olefinic protons of compound **15** (ergosterol-5,8-peroxide)

Optical rotation of compound **15** (+16.5°) and reported ergosterol peroxide (-25°), however, was different, suggesting that compound **15** was an enantiomer of the previously reported compound. The results of anti microbial assay and cytotoxicity test using human cancer cell lines are presented in Table C15.2 and Figure C15.3, respectively.

Table C15.2. Anti microbial activity of compound **15** (ergosterol-5,8-peroxide)

| Compound **15** | *Staphylococcus aureus* | *Bacillus subtilis* | *Escherichia coli* | *Candida albicans* | *Saccharomyces cerevisiae* | *Cladosporium herbarum* |
|---|---|---|---|---|---|---|
| 50 μg | not active | not active | not active | 7 mm | not active | not active |
| 100 μg | 6 mm | 7 mm | not active | 10 mm | not active | not active |

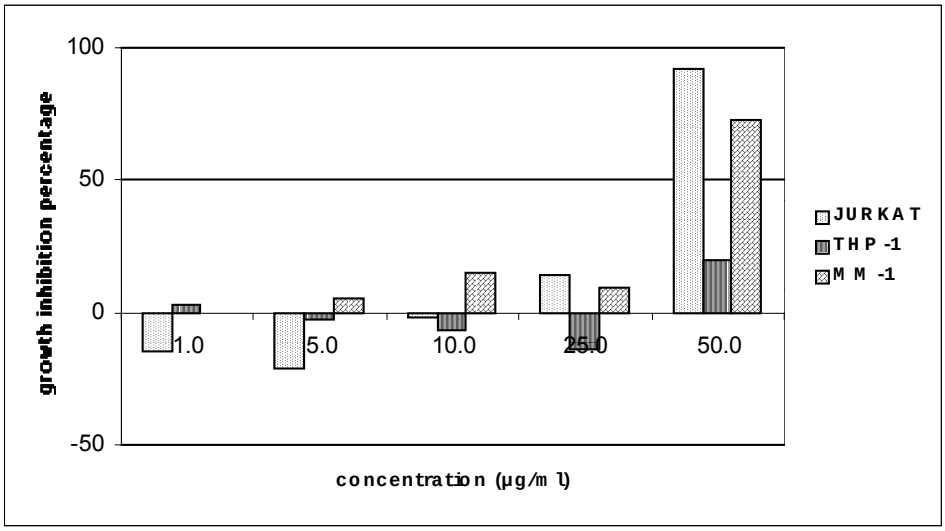



Figure C15.3. Growth inhibition of human cancer cell lines treated with compound **15** (ergosterol-5,8-peroxide)

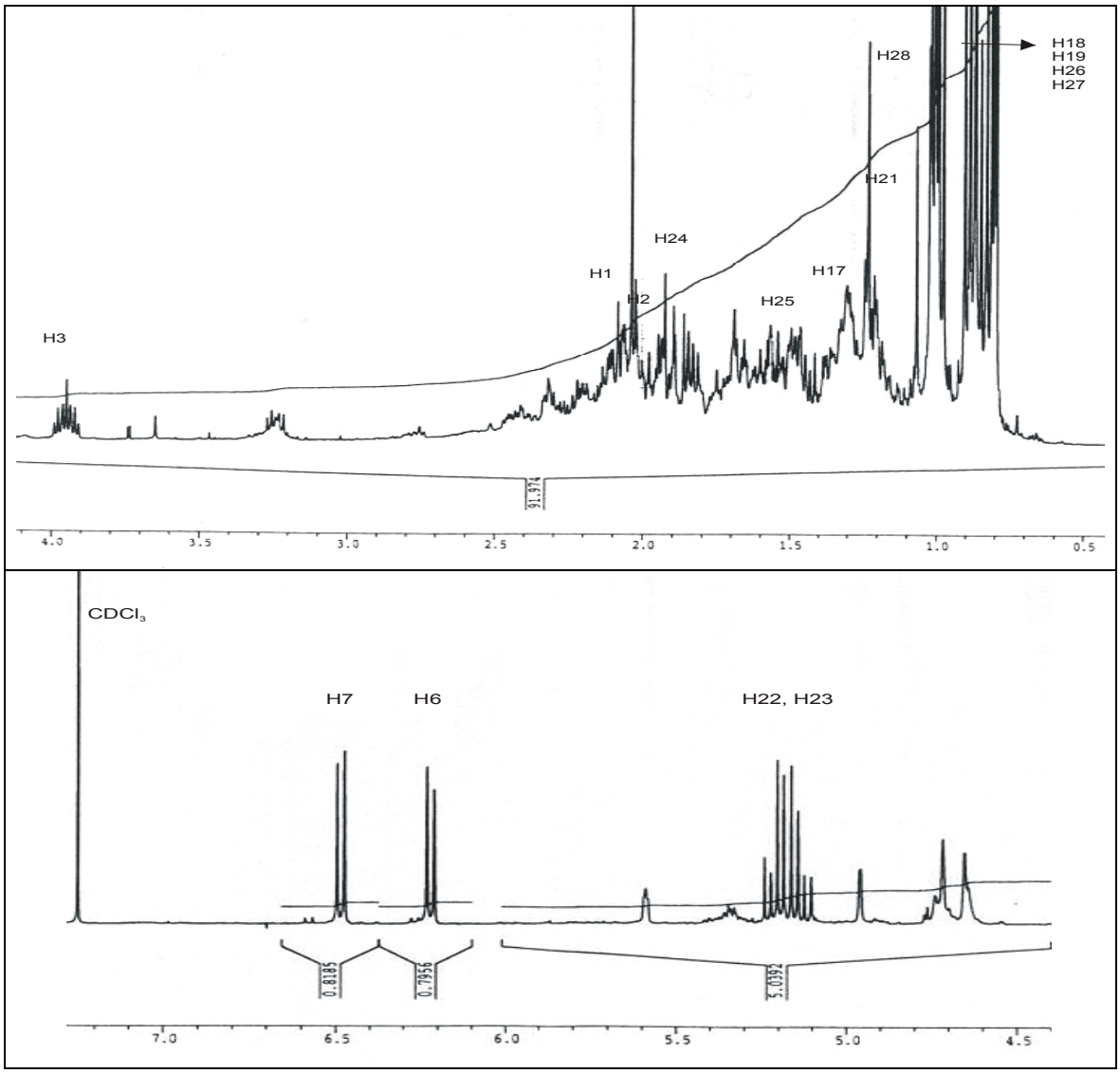



Figure C15.4. $^{1}$H NMR spectrum of compound **15** (ergosterol-5,8-peroxide)

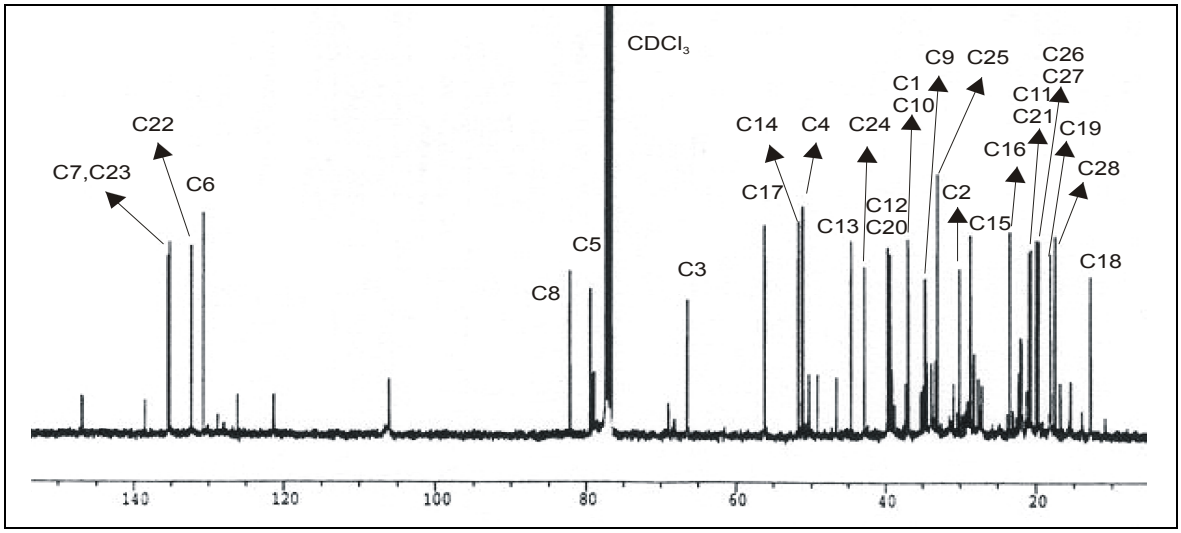



Figure C15.5. $^{13}$C NMR spectrum of compound **15** (ergosterol-5,8-peroxide)

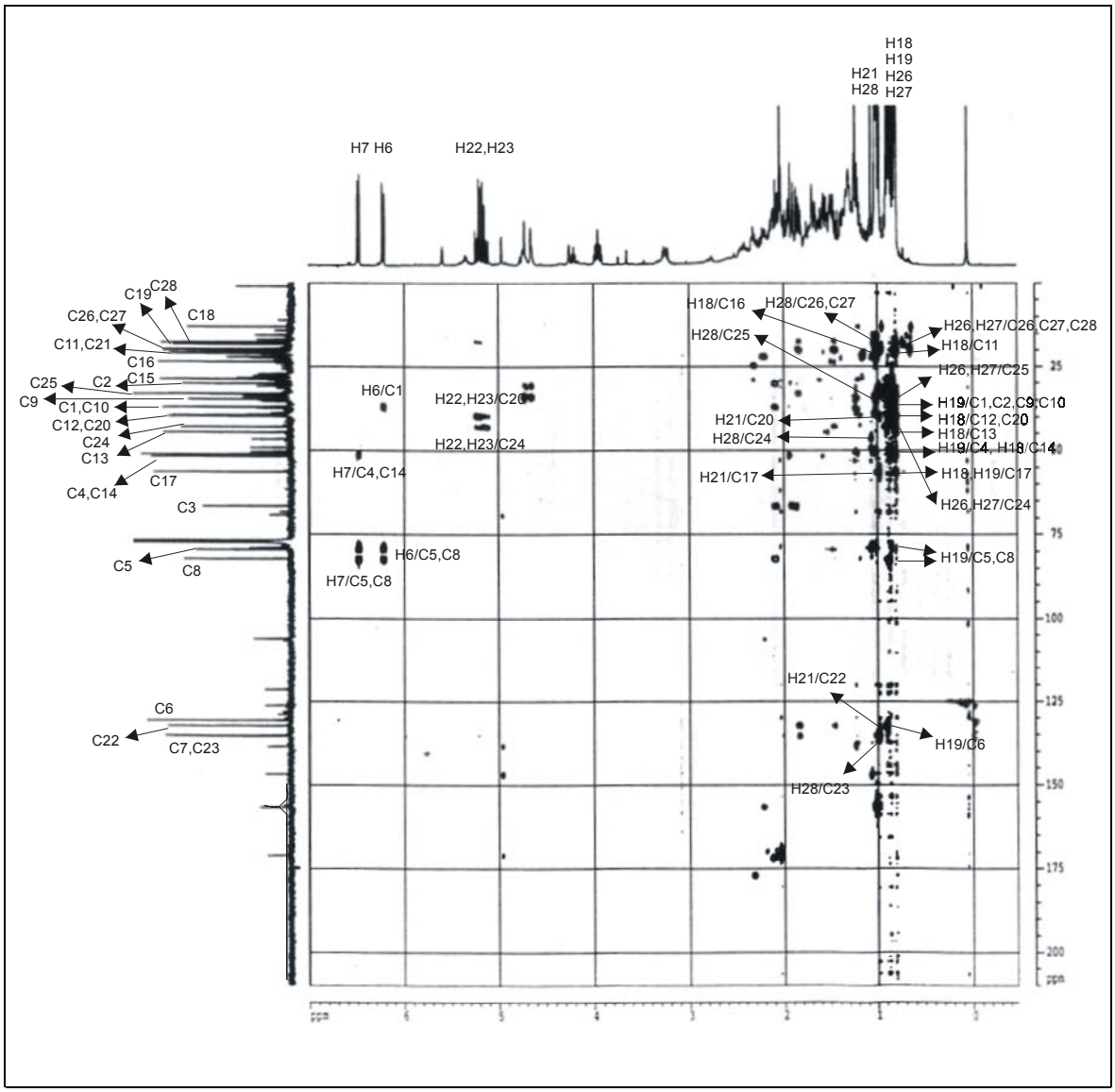



Figure C15.6. HMBC spectrum of methyl groups and olefinic protons of compound **15** (ergosterol-5,8-peroxide)

## 3.3.2. Compound 16 (triterpene acetate)

The FAB-MS spectrum of compound **16** that presented peaks at *m/z* 537 $[M+Na]^+$, 497 $[M-OH]^+$, and 454 $[M\text{-acetoxy}]^+$ suggested a molecular weight of 514 g/mol and an empirical formula of $C_{32}H_{50}O_5$. Molecular fragmentation in the FAB-MS spectrum revealed the loss of a hydroxyl group and acetoxy group.

The $^1$H NMR spectrum of this compound exhibited the presence of four typical low field protons bound to oxygen-bearing carbons [$\delta$ 3.78 (H2), 3.01 (H3), 4.19 (H11), 4.94 (H12)], one acetate [$\delta$ 2.03 (H32)], one olefinic proton [$\delta$ 5.58 (H15)], and one exo-methylene group [$\delta$ 4.64, 4.70 (H28)] (Table C16.1, Figure C16.3).

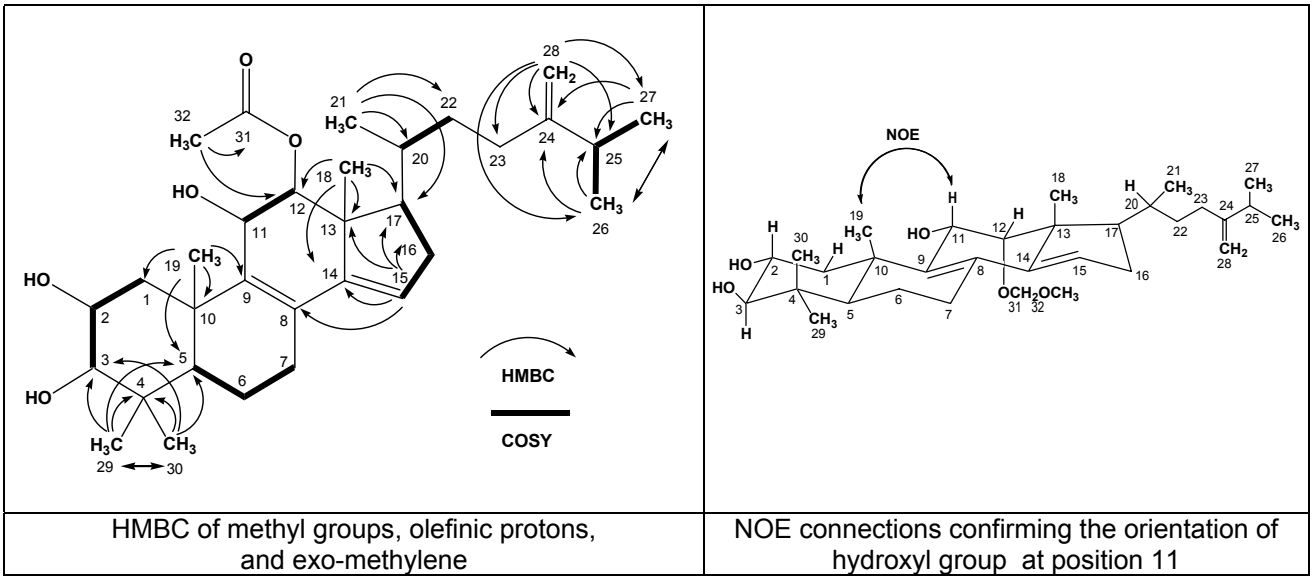
Signals representing 32 carbons in the $^{13}$C NMR spectrum (Table C16.1, Figure C16.4) were in agreement with the molecular formula. DEPT analysis of the $^{13}$C NMR spectrum indicated eight quarternary carbons, nine methine carbons, seven methylene carbons, and eight methyl carbons.

Further examination of the $^{13}$C NMR spectrum proved six olefinic carbons [$\delta$ 125.71 (C8), 138.11 (C9), 146.75 (C14), 121.42 (C15), 156.61 (C24), 106.12 (C28)], one carbonyl [171.43 (C31)], and 25 aliphatic carbons. The presence of H2, H3, H11, H12 at low field in the $^1$H NMR spectrum confirmed the existence of three hydroxyl-carrying carbons (C2, C3, C11) and one acetate group (C12).

The COSY spectrum showed that the proton at position 5 [$\delta$ 5.58] was coupled with methylene protons [$\delta$ 2.04 (H16)] and the side chain-binding proton [$\delta$ 1.94 (H17)]. The nature of the side chain was also confirmed by the presence of several connections among a number of protons (H20, H21, H22, H23, H25, H26, H27, H28) to their adjacent carbons through either two, three, or four bond couplings (Figure C16.1 and C16.5).

The methyl protons [$\delta$ 0.86 (C21)] of the side chain had three bond couplings with C17, confirming that this side chain was connected to the main skeleton through this methine carbon (C17). The assignment of this side chain connection to the triterpene nucleus was also assured by three bond couplings of H16 to C20 and four bond couplings of H17 to C23 (Table C16.1).

The assignment of methyl carbons (C18, C19) attached to the triterpene nucleus was indicated by cross peaks of H18 to C12, C13, C14, C17 and H19 to C1, C5, C9, C10. In the HMBC spectrum the two geminal methyl groups (H29, H30) were coupled to C3, C4, C5 (Figure C16.1).



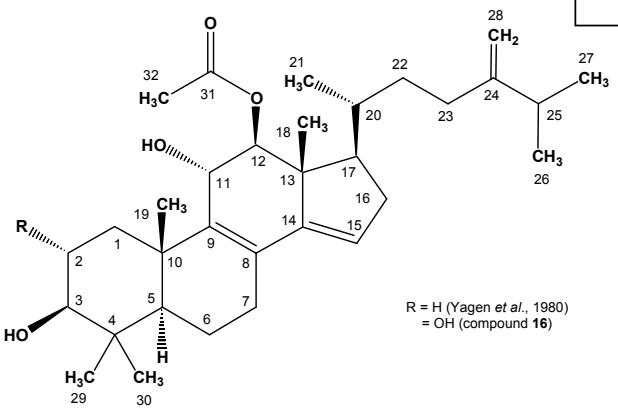

| HMBC of methyl groups, olefinic protons, and exo-methylene | NOE connections confirming the orientation of hydroxyl group at position 11 |
|---|---|

Figure C16.1. COSY, HMBC, and NOE connections of compound **16** (triterpene acetate)

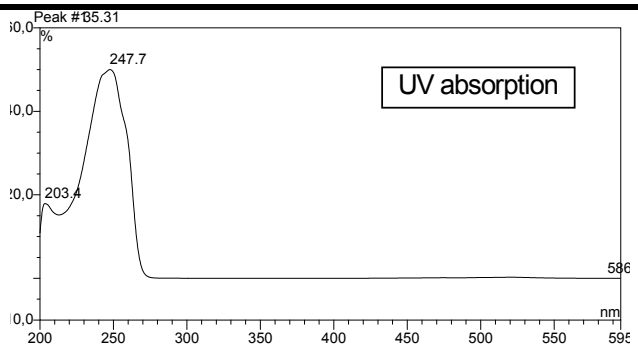
## Triterpene acetate (compound 16)

| | |
|---|---|
| CAS Registry Number | : - |
| Characteristic | : white crystals |
| Formula | : $C_{32}H_{50}O_5$ |
| Molecular Weight | : 514 g/mol |
| Amount | : 72.6 mg |
| Source | : *Fusarium* sp. derived from *Axinella damicornis* |

new compound




| | |
|---|---|
| Retention Time | : 35.36 min |
| $R_f$ | : 0.2 |
| Fluorescence, 254 nm | : + |
| 366 nm | : + |
| Anisaldehyde/$H_2SO_4$ | : brown |




| | |
|---|---|
| Optical Rotation $[\alpha]_D^{20}$ | : experiment = +100° (c=0.1, Aceton) |
| FAB-MS (*m/z*, rel. int.) | : 537 $[M+Na]^+_{(16.8)}$, 497 $[M-OH]^+_{(10.3)}$, 454 $[M\text{-acetoxy}]^+_{(10.3)}$, 439 $[M-5CH_3]^+_{(36.5)}$, $313_{(10.5)}$, $307_{(11.6)}$, $154_{(100)}$, |

Table C16.1. NMR data of compound **16** (triterpene acetate)

| Position | $\delta$ $^{13}$C (ppm) (in $CDCl_3$) | DEPT | $\delta$ $^{1}$H (ppm), multiplicity ($J$ in Hz) | COSY (H → H) | HMBC (H → C) |
|---|---|---|---|---|---|
| 1 | 42.66 (t) | 42.66 (t) | 2.33 (m) | H2, H5 | C2, C3, C5, C10, C11 |
| 2 | 68.95 (d) | | 3.78 (m) | H1, H3 | C3 |
| 3 | 83.29 (d) | | 3.01 (d, 9.5) | H1, H2 | C1, C2, C4, C29, C30 |
| 4 | 39.35 (s) | | | | |
| 5 | 50.14 (d) | | 1.24 (d, 10.1) | H6, H7 | C2, C3, C9, C11 |
| 6 | 18.04 (t) | 18.04 (t) | 1.75 (m) | H5, H7 | C8 |
| 7 | 26.75 (t) | 26.75 (t) | 2.42 (m) | H5, H6 | C8, C9, C11, C14, C15 |
| 8 | 125.71 (s) | | | | |
| 9 | 138.11 (s) | | | | |
| 10 | 38.11 (s) | | | | |
| 11 | 68.95 (d) | | 4.19 | H12 | C8, C9, C12, C13 |
| 12 | 79.39 (d) | | 4.94 | H11 | C9, C11, C13, C14, C18, C31 |
| 13 | 46.64 (s) | | | | |
| 14 | 146.75 (s) | | | | |
| 15 | 121.42 (d) | | 5.58 | H16 | C8, C13, C14, C16, C17 |
| 16 | 35.32 (t) | 35.32 (t) | 2.04 | H17 | C17, C20 |
| 17 | 49.13 (d) | | 1.94 (m) | | C12, C13, C23 |
| 18 | 16.70 (q) | | 1.06 (s) | | C12, C13, C14, C17 |
| 19 | 23.25 (q) | | 1.28 (s) | | C1, C5, C9, C10 |
| 20 | 33.26 (d) | | 1.65 (m) | H22 | C21 |
| 21 | 18.21 (q) | | 0.86 (d, 6.0) | | C17, C20, C22 |
| 22 | 34.48 (t) | 34.48 (t) | 1.88 (m) | H20 | C24, C28 |
| 23 | 30.92 (t) | 30.92 (t) | 2.30 (m) | | C24, C28 |
| 24 | 156.61 (s) | | | | |
| 25 | 33.81 (d) | | 2.20 (m) | H26, H27 | C23, C26, C27 |
| 26 | 22.00 (q) | | 0.99 (d, 7.0) | H25, H27 | C24, C25, C27 |
| 27 | 21.87 (q) | | 1.01 (d, 7.0) | H25, H26 | C24, C25, C26 |
| 28 | 106.12 (t) | 106.12 (t) | 4.64 (s), 4.70 (s) | | C23, C24, C25, C26,C27 |
| 29 | 16.70 (q) | | 0.86 (s) | | C3, C4, C5, C30 |
| 30 | 28.68 (q) | | 1.03 (s) | | C2, C3, C4, C5, C29 |
| 31 | 171.43 (s) | | | | |
| 32 ($CH_3CO$) | 21.31 (q) | | 2.03 (s) 3H | | C12, C31 |

NOE experiments were conducted to confirm the relative stereochemistry of this compound. Irradiation of methyl protons [$\delta$ 1.28 (H19)] resulted in a correlation with H11, suggesting α-configuration of the hydroxyl group at C11. Spatial correlation [equatorial (α configuration)] between H12 and H20 was demonstrated upon irradiation of the methyl protons [$\delta$ 1.06 (H18)], indicating axial ($\beta$) orientation of the acetoxy group at C12 (Figure C16.1).

Comparison of $^{13}$C NMR data of compound **16** with those of a related compound isolated from *Fusarium sporotrichioides* (Yagen *et al*., 1980) exhibited similar chemical shifts with the only exception at position 2. Compound **16** displayed a prominent downfield carbon chemical shift [$\delta$ 68.95 (C2)], confirming that this position should be occupied by an oxygen-bearing carbon instead of a protonated carbon. The coupling constant observed for H3 (9.5 Hz) could only be explained by a diaxial coupling to H2, thus indicating the $\alpha$-configuration of the hydroxyl substituent at C2. Compound **16** has not been reported in the literature before.

Cytotoxicity of compound **16** is presented in Figure C16.2. As much as 7 mm of growth zone of *Staphylococcus aureus* was inhibited by 100 $\mu$g compound **16** (triterpene acetate) in the antimicrobial assay.

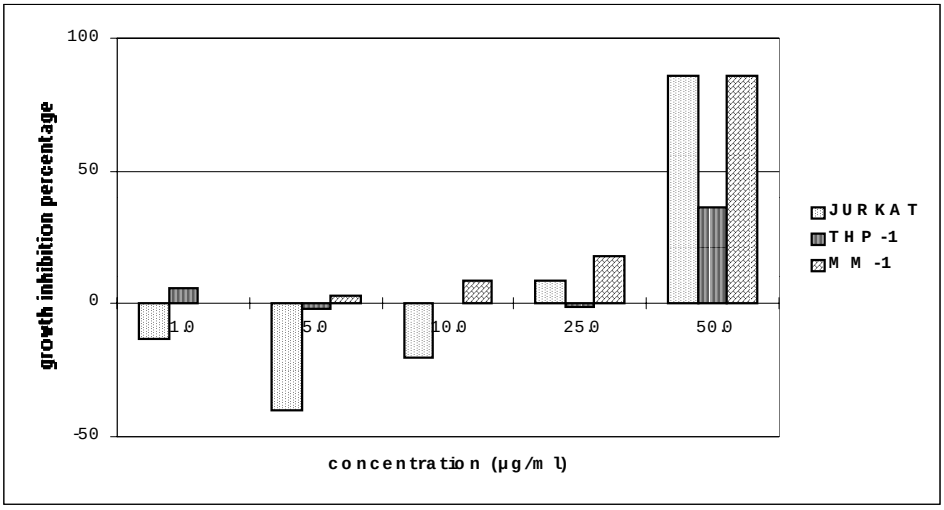



Figure C16.2. Growth inhibition of human cancer cell lines treated with compound **16** (triterpene acetate)

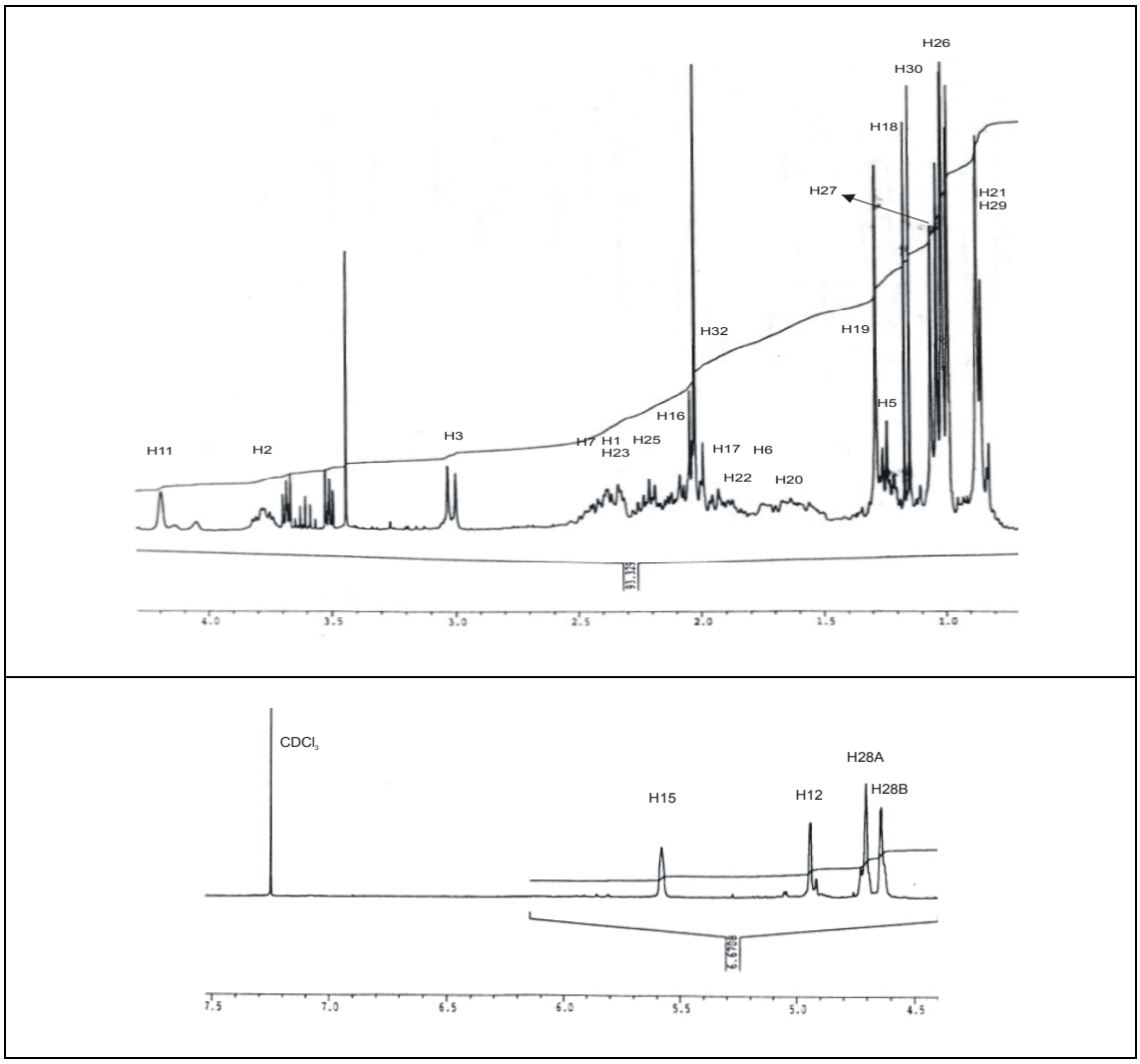



Figure C16.3. $^{1}$H NMR spectrum of compound **16** (triterpene acetate)

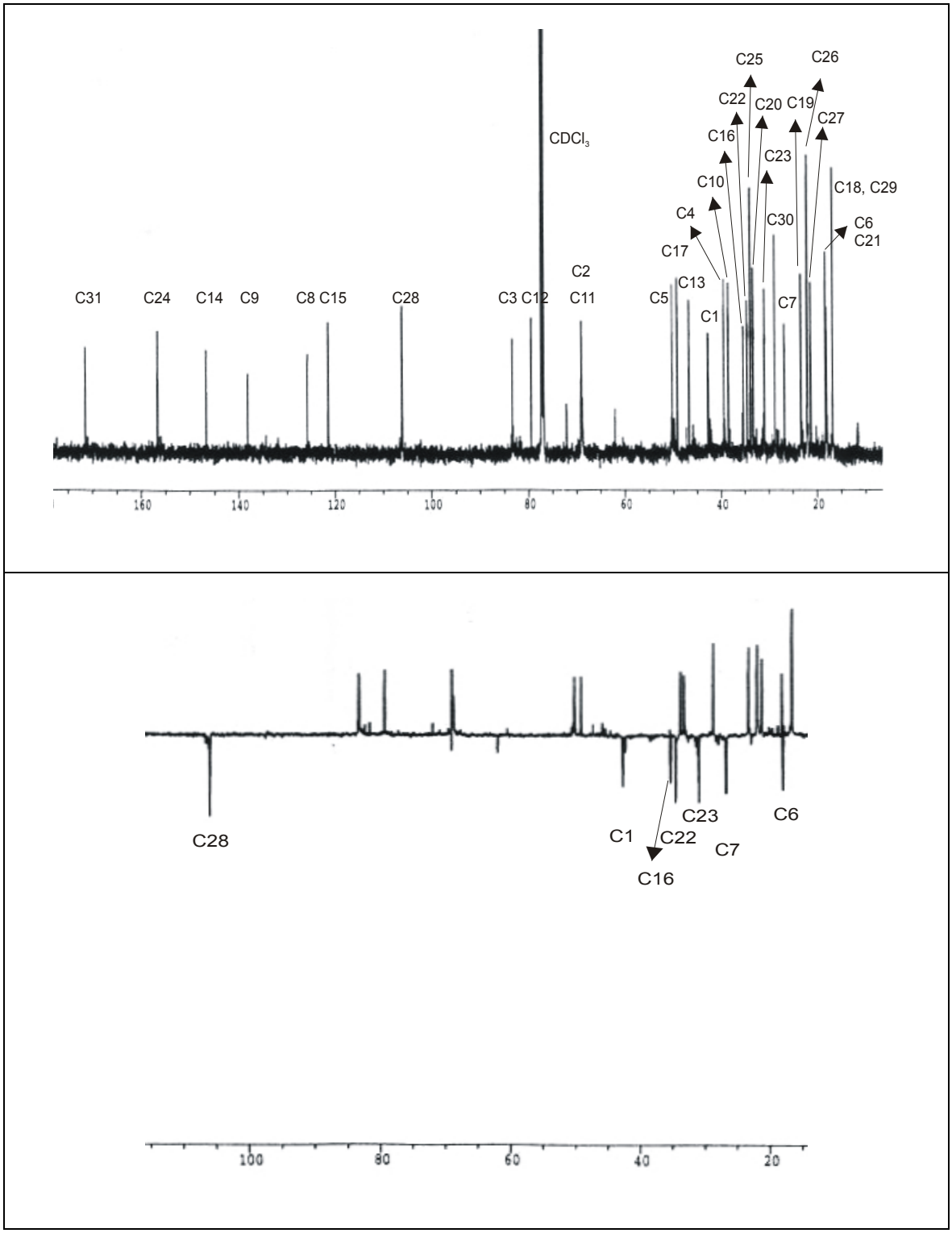



Figure C16.4. $^{13}C$ and DEPT NMR spectra of compound **16** (triterpene acetate)

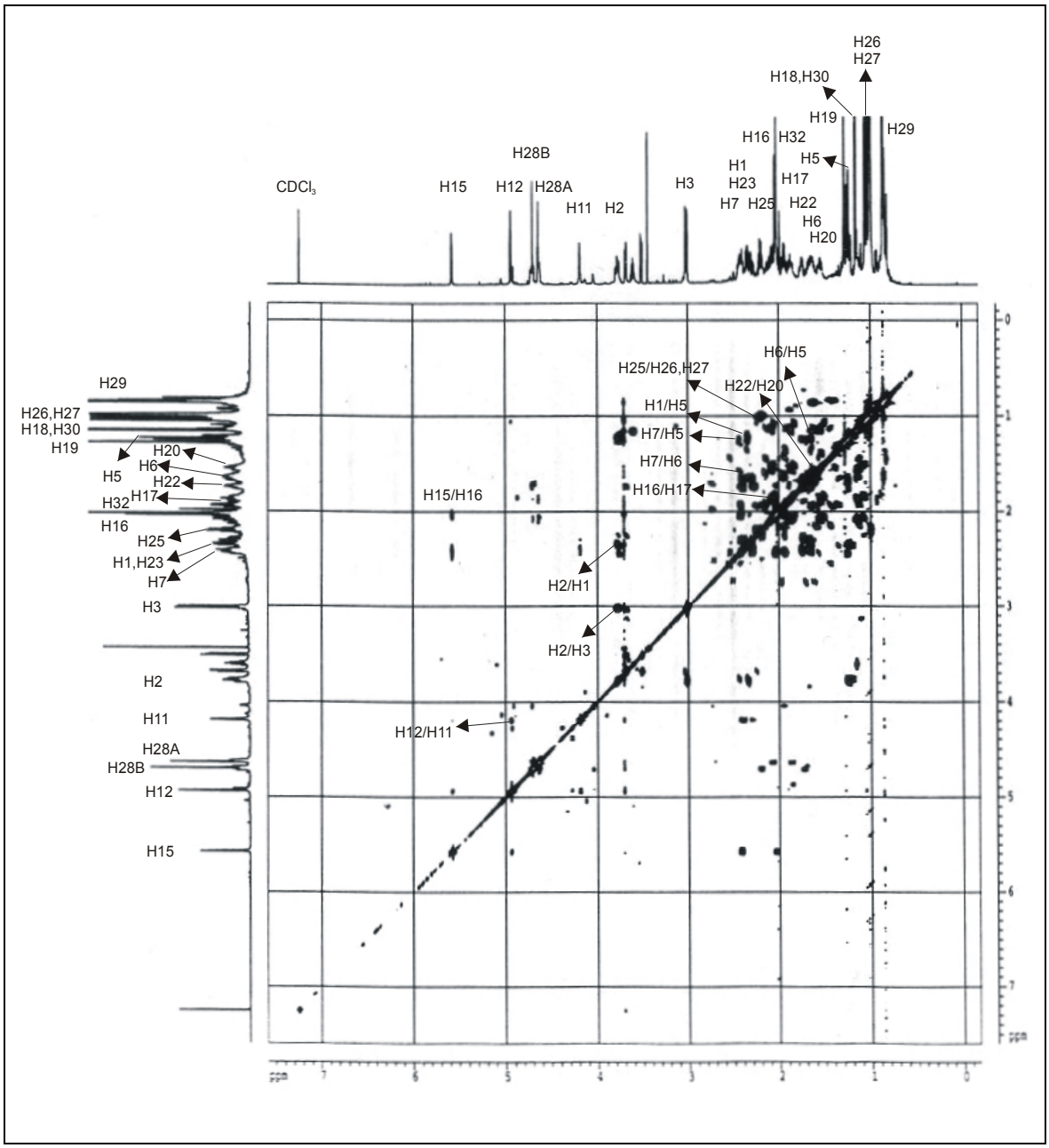



Figure C16.5. COSY spectrum of compound **16** (triterpene acetate)

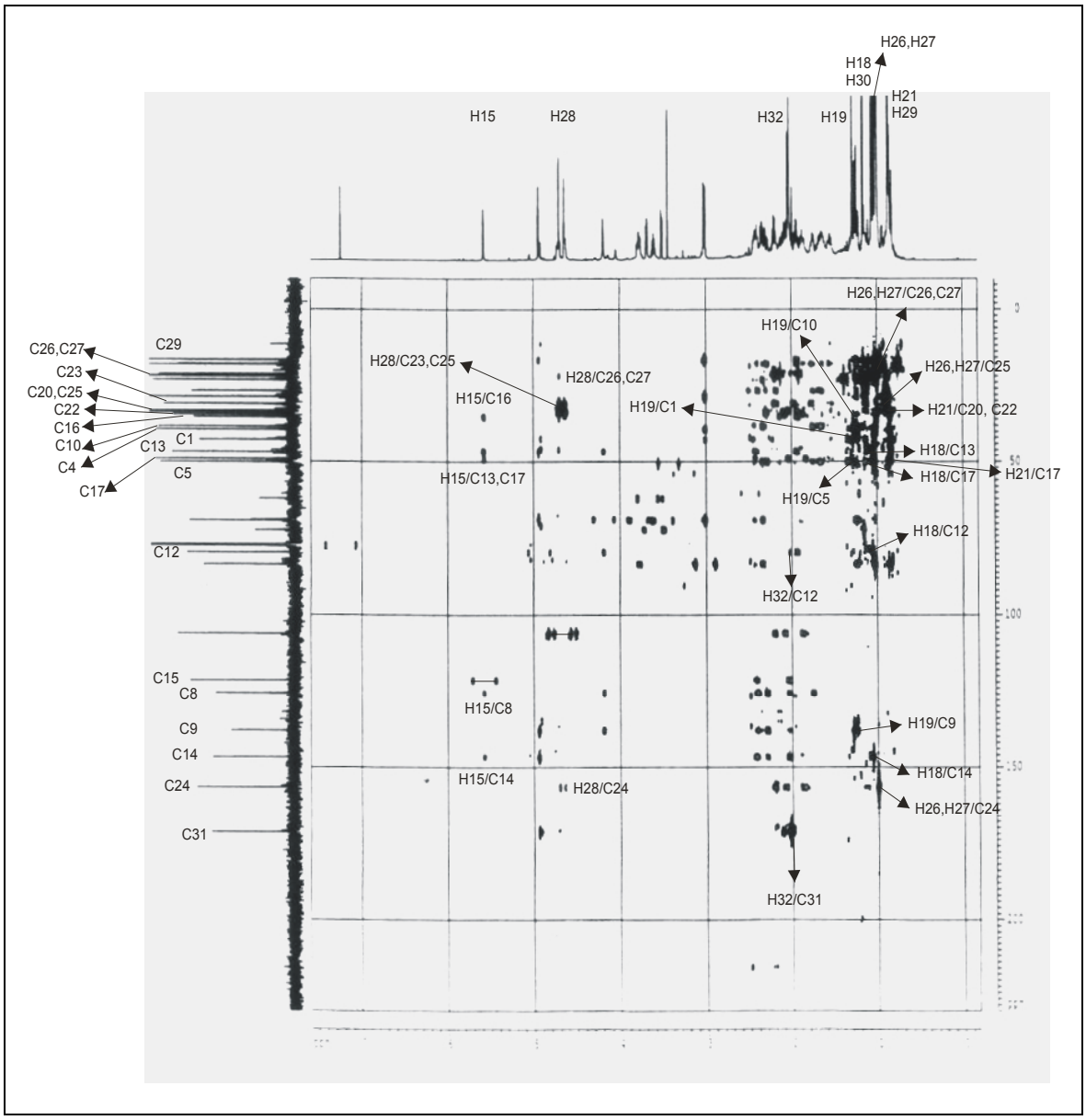


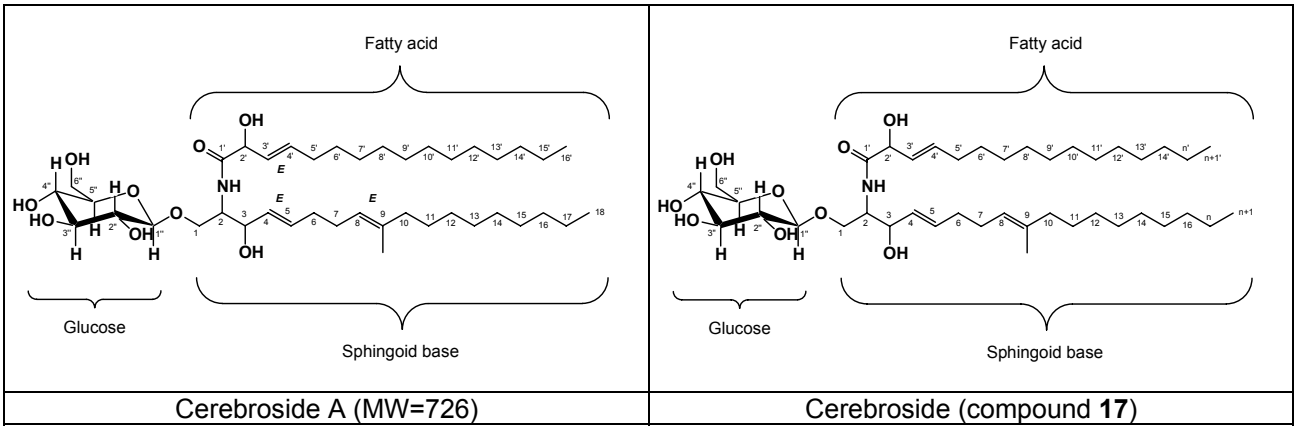

Figure C16.6. HMBC spectrum of methyl groups, olefinic protons, and exo-methylene of compound **16** (triterpene acetate)

## 3.3.3. Compound 17 (cerebroside)

An attempt to determine the molecular weight of this compound through EI, FAB, and ESI-MS analysis was not successful. This was due to the impurity of the compound which was clearly indicated in the ESI-MS spectrum, showing several prominent peaks, representing also several different molecular weights.

Nevertheless, from the comparison of the $^{1}H$ NMR and $^{13}C$ spectra of compound **17** with those of cerebroside A (Koga *et al*., 1998), the chemical shift differences of the isolated compound and the data from the literature varied only at a small range of 0.02 – 0.20 ppm for $^{1}H$ NMR and 0.1 – 0.8 ppm for $^{13}C$ NMR. It was, therefore, concluded that the structure of compound **17** was the same as that of cerebroside A (Figure C17.1).

However, the length of the aliphatic chain of the sphingosine base and the fatty acid remained unresolved, and both portion ends were marked as $CH_2(n)$-$CH_3(n+1)$ for sphingosine base and $CH_2(n')$-$CH_3(n'+1)$ for fatty acids (Figure C17.1).




| Cerebroside A (MW=726) | Cerebroside (compound **17**) |
|---|---|

Figure C17.1. Cerebroside A and cerebroside (compound **17**)

The following elaborations discuss in more detail the three portions (sphingosine base, fatty acid, and glucose) of the isolated cerebroside molecule (compound **17**).

***Sphingosine portion***

The occurrence of the sphingosine base was distinctly characterised by the chemical shift of sphingosine substitution of a glycosylated hydroxyl at C1 (69.7 ppm), an acylamido group at C2 (54.7 ppm) and a hydroxyl group at C3 (72.9 ppm) (Table C17.1).

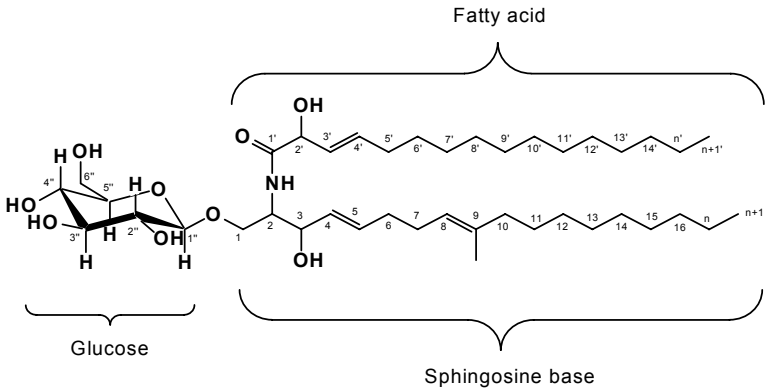
These three typical positions of sphingosine carbons were also supported by the existence of three protons bound to oxygen-carrying carbons [$\delta$ 4.17 (H1A), 3.74 (H1B), 4.17 (H3)] and one proton attached to amide-carrying carbon [$\delta$ 4.03 (H2)]. Amide (NH) signal itself resonated at $\delta$ 7.5 ppm (Figure C17.2).

The assignment of two pairs of double bonds was indicated by three olefinic protons [$\delta$ 5.30 (H4), 5.75 (H5), 5.19 (H8)], suggesting that one double bond carbon had no proton. Four olefinic carbons [$\delta$ 134.4 (C4), 131.0 (C5), 124.9 (C8), 136.1 (C9)] verified also the existence of these double bonds. The methyl protons at $\delta$ 1.64 (H9a) were found to reside at a double bond (C9) (Figure C17.3).

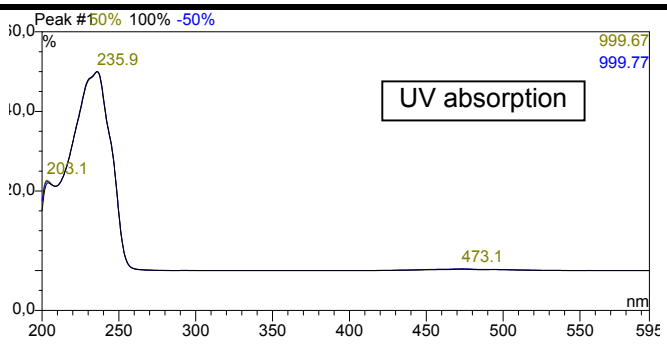
Two isolated methylene protons were encountered at $\delta$ 2.08 (H6) and 2.08 (H7) lying between two pairs of unsaturated carbons (C4-C5, and C8-C9). Other methylene protons of sphingosine, however, were found overlapped in a look-like single peak at $\delta$ 1.33. The aliphatic chain of sphingosine base ended with a methyl group at $\delta$ 0.94 ($H_{n+1}$).

## Cerebroside (compound 17)

| | | |
|---|---|---|
| CAS Registry Number | : | - |
| Characteristic | : | white crystals |
| Formula | : | - |
| Molecular Weight | : | - |
| Amount | : | 5.8 mg |
| Source | : | *Fusarium* sp. derived from *Axinella damicornis* |




| | | |
|---|---|---|
| Retention Time | : | 39.60 min |
| $R_f$ | : | 0.54 |
| Fluorescence, 254 nm | : | - |
| 366 nm | : | - |
| Anisaldehyde/$H_2SO_4$ | : | red |




| | | |
|---|---|---|
| ESI-MS (*m/z*) | : | not available due to impurity of sample |

Table C17.1. NMR data of compound **17** (cerebroside)

| Position | $\delta$ $^{13}$C (ppm) of Cerebroside A (Koga *et al*., 1998 in MeOD) | $\delta$ $^{13}$C (ppm) (in MeOD) | DEPT | $\delta$ $^{1}$H (ppm) of Cerebroside A, multiplicity (*J* in Hz) (Koga *et al*., 1998 in MeOD) | $\delta$ $^{1}$H (ppm) multiplicity (*J* in Hz) (in MeOD ) | COSY (H → H) | HMQC (H → C) direct | HMBC (H→ C) |
|---|---|---|---|---|---|---|---|---|
| **Sphingosine** | | | | | | | | |
| 1 | 69.8 (t) | 69.7 (t) | 69.7 (t) | H1A: 4.11 (dd, 10.3, 3.4)<br>H1B: 3.72 (dd, 10.3, 3.4) | H1A: 4.17 (dd, 10.4, 5.4)<br>H1B: 3.74 (dd, 10.3, 5.3) | H1B, H2 | C1 | C2, C3, C4, C5, C1‘, C1” |
| 2 | 54.9 (d) | 54.7 (d) | | 3.97 (dt, 5.4, 3.4) | 4.03 (dt, 5.5, 3.6) | H1B, H3 | C2 | C3, C4, C1‘ |
| 3 | 73.1 (d) | 72.9 (d) | | 4.14 (dd, 7.3, 5.4) | 4.17 (dd, 10.4, 5.4) | H1B, H2 | C3 | C1, C4, C5 |
| 4 | 134.7 (d) | 134.4 (d) | | 5.47 (dd, 15.1, 7.3) | 5.30 (dd, 15.4, 6.2) | H3, H5, H6 | C4 | C3, C6, C7 |
| 5 | 131.1 (d) | 131.0 (d) | | 5.73 (dt, 15.1, 6.8) | 5.75 (dt, 13.8, 6.3) | H3, H6 | C5 | C3, C6, C7 |
| 6 | 33.9 (t) | 33.8 (t) | 33.8 (t) | 2.02 (m) | 2.08 (m) | H7, H8 | C6 | C4, C5, C7, C8 |
| 7 | 28.9 (t) | 28.8 (t) | 28.8 (t) | 2.08 (m) | 2.08 (m) | H5, H6, H8 | C7 | C4, C5, C6, C8 |
| 8 | 125.0 (d) | 124.9 (d) | | 5.14 (t, 6.8) | 5.19 (m) | H6, H7, H9a, H10 | C8 | C6, C7, C9a, C10, C11 |
| 9 | 136.9 (d) | 136.1 (d) | | | | | | |
| 9a | 16.3 (q) | 16.2 (q) | | 1.60 (s) | 1.64 (s) | H10 | C9a | C7, C8, C11 |
| 10 | 40.9 (t) | 40.8 (t) | 40.8 (t) | 1.98 (m) | 2.09 (m) | H9a | C10 | C8, C9a, C11, C12 |
| 11 | 29.3 (t) | 29.2 (t) | 29.2 (t) | 1.37 – 1.42 (m) | 1.33 (m) | H11 – H (n) | C11 | C11-C(n+1) |
| 12 - 15 | 30.4 – 30.9 (t) | 30.2 – 30.7 (t) | 30.2 – 30.7 (t) | 1.29 (m) | 1.33 (m) | | | |
| 16 | 33.2 (t) | 33.8 (t) | 33.8 (t) | 1.29 (m) | 1.33 (m) | H11 – H (n) | C16 | C11-C(n+1) |
| 17 | 23.8 (t) | 23.7 (t) | 23.7 (t) | 1.29 (m) | 1.33 (m) | H11 – H (n) | C17 | C11-C(n+1) |
| 18 (n) | 14.5 (t) | 14.5 (t) | 14.5 (t) | 1.29 (m) | 1.33 (m) | H11 – H (n) | C (n) | C11-C(n+1) |
| 19 (n+1) | 16.3 (q) | 16.2 (q) | | 0.90 (t, 6.8) | 0.94 (t, 7.0) | H11 – H (n+1) | C (n+1) | C11-C(n+1) |
| **Fatty acid** | | | | | | | | |
| 1‘ | 175.6 (s) | 175.5 (s) | | | | | | |
| 2‘ | 74.3 (d) | 74.1 (d) | | 4.43 (d, 6.4) | 4.48 (d, 6.0) | | C2‘ | C3‘, C4‘, C1” |
| 3‘ | 134.9 (d) | 134.5 (d) | | 5.50 (dd, 15.1, 6.4) | 5.30 (dd, 15.4, 6.2) | H2‘, H4‘, H5‘ | C3‘ | C2‘, C5‘, C6‘ |
| 4‘ | 129.9 (d) | 129.1 (d) | | 5.83 (dt, 15.1, 6.8) | 5.87 (dt, 15.4, 5.5) | H2‘, H5‘ | C4‘ | C2‘, C5‘, C6‘, C7‘ |
| 5‘ | 33.5 (t) | 33.8 (t) | 33.8 (t) | 2.02 (m) | 2.08 (m) | H6‘, H7‘ | C5‘ | C3‘, C4‘, C6‘, C7‘, C8‘ |
| 6‘ - 13‘ | 30.4 – 30.9 (t) | 30.2 – 30.7 (t) | 30.2 – 30.7 (t) | 1.29 (m) | 1.33 (m) | H6‘ – H (n‘) | C6‘ | C6‘-C(n‘+1) |
| 14‘ | 30.4 – 30.9 (t) | 30.2 – 30.7 (t) | 30.2 – 30.7 (t) | 1.29 (m) | 1.33 (m) | H6‘ – H (n‘) | C14‘ | C6‘-C(n‘+1) |
| 15‘ (n‘) | 23.8 (t) | 23.7 (t) | 23.7 (t) | 1.29 (m) | 1.33 (m) | H6‘ – H (n‘) | C (n‘) | C6‘-C(n‘+1) |
| 16‘ (n‘+1) | 14.5 (q) | 14.5 (q) | | 0.90 (t, 6.8) | 0.94 (t, 7.0) | H6‘ – H (n‘) | C (n‘+1) | C6‘-C(n‘+1) |
| **Sugar** | | | | | | | | |
| 1‘‘ | 104.9 (d) | 104.8 (d) | | 4.27 (d, 7.8) | 4.31 (d, 7.8) | H2‘‘ | C1‘‘ | C1, C2“, C3“ |
| 2‘‘ | 75.2 (d) | 75.0 (d) | | 3.20 (dd, 9.3, 7.8) | 3.24 (dd, 9.2, 7.8) | H1‘‘, H3‘‘ | C2‘‘ | C1”, C3”, C4” |
| 3‘‘ | 78.1 (d) | 78.0 (d) | | 3.36 (dd, 9.3) | 3.39 (dd, 9.9, 6.9) | H2‘‘, H4‘‘,H5‘‘ | C3‘‘ | C2”, C4” |
| 4‘‘ | 71.8 (d) | 71.6 (d) | | 3.27 – 3.29 (m) | 3.34 (m) | H2‘‘,H3‘‘,H5‘‘, H6‘‘A,B | C4‘‘ | C2” |
| 5‘‘ | 78.1 (d) | 78.0 (d) | | 3.27 – 3.29 (m) | 3.33 (m) | H2‘‘,H3‘‘,H4‘‘, H6‘‘A,B | C5‘‘ | C3”, C4” |
| 6‘‘ | 62.9 (t) | 62.74 (t) | 62.7 (t) | H6“A: 3.67 (dd, 11.7)<br>H6“B: 3.88 (dd, 11.7) | H6“A: 3.74 (dd, 10.3, 3.1)<br>H6”B: 3.91 (dd, 10.5, 3.3) | H4‘‘, H5‘‘ | C6‘‘ | C3”, C4”, C5” |

The connection of the sphingosine base to the glucose was evident through a prominent three bond coupling of methylene protons (H1) with carbon (C1) of glucose. Likewise the connection of the sphingosine to the fatty acid was indicated by a three bond coupling of methine proton (H2) and a four bond coupling of methylene protons (H1) with carbonyl (C1') of the fatty acid (Figure C17.6).

