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Bioactive secondary metabolites from the culture of the marine sponge-associated fungus *Acremonium persicinum* KUFA 1007

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marine sponge-associated fungus *Acremonium
persicinum* KUFA 1007**

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Author's declaration

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Ana João Sousa Alves

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Abstract

Two previously undescribed compounds, acremine S [(3*S*, 5*R*, 10*S*)-3,10-dihydroxy-2,2,8-trimethyl-1-oxospiro[4.5]dec-8-en-7-one] (**III**) and acremine T [4-(hydroxymethyl)-2-methyl-1-benzofuran-6-ol] (**IV**), together with previously reported metabolites, ergosterol (**I**), ergosterol-5,8-endoperoxide (**II**) and lumichrome (**V**), were isolated from the crude EtOAc extract from the culture of the fungus, *Acremonium persicinum* KUFA 1007, which was isolated from the marine sponge *Mycale* sp.

The structures of the isolated compounds were established on the basis of extensive analysis of the 1D and 2D NMR spectra as well as High Resolution Electrospray Ionization Mass Spectra (HRESIMS) data. In the case of acremine S (**III**), the absolute configurations of its stereogenic carbons were determined by comparison of the calculated and experimental electronic circular dichroism (ECD) spectra as well as ROESY correlations. The structures of the previously described compounds were elucidated by ¹H and ¹³C NMR analysis, as well as by comparison of their ¹H and ¹³C NMR data and other physical data with those reported in the literature.

Acremines S (**III**) and T (**IV**), as well as lumichrome (**V**), were tested for their anticholinesterase and anti-melanogenic activities using the colorimetric assays. All the tested compounds were active in both assays, however, acremine T (**IV**) showed the strongest tyrosinase inhibitory activity, whereas acremines S (**III**) and lumichrome (**V**) showed more potent inhibitory activities toward BChE and AChE, respectively.

Key words: *Acremonium persicinum*, Acremine S, Acremine T, Antityrosinase, Anticholinesterase.

Resumo

Dois compostos, não descritos na literatura, acremina S [(3S, 5R, 10S)-3,10-dihidroxi-2,2,8-trimetil-1-oxospiro[4.5]dec-8-en-7-ona] (**III**) e acremina T [4-(hidroximetil)-2-metil-1-benzofuran-6-ol] (**IV**), juntamente com os metabolitos já conhecidos, ergosterol (**I**), ergosterol-5,8-endoperóxido (**II**) e lumichrome (**V**), foram isolados do extrato bruto de acetato de etilo da cultura do fungo *Acremonium persicinum* KUFA 1007, isolado da esponja marinha *Mycale* sp.

As estruturas dos compostos isolados foram estabelecidas com base na análise extensiva dos espectros 1D e 2D de RMN bem como dos Espectros de Massa de Alta Resolução de Ionização por Eletropulverização. No caso da acremina S (**III**), as configurações absolutas dos carbonos estereogênicos foram determinadas por comparação dos espectros calculados e experimentais de dicroísmo circular eletrônico (ECD), aliada às correlações observadas no espectro de ROESY. As estruturas dos compostos previamente descritos foram elucidadas por análise dos seus espectros de ^1H e ^{13}C de RMN e pela comparação dos dados dos espectros ^1H e ^{13}C de RMN e outros dados físicos com os dados relatados na literatura.

Acreminas S (**III**) e T (**IV**), bem como o lumichrome (**V**), foram testados quanto às suas atividades anticolinesterásica e antimelanogênica, utilizando ensaios colorimétricos. Todos os compostos testados mostraram atividade em ambos os ensaios, destacando-se a acremina T (**IV**) pela capacidade de inibir a tirosinase enquanto que, para os testes anticolinesterásicos, as acreminas S (**III**) e lumichrome (**V**) se mostraram promissoras na inibição da BChE e AChE, respectivamente.

Palavras-chave: *Acremonium persicinum*, Acremina S, Acremina T, Antitirosinase, Anticolinesterase.

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Abbreviation list

Acetyl-CoA - Acetyl coenzyme A
ACh - Acetylcholine
AChE - Acetylcholinesterase
AChEIs - Acetylcholinesterase inhibitors
AD - Alzheimer's disease
Aib - Aminoisobutyric acid
AntiChEs - Anticholinesterases
AntiAChE - Antiacetylcholinesterase
BCh - Butyrylcholine
BChE - Butyrylcholinesterase
BSA - Bovine serum albumin
ChAT - Choline acetyltransferase
CNS - Central nervous system
COSY - Correlation Spectroscopy
DCT - DOPAchrome tautomerase
DEPT - Distortionless Enhancement by Polarization Transfer
DHI - Dihydroxyindole
DHICA - Dihydroxyindole-2-carboxylic
DMSO - Dimethyl sulfoxide
DMSO- d_6 - Deuterated dimethyl sulfoxide
DNA - Deoxyribonucleic acid
DFT - Density Functional Theory
DOPA - Dihydroxyphenylalanine
DQ – DOPAquinone
DTNB - 5,5'-Dithiobis-(2-Nitrobenzoic Acid), Ellman's Reagent
ECD - Electronic circular dichroism
EtOAc - Ethyl acetate
Frs - Fractions
HMBC - Heteronuclear Multiple Bond Correlation
HRESIMS - High-resolution electrospray ionisation mass spectrometry
HRMS - High Resolution Mass Spectrometry
HSQC - Heteronuclear Single Quantum Coherence

IEFPCM - Integral Equation Formalism for Polarizable Continuum Model
ITS - Internal Transcribed Spacer
L-DOPA - Levodopa
MDF - Marine-derived fungi
MITF - Melanocyte Inducing Transcription Factor
MM - Molecular mechanism
NMR - Nuclear Magnetic Resonance
NOESY - Nuclear Overhauser Effect Spectroscopy
N-SMase - neutral sphingomyelinase
OPs - Organophosphates
PD - Parkin's diseases
PDA - Potato dextrose agar
PCR - Polymerase chain reaction
Petrol - Petroleum ether
PNS - Peripheral nervous system
ppt - Parts per trillion
ROESY- Rotating-frame Overhauser Enhancement Spectroscopy
Si - Silica
SFrs - Sub-Fractions
SSFrs - Sub-SFrs
TD-DFT - Time-dependent density functional theory
TBE - Tris/Borate/EDTA
TLC - Thin-layer chromatography
TYR - Tyrosinase
TYRP - Tyrosinase-related protein
UV - Ultraviolet
Yt - Antigen system Cartwright
¹³C NMR - Carbon-13 Nuclear Magnetic Resonance
¹H NMR - Proton Nuclear Magnetic Resonance

Chapter 1 - Introduction

1.1 Natural Products and Biotechnology

The increasing faith of consumers in natural products has given birth to the urge to explore natural wealth so that potent therapeutic natural compounds may be utilized for pharmaceutical and industrial applications ¹. Natural products derived from marine-based extracts are increasingly sought in the industry ². Naturally occurring bioactive compounds as ingredients in various pharmaceuticals have numerous advantages over synthetic counterparts. Marine resources have been demonstrated as rich sources of structurally diverse and biologically active compounds with enormous pharmaceutical and cosmeceutical potential. As more than 70% of the world's surface is covered by oceans, the great diversity of marine organisms offers a rich source of biologically and pharmacologically active natural products ¹.

With so many new marine species still to be discovered, much research effort will be needed to appreciate the vast potentialities marine life has to offer ¹. This diversity has been the source of unique chemical compounds with the potential for industrial development as pharmaceuticals, cosmetics, nutritional supplements, molecular probes, fine chemicals and agrochemicals ³. Marine organisms have evolved biochemical and physiological mechanisms that include the production of bioactive compounds for such purposes as reproduction, communication, and protection against predation, infection and competition ⁴. Although several compounds from marine organisms showed promising activity to be potential drug candidates over the last few decades, a majority has generally not been ready for further development due to the challenge of obtaining continuous and larger supply of the compounds, unless they can be chemically synthesized. Considerable quantity of a drug candidate is vital for clinical trials, but only a few milligrams of most natural products can be isolated from marine samples. One solution is farming marine organisms as sources bioactive metabolites ⁵.

Currently, the search for new marine natural products depends on the harvest of specimens - a limitation to sustainability and replicability. Sustainability issues are associated with large amounts of biomass that are usually required for drug discovery ⁶. Replicability problems are related with environmental variability and community level changes to the chemical ecology of the target organisms. Individuals of the same species sampled in different areas may not display the same chemical composition and therefore may not guarantee the supply of the target metabolite ⁷. Aquaculture of marine

invertebrates may overcome these two issues as animal biomass can be continuously produced using homogenous environmental conditions ⁸. One of the answers to this matter relies on biotechnology: technology that promotes the exploitation of biological processes for industrial or other purposes. Biotechnology is leading the development of new industries of great importance in European Union. These industries will contribute to shape the future of our society ⁹.

Biotechnology involving marine biological resources, whether as the source or target of biotechnology applications, is gaining territory in global biotechnology. Conservative estimations predicted annual growth of 10-12% per year, revealing the enormous potential and high expectations of further development of the marine biotechnology industry on a global scale ⁹. Marine biotechnology presents itself as a multipurpose sector contributing to the solution of the main challenges of society such as sustainable supply of healthy and high-quality food, sustainable energy, etc., securing environmental health and human health. Environmental applications of marine biotechnology are numerous and diverse, ranging from biofouling control, environmental monitoring for ecosystem and human health to marine habitat restoration, bioremediation and more generally natural resource and environment management. Indeed, seas and oceans represent a unique environment with the potential to contribute enormously to the sustainable supply of food, energy, biomaterials and to environmental and human health. During the last decade, it became evident that bioactive molecules are produced by unknown and uncultivated microorganisms (the so-called dark matter), or microorganisms associated with invertebrates, often through symbiosis. The production of microbes, microalgae or invertebrates for bioactive compounds, biorefinery or energy applications is a complex process that needs considerable optimisation a next step that could be made relying on synthetic biology that promises to engineer organisms for the benefit of humanity ¹⁰.

The world's oceans harbour a wide range of environmental niches, host an as yet largely untapped and underutilised source of biodiversity, but if on the one hand, we have the physical and chemical conditions, in the marine environment, to produce a variety of molecules with unique structural features, on the other hand, we have a lack of industrial interest in natural products, attributed to several common problems such as taxonomy, variability, supply, some of which are considered intractable obstacles. All of these, together with a preference for technology-intensive discovery methods, led the pharmaceutical industry to move away from natural products as a source of chemical diversity ¹¹.

Marine natural products, particularly those from bacteria and fungi, should be regarded as favoured sources of chemical diversity for drug discovery and should be compatible with

current industrial and biotechnological fabrication models. Also, the use of organisms from unexplored and extreme environments is an excellent starting point together with the use of reliable taxonomy to source genetically distinct organisms will increase the chances of finding novel chemistry. Extracts and fractions can be screened using biological methods to pinpoint compounds with known activity profiles ⁹.

The use of chemical ecology in the selection of organisms will improve the 'hit rate' in assays where the target and ecology of the organism are carefully matched. For instance, proven antifeedants have been used to derive anticancer, antiviral and anti-aging products; toxins have been used to develop painkillers; and functional products have been obtained from the materials used by sessile organisms to colonise free surfaces. Knowledge of eco-physiological interactions may serve as a platform to facilitate the search for new biotechnological candidates, as well as to optimise culture conditions and achieve production of biomass for industrial applications ⁹⁻¹⁰.

A re-education is necessary to convince industry that marine bioresources are an important source of chemical diversity for drug discovery. The oceans represent a huge reservoir of biomolecules, genetic resources and organisms of which we still know very little. A rational exploitation of these resources for economical purposes, together with adequate preservation and management of marine environments, will be beneficial for the future use of these resources through existing marine biotechnology knowledge and methods or from further developments in this field such as isolation, synthesis or use of proteins and enzymes, food additives, biopolymers and biomaterials that can be applied as wound dressings, bio-adhesives, dental biomaterials, tissue regeneration and 3D tissue culture scaffolds ¹²⁻¹³.

1.2 Secondary Metabolites of Marine Origin

The multiplicity of marine organisms, living in the oceans which account for more than 70% of the world's surface, offers a rich and uncountable source of natural products ¹⁴. Due to special chemical and physical conditions of marine environments, these organisms evolved to develop biochemical and physiological mechanisms that lead to the production of a wide range of bioactive compounds, such as alkaloids, peptides, lipids, proteins and glycosides essential for their survival in terms of reproduction, communication, defence and competition ¹⁵. All these resulted in a diversity of unique chemical compounds with

enormous potential for development of new drugs, pharmaceuticals, cosmeceuticals, nutritional supplements and agrochemicals ¹⁴.

Although the marine environment presents great biodiversity, only a few species are totally described ¹⁶. Various compounds produced by marine organisms show various properties which can be applied in cosmetics, such as antioxidant, anti-inflammatory, anti-allergic, anti-aging, anti-wrinkle and anti-melanogenesis (antityrosinase) ¹⁵. In fact, several compounds from marine organisms showed promising biological and pharmacological activities potential drug candidates over the last few decades. However, many of them have not yet been found in pharmaceutical applications, when compared to compounds obtained from plants and terrestrial organisms. Anyhow, marine organisms continue to provide important molecules that can be considered as promoters for research and development of new products. Nevertheless, there are cases of success with the approval of drugs from marine organisms for pharmaceutical use and of other compounds from sources that are already in clinical trial phase ¹⁷. Having a considerable quantity of a drug candidate is vital for clinical trials, but many times only a few milligrams of most natural products can be isolated from marine samples. As said before, farming those microorganisms, searching for bioactive metabolites as a sustainable source of great amounts of biomass is currently the main focus of researchers ¹⁸. This also can overcome the variability/replicability issues caused by the species origin from various ecological niches ⁷. Also, marine organisms are believed to be the future supply of very interesting therapeutic alternatives ¹⁹.

1.3 Bioactive Metabolites from Fungi

The fungal kingdom encompasses an enormous diversity of taxa with varied ecologies, life cycle strategies, and morphologies ranging from unicellular aquatic chytrids to large mushrooms. Like animals, fungi are heterotrophs. They acquire their food by absorbing dissolved molecules, typically by secreting digestive enzymes into their environment. It is believed that there are about 1.5 million species of fungi in the world, not all of them have yet been described ²⁰. They were believed to be rare in marine environments, but studies based on molecular and metagenomics approaches revealed unexpected diversity of coastal and deep ecosystems ²¹⁻²². Indeed, fungi have a worldwide distribution and grow in a wide range of habitats such as oceans, coastal zones, estuaries, deep water, and sediment with many survival challenges. Marine fungi differ from terrestrial fungi and freshwater fungi. They are an ecologically, physiologically and taxonomically defined

group of microorganisms ²³. They have a great ability to adapt to extreme environments such as high concentrations of salt (33-35 ppt), high sodium levels, low temperatures, low luminosity and oligotrophic nutrient conditions. Some fungi may even survive the intense ionizing radiation and the shortage of oxygen ²⁴⁻²⁷. Also, there are evidences that fungi adaptability induced by these specific adaptations may result in the production of unique secondary metabolites ²⁸. It is well documented that they also play an important ecological role in the marine environment as primary decomposers, as pathogens of marine invertebrates, and as symbionts ²⁹. These ecological interactions can be mutualistic or antagonistic in nature, or in the case of commensal fungi are of no apparent benefit or detriment to the host. So, their ecological activity leads to the production of a range of natural products with antimicrobial or other biological activities. Many species have long been used or are being developed for industrial production of antibiotics, vitamins, anticancer and cholesterol-lowering drugs ³⁰. In the last decade, thousands of new bioactive compounds, including organic acids, polyketides (such as quinones, anthraquinones and xanthenes), monoterpenes to triterpenes, polysaccharides and lipopolysaccharides have been discovered from fungi of marine origin ³¹.