**Fatty acid portion**

A single low field signal (175.5 ppm) in the $^{13}C$ NMR was assigned to the amide carbonyl of fatty acid (C1') moiety. In addition, a resonance at *δ* 74.1 ppm was indicative of the hydroxylated C2' (Table C17.1, Figure C17.3).

The $^{1}H$ NMR spectrum showed a signal at *δ* 4.48 (d, 6.0 Hz, H2'), this low field resonance was in good accordance with its location on an oxygenated carbon lying between a carbonyl group and a double bond (Figure C17.2).

Another hint on the fatty acid of cerebroside A was the presence of two monoprotonated olefinic carbons [*δ* 134.5 (C3'), 129.1 (C4')]. Furthermore, two olefinic protons [*δ* 5.30 (H3'), 5.87 (H4')] also supported the assignment of this unsaturation.

The allylic protons of H5' were found at *δ* 2.08 in the $^{1}H$ NMR spectrum, whereas the other non allylic methylene protons of the fatty acid along with those of the sphingosine base were overlapped in a large signal (*δ* 1.33). The terminal methyl proton of the fatty acid resonated at *δ* 0.94 ($H_{n'+1}$) (Figure C17.2).

**Sugar portion**

The presence of a sugar (*ß*-D-glucose) was proven by the occurrence of a number of low field protons bound to several hydroxyl-carrying carbons. The $^{1}H$ NMR displayed the anomeric proton at *δ* 4.31 (H1") with a coupling constant of 7.8 Hz, typical for the axial-axial configuration of an anomeric proton in *ß*-D-glucose (Figure C17.2).

Through COSY and HMBC analysis the methine H2" (3.24), methine H3"(3.39), methine H4"(3.34), methine H5" (3.33), and the methylene protons H6" (3.74; 3.91) were assigned (Figure C17.5 and C17.8).

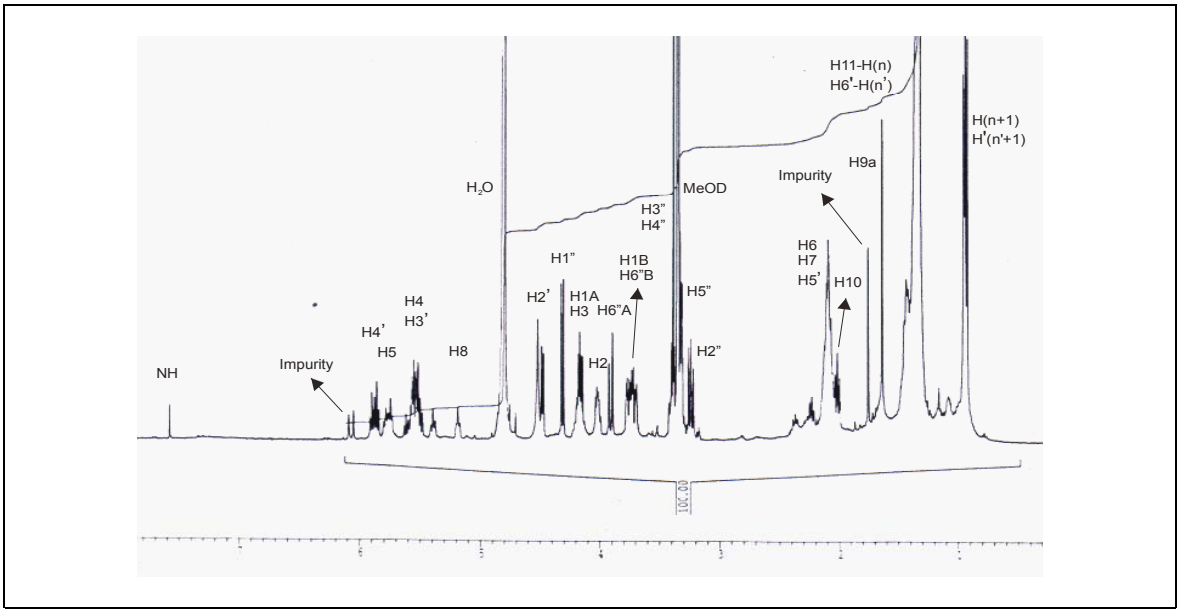



Figure C17.2. $^{1}H$ NMR spectrum of compound **17** (cerebroside)

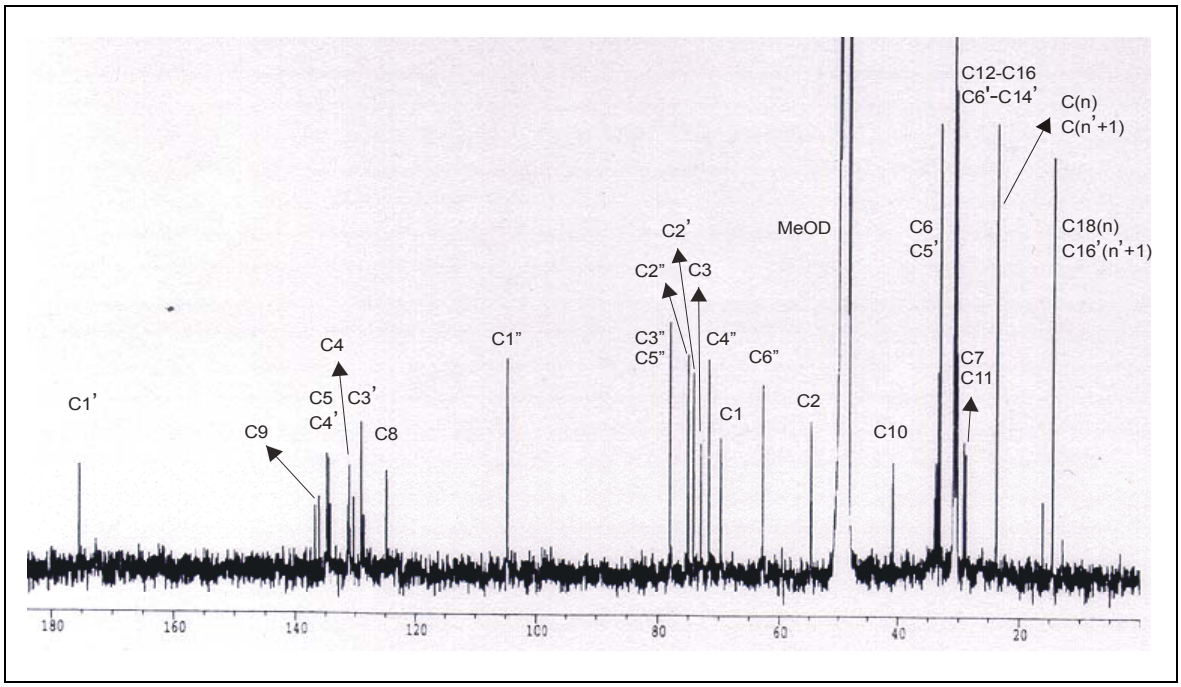



Figure C17.3. $^{13}$C NMR spectrum of compound **17** (cerebroside)

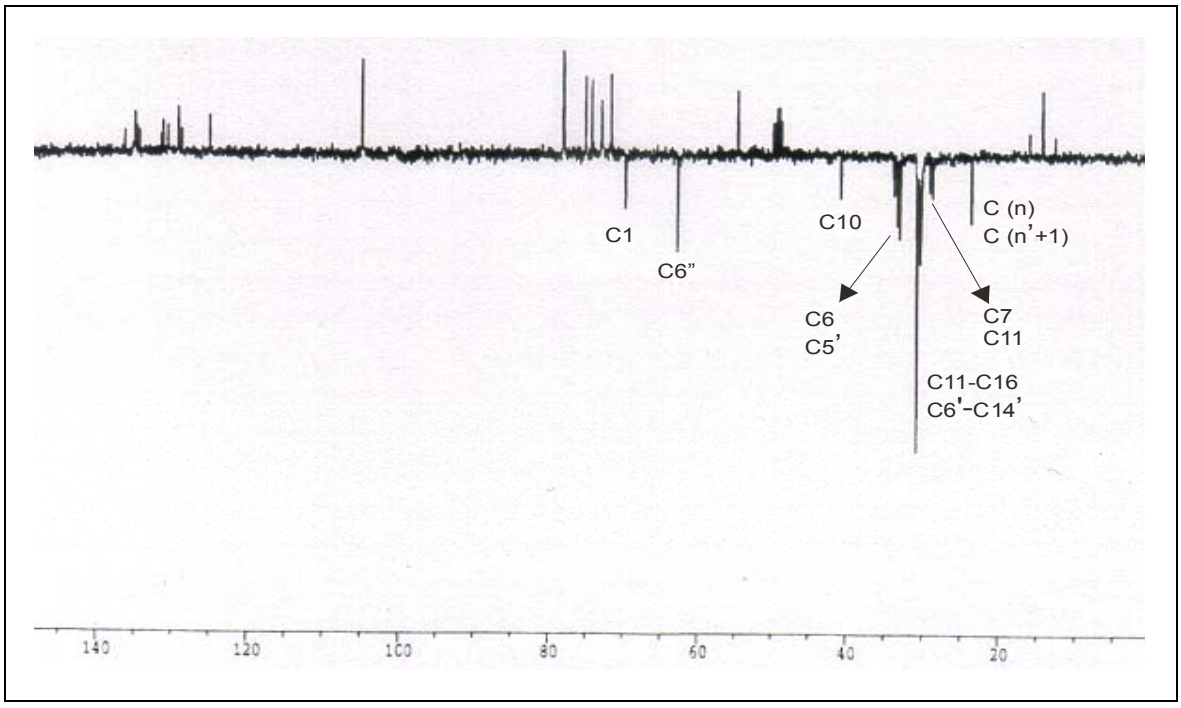



Figure C17.4. DEPT spectrum of compound **17** (cerebroside)

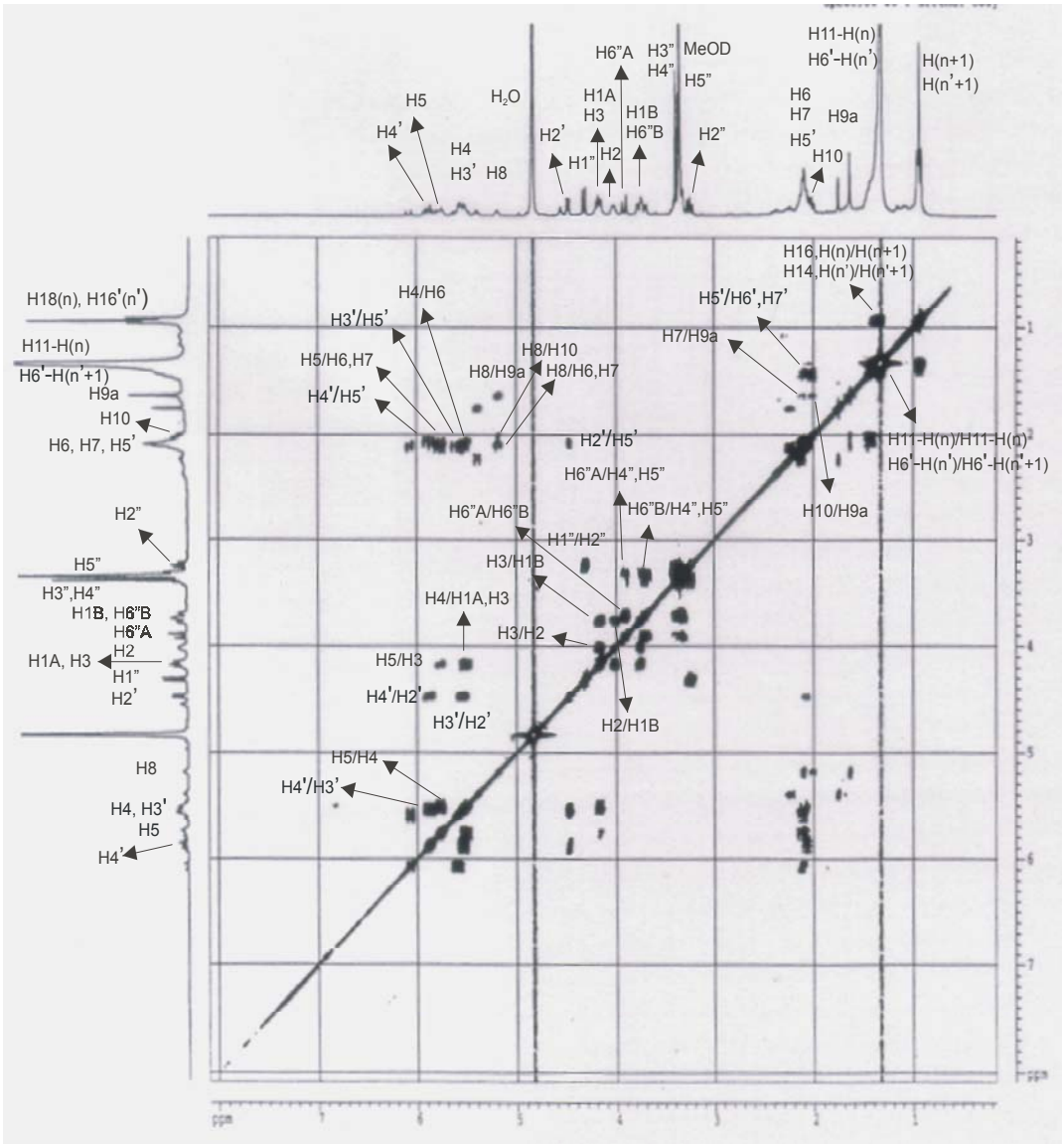



Figure C17.5. COSY spectrum of compound **17** (cerebroside)

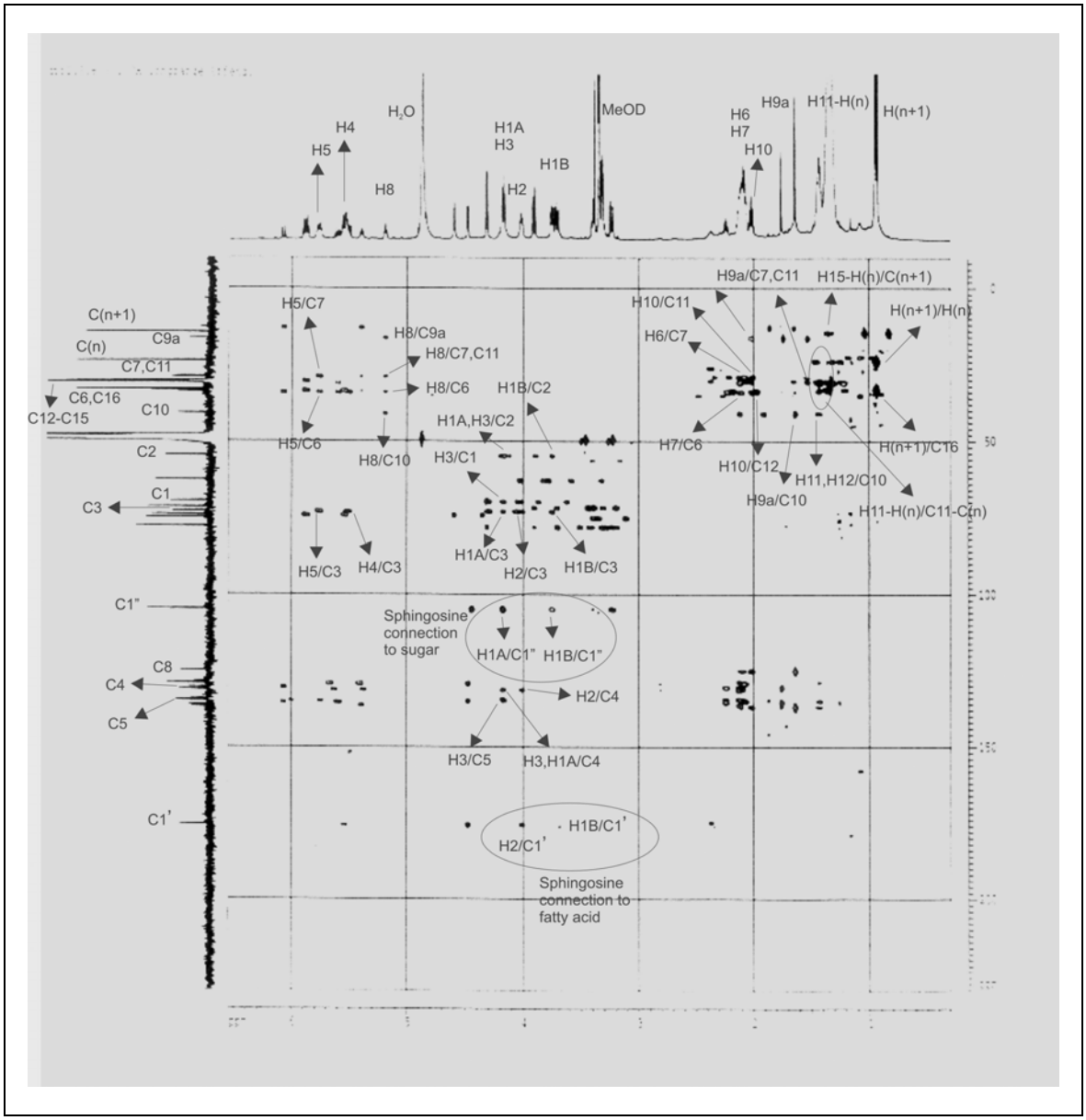



Figure C17.6. Spingosine base HMBC of compound **17** (cerebroside)

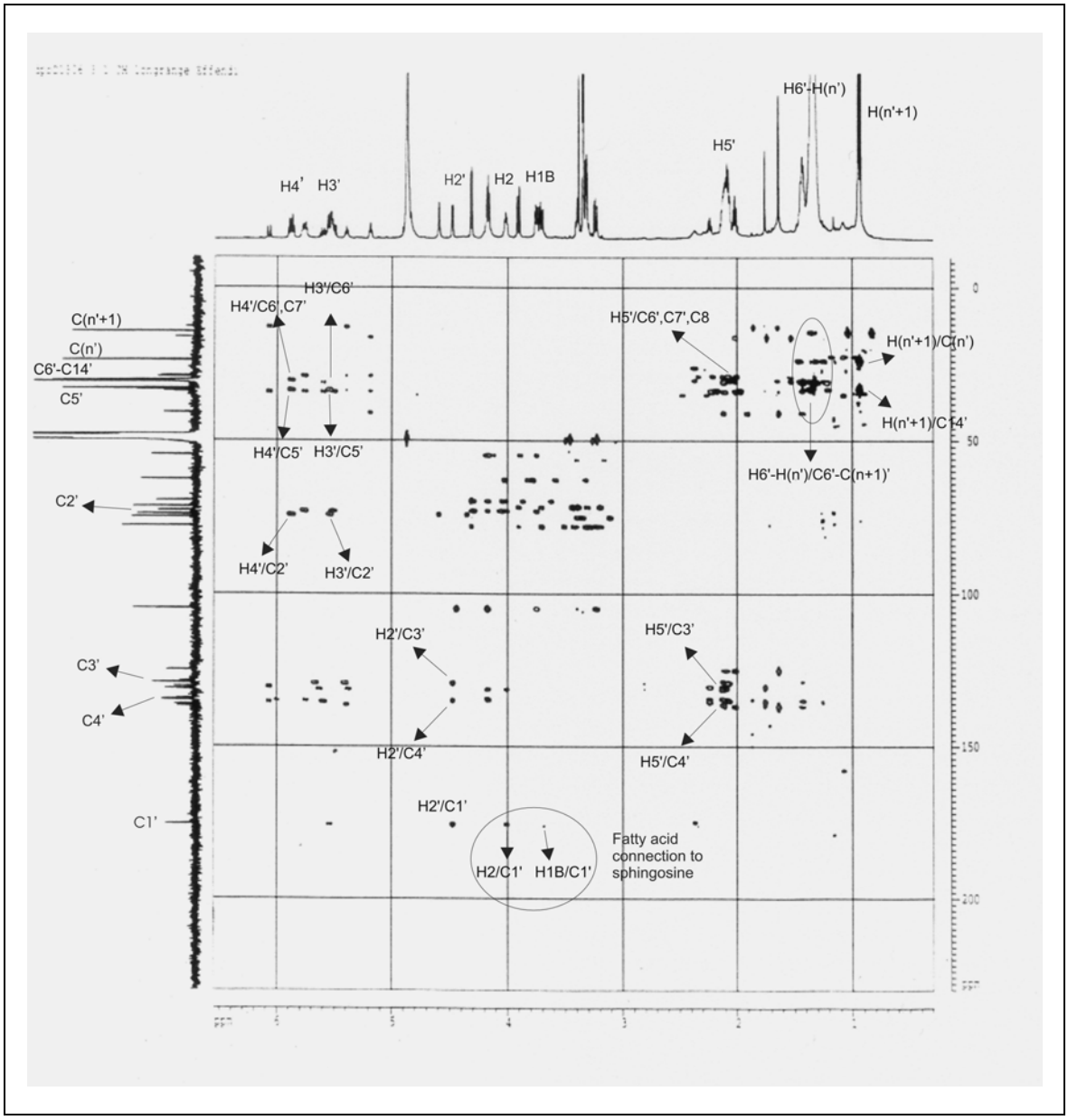



Figure C17.7. Fatty acid HMBC of compound **17** (cerebroside)

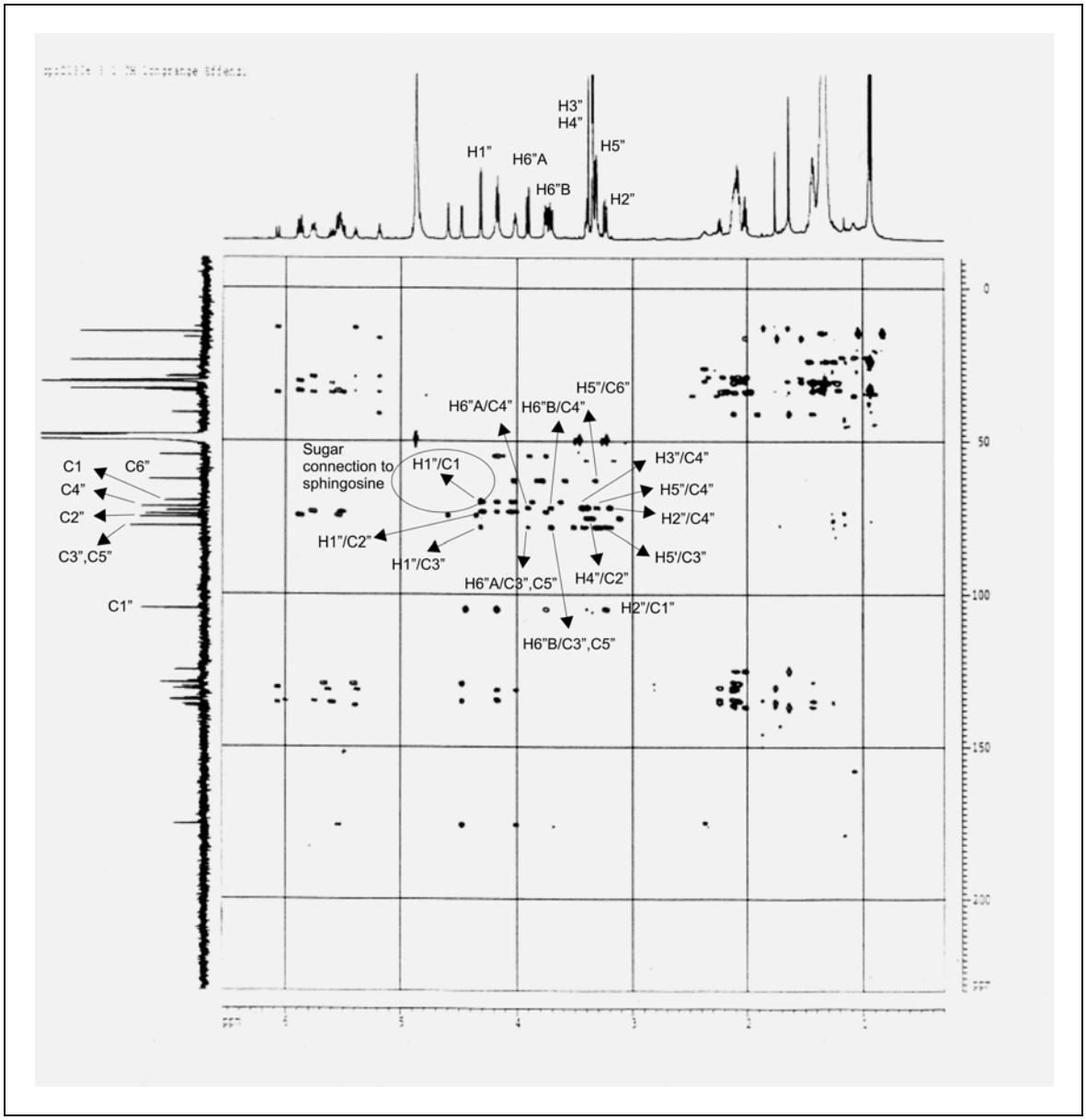



Figure C17.8. Sugar HMBC of compound **17** (cerebroside)

## 3.4. Isolated secondary metabolites of fungus *Lecanicillium evansii* (strain 1)

*L. evansii* strains 1 and 2 isolated from the sponges *Callyspongia* sp. and *Hyrtios* sp., respectively, clearly contained a different variety of secondary metabolites indicated by different peak distribution in the HPLC chromatograms (Figure C18.1). Phenolic compounds dominated the content of strain 1, whereas in strain 2, steroids were the main compounds.

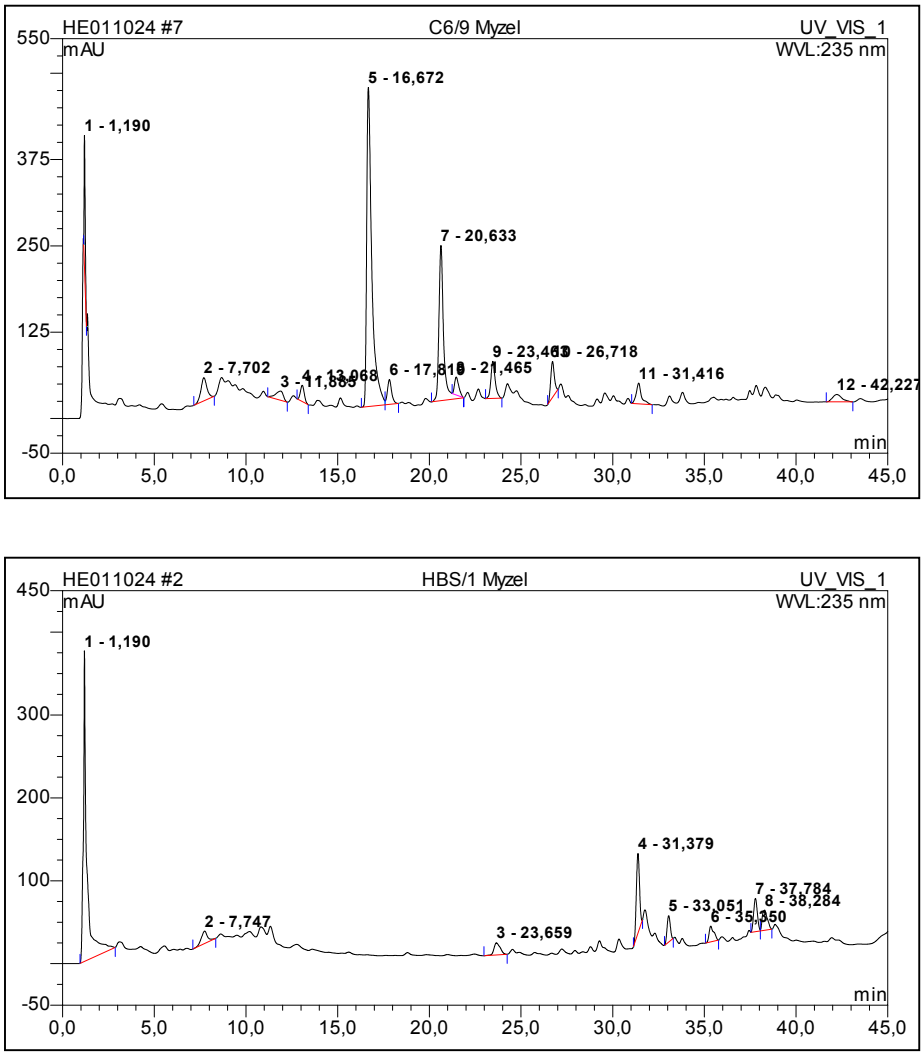



Figure C18.1. Characteristic HPLC chromatograms of *Lecanicillium evansii* (top = strain 1 and bottom = strain 2)

A cultivation experiment of both strains of *L. evansii* in media enriched with or without salt was performed to monitor the growth of both strains. At the onset of the culture period, the media without salt tended to support much faster fungal growth for both strains. After seven days of the culture period, however, both strains indicated no visual difference from the colour and the density of culture. The surface of each flask culture was entirely covered by the white mycellia (Figure C18.2).

The fungi grown in the media without salt contained slightly more diverse secondary metabolites. This was clearly seen in the HPLC charts, particularly crude extract of strain 2 cultivated in the media without salt. It was nevertheless not the case in strain 1.

*L. evansii* (strain 1) day 4,
with salt (left) and without salt (right)

*L. evansii* (strain 1) day 5,
with salt (left) and without salt (right)

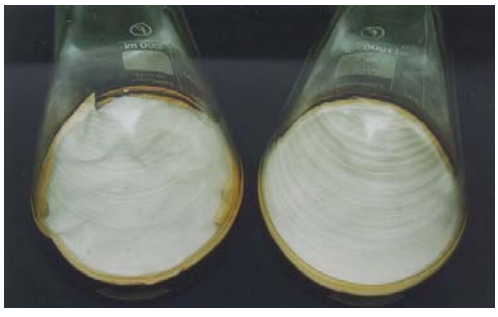
*L. evansii* (strain 1) day 6,
with salt (left) and without salt (right)

*L. evansii* (strain 1) day 7,
with salt (left) and without salt (right)

*L. evansii* (strain 2) day 4,
with salt (left) and without salt (right)

*L. evansii* (strain 2) day 5,
with salt (left) and without salt (right)

*L. evansii* (strain 2) day 6,
with salt (left) and without salt (right)



*L. evansii* (strain 2) day 7,
with salt (left) and without salt (right)

Figure C18.2. *L. evansii* grown under media enriched with or without salt
(top = strain 1, bottom = strain 2)

Seven compounds were successfully isolated from the fungus *L. evansii* (strain 1). The compounds were terphenylin (compound **18**), deoxyterphenylin (compound **19**), terprenin epoxide (compound **20**), terprenin 2 (compound **21**), cyclo(tyrosylprolyl) (compound **22**), acetyl hydroxybenzamide (compound **23**), and 4-hydroxybenzaldehyde (compound **24**).

## 3.4.1. Compound 18 (terphenylin)

Compound **18** (terphenylin) has a molecular formula of $C_{20}H_{18}O_5$ and a molecular weight of 338 g/mol, as derived from the ESI-MS measurement that showed ion peaks at *m*/*z* 339.3 $[M+H]^+$ and 337.5 $[M-H]^-$. Meanwhile, the EI-MS also presented intense ion peaks at *m*/*z* 338 $[M]^+$, 323 $[M-CH_3]^+$ (fragment 1), 308 $[M-2(CH_3)]^+$ (fragment 2), and 292 $[M-CH_3-CH_3O]^+$ (fragment 3) (Figure C18.3).

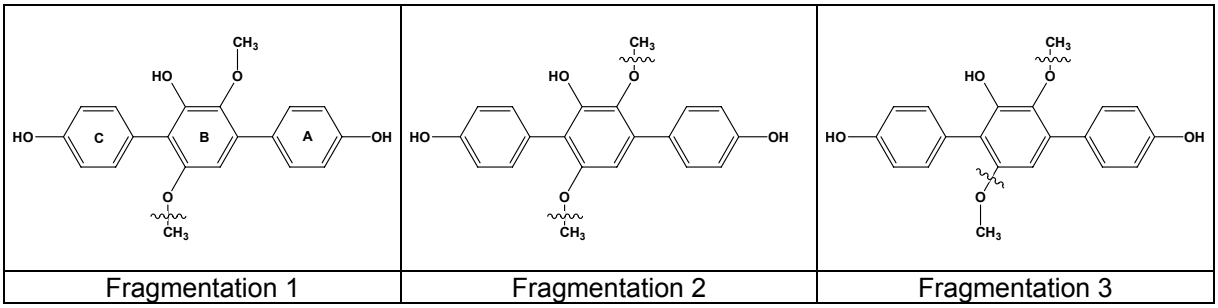



Figure C18.3. Hypothetical fragmentation of compound **18** (terphenylin) in the EI-MS spectrum.

The $^1H$ NMR spectrum of this compound (Table C18.1 and Figure C18.6) disclosed that the molecule consisted of two para-substituted phenyl rings, containing two pairs of ortho-coupled protons, each corresponding to two protons.

These two AA'BB' spin system types in ring A and C were readily assured by the COSY spectrum, through the presence of connections between H2/6 and H3/5 as well as between H2''/6" and H3''/5'', confirmed also by their doublet multiplicity and coupling constants varying from 8.2 and 8.8 Hz (Figure C18.5 and C18.9).

The two pairs of protons exhibited resonances at *δ* 7.43 (H2, H6), 6.84 (H3, H5), 7.09 (H2'', H6''), and 6.75 (H3'', H5''). One singlet proton that appeared at *δ* 6.39 (H6') along with two singlet methoxy groups [*δ* 3.37 (H2'), 3.64 (H5')] suggested a penta-substituted phenyl in ring B. H6' had long range connections with C1, C2', C4', C5' in the HMBC spectrum (Table C18.1 and Figure C18.11).

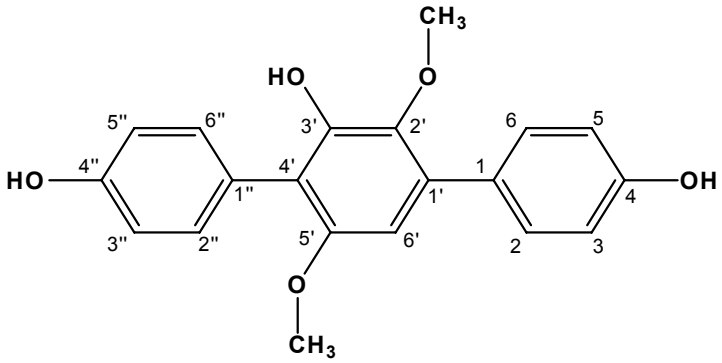
Analysis of the $^{13}C$ NMR spectrum (Table C18.1 and Figure C18.7) pointed out eight non protonated carbons [*δ* 128.50 (C1), 116.12 (C3), 157.14 (C4), 133.15 (C1'), 140.00 (C2'), 118.70 (C4'), 154.93 (C5'), 125.00 (C1''), 157.14 (C4'')], nine methine carbons [*δ* 131.05 (C2, C6), 116.12 (C3, C5), 108.00 (C6'), 133.15 (C2'', C6"), 115.45 (C3'', C5'')], and two methoxy carbons (*δ* 60.75 and 56.43), proving the carbon number of the empirical formula (20 carbons). The $^1H \rightarrow {}^{13}C$ direct connections of the HMQC spectrum conspicuously connected protons to their respective carbons (Figure C18.8).

The assignment of the carbon at position 3' as the most downfield one (*δ* 154.93) in ring B was due to the hydroxyl group directly attached to this position. C2' (*δ* 140.00) was located more upfield than C5' (*δ* 154.93), because of the shielding effect of the hydroxyl group at position 3' (ortho to C2').

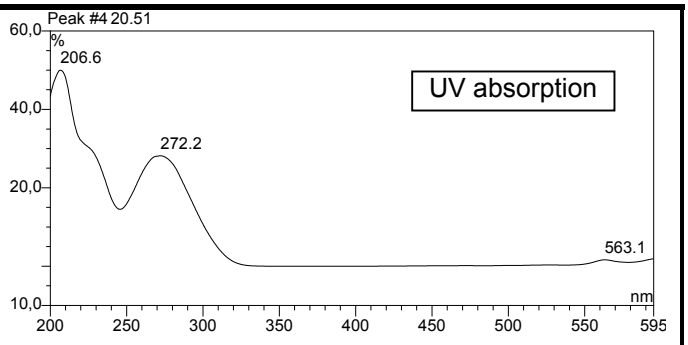
The same argumentation applied to C6' (*δ* 108.00) (ortho to C5') and to C4' (*δ* 118.70) (ortho to both C3' and C5') which was located more upfield than C1' (*δ* 133.15). There are ten possible structures of terphenylin with regard to different positions of the methoxy groups in rings A, B , and C as depicted in Figure C18.4.

## Terphenylin (compound 18)

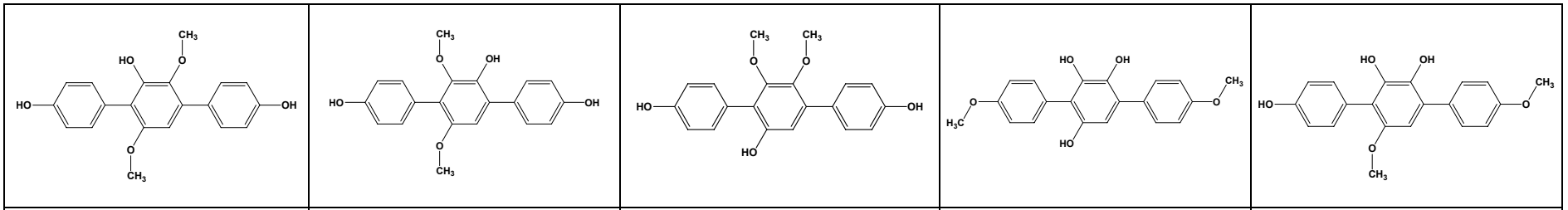
| | | |
|---|---|---|
| CAS Registry Number | : | 52452-60-5 |
| Characteristic | : | white powder |
| Formula | : | $C_{20}H_{18}O_5$ |
| Molecular Weight | : | 338 g/mol |
| Amount | : | 4.9 mg |
| Source | : | *Lecanicillium evansii* (Strain 1) derived from *Callyspongia* sp. |




| | | |
|---|---|---|
| Retention Time | : | 20.57 min |
| $R_f$ | : | 0.56 |
| Fluorescence, 254 nm | : | + |
| 366 nm | : | - |
| Anisaldehyde/$H_2SO_4$ | : | - |




EI-MS (*m/z*, rel. int.) : 338 $[M]^+_{(100)}$, 323 $[M\text{-}CH_3]^+_{(31.5)}$, 308 $[M\text{-}2(CH_3)]^+_{(12.3)}$, 292 $[M\text{-}CH_3\text{-}CH_3O]^+_{(15.0)}$, 263 $[C_{17},H_{11}O_3]^+_{(6.2)}$



| possibility 1 | possibility 2 | possibility 3 | possibility 4 | possibility 5 |
|---|---|---|---|---|
| possibility 6 | possibility 7 | possibility 8 | possibility 9 | possibility 10 |

Figure C18.4. Possible positions of methoxy groups in compound **18** (terphenylin)

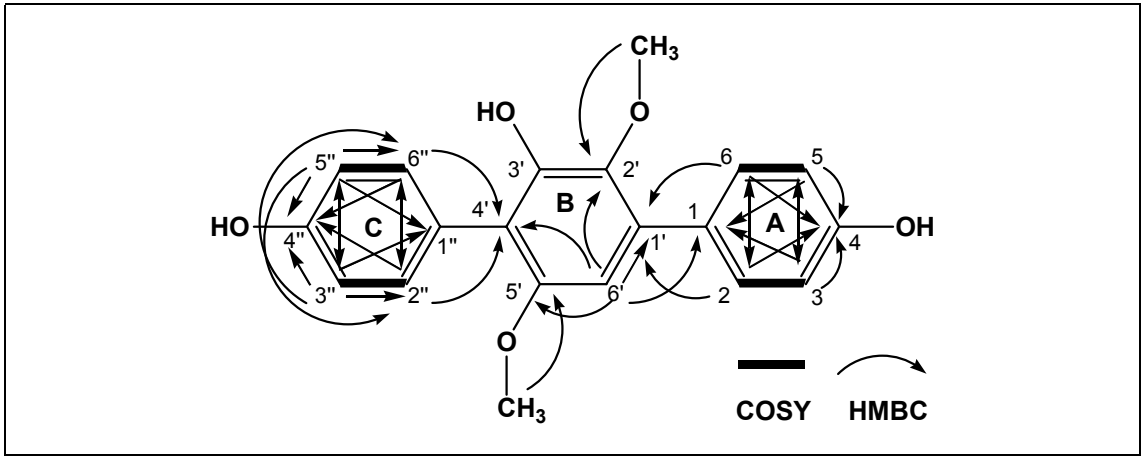



Figure C18.5. COSY and HMBC correlations of compound **18** (terphenylin) in DMSO

Table C18.1. NMR data of compound **18** (terphenylin)

| Position | $\delta$ $^{13}$C (ppm) ( in MeOD) | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) (MeOD) | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) (in DMSO) | COSY (in MeOD) | HMQC direct (in MeOD) | HMBC (in MeOD) | HMBC (in DMSO) |
|---|---|---|---|---|---|---|---|
| 1 | 128.50 (s) | | | | | | |
| 2 | 131.05 (d) | 7.45 (d, 8.8) | 7.43 (d, 8.6) | H3 | C2 | C4, C6, C1‘, | C4, C6, C1‘ |
| 3 | 116.12 (d) | 6.84 (d, 8.8) | 6.84 (d, 8.6) | H2 | C3 | C2, C4, C5, C6 | C1, C4, C5, |
| 4 | 157.14 (s) | | | | | | |
| 5 | 116.12 (d) | 6.84 (d, 8.8) | 6.84 (d, 8.6) | H6 | C5 | C2, C3, C4, C6 | C1, C3, C4 |
| 6 | 131.05 (d) | 7.45 (d, 8.8) | 7.43 (d, 8.6) | H5 | C6 | C2, C4, C1‘ | C2, C4, C1‘ |
| 1‘ | 133.15 (s) | | | | | | |
| 2‘ | 140.00 (s) | | | | | | |
| 2‘ - $OCH_3$ | 60.75 (q) | 3.37 (s) | 3.37 (s) | | C2‘ ($OCH_3$) | C2‘ | C2’ |
| 3‘ | 154.93 (d) | | | | | | |
| 4‘ | 118.70 (s) | | | | | | |
| 5‘ | 149.00 (s) | | | | | | |
| 5‘ - $OCH_3$ | 56.43 (q) | 3.67 (s) | 3.64 (s) | | C5‘ ($OCH_3$) | C5‘ | C5’ |
| 6‘ | 108.00 (d) | 6.44 (s) | 6.39 (s) | | | | C1, C1’, C2’, C4‘, C5’ |
| 1“ | 125.00 (s) | | | | | | |
| 2“ | 133.15 (d) | 7.18 (d, 8.2) | 7.09 (d, 8.6) | H3“ | C2“ | C4‘, C1”, C4“, C6“ | C4‘, C4’’, C6’’ |
| 3“ | 115.45 (d) | 6.79 (d, 8.2) | 6.75 (d, 8.6) | H2“ | C3“ | C1‘, C4“, C5“ | C1”, C2’’, C4’’, C5’’, C6’’ |
| 4“ | 157.14 (s) | | | | | | |
| 5“ | 115.45 (d) | 6.79 (d, 8.2) | 6.75 (d, 8.6) | H6“ | C5“ | C1‘, C3“, C4“ | C1’, C2’’, C3’’, C4’’, C6’’ |
| 6“ | 133.15 (d) | 7.18 (d, 8.2) | 7.09 (d, 8.6) | H5“ | C6“ | C4‘, C1”, C2“, C4“ | C4‘, C2’’, C4” |

Nuclear overhauser effect (NOE) experiments were carried out to determine the correct structure. Through irradiation of methoxy protons at lower field ($\delta$ 3.67), the isolated singlet ring proton at position 6‘ appeared more intense (Figure C18.10). Thus, the isolated ring proton must be located next to methoxy group [$\delta$ 3.67 (H5‘)]. This fact excluded the formula of terphenylin possibilities 3, 4, 7, 8, 9, and 10.

Examination of the HMBC spectrum aided the selection of the structural formula out of the four remaining possibilities. The isolated singlet proton (H6‘) displayed correlation to carbons that in turn were correlated to the two methoxy groups. This fact immediately ruled out possibilities 5 and 6 (Figure C18.11).

A close inspection at the HMBC spectrum revealed that both correlations from H6’ to C2’ and C5’ were indeed strong correlations, thus ruling out the presence of a four bond correlation (as required by possibility 2). Thus, compound **18** was identified as the known terphenylin (possibility 1).

The $^{1}$H NMR data of compound **18** were in good accordance with those of terphenylin isolated from *Aspergillus candidus* (Takahashi *et al.*, 1976). The chemical shift of $^{1}$H NMR data difference only varied in a narrow range of 0.01 – 0.09 ppm.

No microbiological activity of this compound was encountered.

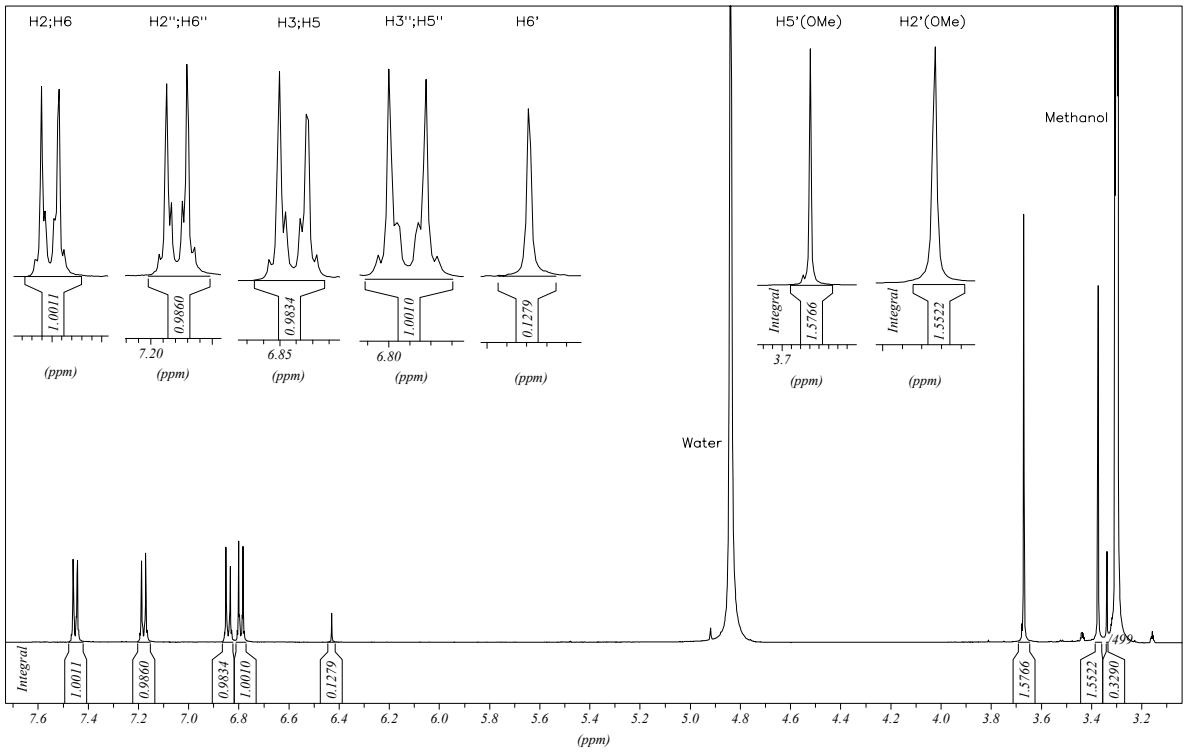



Figure C18.6. $^{1}$H NMR of compound **18** (terphenylin) in MeOD

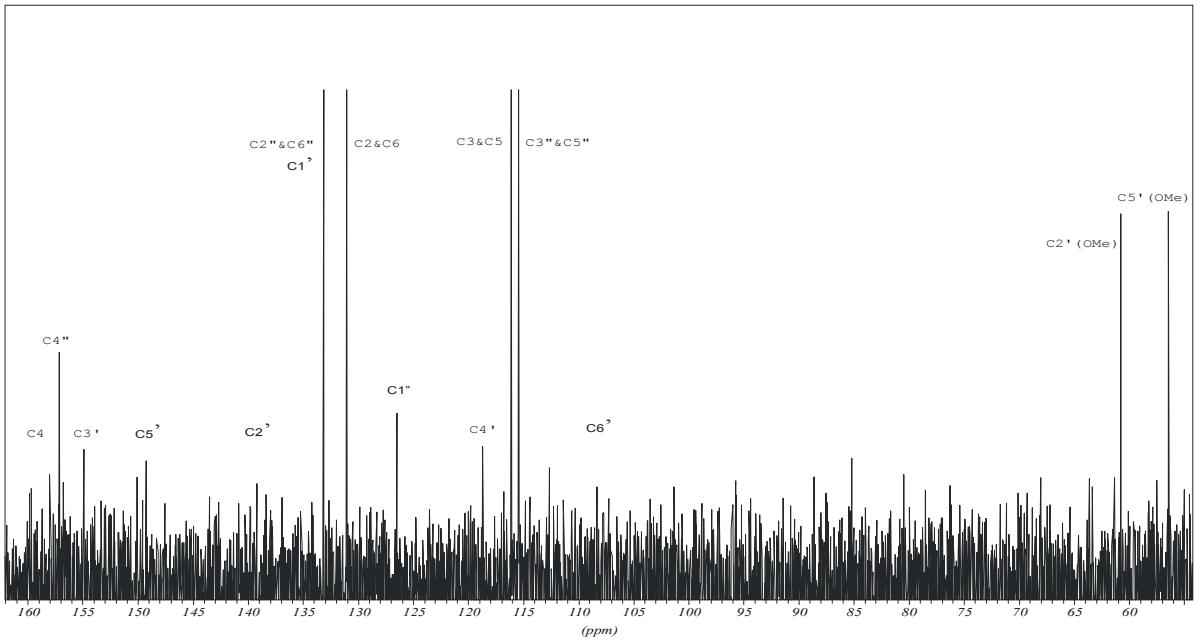



Figure C18.7. $^{13}$C NMR of compound **18** (terphenylin) in MeOD

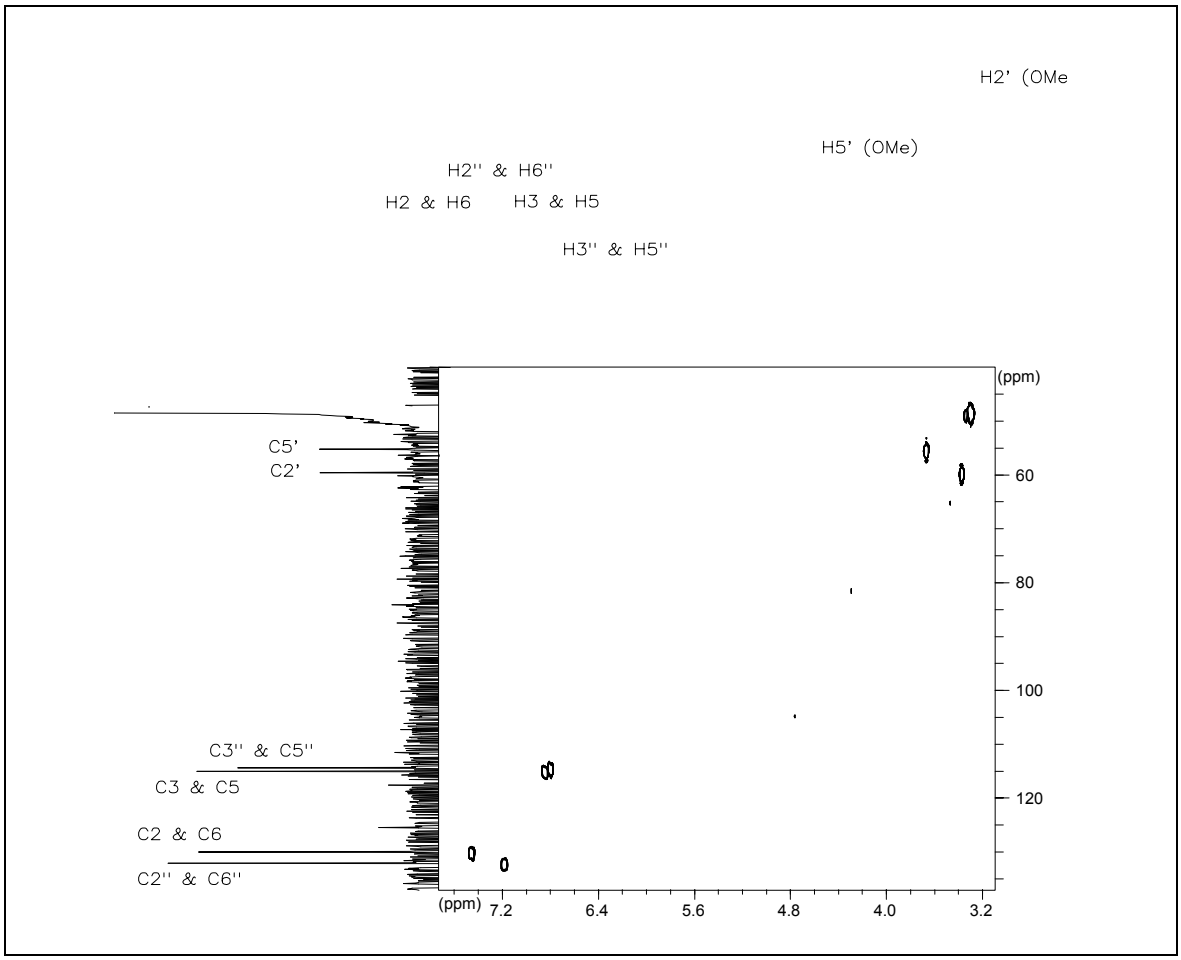



Figure C18.8. HMQC spectrum of compound **18** (terphenylin) in MeOD

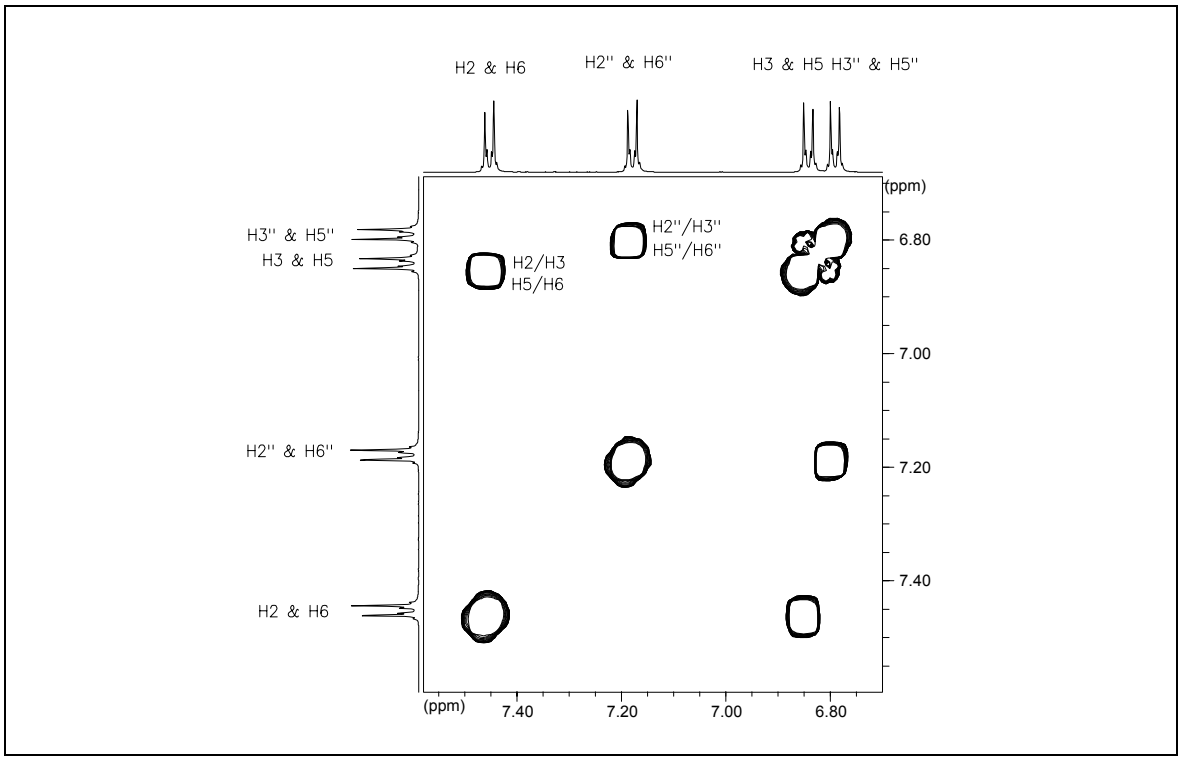



Figure C18.9. COSY spectrum of compound **18** (terphenylin) in MeOD

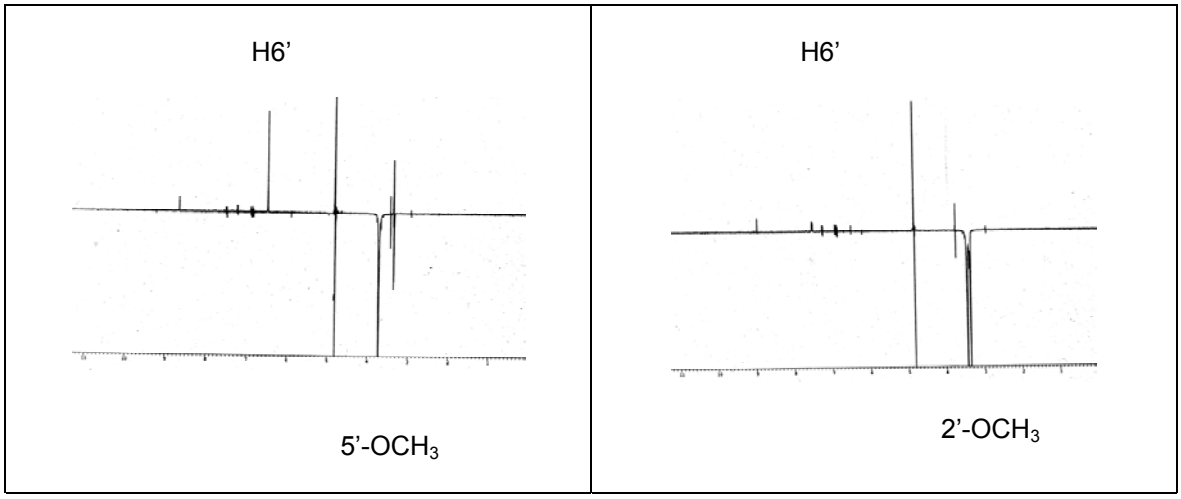



Figure C18.10. NOE spectrum of compound **18** (terphenylin) in MeOD

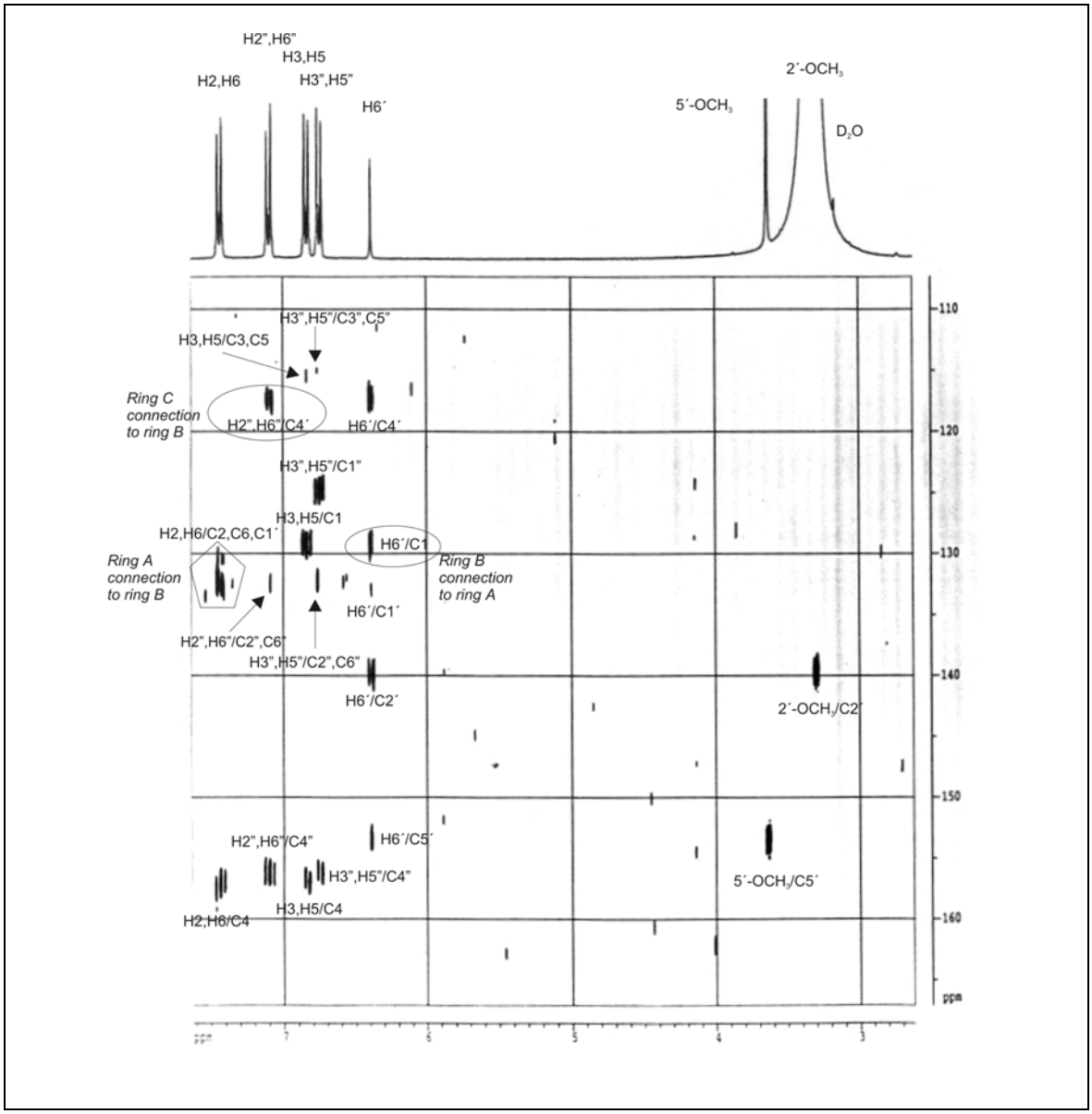



Figure C18.11. HMBC spectrum of compound **18** (terphenylin) in DMSO

## 3.4.2. Compound 19 (deoxyterphenylin)

The ESI-MS spectrum of compound **19** presented intense ions at *m*/*z* 323.4 $[M+H]^+$(positive) and 321.8 $[M-H]^-$ (negative) determining a molecular weight of 322 g/mol and suggesting a molecular formula of $C_{20}H_{18}O_4$ which corresponded to deoxyterphenylin. The EI-MS high resolution spectrum displayed a molecular weight of 322.1195 g/mol.

This was also confirmed by several ion peaks in the EI-MS spectrum at *m*/*z* 322 $[M]^+$, 307 $[M-CH_3]^+$ (fragment 1) 292 $[M-2(CH_3)]^+$ (fragment 2), and 276 $[M-CH_3-CH_3O]^+$ (fragment 3) (Figure C19.1).

The fragmentation was similar to that of terphenylin, with the compound losing one methyl group (fragment 1), followed by another methyl and one methoxy group (fragment 2), and the loss of both methoxy groups (fragment 3).

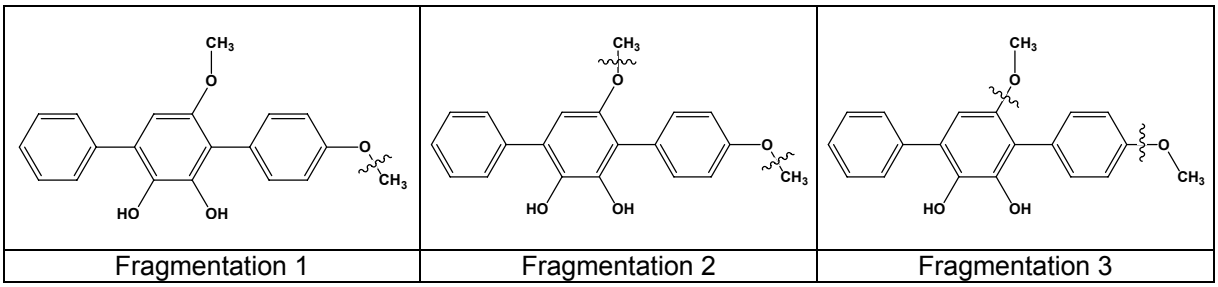



Figure C19.1. Hypothetical fragmentation of compound **19** (deoxyterphenylin) in the EI-MS spectrum.

Through analysis of chemical shifts, signal multiplicities, and coupling constants (7.25 – 8.82 Hz) of protons displayed in the $^1$H NMR spectrum, one mono-substituted benzene (ring A) [$\delta$ 7.61 (H2, H6), 7.42 (H3, H5), 7.34 (H4)], and one para-substituted phenyl (ring C) [ $\delta$ 7.19 (H2‘‘, H6‘‘), 6.80 (H3‘‘, H5‘‘)] (Table C19.1, Figure C19.2) were identified.

The COSY correlations between H2’’/6’’ and H3’’/5’’ and H6’’ pointed out an AA’BB’ spin system and indicated the presence of a para-substituted phenyl in ring C. The second spin system of a mono-substituted benzene in ring A was also distinctly observed in the COSY spectrum (Figure C19.2).

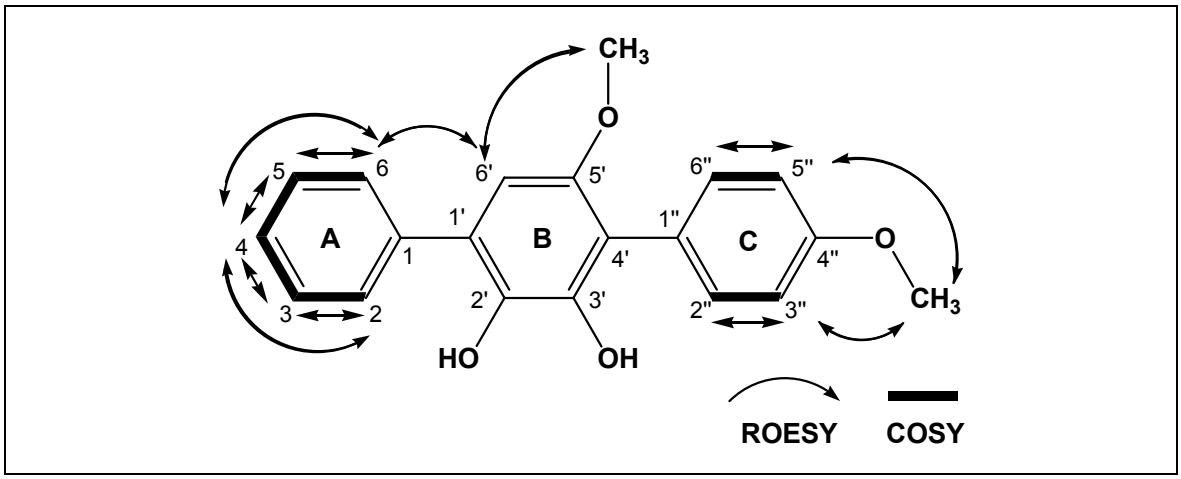



Figure C19.2. COSY and ROESY correlations of compound **19** (deoxyterphenylin)

One penta-substituted phenyl (ring B) was clearly determined by one isolated singlet at $\delta$ 6.46 (H6‘]. In addition, two methoxy singlets ($\delta$ 3.68 and 3.36) were observed.

To determine the positions of the methoxy groups, NOE experiments were performed. Irradiation of methoxy protons ($\delta$ 3.68, H5’) resulted in the intensification of the singlet proton (H6’), assuring that the position of isolated singlet proton (H6‘) was adjacent to this methoxy group (H5‘).

Irradiation of the other methoxy group protons at the higher field ($\delta$ 3.36, H4”) however did not cause any observable effects on other protons. Thus, a 2D-ROESY experiment was conducted (Figure C19.8). Again a correlation between H6’ and 5’-$OCH_3$ was clearly observed, while the correlation of 4”-$OCH_3$ and H3”/5” allowed for positioning of the second methoxy function. Thus, the structure of compound **19** (deoxyterphenylin) was established as depicted in Figure C19.2.

## Deoxyterphenylin (compound 19)

CAS Registry Number : -

Characteristic : white powder

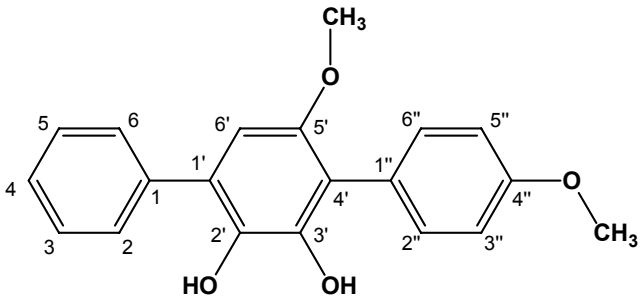
Formula : $C_{20}H_{18}O_4$

Molecular Weight : 322 g/mol

Amount : 4.3 mg

Source : *Lecanicillium evansii* (strain 1) derived from *Callyspongia* sp.

new compound

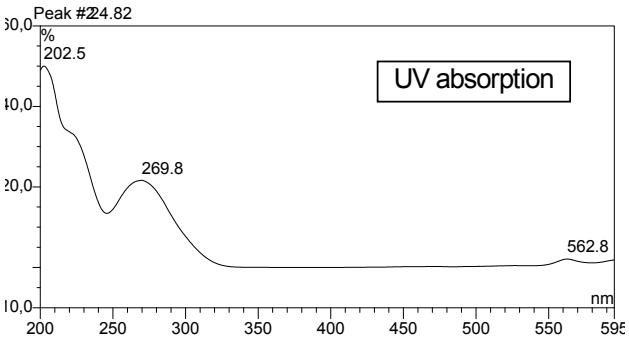



Retention Time : 24.76 min

$R_f$ : 0.66

Fluorescence, 254 nm : +
366 nm : -

Anisaldehyde/$H_2SO_4$ : -




EI-MS (*m/z*, rel. int.) : 322 $[M]^+_{(100)}$, 340 $[M+H_2O]^+_{(31.5)}$, 307 $[M-CH_3]^+_{(6.2)}$
292 $[M-2(CH_3)]^+_{(15.6)}$, 276 $[M-CH_3-CH_3O]^+_{(16.8)}$

Table C19.1. NMR data of compound **19** (deoxyterphenylin)

| Position | $\delta$ $^1$H (ppm), multiplicity (*J* in Hz) (in MeOD) | $\delta$ $^1$H (ppm), multiplicity (*J* in Hz) (in DMSO) | COSY (H → H) (in MeOD) | ROESY (H → H) (in DMSO) |
|---|---|---|---|---|
| 1 | | | | |
| 2 | 7.61 (d, 7.3) | 7.61 (d, 8.5) | H3 | H3, H4 |
| 3 | 7.42 (t, 7.3, 7.9) | 7.46 (t, 7.4, 7.8) | H2, H4 | H2, H4 |
| 4 | 7.34 (t, 7.3, 7.6) | 7.37 (t, 7.3, 7.5) | H3, H5 | H3, H5 |
| 5 | 7.42 (t, 7.3, 7.9) | 7.46 (t, 7.4, 7.8) | H4, H6 | H4, H6 |
| 6 | 7.61 (d, 7.3) | 7.61 (d, 8.5) | H5 | H4, H5, H6' |
| 1‘ | | | | |
| 2‘ | | | | |
| 3‘ | | | | |
| 4‘ | | | | |
| 5‘ | | | | |
| 5‘-$OCH_3$ | 3.68 (s) | 3.65 (s) | | |
| 6‘ | 6.46 (s) | 6.45 (s) | | H5'-$OCH_3$ |
| 1“ | | | | |
| 2“ | 7.19 (d, 8.8) | 7.11 (d, 8.5) | H3“ | H3” |
| 3“ | 6.80 (d, 8.8) | 6.76 (d, 8.6) | H2“ | H2” |
| 4“-$OCH_3$ | 3.36 (s) | 3.30 (s) | | H3”, H5” |
| 5“ | 6.80 (d, 8.8) | 6.76 (d, 8.6) | H6“ | H6” |
| 6“ | 7.19 (d, 8.8) | 7.11 (d, 8.5) | H5“ | H5” |

This compound has not been quoted in the literature before. Takahashi *et al*. (1976) isolated a related deoxyterphenylin from *Aspergillus candidus*. Both methoxy groups of this compound, however, are located in positions 2' and 5', whereas the methoxy groups of the new compound **19** are bound to positions 5' and 4” instead.

Antimicrobial assay conducted using this newly elucidated compound **19** demonstrated no biological activity.

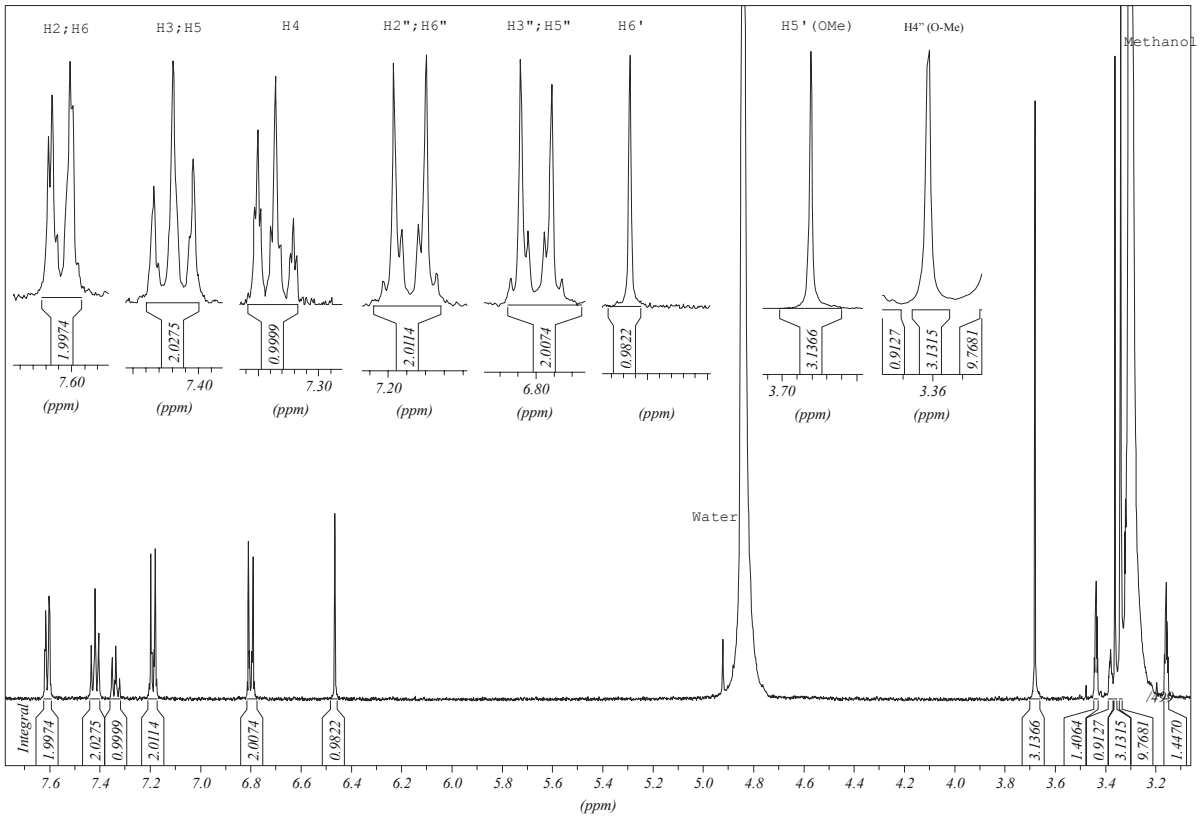



Figure C19.4. $^{1}$H NMR spectrum of compound **19** (deoxyterphenylin) in MeOD

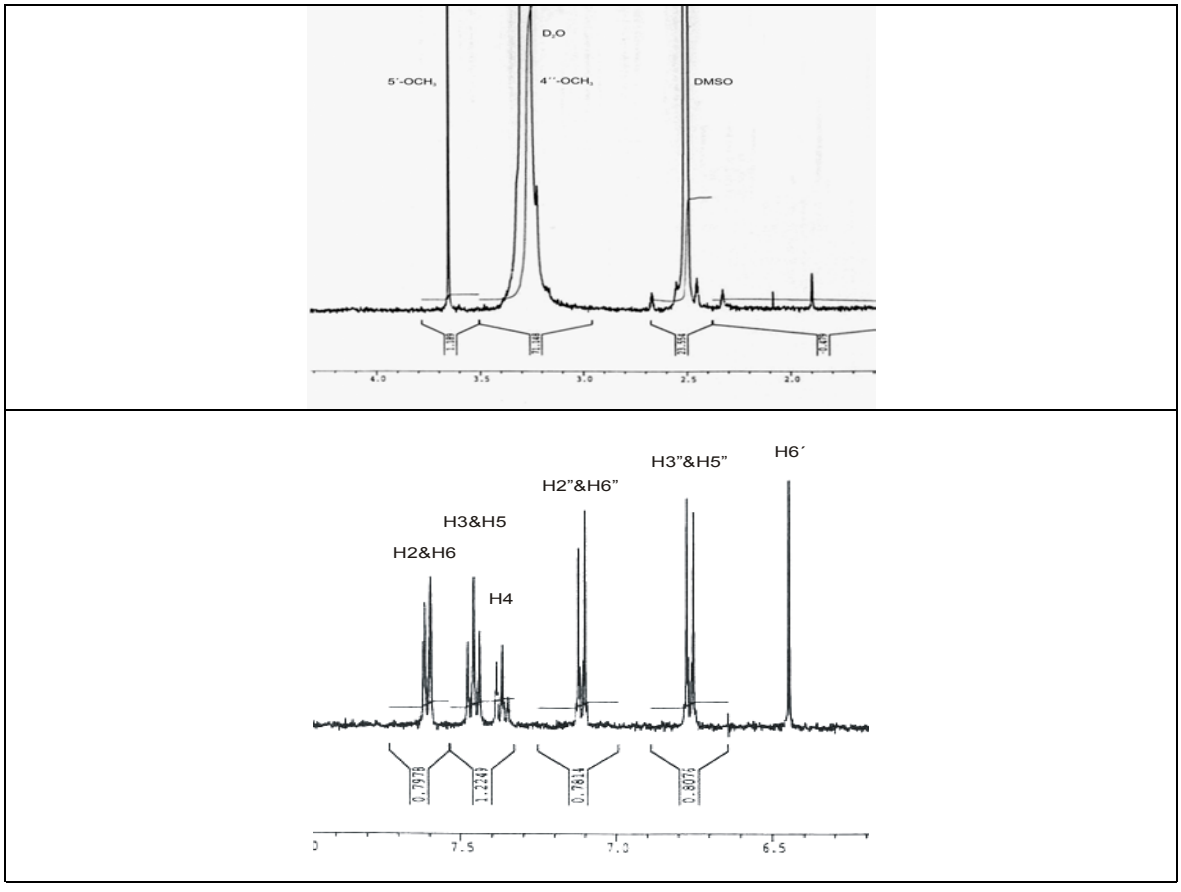



Figure C19.5. $^{1}$H NMR spectrum of compound **19** (deoxyterphenylin) in DMSO

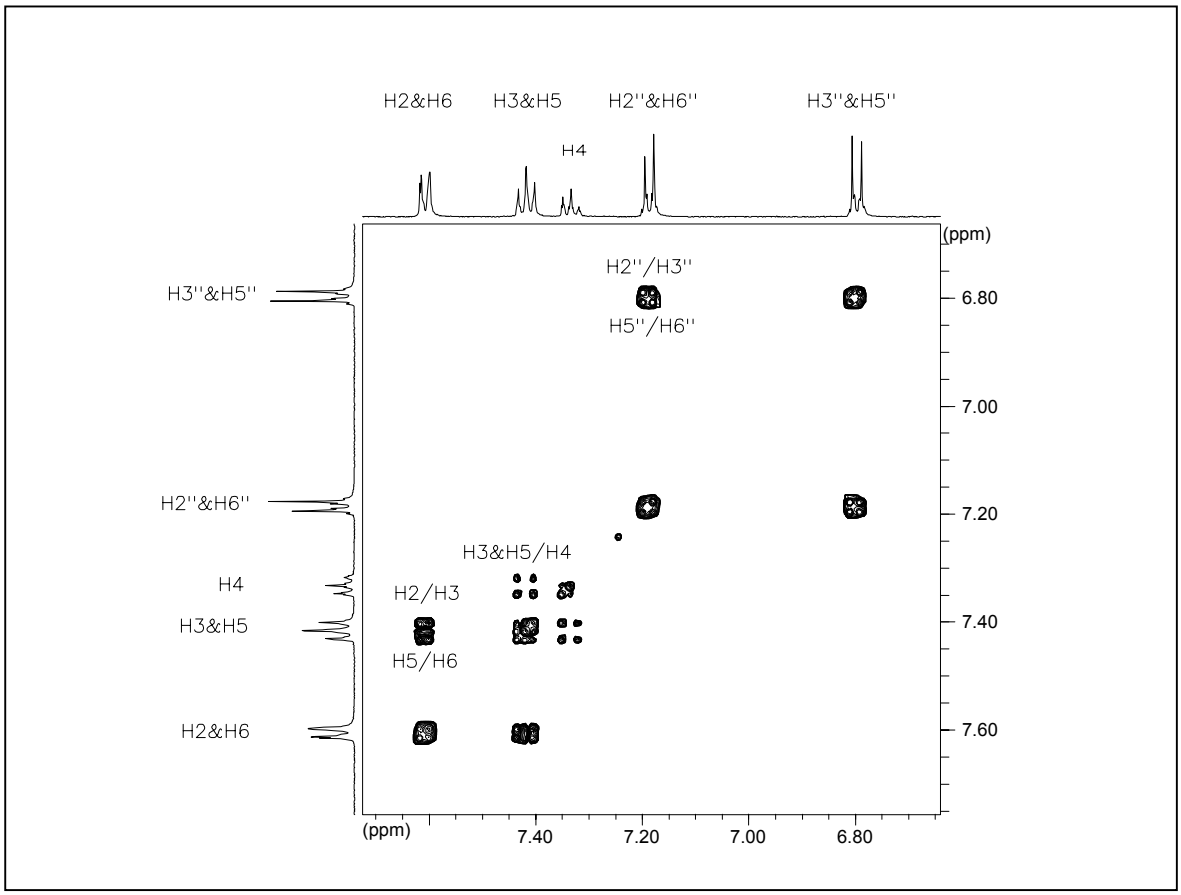



Figure C19.6. COSY spectrum of compound **19** (deoxyterphenylin)

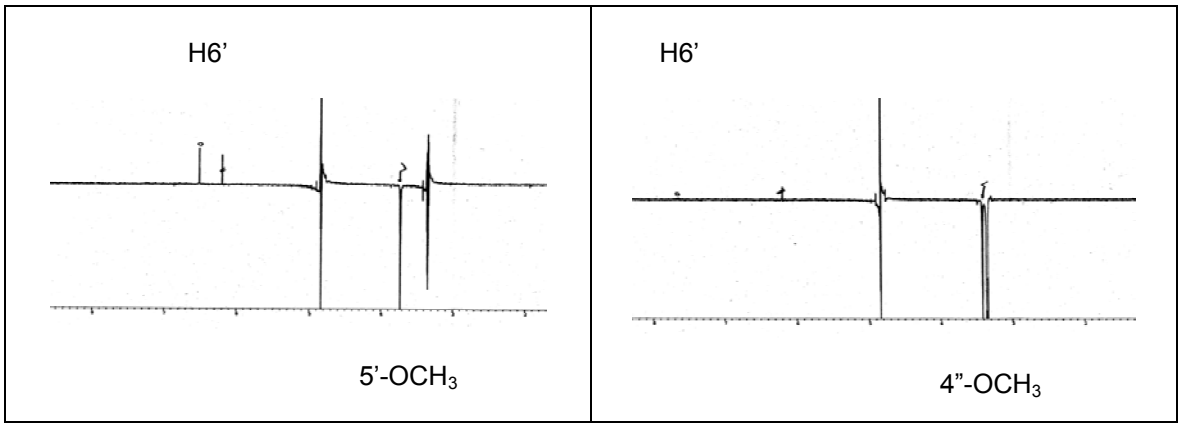



Figure C19.7. NOE spectrum of compound **19** (deoxyterphenylin)

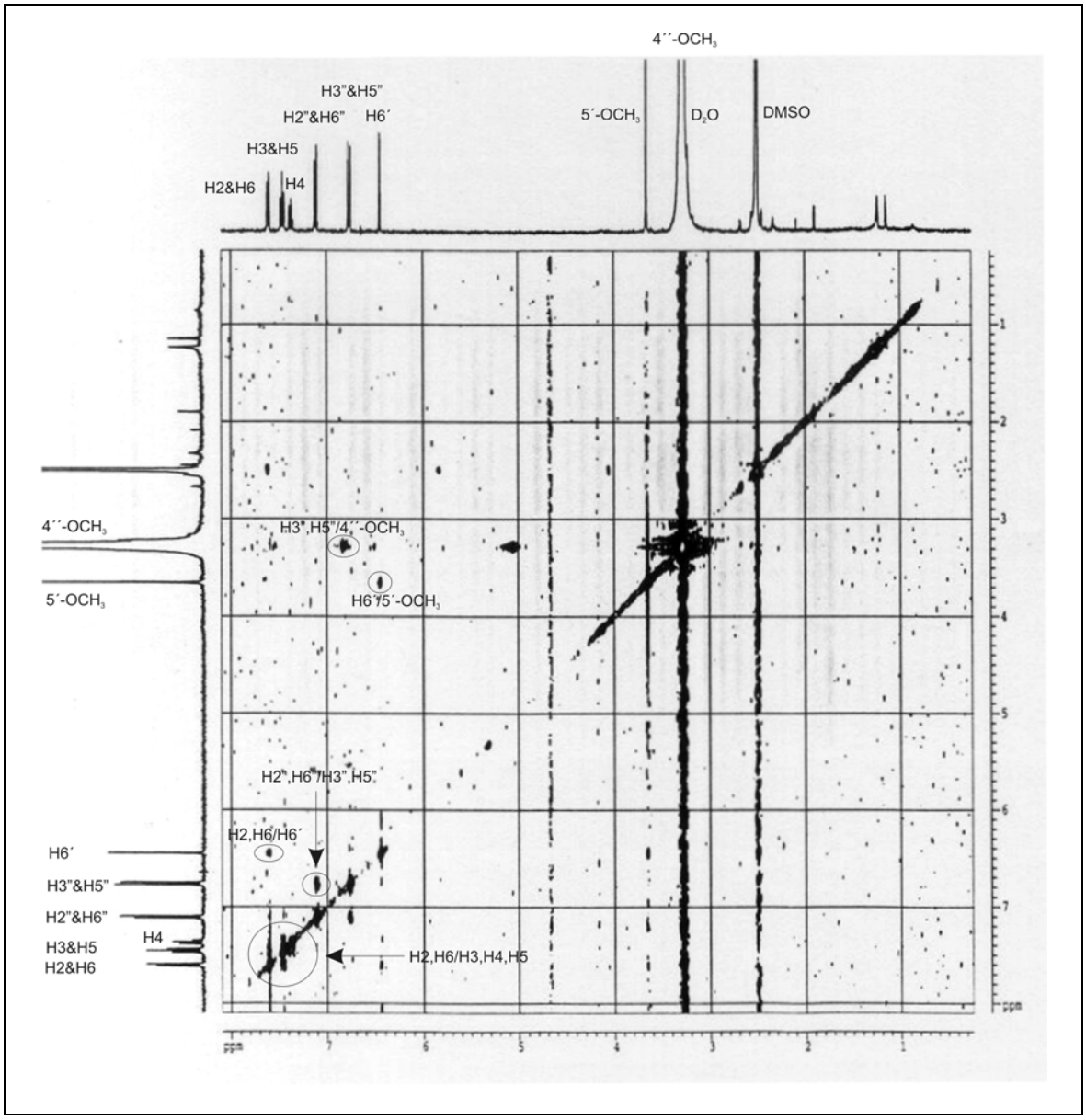


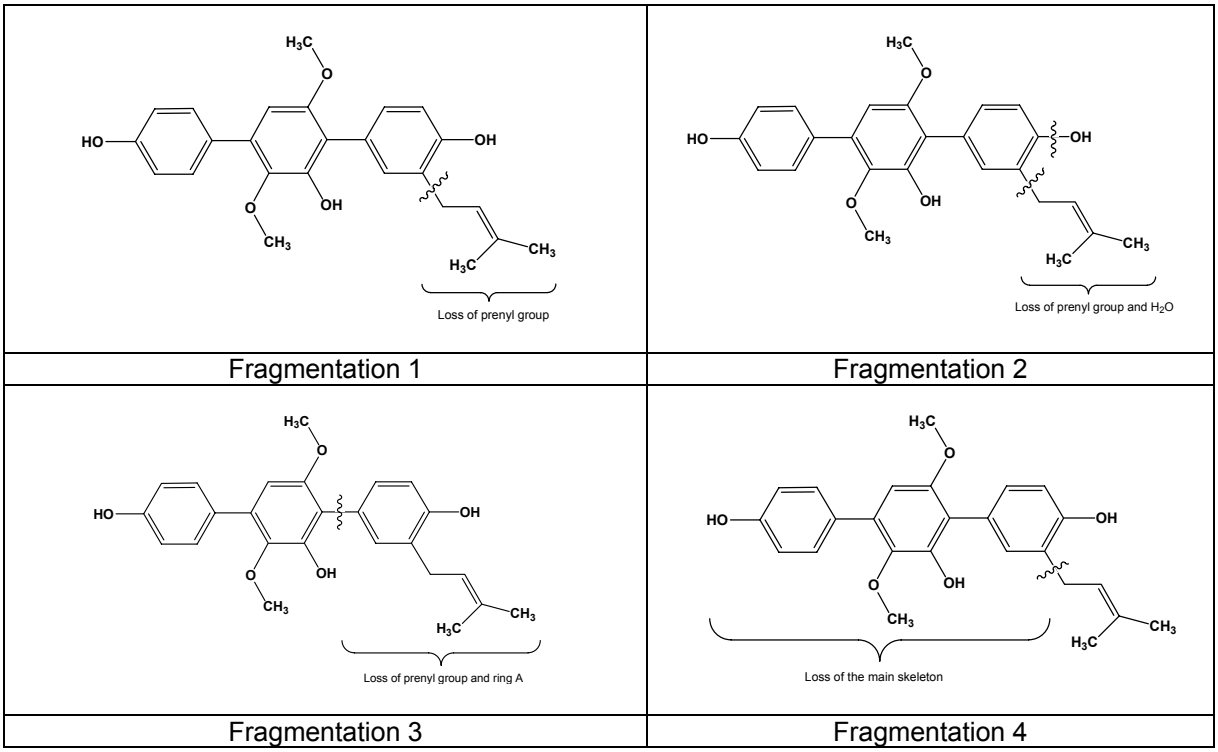

Figure C19.8. ROESY spectrum of compound **19** (deoxyterphenylin) in DMSO

## 3.4.4. Compound 20 (terprenin 2)

The ESI-MS spectrum of compound **20** showed the ion peak at *m*/*z* 407$[M+H]^+$ suggesting a molecular weight of 406 g/mol and a molecular formula of $C_{25}H_{26}O_5$.

The molecular weight was also confirmed by the EI-MS spectrum with several intense ion peaks at *m*/*z* 406 $[M]^+$, 340 [M-prenyl group]$^+$ (fragment 1), 322 [M-prenyl group-$H_2O$]$^+$ (fragment 2), 177 [M-prenyl group-ring A]$^+$ (fragment 3), 69 [M of prenyl group]$^+$ (fragment 4) (Figure C20.1).

The ESI-MS high resolution spectrum displayed an ion peak at *m*/*z* 429.1673 $[M+Na]^+$, supporting the assignment of molecular weight and molecular formula.




| Fragmentation 1 | Fragmentation 2 |
|---|---|
| Fragmentation 3 | Fragmentation 4 |

Figure C20.1. Hypothetical fragmentation of compound **20** (terprenin 2) in the EI-MS spectrum.

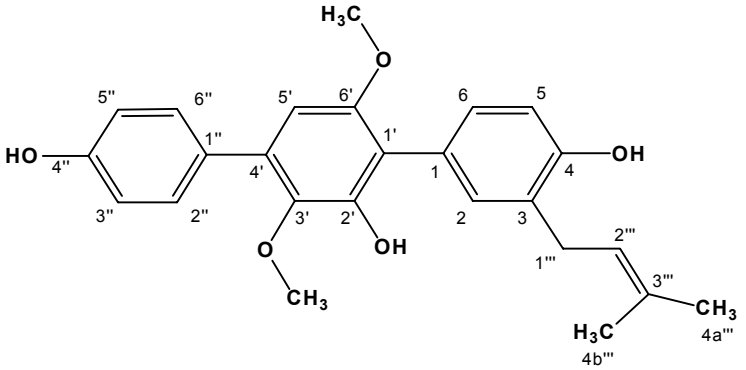
The $^1$H NMR spectrum analysis (Table C20.1, $CDCl_3$) indicated that terprenin 2 had a prenyl side chain [$\delta$ 3.41(H1‘‘‘/aliphatic proton directly attached to benzene ring), 5.40(H2‘‘‘/olefinic proton), 1.78(H4a‘‘‘), 1.77(H4b‘‘‘)], two methoxy groups [$\delta$ 3.46 (3’-$OCH_3$) and 3.74 (6’-$OCH_3$)], three phenolic hydroxyl groups [$\delta$ 5.15 (4-OH), 5.88 (2’-OH), 4.86 (4”-OH)], and contained three phenyl rings with three different types of spin systems.

The first spin system was AA'BB' in ring C [ $\delta$ 7.53 (d, *J*=6.6, H2”,H6”), 6.92 (d, *J*=6.5, H3”, H5”) ], the second spin system was the penta-substituted phenyl (ring B) possessing only one proton [$\delta$ 6.45 (s, H5‘)], and the third spin system was the ABX in ring A consisting of three protons [$\delta$ 7.25 (d, *J*=2.1, H2), 6.89 (d, *J*=8.7, H5), 7.22 (dd, *J*=2.1, 8.5, H6)] (Table C20.2, Figure C20.4). The assignment of these three spin systems was also clearly verified by the proton signals displayed in the $^1$H NMR spectrum recorded in MeOD and acetone. The correlations appearing in the COSY spectrum supported the three spin systems in the phenyl rings (Figure C20.3).

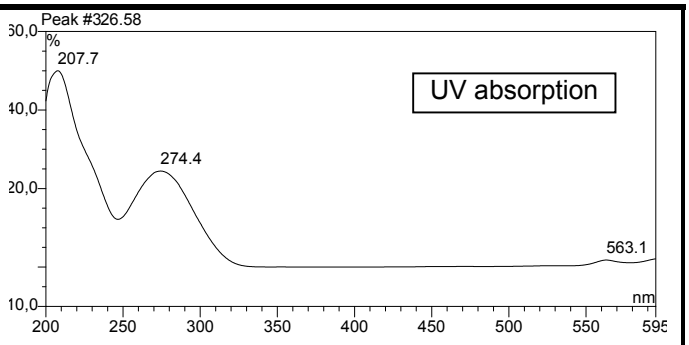
## Terprenin 2 (compound 20)

| | |
|---|---|
| Chemical abstract | : - |
| Characteristic | : white powder |
| Formula | : $C_{25}H_{26}O_5$ |
| Molecular Weight | : 406 g/mol |
| Amount | : 5.0 mg |
| Source | : *Lecanicillium evansii* (strain 1) derived from *Callyspongia* sp. |

new compound




| | |
|---|---|
| Retention Time | : 26.52 min |
| $R_f$ | : 0.72 |
| Fluorescence, 254 nm | : + |
| 366 nm | : + |
| Anisaldehyde/$H_2SO_4$ | : + |




EI-MS (*m*/*z*, rel. int.) : 406 $[M]^+$ (100), 340 [M-prenyl group]$^+$ (10.3), 69 [prenyl group]$^+$ (75.1)

Table C20.1. NMR data of compound **20** (terprenin 2)

| Position | $\delta$ $^{13}$C(ppm) (in $CDCl_3$) | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) (in MeOD) | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) (in Acetone) | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) (in $CDCl_3$) | COSY (H → H) (in $CDCl_3$) | HMBC (H → C) (in $CDCl_3$) | ROESY (H → H) (in Acetone) |
|---|---|---|---|---|---|---|---|
| 1 | 129.94 (s) | | | | | | |
| 2 | 132.36 (d) | 6.95 (d, 1.9) | 7.13 (d, 2.5) | 7.25 (d, 2.1) | H6 | C1, C4, C1‘‘‘ | H1‘‘‘ |
| 3 | 126.40 (s) | | | | | | |
| 4 | 153.72 (s) | | | 5.15 (OH) | | C3, C4, C5 | |
| 5 | 115.50 (s) | 6.67 (d, 8.2) | 6.83 (d, 8.2) | 6.89 (d, 8.7) | H6 | C3, C4, C6, C1‘‘‘ | H6 |
| 6 | 125.23 (d) | 6.91 (dd, 1.9; 8.2) | 7.05 (dd, 1.9; 8.2) | 7.22 (dd, 2.1; 8.5) | H2, H5 | C1, C2 | H5 |
| 1‘ | 115.64 (s) | | | | | | |
| 2‘ | 147.31 (s) | | | 5.88 (OH) | | C1’, C2’, C3’ | |
| 3‘ | 138.50 (s) | | | | | | |
| 3‘-$OCH_3$ | 56.04 (q) | 3.35 (s) | 3.38 (s) | 3.46 (s) | | C3’ | |
| 4‘ | 130.00 (s) | | | | | | |
| 5‘ | 103.83 (d) | 6.34 (s) | 6.46 (s) | 6.45 (s) | H6’($OCH_3$) | C6, C1’, C2’, C3’, C6’, C1“, C2’’, C6’’ | H6’($OCH_3$), |
| 6‘ | 153.72 (s) | | | | | | |
| 6‘-$OCH_3$ | 60.73 (q) | 3.66 (s) | 3.69 (s) | 3.74 (s) | H5’ | C6’ | H5’ |
| 1“ | 130.16 (s) | | | | | | |
| 2“ | 132.12 (d) | 7.36 (d, 8.2) | 7.50 (d, 6.3) | 7.53 (d, 6.6) | H3’’ | C4’, C4’’, C6’’ | H3’’, H4’’,H5’’ |
| 3“ | 115.41 (d) | 6.75 (d, 8.2) | 6.92 (d, 6.3) | 6.92 (d, 6.5) | H2’’ | C1’’, C4’’, C5’’ | |
| 4“ | 155.13 (d) | | | 4.86 (OH) | | C3’’, C4’’, C5’’ | |
| 5“ | 115.41 (d) | 6.75 (d, 8.2) | 6.92 (d, 6.3) | 6.92 (d, 6.5) | H6’’ | C1’’, C3’’,C4’’ | |
| 6“ | 132.28 (d) | 7.36 (d, 8.2) | 7.50 (d, 6.3) | 7.53 (d, 6.6) | H5’’ | C4’, C2’’, C4’’ | H5’, H3’’, H4’’, H5’’ |
| 1‘‘‘ | 30.02 (t) | 3.35 | 3.36 (d, 6.9) | 3.41 (d, 7.3) | H2’’’, H4a’’’, H4b’’’ | C2, C3, C4, C2’’’, C3’’’ | H2, H2’’’ |
| 2‘‘‘ | 121.90 (d) | 5.35 (m) | 5.39 (m) | 5.40 (m) | H1’’’, H4a’’’, H4b’’’ | C4a’’’ | H1’’’ |
| 3‘‘‘ | 135.00 (s) | | | | | | |
| 4a‘‘‘ | 25.85 (q) | 1.72 (s) | 1.71 (s) | 1.78 (s) | H1’’’, H2’’’, H4b’’’ | C2’’’, C3’’’, C4b’’’ | H2’’’ |
| 4b‘‘‘ | 17.93 (q) | 1.71 (s) | 1.70 (s) | 1.77 (s) | H1’’’, H2’’’, H4a’’’ | C2’’’, C3’’’, C4a’’’ | H2’’’ |

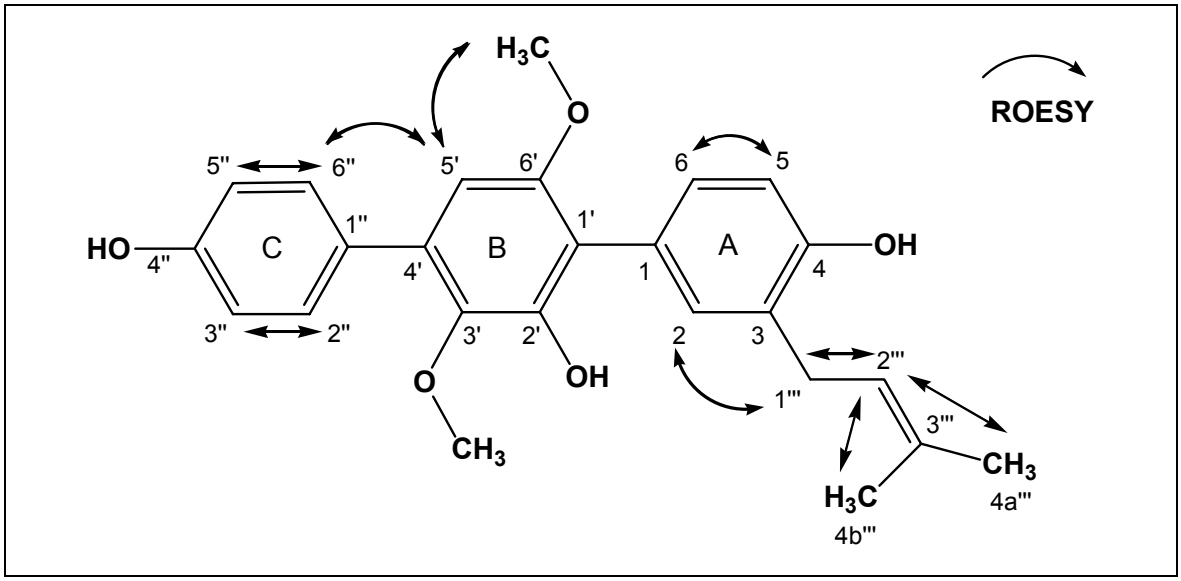



Figure C20.2. ROESY correlations of compound **20** (terprenin 2) in Acetone

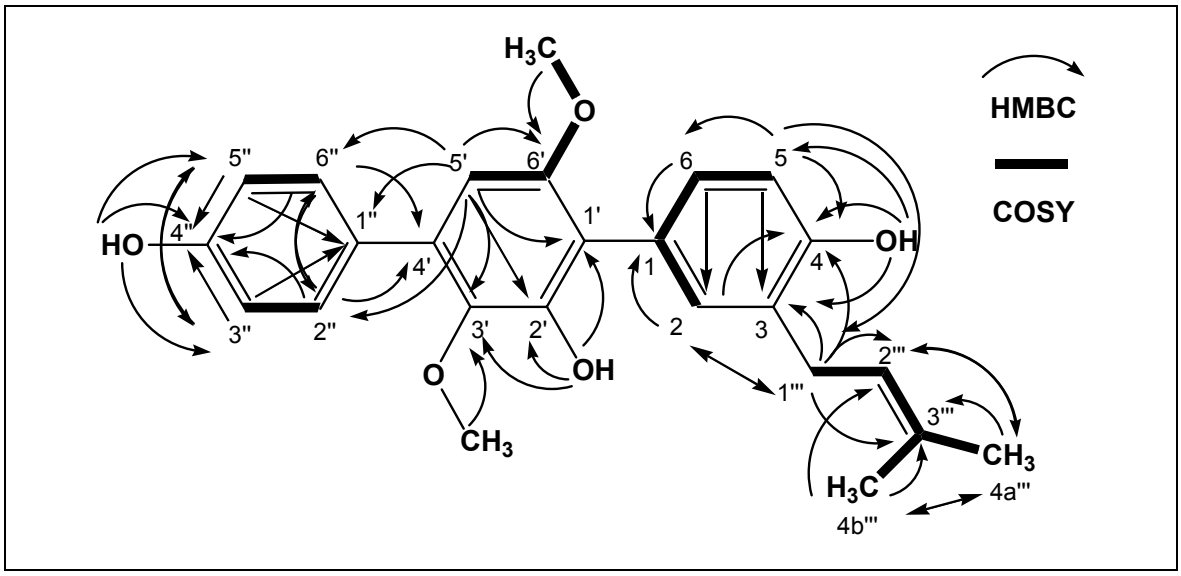



Figure C20.3. COSY and HMBC correlations of compound **20** (terprenin 2) (in $CDCl_3$)

The placement of the two methoxy groups in ring B (positions 3' and 6') was identified by close inspection of the HMBC spectrum. Similar to compound **18**, also in compound **20** H5' displayed correlations to both oxygenated aromatic carbons ($\delta$ 138.50 and $\delta$ 153.72) that in turn were correlated to the methoxy signals. Following the same argumentation as outlined above in detail, the positions of the two methoxy groups were identified as depicted in Figure C20.3.