Generally, and as mentioned earlier, marine fungi live in symbiotic association with other marine organisms such as algae, corals, anemones, starfish, sea grasses, sea urchins and especially with marine sponges ^{23, 32}. Approximately 75% of the new bioactive compounds are derived from marine invertebrates, especially sponges. The sponges are highlighted by the important role they play as the most productive source of new compounds in preclinical development and they are known for hosting not only fungi but also a large community of other microorganisms ³³. The different symbiotic relationships, that fungi establish, influence the number and nature of the secondary metabolites they produce. Their metabolites may act specifically on interspecies interactions to protect the host against competitors or diseases ³⁴. Lately, many studies have shown that fungi associated with marine sponges are a source of many compounds of interest ³⁵.

Fungi are among the marine organisms with the highest potential of producing bioactive compounds which can be exploited for several commercial purposes, including pharmaceutical, cosmetic and cosmeceuticals. There are many cosmetics incorporating fungi-derived molecules, including those for skin care such as anti-aging, anti-acne, antioxidant, skin revitalizing, skin whitening and hair products ³⁶.

On the other hand, almost half of this molecules/drugs, currently in clinical use, are enzyme inhibitors ³⁷. Several marine natural products with potent cholinesterase inhibitory properties have been isolated from a variety of marine algae, fungi, bacteria and other marine microorganisms. Thus, most of the compounds used as inhibitors of

cholinesterase are natural products or derivatives thereof. The advantage is that, unlike potent inhibitors already identified by *in vitro* screening that are often accompanied by poor pharmaceutical properties or off-target activities leading to unwanted side effects, these factors can be eased by the chemical diversification of the natural pharmacophore. Cholinesterase inhibitors are used to ease the symptoms of neurological disorders, such as dementia, Alzheimer's disease, myasthenia gravis and glaucoma ³⁷.

1.4 *Acremonium* sp.

The genus *Acremonium*, NCBI txid:159075, contains about 200 recognized and nomadic species but few are properly named ³⁸. Up to 2011, the taxonomy of *Acremonium* has been almost exclusively based on morphological criteria. However, Perdomo *et al.* have demonstrated that certain *Acremonium* species cannot be reliably identified using only phenotypic features ³⁹. Actually, the phonological taxonomic bank administered by the International Mycological Association has 205 registered strains, 197 of which are denominated species ⁴⁰⁻⁴¹. The genus *Acremonium* is highly polyphyletic comprising phylogenetically distant fungi ⁴².

In the anamorphic polyphyletic taxon *Acremonium*, 100 species have been described, many of which are responsible for producing biologically active metabolites, including β -lactam antibiotics and cephalosporins, the immunosuppressants cyclosporins, the tremorgenic indole diterpenoids lolitrems and prenylated phenols inhibitors of neutral sphingomyelinase (N-SMase) ⁴³. Endophytic fungi are a good source of novel secondary metabolites and the endophyte behaviour of many *Acremonium* spp. in *Gramineae* has been positively related to resistance of the host grass against aphids and other important insect pasture pests, but it is also at the basis of very potent toxicosis in humans and animals ⁴⁴.

Acremonium has a worldwide distribution being frequently found in soil and dead plants and some species can also be found in food. A number of species of *Acremonium* have rather specialized relationships to other living organisms ⁴¹. *Acremonium* species utilize a range of complex nitrogenous compounds, and a number of species have proteolytic activity ⁴¹. *Acremonium* species is mostly saprophytic and non-pathogenic; however, some species have been recognized as opportunistic as man and animal's pathogens, causing eumycetoma, onychomycosis and hyalohyphomycosis. The clinical manifestations of hyalohyphomycosis may include arthritis, osteomyelitis, peritonitis, endocarditis, pneumonia, cerebritis, and subcutaneous infections ^{41, 45-46}. Gams, for the first time in

1971, divide the genus into three sections: *Simplex*, *Gliomastix* and *Nectrioidea*. The characters employed in identification of the species are: appearance of the culture on malt extract or oatmeal agars; presence and form of the conidiophore or its absence; form of the conidiogenous cell; presence or absence of a collarete after the first conidium has seceded; way in which the conidia collect at the tip of the conidiogenous cell, i.e., in "heads" or "false chains"; presence and form of chlamydospores and of sclerotia ^{41, 47}. Colonies of *Acremonium* are characterized by their pale colors often turning pink or orange, and the production of wet or slimy spores ⁴¹. The metabolic competence of *Acremonium* is reflected in the fact that the majority of species grows well on a relatively simple medium such as malt extract agar, and many will grow on a defined medium such as Czapek's solution agar ⁴¹. Species of *Acremonium* grow well in submerged culture, and most physiological investigations and industrial exploitations use liquid media. Filamentous fungi growing in shaken or stirred liquid culture can exhibit a range of morphologies, from dispersed mycelium forming a homogeneous suspension of high viscosity to spherical mycelial pellets forming a suspension of relatively low viscosity ⁴¹. Although the full expression of differentiation and subsequent dispersal of *Acremonium* and *Penicillium* are usually associated with growth on solid substrates, sporulation of at least some species in both genera can occur in liquid culture ⁴⁸.

1.4.1 *Acremonium persicinum*

Acremonium persicinum (NCBI txid: 5051), named by Nicot W. Gams, is an ascomycota fungus belonging to the Hypocreaceae family (Figure 1) ⁴⁷. It was previously named *Paecilomyces persicinus* by the same author, in 1958, and referred as a *Cephalosporium* fungus ⁴¹. Actually, *Cephalosporium* was introduced by Corda in 1839, describing colorless molds with simple unbranched conidiophores and conidiogenous cells bearing at the tip a group or "head" of unicellular conidia, from whence the name was derived ⁴¹.

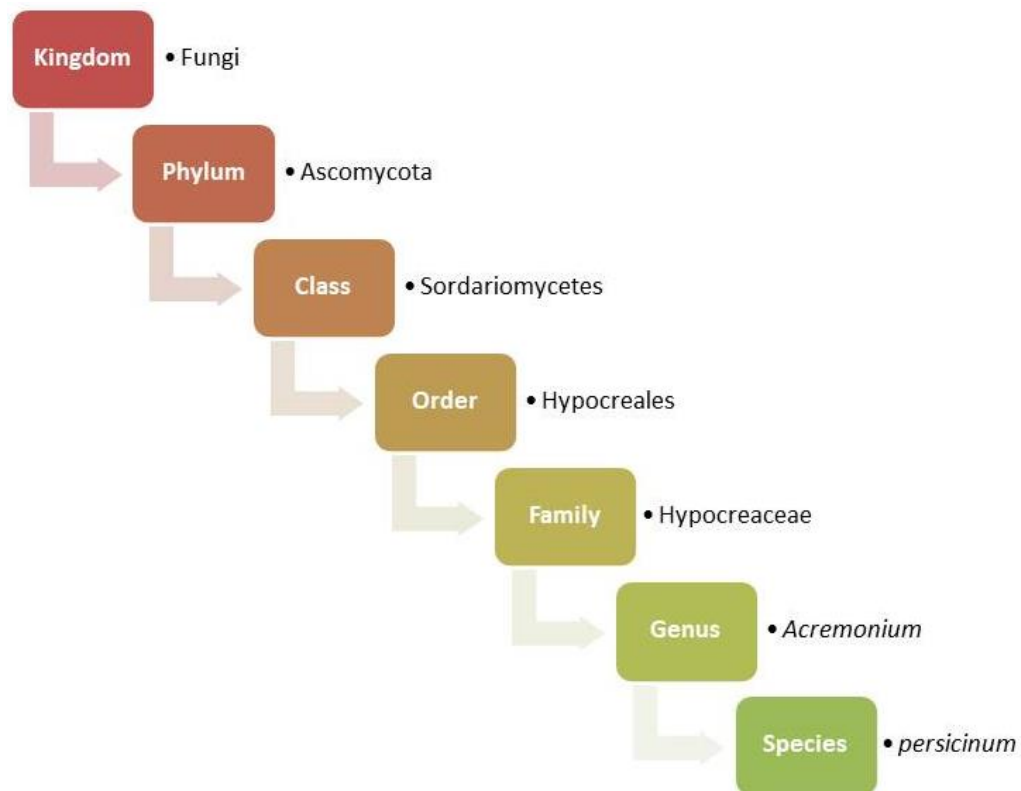


Figure 1 - *Acremonium persicinum* Taxonomic Tree (Adapted from Gams, 1971)

At first sight, the morphology of *Acremonium* is simple. It is only when the arrangement, form, and function of the conidiogenous cells are compared in the individual species that an underlying complexity is revealed. The conidiogenous cells that generate conidia in *Acremonium* do so by an active wall-building region at the tip of the cell budding off the first conidium, both inner and outer wall layers being involved; a basal cross wall is laid down, and the process is described as "holoblastic". Thereafter, this conidium becomes detached by the separation of the two layers of wall between conidiogenous cell and conidium (schizolysis), and a further conidium is formed at the tip of the conidiogenous cell, the original inner wall layer becoming the outer wall and a new inner wall layer being laid down within it. These and subsequent conidia are said to be formed "enteroblastically", and after they secede, they may remain either clustered in the typical "head" of a "Cephalosporium" or loosely connected end-to-end in a "false chain" ⁴¹.

Acremonium persicinum (Figure 2) colonies slow-grow on malt extract or oatmeal agar culture media, attaining the diameter of less than 25 mm in 10 days at 20 °C ⁴¹. This fungus also requires wet growth conditions and is expected to be mesophilic to hydrophilic. Initially the colonies are compact and humid, becoming powdery, suede-like, with the passage of time, and may be whitish to ochre-brown. Their hyphae are thin-walled and hyaline and produce mainly simple eroded phialides. Conidia are often

obovoid, with slightly protruding and truncate base, usually smooth, hyaline, slimy heads or in chains ³⁹. In 1975, Seviour and Codner ⁴⁹ determined that its maximum specific growth rate was at pH 6.0 with significant changes in morphology, from thick, short, sporulating hyphal fragments at pH 3.0 to branched mycelial clumps at pH 6.0, and long, thin, unbranched filaments at pH 9.0. Light may have a profound influence on the growth and development of fungi, but in this case, the most obvious effects of light are the pigmentation of the colonies. This species showed enhanced pigmentation when grown in the light ⁵⁰. Also, they produced carotenoids only when growing in the filamentous form after exposure to light ⁴⁹. The single-celled yeast-like form, on the other hand, is able to produce pigments even when grown in the dark ⁵¹.



Figure 2 - Colony of *Acremonium persicinum* KUFA 1007 on potato dextrose agar medium.

Due to its biological characteristics, *Acremonium persicinum* could inhabit in the marine environment, and therefore can be identified as a marine-derived fungus.

Generally, marine fungi form an ecological, but not a taxonomic group that can be classified as “obligate marine” (i.e. grow and sporulate exclusively in marine habitats) and “facultative marine” (i.e. from freshwater and terrestrial milieus, but are able to grow and possibly sporulate in marine environments) ⁵². As more studies emerge, marine fungi are increasingly considered to play an important ecological role as saprotrophs, parasites, commensals or mutualists in marine ecosystems, and have been reported to be associated with higher marine organisms, such as macroalgae, corals and sponges ⁵³.

Fungi occupy an important ecological niche in the marine environment, playing a critical role in the ecosystem but their interactions and functions in marine hosts, and in particular marine sponges, is poorly understood ⁵⁴. Interactions between fungi and marine

sponges are far less studied when compared with interactions between sponges and bacteria in terms of host specificity and microbial diversity⁵⁵. Many marine-derived fungi, isolated from various marine habitats and substrata, produce an array of novel secondary metabolites. Among the marine-derived fungi from various marine sources, those associated with marine sponges have higher species diversity and are prolific producers of novel secondary metabolites⁵⁶⁻⁵⁷.

Marine sponges (Phylum Porifera) represent the most primitive multicellular animals, with origin dating back to the Precambrian⁵⁸. Sponges are sessile, filter-feeding organisms that play a crucial role in benthic communities through nutrient cycling, including carbon, silicon, oxygen and nitrogen, and the provision of habitats for a range of fauna and flora⁵⁹. There are about 9000 living species of sponges variously distributed among all the oceans and at various depths⁶⁰. They can harbour microbial communities from all three domains of life, archaea, bacteria and eukaryotes (fungi, microalgae and protozoa), which can account for up to 40–60% of the sponge's volume in some species⁶¹.

The presence of fungi in sponges is rather “accidental”⁶². The ability of many sponge-derived fungi to produce bioactive compounds has been suggested to contribute to host defence^{35, 63-65}. Ubiquitous fungal genera (e.g., *Aspergillus*, *Penicillium*, *Trichoderma*, and *Acremonium*) have been isolated from various sponge species worldwide⁶⁶. Recent investigations have established that, given the high species diversity and wide distribution of sponges, marine-derived fungi associated with them are an excellent source of novel bioactive metabolites with the potential to function as drugs or drug leads⁶⁷. There are already many described marine sponge-associated fungal metabolites such as antitumor, antibiotic, antifungal, anti-algal, anti-insect, antioxidant and acetylcholinesterase inhibitors⁶⁸. Of note is that fungi, isolated from different sponge species, produce different secondary metabolite profiles⁶⁹.

Although some terrestrial strains of *Acremonium persicinum* were found to produce secondary metabolites with pharmacological-interest such as antifungal hexapeptide⁷⁰ and peptaibiotics⁷¹, currently, there are no references supporting the isolation of secondary metabolites with pharmaceutical interest from sponge-associated *A. persicinum*. Therefore, this thesis describes isolation and a complete structure elucidation, including the stereochemistry, of previously undescribed secondary metabolites and some previously reported metabolites together with the evaluation of their inhibitory activities toward acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) as well as tyrosinase enzymes which can be potential for development of therapeutic agents for

treatment of neurological disorders as well as for application in cosmetics as skin whitening agent.

1.4.2 Importance of secondary metabolites produced by *Acremonium persicinum*

Very few fungi with secure taxonomic status within the genus *Acremonium* have been reported to produce secondary metabolites. *Cephalosporium* is the well-known and unique in this respect, since it produces the famous penicillin and cephalosporins though it has more recently been properly included in the *Acremonium* genus⁴¹.

Acremonium species have proved to be a rich source of other new and bioactive secondary metabolites. There are more than known 356 metabolites, classified as steroids, terpenoids, meroterpenoids, polyketides, alkaloids and peptides, isolated from *Acremonium* species. These metabolites exhibited a wide range of biological activities, including antimicrobial, cytotoxic, antitumor, immunosuppressive, antioxidant, anti-inflammatory, antimalarial, phytotoxic, tremorgenic, antiviral, neurotoxicogenic, insecticidal and enzyme-inhibiting activities⁷².

Fungi from the genus *Acremonium* are well-known as producers of the antibiotic cephalosporin C⁷³. Other substances, such as antioxidant hydroquinone derivatives and a chlorinated polyketide have been reported from an algal-derived *Acremonium* sp.³⁰. Also, isolates derived from marine sponges have yielded alkaloids, peptides or oxygenated metabolites⁷³. A fermentation extract of the strain SCSIO 115, identified as *A. persicinum*, showed strong lethality against brine shrimp (*Artemia salina*) and cytotoxicity toward a panel of tumour cell lines⁷⁴. Fungi from the genus *Acremonium*, isolated from both terrestrial and marine environments, have been reported to produce meroterpenoids, alkaloids, peptides or oxygenated metabolites^{44, 74-76}.

In 1986, Rowan *et al.* have characterized peramine (**1**, Figure 3), an insect antifeedant, from *A. loliae*, which is probably derived from proline and arginine, and is essentially a modified diketopiperazine⁷⁷. Moreover, *A. loliae* has been found very recently to elaborate paxilline (**2**, Figure 3)⁷⁸. Also, recently, the same authors have reported new fungal metabolites, consisting of tryptophanol, tyrosol, and a tyrosol dimer (unpublished observations) from the same fungus⁴¹. A diketopiperazine, based on cyclic Phe-Pro, is also produced by this fungal species, together with several sterols⁷⁹. Thus, it is now also clear that *A. loliae* can biosynthesize an indole-isoprenoid which equates to the principal part of the lolitrem B molecule (**3**, Figure 3). Other studies identified amino acid-derived secondary metabolites that established a link between *Acremonium* and *Penicillium*

genera, indicating that the biosynthetic potential in *Acremonium* may be richer than is yet evidenced and that, in addition to penicillins, peramine, and paxilline, other biomolecules await discovery ⁴¹.