Long range correlation in the HMBC spectrum confirmed the position of prenyl group through a number of prominent connections between H1''' and C3, C2, C4 (Figure C20.3 and C20.7).

The correlation of H1''' and H2 in the ROESY spectrum also assured the location of the prenyl group. Likewise, the isolated proton of H5' displayed correlations with H2"/H6", and 6'-$OCH_3$, thus supporting the structure of terprenin 2 (Figure C20.2 and C20.8).

This compound has not been reported in the literature. A related compound with the prenyl group attached to the benzene ring at the position four through an oxygen bridge (terprenin) was reported by Kamigauchi *et al*. (1998) and Stead *et al*. (1999).

There was no microbiological activity of this compound identified after carrying out a number of anti-microbiological assays.

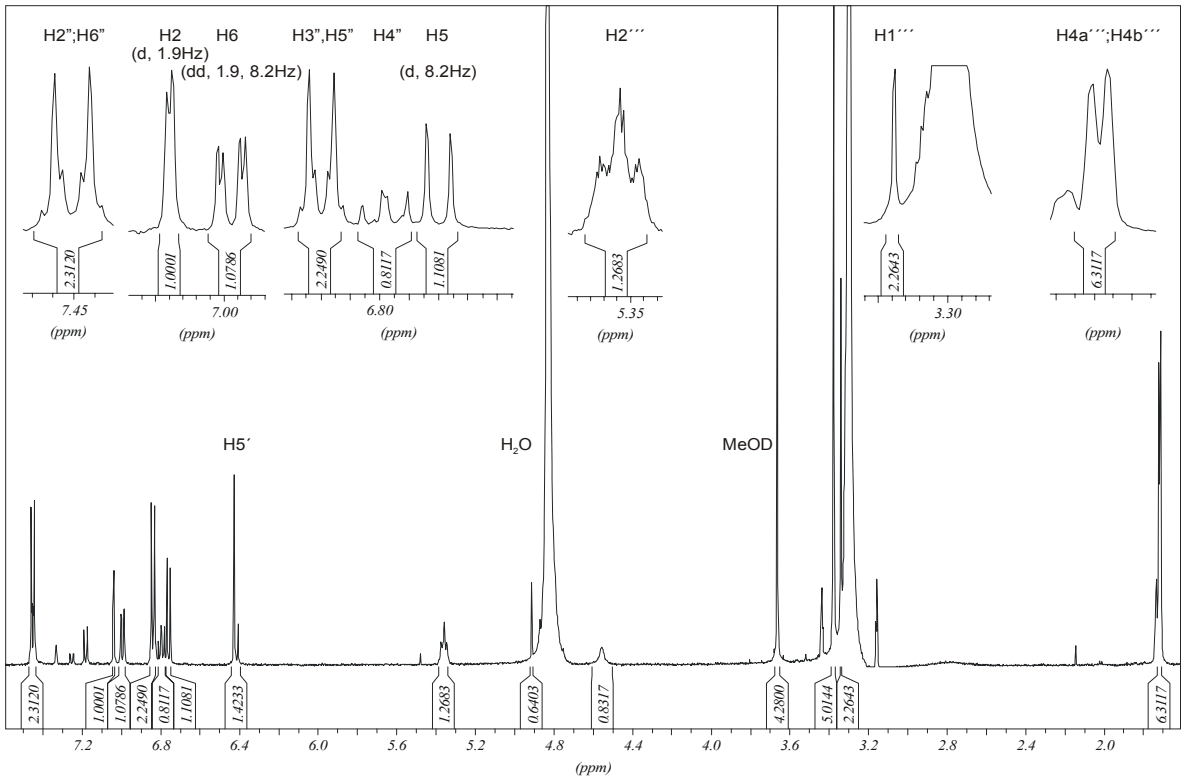



Figure C20.4. $^{1}$H NMR spectrum of compound **20** (terprenin 2) in MeOD

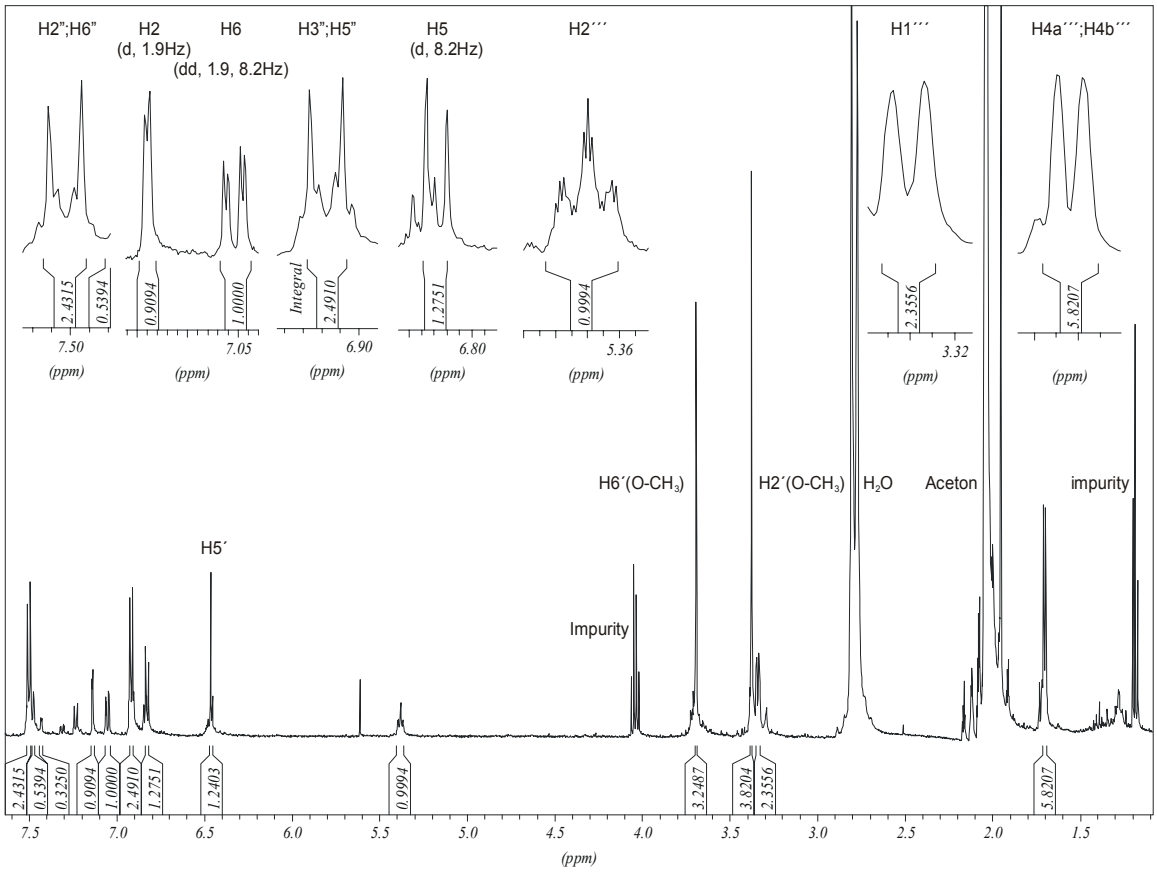



Figure C20.5. $^{1}$H NMR spectrum of compound **20** (terprenin 2) in Acetone

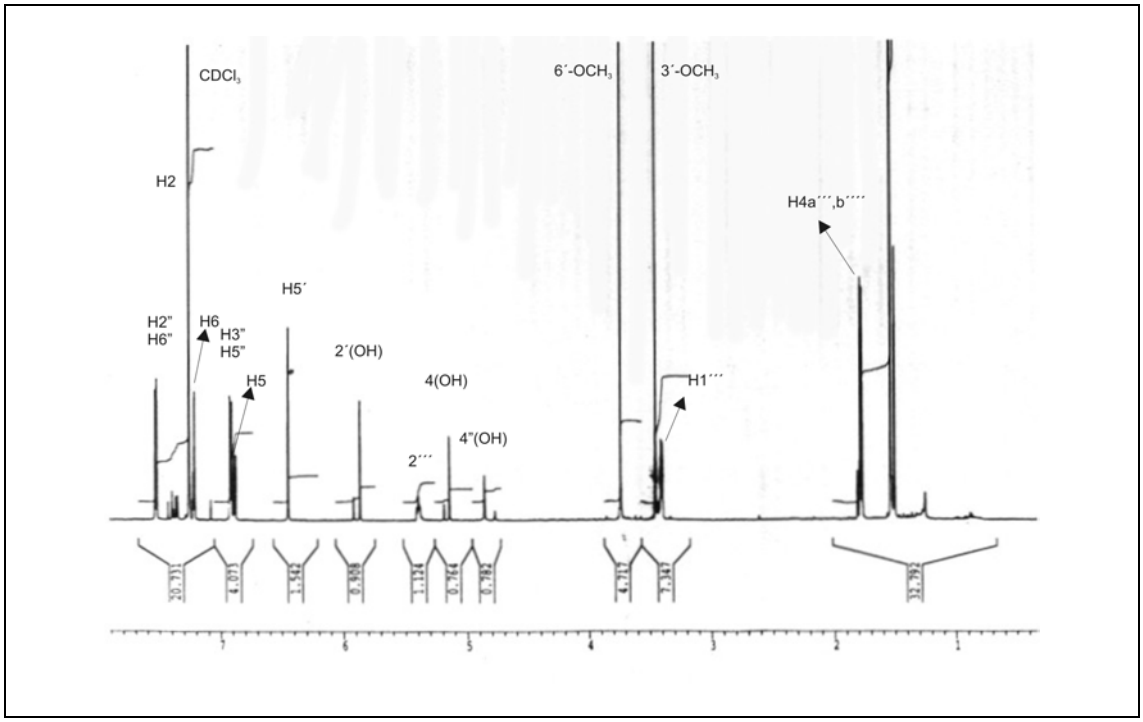



Figure C20.6. $^{1}$H NMR spectrum of compound **20** (terprenin 2) in $CDCl_3$

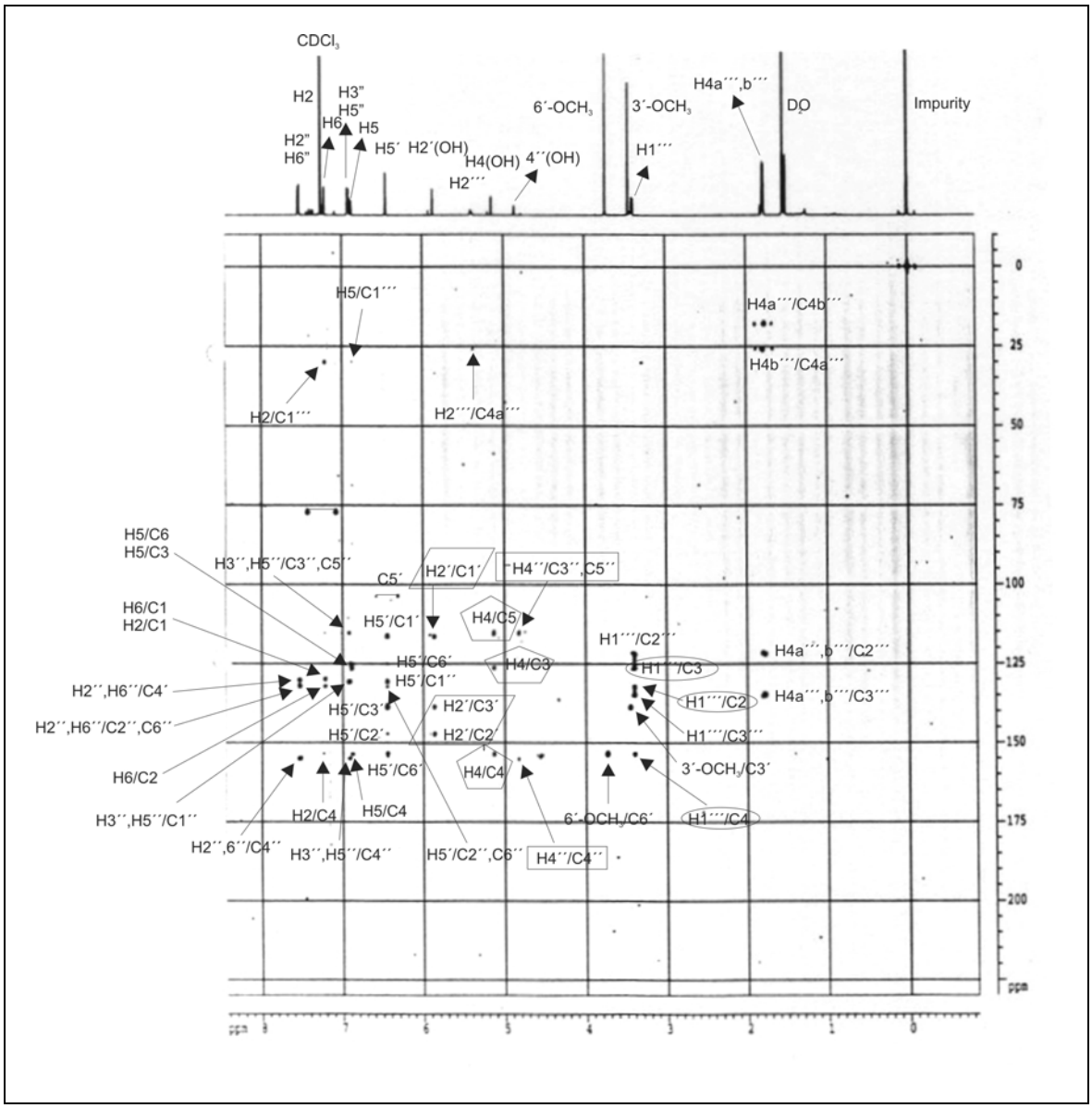



Figure C20.7. HMBC spectrum of compound **20** (terprenin 2) in $CDCl_3$

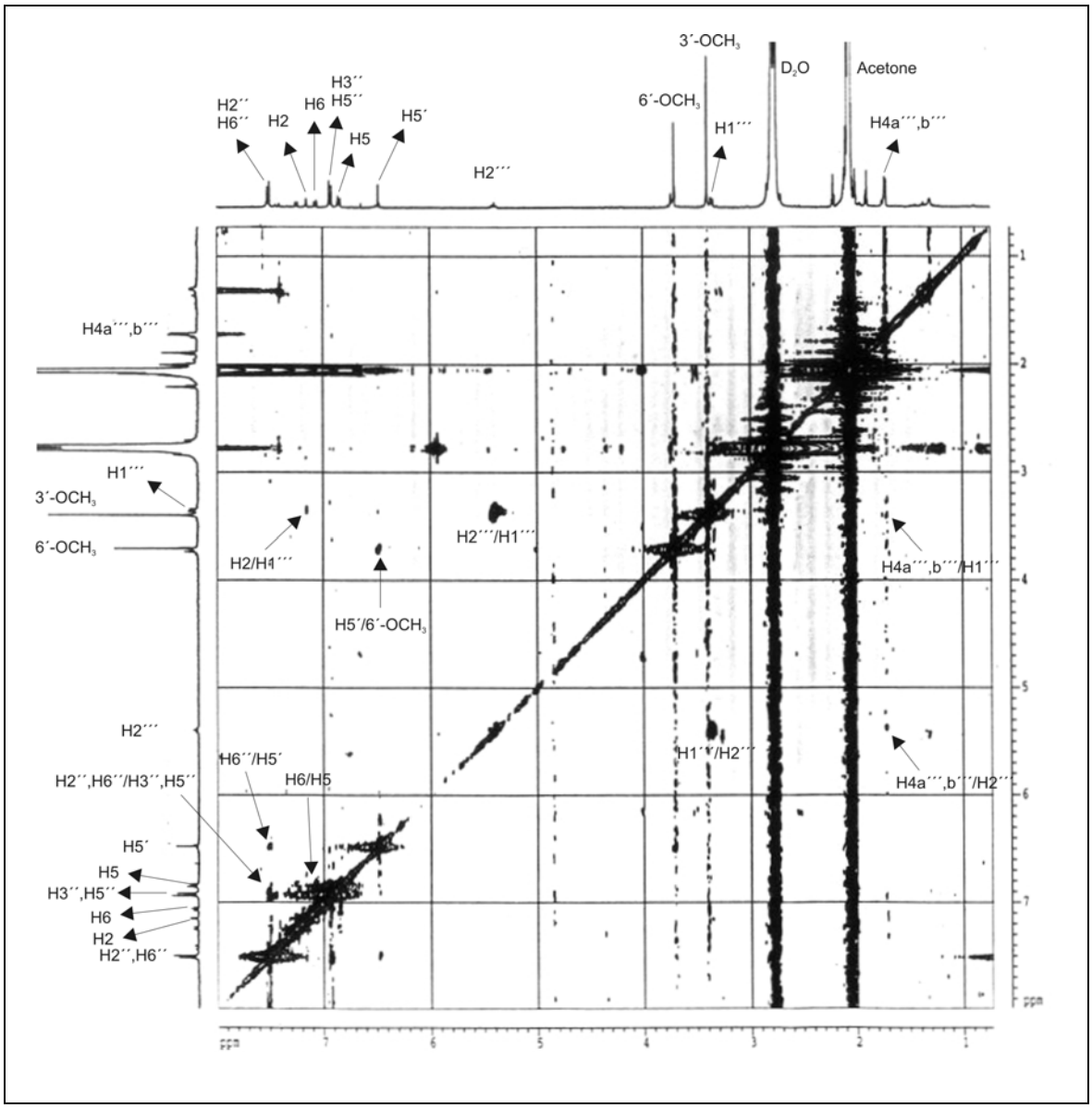



Figure C20.8. ROESY spectrum of compound **20** (terprenin 2) in Acetone

## 3.4.3. Compound 21 (terprenin epoxide)

The molecular formula of compound **21** and molecular weight were established as $C_{25}H_{26}O_6$ and 422 g/mol, respectively, by the ESI-MS (*m/z* 423.3 $[M+H]^+$, 421.6 $[M-H]^-$). The high resolution of ESI-MS indicated the molcular weight of 422.1713 g/mol.

The $^1$H NMR data analysis indicated that compound **21** had an epoxy prenyl side chain [$\delta$ 3.28 (H1‘‘‘A), 3.15 (H1‘‘‘B), 4.63 (H2’’’), 1.19 (H4a‘‘‘), 1.14 (H4b‘‘‘)], two methoxy groups [$\delta$ 3.37 (3’-$OCH_3$), 3.70 (6’-$OCH_3$)], three phenolic hydroxyl groups [$\delta$ 4.85 (4-OH), 5.90 (2’-OH), 4.85 (4’’-OH)], and contained three phenyl rings with different substitution patterns as described above for compound **20** (Table C21.1, Figure C21.2).

The presence of an epoxy group was assured by the molecular formula and by the HMBC correlations between H4a’’’ and H4b’’’ with C2’’’ and C3’’’. The downfiled shifts of the latter could only be explained by oxygen substitution, while the molecular formula only accounted for one additional oxygen atom in comparison to compound **20**.

With the aid of proton multiplicity and coupling constants observed in the $^1$H NMR spectrum, the three phenyl rings were determined as possessing an ABX spin system on ring A [$\delta$ 7.17 (H2), 6.67 (H5), 7.08 (H6)], a one proton system in ring B ($\delta$ 6.46 (H5‘),an AA’BB’ spin system on ring C [$\delta$ 6.92 (H3‘‘, H5‘‘), 7.50 (H2‘‘, H6‘‘)], and an ABX spin system was found in the side chain of epoxy prenyl group (Table C21.1, Figure C21.2). Proton signals of the $^1$H NMR spectrum recorded in $CDCl_3$ and methanol also supported the assignment of these three spin system types.

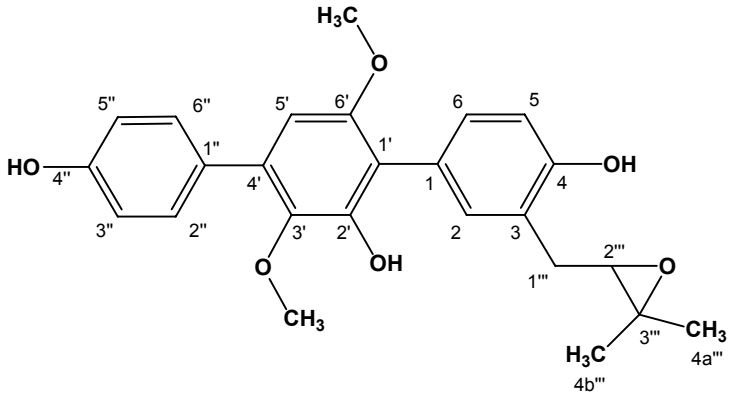
Table C21.1. NMR data of compound **21** (terprenin epoxide)

| Position | $\delta$ $^{13}$C (ppm) (in Acetone) | $\delta$ $^1$H (ppm), multiplicity (*J* in Hz) (in Acetone) | $\delta$ $^1$H (ppm), multiplicity (*J* in Hz) (in $CDCl_3$) | COSY (H $\rightarrow$ H) (in Acetone) | HMBC (H $\rightarrow$ C) (in $CDCl_3$) |
|---|---|---|---|---|---|
| 1 | | | | | |
| 2 | 128.0 | 7.17 (br s) | 7.25 (d) | H5, H6, H1‘‘‘ | |
| 3 | 136.0 | | | | |
| 4 | | | 4.85 (OH) | | |
| 5 | | 6.67 (d, 8.2) | 6.87 (d, 8.2) | H6 | |
| 6 | | 7.08 (d, 8.2) | 7.20 (dd, 8.2, 2.0) | H2, H5 | |
| 1‘ | 131.0 | | | | |
| 2‘ | | | 5.90 (OH) | | |
| 3‘ | 139.0 | | | | |
| 3‘-$OCH_3$ | | 3.37 (s) | 3.45 (s) | | C3‘ |
| 4‘ | | | | | |
| 5‘ | | 6.46 (s) | 6.45 (s) | 6‘-$OCH_3$ | C1‘, C3‘, C6‘ |
| 6‘ | 154.0 | | | | |
| 6‘-$OCH_3$ | | 3.70 (s) | 3.74 (s) | H5‘ | C6‘ |
| 1‘‘ | | | | | |
| 2‘‘ | | 7.50 (d, 8.8) | 7.53 (d, 6.7) | H3‘‘ | |
| 3‘‘ | | 6.92 (d, 8.8) | 6.90 (d, 6.7) | H2‘‘ | |
| 4‘‘ | | | 4.85 (OH) | | |
| 5‘‘ | | 6.92 (d, 8.8) | 6.90 (d, 6.7) | H6‘‘ | |
| 6‘‘ | | 7.50 (d, 8.8) | 7.53 (d, 6.7) | H5‘‘ | |
| 1‘‘‘ | | H1‘‘‘A: 3.28 (dd, 15.7, 8.8)<br>H1‘‘‘B: 3.15 (dd, 15.7, 9.5) | 3.21 (dd, 19.6, 9.8) | H2‘‘‘ | C2 |
| 2‘‘‘ | 72.0 | 4.63 (t, 9.5, 8.8) | 4.65 (t, 9.3) | H1‘‘‘ | C3 |
| 3‘‘‘ | 91.0 | | | | |
| 4a‘‘‘ | 24.0 | 1.19 (s) | 1.38 (s) | H4b‘‘‘ | C2‘‘‘, C3‘‘‘, H4b‘‘‘ |
| 4b‘‘‘ | 26.0 | 1.14 (s) | 1.25 (s) | H4a‘‘‘ | C2‘‘‘, C3‘‘‘, H4a‘‘‘ |

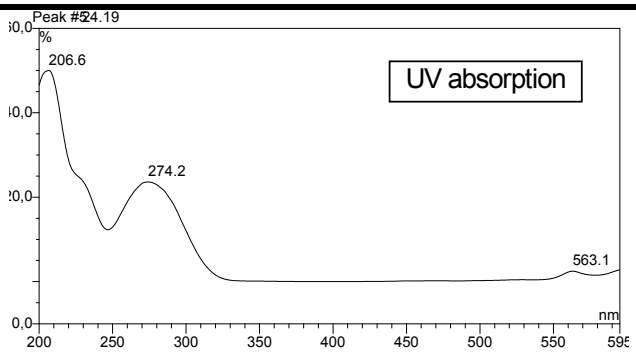
## Terprenin epoxide (compound 21)

| | |
|---|---|
| CAS Registry number | : - |
| Characteristic | : white |
| Formula | : $C_{25}H_{26}O_6$ |
| Molecular Weight | : 422 g/mol |
| Amount | : 6.0 mg |
| Source | : *Lecanicillium evansii* (strain 1) derived from *Callyspongia* sp. |

new compound




| | |
|---|---|
| Retention Time | : 24.12 min |
| $R_f$ | : 0.69 |
| Fluorescence, 254 nm | : + |
| 366 nm | : + |
| Anisaldehyde/$H_2SO_4$ | : + |




| | |
|---|---|
| Optical Rotation $[\alpha]_D^{20}$ | : - 4.0° (c=0.1, Aceton) |
| ESI-MS (*m*/*z*) | : 423.3 $[M+H]^+$ (positive), 421.6 $[M-H]^-$ (negative) |

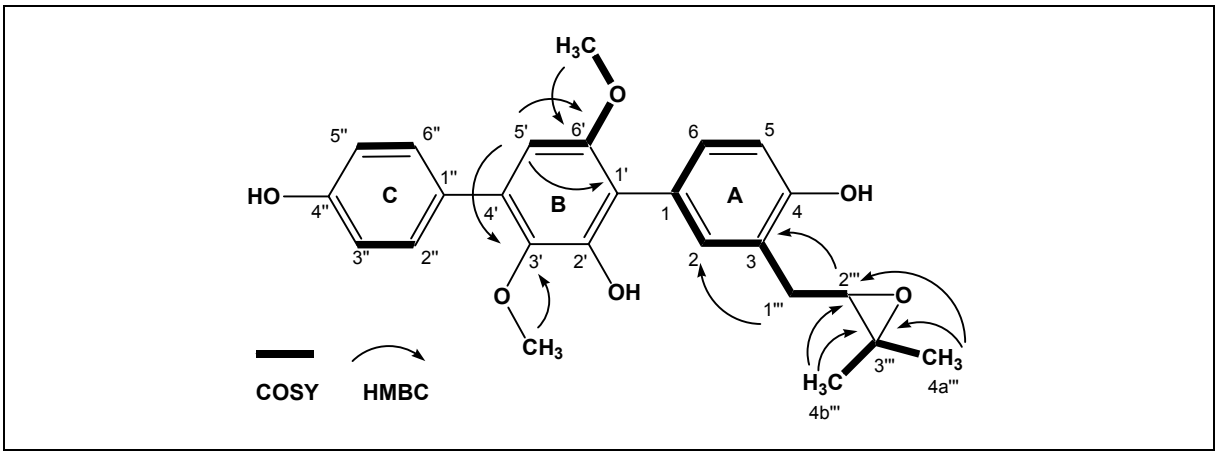



Figure C21.1. COSY and HMBC correlations of compound **21** (terprenin epoxide)

The epoxy group brought about a centre of asymmetric at position 2‘‘‘. Consequently methylene protons at the position 1‘‘‘resonated as double doublet signals as clearly seen in the $^{1}$H NMR spectrum when recorded in acetonee (Figure C21.2).

An H/D exchange experiment with methanol as a solvent to eliminate the hydroxyl protons that existed in the $^{1}$H NMR spectrum recorded in chloroform was conducted. Signals of the hydroxyl groups at positions 4, 2’ , and 4” disappeared.

COSY correlations also supported the assignment of the four spin systems in the molecule (Figure C21.1). Likewise, HMBC correlations assigned the position of the isolated proton H5‘ and the position of two methoxy groups in ring B. The side chain of epoxy prenyl group was bound to ring A through the position 3. This side connection was verified by the COSY correlations of H1‘‘‘, H2‘‘‘ and H2. The long range correlations between H1’’’ and C2 as well as between H2’’’ and C3 in the HMBC spectrum also clarified the side chain position (Figure C21.1, 3, 4).

This terprenin epoxide has never been quoted in the literature. Antimicrobial assay conducted using compound **21** as the test substance demonstrated no biological activity.

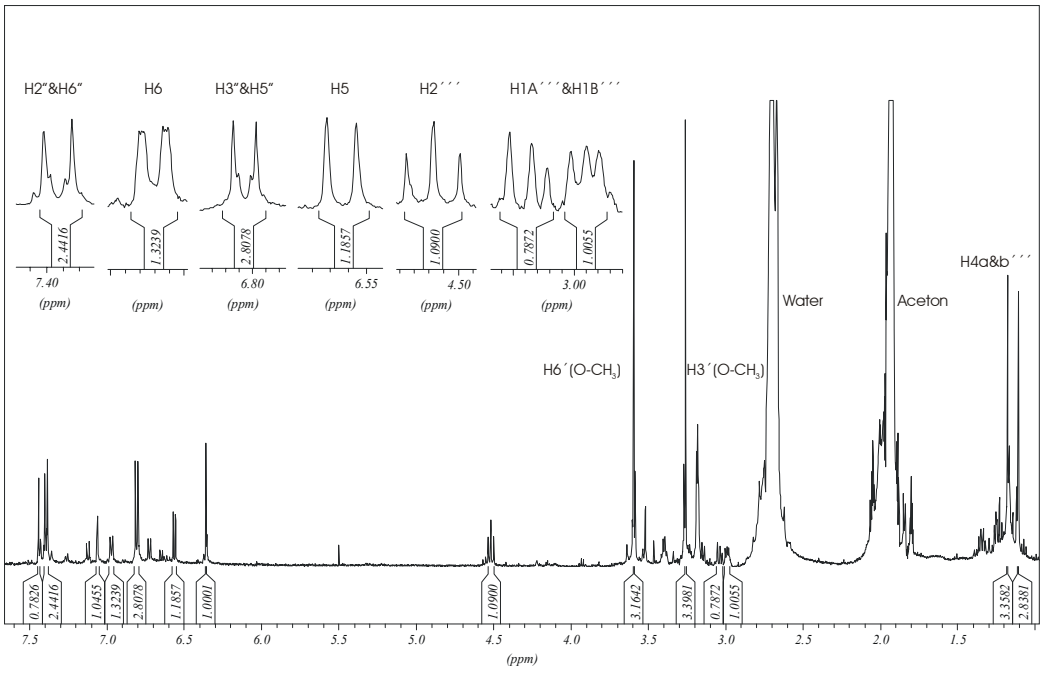



Figure C21.2. $^{1}$H NMR spectrum of compound **21** (terprenin epoxide) in Acetone

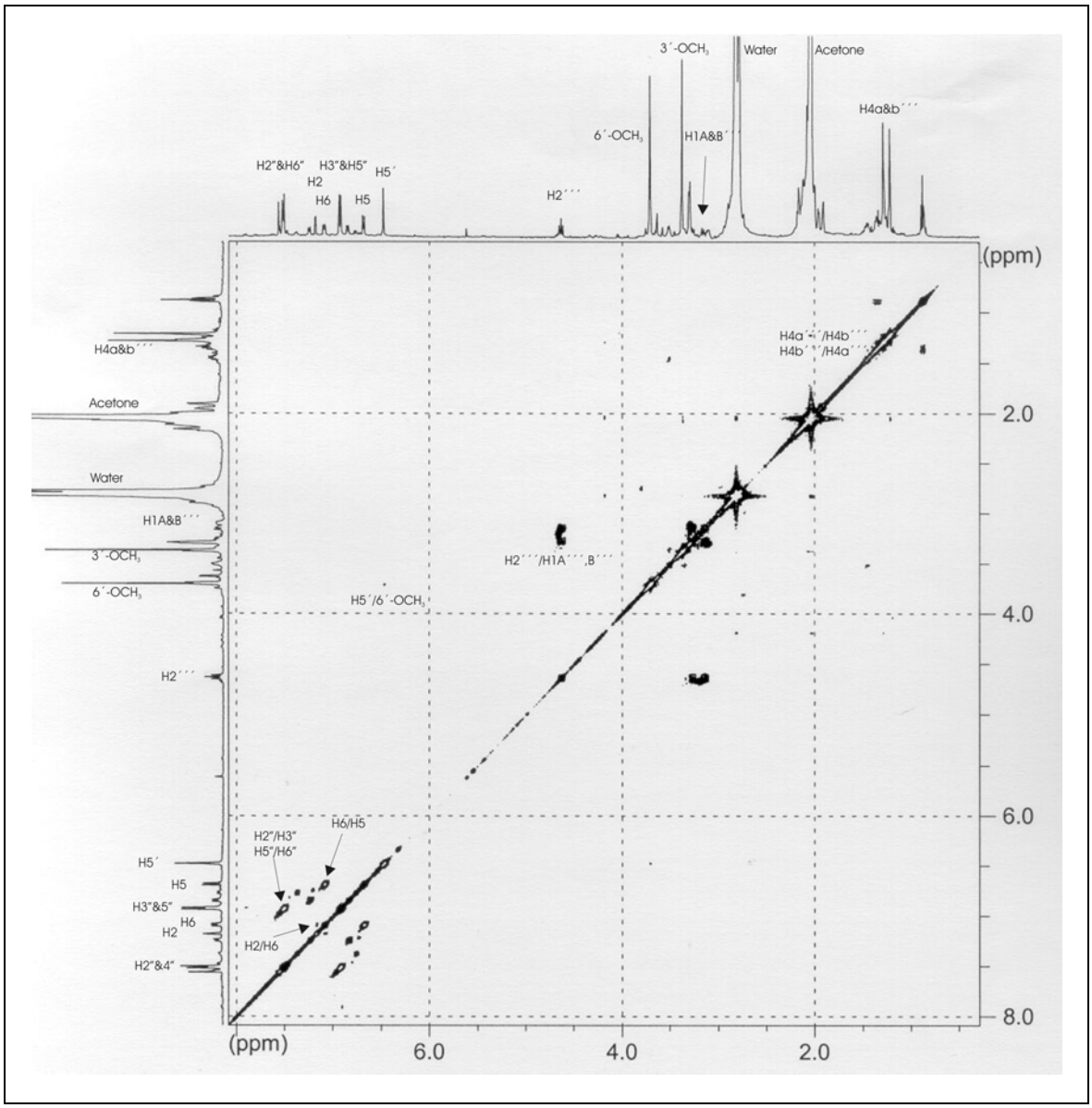



Figure C21.3. COSY spectrum of compound **21** (terprenin epoxide) in Acetone

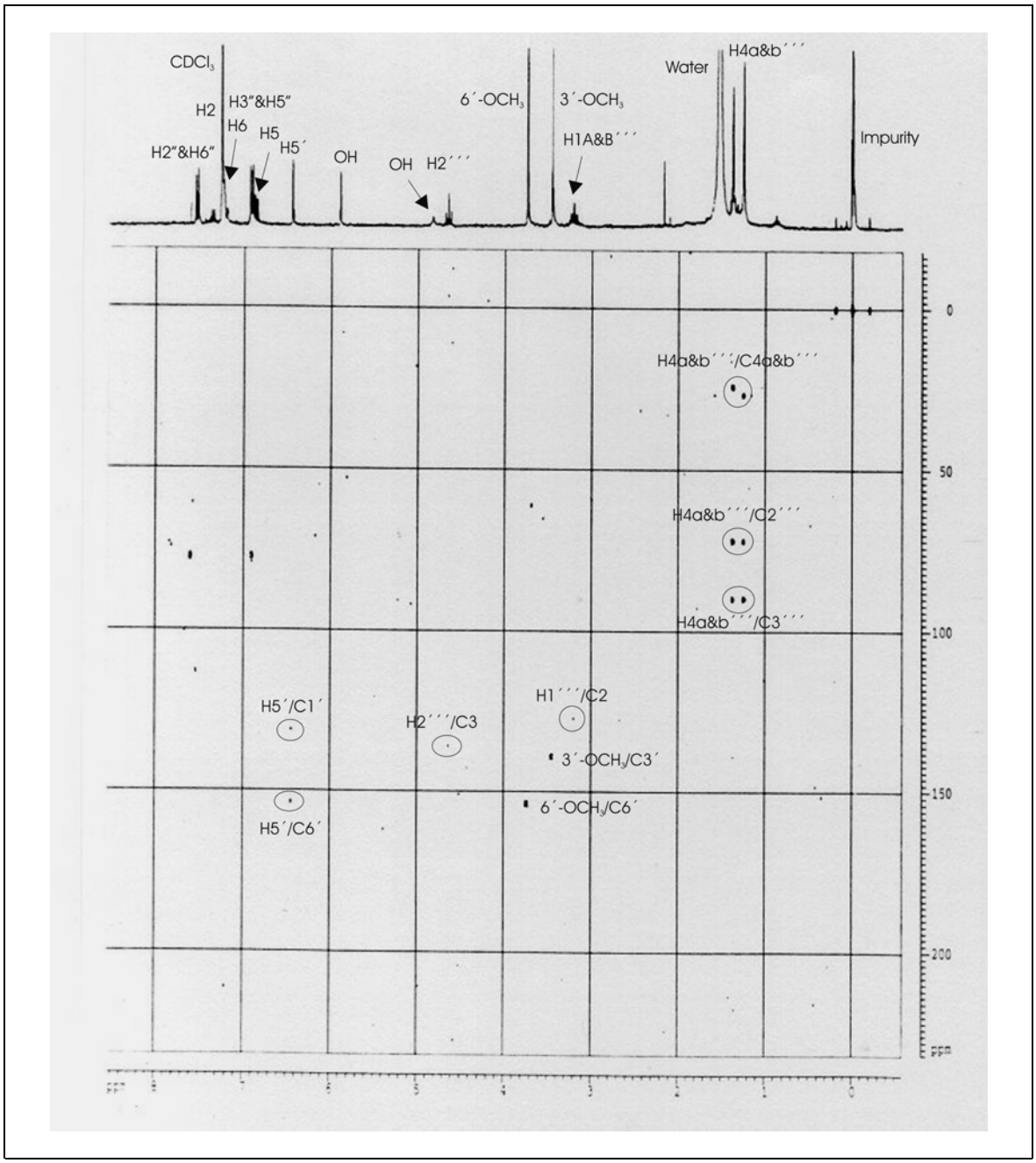



Figure C21.4. HMBC spectrum of compound **21** (terprenin epoxide) in $CDCl_3$

## 3.4.5. Compound 22 (cyclo-tyrosylprolyl)

This compound has a molecular weight of 260 g/mol and a molecular formula of $C_{14}H_{16}N_2O_3$. These chemical characteristics were indicated by the ESI-MS spectrum with the pseudomolecular peak at *m*/*z* 261 $[M+H]^+$. The EI-MS spectrum confirmed this molecular weight. Two intense peaks appeared at *m*/*z* 260 $[M]^+$, 154 $[M\text{-}C_7H_7O_1]^+$ (fragment 1), and 107 $[M\text{-}C_7H_9O_2N_2]^+$ (fragment 2) in the EI spectrum (Figure C22.1).

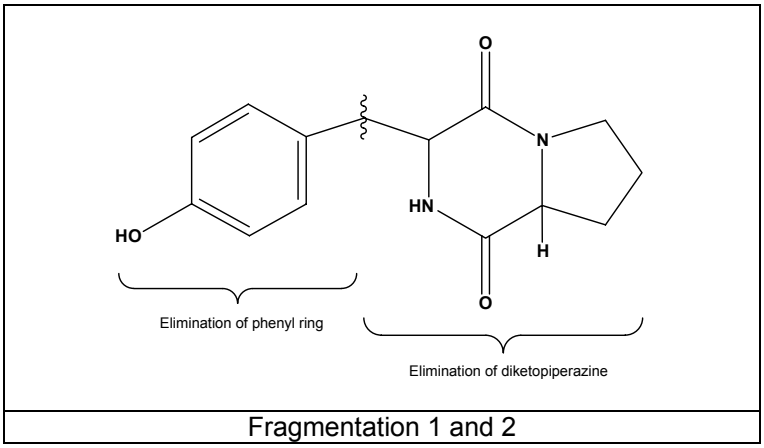



Figure C22.1. Hypothetical fragmentation of compound **22** (cyclo-tyrosylprolyl) in the EI-MS spectrum.

From $^1$H, COSY, and HMBC spectra (Table C22.1, Figure C22.2 - 6), the existence of an AA'BB' spin system indicating a para-substituted phenyl ring [$\delta$ 7.03 (d, *J*=8.2 Hz, H2', H6'), $\delta$ 6.69 (d, *J*=8.8 Hz, H3', H5')] was observed.

This para-substituted phenyl ring was connected to a diketopiperazine molecule through position 10 with protons ($CH_2$) at $\delta$ 3.05 (dd, *J*=15.1, 15.1 Hz, 2H). Two downfield protons at 4.35 (H3) and 4.04 (H6), due to paramagnetic effects of nitrogen atom and ketone group, were observed verifying the presence of a diketopiperazine nucleus (Figure C22.3).

Two singlet carbons representing a ketone group were evident in the $^{13}$C spectrum at $\delta$ 182.00 (C1) and $\delta$ 179.77 (C4) (Table C22.1, Figure C22.4). Further analysis of the $^{13}$C spectrum revealed two other nonsubstituted carbons [$\delta$ 129.00 (C1'), 156.00 (C4'/oxygen-binding aromatic carbon)], six methine carbons [$\delta$ 57.91 (C3/nitrogen-carrying aliphatic carbon), 60.06 (C6/nitrogen-carrying aliphatic carbon), 132.10 (C2'), 116.19 (C3'), 116.19 (C5'), 132.10 (C6')], and four methylene carbons [$\delta$ 29.40 (C7), 22.72 (C8), 45.91 (C9/nitrogen-carrying aliphatic carbon), 37.68 (C10)].

All protons were clearly assigned to their corresponding carbon positions as seen in the HMQC spectrum (Figure C22.5).

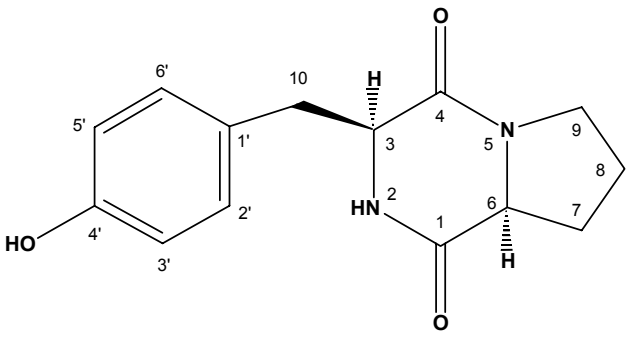
Carbons at position 3' and 5' appeared more upfield than C2' and C6'. This was due to the shielding effect of the hydroxyl group at C4' toward its ortho coupled carbon (C3' and C5'). A similar phenomenon occurred on C1' (para coupled with C4') which shifted more upfield than C2' and C6'.

Comparison of $^1$H NMR spectrum of compound **12** with that of a compound reported by Tatsuno *et al*. (1971) isolated from *Fusarium nivale* proved that both compounds were identical in terms of their chemical shifts.

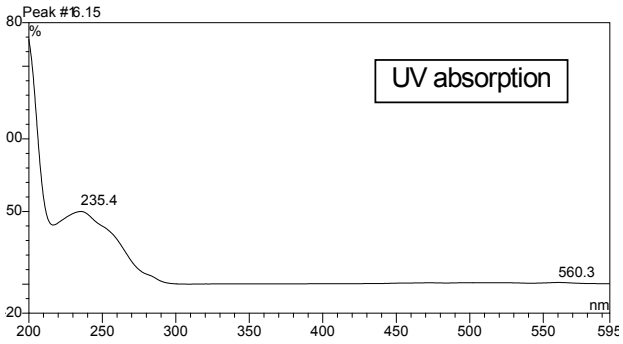
Since the optical rotation (-54.4$^o$) of compound **12** is almost the same as that (-58.6$^o$) reported in the literature (Tatsuno *et al*., 1971), the stereochemistry of compound **12** at the stereogenic centres of C3 and C6 was assumed to be identical with that in the literature namely cyclo(*L*-tyrosyl-*L*-prolyl). Antimicrobial assay of this compound indicated no biological activity.

## Cyclo-tyrosylprolyl (compound 22)

| | |
|---|---|
| CAS Registry Number | : 4549-02-4 |
| Characteristic | : white powder |
| Formula | : $C_{14}H_{16}N_2O_3$ |
| Molecular Weight | : 260 g/mol |
| Amount | : 7.7 mg |
| Source | : *Lecanicillium evansii* (strain 1) derived from *Callyspongia* sp. |




| | |
|---|---|
| Retention Time | : 6.33 min |
| $R_f$ | : 0.45 |
| Fluorescence, 254 nm | : + |
| 366 nm | : - |
| Anisaldehyde/$H_2SO_4$ | : - |




| | |
|---|---|
| Optical Rotation $[\alpha]_D^{20}$ | : experiment = -54.4° (c=0.1, MeOH) |
| | literature = -58.6° (c=0.5, MeOH) (Tatsuno *et al*., 1971) |
| ESI-MS (*m/z*) | : 261.3 $[M+H]^+$ (positive) |
| EI-MS (*m/z*, rel. int.) | : 260 $[M]^+$(14.4), 154 $[M-C_7H_7O_1]^+$(100), 107 $[M-C_7H_9O_2N_2]^+$(53.9) |

Table C22.1. NMR data of compound **22** (cyclo-tyrosylprolyl)

| Position | $\delta$ $^{13}$C (ppm) | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) (in MeOD) | COSY (H → H) | HMQC (H → C) direct | HMBC (H → C) |
|---|---|---|---|---|---|
| 1 | 182.00 (s) | | | | |
| 2 | | | | | |
| 3 | 57.91 (d) | 4.35 (t, 3.2, 3.2, 1.3) | H10 | C3 | |
| 4 | 179.77 (s) | | | | |
| 5 | | | | | |
| 6 | 60.06 (d) | 4.04 (dd, 6.3, 1.9 ) | H7 | C6 | |
| 7 | 29.40 (t) | 2.08 (m, 2H) | H8, H6 | C7 | |
| 8 | 22.72 (t) | 1.80 (m, 2H) | H7, H9 | C8 | |
| 9 | 45.91 (t) | 3.54 (dd, 8.2, 3.8; 2H) | H8, H7 | C9 | |
| 10 | 37.68 (t) | 3.05 (dd, 15.1, 5.0; 2H) | | C10 | C2‘, C6’ |
| 1‘ | 129.00 (s) | | | | |
| 2‘ | 132.10 (d) | 7.03 (d, 8.2) | H3‘ | C2’ | C4‘,C6‘ |
| 3‘ | 116.19 (d) | 6.69 (d, 8.8) | H2‘ | C3’ | C1‘ |
| 4‘ | 156.00 (s) | | | | |
| 5‘ | 116.19 (d) | 6.69 (d, 8.8) | H6‘ | C5‘ | C1’ |
| 6‘ | 132.10 (d) | 7.03 (d, 8.2) | H5‘ | C6‘ | C2‘, C4‘ |

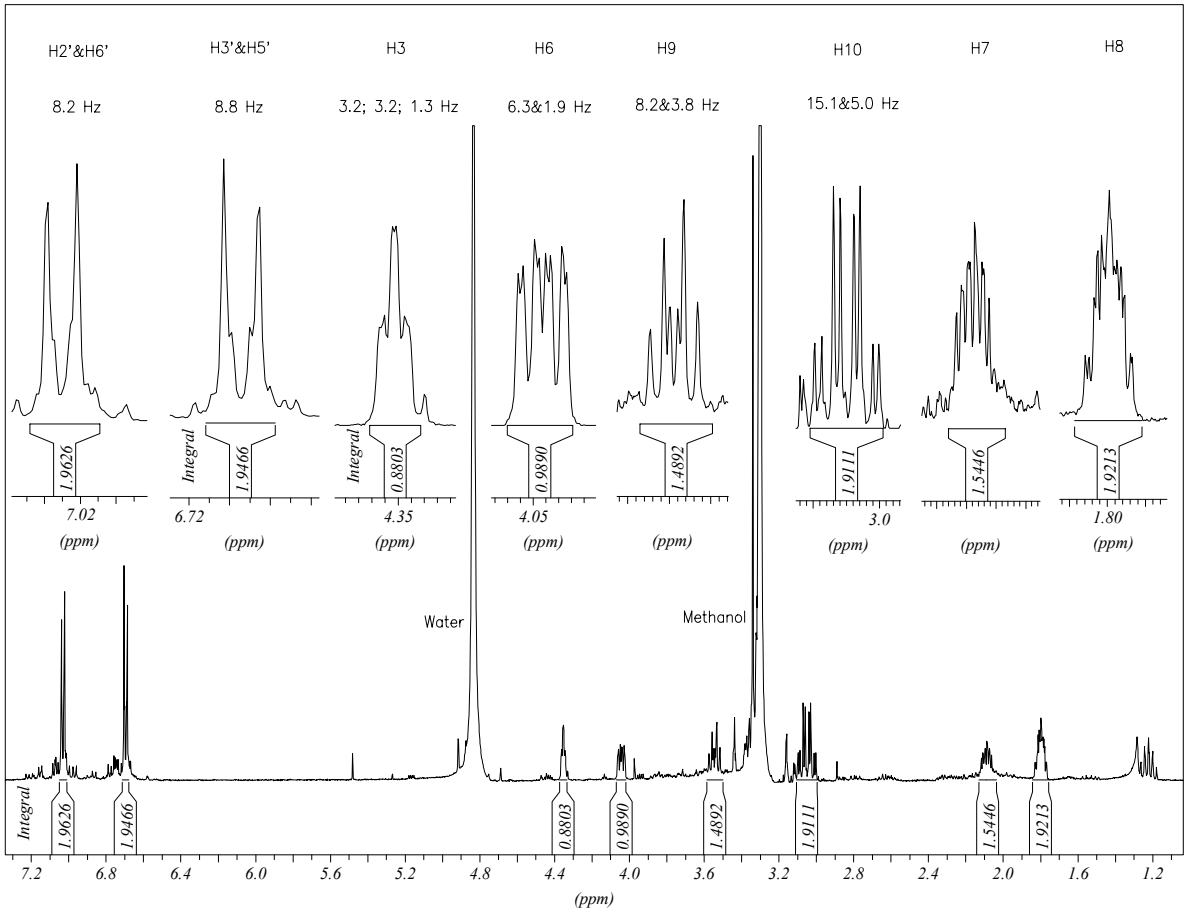



Figure C22.2. $^{1}$H NMR spectrum of compound **22** (cyclo-tyrosylprolyl) in MeOD

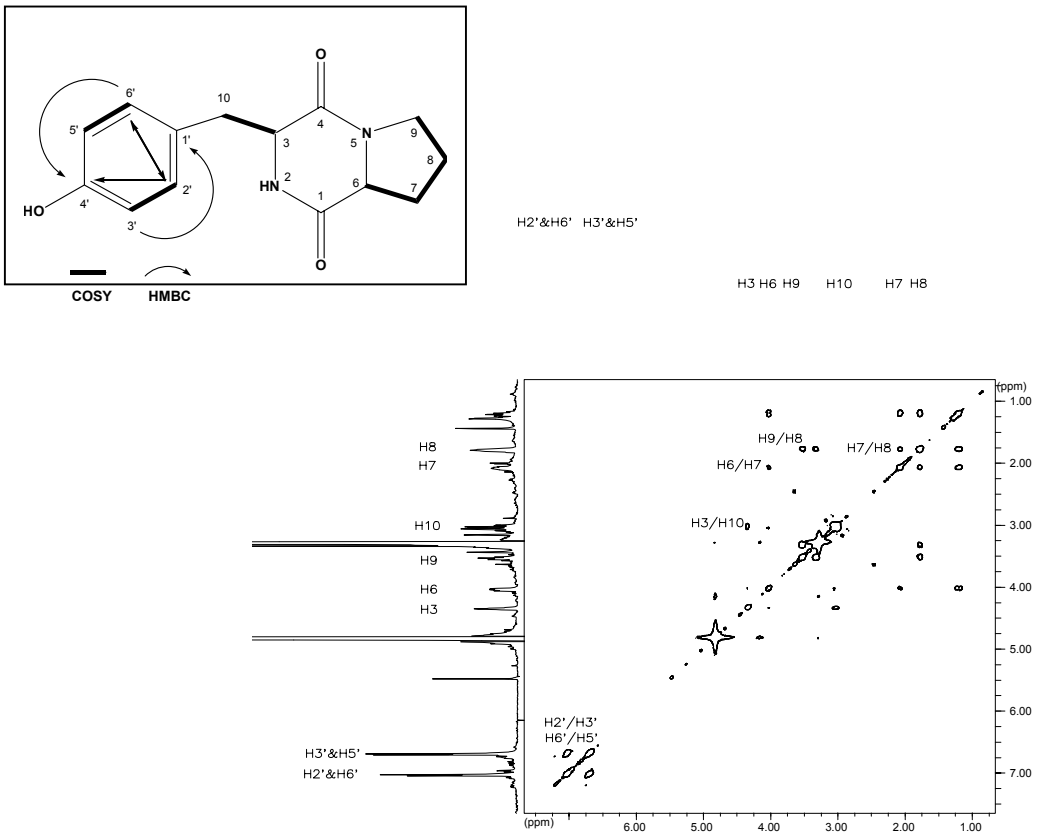



Figure C22.3. COSY spectrum of compound **22** (cyclo-tyrosylprolyl)

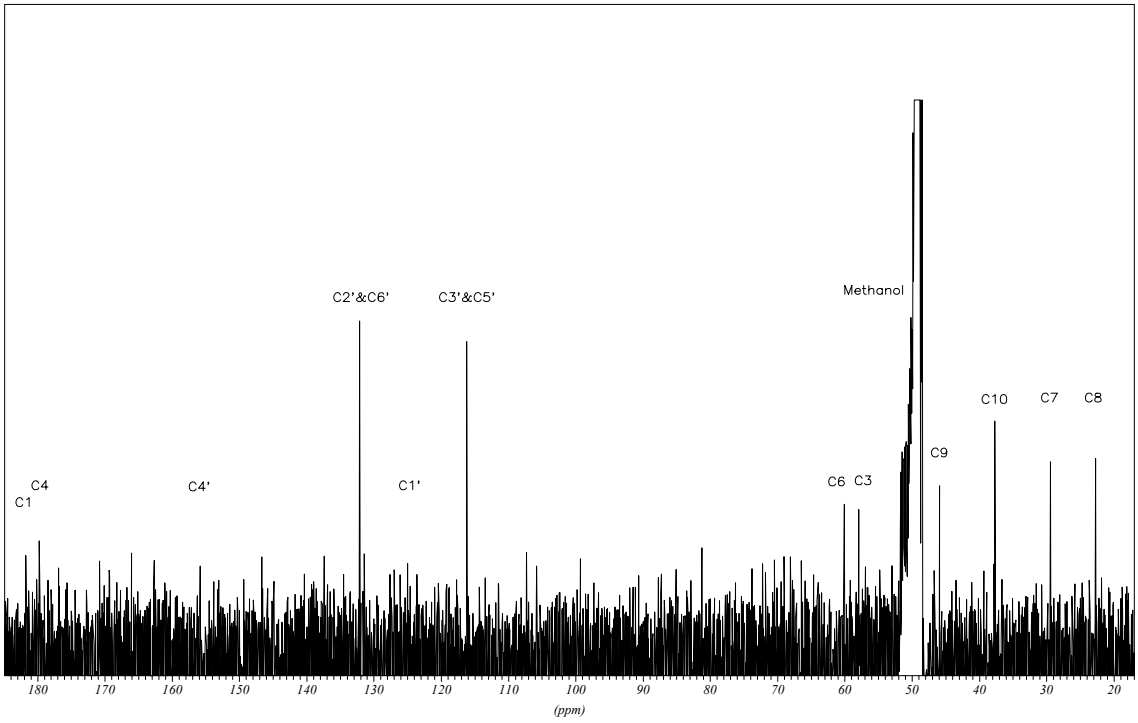



Figure C22.4. $^{13}$C NMR spectrum of compound **22** (cyclo-tyrosylprolyl) in MeOD

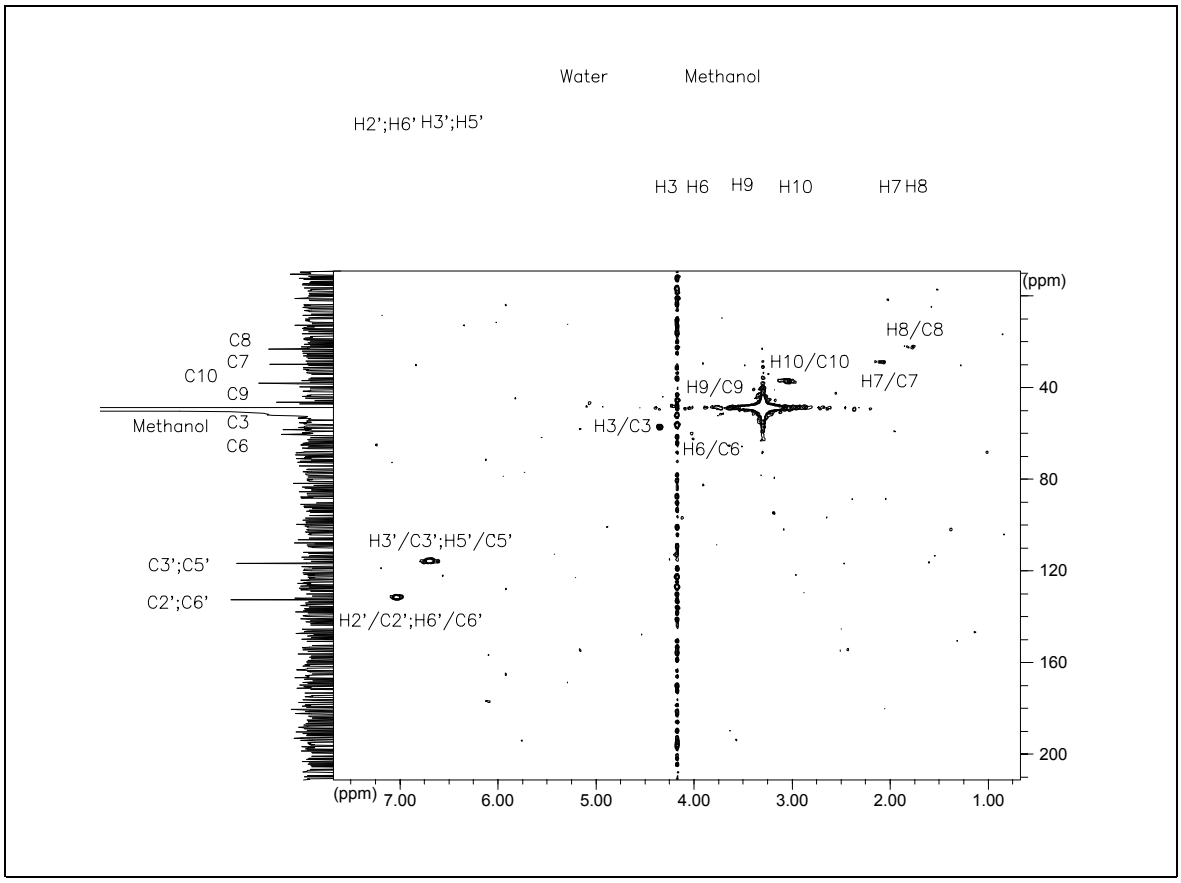



Figure C22.5. HMQC spectrum of compound **22** (cyclo-tyrosylprolyl)

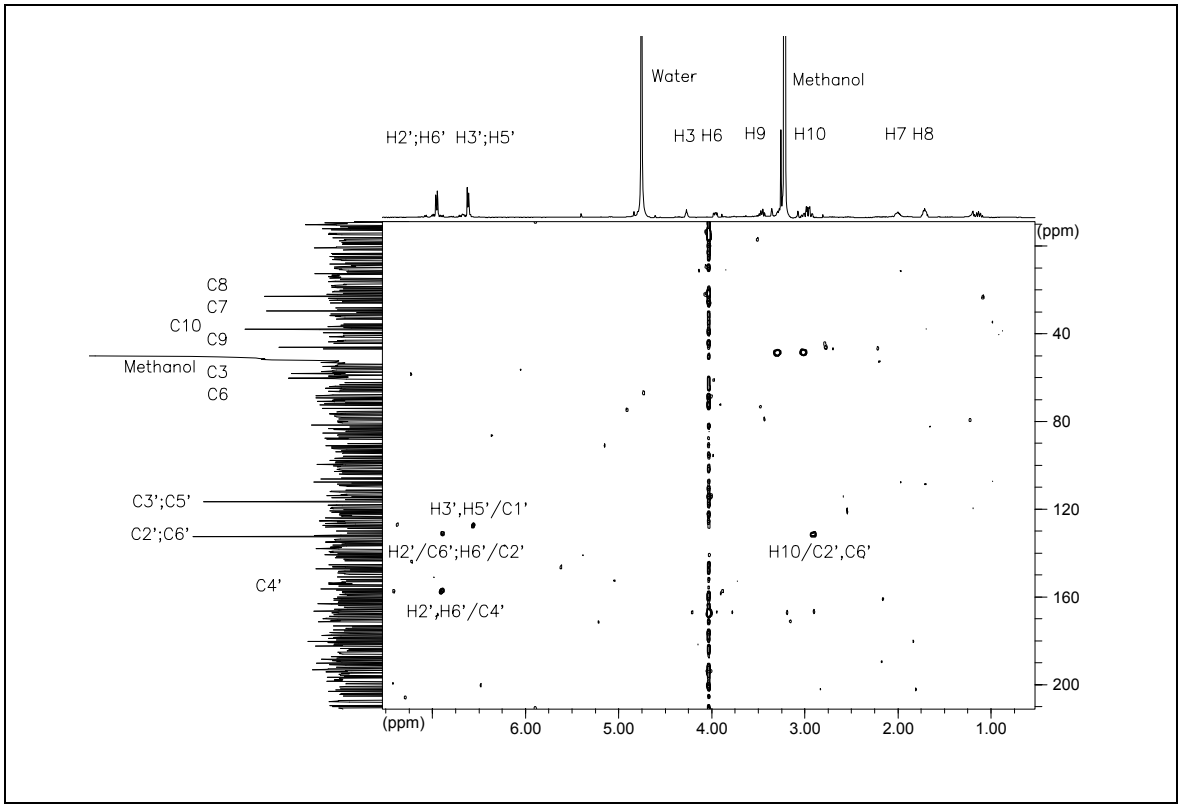



Figure C22.6. HMBC spectrum of compound **22** (cyclo-tyrosylprolyl)

## 3.4.6. Compound 23 (acetyl hydroxybenzamide)

An intense ion peak in the ESI-MS spectrum observed at *m*/*z* 180 $[M+H]^+$ indicated a molecular weight of 179 g/mol and a molecular formula of $C_9H_9NO_3$. The EI-MS spectrum indicated several clear-cut fragmentations at *m*/*z* 179 $[M]^+$, 161 $[M-H_2O]^+$ (fragment 1), 164 $[M-CH_3]^+$ (fragment 2), 137 $[M-COCH_3]^+$ (fragment 3), 120 $[M-NHCOCH_3]^+$ (fragment 4), and 93 $[M-CONHCOCH_3]^+$ (fragment 5) (Figure C23.1).

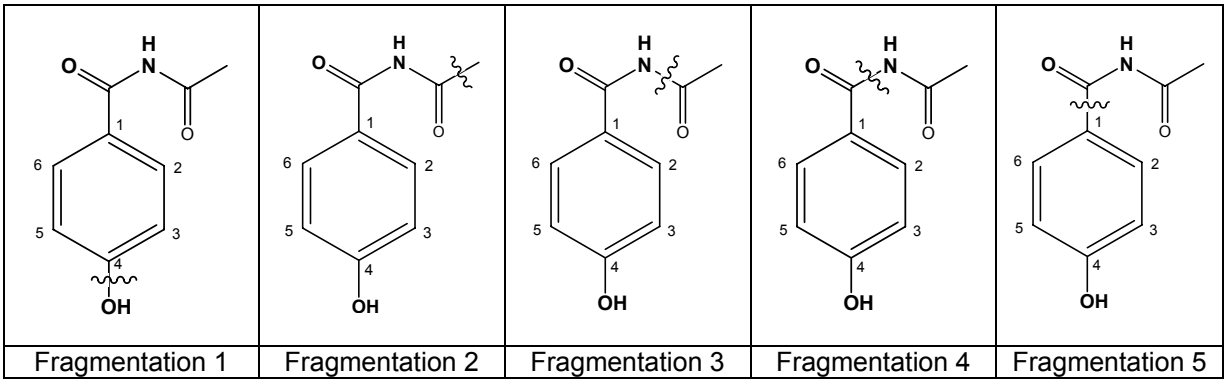



Figure C23.1. Hypothetical fragmentation of compound **23** (acetyl hydroxybenzamide) in the EI-MS spectrum

An AA'BB' spin system was pointed out clearly by two pairs of protons [$\delta$ 7.82 (d, *J*=8.3 Hz, H2, H6) and $\delta$ 7.59 (d, *J*=8.0 Hz, H3, H5)] (Table C23.1, DMSO). The $^1H \rightarrow {}^1H$ direct connections between H2 and H3, as well as between H5 and H6, appeared in the COSY spectrum, also confirmed this spin system assignment (Figure C23.5).

Methyl protons and NH attached to carbonyl group were distinctly seen at $\delta$ 2.06 (s) and $\delta$ 10.06 (br s), respectively. The existence of the hydroxyl group caused diamagnetic (shielding) effect toward ortho protons (H3, H5), resulting in these positions being shifted more upfield than the other aromatic protons (H2, H6)

This compound (*N*-acetyl-4-hydroxy-benzamide) has not been quoted as a natural product. A related compound namely 4-hydroxybenzamide was reported as a natural product, isolated from *Streptomyces tendae* (Blum *et al*., 1996) (Figure C23.2). There was no antimicrobial activity of compound **23** found in this study.

Another related compound [5-acetyl-2-hydroxybenzamide (5-acetyl salicylamide)] is produced commercially as an insecticide.

Table C23.1. NMR data of compound **23** (acetyl hydroxybenzamide)

| Position | $\delta$ $^1$H (ppm), multiplicity (*J* in Hz) (in MeOD) | $\delta$ $^1$H (ppm), multiplicity (*J* in Hz) (in DMSO) | COSY (H→ H) |
|---|---|---|---|
| 1 | | | |
| 2 | 7.94 (dd, 8.8, 1.9) | 7.82 (d, 8.3) | H3 |
| 3 | 7.62 (d, 8.8) | 7.59 (d, 8.0) | H2 |
| 4 | | | |
| 5 | 7.62 (d, 8.8) | 7.59 (d, 8.0) | H6 |
| 6 | 7.94 (dd, 8.8, 1.9) | 7.82 (d, 8.3) | H5 |
| CONH | | 10.06 (br s) | |
| $COCH_3$ | 2.13 (s) | 2.06 (s) | |

## Acetyl hydroxybenzamide (compound 23)

CAS Registry Number : -

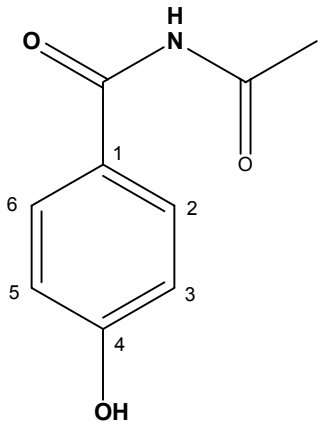
Characteristic : white powder

Formula : $C_9H_9O_3$

Molecular Weight : 179 g/mol

Amount : 5.1 mg

Source : *Lecanicillium evansii* (strain 1) derived from *Callyspongia* sp.

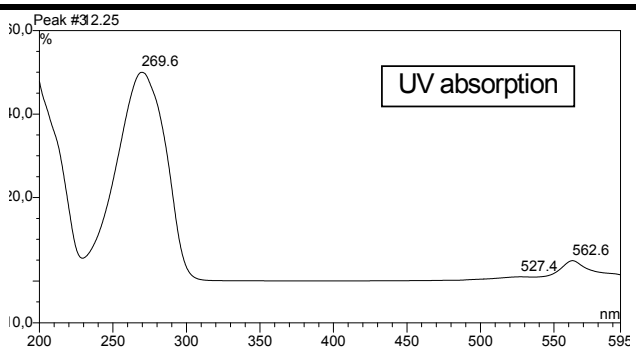



Retention Time : 12.33 min

$R_f$ : 0.64

Fluorescence, 254 nm : +
366 nm : -

Anisaldehyde/$H_2SO_4$ : -




EI-MS (*m*/*z*, rel. int.) : 179 $[M]^+$ (46.8), 161 $[M-H_2O]^+$ (14.5), 164 $[M-CH_3]^+$ (7.7)
137 $[M-COCH_3]^+$ (100), 120 $[M-NHCOCH_3]^+$ (52.8)
93 $[M-CONHCOCH_3]^+$ (10.5)

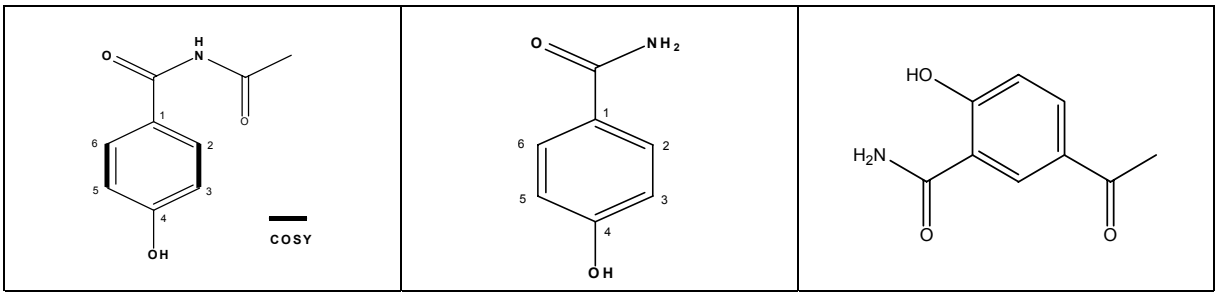



Figure C23.2. COSY correlations of compound **23** (acetyl hydroxybenzamide) (left), structure of 4-hydroxybenzamide (middle), and 5-acetyl-2-hydroxybenzamide (right)

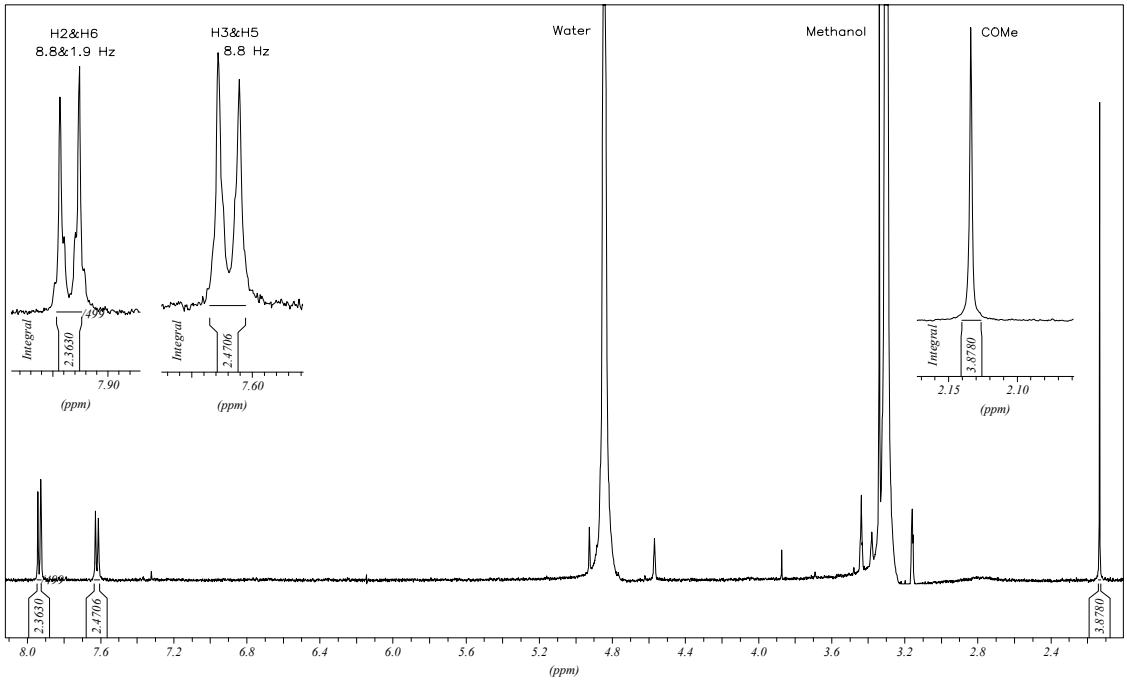



Figure C23.3. $^{1}$H NMR spectrum of compound **23** (acetyl hydroxybenzamide) in MeOD

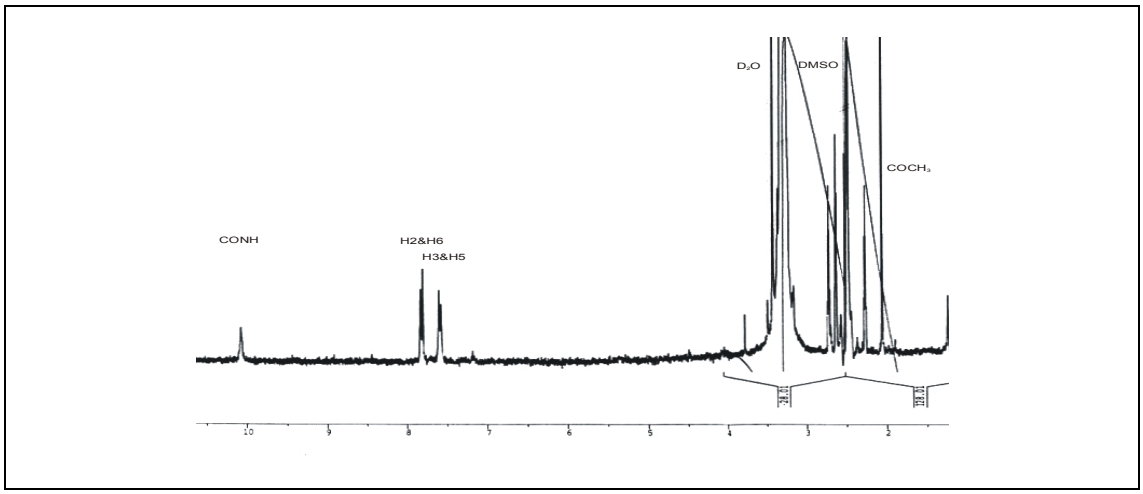



Figure C23.4. $^{1}$H NMR spectrum of compound **23** (acetyl hydroxybenzamide) in DMSO

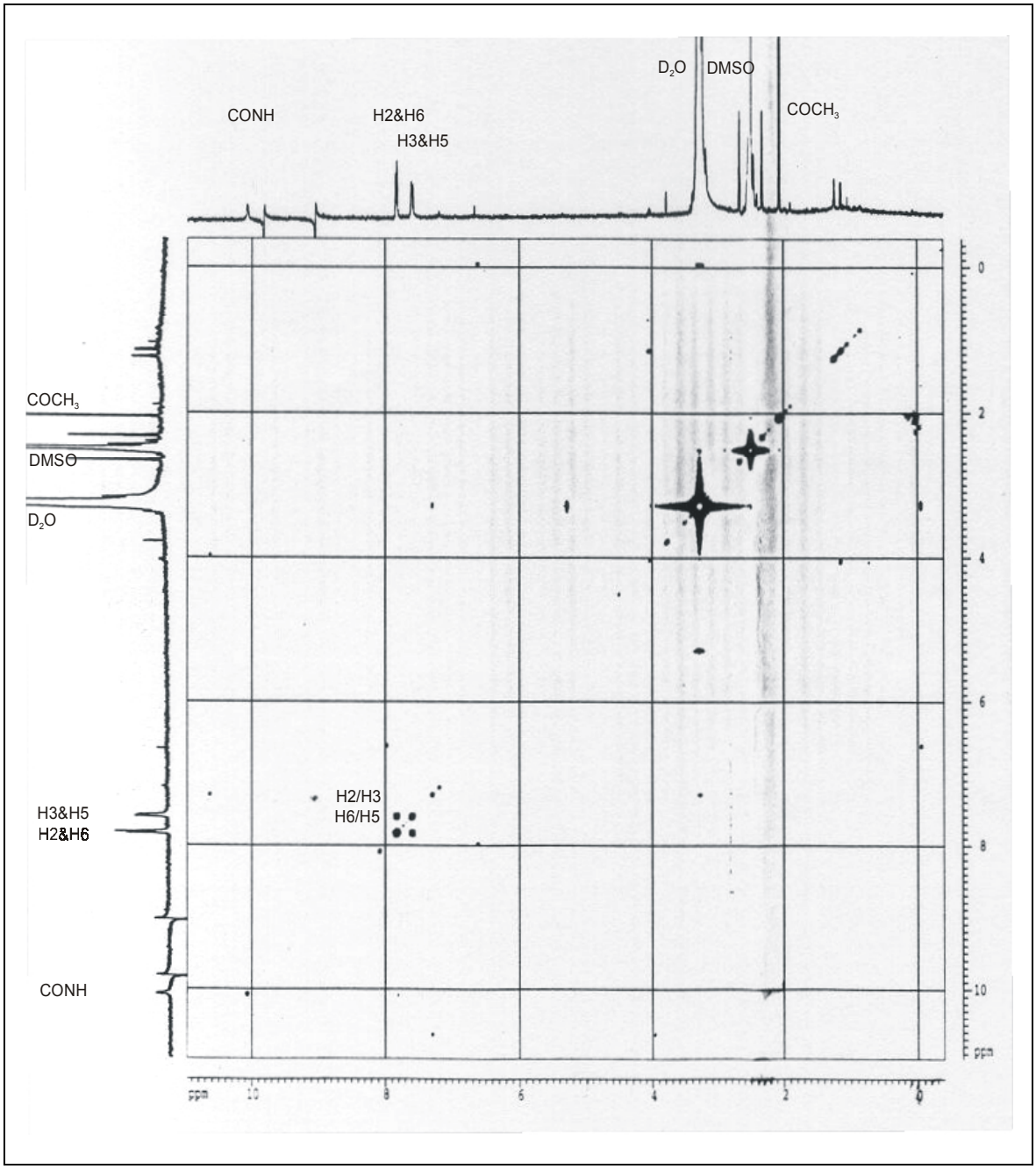



Figure C23.5. COSY spectrum of compound **23** (acetyl hydroxybenzamide) in DMSO

## 3.4.7. Compound 24 (4-hydroxybenzaldehyde)

Molecular weight and molecular formula of this compound were 122 g/mol and $C_7H_6O_2$, respectively. It was determined by the ESI-MS spectrum showing an ion peak at *m*/*z* 121 $[M-1]^-$.

A para-substituted phenyl ring was evident from proton signals at *δ* 7.78 (d, H2, H6) and *δ* 6.91 (H3, H5). This AA'BB' spin system was apparently displayed by two pairs of doublet protons with ortho coupling magnitude of 8.8 Hz. Carbonyl proton found at low field [*δ* 9.75 (s, H7)] also distinctly appeared in the $^1$H NMR spectrum, and represented an aldehyde proton (Table C24.1, Figure C24.1).

Table C24.1. NMR data of compound **24** (4-hydroxybenzaldehyde)

| Position | $\delta$ $^{13}$C (ppm), (Standard in MeOD) | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) (Standard in MeOD) | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) (in MeOD) |
|---|---|---|---|
| 1 | 129.43 (s) | | |
| 2 | 133.84 (d) | 7.76 (d, 8.5) | 7.78 (d, 8.8) |
| 3 | 117.16 (d) | 6.91 (d, 8.5) | 6.91 (d, 8.8) |
| 4 | 165.57 (s) | | |
| 5 | 117.16 (d) | 6.91 (d, 8.5) | 6.91 (d, 8.8) |
| 6 | 133.84 (d) | 7.76 (d, 8.5) | 7.78 (d, 8.8) |
| 7 | 193.22 (d) | 9.75 (s) | 9.75 (s) |

Comparison of chemical shifts of the $^{1}$H NMR data of compound **24** with those of a standard compound (para-hydroxybenzaldehyde) in the same NMR solvent (MeOD) proved that both compounds are identical.

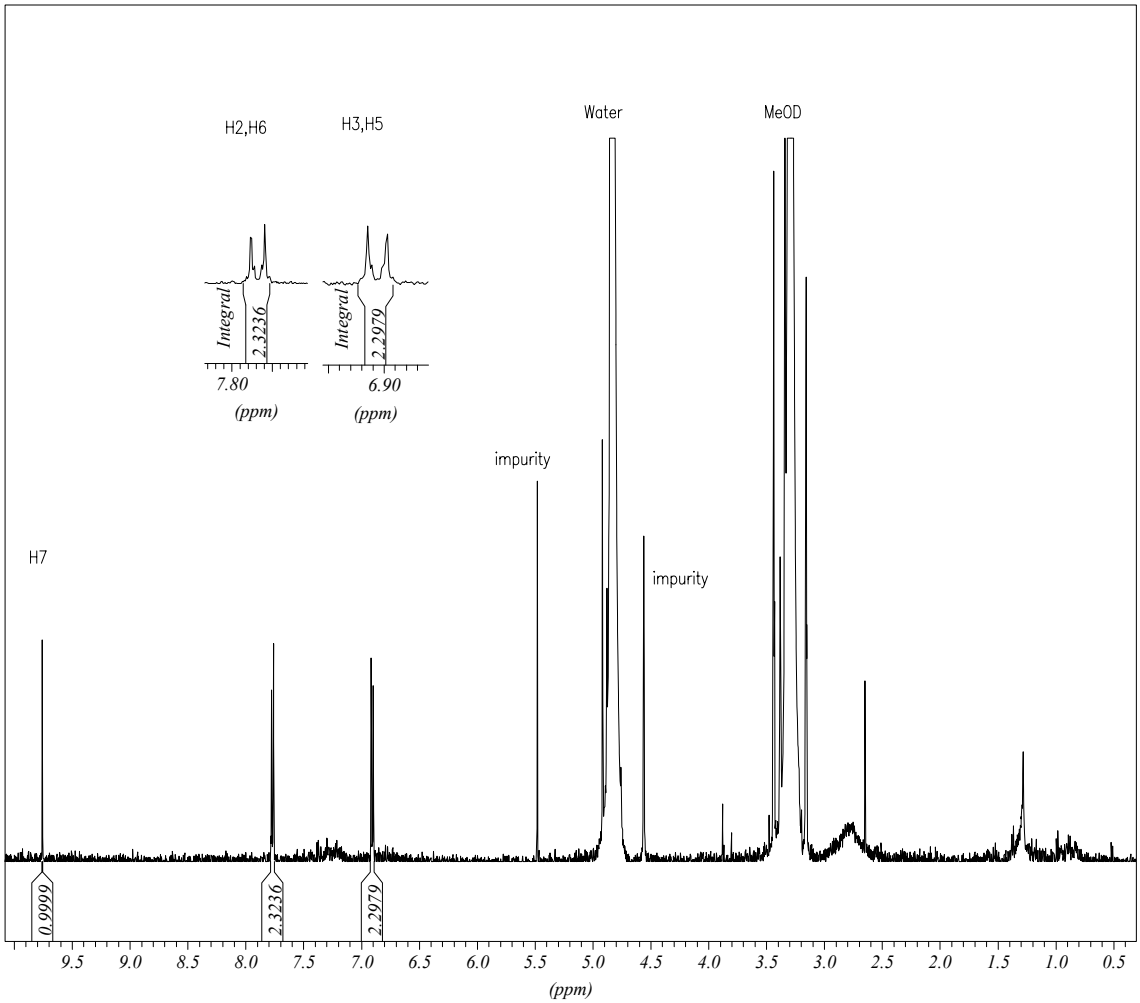



Figure C24.1. $^{1}$H NMR spectrum of compound **24** (4-hydroxybenzaldehyde)

## 3.5. Isolated secondary metabolites of fungus *Lecanicillium evansii* (strain 2)

Eight compounds could be isolated from the fungus *Lecanicillium evansii* (strain 2) that had been derived from the sponge *Hyrtios* sp. The compounds were cytosine riboside (compound **25**), cytosine deoxyriboside (compound **26**), adenosine riboside (compound **27**), adenosine deoxyriboside (compound **28**), ergosterol-5,8-peroxide (compound **29**), dehydroergosterol-5,8-peroxide (compound **30**), and cerebroside C (compound **31**).

### 3.5.1. Compound 25 (cytosine riboside)

In the ESI-MS spectrum, this compound exhibited a protonated molecular ion peak $[M+H]^+$ at *m*/*z* 244, suggesting a molecular weight of 243 g/mol and a molecular formula of $C_9H_{13}N_3O_5$. The $^1$H NMR spectrum (Table C25.1, Figure C25.1) exhibited two doublet signals at *δ* 5.90 and *δ* 8.05 representing heterocyclic protons of the pyrimidine unit of cytosine moiety.

Anomeric proton (H1') of sugar was overlapped with H5. Furthermore, the only proton at 4.75 (H2') confirmed the ribose moiety along with a series of signals at *δ* 3.80 – 4.75 due to hydroxyl groups, indicated that this was nucleoside (cytosine riboside).

Table C25.1. NMR data of compound **25** (cytosine riboside)

| Position | *δ* $^1$H (ppm), multiplicity (*J* in Hz) (in MeOD) |
|---|---|
| 5 | 5.90 (d) |
| 6 | 8.05 (d) |
| 1‘ | 5.90 (d) |
| 2‘ | 4.75 |
| 3‘ | 4.15 (d) |
| 4‘ | 4.05 (m) |
| 5‘A | 3.90 (dd) |
| 5‘B | 3.80 (dd) |

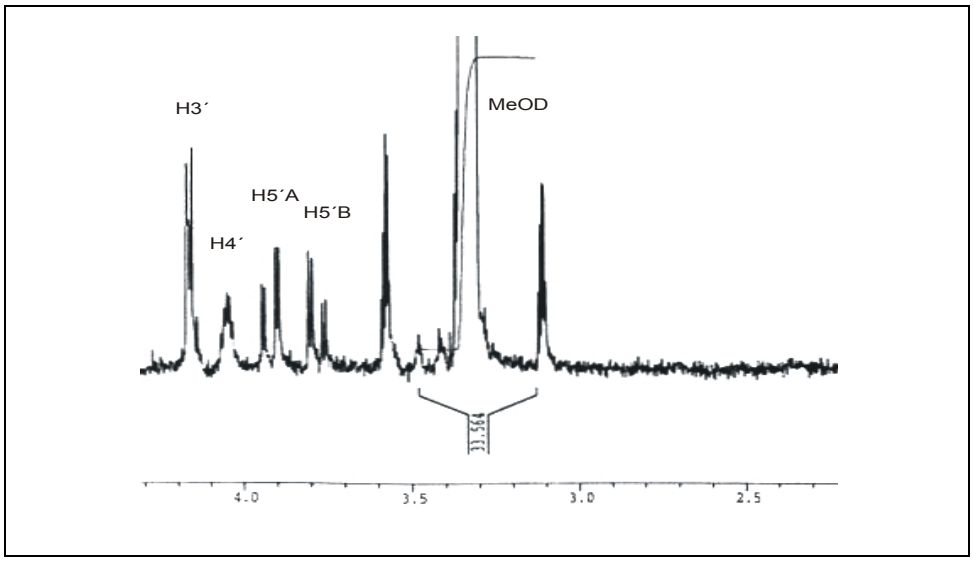



Figure C25.1. $^1$H NMR spectrum of compound **25** (cytosine riboside)

## Cytosine riboside (compound 25)

CAS Registry Number : 65-46-3

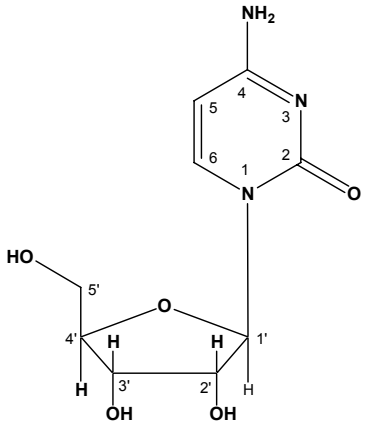
Characteristic : colourless

Formula : $C_9H_{13}N_3O_5$

Molecular Weight : 243 g/mol

Amount : 7.1 mg

Source : *Lecanicillium evansii* (strain 2) derived from *Hyrtios* sp.



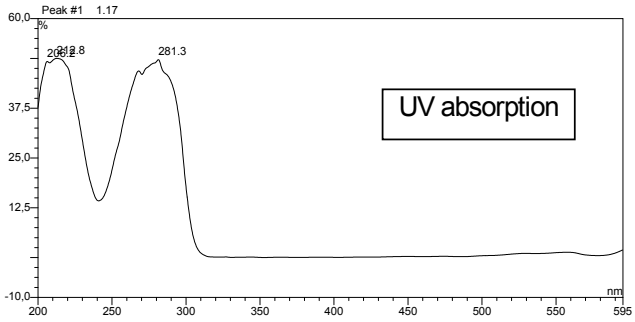

Retention Time : 1.20 min

$R_f$ : 0.75

Fluorescence, 254 nm : -

366 nm : -

Anisaldehyde/$H_2SO_4$ : +




Optical Rotation $[\alpha]_D^{20}$ : experiment = $+20.6^o$ (c=0.1, $H_2O$)

literature = $+34.2^o$ ($H_2O$) (Antibase, 2002)

EI-MS (*m/z*, rel. int.) : 244 $[M+H]^+$ (14)

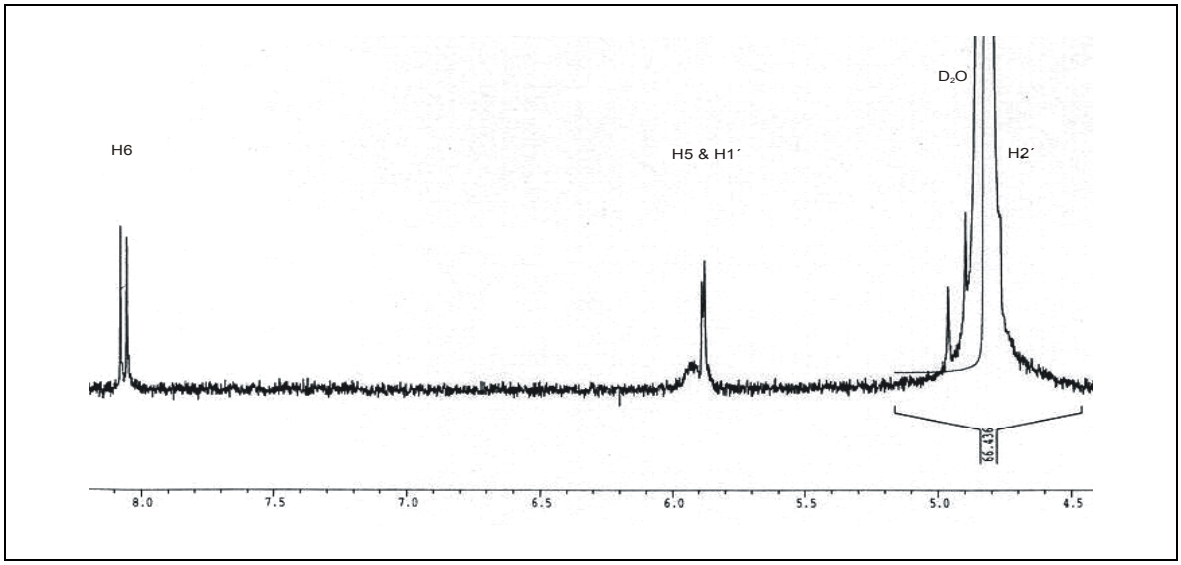



Figure C25.1. $^{1}$H NMR spectrum of compound **25** (cytosine riboside) (continued)

## 3.5.2. Compound 26 (cytosine deoxyriboside)

This compound has a molecular weight of 227 g/mol and a molecular formula of $C_9H_{13}N_3O_4$. This was compatible with the ESI-MS spectrum which exhibited a protonated molecular ion peak $[M+H]^+$ at *m*/*z* 228 and 251 $[M+Na]^+$.

The $^{1}$H NMR spectrum clearly presented a couple of doublet signals at $\delta$ 5.93 (d, *J*=6.6 Hz, H5) and 8.02 (d, *J*=7.5 Hz, H6) representing heterocyclic protons of pyrimidine of the cytosine moiety. Triplet signal at $\delta$ 6.29 (t, *J*=6.5, 6.5 Hz, H1') assignable to the anomeric proton was also evident (Table C26.1, Figure C26.1 and C26.2).

The existence of two pairs of diastereomeric protons at position 2' and 5' indicated the presence of a deoxyribose. Two other signals at $\delta$ 3.35 (H3') and 3.97 (H4') corroborated the presence of a sugar moiety. Methylene protons at position 2' and 5' were split to 2' (A, B) and 5' (A, B) because of the effect of chiral centres at position H1', H3' and H4' (Table C26.1).

Table C26.1. NMR data of compound **26** (cytosine deoxyriboside)

| Position | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) (in MeOD) | COSY (H $\rightarrow$ H) |
|---|---|---|
| 5 | 5.93 (d, 6.6) | H6 |
| 6 | 8.02 (d, 7.5) | H5 |
| 1‘ | 6.29 (dd, 6.5) | H2’A, H2’B |
| 2’A | 2.40 (ddd, 7.5, 6.2, 6.0) | H2’B, H1’ |
| 2‘B | 2.18 (ddd, 6.8, 6.8, 6.6) | H2’A, H1’ |
| 3‘ | 3.35 (m) | |
| 4‘ | 3.97 (dd, 7.4, 3.6) | H3’, H5’A, H5’B |
| 5‘A | 3.83 (dd, 11.0, 3.6) | H5’B, H4’ |
| 5‘B | 3.77 (dd, 12.0, 4.0) | H5’A, H4’ |

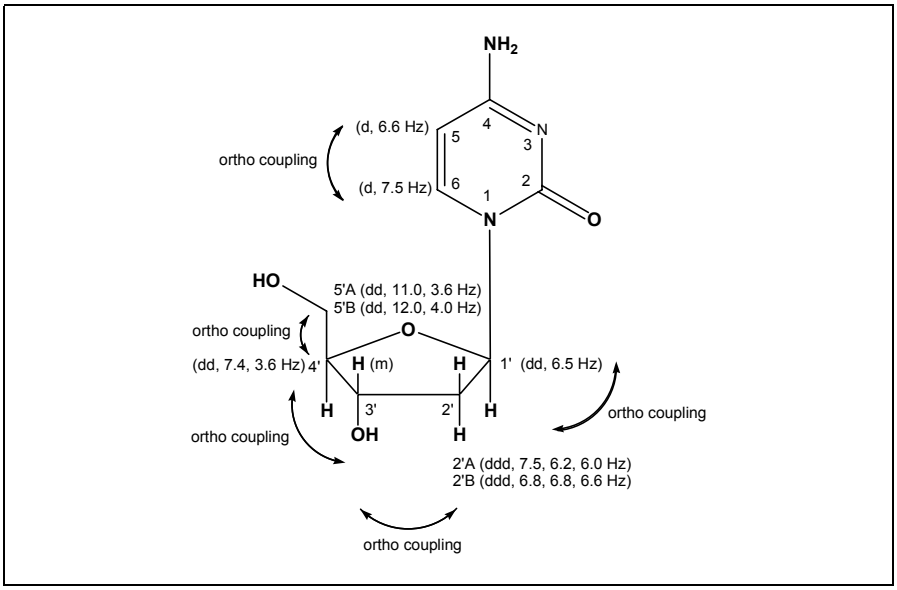



Figure C26.1. Coupling constants of compound **26** (cytosine deoxyriboside)

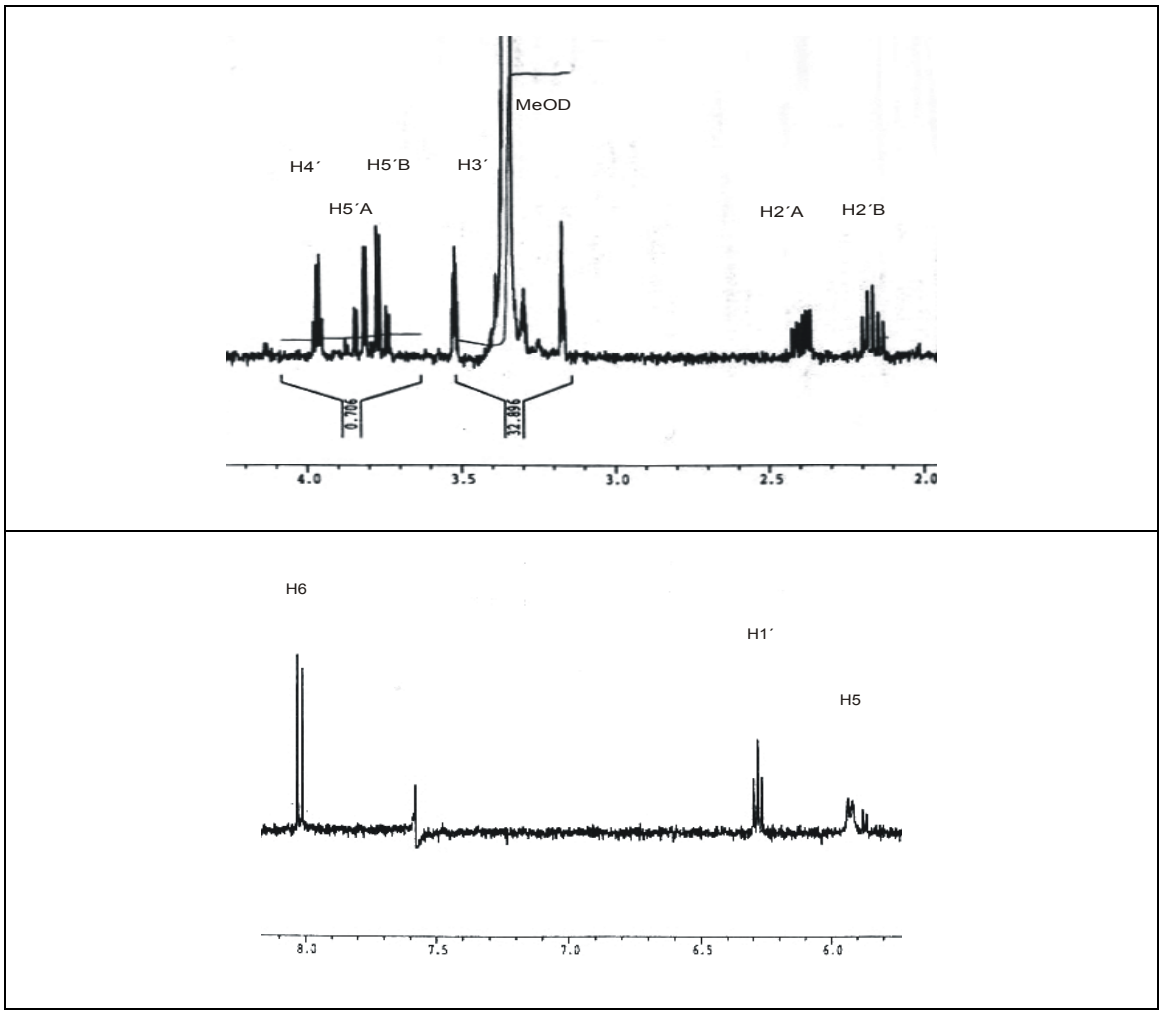
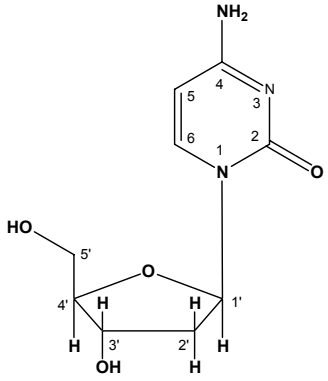


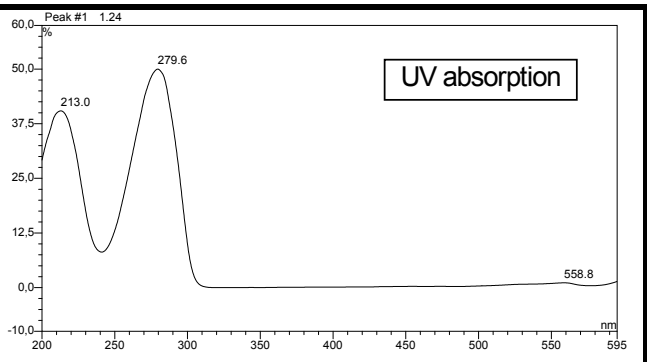

Figure C26.2. $^{1}$H NMR spectrum of compound **26** (cytosine deoxyriboside)

## Cytosine deoxyriboside (compound 26)

| | |
|---|---|
| CAS Registry Number | : - |
| Characteristic | : colourless |
| Formula | : $C_9H_{13}N_3O_4$ |
| Molecular Weight | : 227 g/mol |
| Amount | : 3.3 mg |
| Source | : *Lecanicillium evansii* (strain 2) derived from *Hyrtios* sp. |




| | |
|---|---|
| Retention Time | : 1.21 min |
| $R_f$ | : 0.60 |
| Fluorescence, 254 nm | : + |
| 366 nm | : - |
| Anisaldehyde/$H_2SO_4$ | : + |




| | |
|---|---|
| Optical Rotation $[\alpha]_D^{20}$ | : experiment = +140° (c=0.1, $H_2O$)<br>literature = +152° (c=0.15, $H_2O$) (Takahashi *et al*., 1992) |
| ESI-MS (*m*/*z*) | : 228 $[M+H]^+$ (positive) |
| EI-MS (*m*/*z*, rel. int.) | : 228 $[M+H]^+_{(19)}$, 251 $[M+Na]^+_{(6)}$ |

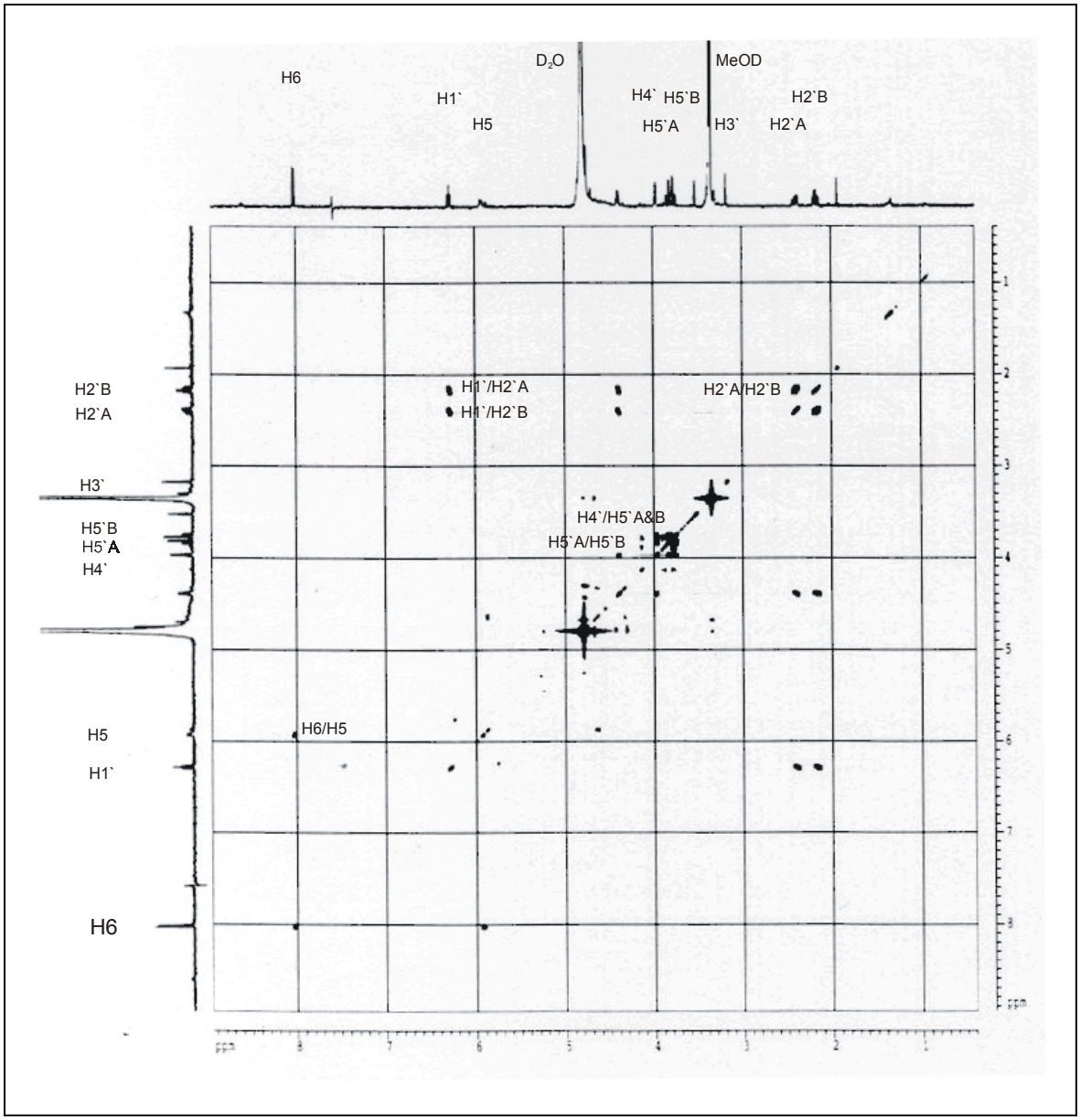



Figure C26.3. COSY spectrum of compound **26** (cytosine deoxyriboside)

## 3.5.3. Compound 27 (adenosine riboside)

The existence of a sugar moiety in this compound was evident by a number of protons resonating between 3.75 ppm and 5.90 ppm. Doublet proton signal at *δ* 5.90 represented the anomeric proton (H1'). There was only one H2' signal appearing at *δ* 4.65, implying the existence of ribose. Two singlet protons encountered in the low field of the $^{1}$H NMR spectrum were assigned to positions 2 and 8 of the purine moiety.