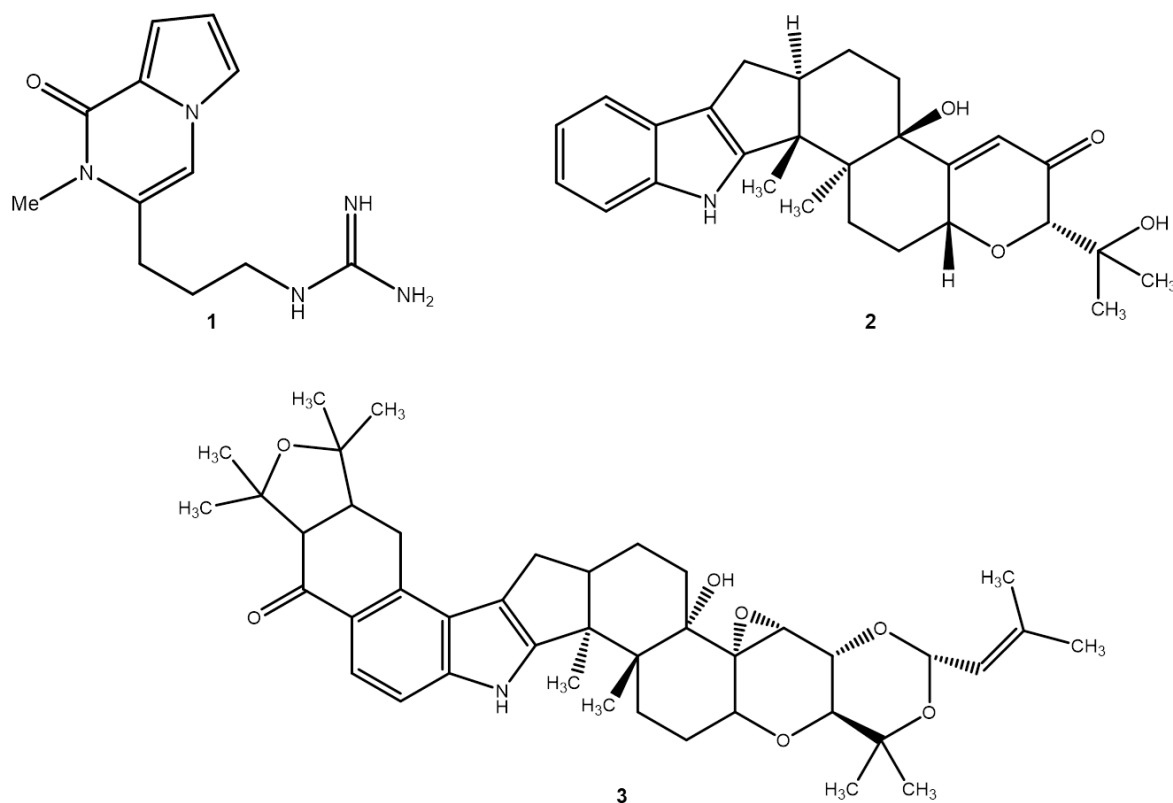


Figure 3 - Chemical structures of peramine (**1**), paxilline (**2**), lolitrem B (**3**).

In 1996, *A. persicinum*, was reported as a producer of a peptide, named XR586 (**4**, Figure 4). This peptide derived from a *Xenova* culture collection X21488 fermentation, designated as *Acremonium persicinum* by Nicot W. Gams in 1971, amino acid is a peptaibol-like antibiotic particularly identical to zervamicins. Peptaibol antibiotics are peptides, typically of 15 to 20 residues, containing a large proportion of aminoisobutyric acid (Aib) residue. These peptides display antibacterial and cytotoxic properties due to their ability to form pores in membranes. Their helices are amphipathic, i.e. they are hydrophilic on one side and hydrophobic on the other, thus allowing them to cluster together in membranes, forming voltage gated ion channels ⁸⁰.

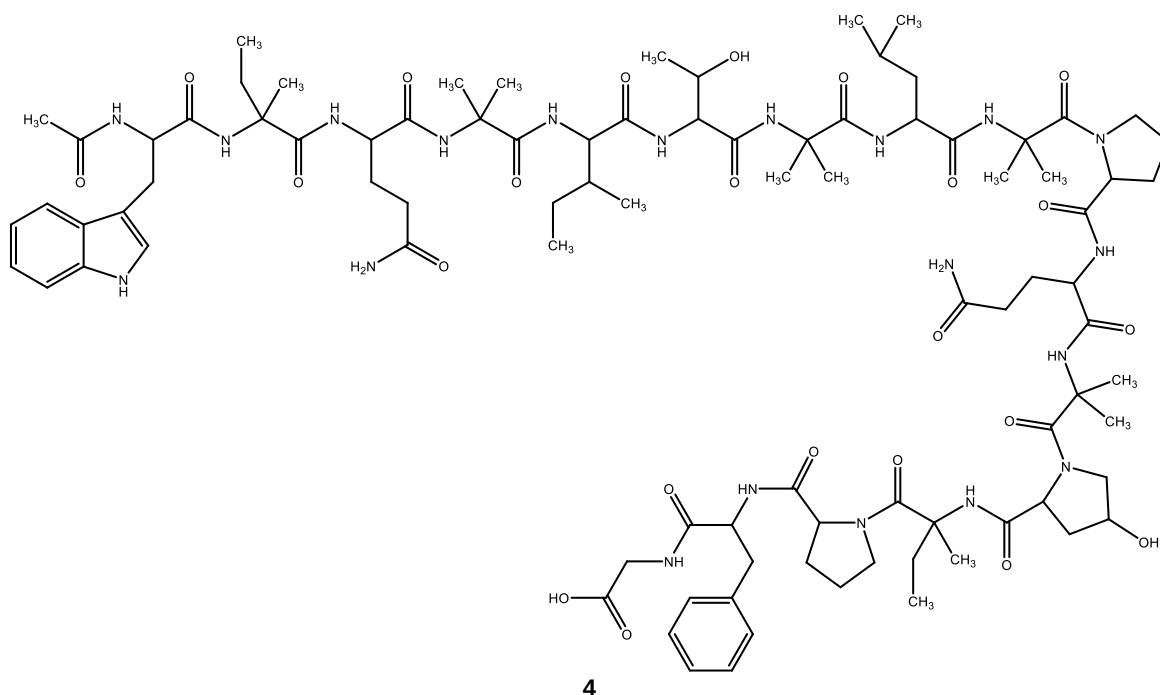


Figure 4 - The structure of the peptide XR586 (**4**)

However, many other compounds produced by the member of the genus *Acremonium* were isolated and characterized. For example, an article published in 2000 by Belofsky *et al.* reported new natural products containing oxepinamides (**5-7**, Figure 5) and two new fumiquinazolines (**8-9**, Figure 5) metabolites that were isolated from organic extracts from the culture broth and mycelia of *Acremonium* sp., which was isolated from the surface of the Caribbean tunicate *Ecteinascidia turbinata*, collected in the Bahamas²⁸.

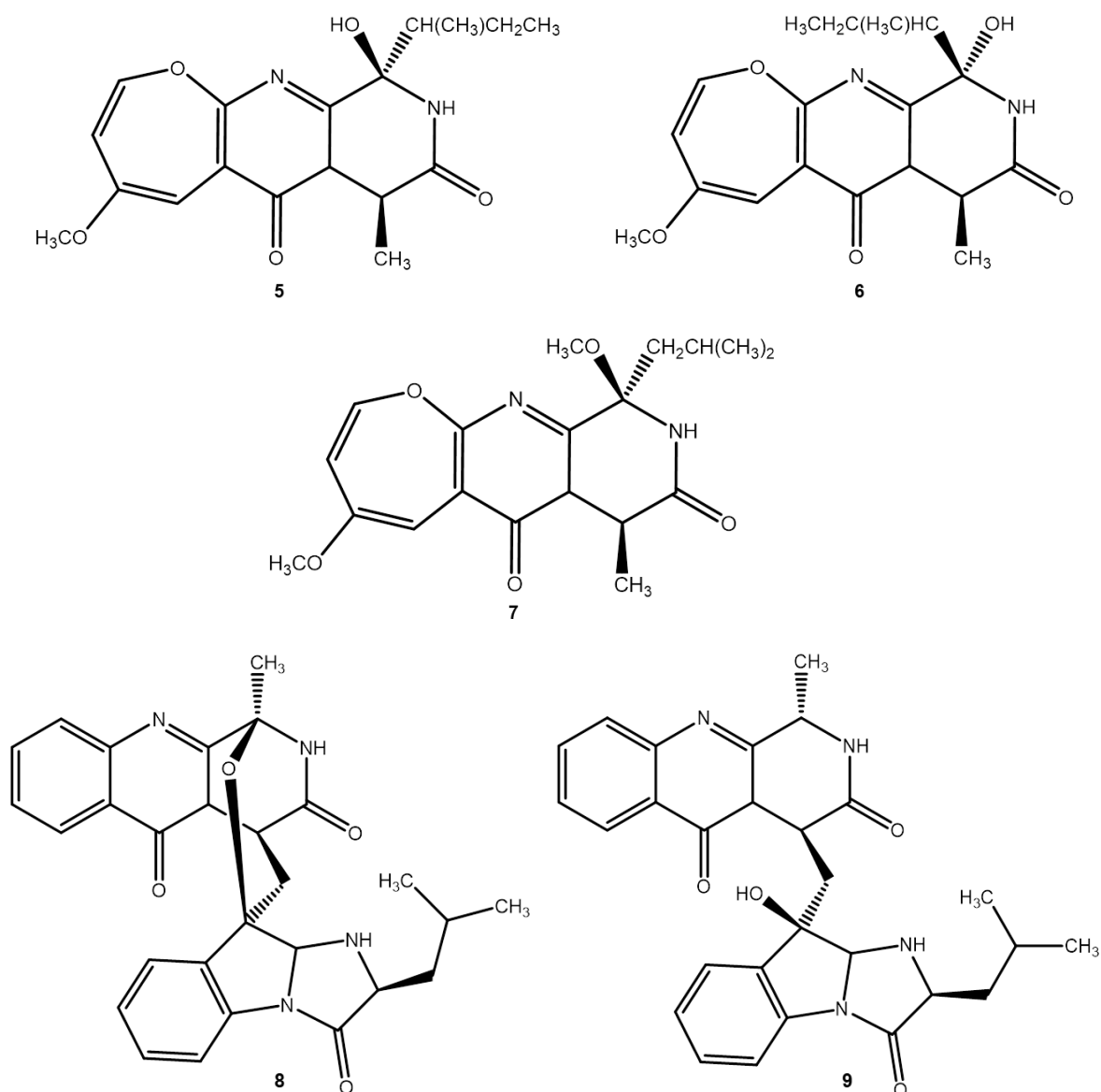


Figure 5 - Chemical structures of oxepinamide A (**5**), oxepinamide B (**6**), oxepinamide C (**7**), fumiquinazoline H (**8**) and fumiquinazoline I (**9**).

Later, in 2005, Assante *et al.* reported the isolation of six new metabolites named, acremines A-F (**10-15**, Figure 6) from agar cultures of *Acremonium sp.*⁴⁴.

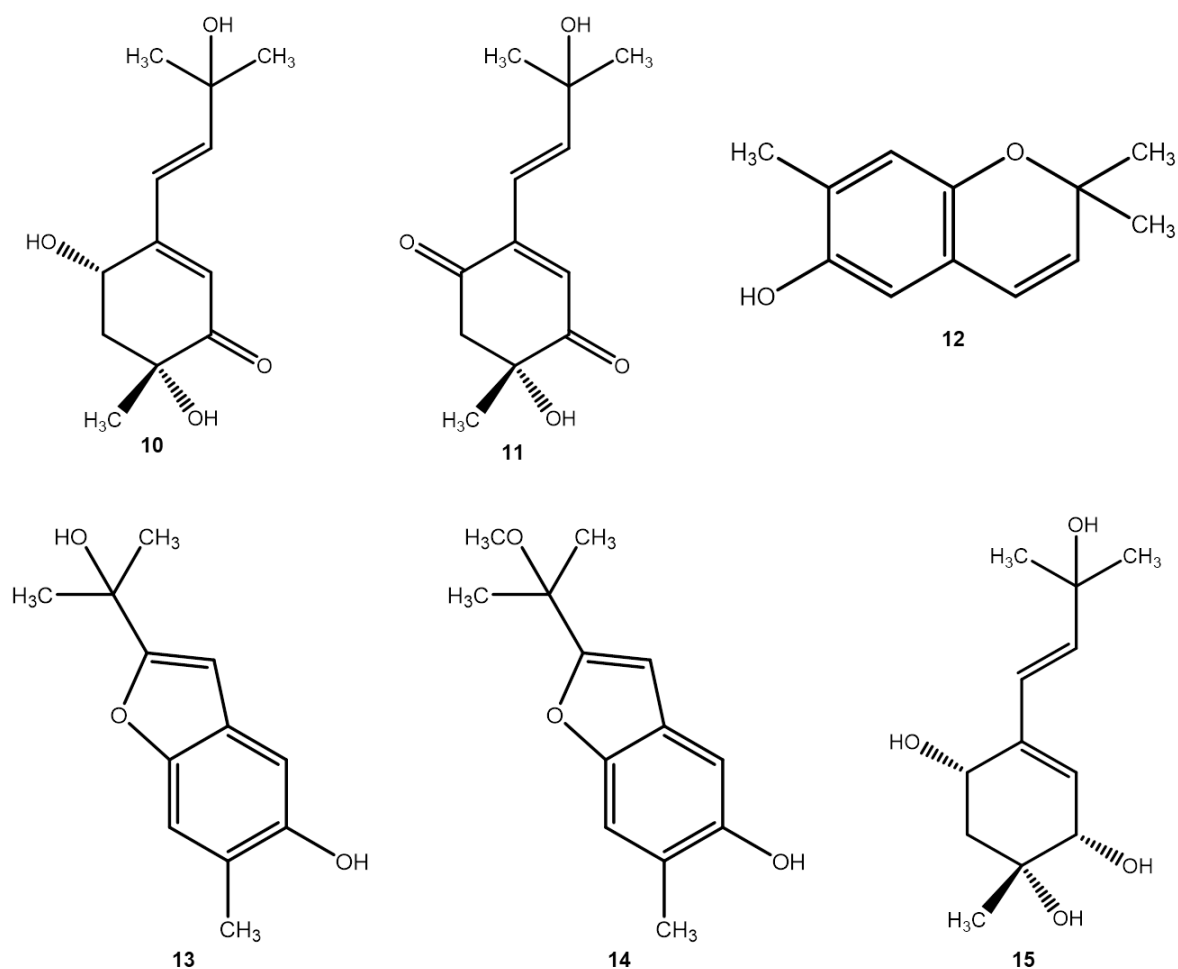


Figure 6 - Chemical structures of acremine A (**10**), acremine B (**11**), acremine C (**12**), acremine D (**13**), acremine E (**14**) and acremine F (**15**).

Later on, Arnone *et al.* reported isolation of acremine G (**16**, Figure 7), a new dimeric racemic metabolite from cultures of *A. byssoides* A20, together with the previously reported acremines A-F⁴³. The acremines are meroterpenes, biosynthetically derived from a monoterpene unit and a polyketide moiety. In continuation of their work, Arnone *et al.* isolated more five new acremine derivatives, acremines H-N (**17-21**, Figure 7) from the malt extract–peptone–glucose agar cultures of the same fungus⁷⁵.