The asymmetric centre at position 4' caused the signal of H5' to split into H5'A and H5'B (Table C27.1, Figure C27.1). Multiplicity of each sugar proton was the same with the one reported in Antibase 2002. Likewise the chemical shifts were similar, leading to the conclusion that this compound is adenosine riboside.

Table C27.1. NMR data of compound **27** (adenosine riboside)

| Position | $\delta$ $^{13}$C (ppm), multiplicity (*J* in Hz) (Antibase, 2002 in DMSO) | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) (Antibase, 2002 in DMSO) | $\delta$ $^{1}$H (ppm), multiplicity (*J* in Hz) (in MeOD) |
|---|---|---|---|
| 1 | | | |
| 2 | 152.30 (d) | | 8.60 (s) |
| 3 | | | |
| 4 | 149.00 (s) | | |
| 5 | 119.30 (s) | | |
| 6 | 156.10 (s) | | |
| 7 | | | |
| 8 | 139.90 (d) | | 8.00 (s) |
| 9 | | | |
| 10 | | | |
| 1‘ | 87.90 (d) | 5.87 (d, 6.0) | 5.90 (d) |
| 2‘ | 70.60 (d) | 4.59 (t, 10.0) | 4.65 (t) |
| 3‘ | 73.40 (d) | 4.13 (dd, 10.0, 8.0) | 4.35 (dd) |
| 4‘ | 85.80 (d) | 3.95 (dd, 8.0, 3.0) | 4.05 (dd) |
| 5‘A | 61.60 (t) | 3.65 (dd, 12.0, 4.0) | 3.85 (dd) |
| 5‘B | | 3.55 (dd, 12.0, 4.0) | 3.75 (dd) |

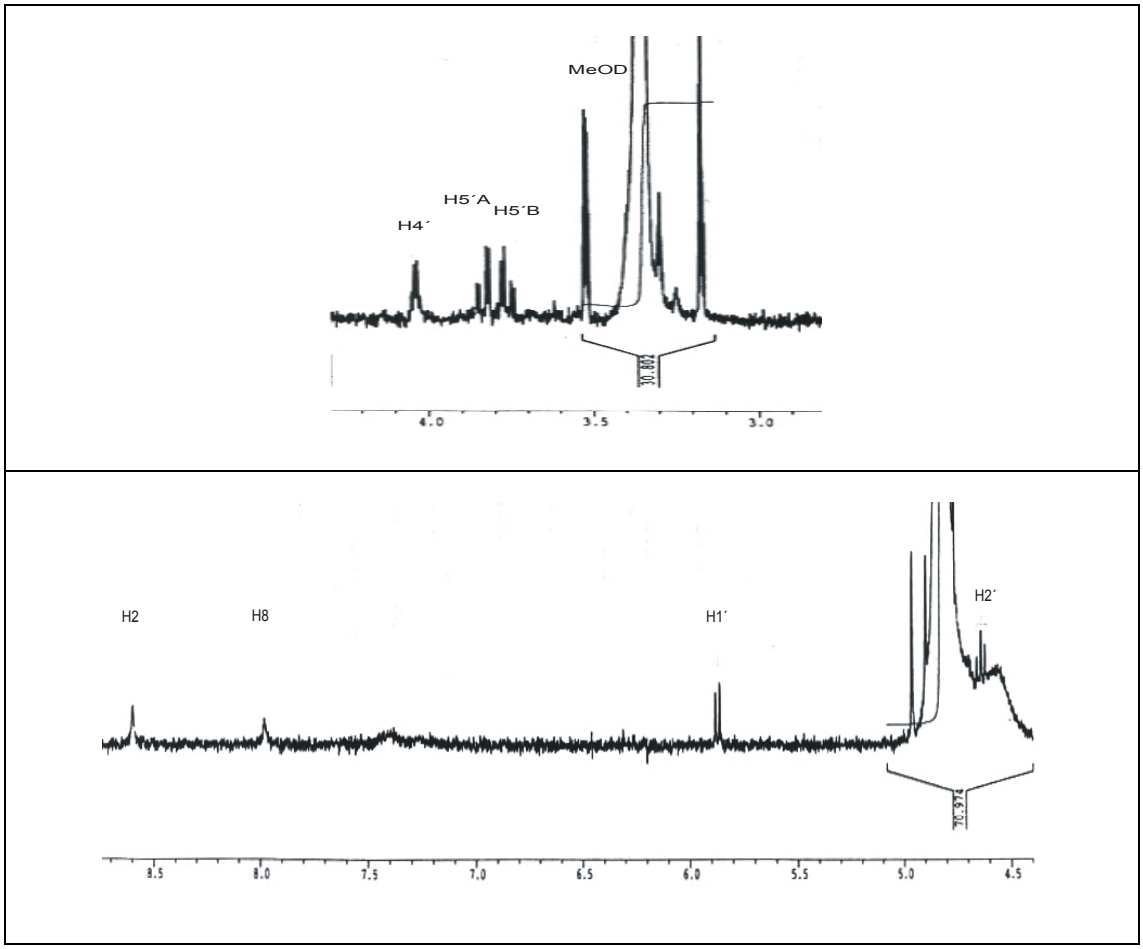



Figure C27.1. $^{1}$H NMR spectrum of compound **27** (adenosine riboside)

## Adenosine riboside (compound 27)

CAS Registry Number : -

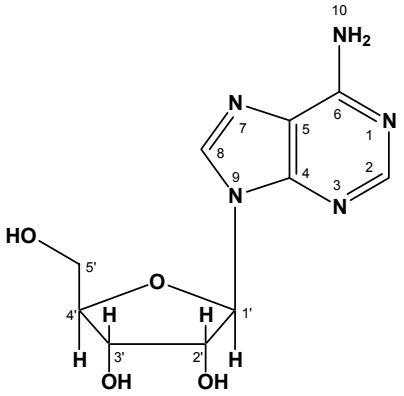
Characteristic : transparent

Formula : $C_{10}H_{13}N_5O_4$

Molecular Weight : 267 g/mol

Amount : 4.4 mg

Source : *Lecanicillium evansii* (strain 2) derived from *Hyrtios* sp.

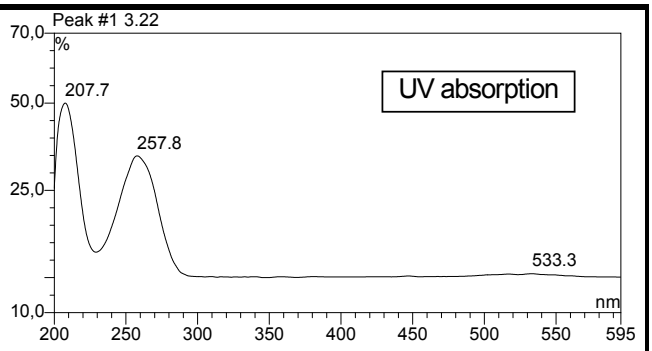



Retention Time : 3.07 min

$R_f$ : 0.75

Fluorescence, 254 nm : -
366 nm : -

Anisaldehyde/$H_2SO_4$ : +




Optical Rotation $[\alpha]_D^{20}$ : experiment = -46.0 (c=0.1, $H_2O$)
literature = -61.7 (c=0.706, $H_2O$)

EI-MS (*m*/*z*, rel. int.) : 252 $[M\text{-}NH_2]^+$ (8)

## 3.5.4. Compound 28 (adenosine deoxyriboside)

The examination of signal systems of the sugar moiety in this compound indicated the presence of deoxyribose linked to position 9 of the purine ring. Two downfield singlet signals at *δ* 8.20 and 8.40, representing protons at position 2 and 8 of purine ring, respectively, indicated the presence of adenosine moiety. Another downfield signal at *δ* 6.50 belonged to the anomeric proton of the sugar at position 1'. Six other signals assigned to H2'A, H2'B, H3', H4', H5'A, and H5'B were also observed (Table C28.1, Figure C28.1).

The chemical shift of $^{1}$H NMR differences of the previously reported compound (Evidente *et al*., 1989) and compound **28** varied at range 0.02 – 0.13 ppm. It was therefore concluded that both compounds are identical.

Table C28.1. NMR data of compound **28** (adenosine deoxyriboside)

| Position | *δ* $^{13}$C (ppm), multiplicity (*J* in Hz) (Evidente *et al*., 1989 in MeOD) | *δ* $^{1}$H (ppm), multiplicity (*J* in Hz) (Evidente *et al*., 1989 in MeOD) | *δ* $^{1}$H (ppm), multiplicity (*J* in Hz) (in MeOD) |
|---|---|---|---|
| 1 | | | |
| 2 | 153.5 (d) | 8.17 (s) | 8.20 (s) |
| 3 | | | |
| 4 | 149.9 (s) | | |
| 5 | 120.8 (s) | | |
| 6 | 157.5 (s) | | |
| 7 | | | |
| 8 | 141.5 (d) | 8.30 (s) | 8.40 (s) |
| 9 | | | |
| 10 | | | |
| 1' | 87.1 (d) | 6.43 (dd, 8.0, 6.1) | 6.50 (dd) |
| 2'A | 41.5 (t) | 2.81 (ddd, 13.6, 8.0, 5,9) | 2.90 (ddd) |
| 2'B | | 2.40 (ddd, 13.6, 6.1, 2.8) | 2.50 (ddd) |
| 3' | 73.0 (d) | 4.58 (ddd, 5.9, 2.9, 2.8) | 4.70 (ddd) |
| 4' | 89.9 (d) | 4.08 (ddd, 2.9, 2.9, 2.8) | 4.10 (ddd) |
| 5'A | 63.6 (t) | 3.85 (dd, 12.3, 2.9) | 3.90 (dd) |
| 5'B | | 3.75 (dd, 12.3, 2.9) | 3.80 (dd) |

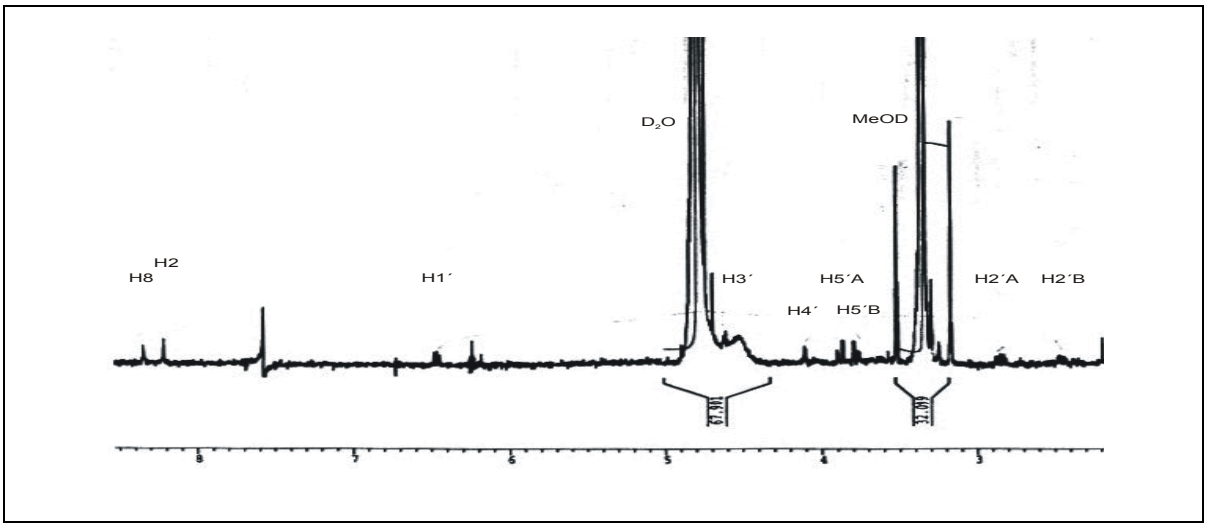
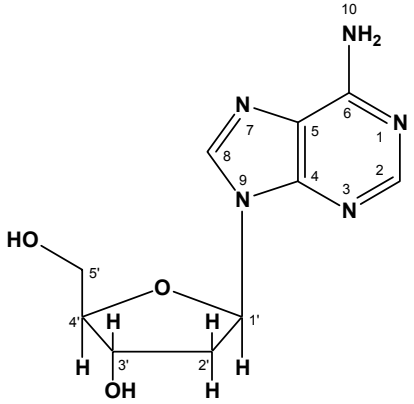


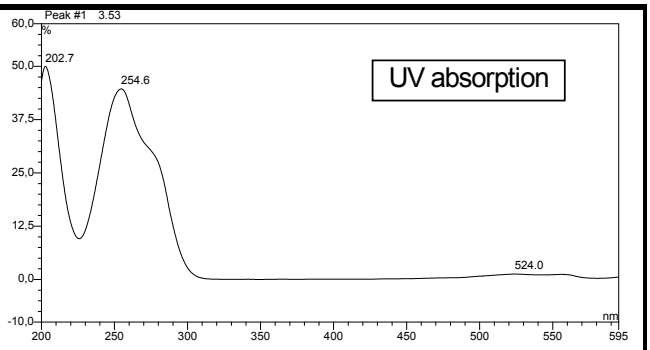

Figure C28.1. $^{1}$H NMR spectrum of compound **28** (adenosine deoxyriboside)

## Adenosine deoxyriboside (compound 28)

| | |
|---|---|
| CAS Registry Number | : - |
| Characteristic | : colourless |
| Formula | : $C_{10}H_{13}N_5O_3$ |
| Molecular Weight | : 251 g/mol |
| Amount | : 3.2 mg |
| Source | : *Lecanicillium evansii* (strain 2) derived from *Hyrtios* sp. |




| | |
|---|---|
| Retention Time | : 3.76 min |
| $R_f$ | : 0.76 |
| Fluorescence, 254 nm | : - |
| 366 nm | : - |
| Anisaldehyde/$H_2SO_4$ | : + |




| | |
|---|---|
| Optical Rotation $[\alpha]_D^{20}$ | : experiment = -15 (c=0.1, $H_2O$)<br>literature = -26.0 (c=1.0, $H_2O$) (Evidente *et al*., 1989) |
| EI-MS (*m*/*z*, rel. int.) | : not measured |

## 3.5.5. Compound 29 (ergosterol-5,8-peroxide)

Through spectroscopic data analysis, compound **29** was identified as ergosterol peroxide with the molecular formula of $C_{28}H_{44}O_3$. Its molecular weight of 428 g/mol was established by the FAB-MS spectrum implying ion peaks at *m*/*z* 451and 396 corresponding to $[M+Na]^+$ and $[M-O_2]^+$, respectively.

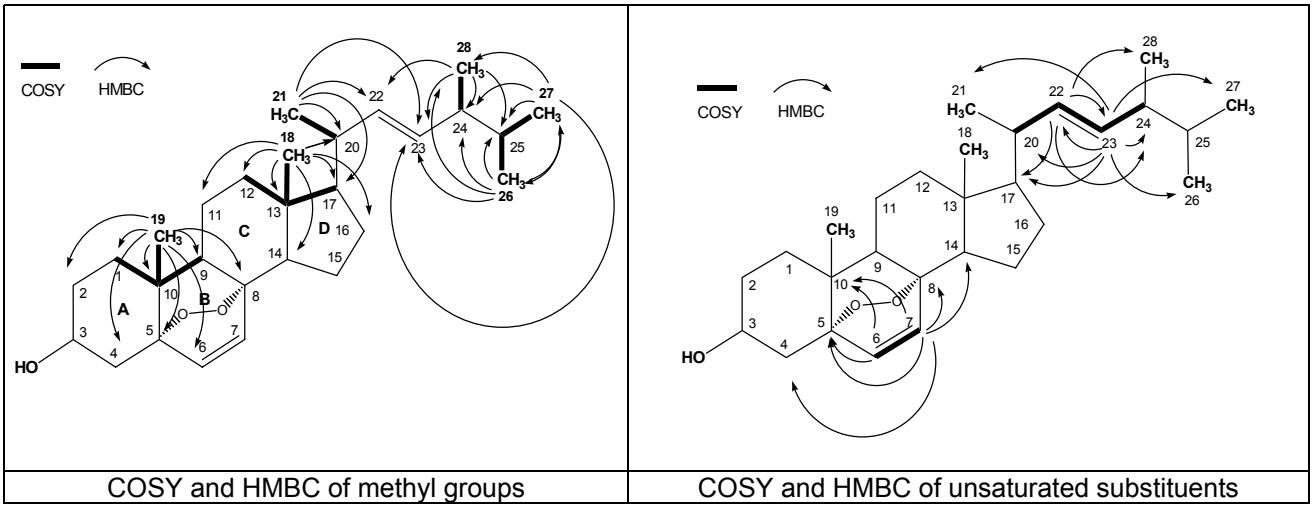
Downfield signals at $\delta$ 6.20 (H6), 6.50 (H7), 5.19 (H22, H23) in the $^1$H NMR spectrum (Table C29.1, Figure C30.2) revealed the presence of two pairs of disubstituted double bonds. Carbon signals at $\delta$ 130.80 (C6), 135.46 (C7), 132,38 (C22), and 135.25 (C23) in the $^{13}$C NMR spectrum (Table C29.1, Figure C29.3) confirmed the existence of these two pairs of double bonds.

The location of the double bonds was assigned through analysis of COSY and HMBC spectra (Figure C29.4,5,6) in which one pair (C6 and C7) was positioned in ring B, and another double bond (C22 and C23) was in the sterol side chain (Figure C29.1). Two non-protonated carbon signals at $\delta$ 82.19 (C5) and 79.46 (C8) were characteristic of oxygen-bearing carbons, implying a peroxide group attached to position 5 and 8. Another oxygen-carrying carbon signal was observed at $\delta$ 66.52 (C3) where a hydroxyl group was attached.

Other specific signals of ergosterol peroxide in the $^1$H NMR spectrum were six methyl signals [$\delta$ 0.81 (H18), 0.88 (H19), 1.00 (H21), 0.82 (H26), 0.83 (H27), 1.00 (H28)]. Further interpretation of the COSY spectrum led to the assignment of these methyl group positions. Two methyl groups (H18, H19) were located in the sterol nucleus. These positions were elucidated on the basis of HMBC cross peaks between H18 and C11, C12, C13, C14, C16, C17, C20; between H19 and C1, C2, C4, C5, C6, C8, C9, C10.

Four other methyls (C21, C26, C27, C28) were positioned in the sterol side chain. Long range correlation (HMBC) among these methyl groups and their adjacent carbons through either two, three, or four bond couplings readily confirmed these methyl group assignments (Figure C29.1). Direct correlation of $^1$H and $^{13}$C in the HMQC spectrum connected all protons to their respective carbons (Figure C29.5).

The large coupling constant ($J$ = 15.2 Hz) between H22 and H33 indicated *trans* (*E*) configuration of the double bond at C22. Comparison of $^1$H NMR data of compound **29** with those reported by Bok *et al*. (1999) proved both compounds to be identical.

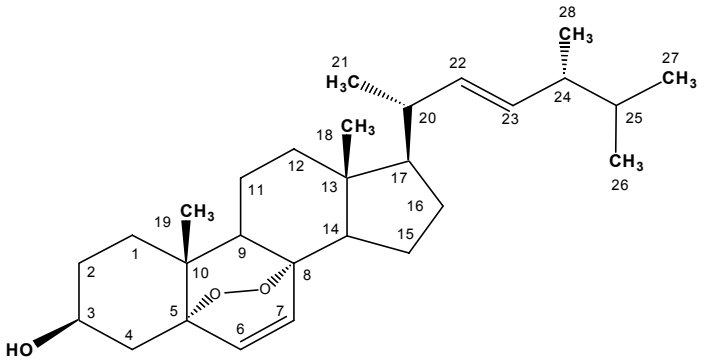



| COSY and HMBC of methyl groups | COSY and HMBC of unsaturated substituents |
|---|---|

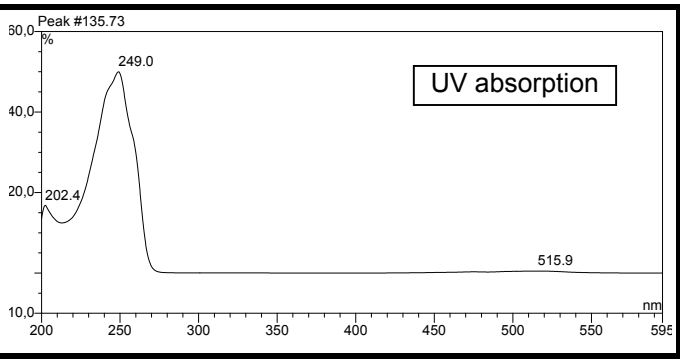
Figure C29.1. COSY and HMBC correlations of methyl groups and unsaturated substituents of compound **29** (ergosterol-5,8-peroxide)

## Ergosterol-5,8-peroxide (compound 29)

| | | |
|---|---|---|
| CAS Registry Number | : | 2061-64-5 |
| Characteristic | : | white crystals |
| Formula | : | $C_{28}H_{44}O_3$ |
| Molecular Weight | : | 428 g/mol |
| Amount | : | 6.3 mg |
| Source | : | *Lecanicillium evansii* (strain 2) derived from *Hyrtios* sp. |




| | | |
|---|---|---|
| Retention Time | : | 35.69 min |
| $R_f$ | : | 0.41 |
| Fluorescence, 254 nm | : | + |
| 366 nm | : | + |
| Anisaldehyde/$H_2SO_4$ | : | brown |




| | | |
|---|---|---|
| Optical Rotation $[\alpha]_D^{20}$ | : | experiment = $-21^o$ (c=0.1, $CHCl_3$)<br>literature = $-25^o$ ($CHCl_3$) (Gunatilaka *et al*., 1981) |
| FAB-MS (*m/z*, rel. int.) | : | 451$[M+Na]^+_{(6.0)}$, 396 $[M-O_2]^+_{(42.7)}$, 377$_{(23.3)}$,<br>363$[M-H_2O-O_2-CH_3]^+_{(10.1)}$, 337$[M-O_2-C_3H_7O]^+_{(5.3)}$,<br>303$[M\text{-side chain}]^+_{(2.1)}$ |

Table C29.1. NMR data of compound **29** (ergosterol-5,8-peroxide)

| Position | $\delta$ $^{13}$C(ppm) (in $CDCl_3$) | DEPT | $\delta$ $^{1}$H (ppm) multiplicity (*J* in Hz) ( in $CDCl_3$ ) | COSY (H → H) | HMQC (H→ C) direct | HMBC (H→ C) |
|---|---|---|---|---|---|---|
| 1 | 34.76 (t) | 34.76 (t) | H1A: 1.95 (m)<br>H1B: 1.72 (m) | H1B, H2B, H3<br>H1A, H2B, H3 | C1 | C2, C3, C5, C10, C19<br>C2, C3, C5, C10, C19 |
| 2 | 30.19 (t) | 30.19 (t) | H2A: 1.60 (m)<br>H2B: 1.90 (m) | H3, H4A, H5<br>H1B, H3, H4A, H4B | C2 | C3, C5<br>C1 |
| 3 | 66.52 (d) | | 4.00 (m) | H1A, H1B, H2A, H2B, H4A, H4B | C3 | |
| 4 | 51.19 (t) | 51.19 (t) | H4A: 1.95 (dd, 8.3, 3.4)<br>H4B: 2.10 (dd, 2.2) | H2A, H2B, H3<br>H2B, H3, H4A | C4 | C2, C3, C5<br>C2, C3, C5, C10 |
| 5 | 82.19 (s) | | | | | |
| 6 | 130.80 (d) | | 6.20 (d, 8.5) | H7 | C6 | C5, C8, C10 |
| 7 | 135.46 (d) | | 6.50 (d, 8.5) | H6 | C7 | C4, C5, C8, C10, C14 |
| 8 | 79.46 (s) | | | | | |
| 9 | 34.76 (d) | | 1.60 (t, 3.5) | H11, H12, H19 | C9 | C5, C6, C13 |
| 10 | 37.02 (s) | | | | | |
| 11 | 20.93 (t) | 20.93 (t) | 1.60 (m) | H9, H12 | C11 | C5, C13, C18 |
| 12 | 39.42 (t) | 39.42 (t) | 2.00 (t) | H11, H17 | C12 | C8, C10, C13, C14 |
| 13 | 44.63 (s) | | | | C13 | |
| 14 | 51.75 (d) | | 1.55 (t) | H12, H17 | C14 | C6 |
| 15 | 28.67 (t) | 28.67 (t) | 1.84 (m) | H16, H17 | C15 | C13, C17 |
| 16 | 23.46 (t) | 23.46 (t) | 1.47 (m) | H15, H17 | C16 | C22 |
| 17 | 56.29 (d) | | 1.26 (m) | H15, H16, H18 | C17 | C18 |
| 18 | 12.92 (q) | | 0.81 (s) | H12, H17 | C18 | C11, C12, C13, C14, C16, C17, C20 |
| 19 | 18.21 (q) | | 0.88 (s) | H1A, H9 | C19 | C1, C2, C4, C5, C6, C8, C9, C10 |
| 20 | 39.74 (d) | | 2.20 (m) | H17, H21, H22 | C20 | C22, C23 |
| 21 | 19.98 (q) | | 1.00 (d, 6.6) | H20 | C21 | C13, C16, C17, C20, C22, C23 |
| 22 | 132.38 (d) | | 5.19 (dd, 15.2, 7.7) | H20, H23 | C22 | C17, C23, C24, C26, C27, C28 |
| 23 | 135.25 (d) | | 5.19 (dd, 15.2, 7.7) | H22, H24 | C23 | C17, C20, C21, C22, C24, C26, C27, C28 |
| 24 | 42.83 (d) | | 1.90 (d, 2.0) | H23, H25, H26, H27,H28 | C24 | C22, C23, C25, C26, C28 |
| 25 | 33.12 (d) | | 1.55 (m) | H24, H26, H27 | C25 | C23, C24, C26 |
| 26 | 19.68 (q) | | 0.82 (d, 6.8) | H25, H27 | C26 | C23, C24, C25, C27, C28 |
| 27 | 20.68 (q) | | 0.83 (d, 6.8) | H25, H26 | C27 | C23, C24, C25, C26, C28 |
| 28 | 17.60 (q) | | 1.00 (d, 6.8) | H24 | C28 | C23, C24, C25 |

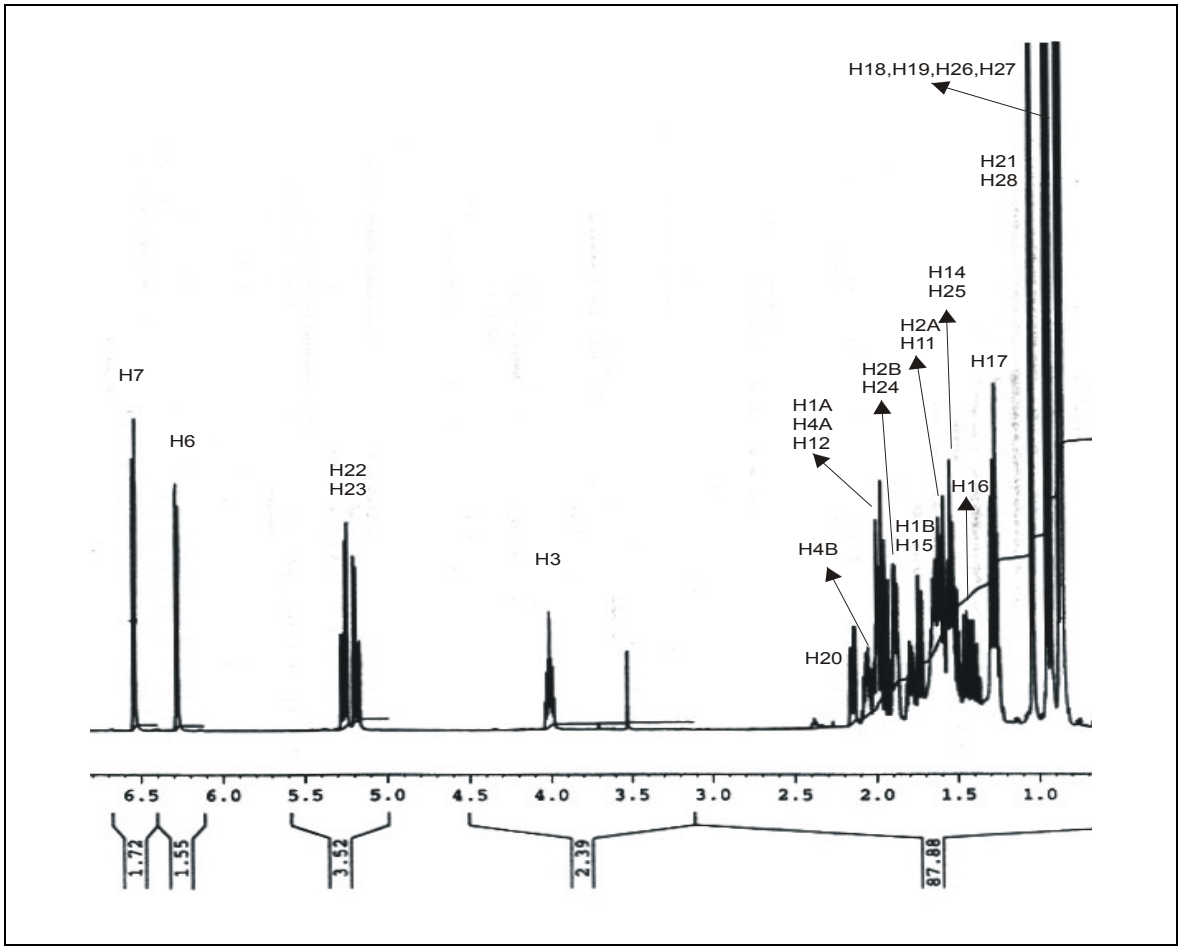



Figure C29.2. $^{1}$H NMR spectrum of compound **29** (ergosterol-5,8-peroxide)

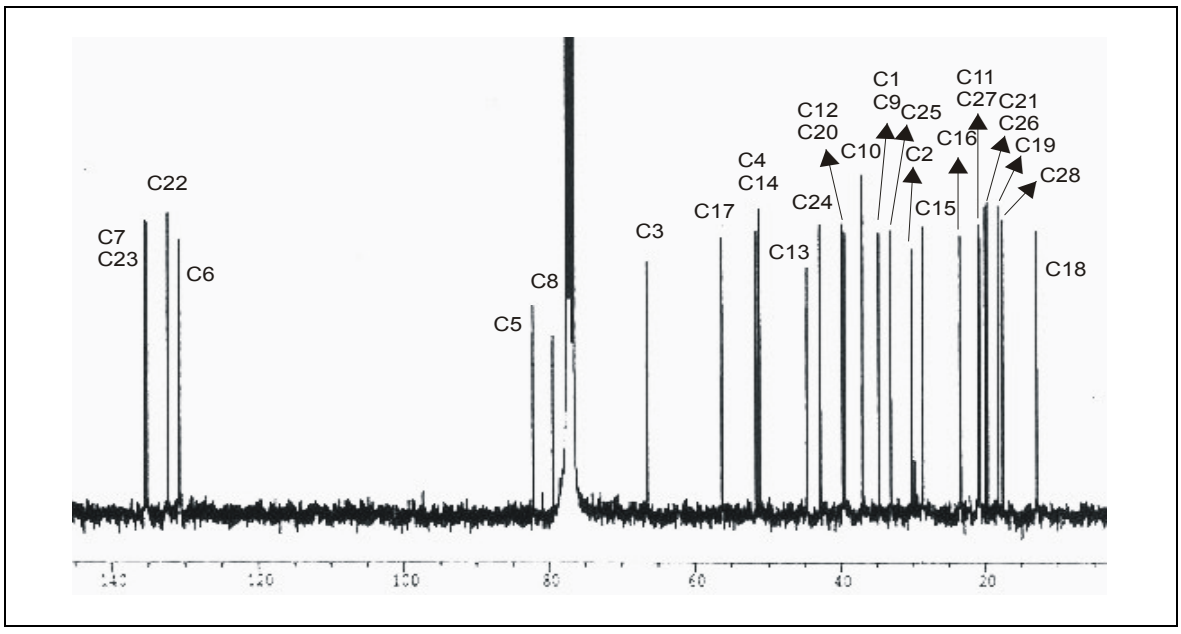



Figure C29.3. $^{13}$C NMR spectrum of compound **29** (ergosterol-5,8-peroxide)

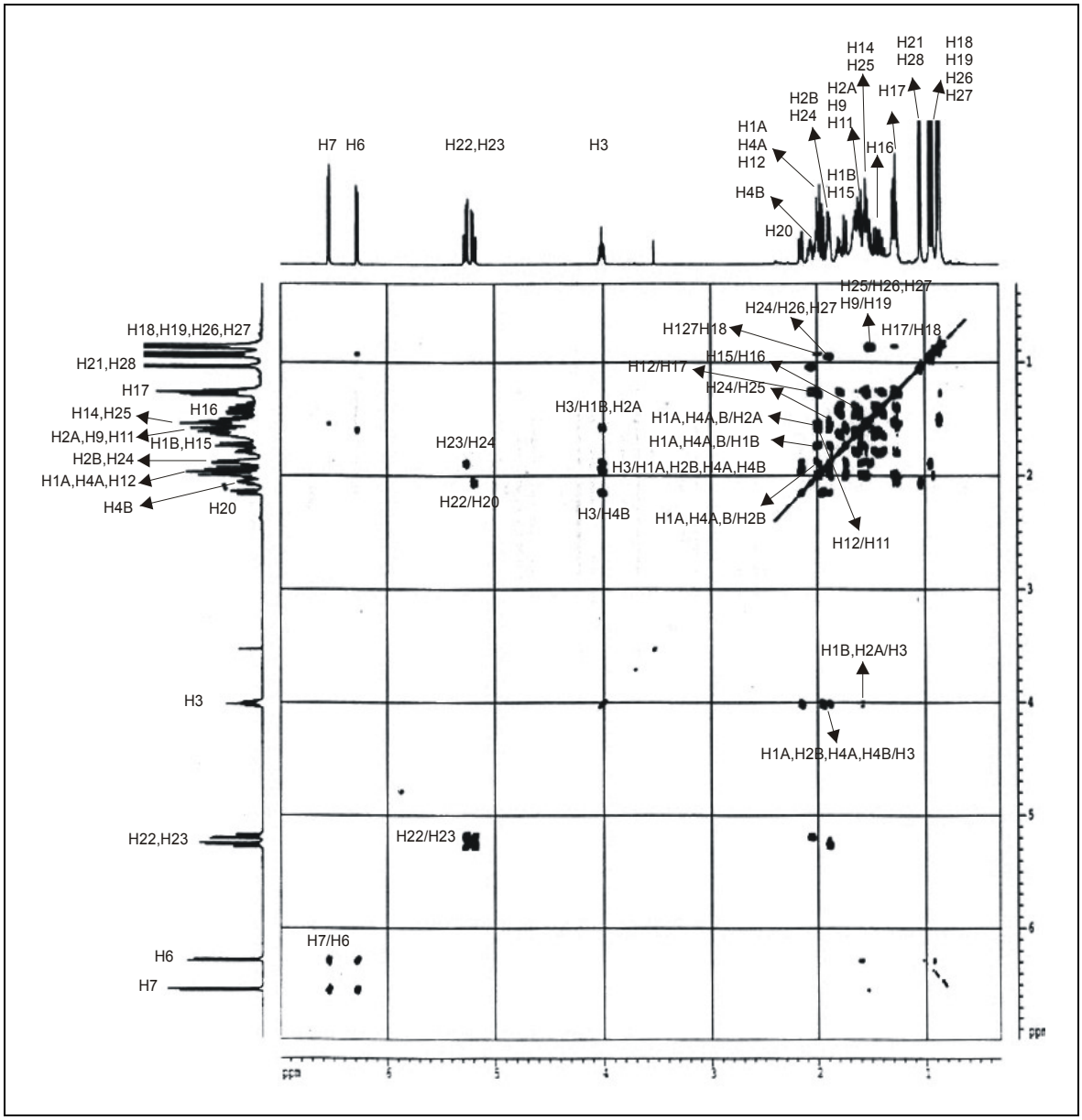



Figure C29.4. COSY spectrum of compound **29** (ergosterol-5,8-peroxide)

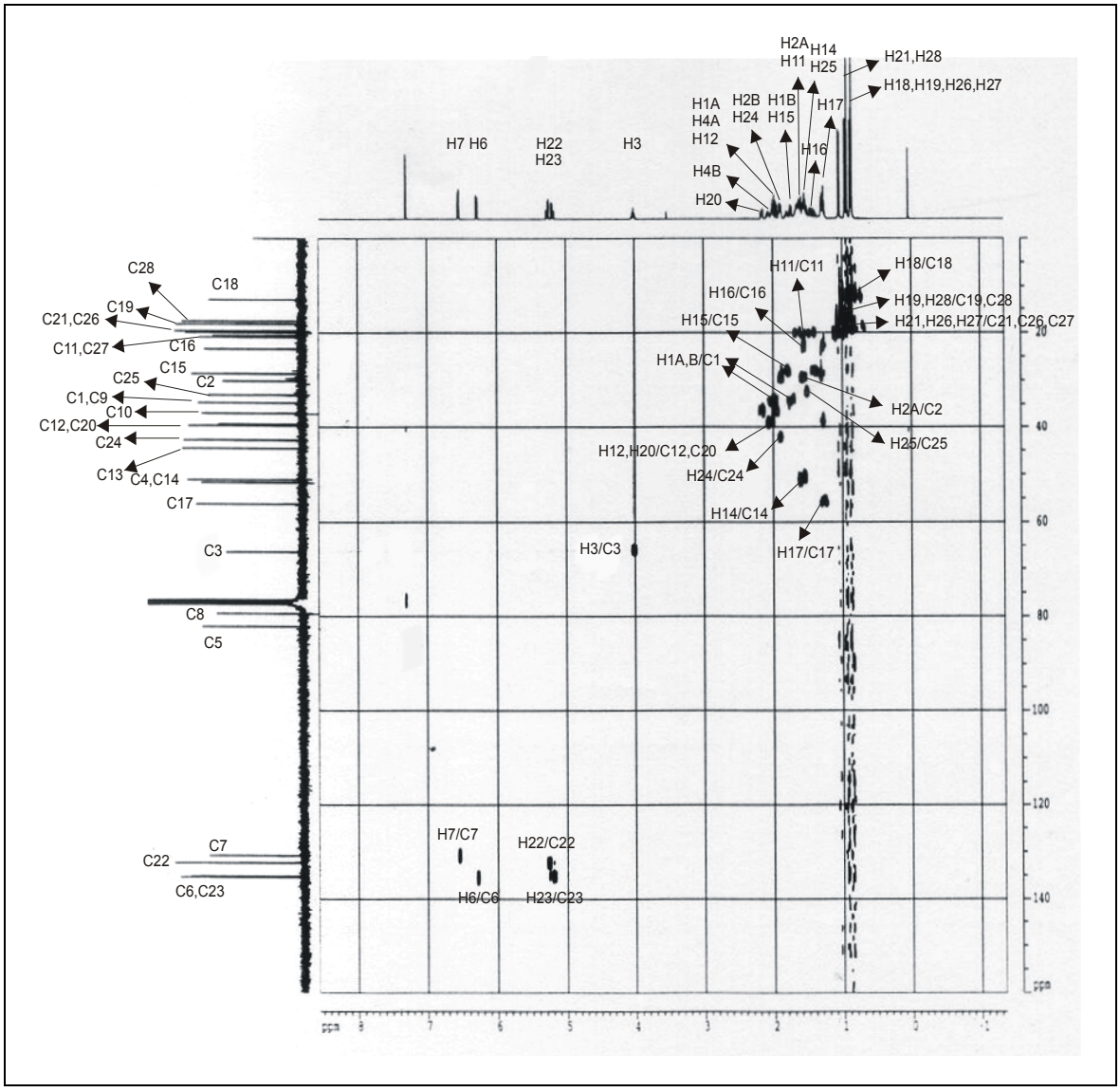



Figure C29.5. HMQC spectrum of compound **29** (ergosterol-5,8-peroxide)

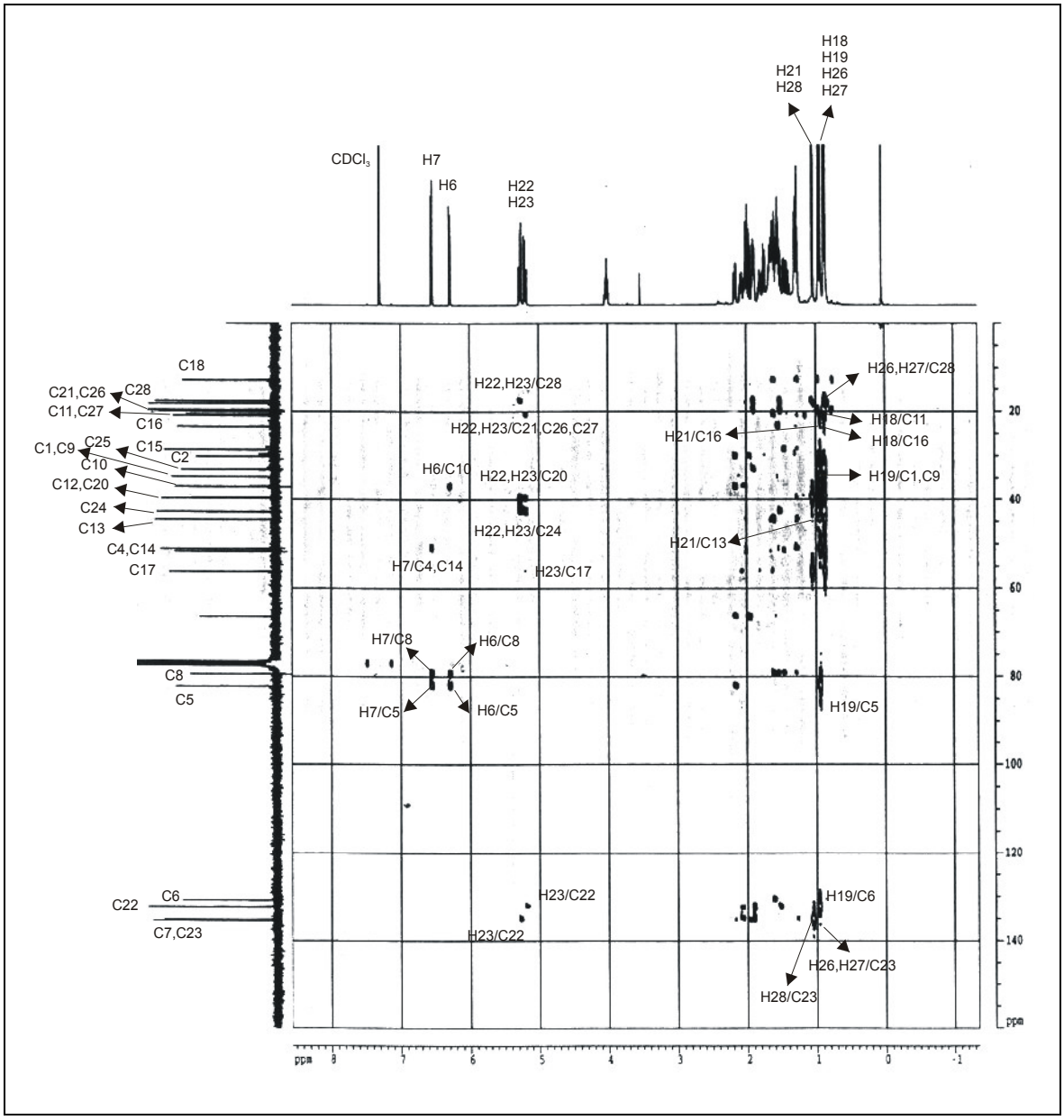



Figure C29.6. HMBC spectrum of methyl and olefinic proton of compound **29** (ergosterol-5,8-peroxide)

## 3.5.6. Compound 30 (dehydroergosterol-5,8-peroxide)

This compound had a molecular weight of 426 g/mol and an empirical formula of $C_{28}H_{42}O_3$ as established by the ESI-MS spectrum. Molecular ion fragmentation occurred at *m*/*z* 449 $[M+Na]^+$, 409 $[M-OH]^+$, 381 $[M-3CH_3]^+$ and 375 $[M-H_2O-O_2]^+$. Ion peaks of *m*/*z* 409 and 375 represented the loss of hydroxyl group and peroxide group, respectively.

Dehydroergosterol peroxide is characterised by having a steroid nucleus (19 carbons atoms) (Table C30.1). This compound possesses two double bonds at C6, C7, C9, C11, and an ergostane side chain having C22 and C23 unsaturation.

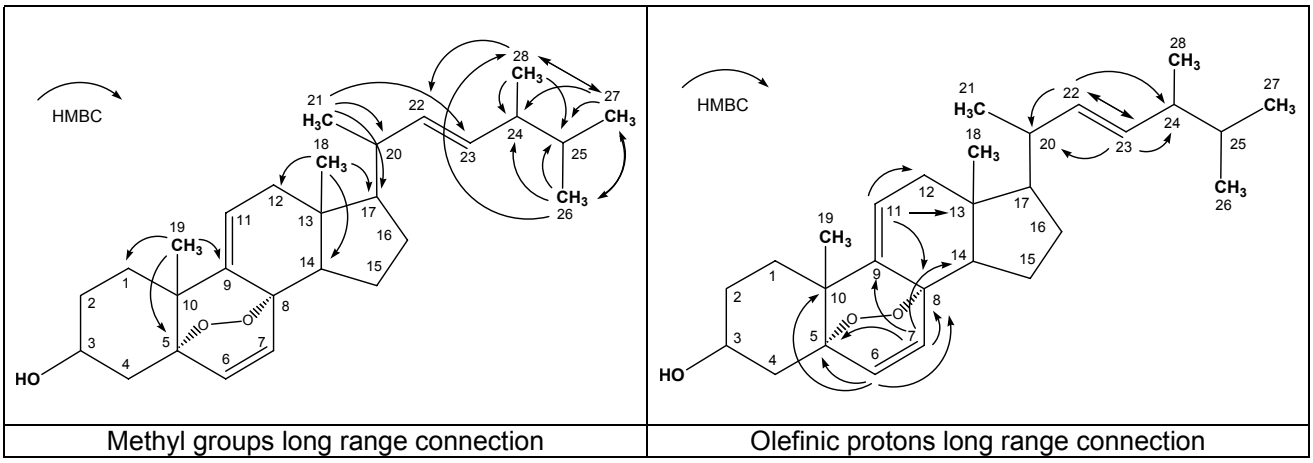
Unsaturation of position 6 and 7 was conspicuously detected by the existence of the two mutually coupled olefinic protons at *δ* 6.28 (H6), 6.60 (H7) and their relations to the adjacent carbon as exhibited in the long range HMBC spectrum. The chemical shifts of *δ* 6.28 (H6) and 6.60 (H7) were indicative of a 5α,8α-epidioxy orientation, as normally encountered at around *δ* 6.3 (H6) and *δ* 6.5 (H7) (Seo *et al*., 1996).

Further HMBC spectrum analysis revealed another double bond (C9 and C11) within the sterol nucleus. This double bond assignment in ring C was supported by the long range correlation between H11 and C8, C12, and C13. The assignment of the double bond in the side chain was deduced by the existence of two double doublets of olefinic protons [*δ* 5.23 (H22), 5.15 (H23)], verified by their long range correlation to C20 and C24 in the HMBC spectrum (Figure C30.5 and C30.6).

The existence of unsaturation of position 9 and 11 was also shown by the olefinic proton at *δ* 5.42 (H11). Asymmetric effect of position 13 brought about the splitting of H12 which resonated as two multiplet signals at *δ* 2.10 (H12A) and 2.28 (H12B).

The deshielding effect of the double bond (C9, C11), extending to the allylic proton H12, resulted in the shifting of this proton more downfield [(*δ* 2.10, H12A), (2.28, H12B)] compared to H12 (*δ* 2.00) of ergosterol peroxide. The proton signal at *δ* 4.02 was assigned to H3, pointing out that a hydroxyl group was bound to the C3 position.

Four doublet methyl signals at *δ* 0.99 (H21), 0.85 (H26), 0.83 (H27), 0.99 (H28) belonged to side chain methyls, whereas other two singlet methyl signals at *δ* 0.74 (H18) and 1.09 (H19) were attached to the sterol nucleus. These methyl position assignments were explained by their connection to the adjacent carbons in the HMBC spectrum (Figure C30.1 and C30.5)

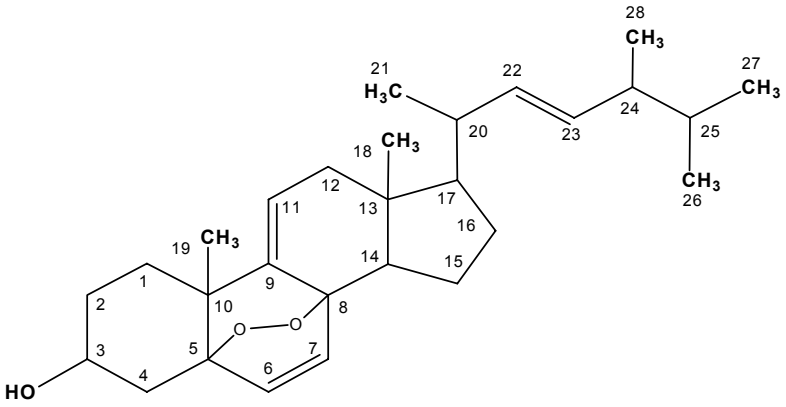



Methyl groups long range connection | Olefinic protons long range connection

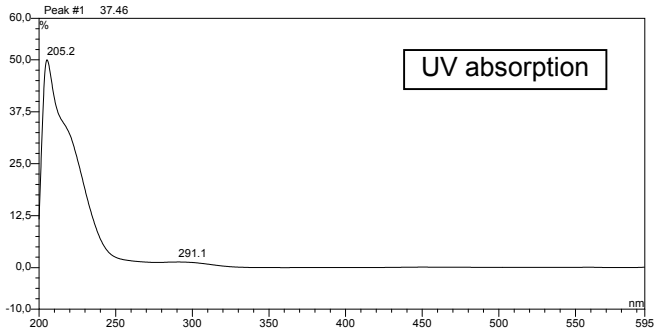
Figure C30.1. Long range correlation (HMBC) of methyl groups and olefinic protons of compound **30** (dehydroergosterol-5,8-peroxide)

## Dehydroergosterol-5,8-peroxide (compound 30)

| | | |
|---|---|---|
| CAS Registry Number | : | 86363-50-0 |
| Characteristic | : | white crystals |
| Formula | : | $C_{28}H_{42}O_3$ |
| Molecular Weight | : | 426 g/mol |
| Amount | : | 0.7 mg |
| Source | : | *Lecanicillium evansii* (strain 2) derived from *Hyrtios* sp. |




| | | |
|---|---|---|
| Retention Time | : | 37.56 min |
| $R_f$ | : | 0.41 |
| Fluorescence, 254 nm | : | - |
| 366 nm | : | - |
| Anisaldehyde/$H_2SO_4$ | : | + |




| | | |
|---|---|---|
| Optical Rotation $[\alpha]_D^{20}$ | : | experiment = -24.2° (c=0.05, $CHCl_3$)<br>literature = +72.9° (c=1.0, $CHCl_3$) (Gauvin *et al*., 2000) |
| ESI-MS (*m/z*, rel. int.) | : | 449$[M+Na]^+_{(24)}$, 409$[M-OH]^+_{(19)}$, 381$[M-3CH_3]^+_{(100)}$, 375$[M-H_2O-O_2]^+_{(42)}$ |

Table C30.1. NMR data of compound **30** (dehydroergosterol-5,8-peroxide)

| Position | $\delta$ $^{13}$C (ppm) (Gauvin *et al*., 2000 in $CDCl_3$ ) | $\delta$ $^{13}$C (ppm) (in $CDCl_3$) | DEPT | $\delta$ $^{1}$H (ppm) multiplicity (*J* in Hz) (Gauvin *et al*., 2000 in $CDCl_3$ ) | $\delta$ $^{1}$H (ppm) multiplicity (*J* in Hz) (in $CDCl_3$ ) | HMQC (H → C) direct | HMBC (H → C) |
|---|---|---|---|---|---|---|---|
| 1 | | 33.13 (t) | 33.13 (t) | | H1A: 2.35 (t, 15.0, 7.5) ; H1B : 1.47 (m) | C1 | C2, C19 |
| 2 | | 30.71 (t) | 30.71 (t) | | H2A :1.55 (m); H2B : 1.90 (m) | C2 | C1, C19 |
| 3 | | 66.75 (d) | | 3.93 (m)* | 4.02 (m) | C3 | C2 |
| 4 | | 51.50 (t) | 51.50 (t) | | H4A: 2.12 (dd, 8.3, 3.5) ; H4B: 1.91 (dd, 7.7, 2.2) | | C3, C5, C9, C11, C2, C3 |
| 5 | 82.80 (s) | 83.00 (s) | | | | | |
| 6 | | 130.82 (d) | | 6.27 (d, 8.5) | 6.28 (d, 8.4) | C6 | C5, C8, C10 |
| 7 | | 135.53 (d) | | 6.58 (d, 8.5) | 6.60 (d, 8.6) | C7 | C5, C8, C9, C14 |
| 8 | 79.52 (s) | 78.40 (s) | | | | | |
| 9 | 142.60 (s) | 143.00 (s) | | | | | |
| 10 | | 36.19 (s) | | | | | |
| 11 | 119.80 (d) | 119.73 (d) | | 5.40 (dd, 6.0, 2.0) | 5.42 (dd, 6.0, 2.0) | C11 | C8, C12, C13 |
| 12 | | 41.28 (t) | 41.28 (t) | | H12A: 2.28 (m)<br>H12B: 2.10 (m) | C12 | C9, C11, C13, C18<br>C9, C11, C13, C17, C18 |
| 13 | | 44.00 (s) | | | | | C7, C22 |
| 14 | | 51.75 (d) | | | 1.58 (m) | | C15 |
| 15 | | 28.65 (t) | 28.65 (t) | | 1.82 (m) | C15 | C7, C8, C13, C18 |
| 16 | | 20.97 (t) | 20.97 (t) | | 1.60 (m) | C16 | C17, C22 |
| 17 | | 55.98 (d) | | | 1.35 (m) | C17 | C15 |
| 18 | | 13.03 (q) | | 0.79 (s) | 0.74 (s) | C18 | C12, C14, C17 |
| 19 | | 25.60 (q) | | 1.07 (s) | 1.09 (s) | C19 | C1, C5, C9 |
| 20 | | 39.90 (d) | | | 2.08 (m) | C20 | C9, C11, C13, C17, C18 |
| 21 | | 20.77 (q) | | 0.97 (d, 6.6) | 0.99 (d, 6.9) | C21 | C17, C20, C23 |
| 22 | 134.40 (d) | 132.54 (d) | | 5.22 (dd, 15.1, 7.9)* | 5.23 (dd, 15,2, 7.0) | C22 | C20, C23, C24 |
| 23 | 135.50 (d) | 135.18 (d) | | 5.09 (dd, 15.5, 8.8)* | 5.15 (dd, 15.3, 7.7) | C23 | C20, C22, C24 |
| 24 | | 43.00 (d) | | | 1.86 (d, 6.8) | C24 | C25, C26, C27 |
| 25 | | 32.67 (d) | | | 1.70 (m) | C25 | |
| 26 | | 19.98 (q) | | 0.88 (d, 6.4) | 0.85 (d, 7.0) | C26 | C24, C25, C27, C28 |
| 27 | | 19.68 (q) | | 0.89 (d, 6.8) | 0.83 (d, 8.0) | C27 | C24, C25, C26, C28 |
| 28 | | 17.60 (q) | | 0.99 (d, 6.8)* | 0.99 (d, 7.0) | C28 | C22, C24, C25, C27 |

* Gunatilaka *et al*. (1981) in $C_6D_6$

The presence of peroxide group was indicated by two typical carbon signals carrying oxygen atoms at *δ* 83.00 (C5) and *δ* 78.40 (C8). Two pairs of double bonds in the sterol nucleus previously identified by analysing the $^{1}$H NMR spectrum were also denoted clearly by four downfield signals of carbons at *δ* 130.82 (C6), 135.53 (C7), 143.00 (C9), and 119.73 (C11). The other two downfield $^{13}$C NMR signals *δ* 132.54 and 135.18 were assigned to C22 and C23, respectively, verifying the unsaturation in the side chain (Figure C30.3).