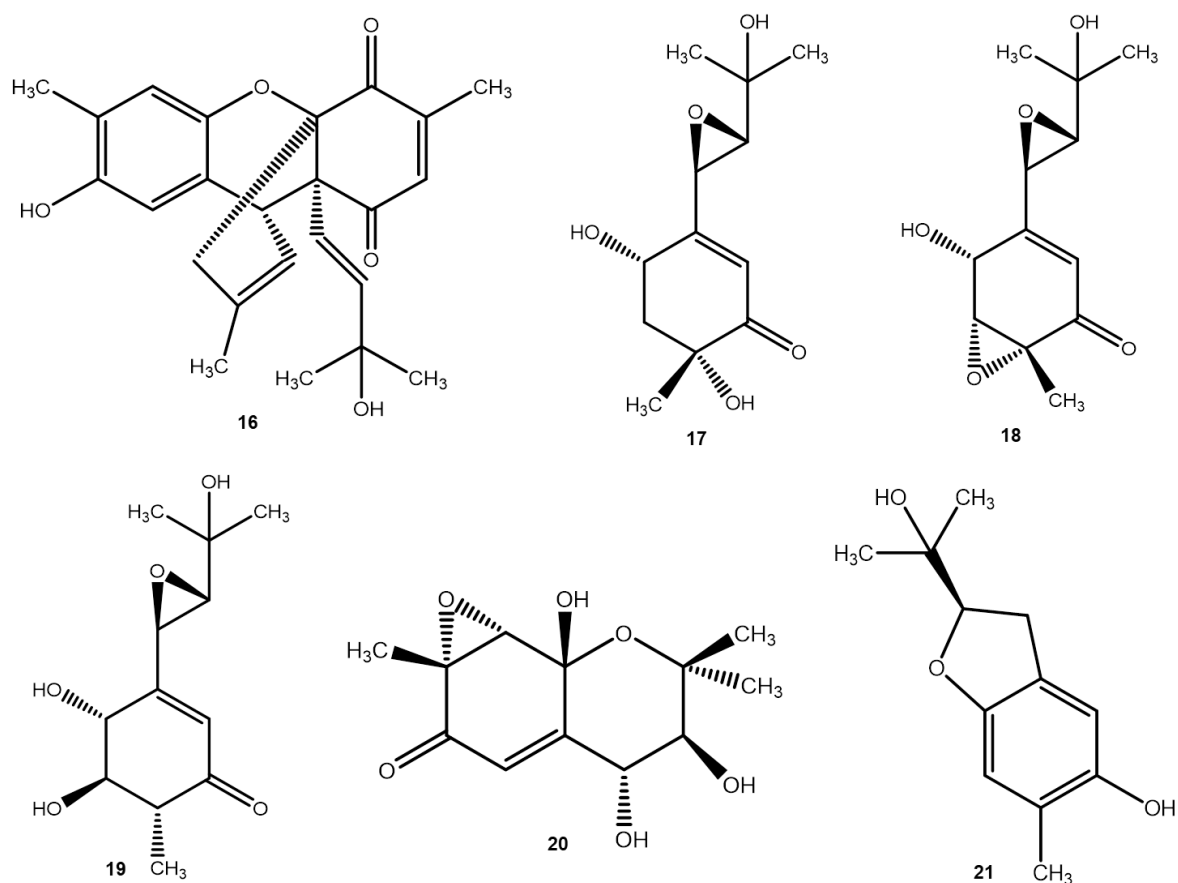


Figure 7 - Chemical structures of acremine G (**16**), acremine H (**17**), acremine I (**18**), acremine L (**19**), acremine M (**20**) and acremine N (**21**).

In 2012, Chen *et al.* reported the isolation of three new cycloheptapeptides, cordyheptapeptides C, D and E (**22-24**, Figure 8), from the fermentation extract of the marine-derived fungus *A. persicinum* SCSIO 115. Compounds **22** and **24** exhibited cytotoxicity against three tumor cell lines (CNS glioma cell line SF-268, breast cancer cell line MCF-7 and lung cancer cell line NCI-H460) ⁷⁴.

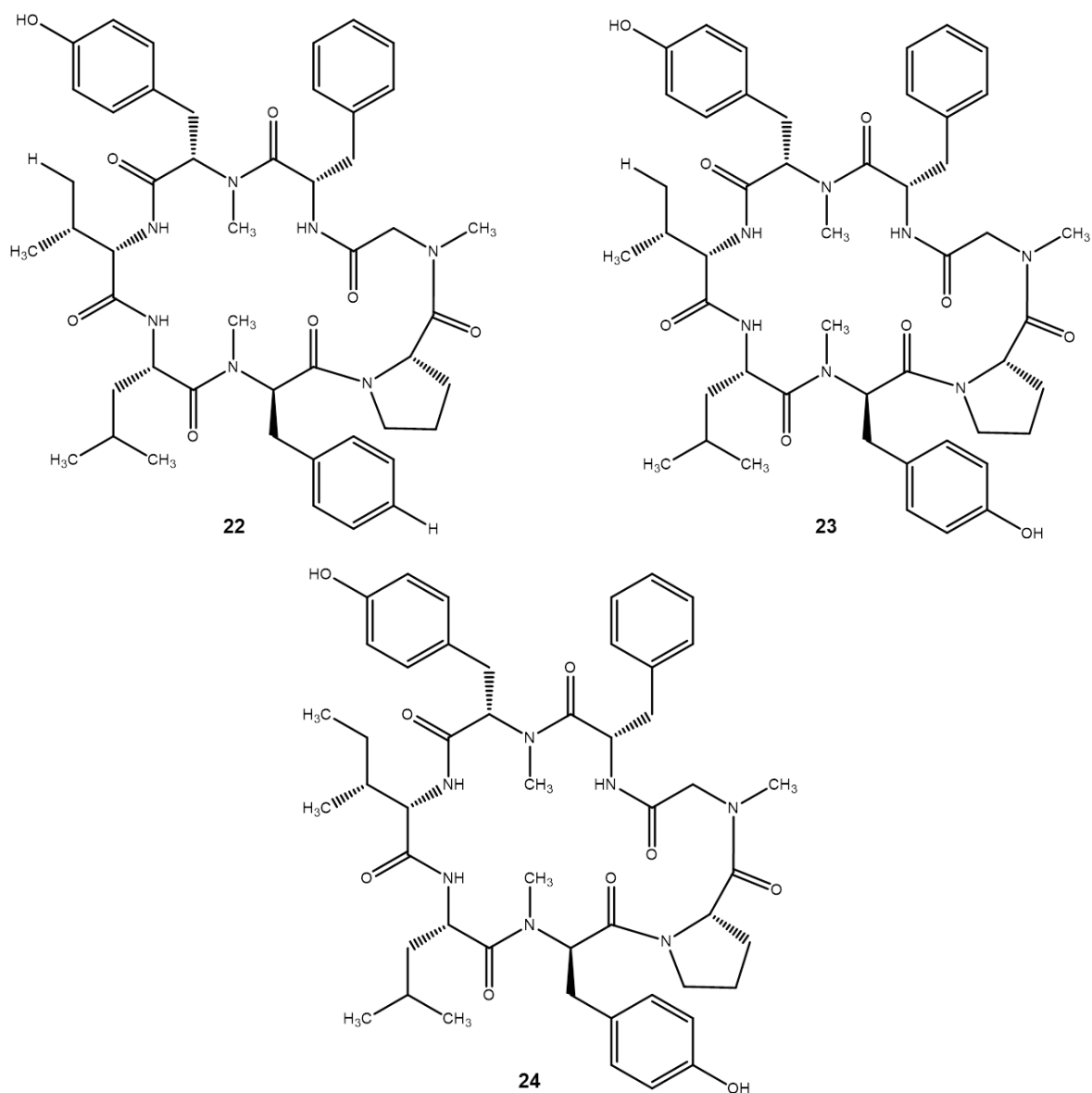


Figure 8 - Chemical structures of cordyheptapeptides C (**22**), cordyheptapeptides D (**23**) and cordyheptapeptides E (**24**).

Suciati *et al.*, reported the isolation and characterization of six new acremine metabolites, 5-chloroacremines A and 5-chloroacremines H (**25-26**, Figure 9), acremines O, P, Q and R (**27-30**, Figure 9), together with the known acremines A, F (**10** and **15**, Figure 6) and N (**21**, Figure 7) from the culture of *Acremonium persicinum*, which was isolated from the marine sponge *Anomoianthella rubra*⁷³.

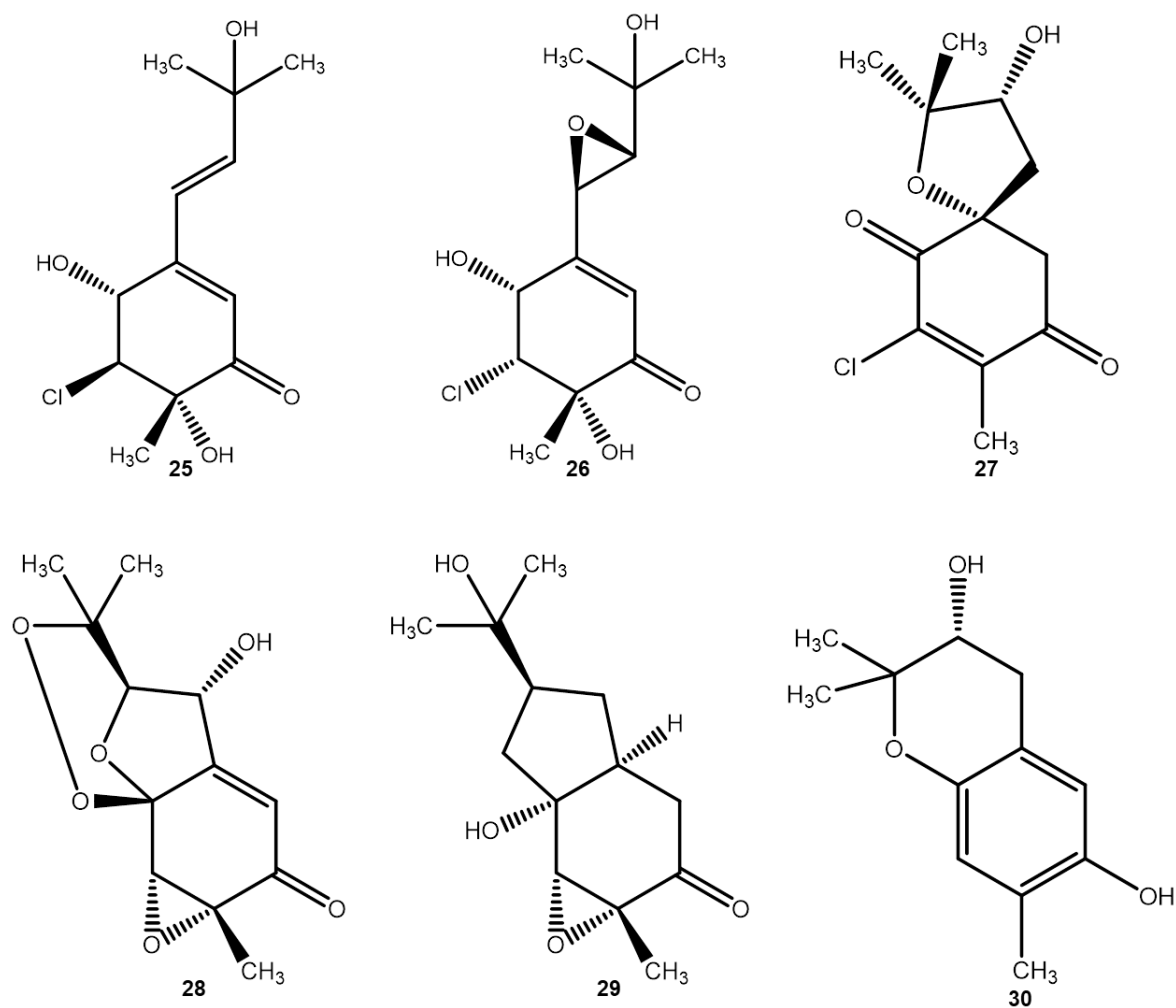


Figure 9 - Chemical structures of 5-chloroacremine A (**25**), 5-chloroacremine H (**26**), acremine O (**27**), acremine P (**28**), acremine Q (**29**) and acremine R (**30**).

In 2017, Garson *et al.*, reported the structure elucidation of the meroterpenoid acremine P (**28**) from a strain of *A. persicinum*, isolated from the marine sponge *Anomoianthella rubra*, collected offshore from Mooloolaba in southeast Queensland, Australia. In this article the authors concluded that the previously published structure of acremine P (**28**) was not correct based on a comparison of calculated and experimental NMR data. A conversion of the acremine P (**28**) into acremine A (**10**) by hydrogenation has been demonstrated⁸¹.

1.5 Biological activities

1.5.1 Antityrosinase activity

The pharmaceutical and cosmeceutical industries are in a constant search for natural compounds or extracts with relevant bioactivities, leading to the design of new formulations ⁸². Specifically, natural ingredients for skin care are becoming popular due to their protective and defensive roles against degeneration and aging ⁸³. Thus, an increasing interest in finding natural resources able to inhibiting tyrosinase, and using them as potential cosmetic ingredients in creams or lotions for topical application is an interesting topic ⁸⁴.

Tyrosinase (TYR) is a multifunctional, glycosylated and copper-containing oxidase, which catalyses the early stages of melanogenesis ⁸⁵. Afterwards, this process consists of a sequential enzymatic process in which the melanosomes are transferred via dendrites to the surrounding keratinocytes, where they play a critical role in photoprotection ⁸⁶⁻⁸⁷. Interestingly, the number of tyrosinase molecules present in black or white skin colour is equal; therefore, the level of tyrosinase activity in melanocytes will regulate cutaneous pigmentation ⁸⁸. Indeed, pigmentation is highly heritable, being regulated by genetic, environmental, and endocrine factors that modulate the amount, type, and distribution of melanin in the skin, hair, and eyes. It has been believed that skin pigmentation is the most important photoprotective factor, since melanin, besides functioning as a broadband UV absorbent, has antioxidant and radical scavenging properties. In addition, it also has an important role in camouflage, heat regulation and cosmetic interaction. Melanin also plays an important role in protecting against environmental oxidative stress. Being the largest organ of the body that is always under the influence of internal and external factors, the skin often reacts to those agents by modifying the constitutive pigmentation pattern ⁸⁹⁻⁹⁰. Melanin contributes for the skin colour and plays a crucial protective role against skin photo-carcinogenesis. However, the abnormal production or lack of melanin pigment is a serious cosmetic issue ⁹¹. Minor changes in the physiological status of the human body or exposure to harmful external factors can affect pigmentation patterns either in transitory (such as in pregnancy) or permanent (e.g., age spots) manners ⁹⁰. In this way, there is also a great demand for whitening cosmetics for the care of lentigo, pregnancy mask or even hyperpigmentation following medicine poisoning ⁹².

Melanin biosynthesis is a complex pathway that appears in highly specialized cells, called melanocytes, within membrane-bound organelles called melanosomes ⁹⁰. Once

established, *in situ*, melanocytes start producing melanosomes in which melanin synthesis takes place. Melanosomes are typically divided into four maturation stages (I–IV), determined by their structure and the quantity, quality, and arrangement of the melanin produced (Figure 10) ⁹³⁻⁹⁴. Nascent melanosomes are assembled in the perinuclear region, near the Golgi system, receiving all enzymatic and structural proteins required for melanogenesis. Stage I melanosomes are spherical vacuoles lacking tyrosinase ⁹⁵ activity (the main enzyme involved in melanogenesis) and have no internal structural components. TYR can be detected in Golgi vesicles, but it has been shown that it is subsequently trafficked to stage II melanosomes (elongated fibrillar organelles – PME117) that contain tyrosinase and exhibit minimal melanin deposition. Thereafter, the melanin synthesis begins, and the pigment is uniformly deposited on the internal fibrils, when the melanosomes are called stage (III). In this last stage of development (IV), these melanosomes are detected in highly pigmented melanocytes (Figure 10). They are elliptical or ellipsoidal, electronically opaque due to complete melanization and have minimal TYR activity. These developmental stages refer mainly to eumelanosomes but are similar to pheomelanosomes, the only difference being that the latter remain round and are not fibrillary during maturation ⁹³. Within melanosomes, at least three enzymes are absolutely required to synthesize different types of melanin: tyrosinase-related protein-1 (TYRP1), tyrosinase-related protein-2 (TYRP2) and DOPAchrome tautomerase (DCT) that are further involved in modifying melanin into different types of pigments: eumelanin (brown-black or dark insoluble polymer), which is found in dark skin and black hair, and pheomelanin (red-yellow soluble polymer), which is connected to the red hair/freckled skin type (Figure 10) ^{86, 96}.