The HMQC spectrum connected protons to their respective carbons with the exception for H14 due to its missing cross peak (Figure C30.4). The $^{1}$H NMR data difference of compound **30** and dehydro ergosterol-5,8-peroxide reported by Gauvin *et al*. (2000) varying at range 0.01 – 0.09 ppm, indicated that both compounds are identical with regard to their conformation.

No antimicrobial activity was found in this compound after conducting a series antimicrobial assay.

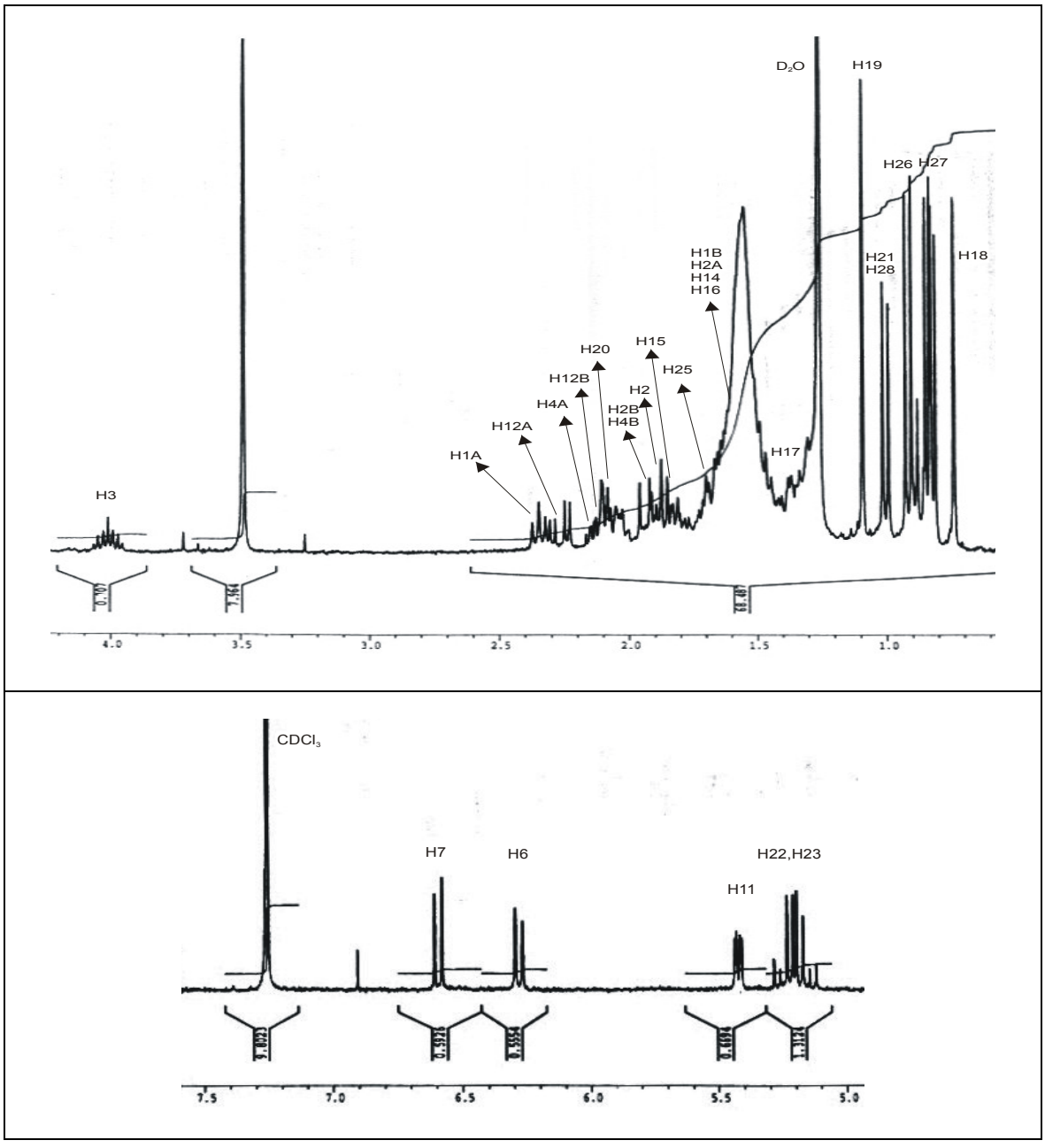



Figure C30.2. $^{1}$H NMR spectrum of compound **30** (dehydroergosterol-5,8-peroxide)

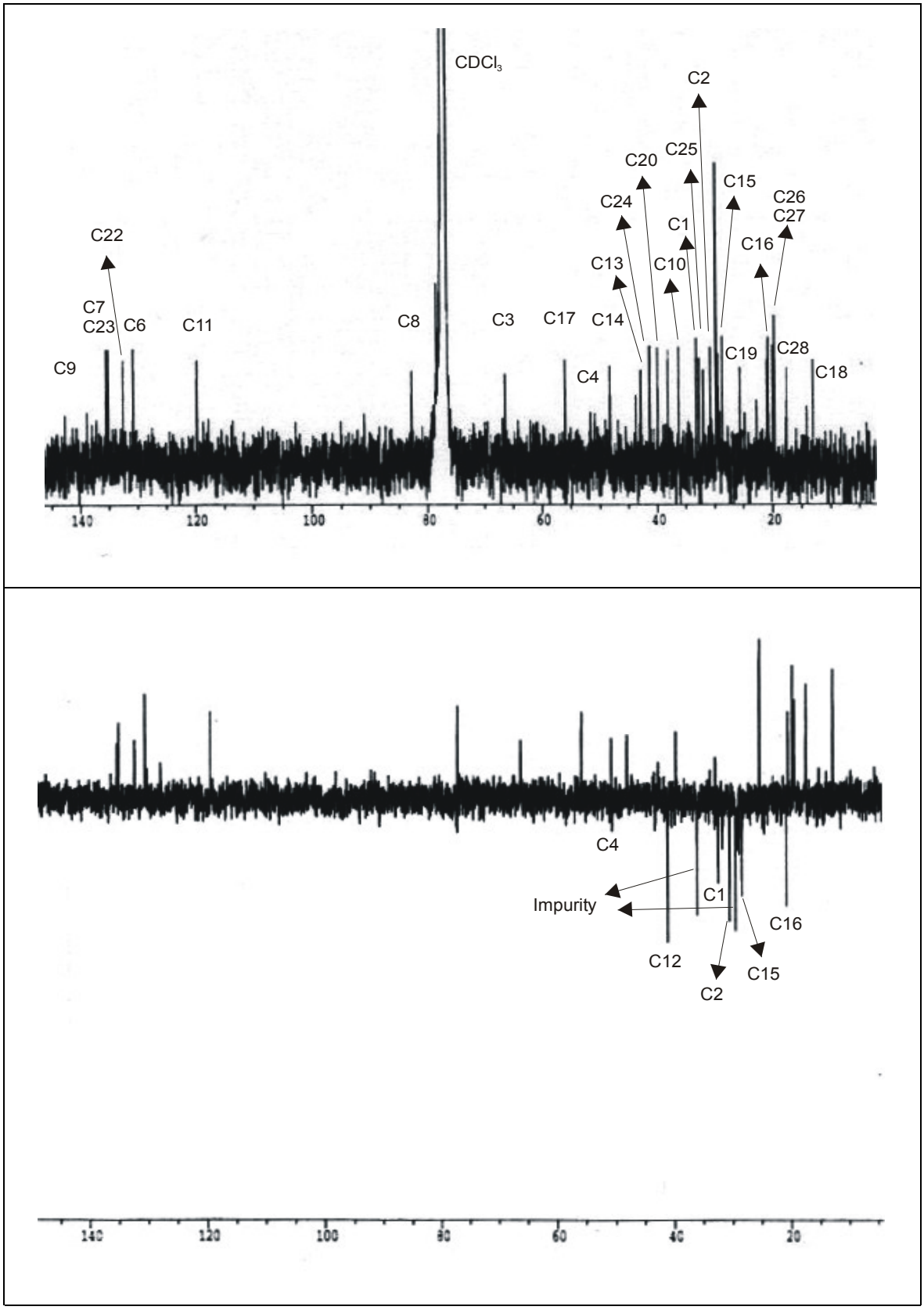



Figure C30.3. $^{13}C$ NMR and DEPT spectra of compound **30** (dehydroergosterol-5,8-peroxide)

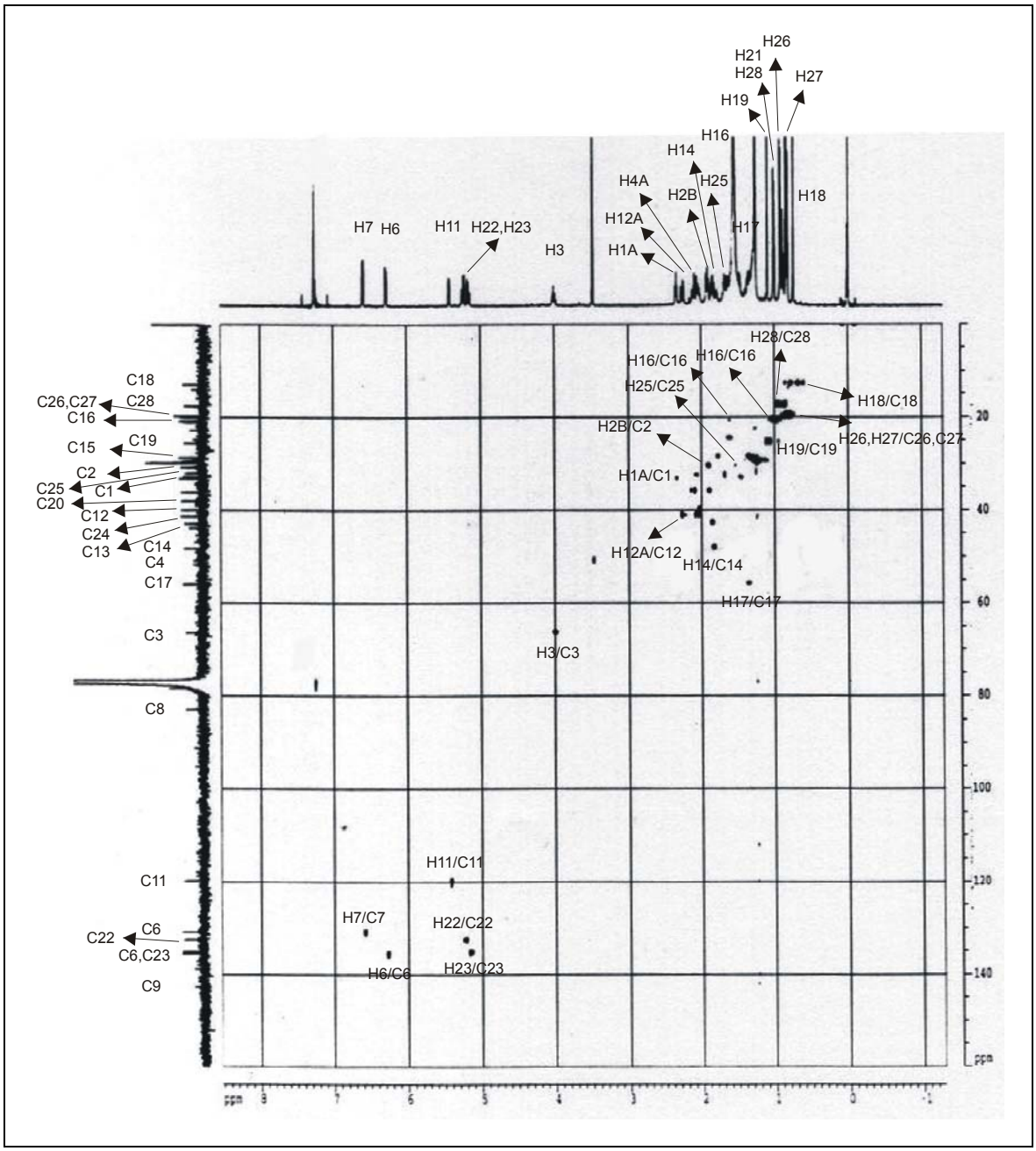



Figure C30.4. HMQC spectrum of compound **30** (dehydroergosterol-5,8-peroxide)

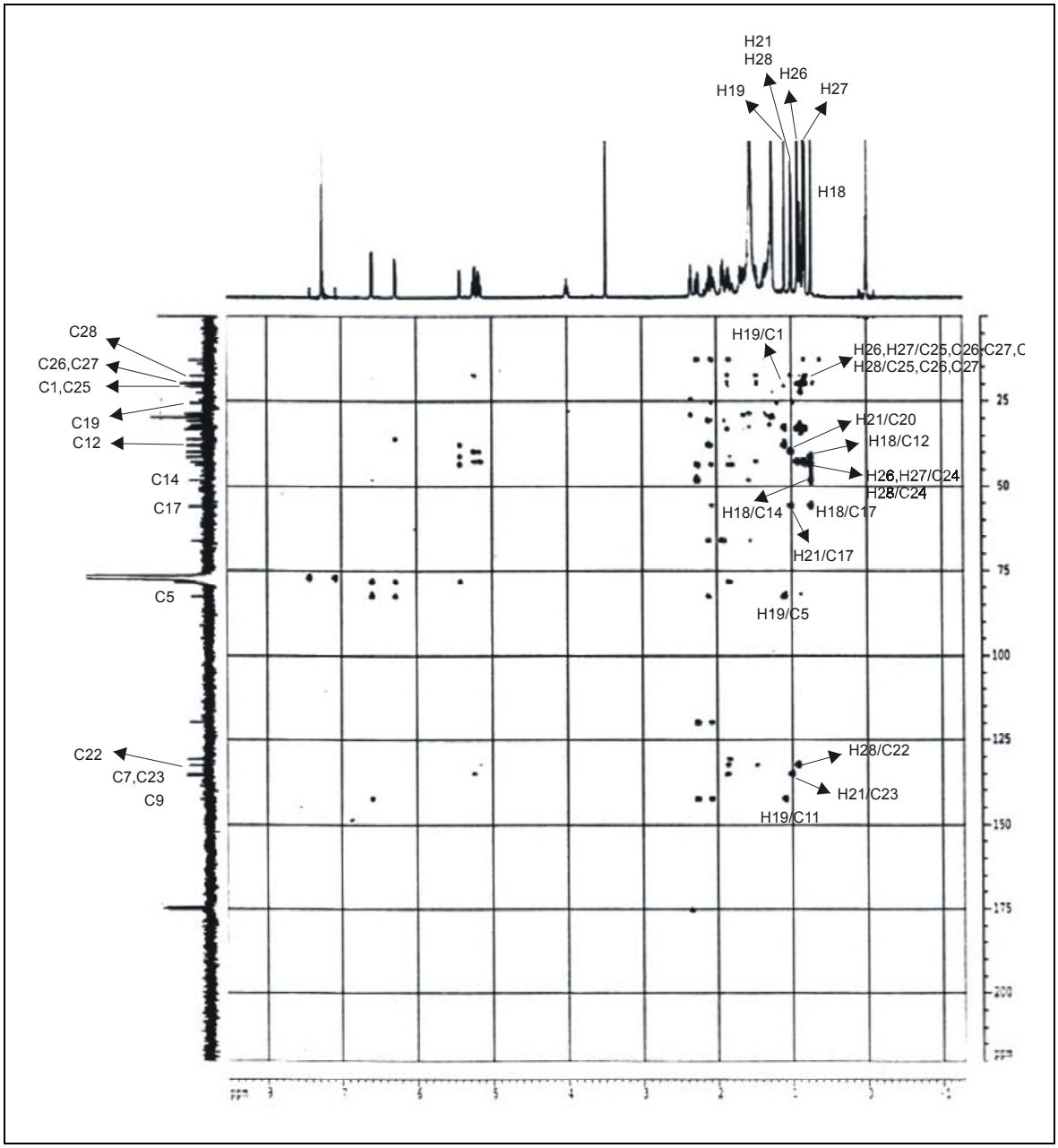



Figure C30.5. Methyl HMBC spectrum of compound **30** (dehydroergosterol-5,8-peroxide)

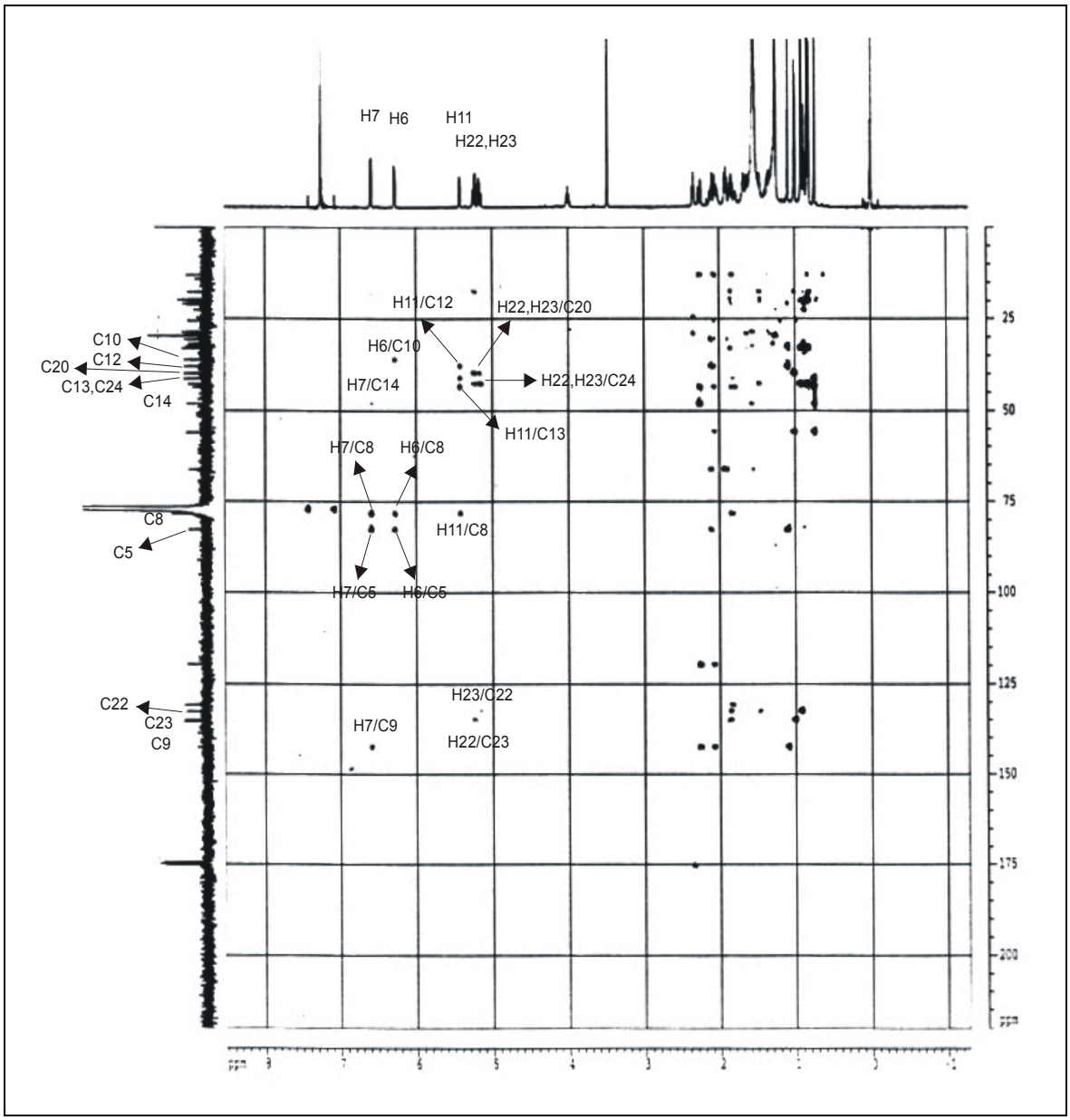



Figure C30.6. Olefinic proton HMBC spectrum of compound **30** (dehydroergosterol-5,8-peroxide)

## 3.5.7. Compound 31 (cerebroside C)

The molecular formula $C_{43}H_{79}NO_9$ and molecular weight 753 g/mol for this compound were deduced on the basis of the ESI-MS spectrum. This compound exhibited a pseudomolecular molecular ion at *m*/*z* 754 $[M+H]^+$ and major peaks at *m*/*z* 776.58 $[M+Na]^+$ and 736.59 $[M-H_2O]^+$. Analysis of NMR data (Table C31.1) proposed that this compound was a glycosphingolipid consisting of a sphingosine base, a fatty acid, and a *β*-glucose (Figure C31.1).

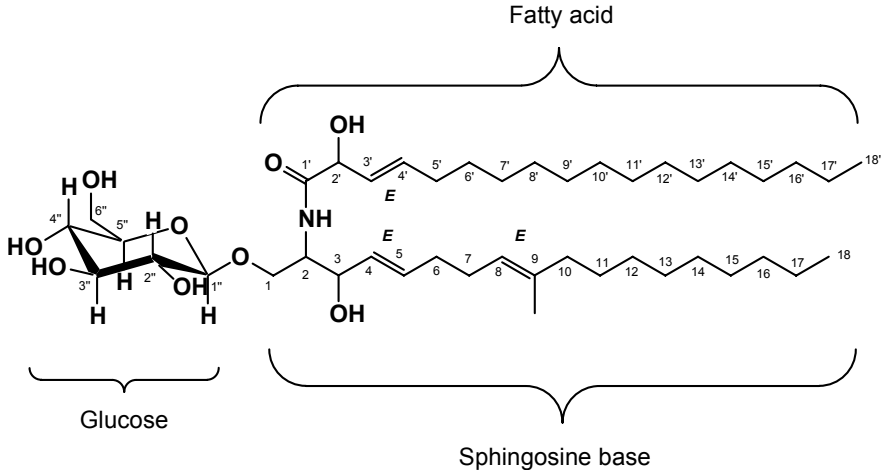



Figure C31.1. Cerebroside C composed of sphingosine base, fatty acid, and glucose

### *Sugar part*

An anomeric proton at *δ* 4.27 (H1'') with a large coupling constant of 7.8 Hz were seen in the $^1$H NMR spectrum indicating the *β*-glucose configuration. The other sugar protons were found in the congested region at *δ* 3.21 – 3.36 of the $^1$H NMR spectrum. Assignments of H2'', H3'', H4'', H5'' were based on the COSY and HMBC spectra (Figure C31.2, partial structure 4). The splitting of H6" to H6"A (*δ* 3.84) and H6"B (*δ* 3.74) due to a chiral centre at position 5'' resulted in these protons shifted downfield in comparison to the other sugar protons (Table C31.1, Figure C31.3).

The multiplet H5'' (*δ* 3.30) was assigned due to coupling with methylene protons of H6A and H6B in the COSY spectrum. Six signals of oxygenated carbons at [*δ* 103.4 (C1''), 73.4 (C2''), 76.7 (C3''), 70.4 (C4''), 76.5 (C5''), 61.8 (C6'')] proved the existence of a *β*-glucose moiety (Figure C31.4). The HMBC correlation between methylene proton (H1) and the anomeric proton of glucose (C1'') and vice versa led to the connectivity of this glucose part and the long-chain base (sphingosine part) (Figure C31.2, partial structure 1)

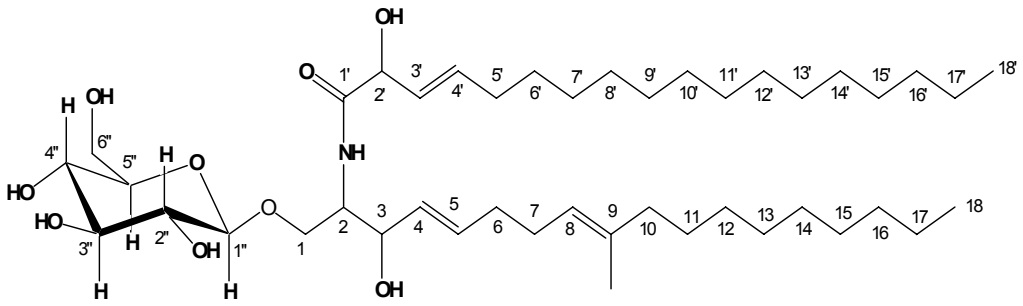
### *Sphingosine part*

An intense singlet signal at *δ* 1.26 in the $^1$H NMR spectrum, integrating for 16 methylene protons of H17 – H17 and H6' – H17', was characteristic of the presence of an aliphatic long chain (Figure C32.3). Large signal of methylene carbons in the DEPT spectrum also supported the long aliphatic chain presence.

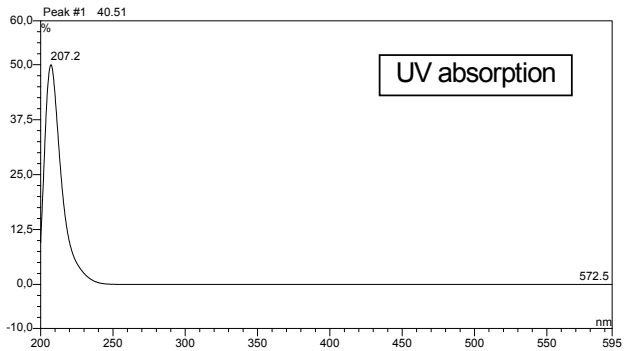
A triplet at *δ* 0.88 (C18, C18') was assigned for two terminal methyl groups of sphingosine base and fatty acid. Signal of methylene at *δ* 68.6 (C1) of glycosylated hydroxyl, methine carbon at *δ* 53.7 (C2) characteristic of a carbon attached to nitrogen (acylamido group), and a carbon at *δ* 72.3 (C3) (an oxygen carrying methine), suggested the usual sphingosine substitution pattern. Long range correlations between H3 with C2, C3, C5 in the HMBC spectrum also supported the presence of sphingosine (Figure C31.7).

## Cerebroside C (compound 31)

| | | |
|---|---|---|
| CAS Registry Number | : | 86363-50-0 |
| Characteristic | : | white crystals |
| Formula | : | $C_{43}H_{79}NO_9$ |
| Molecular Weight | : | 753 g/mol |
| Amount | : | 13.2 mg |
| Source | : | *Lecanicillium evansii* (strain 2) derived from *Hyrtios* sp. |




| | | |
|---|---|---|
| Retention Time | : | 40.59 min |
| $R_f$ | : | 0.63 |
| Fluorescence, 254 nm | : | - |
| 366 nm | : | - |
| Anisaldehyde/$H_2SO_4$ | : | + |




| | | |
|---|---|---|
| Optical Rotation $[\alpha]_D^{20}$ | : | experiment = -55.5° (c=0.1, MeOH) |
| | | literature = -6.2° (c=1.0, MeOH) (Sitrin *et al*., 1988) |
| ESI-MS (*m/z*, rel. int.) | : | 776.58 $[M+Na]^+$ (47), 754$[M+H]^+$ (47), 736.59$[M-H_2O]^+$ (28) |

Table C31.1. NMR data of compound **31** (cerebroside C)

| Position | $\delta$ $^{13}$C (ppm) (Koga *et al*., 1998 in MeOD) | $\delta$ $^{13}$C (ppm) (in MeOD) | DEPT | $\delta$ $^{1}$H (ppm) multiplicity (*J* in Hz) (Koga *et al*., 1998 in MeOD) | $\delta$ $^{1}$H (ppm) multiplicity (*J* in Hz) (in MeOD) | COSY (H → H) | HMBC (H → C) |
|---|---|---|---|---|---|---|---|
| 1 **Sphingosine** | 69.8 (t) | 68.6 (t) | 68.6 (t) | H1A: 4.11 (dd, 10.3, 3.4)<br>H1B: 3.72 (dd, 10.3, 3.4) | H1A: 4.07 (dd, 10.0, 3.0)<br>H1B: 3.84 (dd, 10.0, 3.0) | H1B<br>H1A, H2 | C1'', C2, C3<br>C2, C3 |
| 2 | 54.9 (d) | 53.7 (d) | | 3.97 (dt, 5.4, 3.4) | 3.98 (dt, 5.9, 3.0) | H1A, H1B | C1, C1'' |
| 3 | 73.1 (d) | 72.3 (d) | | 4.14 (dd, 7.3, 5.4) | 4.07 (dd, 7.1, 5.8) | H2 | C1, C2, C4, C5 |
| 4 | 134.7 (d) | 134.6 (d) | | 5.47 (dd, 15.1, 7.3) | 5.44 (dd, 15.0, 7.1) | H5, H6 | C3, C5 |
| 5 | 131.1 (d) | 129.2 (d) | | 5.73 (dt, 15.1, 6.8) | 5.74 (dt, 15.4, 6.7) | H4, H6 | C4 |
| 6 | 33.9 (t) | 33.0 (t) | 33.0 (t) | 2.02 (m) | 2.05 (m) | H4, H5, H7 | C4, C7, C8, C9, C5 |
| 7 | 28.9 (t) | 27.9 (t) | 27.9 (t) | 2.08 (m) | 2.05 (m) | H5, H8 | C4, C5, C6, C8, C9, C10 |
| 8 | 125.0 (d) | 123.6 (d) | | 5.14 (t, 6.8) | 5.11 (br s) | H6, H7, H9 | |
| 9 | 136.9 (s) | 136.3 (s) | | | | | |
| 9a | 16.3 (q) | 16.0 (q) | | 1.60 (s) | 1.59 (s) | H8 | C8, C9, C10 |
| 10 | 40.9 (t) | 40.0 (t) | 40.0 (t) | 1.98 (m) | 1.96 (m) | H11 | C8, C9, C9a, C11, C12, C13 |
| 11 | 29.3 (t) | 28.3 (t) | 28.3 (t) | 1.37 – 1.42 (m) | 1.26 (m) | H10 | C11-17 |
| 12 | 30.4 – 30.9 (t) | 29.3 (t) | 29.3 (t) | 1.29 (m) | 1.26 (m) | | C11-17 |
| 13 | 30.4 – 30.9 (t) | 29.3 – 32.9 (t) | 29.3 – 32.9 (t) | 1.29 (m) | 1.26 (m) | | C11-17 |
| 14 | 30.4 – 30.9 (t) | 29.3 – 32.9 (t) | 29.3 – 32.9 (t) | 1.29 (m) | 1.26 (m) | | C11-17 |
| 15 | 30.4 – 30.9 (t) | 29.3 – 32.9 (t) | 29.3 – 32.9 (t) | 1.29 (m) | 1.26 (m) | | C11-17 |
| 16 | 33.2 (t) | 29.3 – 32.9 (t) | 29.3 – 32.9 (t) | 1.29 (m) | 1.26 (m) | | C11-17 |
| 17 | 23.9 (t) | 22.9 (t) | 22.9 (t) | 1.29 (m) | 1.26 (m) | H16, H18 | C11-18 |
| 18 | 14.6 (q) | 14.2 (q) | | 0.90 (t, 6.8) | 0.88 (t, 6.8) | H17 | C16', C17' |
| 1' **Fatty acid** | 175.6 (s) | 174.4 (s) | | | | | |
| 2' | 74.3 (d) | 73.2 (d) | | 4.43 (d, 5.9) | 4.48 (d, 6.1) | H3', H4'(*) | C1', C3', C4' |
| 3' | 134.9 (d) | 134.2 (d) | | 5.50 (dd, 15.6, 5.9) | 5.52 (dd, 15.5, 6.1) | H2', H4', H5'(*) | C2' |
| 4' | 129.2 (d) | 127.3 (d) | | 5.83 (dt, 15.6, 6.8) | 5.85 (dt, 15.0, 6.7) | H2', H3', H5' | C2' |
| 5' | 33.5 (t) | 32.6 (t) | 32.60 (t) | 2.02 (m) | 2.05 (m) | H3', H4', H6' | C3', C4', C7', C8' |
| 6' | 30.4 – 30.9 (t) | 29.3 – 32.9 (t) | 29.3 – 32.9 (t) | 1.37 – 1.42 (m) | 1.26 (m) | H5' | C6'-17' |
| 7' | 30.4 – 30.9 (t) | 29.3 – 32.9 (t) | 29.3 – 32.9 (t) | 1.29 (m) | 1.26 (m) | | C6'-17' |
| 8' – 15' | 30.4 – 30.9 (t) | 29.3 – 32.9 (t) | 29.3 – 32.9 (t) | 1.29 (m) | 1.26 (m) | | C6'-17' |
| 16' | 33.2 (t) | 32.2 (t) | 32.2 (t) | 1.29 (m) | 1.26 (m) | H17', H18' | C6'-17' |
| 17' | 23.9 (t) | 22.9 (t) | 22.9 (t) | 1.29 (m) | 1.26 (m) | H16', H18' | C6'-18' |
| 18' | 14.6 (q) | 14.2 (q) | 16.0 (q) | 0.90 (t, 6.8) | 0.88 (t, 6.8) | H17' | C16', C17' |
| 1'' **Sugar** | 104.9 (d) | 103.4 (d) | | 4.27 (d, 7.8) | 4.27 (d, 7.8) | H2'', H3'' | C1, C2'' |
| 2'' | 75.2 (d) | 73.4 (d) | | 3.20 (dd, 9.3, 7.8) | 3.24 (dd, 9.4, 8.0) | H1'', H3'' | C3'' |
| 3'' | 78.1 (d) | 76.7 (d) | | 3.36 (dd, 9.3) | 3.31 (dd, 9.4) | H2'', H4'' | C2'', C4", C5'' |
| 4'' | 71.8 (d) | 70.4 (d) | | 3.29 (m) | 3.34 (m) | H3'', H5'' | C2", C3'' |
| 5'' | 78.1 (d) | 76.5 (d) | | 3.27 (m) | 3.30 (m) | H4'', H6''A, H6''B | C2", C3'' |
| 6''A | 62.9 (t) | 61.8 (t) | 61.8 (t) | 6''A: 3.88 (d, 12.2) | 6''A: 3.74 (d, 12.5) | H5'', H6''B | C4'', C5'' |
| 6''B | 62.9 (t) | | | 6''B: 3.67 (dd, 12.2) | 6''B: 3.84 (dd, 12.5) | H5'', H6''A | C4'',C5'' |

(*) coupling (4 bond)

Five double bond proton signals [$\delta$ 5.44 (H4), $\delta$ 5.74, (H5), 5.11 (H8), 5.52 (H3'), 5.85 (H4')] were observed in the molecule. This proposed the existence of three double bonds in which one of the six carbons was nonprotonated. The methyl signal at $\delta$ 1.59 (H9a) indicated it was bound to one of the double bond. The presence of these double bonds was also apparent in the $^{13}$C NMR spectrum at $\delta$ 134.6 (C4), 129.2 (C5), 123.6 (C8), 136.3 (C9), 134.2 (C3'), 127.3 (C4') (Figure C31.4). Correlation of H9a with H8 in the COSY spectrum as well as correlation of H9a with C8, C9, C10 verified the assigment of this methyl (H9a) in the position 9 (Table C32.1).

Through COSY and HMBC spectra analysis, it was revealed that H1A and H1B correlated with H2 (assumed to be geminal with the nitrogen of the amide group), H3, and further correlation with H4, H5, H6, H7, olifenic proton H8, H9a, H10, and H12 – H16, resulted in the establishment of partial structure 1, representing sphingosine part of this compound, connected to partial structure 2A as the end of aliphatic chain (Figure C31.2, C31.5, and C31.7).

The geometry of the double bond in the long-chain alkene can be determined on the basis of the $^{13}$C NMR chemical shift of the methylene carbon adjacent to the olefinic carbon. Bohlmann *et al.* (1975) reported that in comparing the terpenes nerol and geraniol, a similarly situated methyl carbon appears at $\delta$ 16.0 ppm when it is situated *trans* to an olefinic proton, and at $\delta$ 23.5 ppm when *cis*. Thus, the methyl group ($\delta$ 16.0, C9a) must be *trans* to H8 and *cis* to the C$H_2$-7.

The stereochemistry for the C4 and C5 double bond was assigned *trans* due to large coupling constant (15.4 Hz) which was verified by non-shielded chemical shift of the adjacent methylene carbon at $\delta$ 33.0 (C6). The chemical shift values for the *trans* (*E*) isomers are around $\delta$ 32 - 33 ppm and for the *cis* (*Z*) isomers around $\delta$ 27 - 28 ppm (Kim *et al*., 1997 and Liu *et al*., 1998).

***Fatty acid part***

A downfield doublet proton signal at $\delta$ 4.48 (d, 5.9 Hz, H2') suggesting a proton attached to oxygen-carrying carbon [$\delta$ 73.2 (C2')] was displayed in the $^{1}$H NMR spectrum. This low field chemical shift was in accordance with its location between a carbonyl group (C1') and a double bond (C3').

The signal of an amide carbonyl group was observed at $\delta$ 174.4 (C1') in the $^{13}$C NMR spectrum. Assignment of carbonyl group at position C1' was based on HMBC correlations between H2' and C1' as well as between H2' and C3' (Figure C31.2, partial structure 3).

Partial structure 3 was established by cross peak signals between H2' and H3', H4', H5', H6' in the COSY spectrum. Long range HMBC connections were observed between H2' and C1' (carbonyl group), C3' ($\delta$ 134.19, double bond), C4' ($\delta$ 127.31, double bond). The presence of a cross peak signal between H5' and H4', H7' – 16' also supported the partial structure 3 (Figure C31.2 and C31.8).

The assignment of partial structure 3 suggested the fatty acid part of this compound. The multiplet signal at $\delta$1.26 was due to the H6'until H15' methylene protons of the fatty acid part and signals for the non allylic methylene protons of the sphingosine part.

The same with sphingosine, this fatty acid part terminated with methyl group coincided in a signal at $\delta$ 0.88 (H18'), which was also confirmed by the partial structure 2B (Figure C31.2). The geometry of the double bond (C3' and C4') of the fatty acid moiety was determined as *trans*. It was explained by a large vicinal coupling constant of 15.5 Hz, supported also by the chemical shift of adjacent carbon (C5') which was encountered at resonance of $\delta$ 32.6, in agreement with the chemical shift of the *trans* vinylic methylene ($\delta$ 32 - 33 ppm).

Comparison of $^{1}$H data of compound **31** with those of cerebroside C isolated from rice pathogenic fungus (*Magnoporthe grisea*) (Koga *et al.,* 1998) showed comparable chemical shifts leading to the conclusion that both compounds are the same.

However, the stereochemistry at the asymmetric centres C2 and C3 might be different due to the difference in optical rotation. The optical rotation of cerebroside C with its stereochemistry *2R* and *3S*, and of compound **31** were –6.2$^{o}$ (c=1.0, MeOH) and –55.5$^{o}$ (c=0.1, MeOH) respectively.

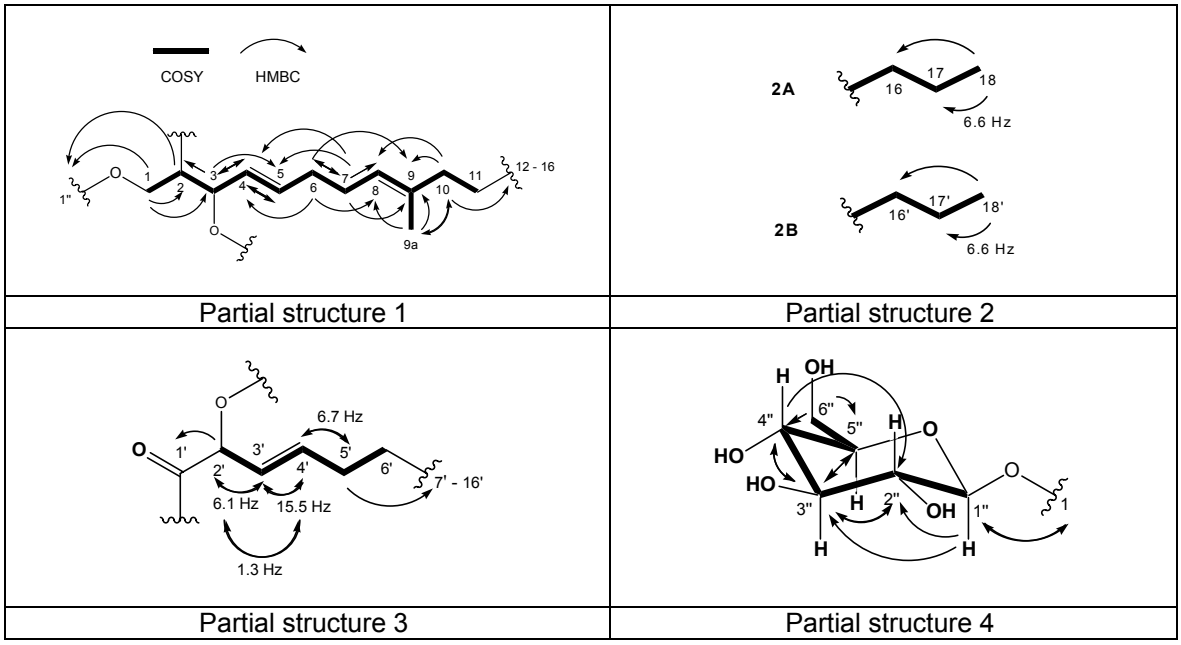



Figure C31.2. Partial structures of compound **31** (cerebroside C) established from COSY and HMBC correlations

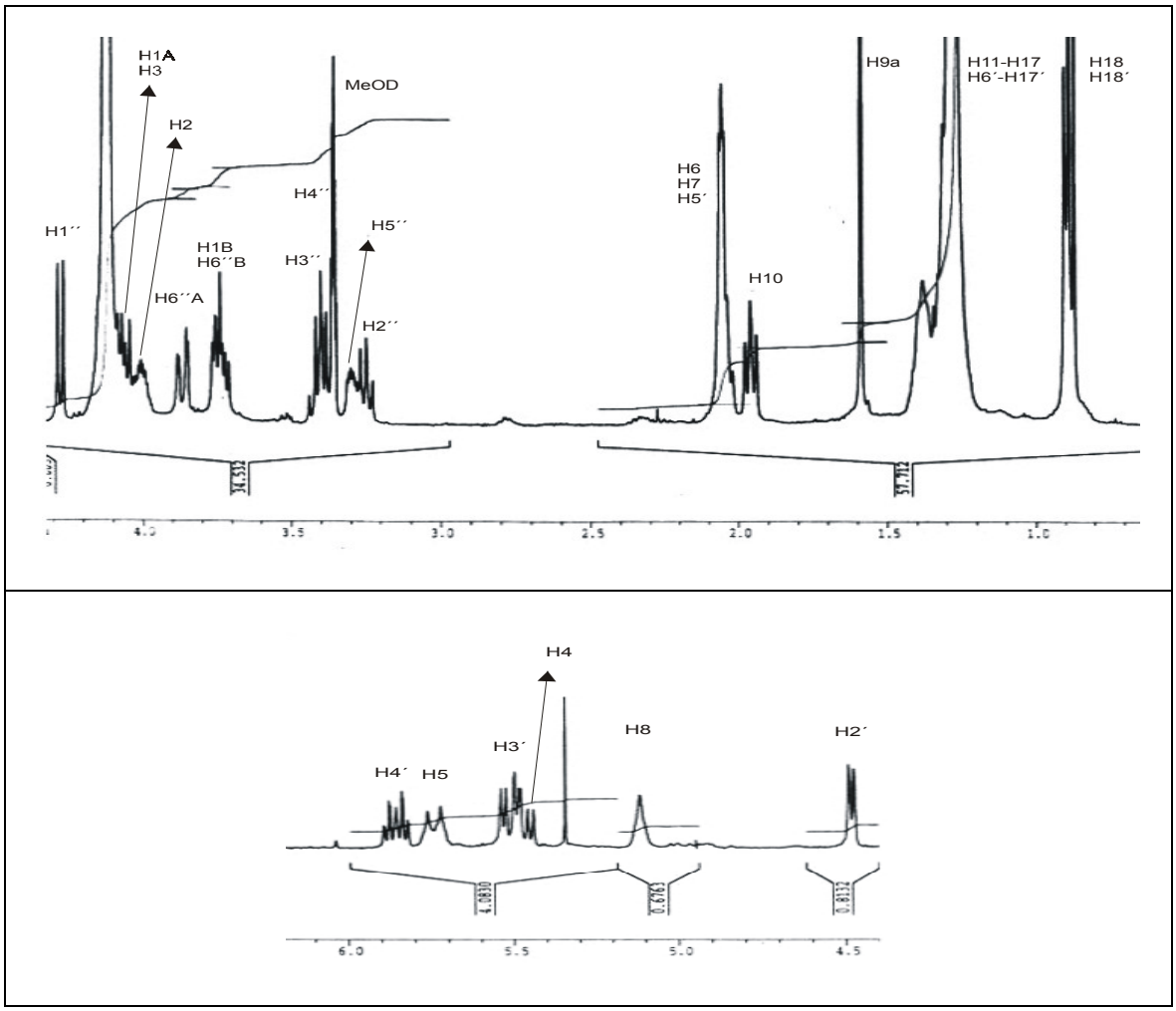



Figure C31.3. $^{1}$H NMR spectrum of compound **31** (cerebroside C)

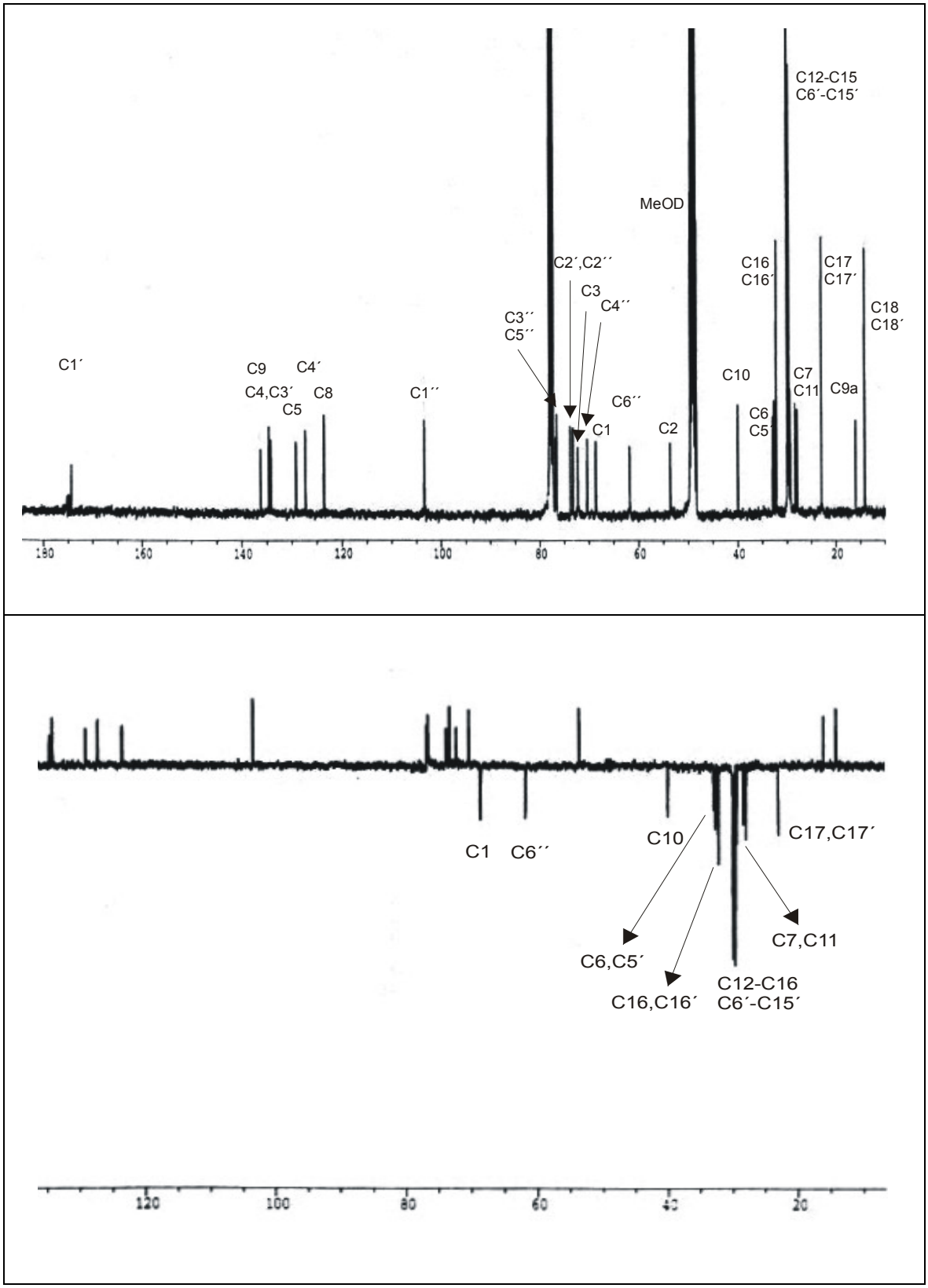



Figure C31.4. $^{13}$C NMR and DEPT spectra of compound **31** (cerebroside C)