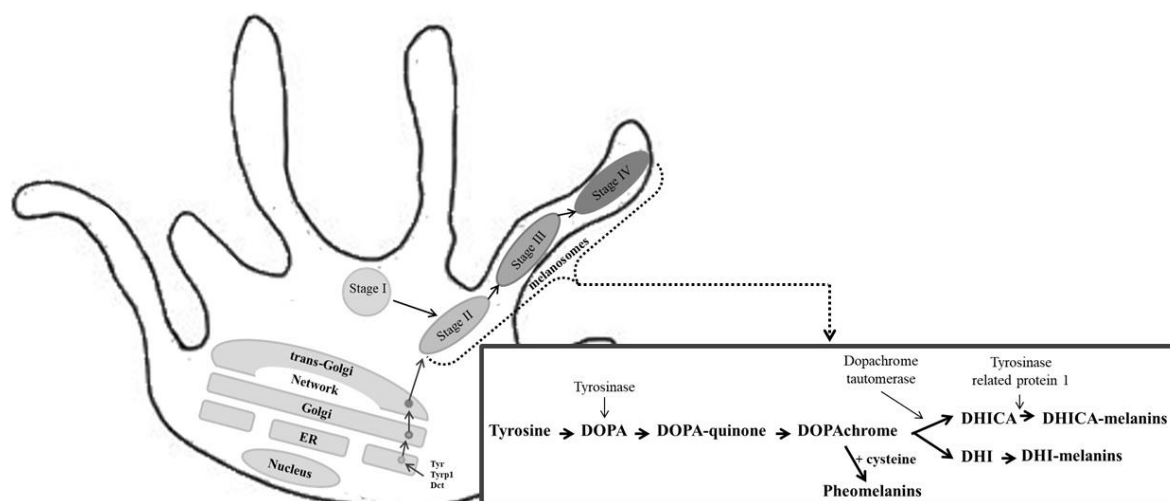


Figure 10 - Schematic presentation of a melanocyte: the engine for melanin production. Melanosomes are divided into four maturation stages (I–IV). Nascent melanosomes (Stage II) are assembled in the perinuclear region, near the Golgi system, receiving all enzymatic (TYR, TYRP1, DCT) and structural proteins required for melanogenesis. The melanin synthesis begins at Stage III and in Stage IV the melanocytes acquire the high amount of pigment. (Adapted from Costin *et al.*, 2007)

Biochemically, the synthesis of melanin is initiated with the oxidation of L-tyrosine by TYR to form DOPAquinone (DQ), which is a substrate for both eumelanin and pheomelanin synthesis (Figure 10). Briefly, after its formation, DQ reacts with equimolar cysteine, if available, to form 3- or 5-cysteiny DOPAs, which then oxidized and polymerize, giving rise to red-yellow soluble pigments, known collectively as pheomelanins. On the other hand, DQ can cyclize to form an orange intermediate DOPACHROME. The spontaneous decarboxylation of DOPACHROME generates 5,6-dihydroxyindole (DHI), which is rapidly oxidized and polymerizes to form dark brown/black, high molecular weight insoluble polymers, known as DHI-melanin⁹⁷. Finally, eumelanin is synthesized through a series of oxidation reactions from DHIs (dihydroxyindole and dihydroxyindole-2-carboxylic acid or DHICA), which are the reaction products of DOPACHROME. In addition to eumelanin and pheomelanin, other “melanin” relying on phenolic monomers different from tyrosine is termed allomelanin⁸⁵. Tyrosinase, when no longer needed, is degraded endogenously by proteasomes⁹⁸.

So, tyrosinase inhibitors have received great attention in the field of cosmetics due to the enhancement of skin pigmentation disorders⁹⁹. There are many *in vitro* tyrosinase inhibitors, but only a few showed positive activities in clinical trials¹⁰⁰⁻¹⁰¹. Moreover, a lot of disorders in melanin biosynthesis pathway, for example the neurotoxicity and neurodegeneration of dopamine, are related to some neurodegenerative diseases such as Alzheimer's (AD), Parkinson's (PD) and Huntington's diseases⁹⁶. AD is often seen in PD

patients, and therefore, the inhibition of TYR as well as cholinesterases is also critical for the treatment of these diseases ¹⁰². Different types of tyrosinase inhibitors have been obtained from marine resources ¹⁰³.

It is well known that enzymatic inhibition mechanisms generally comprise competitive inhibition (a competitive inhibitor prevents binding with substrate, binding it first), non-competitive inhibition (the inhibitor binds the enzyme, inactivating it), mixed inhibition (the inhibitor can bind not only to a free enzyme but also to the enzyme/substrate complex) and anti-competitive inhibition (the inhibitor is capable of binding to the enzyme/substrate complex, but not to the free enzyme). In most studies conducted to discover new tyrosinase inhibitors, a well-known tyrosinase inhibitor such as kojic acid (**31**, Figure 11) is often used as a positive standard ⁸⁵. Kojic acid is the most intensively studied inhibitor of tyrosinase, and is a fungal metabolite currently used as a cosmetic skin-whitening agent and as a food additive for preventing enzymatic browning reaction ¹⁰⁴.

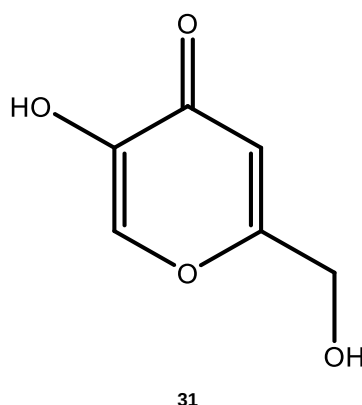


Figure 11 - Chemical structure of kojic acid (**31**)

Anyhow, the designation of "tyrosinase inhibitor" is not always very consensual because some authors use the same terminology to refer inhibitors of melanogenesis whose action mainly resides in some interference in melanin formation without direct tyrosinase-inhibitor interaction. Only specific inactivators and specific inhibitors of tyrosinase are considered "true inhibitors", since they bind to the enzyme and inhibit its activity. The "true inhibitors" of tyrosinase are then divided into two classes: specific tyrosinase inhibitors which bind reversibly to the enzyme and reduce its catalytic capacity ⁸⁵, and specific tyrosinase inactivators, also known as irreversible inhibitors or "suicide substrates" - forming a covalent bond, altering the active site, inactivating the enzyme irreversibly during the catalytic reaction (e.g. L-DOPA and catechol). They are generally specific for tyrosinase and do not inactivate other proteins ¹⁰⁵⁻¹⁰⁶.

Numerous natural compounds from marine organisms have already been employed as tyrosinase inhibitors, although some of them (hydroquinones) presented adverse effects on human health ¹⁰⁷. The demands for new inhibitors leading to those discovered in the last years are classified into five major classes: polyphenols, benzaldehyde and benzoate derivatives, long-chain lipids and steroids, other natural or synthetic inhibitors, and irreversible inactivators, based on either the chemical structures or the inhibitory mechanism ⁸⁵.

For this study we will address the antityrosinase activity of the secondary metabolites isolated from the extract of the culture of the marine sponge-associated *A. persicinum* KUFA1007. 'Till this date there are no reports in the literature on antityrosinase compounds or extracts from the marine sponge-derived fungus *Acremonium persicinum*.

1.5.2 Antiacetylcholinesterase and anti-butyrylcholinesterase activities

Nerve cells communicate with each other through the release of chemicals, called neurotransmitters. Acetylcholine (ACh) is the most abundant neurotransmitter in the body and the primary neurotransmitter in the brain which is responsible for cholinergic transmission ¹⁰⁸. During the neurotransmission, ACh is released from the nerve into the synaptic cleft and binds to ACh receptors (nicotinic and muscarinic) on the post-synaptic membrane, relaying the signal from the nerve. Specifically, acetylcholine transmits information from somatic and autonomic neurons. On the somatic side, the lower motor neurons use ACh to produce excitatory impulses on voluntary muscle ¹⁰⁹. In the autonomic nervous system, ACh is the neurotransmitter in the preganglionic sympathetic and parasympathetic neurons, as well as at the adrenal medulla and serves as the neurotransmitter in all the parasympathetic innervated organs. ACh is also the neurotransmitter at the sweat glands, and at the piloerector muscle of the sympathetic autonomic nervous system. In the peripheral nervous system, ACh is the neurotransmitter at the neuromuscular junction between the motor nerve and skeletal muscle. In the central nervous system, ACh is found primarily in interneurons, and a few important long-axon cholinergic pathways have also been identified ¹¹⁰.

ACh is synthesized by a single step reaction, catalysed by the biosynthetic enzyme choline acetyltransferase (ChAT) ¹⁰⁹, and the presence of this enzyme is a "marker" of cholinergic neurons. Acetylcholinesterase (AChE) is a secretory carboxylesterase present in the central and peripheral nervous systems ¹¹¹. Thus, AChEs are enzymes that

hydrolyse the neurotransmitter acetylcholine to acetate and choline (Figure 12). This enzyme rapidly hydrolyses the neurotransmitter ACh into choline and ultimately terminates the excitatory effects of ACh on the postsynaptic membrane, ensuring the proper transduction of nervous signals and critically affecting the cholinergic nervous system ¹¹². AChE is located at the synaptic cleft and whose functions are terminating synaptic transmission, catalysing the degradation of ACh, allowing cholinergic neurons to return to a resting state upon activation. The liberated choline from the ACh decomposition is taken up again by the pre-synaptic nerve and the neurotransmitter is synthesized by combining with acetyl-CoA through the action of choline acetyltransferase ¹¹³⁻¹¹⁴ (Figure 12). Drugs that inhibit or inactivate the AChE enzyme or Antiacetylcholinesterase (AntiAChE) agents cause ACh to accumulate at cholinceptive sites, producing a continuous stimulation effect ¹¹⁵.

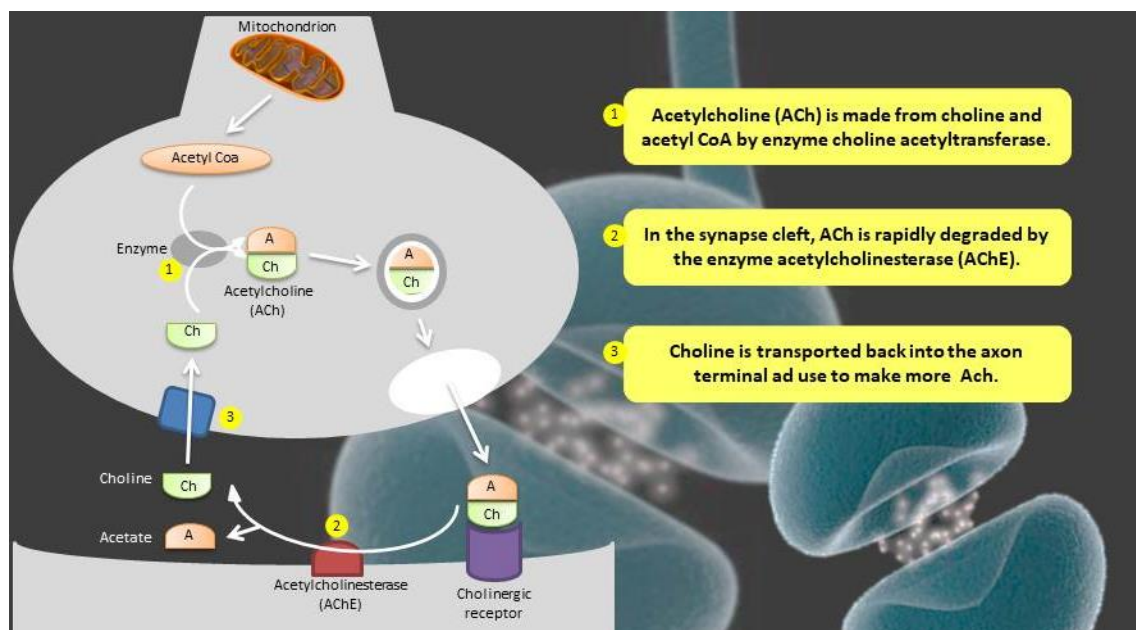


Figure 12 - Acetylcholinesterase quickly hydrolyses the neurotransmitter acetylcholine to choline by avoiding the constant excitatory effects of acetylcholine on the postsynaptic membrane, ensuring proper transduction of nerve signals. (Adapted from Moghul and Wilkinson, 2001)

Abnormal cholinergic activity is characterized by a short-term deficit or decrease in cholinergic transmissions at synapses, and has been attributed to the reduction of ACh production or AChE deactivation/hydrolysis ¹¹⁶⁻¹¹⁷. The etiologies of cognitive problems highlight problems with signal transduction through synapses, so the regulation of AChE activities has become an important focus of research ¹¹⁸⁻¹¹⁹. Actually, AChE tends to become deposited within the neurofibrillary tangles and amyloid plaques associated with AD ¹²⁰.

AChE is an esterase member of the superfamily of proteins termed the α,β -hydrolase-fold family. These superfamilies of proteins have multiple catalytic functions involving H_2O addition, as well as having a recognition role in adhesion with other partnering proteins. Although other non-catalytic and catalytic functions of AChE have been proposed, the only established physiologic function of this enzyme is catalysis of the hydrolysis of Ach in localized synaptic areas in the central and peripheral nervous systems ¹²¹. In addition to its role in transducing nervous signals, AChE is also involved in the other cellular processes, such as cell differentiation, apoptosis, and cell adhesion ¹²².

There are two AChE isoenzymes in blood: AChE_T and AChE_H. AChE_T is found in many types of conductive tissue such as nerves and muscle, central and peripheral tissues, motor and sensory fibers, and cholinergic and non-cholinergic fibers. The activity of AChE is higher in motor neurons than in sensory neurons ¹²³⁻¹²⁴. AChE_H is found in the red blood cell membranes, where it constitutes the Yt blood group antigen (also known as antigen system Cartwright). The enzyme exists in multiple molecular forms, which possess similar catalytic properties but differ in their oligomeric assembly and mode of attachment to the cell surface. In the mammalian central nervous system (CNS), the majority of AChE occurs as globular monomers G1 or in its globular tetrameric G4 form. In peripheral nervous system (PNS), AChE is found in skeletal muscle as A12 (asymmetric form), and in erythrocytes as globular dimer or G2 form ¹²⁵. Cholinesterase inhibitors represent the most promising therapeutic agents for Alzheimer's type dementia patients, as shown by the clinical studies on the effects of these drugs on cognition (memory and concentration) and behavioral symptoms (apathy and motor agitation) ¹²⁶.

There is another (pseudo)cholinesterase known as butyrylcholinesterase (BChE) or plasma cholinesterase. It is produced in the liver and found in plasma and tissues ¹²⁷⁻¹²⁸. BChE (EC 3.1.1.8) is a nonspecific cholinesterase enzyme that hydrolyses many different choline-based esters ¹²⁹. BChE is an activating enzyme that converts prodrugs into its active forms. It is also an endogenous eliminator of anticholinesterase (antiChE) compounds such as organophosphates. BChE may be a physiological ghrelin regulator ¹³⁰.

Although the precise role of BChE in the pathogenesis of most dementia disorders have not been unequivocally delineated, it has, however, been observed that BChE increases in the brain of AD patients and may be the first line of defence against toxic compounds that inhibit AChE. It has been suggested that inhibition of both cholinesterase enzymes may be important in the management of cognitive deficits associated with the AD ¹³¹⁻¹³². Butyrylcholine (BCh) has been found to be present in significantly greater amounts in Alzheimer's plaques than in age-related normal dementia of the brains. Thus, the search

for cholinesterase inhibitors is considered an important, and an ongoing strategy to introduce new candidates for the treatment of AD and other related diseases ¹³³.

AChE or BChE activity could be selectively reduced by exposing them to poisonous chemical agents such as insecticides such as organophosphates or carbamates, anaesthetics, and a variety of therapeutic drugs, including donepezil or rivastigmine which are used for treating AD. AChE is over activated in the synapses of patients with AD, so levels of ACh in their brains is significantly diminished, leading to memory loss and other adverse effects ¹³⁴. AChE inhibitors are also used to treat certain conditions, such as dementia ¹³⁵. Inhibition of AChE leads to accumulation of acetylcholine at smooth muscle M3 muscarinic receptors and M1/M3 muscarinic receptors in salivary glands, stomach, exocrine pancreas, and intestine to produce hypermotility and hypersecretion, respectively ¹³⁶. Drugs such as neostigmine, edrophonium, and pyridostigmine, organophosphate insecticides that include parathion, malathion, and paraoxon, and toxic nerve gases including tabun and sarin, all are capable of causing severe diarrhoea and death as a result of reversible or irreversible blockage of AChE ¹³⁷. This non-selectivity of some antiChE, with the dual ability to inhibit AChE and BChE are implicated in several side effects. However, it is speculated that this duality could lead to better clinical outcomes, particularly with regard to the treatment of AD ¹³¹. Thus, the development of selective AChE inhibitors has been the major challenge for pharmaceutical chemists to date. AChE is very specific in its recognition and substrate requirement whereas BChE recognizes a wide range of substrates. For this reason, it has been suggested that circulating plasma BChE could possibly serve as an elimination function, thus protecting AChE from naturally occurring inhibitors. In this regard, BChE may also eliminate anticholinesterase drugs, thereby raising the dose required to achieve reasonable inhibitions in the target region of the brain for effective therapeutic relief ¹³⁸.

AChE inhibitors can be divided into two groups: irreversible and reversible. Reversible inhibitors, competitive or non-competitive, mostly have therapeutic applications, while toxic effects are associated with irreversible AChE activity modulators. Strong inhibitors are primarily used as neural toxins and pesticides, while weak inhibitors have been used in the treatments of AD, dementia with Lewy bodies, PD, myasthenia gravis, glaucoma, postural tachycardia syndrome, autism, insomnia, and cognitive disorder ¹³⁵. Reversible AChE Inhibitors include compounds with different functional groups (carbamate, quaternary or tertiary ammonium group) ¹³⁵. Irreversible AChE Inhibitors are organophosphorus compounds or organophosphates (OPs), esters or thiols derived from phosphoric, phosphonic, phosphinic or phosphoramidic acid. OPs are substrate analogues to ACh and, like natural substrate, enter the active site binding covalently ¹³⁹.

The inhibition of the enzyme leads to accumulation of ACh in the synaptic cleft, resulting in over-stimulation of the receptors obstructing neurotransmission. The typical symptoms of acute poisoning are agitation, muscle weakness, muscle fasciculations, miosis, hypersalivation, sweating. Severe poisonings may cause respiratory failure, unconsciousness, confusion, convulsions and/or death^{109, 140}. Irreversible inhibition occurs in two steps; the first one is fast, short-term reversible enzyme inactivation, and its influence is dominant in the beginning of the inhibition. The next step is slow irreversible inhibition, producing a very stable enzyme-inhibitor complex (phosphorylated enzyme)-inhibitor which is covalently bonded to the enzyme¹³⁵. OPs can produce delayed neurotoxic effect in humans, called OP-induced delayed neuropathy. It is associated with phosphorylation and further dealkylation (aging) of a protein in neurons, called neuropathy target esterase, subsequently leading to this syndrome. The symptoms of this neuropathy are paralysis and ataxia, and appear in 14-24 days after the poisoning¹⁴¹⁻¹⁴². OP poisoning can be treated non-pharmacologically and pharmacologically¹⁴³. The non-pharmacologic treatment includes resuscitation, oxygen supply or decontamination, depending on the OP entrance to the human body (e.g. skin, eye, gastric decontamination), whereas the pharmacologic treatment is based on the administration of symptomatic and causal drugs¹⁴⁴. Parasympatolytics, which reduce the effects of the accumulated ACh on the cholinergic receptors¹⁴⁵, and anticonvulsives which diminish neuromuscular seizures¹⁴⁶, are used as the symptomatic treatment.

The mechanisms of action of the compounds that typify the classes of antiChEs agents differ mainly in quantitative aspects of the reaction between AChE and its normal substrate, ACh. Stages involved are the hydrolysis of ACh by AChE (I) and in the inhibition of AChE by reversible agents (II), carbamyl ester (III) and organophosphates (IV)¹¹⁵.

Physostigmine and neostigmine are among the principal antiChEs. These drugs have only a few clinical uses, mainly in augmenting gastric and intestinal contractions (in treatment of obstructions of the digestive tract) and in augmenting muscular contractions in general (in the treatment of myasthenia gravis). AntiAChE drugs that are used more widely in the clinic are those that inhibit AChE in the brain. The most useful application of such agents is in the treatment of AD, in which reduced transmission of ACh contributes to the neuropathology of the disease. When the breakdown of ACh is inhibited, levels of the neurotransmitter can return to near normal and the degeneration of neurons, and hence the degeneration of cognitive ability, is slowed down. Agents that have been developed for this purpose include donepezil, tacrine, and galantamine¹⁴⁷. However, the potentially dangerous side effects of these drugs have limited their use. For example, liver toxicity

caused by tacrine has restricted its availability by prescription. In addition, although donepezil, which is marketed as Aricept®, was found to marginally benefit some persons with early-onset AD, its use has been primarily limited to individuals with late-stage disease, for whom the benefits outweigh the risks of side effects ¹⁴⁸. Commercially available synthetic acetylcholinesterase inhibitors (AChEIs) include donepezil (Aricept®), rivastigmine (Exelon®), galantamine (Razadyne®), and tacrine (Cognex®) which influence the dynamics of ACh by inhibiting the activity of AChE, thus increasing the availability and interaction time of ACh with cholinergic receptors of synaptic cells ¹⁴⁸.

AChEIs are an attractive research subject owing to their potential applications in the treatment of neurodegenerative diseases. Fungi and bacteria are major producers of AChEIs ^{111, 147}. Considering the disadvantages of synthetic AChEIs, which include gastrointestinal disturbances, moderate effectiveness, high cost and short half-life ¹⁴⁹⁻¹⁵⁰, natural product-based compounds have been increasingly explored for better effects ¹⁵¹⁻¹⁵². Desirable properties of botanical extracts or natural product-based compounds include a comparatively better penetration of the blood–brain barrier than the pharmaceutical options and better specificity for human type AChE (G4) ¹⁵⁰. Thus, the chemical inhibition of AChE is a potent strategy for addressing signal-related neuropathology and natural products are potential sources of compounds with such properties ¹⁵³.

Wang *et al.* ¹⁵⁴, and more recently Su *et al.* ¹⁵⁵, have reported AChEIs from fungi, including *Acremonium* species. Huperzine A (**32**, Figure 13) is the most studied AChEI alkaloid. This compound possesses a multitude of pharmacological effects, being highly selective and efficient as an AChEI in the central nervous system. Huperzine A (**32**) can also provide protection for cells by inhibiting excitotoxicity induced by glutamate and blocking oxidative stress and apoptosis. Additionally, huperzine A (**32**) may exert therapeutic effects in a variety of degenerative diseases ¹⁵⁴⁻¹⁵⁵.

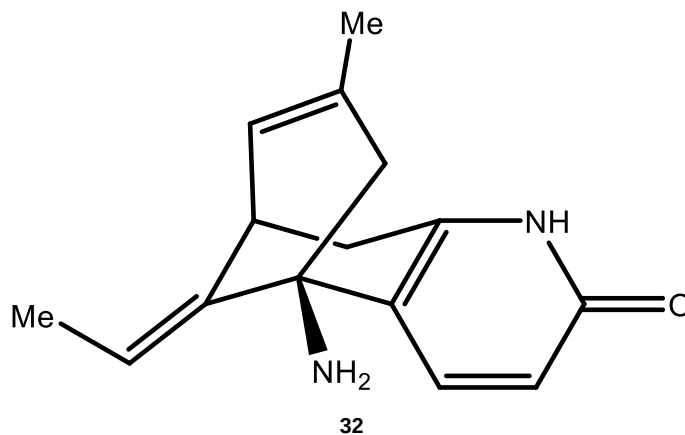


Figure 13 - Chemical structure of huperzine A (**32**)

Chapter 2 – Objectives

The main objective of this dissertation consists of two parts:

- I. Isolation and structure elucidation of the secondary metabolites from the EtOAc extract from the cultures of the fungus *Acremonium persicinum* KUFA 1007, isolated from the marine sponge *Mycale* sp., collected from the Coast of Samaesan Island in the Gulf of Thailand.
- II. Evaluation of the antityrosinase, antiacetylcholinesterase and anti-butyrylcholinesterase activities of the isolated compounds.

Chapter 3 – Materials and Methods

3.1 Materials and Solvents

3.1.1 Material and solvents used in the isolation and purification of secondary metabolites from the Fungal Crude Extracts

- ✓ Compressed air
- ✓ Nitrogen air
- ✓ 100, 250, 500, 1000 mL Erlenmeyer
- ✓ 10, 100 e 500mL beakers
- ✓ 100, 250 e 1000mL Round bottom balloon
- ✓ 2000mL separating funnel
- ✓ Heating bath B-491, rotavapor R-210, vacuum module V-801 EasyVac and Vacuum pump V-700 (Büchi)
- ✓ UV detector, 254 nm and 365 nm (Vilber Lourmat)
- ✓ Paper filtrate No. 1 (Whatman)
- ✓ Microcapillary pipettes, calibrated, sized 10 μ L (Marienfeld)
- ✓ TLC aluminium sheets 20x20 cm silica gel 60 GF₂₅₄ (Macherey-Nagel)
- ✓ Silica gel 60 GF₂₅₄ (0.063-0.2 mm), Merck for column chromatography
- ✓ Silica gel 60 GF₂₅₄, Merck for thin layer chromatography
- ✓ Silica gel 60 (40-63 μ m) for flash chromatography, Fluka Analytical
- ✓ Sephadex LH-20 for column chromatography
- ✓ Column chromatography (NS 45/40, NS 29/32 and NS 14/23)
- ✓ 20x20 cm glass plates
- ✓ Chamber for TLC
- ✓ Iodine chamber
- ✓ Distilled water
- ✓ Iodine (I₂), (BDH Prolabo Chemicals)
- ✓ Petroleum ether (Petrol) 40-60 °C, Analytical reagent grade (Fisher Chemical)
- ✓ Chloroform (CHCl₃), Analytical reagent grade (Fisher Chemical)
- ✓ Acetone (Me₂CO), Analytical reagent grade (Fisher Chemical)
- ✓ Methanol (MeOH), Analytical reagent grade (Fisher Chemical)
- ✓ Ethyl acetate (EtOAc), Analytical reagent grade (Fisher Chemical)

- ✓ Formic acid (HCO₂H), 89.91%, (Acros Organics)
- ✓ Dichloromethane (CH₂Cl₂), Analytical reagent grade (Fisher Chemical)
- ✓ Anhydrous sodium sulphate (Na₂SO₄) (PanReac)

3.1.2 Material and solvents used in the structure elucidation of the isolated secondary metabolites

- ✓ ADP410 Polarimeter
- ✓ Stuart Scientific Melting Point Apparatus SMP3
- ✓ NMR spectrometer (Bruker AMC instrument)
- ✓ HRM spectrometer (Waters Xevo QToF coupled to a Waters Aquity UPLC system)
- ✓ Circular dichroism spectropolarimeter (Jasco J-815 CD)
- ✓ Deuterated dimethyl sulfoxide (DMSO-*d*₆)
- ✓ Deuterated chloroform (CDCl₃)

3.1.3 Material and solutions used in biologic activity assay

- ✓ Tris(hydroxymethyl)aminomethane (TCI)
- ✓ Water, LC-MS Grade (Fisher Chemical)
- ✓ Albumin, from bovine serum (Sigma-Aldrich)
- ✓ Galantamine hydrobromide, from *Lycoris* sp. (Sigma-Aldrich)
- ✓ Acetylthiocholine iodide, minimum 98% TLC (Sigma-Aldrich)
- ✓ Acetylcholinesterase (EC 3.1.1.7), from *electrophorus electricus* (Sigma-Aldrich)
- ✓ Butyrylcholinesterase (EC 3.1.1.8), from lyophilized horse serum (Merck)
- ✓ Di-Sodium hydrogen phosphate dodecahydrate (Merck)
- ✓ Sodium dihydrogen phosphate (VWR)
- ✓ Kojic acid (Sigma-Aldrich)
- ✓ Quercetin dihydrate (Sigma-Aldrich)
- ✓ 3,4-Dihydroxy-L-phenylalanine (Sigma-Aldrich)
- ✓ Tyrosinase (EC 1.14.18.1), from lyophilized mushroom (Sigma-Aldrich)

Tris buffer (50 mM, pH 8.0) - Weigh 3.028 g of Tris(hydroxymethyl)aminomethane and dissolve in 500mL of distilled water. Adjust the solution to the desired pH 8.0 using HCl or NaOH. Store at 5 °C.

AChE and BChE stock solution - The original lyophilizate was dissolved in the tris buffer (pH 8) and this stock solution was kept at -20 °C. A suitable amount of the stock solution was defrosted each working day and AChE/BChE activity was measured.

DTNB and acetylthiocholine iodide stock solutions - Appropriate amounts of the substances were dissolved in phosphate buffer, pH 8 and these solutions were kept at 5 °C not longer than 2-3 days. The suitable working concentrations of DTNB and acetylthiocholine iodide were prepared immediately before use by dilution with buffer solution.

Galantamine stock solution (10 mM) - Weigh 1.84 mg galantamine and dissolve in 500 µL MeOH or DMSO. Store at 5 °C. Before using, prepare solutions of 600, 300, 150, 75 and 37.5 µM galantamine in MeOH/DMSO.

Sample stock solution (200µM) - To evaluate the inhibition rate of AChE/BChE different concentrations of the test sample were prepared in MeOH or DMSO.

Phosphate buffer (20 mM, pH 6.8) - Weigh 1.312 g of disodium hydrogenphosphate dodecahydrate and 0.704 g of sodium di-hydrogenphosphate and dissolve in 500 ml of distilled water. Adjust the solution to the desired final pH 6.8 using HCl or NaOH. Equilibrate to room temperature before use. Store at 5 °C.

L-DOPA and tyrosinase stock solution - The original lyophilizate was dissolved in phosphate buffer (pH 6.8). The same was true for L-DOPA. Store at 5 °C.

Kojic acid stock solution (10 mM) - Weigh 0.71 mg kojic acid and dissolve in 500 µL DMSO. Store at 5 °C. Before using, prepare solutions of 600, 300, 150, 75 and 37.5 µM kojic acid in DMSO.

Sample stock solution (600µM) - To evaluate the inhibition rate of tyrosinase, different concentrations of the test sample were prepared in DMSO.

Statistical analyses for AChE/BChE inhibitory assays - Data were reported as means $\pm$ standard error of the mean (SEM) of at least three independent experiments. Statistical analysis of the results was performed with GraphPad Prism (GraphPad Software, San Diego, CA). Unpaired T-tests were carried out to test for any significant differences between the means. Differences at the 5% confidence level were considered significant. The half maximal inhibitory concentration (IC₅₀) values were obtained by linear regression.