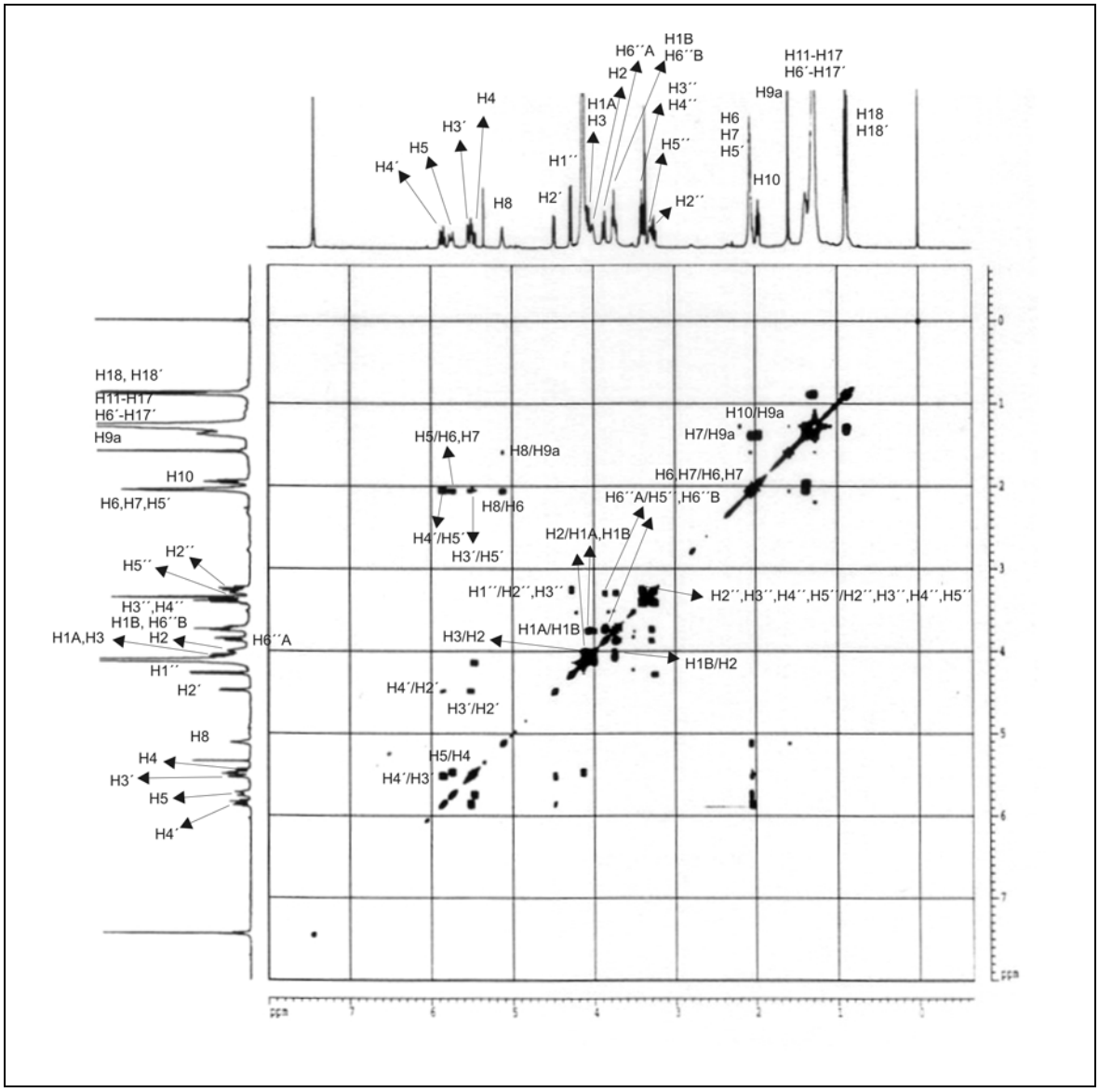



Figure C31.5. COSY spectrum of compound **31** (cerebroside C)

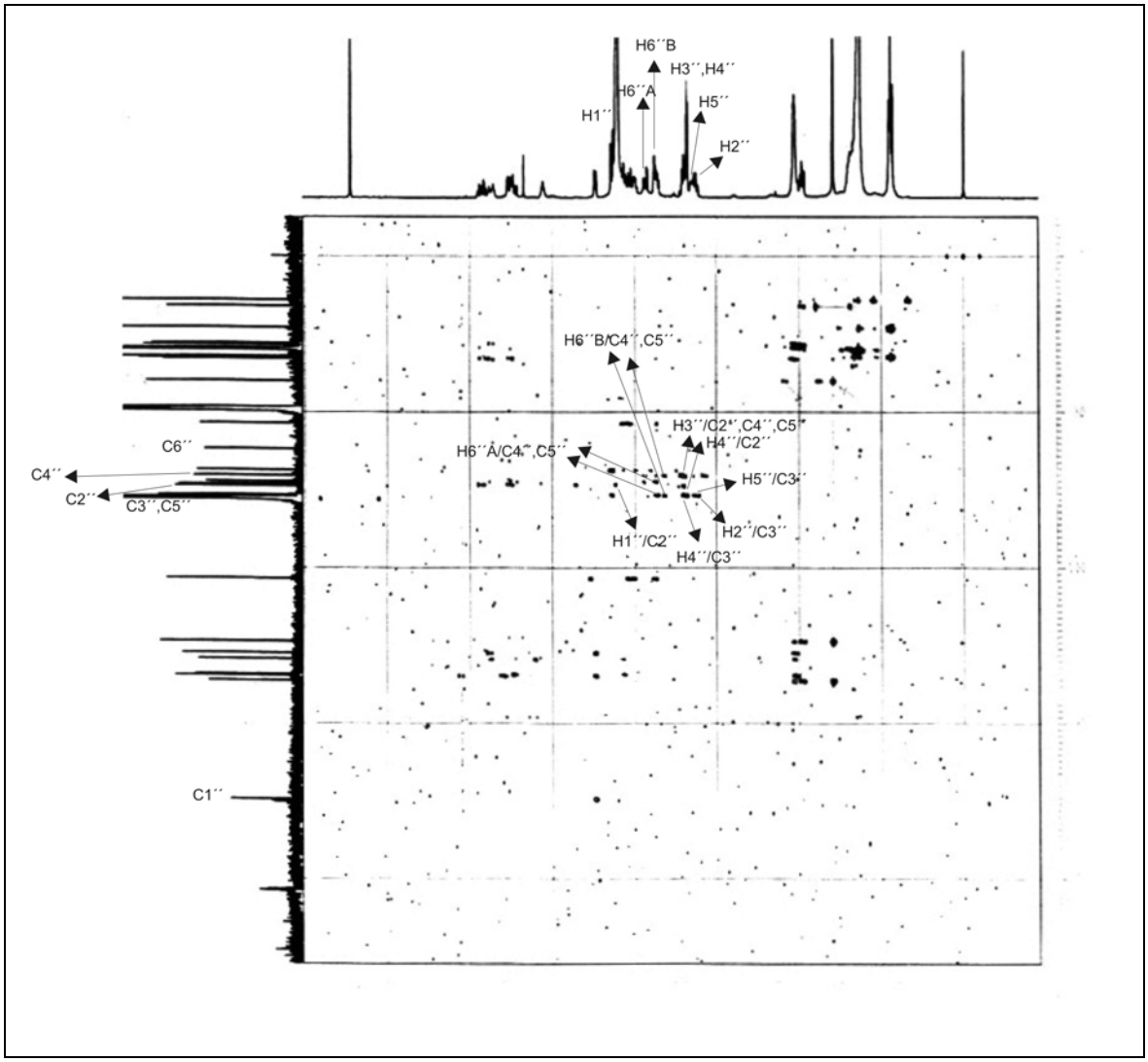



Figure C31.6. Sugar HMBC spectrum of compound **31** (cerebroside C)

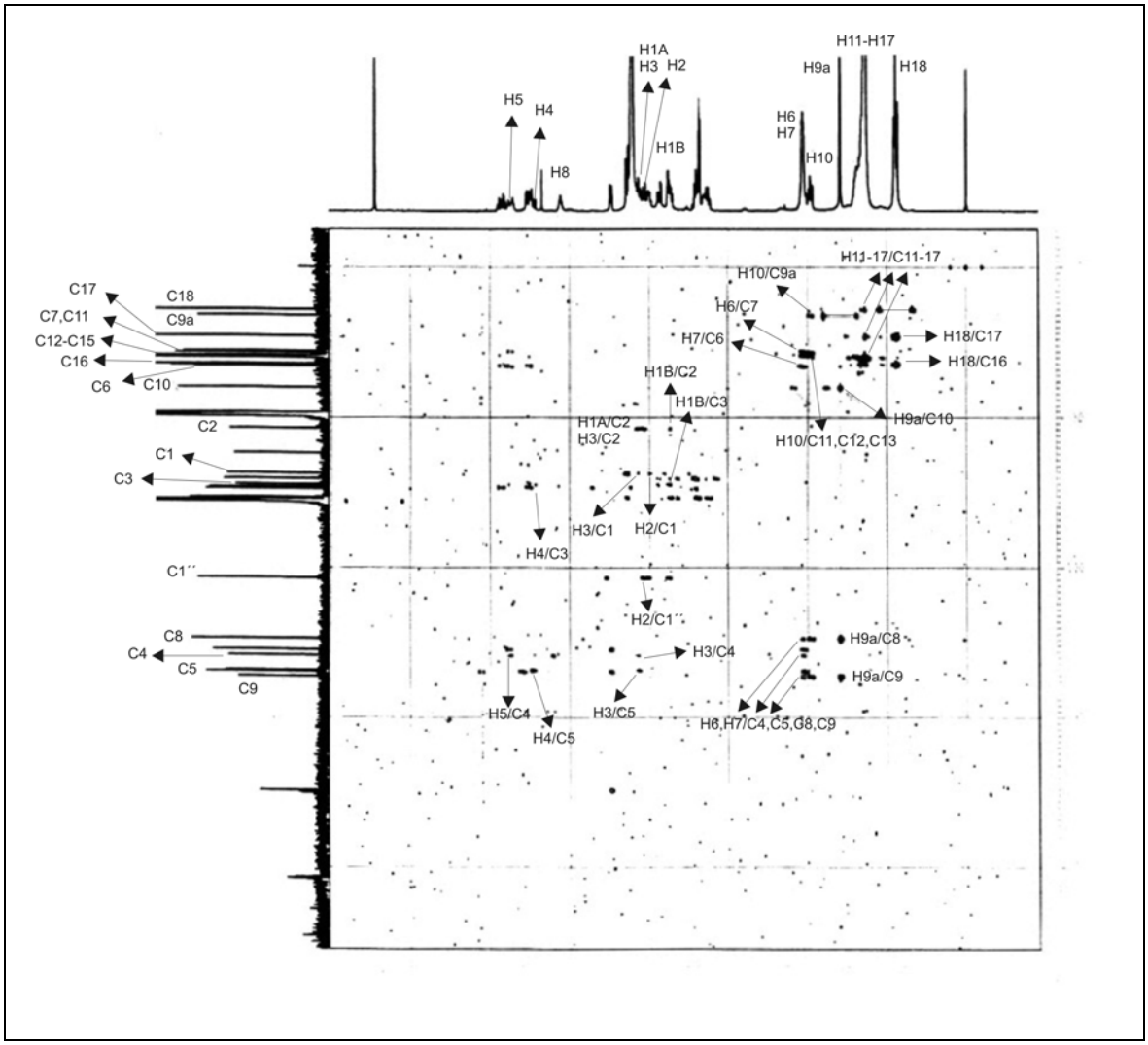



Figure C31.7. Sphingosine base HMBC spectrum of compound **31** (cerebroside C)

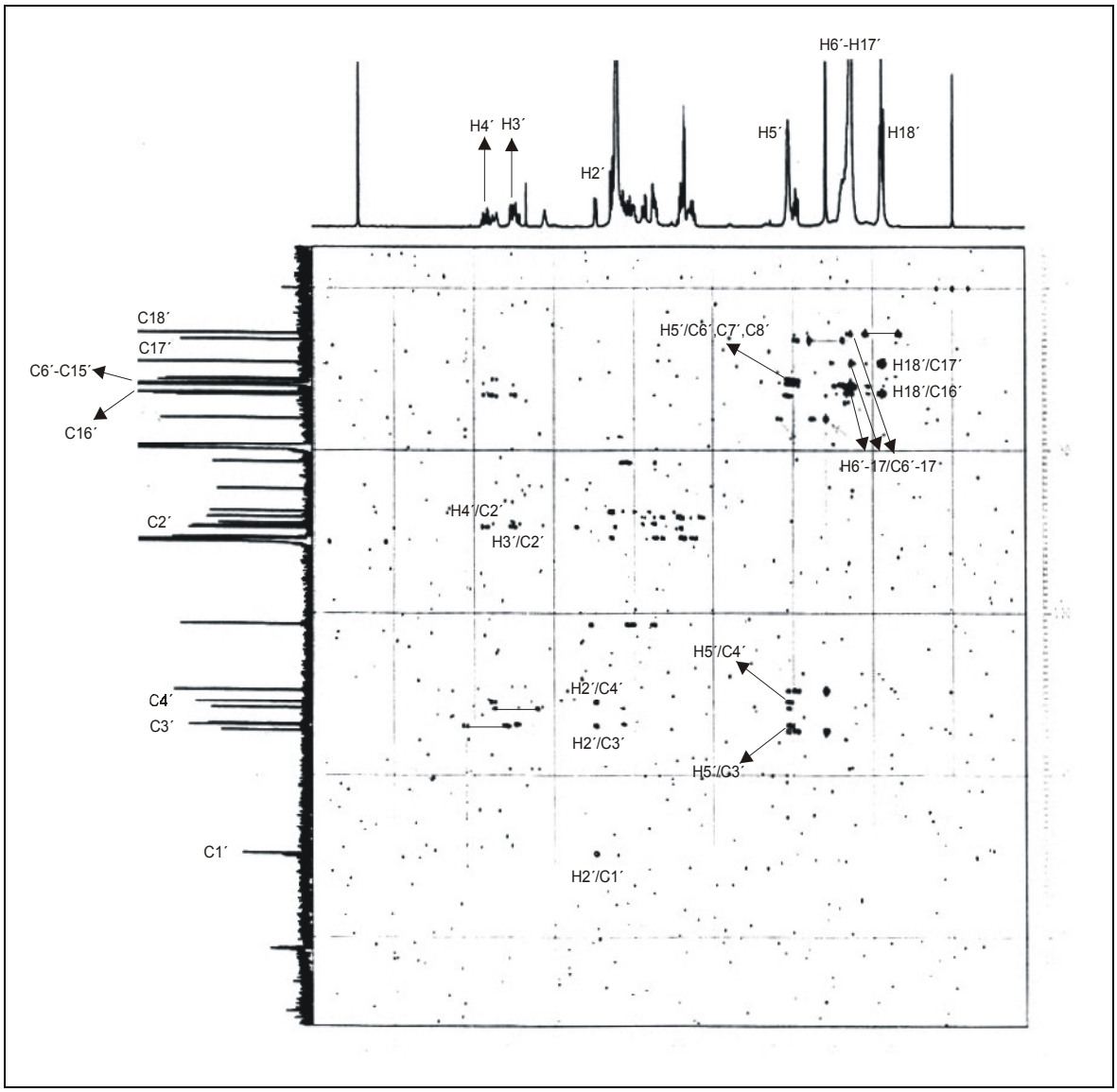



Figure C31.8. Fatty acid HMBC spectrum of compound **31** (cerebroside C)

# IV. DISCUSSION

## 4.1. Selected fungi

Three of the major natural product sources examined by pharmaceutical industry are microorganisms, plants and marine macrooorganisms. In general, microorganisms have today demonstrated a greater diversity of producer genera and species per lead compound in comparison to the others. Studies of actinomycetes from a variety of diverse Australian environments have shown that at least one-third of actinomycete types isolated appear to be area specific (Wildman, 1997).

This suggests that habitat conservation for microbes is an important consideration if the pharmaceutical industry is going to be able to access the remaining 90 – 99% of the microorganisms that are always reputed to have not been cultivated in most ecosystems (Wildman, 1997).

It is also reported that sponges contain large numbers of bacteria and other microorganisms, including cyanobacteria, actinomycetes, and fungi. There has been speculation that many bioactive compounds isolated from sponges may, in fact, be of microbial origin and this has been confirmed in a number of cases (Proksch *et al*., 2002; Wildman, 1997).

Four species of fungi [*Penicillium* sp., *Verticillium* cf *cinnabarium*, *Fusarium* sp., *Lecanicillium evansii* (strain 1 and 2)] were thoroughly analysed for their secondary metabolite contents. These species belong to the following taxonomical classification.

| | |
|---|---|
| Kingdom : Fungi<br>Phylum : Ascomycota<br>Class : Euascomycetes<br>Order : Eurotiales<br>Family : Trichomaceae<br>Genus : *Penicillium* | Kingdom : Fungi<br>Phylum : Ascomycota<br>Class : Sordarimycetes<br>Order : Hypocreales<br>Family : Hypocreaceae<br>Genus : *Fusarium*<br>*Verticillium*<br>*Lecanicillium* |

Both strains of *L. evansii* contained different types of metabolites. The secondary metabolite content of strain 1 included phenolic compounds (6 structures) and a bipeptide (1 structure), whereas in strain 2 four nucleosides, two steroids, and one cerebroside were encountered.

Wildman (1998) reported that there are variations in secondary metabolite production between different samples of the same species from different habitats. It is not clear, however, whether these differences mirror the diversity of genotypes within a sampling site, or if the diversity is a result of habitat variability and its influence on gene expression. However the latter possibility seems unlikely in this case as both fungi were grown under identical conditions.

Furthermore, he explained also that it is rare to find a fungal natural product that is known from only one taxon or single strain. Several taxa can produce the same metabolites, even though the producing fungi might not be taxonomically closely related.

This phenomenon occurred also in this study in which ergosterol peroxide was found in two different species namely *Fusarium* sp. and *L. evansii* (strain 2). Secondary metabolites produced exclusively by only one taxon or a single strain are in most cases the exception rather than the rule.

Both strains of *L. evansii* were cultivated in media enriched with salt or without salt. There were no conspicuously visual differences between the two cultures. At the end of the culture period, a bulk of fungi mycellia of both strains completely covered the surface of both media. The content of fungal secondary metabolites in media with or without salt tended to be similar. Dix and Webster (1995) reported that many physiological studies have focused on the effect of salinity. It is surprising that in pure culture many marine fungi grow equally well on media made up with sea or fresh water.

## 4.2. Isolated compounds

There were 31 compounds isolated from four species of fungi. These compounds were grouped into anthraquinones (2 compounds), alkaloids (2 compounds), terpenoids (3 compounds), simple aromatic compounds (4 compounds), bipeptides (4 compounds), steroids (3 compounds), cerebrosides (2 compounds), phenolic compounds (4 compounds), and nucleosides (4 compounds).

### 4.2.1. Emodin and Hydroxyemodin

Anthraquinone derivatives, the largest class of naturally occurring quinones, have widely been found in higher plants, mosses, lichens, fungi as well as in sea animals and algae.

Natural anthraquinones are distinguished by a large structural variety, wide range of biological activity, and low toxicity. They possess purgative, antioxidant, anti-inflammatory, antitumor, bactericide effects, antimutagenic, immunosuppressive, and enzyme-inhibitory activities.

They are also involved in the process of metabolism, respiration, cell division, oxidative phosphorylation, complexation with DNA and RNA, and in others physiological processes of vital importance. They are parts of many medicines of plant origin. The anthraquinones have also found application as dyes, pigments, luminophores, analytical reagents, or chemical means for plant protection (Choi *et al*., 2000; Muzychkina, 1998).

Emodin was found to inhibit lipid peroxidation in the linoleic acid system. Anthraquinone glycosides have long been used medicinally as cathartics and laxatives. Plant-derived drugs of this type include aloe (*Aloe vera*), cascara sagrada (*Rhamus purshianus*), frangula (*Rhamnus frangula*), rhubarb (*Rheum officinale*), rumex or yellow dock (*Rumex crsipus*) and senna (*Cassia* sp.). Many of the commercial preparations based on these plants are readily available (Harborne, 1973; Seigler, 1998).

Some natural anthraquinones in plants are derived from the shikimic acid pathway. However, emodin, as an important fungi anthraquinone, particularly in the genera *Penicillium* and *Aspergillus,* is synthesised from acetate-malonate (octaketide) pathway. Acetate malonate derived anthraquinones usually can be distinguished by their structure because they possess substituents in both benzene rings of the anthraquinone nucleus. Emodin serves as an important intermediate in the biosynthesis of many other fungal metabolites (Sanakawa, 1980; Seigler, 1998).

Pure anthraquinones as drugs are rarely prescribed, but mostly simple extracts of the crude drugs. The sugar moiety in the glycosides increases solubility and facilitates transport to the site of action, but it is mainly the anthrone form that is effective. The use of anthraquinone drugs should be restricted to short-term treatment of constipation only, as frequent or long term use has been associated with increased risk of intestinal tumours (Samuelsson, 1999).

### 4.2.2. Bipeptides

All four cyclic bipeptides found in this study possess a diketopiperazine (DKP) skeleton. DKP are ubiquitous throughout nature and are most commonly isolated from terrestrial yeasts, lichens and fungal culture filtrates and are also observed in the culture broth of marine bacteria and marine actinomycetes.

Some examples of DKP from marine sources include the isolation of cyclo-(Gly-*S*-Pro) from the starfish *Luidia clatharata*, cyclo-(Ala-Pro) from marine bacteria associated with sponges, cyclo-(Leu-Pro) from sponge *Tedania ignis* and from marine fungi. DKPs have also been isolated from the following marine sponges: *Jaspis* sp. *Tedania ignis*, *Dysidea fragilis*, *D. erbacea*, *Geodia baretti*, and *Leucophloeus fenestrata* (Adamczeski *et al*., 1995).

Tryptophan is produced by a strain of yeasts closely related to *Candida tenuis* and *C. parapsilosis*, and some strains of *Hansenula* can produce tryptophan when growing on beet molasses as substrate and anthranilic acid as a precursor. Other amino acid-producing fungi include *Torulopsis utilis* and *Fusarium roseum* (Wainwright, 1992).

A number of peptides, especially those from microorganisms, have antibiotic properties. These compounds often have molecular weights in the range of 300 – 2000. The series of medicinal antibiotics such as bacitracin A, gramicidin S, actinomycin D, and penicillin, cephalosporins are produced by *Bacillus licheniformis*, *Bacillus brevis*, *Streptomyces*, *Penicillium*, and *Cephalosphorium* and serve as self-protection for organisms, are peptides or derivatives of peptides (Sasaki *et al*., 1982; Seigler, 1995).

Cyclosporine A produced by certain fungi imperfecti, was shown to prevent attack of host tissue towards transplanted organs. Bleomycin A2, a peptide isolated from *Streptomyces verticillus* is the main component used clinically, and has pronounced antitumor activity. Pathogenic bacteria and fungi synthesise a number of compounds that help to break down host tissue and to weaken the host plant. Similarly phytotoxins, especially those produced by bacteria, are peptides typically with molecular weights less than 600 (Seigler, 1995)

Barrow and Sun (1994) reported the isolation of 11 secondary metabolites from the fungus *Aspergillus flavipes*. Among those 11 metabolites were 7 diketopiperazines such as: cyclo(*L*-phe-*L*-pro), cyclo (*L*-phe-*D*-pro), cyclo (*L*-leu-*L*-pro), cyclo(*L*-tyr-*L*-pro), cyclo(*L*-tyr-*D*-pro), cyclo(*L*-val-*L*-pro), and cyclo(*L*-val-*D*-pro).

An example of marine-derived compounds is the peptide ziconotide that has successfully underwent phase III clinical trials for two therapeutic applications, to alleviate pain associated with malignant diseases (cancer and AIDS) and as an analgesic for non malignant neuropathic pain. Ziconotide is a 25 amino acid linear peptide exhibiting three sulphide bonds. It occurs along with other peptides in the venom of the predatory Indo Pacific marine mollusc *Conus magus* (Olivera, 2000; Proksch *et al*., 2002).

### 4.2.3. Meleagrine

Meleagrine is an alkaloid and was isolated from *Penicillium* sp. Alkaloids are characterised by having nitrogen in the molecule. This nitrogen atom originating from amino acids is usually incorporated in a heterocyclic ring, and is basic. Alkaloids are cyclic compounds containing nitrogen in a negative oxidation state, which is of limited distribution among living organisms. Although alkaloids largely are plant products, a number of these compounds are found in animals such as insect, amphibians, and marine invertebrate (Bick, 1985, Seigler, 1995).

Some alkaloids (nicotine and anabasine) are sufficiently toxic to animals to cause death if eaten. The alkaloids such as: atropine, berberine, caffeine, cocaine, codeine, colchicine, emetine, ephedrine, ergotamine, hyoscymine, morphine, physostigmine, pilocarpine, quinidine, quinine, reserpene, scopolamine, strychnine, theophylline, tubocurarine, and yohimbine, are important as therapeutic agents (Seigler, 1995).

Alkaloids are derived from many biosynthetic pathways, including those of amino acids, polyketide, shikimic acid, acetate, and terpenoid metabolism. Most alkaloids are derived biosynthetically from several amino acids such as: ornithine, lysine, nicotinic acid, anthranilic acid, phenylalanine, tyrosine, and tryptophan (Bick, 1985; Seigler, 1995).

Plants containing alkaloids have been used by man for at least 3000 years as medicines, teas, and potions. However, the compounds responsible for activity were not isolated and characterised until the 19$^{th}$ century. At present there are at least 10,000 characterised alkaloids. About 20 – 30% of higher plant accumulate alkaloids. Around 30 alkaloids account for most commercial interest, primarily as medicines, flavourings, or poisons (Seigler, 1995).

Meleagrine has some unusual structural features, e.g. an N-OMe group, a dehydroxyhistidine unit, a reversed isopentenyl group and a single carbon atom possessing three nitrogen functionalities (Konda *et al*., 1980). Two nitrogen-containing amino acids with one isoprenyl group were regarded as the origin of meleagrine. The fact that the structure contains a diketopiperazine ring proposed that this compound should be formed by two nitrogen-containing amino acids (tryptophan and histidine) (Nozawa and Nakajima,1979).

Meleagrine and oxaline posses several unique structural features. Oxaline was regarded as the first fungal metabolite having the N-methoxylindoline moiety. Other uncommon features include the position of the isoprene unit attached to C3 of tryptophan (C2 is the common location). Position two carries three nitrogen functionalities.

Nozawa and Nakajima (1979) reported that the culture broth of *Penicillium meleagrinum* had antibacterial activity. Meleagrine along with fumitremorgin A, and B, verruculogen, roquefortin C and D belong to the category of the tremorgenic mycotoxins.

Furthermore, alkaloids are also well known from marine invertebrates such as sponge. The calcareous sponge *Leucetta* contains a number of imidazole alkaloids such as naamines, isonaamines, naamidines, isonaamidines, and calcaridines. Imidazole alkaloids exhibited antimicrobial activities and cytotoxicity (Kong and Faulkner 1993; Mancini *et al*., 1995). A series of brominated dimeric pyrrole alkaloids were isolated from the sponge *Agelas*. Bromopyrrole alkaloids are well known in marine sponge of the genus *Agelas* (Assmann, 2002).

## 4.2.4. Citreohybridonol and Andrastin A

Citreohybridonol has a γ-lactone ring bridge from C6 to C23 of andrastin A. It was speculated that andrastin A had to be a precursor of citreohybdridone metabolites. Citreohybridonol is biosynthesised from a sesquiterpene and a tetraketide (Kosemura *et al.*, 1992 and 1994).

It has been reported that citreohybridone A, B, C, citreohybriddiones A, B, and isocitreohybridones A, B, isolated from *Penicillium citreo-viride* B. IFO 6200 and 4692 exhibited potent antifeeding activity against *Plutella xylostella*. Furthermore, citreohybridonol, isolated from the mycelium of *Penicillium* sp. FO-3929 has antifeedant and insecticidal activity (Kosemura and Yamamura, 1997).

Andrastin A, B, and C derived from *Penicillium* sp. were reported as protein farnesyltransferase inhibitors (Kosemura and Yamamura, 1997). Rho *et al*. (1998) reported that andrastin A directly interacts with P-glycoprotein and inhibits the efflux of antitumor agents from drug resistant cells.

It has been reported that that citreohybridones are likely formed by successive methyl migration and skeletal rearrangement of sesterterpenoid containing five isoprene units. Nevertheless, terretonin (terpenoid compound), a mycotoxin isolated from *Aspergillus terreus* (McIntyre *et al*.,1989) was proposed to originate from a mixed polyketide-terpenoid (meroterpenoid) biosynthetic pathway.

## 4.2.5. Triterpene acetate

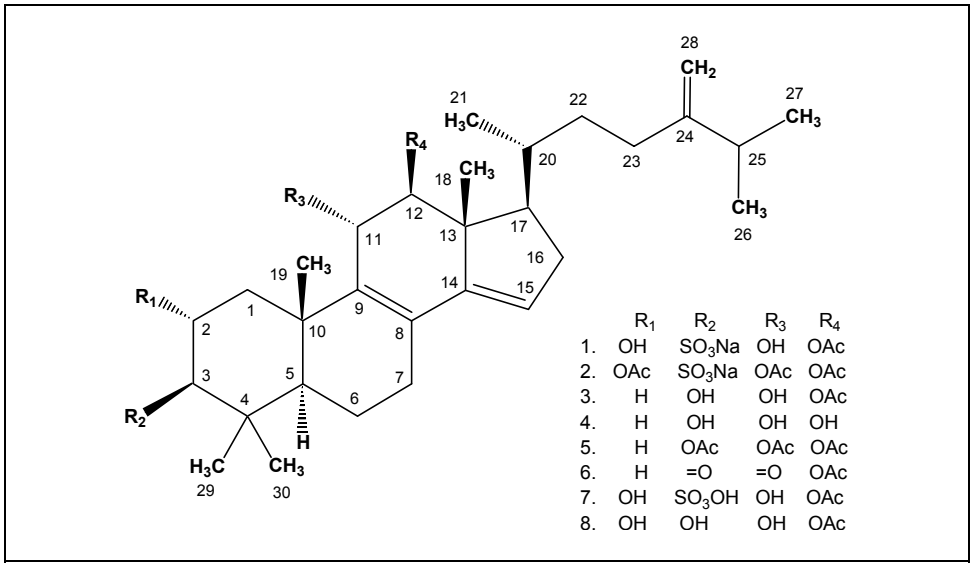
A number of triterpene acetates with different substituents at positions 2, 3, 11, and 12 have been reported in the literature (Figure 4.2.5.1).

Triterpene acetates 1 and 2 were isolated from *Fusarium graminearum* (Vesonder *et al*., 1990). Yagen *et al*. (1980) isolated triterpene acetates 3, 4, 5, and 6 from *Fusarium sporotrichioides* 921. Compound **7** was reported by Brill *et al*. (1996), isolated from terrestrial *Fusarium compactum*. The triterpene acetate **8** (new triterpene acetate) was isolated from *Fusarium* sp. (Figure 4.2.5.1).

*Fusarium sporotrichioides* which produces triterpene acetates 3 – 6 is a toxic species responsible for alimentary toxic aleukia (ATA). ATA is a disease that has been recorded in Russia since the beginning of the 19$^{th}$ century. This disease has been associated with the consumption of food made

from grain which remained unharvested under snow and became mouldy from contamination with a variety of microorganisms. The most prevalent of the organisms are toxic species of *Fusarium sporotrichioides* (Yagen *et al*., 1980).

Triterpene acetate **7** has been shown to be a phytotoxic compound which inhibits seed germination of wheat and tomato, and has some activity against fungi (Vesonder *et al*., 1990). This compound has also weak activity against *Staphylococcus aureus* and *Streptococcus* strain in the range of 6 – 50 µg/ml (Brill *et al*., 1996).




| | $R_1$ | $R_2$ | $R_3$ | $R_4$ |
|---|---|---|---|---|
| 1. | OH | $SO_3Na$ | OH | OAc |
| 2. | OAc | $SO_3Na$ | OAc | OAc |
| 3. | H | OH | OH | OAc |
| 4. | H | OH | OH | OH |
| 5. | H | OAc | OAc | OAc |
| 6. | H | =O | =O | OAc |
| 7. | OH | $SO_3OH$ | OH | OAc |
| 8. | OH | OH | OH | OAc |

Figure 4.2.5.1. Triterpene acetate and triterpene sulphate

As much as 100 µg/ml of the new triterpene acetate 8 was found to be active against *Staphylococcus aureus*, causing a 7 mm inhibition zone. The triterpene acetate 8 also showed cytotoxicity toward a human cancer cell lines with growth inhibition of 91.25% (JURKAT), 36.00% (THP-1), and 85.67% (MM-1).

## 4.2.6. Simple aromatic compounds

The major source of carbon and energy for most heterotrophic organisms is glucose, usually supplied in laboratory cultures and derived from polymer carbohydrates in nature. A few fungal metabolites are derived directly from glucose. Fungi sometimes accumulate simple derivatives of glucose, such as mannitol and gluconic acid in large quantity. Simple disaccharides have been obtained from some fungi and lichens and a few glycosides have been isolated from fungi (Turner, 1971).

The shikimic acid pathway which plays a primary role in producing the essential aromatic amino acids phenylalanine, tyrosine and tryptophan can also provide intermediates for biosynthesis of simple benzene derivatives which are widely distributed in nature (Turner, 1971).

### 4.2.6.1. 3-Hydroxyanthranilic acid

3-hydroxyanthranilic acid, a metabolite of 3-hydroxykynurenine, is an intermediate product of catabolism of tryptophan through the kynurenine pathway. A diverse set of findings implicates the kynurenine pathway and tryptophan catabolites to be involved in a variety of neurodegenerative, inflammatory, and immunologic phenomena including Huntington's disease, Parkinson's disease, HIV ecephalophaty, cerebral malaria, poliomyelitis, and fetomaternal tolerance (Goldstein *et al*., 2000).

Yokoi *et al.* (1998) examined the effects of kynurenine metabolites administered into the right cerebroventricle (1 μmole) on the electrocortigram (EcoG) of rats to establish the role of kynurenines on brain function. Kynurenine, anthranilic acid, quinaldic acid, xanthurenic acid, and 8-hydroxyquinaldic acid showed no effect on EcoG throughout the recording periods of 4 hours.

### 4.2.6.2. Tyramine

Tyramine as a natural substance, is formed from the breakdown of protein, and is directly biosynthesised from the essential amino acid tyrosine. Tyramine is commonly found in aged, fermented, or spoiled food. Generally speaking, the longer a high-protein food ages, the greater the tyramine content.

Aged cheese, fermented sausage, pickled herring, dried fish, broad beans, yeast extract, etc. are among foods with high tyramine content. Foods that are riper or overripe tend to have higher tyramine content than fresh or freshly prepared foods. Furthermore, bacterial and fungal actions on protein sources such as meat and soy products can cause an increase in the food tyramine level.

Excessive level of tyramine can cause headache, palpitations, nausea, vomiting, and hypertensive crisis (dangerously high blood pressure). A tyramine-free diet is prescribed for people who are sensitive to tyramine, such as migraine sufferer, or those taking prescription monoamine oxidase (MAO) antidepressants (Alpers *et al.,* 1995; Mahan and Escott, 2000).

Under normal circumstances, tyramine and dopamine are metabolised to harmless metabolites by the enzyme monoamine oxidase (MAO). Drugs that inhibit MAO also inhibit the metabolism of tyramine and dopamine leading to elevated levels of these substances in the bloodstream (Alpers *et al.,* 1995; Mahan and Escott, 2000).

### 4.2.6.3. Acetyl hydroxybenzamide

The substituent of $CONH_2$ bound to aromatic ring characterises the benzamide molecule with its synonyms benzolamide, benzoic acid amide, and phenylcarboxyamide. The acetyl hydroxy benzamide found in this research has not been quoted as a natural product before.

Para hydroxybenzamide and anhydroshikimate were isolated from *Streptomyces tendae*. Both compounds possess no antimicrobial activity (Blum *et al*., 1996). Some derivatives of benzamides (benzohydroxamic acid, tioxymid, trichlamide, zarilamid, and zoxamide) are available commercially as fungicides.

### 4.2.7. Lichesterol, Ergosterol-5,8-peroxide, and Dehydroergosterol-5,8-peroxide

Sterols from the marine environment differ from their terrestrial counterparts mainly in the side chain. While the latter rarely contain more than 10 carbon atoms, frequent discoveries of new C11 side chains have been reported from marine steroids. C12 side chain sterols were also found in tunicates and sponges (Li *et al*., 1981). Marine organisms have been the source of numerous 3*β*-hydroxy sterols and their oxygenated analogues (Gunatilaka *et al*., 1981).

Ergosterol, the principal steroid from yeast, is a mycosterol which is converted to vitamin $D_2$ on irradiation via *cis*-tachsterol as an intermediate. It was reported that carbon atoms of ergosterol skeleton apparently arise from the cyclization of squalene (cholesterol) (Griffin, 1969; Staple, 1967).

Ergosterol peroxide has been found in lower terrestrial organisms such as: fungi and lichens but in contrast to the variety of sterol peroxides in sponges, only ergosterol peroxide has been encountered in these terrestrial sources. The dehydro ergosterol peroxide has been observed as a natural product

isolated from *Rhizoctonia repens*, *Fusarum monoliforme*, *Ascidia nigra*, *Dendrogyrus cylindrus*, *Thalysias juniperina*, *Aplysia dactylomela* , and *Armillariella mellea* (Gunatilaka *et al*., 1981; Kim *et al*., 1999).

Singlet and oxygen radicals have been regarded as playing a crucial role in numerous biological processes, such as: enzymatic reactions occurring in the mithocondrial respiratory chain, detoxifying reaction of the cytochrome system, and prostaglandin synthesis. Several mushrooms (*Armillariella mellea, Daedalea dickinsi*, and *Fomitella fraxinea*) containing ergosterol peroxide showed remarkable inhibition of lipid peroxidation of rat liver microsomes and hepatic aldehyde oxidase (Kim *et al*., 1999).

It was reported that ergosterol peroxide isolated from *Inonotus obliquus* not only inhibited the growth of cancer cells but also killed them in the antitumor test. Recent study also revealed its inhibitory effects on induced inflammation and tumor promotion in mouse skin. Ergosterol peroxide showed stronger inhibition than $\alpha$-tocopherol and thiourea by 19.2%, and 21.5%, respectively, while less activity than butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA) were observed (Kim *et al*., 1999).

Ergosterol peroxide and dehydro ergosterol peroxide isolated from mushrooms (*Pisolithus tinctorius*, *Microporus flabelliformis* and *Lenzites betulina*) were also found to be immunosupressive agents (Fujimoto *et al*., 1994).

## 4.2.8. Phenolic compounds

### 4.2.8.1. Terphenylin and Deoxyterphenylin

Deoxyterphenylin found in this study differs from the known deoxyterphenylin with regard to the methoxy group position. The methoxy group of the new deoxyterphenylin is bound to position 4" unlike the methoxy group of the known deoxyterphenylin which is attached to position 3'.

Para-terphenyl metabolites showing a typical phenolic nature are found rarely as natural products. However, terphenylquinones have mostly been isolated from Basidiomycetes (Thomson, 1971). Terphenyllin was the first of this type compound isolated from *Aspergillus candidus*. Takahashi *et al*. (1976) working also with *A. candidus* reported five *p*-terphenyl derivates

Terphenylin (3 – 10 $\mu$g/ml) caused a specific toxicity on Hela cells (Takahashi *et al*., 1974). Previous literature indicated that certain quinones containing alkylating group have anti tumor activity. Cain (1961) performed several cytotoxicity test on some quinone derivates, in an attempt to encounter the features of the quinone molecule responsible for antitumor activity.

Compounds without the central part of 2,5-dihydroxy group proved inactive against the L1210 leukaemia in mice and no antibacterial activity. Likewise, the simple molecules 2,5-dihydroxyquinone and 2,5-dihydroxy-3-phenyl-1,4-benzoquinone exhibited also no bioactivity.

It was therefore an ambiguity in precisely determining the part of molecule responsible for cytotoxicity. However, the need of retaining the dihydroxyquinone group for keeping the cytotoxicity potential, proposed that biological efficacy might be effected by the following properties: the chelating power, the redox potential, or the acidity of the α-hydroxyquinone system (Cain, 1961 and 1964).

### 4.2.8.2. Terprenin 2 and Terprenin epoxide

Terprenin 2 and terprenin epoxide differ from the known terprenin derivatives in their side chains. The prenyl side chain of the new terprenin 2 directly attaches to benzene ring C through the position C3, without an oxygen bridge as occurred in the known terprenin. The epoxy group in the side chain marked the terprenin epoxide to be a new natural product. This unusual characteristic of prenyl side chain and epoxy group side chain in the terprenin have not been quoted in the literature before.

Terprenins possessed very strong proliferations against mouse spleen lymphocytes stimulated with Con A and LPS. The $IC_{50}$ values of terprenin, 3-methoxyterprenin, and 4''-deoxyterprenin were calculated as 1.2, 2.0, and 5.6 ng/ml against Con A-induced proliferation and 4.5, 8.0, and 15.6 ng/ml against LPS-induced proliferation (Kamigauchi *et al*., 1998).

Stead *et al*. (1999) quoted that terprenin possesses potent cytotoxicity against BALB/MK and other hyperproliferative cell lines. It is assumed that the existence of an oxygen-linked isoprene substituent brings about such an effect on cytotoxicity potency of this compound. This effect likely occurs through the inhibition of pyrimidine biosynthesis. However no antimicrobial activity against bacteria and fungi was reported.

## 4.2.9. Nucleosides

Adenosine deoxyriboside, cytosine deoxyriboside, adenosine riboside, and cytosine riboside, one deoxyribose were found in the fungus *Lecanicillium evansii* (strain 2).

Nucleoside antibiotics are found as diverse groups of secondary metabolites of microbial origin. 163 nucleoside antibiotics have been classified on the basis of their structures. The biological activity of nucleoside antibiotics is also wide ranging, comprising antibacterial, antifungal, antitrypanosomal, antitumor, antiviral, herbicidal, insecticidal, immunostimulating, and often immunosuppressive properties.

Not only nucleic acid synthesis but also protein synthesis, glycan synthesis, and glycoprotein synthesis are targets of nucleoside antibiotics. Nucleoside antibiotics are, thus, potential candidates for the regulation of all aspects of cell growth and differentiation (Isono,1988).

In the early 1950s, Bergmann discovered the nucleosides spongouridine and spongothymidine (Arabinose A and B). These compounds had antiviral activity. These discoveries aided in the development of antitumor agents and a generation of nucleoside antivirals.

Through synthetic efforts, cytosine arabinoside (Ara C) was first synthesised by Walwick *et al*. (1959) as a clinically useful antitumor agent, and later, adenine arabinoside (Ara D) was approved for use as an antiviral agent (Ara C and D) (McConnel *et al*., 1994). Takahashi *et al*. (1992) first reported synthetic anticancer drug (Ara C) as a natural product. It was isolated from a mushroom (*Xerocomus nigromaculatus*).

Adenine arabinoside was derived from fermentation cultures of *Streptomyces antibioticus*. Adenine arabinoside (Ara D) is applied for the treatment of *Herpes simplex* virus encephalitis (McConnel *et al*., 1994).

## 4.2.10. Cerebrosides

Two cerebrosides were isolated in this study. They were isolated from *Fusarium* sp. and *Lecanicillium evansii* (strain 2).

Cerebrosides are widely distributed in nature as constituents of brain, nerves, and also contained in milk, oyster, some plants, etc. Sphingolipids are ubiquitous components of the membranes of all eukaryotic cells, are particularly abundant in plasma membranes, and are thought to participate in antigen-antibody reactions and transmission of biological information (Koga *et al.*, 1998; Okuyama and Yamazaki, 1983; Shibuya *et al.*, 1990).

In fungi, sphingolipids are known to function as inducers of cell differentiation. Fungal cerebrosides, including cerebroside A, B, and C induce cell differentiation in the fungus *Schizophyllum commune* (Koga *et al.*, 1998; Okuyama and Yamazaki, 1983; Shibuya *et al.*, 1990).

Furthermore, it was reported that the methyl group at C9 and the 4*E*-double bond in the sphingolipid base moiety of cerebrosides A and C are the key elements determining the elicitor activity of these compounds. When plants interact with certain pathogens, they protect themselves by generating various chemical and physiological barriers called the hypersensitive response. These barriers are induced by molecules called elicitors that are produced by pathogens. (Koga *et al.,* 1998; Okuyama and Yamazaki, 1983).

A cerebroside mixture obtained from an Australian sponge was reported to inhibit histidine decarboxylase (Kawano *et al*., 1988). Antimicrobial assay of cerebroside C towards *Candida albicans* with 0.05 μg/ml aculeacin displayed an activity with zone inhibition of 17 mm. However if the antimicrobial assay was conducted only on the basis of *C. albicans* as target fungus and cerebroside C as a test substance, no activity was found (Sitrin *et al*., 1988).

# V. SUMMARY

After performing biological screening tests on 79 fungal strains, isolated from several different sponges, collected from Elba (Italy) and West Bali sea (Indonesia), five different sponge-derived fungi were chosen for isolation of their secondary metabolites on the basis of bioassay-guided fractionation.

Elba and Bali fungal samples were stored in the Alfred Wegener Institute (AWI) for Marine and Polar Ecology, Bremerhaven, and in the Institute for Pharmaceutical Biology, Heinrich-Heine University, Düsseldorf, Germany, respectively.

The selected fungi included *Penicillium* sp. (1) isolated from the sponge *Ircinia fasciculata* collected from Elba sea, *Verticillium* cf *cinnabarium* (2) derived from *Ircinia fasciculata* collected from Elba sea, *Fusarium* sp. (3) isolated from *Axinella damicornis* also originated from Elba sea, *Lecanicillium evansii* (strain 1) (4) derived from *Callyspongia* sp., and *L. evansii* (strain 2) (5) separated from *Hyrtios* sp. Both strains of *L. evansii* were collected from west Bali sea.

The selection of these five fungi was based on their biological activities toward brine shrimp (*Artemia salina*), insects (*Spodoptera littoralis*), and on their antimicrobial activity [two gram positive bacteria (*Bacillus subtilis*, *Staphylococcus aureus*), gram negative bacteria (*Escherichia coli*), and three fungal strains (*Saccharomyces cerevisiae*, *Candida albicans, Cladosporium herbarum*)].

The uniqueness of their UV absorption patterns displayed by the HPLC chromatogram and the distribution of molecular weights in the crude extracts prevailed by the ESI-MS were also taken into account.

Mass stand cultivations (10 litre) of fungi 1, 2 and 3 in Wickerham medium and taxonomical identification were performed in AWI. Fungi 4 and 5, identified by *Centraalbureau voor Schimmelcultures*, Baarn, Netherlands, were new fungal species. Stand cultures of these new fungi species in Wickerham medium were conducted at the Institute for Pharmaceutical Biology, Heinrich-Heine University.

Thirty one compounds were identified in this study. Six compounds were isolated from *Penicillium* sp. They consisted of anthraquinones (emodin, hydroxyemodin), an alkaloid (meleagrine), a bipeptide (cyclo-leucylprolyl), and triterpenes (citreohybridonol, andrastin A).

Eight compounds were derived from *Verticillium* cf *cinnabarium*. These compounds included simple aromatic compounds (3-hydroxyanthranilic acid, 4-hydroxybenzaldehyde, tyramine), bipeptides (cyclo-alanyltryptophan, cyclo-prolylvalyl, cyclo-leucylprolyl), an alkaloid (verticillin B), and a steroid (lichesterol).

Only three compounds [steroid (ergosterol-5,8-peroxide), triterpene acetate, and cerebroside] could be derived from *Fusarium* sp.

*L. evansii* strain 1 and 2 contained different metabolites. This was conspicuously indicated by different HPLC peak distributions. Both strains of *L. evansii* cultivated in media without salt tended to grow much faster at the onset of the culture period. After seven days of the culture period, however, both strains indicated no visual colour and density differences. The surface of each culture flask was entirely covered by the white mycellia.

*L. evansii* when grown in the media without salt contained slightly more diverse secondary metabolites. This was supported by the HPLC spectrum of strain 2 crude extract, that possessed much more peaks. But it was not the case in strain 1.

Seven compounds were isolated from *L. evansii* (strain 1). Those compounds comprised phenolic compounds (terphenylin, deoxyterphenylin, terprenin 2, terprenin epoxide), bipeptide (cyclo-tyrosylprolyl), and simple aromatic compounds (acetyl hydroxybenzamide, 4-hydroxybenzaldehyde).

Meanwhile, strain 2 of *L. evansii* possessed seven compounds constituting nucleosides (cytosine riboside, cytosine deoxyriboside, adenosine riboside, adenosine deoxyriboside), steroids (ergosterol-5,8-peroxide, dehydro ergosterol-5,8-peroxide), and cerebroside C.

Among the isolated metabolites, four of them (triterpene acetate, deoxyterphenylin, terprenin 2, and terprenin epoxide) were new compounds.

The triterpene acetate was new because of the existence of a hydroxyl group at position C2 (Figure 5.1). As much as 100 µg of triterpene acetate was found active against *S. aureus*, which inhibited 7 mm diameter of its growth zone. In addition, the triterpene acetate also exhibited cytotoxicity toward human cancer cell lines with growth inhibition of 91.25% (JURKAT), 36.00% (THP-1), and 85.67% (MM-1).

The methoxy group of the new deoxyterphenylin is located at position 4", whilst in the reported deoxyterphenylin, it is attached to position 2' (Figure 5.1). The unusual prenyl side chain which was directly bound to phenyl ring through position 3, without an oxygen bridge as normally reported, marked the characteristic of the new terprenin 2 (Figure 5.2). The new terprenin epoxide is characterised by the epoxy group in the side chain (Figure 5.2).

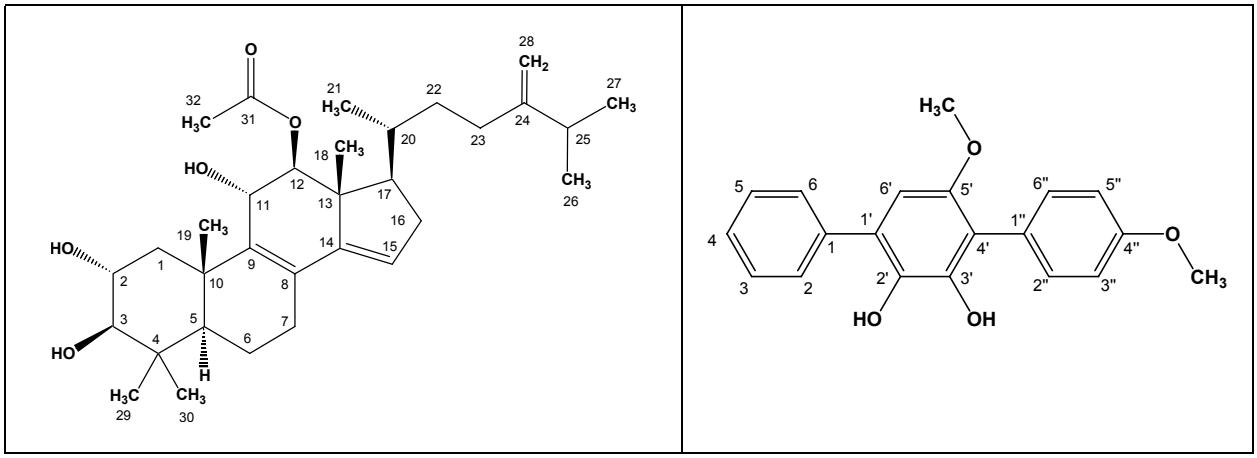


Figure 5.1. Triterpene acetate (compound **16**) (left) and deoxyterphenylin (compound **19**) (right)

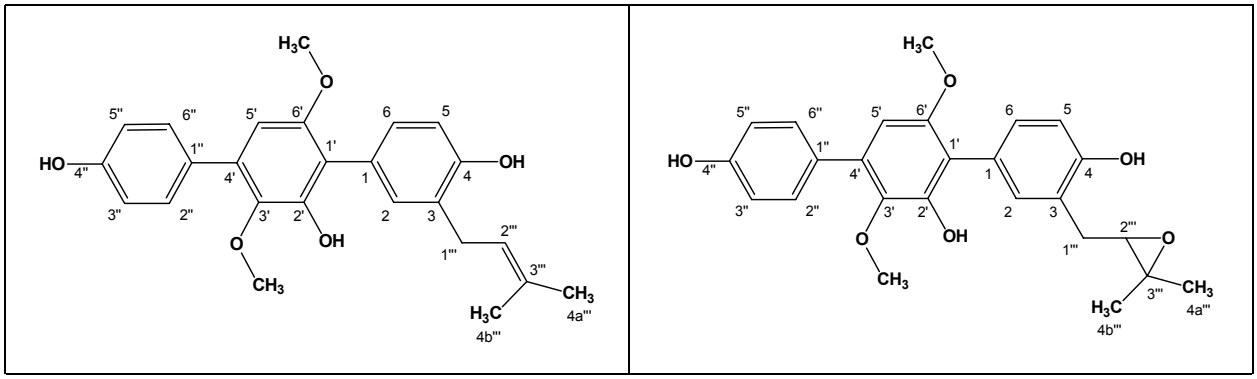


Figure 5.2. Terprenin 2 (compound **20**) (left) and terprenin epoxide (compound **21**) (right)

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# List of Abbreviations

| | |
|---|---|
| $[\alpha]_D$ | : specific rotation at the sodium D-line |
| br | : broad |
| $^{13}C$ | : carbon |
| CI | : chemical ionisation |
| COSY | : correlation spectroscopy |
| *δ* | : chemical shift |
| d | : doublet |
| dd | : double doublet |
| 1D | : one dimension |
| 2D | : two dimension |
| DEPT | : distortionless enhancement by polarisation transfer |
| DMSO | : dimethylsulfoxide |
| DNA | : deoxyribonucleic acid |
| EI | : electron impact |
| ESI | : electron spray ionisation |
| *et al.* | : *et altera* (and others) |
| EtOAC | : ethyl acetate |
| EV | : electron volt |
| FAB | : fast atom bombardment |
| g | : gram |
| $^{1}H$ | : hydrogen (proton) |
| HMBC | : heteronuclear multiple bond connectivity |
| HMQC | : heteronuclear multiple quantum coherence |
| HPLC | : high performance liquid chromatography |
| Hz | : herz |
| H/D | : $^{1}H$ (hydrogen) / $^{2}H$ (deuterium) |
| m | : multiplet |
| MALDI | : matrix assisted laser desorption |
| MeOD | : deuteurated methanol |
| MeOH | : methanol |
| mg | : milligram |
| mL | : millilitre |
| µg | : microgram |
| µL | : microlitre |
| mRNA | : messenger ribose nucleic acid |
| MS | : mass spectrometry |
| *m*/*z* | : mass per charge |
| NMR | : nuclear magnetic resonance |
| NOE | : nuclear overhauser effect |
| ppm | : parts per million |
| q | : quartet |
| rel. int. | : relative intensity |
| RP-18 | : reversed phase C-18 |
| ROESY | : rotating frame overhauser enhancement spectroscopy |
| s | : singlet |
| t | : triplet |
| TFA | : trifluoroacetic acid |
| TLC | : thin layer chromatography |
| UV | : ultra violet |
| VLC | : vacuum liquid chromatography |

# Biodata

| | | |
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