Statistical Analysis for TYR inhibitory assay - Data were reported as means $\pm$ standard error of the mean (SEM) of at least three independent experiments. Statistical analysis of the results was performed with GraphPad Prism (GraphPad Software, San Diego, CA). The half maximal inhibitory concentration (IC₅₀) values were obtained by non-linear regression. Activities have been compared using a one-way analysis of variance

(ANOVA). Statistical differences were considered as follows: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

3.2 Methodology

3.2.1 General experimental procedures

- A Merck (Darmstadt, Germany) silica gel GF₂₅₄ was used for preparative TLC, and a Merck Silica gel 60 (0.2–0.5 mm) was used for column chromatography.
- ¹H and ¹³C NMR spectra were recorded at ambient temperature on a Bruker AMC instrument (Bruker Biosciences Corporation, Billerica, MA, USA) operating at 300 or 500 and 75 or 125 MHz, respectively.
- The solvents were evaporated under reduced pressure, using Büchi heating bath B-491, rotavapor R-210, vacuum module V-801 EasyVac and vacuum pump V-700.
- High resolution mass spectra were measured with a Waters Xevo QToF mass spectrometer (Waters Corporations, Milford, MA, USA) coupled to a Waters Aquity UPLC system.
- Melting points were determined on a Stuart Scientific Melting Point Apparatus SMP3 (Bibby Sterilin, Stone, Staffordshire, UK) and are uncorrected.
- Optical rotations were measured on a ADP410 Polarimeter (Bellingham + Stanley Ltd., Tunbridge Wells, Kent, UK).
- Electronic circular dichroism spectrum was measured with a Jasco J-815 CD spectropolarimeter. Conformational search, including dihedral driver and molecular mechanics MM2 minimizations, was performed in Chem3D Ultra (Perkin-Elmer Inc.).
- Biological activities were tested using BioTek SynergyTM HT Microplate Reader (Winooski, Vermont, USA).



Figure 14 – Equipments used through the stage: **A** - Bruker AMC instrument; **B** - Büchi heating bath B-491, rotavapor R-210, vacuum module V-801 EasyVac and vacuum pump V-700; **C** - Waters Xevo QToF mass spectrometer; **D** - Waters Aquity UPLC system; **E** - ADP410 Polarimeter; **F** - Jasco J-815 CD spectropolarimeter and **G** - BioTek SynergyTM HT Microplate Reader, **H** - Stuart Scientific Melting Point Apparatus SMP3.

3.2.2 Fungal material

The strain KUFA 1007 was isolated from the marine sponge *Mycale* sp., which was collected by scuba diving at a depth of 5-10 m, from the coral reef at Samaesan Island (12°34'36.64"N 100°56'59.69"E), in the Gulf of Thailand, in September 2016. The sponge was washed with 0.01% sodium hypochlorite solution for 1 min, followed by sterilized seawater three times, and then dried on a sterile filter paper under sterile aseptic conditions. The sponge was cut into small pieces (5×5 mm) and placed on Petri dish plates containing 15 mL potato dextrose agar (PDA) medium mixed with 300 mg/L of streptomycin sulphate, and incubated at 28 °C for 7 days. The hyphal tips emerging from sponge pieces were individually transferred onto PDA slant and maintained as pure cultures at Kasetsart University Fungal Collection, Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand, for further identification.

The fungal strain KUFA 1007 was identified as *Acremonium persicinum*, based on morphological characteristics such as colony growth rate and growth pattern on standard media, namely Czapek's agar, Czapek yeast autolysate agar, and malt extract agar. Microscopic characteristics including size, shape, ornamentation of conidiophores and spores were examined under light and scanning electron microscopes. This identification was confirmed by molecular techniques using Internal Transcribed Spacer (ITS) primers. DNA was extracted from young mycelia following a modified Murray and Thompson method ¹⁵⁶. Primer pairs ITS1 and ITS4 ¹⁵⁷ were used for ITS gene amplification. Polymerase chain reactions (PCR) were conducted on Thermal Cycler and the amplification process consisted of initial denaturation at 95 °C for 5 min, 34 cycles at 95 °C for 1 min (denaturation), at 55 °C for 1 min (annealing) and at 72 °C for 1.5 min (extension), followed by final extension at 72 °C for 10 min. The PCR products were examined by agarose gel electrophoresis (1% agarose with 1×TBE buffer) and visualized under UV light after staining with ethidium bromide. DNA sequencing analyses were performed using dideoxyribonucleotide chain termination method ¹⁵⁸ by Macrogen Inc. (Seoul, Korea). The DNA sequences were edited using the FinchTV software (version 1.4, Geospiza Inc., Seattle, WA, USA) and submitted into the BLAST program for alignment and compared to fungal species in the NCBI database (<http://www.ncbi.nlm.nih.gov/>). Its gene sequences were deposited in GenBank with accession number MG755248.

3.2.3 Extraction of secondary metabolites

The fungus was cultured for one week at 28 °C in five Petri dishes (i.d. 90 mm) containing 20 mL of potato dextrose agar (PDA) per dish. The mycelial plugs (5 mm in diameter) were transferred to two 500 mL Erlenmeyer flasks containing 200 mL of potato dextrose broth, and incubated on a rotary shaker at 120 rpm at 28 °C for one week. Fifty 1000 mL Erlenmeyer flasks, each containing 300 g of cooked rice, were autoclaved at 121 °C for 15 min. After cooling to room temperature, 20 mL of a mycelial suspension of the fungus was inoculated per flask and incubated at 28 °C for 30 days, after which 500 mL of EtOAc was added to each flask of the moldy rice and macerated for 7 days, and then filtered with Whatman No. 1 filter paper (GE Healthcare UK Limited, Buckinghamshire, UK). The EtOAc solution were combined and concentrated under reduced pressure to yield 59.8 g of crude EtOAc extract which was dissolved in 500 mL of EtOAc and then filtered with Whatman No. 1 filter paper. The EtOAc solution was then washed with H₂O (3×500 mL)

and dried with anhydrous Na_2SO_4 , filtered and evaporated under reduced pressure, to give 46.3 g of crude EtOAc extract.

3.2.4 Isolation and purification of the secondary metabolites

The crude EtOAc extract was applied on a column chromatography of silica gel (390 g) and eluted with mixtures of petrol: CHCl_3 , CHCl_3 : Me_2CO , Me_2CO and MeOH, wherein 250 mL fractions were collected as follow: Fractions (Frs) 1-23 (petrol: CHCl_3 , 1:1), 24-49 (petrol: CHCl_3 , 3:7), 50-177 (petrol: CHCl_3 , 1:9), 178-207 (CHCl_3), 208-453 (CHCl_3 : Me_2CO , 9:1), 454-546 (CHCl_3 : Me_2CO , 7:3), 547-596 (CHCl_3 : Me_2CO , 1:1) and 597 (MeOH). The fractions were monitored by TLC and the fractions with the same or similar TLC profiles were combined for further purification.

- i. Frs 54-75 were combined (296.5 mg) and recrystallized in MeOH to give **I** (125.3 mg).
- ii. Frs 213 (264.3 mg) was crystallized in MeOH to give **II** (47.8 mg).
- iii. Frs 214-285 were combined (2.10 g) and applied on a column chromatography of silica gel (40 g) and eluted with mixtures of petrol, CHCl_3 , Me_2CO and MeOH, wherein 100 mL sub-fractions (sfrs) were collected as follows: sfrs 1-52 (petrol: CHCl_3 , 3:7), 53-74 (petrol: CHCl_3 , 1:9), 75-158 (CHCl_3), 159-218 (CHCl_3 : Me_2CO , 19:1), 219-254 (CHCl_3 : Me_2CO , 9:1), 255-306 (CHCl_3 : Me_2CO , 8:2), 307-325 (CHCl_3 : Me_2CO , 7:3), 326-332 (CHCl_3 : Me_2CO , 5:5), 333-334 (Me_2CO) and 335 (MeOH). Sfrs 122-160 were combined (314.4 mg) and recrystallized in MeOH to give **II** (60.2 mg).
- iv. Frs 246-255 were combined (174.7 mg) and purified by TLC (silica gel G_{254} , CHCl_3 : Me_2CO : HCO_2H , 8:2:0.1) to give 10.4 mg of **IV** (with impurities), which was further applied on a Sephadex LH-20 column (5 g) and eluted with MeOH, wherein 67 sfrs of 1 mL were collected. Sfrs 40-50 were combined and, after evaporation of solvent, gave 2.4 mg of **IV** as a pure compound.
- v. The other part of the TLC separation of frs 246-255 was combined with frs 256-285 (318.1 mg) and applied on a column chromatography of silica gel (30 g), and eluted with a mixture of petrol, CHCl_3 , Me_2CO and MeOH, wherein 100 mL fractions were collected as follows: Sfrs 1-63 (petrol: CHCl_3 , 3:7), 64-127 (CHCl_3 : Me_2CO , 9:1), 128-161 (CHCl_3 : Me_2CO , 7:3), 162-169 (Me_2CO) and 170 (MeOH). Sfrs 71-75 were combined (11.9 mg) and purified by TLC (silica Gel G_{254} , CHCl_3 : Me_2CO : HCO_2H , 8:2:0.1) to give 30.9 mg of a compound that was applied

on Sephadex LH-20 column (5 g), and eluted with a 1:1 mixture of CH_2Cl_2 :MeOH, wherein 62 sfrs of 1 mL were collected. By using TLC to monitor the profile of the eluted compounds, these sub-fractions were divided in two groups: Group I (sfrs 8-19) and Group II (sfrs 19-30). Sfrs 8-19 (group I) were combined (19.7 mg), and applied on a Sephadex LH-20 column (5 g), and eluted with a 1:1 mixture of CH_2Cl_2 : MeOH, wherein 40 sub-sfrs (ssfrs) of 1 mL were collected. Ssfrs 15-36 were combined and, after evaporation of solvent, gave 15.0 mg of **III**. Sfrs 19-30 (group II) were combined (5.1 mg), and applied on a Sephadex LH-20 column (5 g), and eluted with MeOH, wherein 20 ssfrs of 1 mL were collected. Ssfrs 5-15 were combined and, after evaporation of solvent, gave 2.3 mg of **IV**.

- vi. Frs 286-315 were combined (180.3 mg) and crystallized in Me_2CO to give 6.0 mg of **V**. The mother liquid was combined with frs 316-365 (291.9 mg) and applied on a column chromatography of Silica Gel (10 g), and eluted with mixtures of petrol, CHCl_3 , Me_2CO and MeOH, wherein 100 mL sfrs were collected as follows: sfrs 1-22 (petrol: CHCl_3 , 5:5), 23-41 (petrol: CHCl_3 , 3:7), 42-53 (petrol: CHCl_3 , 1:9), 54-73 (CHCl_3), 74-162 (CHCl_3 : Me_2CO , 19:1), 163-178 (CHCl_3 : Me_2CO , 9:1), 195-201 (CHCl_3 : Me_2CO , 7:3), 202-210 (CHCl_3 : Me_2CO , 1:1), 211-214 (Me_2CO) and 215-216 (MeOH). Sfrs 79-90 were combined (98.8 mg) and recrystallized in MeOH to give an additional 20.1 mg of **V**.

3.2.5 Electronic circular dichroism experimental

The Electronic circular dichroism (ECD) spectrum of **III** (7 mM in MeOH) was obtained in a Jasco J-815 CD spectropolarimeter (Jasco, Mary's Court, Easton, MD, USA) with a 0.1 mm cuvette and eight accumulations. Conformational search, including dihedral driver and molecular mechanics MM2 minimizations, was done in Chem3D Ultra (Perkin-Elmer Inc., Waltham, MA, USA). Quantum chemical density functional theory (DFT) minimizations were performed with Gaussian 16W (Gaussian Inc., Wallingford, CT, USA) using the APFD method with a 6-31G basis set, for a pre-selection of the most stable conformations, and a 6-311+G(2d,p) basis set for final minimizations and ECD spectral calculations Time-dependent density functional theory (TDDFT, 50 transitions)¹⁵⁹. All DFT calculations included an Integral Equation Formalism for Polarizable Continuum Model (IEFPCM) solvation model of methanol. The spectrum of each relevant configuration/conformation was constructed by applying a Gaussian line broadening of 0.3 eV to each computed transition; no UV-shift was applied.

The final theoretical spectrum of each possible configuration of **III** was obtained by a Boltzmann-weighted sum of the ECD spectra of its most abundant conformations, accounting for at least 90% of the theoretical conformer population ¹⁶⁰.

3.2.6 Antityrosinase assay

Today, many researchers are focusing on the identification, isolation, synthesis and characterisation of new and potent tyrosinase inhibitors for various applications in food, cosmetics and pharmaceutical industries ¹⁶¹. Natural sources, including plants, bacteria and fungi, are becoming of interest for their capacity of producing a variability of bioactive compounds. Several researchers prefer to identify inhibitors of natural sources due to their lower toxicity and better bioavailability, especially for food, cosmetics and biomedical applications ¹⁶². Tyrosinase (EC 1.14.18.1) is a crucial enzyme in melanin biosynthesis in mammals, bacteria, plants, and fungi. It is a bi-functional copper containing monooxygenase enzyme and catalyses hydroxylation of monophenol to *o*-diphenol and oxidation of *o*-diphenol to *o*-quinones, which generates melanin ¹⁶³. Furthermore, tyrosinase is a key rate-limiting enzyme that can catalyse enzymatic browning and melanin synthesis ¹⁶⁴. Therefore, controlling the activity of enzyme by tyrosinase inhibitors is an essential endeavour for treating mammalian hypopigmentary disorders as well as the enzymatic browning of fruits and fungi. To date, numerous effective inhibitors are identified and developed for using in the medicinal and cosmetic products, as well as food bioprocessing and agricultural and environmental industries. However, in medicine, tyrosinase inhibitors are a class of important clinical antimelanoma drugs, however, only a few compounds are known to serve as effective and safe tyrosinase inhibitors ⁸⁵.

In man, potent tyrosinase inhibitors, such as kojic acid and related compounds, have been used as whitening agents in cosmetic products, due to their ability to suppress melanin production ¹⁶⁵. Kojic acid has been reported as skin-whitening agent in the cosmetic industry but some reports failed to confirm their effect as an agent to lighten skin in clinical trials ¹⁶⁶. Also the hydroquinone and arbutin are known tyrosinase inhibitors but they have serious side effects such as permanent depigmentation, erythema, and contact dermatitis, etc. ¹⁶⁷. According to an article published in 2017 by Quispe *et al.* ¹⁶⁸, quercetin also showed strong inhibition of tyrosinase with an IC_{50} of $14.29 \pm 0.3 \mu M$, slightly lower than kojic acid (IC_{50} $8.0 \pm 0.5 \mu M$). Thus, this polyphenolic flavonoid compound has gained prominence in the field of cosmetics due to its anti-melanogenic abilities. It was reported that quercetin binds to the active site of tyrosinase through hydrophobic interaction,

promoting a conformational change and preventing the entry of the substrate, inhibiting its catalytic activity. Known for its antityrosinase activity, quercetin has potential in the treatment of pigmentation disorders ¹⁶⁹⁻¹⁷⁰.

Compounds with strong antioxidant and radical scavenging activities could inhibit tyrosinase ¹⁶⁴. Many potent tyrosinase inhibitors, already described in the literature, bear the same phenyl group as L-tyrosine and L-dopa which are natural substrates for tyrosinase ¹⁷¹⁻¹⁷². High affinity of such compounds to the enzyme is the result of the *van der Waals* interactions of the phenyl group with the residues present in the hydrophobic tyrosinase cavity ¹⁷³. Diverse tyrosinase inhibitors isolated from plant sources and fungi are mostly phenolic compounds, steroids, and alkaloids which are structurally similar to each other ¹⁷⁴.

In fungi, tyrosinases are associated with melanin production, which constitutes a mechanism of defence to stress such as UV radiation, free radicals, gamma rays, dehydration and extreme temperatures and contribute to the fungal cell-wall resistance against hydrolytic enzymes, avoiding cellular lysis ¹⁷⁵. The discovery of novel tyrosinase inhibitors for the development of anti-melanogenic drug has received great attention in the field of cosmeceutical. For anti-melanogenic drugs screening, various methods have been established from *in vitro* to *in vivo* assays ¹⁷⁶.

The tyrosinase inhibitory activity was determined by a colorimetric method. In our study we used the modified microplate assay, previously described by Likhitwitayawuid and Sritularak, to measure the tyrosinase inhibitory activity of the isolated compounds in assorted concentrations ¹⁶⁵. Briefly, the assays were conducted in a 96-well microtiter plate, and a BioTek Synergy™ HT Microplate Reader (Winooski, Vermont USA) was used to measure the absorbance at 475 nm. For each concentration of the sample solution, four wells designated as A, B, C, and D, each containing a reaction mixture (190 µL) as follows: (A) 20 µL of mushroom tyrosinase in 20 mM phosphate buffer (480 U/mL), 140 µL of 20 mM phosphate buffer (pH 6.8), and 10 µL of methanol; (B) 160 µL of 20 mM phosphate buffer (pH 6.8) and 10 µL of methanol; (C) 20 µL of mushroom tyrosinase solution (480 U/ml), 140 µL of 20 mM phosphate buffer (pH 6.8), and 10 µL of sample solution; (D) 160 µL of 20 mM phosphate buffer (pH 6.8) and 10 µL of sample solution. Each well was mixed and incubated at 25 °C for 10 min. Then, 20 µL of 0.85 mM L-DOPA in phosphate buffer (pH 6.8) was added incubated at 25 °C and read the amount of DOPACHrome in each reaction mixture for 9 min at 1.30 min. intervals. The percentage inhibition of tyrosinase activity was calculated using the equation:

$$\text{percentage of inhibition} = 100 \left[\frac{(A - B) - (C - D)}{(A - B)} \right]$$

Where A-represents the difference of optical density before and after incubation without test sample; B-the difference of optical density before and after incubation without test sample and without enzyme; C-the difference of optical density before and after incubation with test sample; D-the difference of optical density before and after incubation with test sample, but without enzyme. Kojic acid and quercetin were used as positive controls.

3.2.7 Anticholinesterase assay

As the clinical demand for AChEIs continues to increase, *in vitro* assays for AChEIs are also undergoing rapid development, particularly with the development of Ellman's method, which allows for the *in vitro* high-throughput screening. Enzymatic hydrolysis of ACh is an important step in the nerve transport, and its defect course is one of the possible reasons for AD. Ellman's procedure is commonly used for the determination of cholinesterase activity and also for monitoring of the ACh hydrolysis by AChE or BChE *in vitro* ¹⁷⁷.

The development of the modified Ellman's method, TLC bioautography, combined LC-MS and modified Ellman's method, and other methods has significantly improved isolation and screening techniques for AChEI-producing microorganisms, which predominantly consist of fungi and bacteria ¹¹¹. Ellman's assay and its modifications are currently extensively used to determine the AChE inhibitory activity of both naturally occurring and synthetic compounds. A great number of adherents to this methodology is related to the practicality, sensitivity and reproducibility of the method. It is not only a very fast, simple and inexpensive method ¹⁷⁸ but also an extremely sensitive and applicable to small amounts of tissue or to low concentrations of enzyme. This makes detailed kinetic studies of acetylcholinesterase activity possible ¹⁷⁷.

The methods used initially to evaluate AChE activity were based on acid-base titration. These tests quantify acetic acid produced during the hydrolysis of ACh. In the first case, the hydrolysis occurs in NaHCO₃/Na₂CO₃ buffer leading to the formation of acetic acid, which in turn reacts with the base of the buffer and promotes the release of CO₂, which is subsequently measured by a manometric apparatus. The acetic acid produced from the hydrolysis of ACh can also be titrated with alkaline solution of known concentration, where several acid/base indicators can be used ¹³⁵. A more convenient method to indicate pH

change is potentiometric titration using quinine/hydroquinone electrode, glass electrode or antimony electrode ¹⁷⁹⁻¹⁸⁰. Many other methods have been developed and improved, however, the methodology developed by Ellman and his collaborators remains the most widely used colorimetric method for the determination of AChE activity ¹⁷⁷. This procedure was developed by combining the knowledge published by Koelle in 1951, using DTNB known as Ellman's reagent ¹⁸¹. The Ellman's method uses a photometric method for the determination of anticholinesterase activity. This assay is based on measuring the rate of production of thiocholine, as ACh is hydrolysed by AChE. This is possible because the thiol group of the thiocholine reacts with Ellman's reagent to produce a yellow anion of 5-thio-2-nitrobenzoic acid (Figure 15). In turn, the presence of this anion can be detected by reading the absorbance at 412 nm. However, this method requires that all reagents and samples have high-water solubility. Also, the reaction with the thiol has been shown to be sufficiently rapid so as not to be rate limiting in the measurement of the enzyme and in the concentrations used does not inhibit the enzymatic hydrolysis ¹⁷⁷.

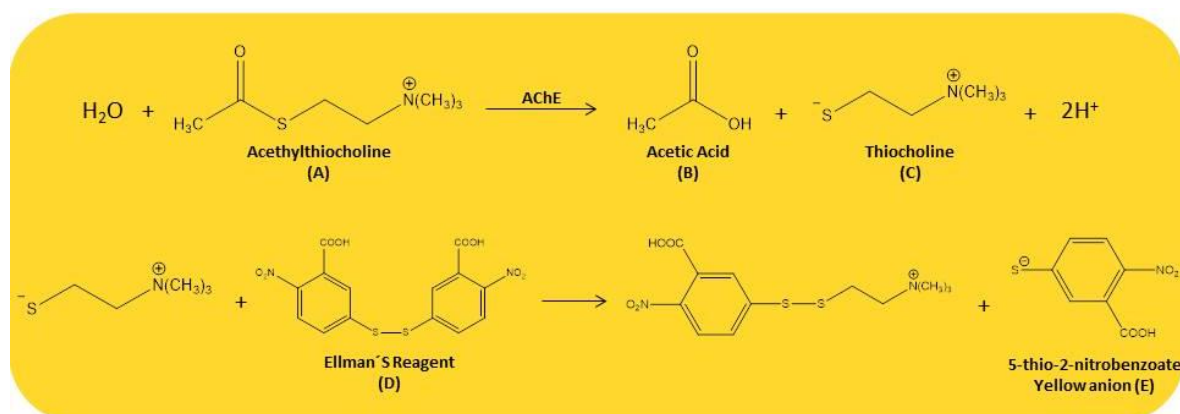


Figure 15 - Production of thiocholine (C) as acetylthiocholine (A) is hydrolysed. This is accomplished by the continuous reaction of the thiol with DTNB (D) ion to produce the yellow anion (E) (Adapted by Ellman *et al.*, 1961).

The enzymatic activity could be calculated from the angular coefficient of the linear region of the kinetic curve obtained by molecular absorption spectrophotometry, which corresponds numerically to the variation of absorbance over time ($\Delta A/\Delta t$). The linear portion of the curve describing the hydrolysis observed during the first 5 min of reaction; the slope is the rate in units of absorbance/min ¹⁷⁷.

In 2001, this technique was also adapted for the microscale, the methodology developed by Ellman *et al.*, by simply monitoring the production of colored compound in 96-well plates using a microplate reader. This assay revealed to be more sensitive than the TLC

method and allowed greater automation and a faster analysis of a larger number of samples¹⁸².

In our study we used the modified Ellman's microplate assay to measure acetylcholinesterase inhibitory activity of our compounds in assorted concentrations¹⁷⁷. Briefly, for AChE inhibitory assay, 20 µL of 0.22 U/mL AChE of *Electrophorus electricus* (EC 3.1.1.7, Sigma-Aldrich, Saint Louis, MO, USA) was added to the wells containing 10 µL of the tested compounds (200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.563 µM in methanol or DMSO), 100 µL of 3 mM of 5,5'-dithiobis 2-nitrobenzoic acid (DTNB) and 20 µL of 15 mM acetylthiocholine iodide. Absorbance of the colored-end product was measured 11 times, during 5 minutes, with 30 sec. intervals at 412 nm, controlled by a BioTek SynergyTM HT Microplate Reader (Winooski, Vermont USA). The controls containing 10 µL of methanol or DMSO instead of compounds, and blanks containing 20 µL of buffer [0.1% (w/v) bovine serum albumin (BSA) in 50 mM Tris-HCl] instead of enzyme. In this assay, blank samples containing 20 µL of buffer (0.1% (w/v) BSA in 50 mM Tris-HCl) instead of AChE were also performed. The percentage of enzymatic inhibition was calculated as:

$$\text{percentage of inhibition} = 100 - \left[\left(\frac{S - S_0}{C - C_0} \right) \times 100 \right]$$

Where C is the absorbance of the control, C_0 is the absorbance of blank, S is the absorbance in the presence of the sample compounds and S_0 is the absorbance of sample's blank. Each of the experiments was performed in triplicate and galantamine was used as a positive control to validate the method. The inhibitory activities of the tested compounds towards AChE were expressed as IC_{50} , which was determined to be the effective concentration at which AChE was inhibited by 50%. The IC_{50} value was obtained by interpolation from linear regression analysis. In the case that when the insolubility of the compound occurred and thus the IC_{50} value could not be determined, the percentage of inhibition for the maximum tested concentration was used.

Note: For analysis of butyrylcholinesterase from the lyophilized horse serum (EC 3.1.1.8, Merck, Darmstadt, Germany) all other reagents and conditions were maintained, except for the tested compounds which were used 20 µL per well.

Chapter 4 – Results and Discussion

4.1 Secondary metabolites isolated from *Acremonium persicinum* KUFA 1007

Chromatographic fractionation and further purification of the crude ethyl acetate extract of the culture of the marine-derived fungus *Acremonium persicinum* KUFA 1007 furnished two previously undescribed compounds, dihydroxy-2,2,8-trimethyl-1-oxospiro[4.5]dec-8-en-7-one (III) and 4-(hydroxymethyl)-2-methyl-1-benzofuran-6-ol (IV), as well as three previously reported metabolites, including ergosterol (I), ergosterol-5,8-endoperoxide (II), and lumichrome (V). The structures of these compounds are showed in Figure 16.

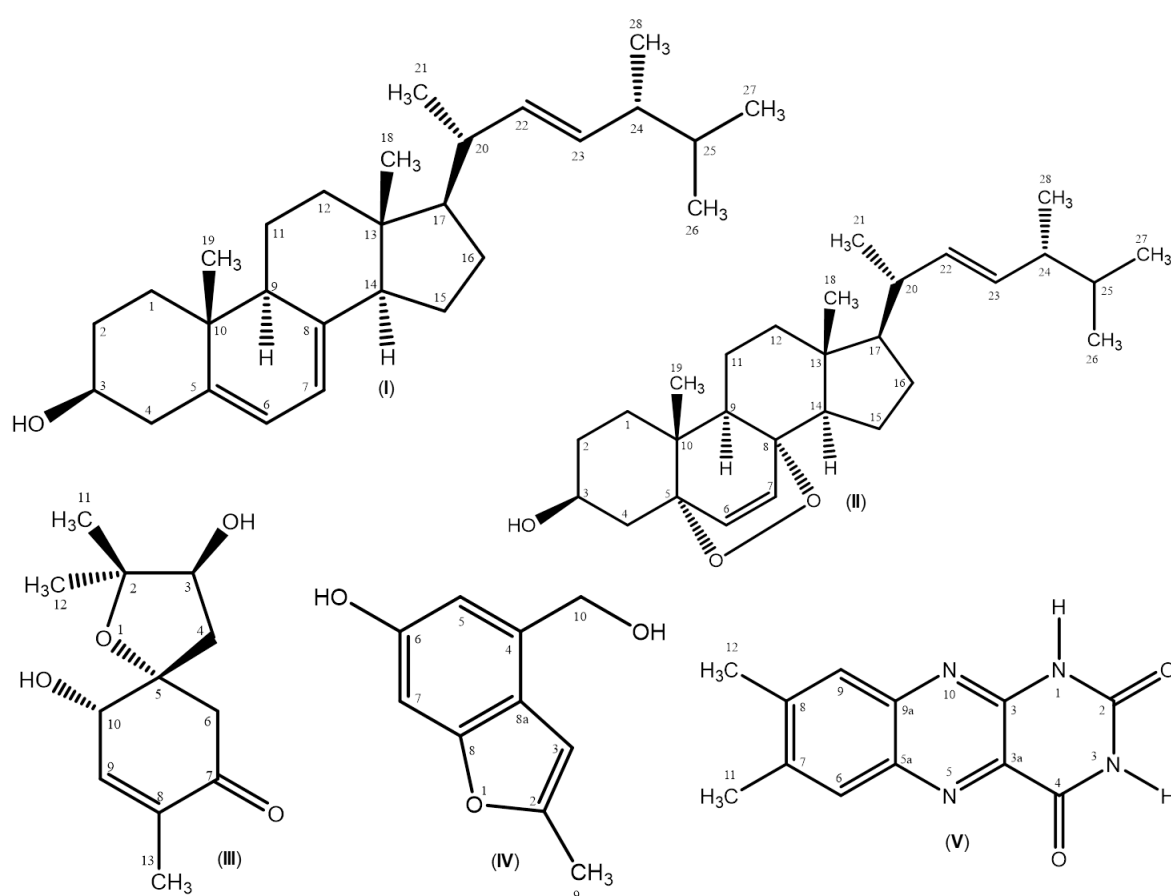


Figure 16 - Structures of the compounds isolated from the marine-derived fungus *Acremonium persicinum* KUFA 1007.

4.1.1 Ergosterol (I)

Compound **I** was isolated as colorless crystals (mp. 148-150 °C). Its ^{13}C NMR spectrum (Table 1, Supplementary materials - Figure 38) exhibited twenty-eight carbon signals which, in combination with DEPT and HSQC spectra, can be identified as two quaternary sp^2 (δ_{C} 141.4 and 139.8), four methine sp^2 (δ_{C} 135.5, 132.0, 119.6 and 116.3), two quaternary sp^3 (δ_{C} 42.8 and 37.0), seven methine sp^3 (δ_{C} 70.5, 55.7, 54.6, 46.2, 42.8, 40.4 and 33.1), seven methylene sp^3 (δ_{C} 40.8, 39.1, 38.4, 32.0, 31.6, 28.3 and 23.0) and six methyl (δ_{C} 21.1, 20.0, 19.7, 17.6, 16.3 and 12.0) carbons.

Table 1 - ^1H and ^{13}C NMR (CDCl_3 , 300.13 MHz and 75.48 MHz) and HMBC assignment for ergosterol (**I**)

Position	δ_{C} , type	δ_{H} , mult. (J in Hz)	COSY	HMBC
1	38.4, CH_2	1.28, m 1.90, m	H - 2	---
2	32.0, CH_2	1.88, m 1.50, m	H - 1, 3	---
3	70.5, COH	3.64, m	H - 2, 4	C - 4
4	40.8, CH_2	2.28, m 2.45, m	H - 3, 6	---
5	139.8, C	---	---	---
6	119.6, CH	5.57, dd (5.6, 2.4)	H - 4, 7	C - 8
7	116.3, CH	5.38, m	H - 6, 14	---
8	141.4, C	---	---	---
9	46.2, CH	1.96, m	---	---
10	37.0, C	---	---	---
11	23.0, CH_2	1.33, m 1.66, m	H - 12	---
12	39.1, CH_2	1.18, m 2.04, m	H - 11	C - 18
13	42.8, C	---	---	C - 11,18,
14	54.6, CH	1.87, m	H - 7	C - 9, 17, 18
15	28.3, CH_2	1.31, m 1.74, m	---	C - 17

Position	δ_C , type	δ_H , mult. (J in Hz)	COSY	HMBC
16	31.6, CH ₂	1.84, m	---	---
		1.53, m		
17	55.7, CH	1.24, m	H – 20	C – 12, 13, 18, 21
18	12.0, CH ₃	0.63, s	---	C - 12, 14, 17
19	16.3, CH ₃	0.95, s	---	---
20	40.4, CH	1.99, m	H - 17, 21, 22	C - 21
21	21.1, CH ₃	1.02, d (6.4)	H – 20	C - 17
22	135.6, CH	5.16, dd (15.5, 6.7)	H - 20, 23	C - 21, 24
23	132.0, CH	5.23, dd (15.5, 7.2)	H - 22, 24	C - 24
24	42.8, CH	1.85, m	H - 23, 25, 28	C - 22, 23, 25, 26, 27, 28,
25	33.1, CH	1.47, m	H - 24, 26, 27,	C - 24, 26, 27, 28
26	20.0, CH ₃	0.84, d (6.6)	H - 25	C - 24, 25, 27
27	19.7, CH ₃	0.82, d (6.6)	H - 25	C - 24, 25, 26
28	17.6, CH ₃	0.92, d (6.6)	H - 24	C - 23, 24, 25

The presence of four protonated sp^2 (δ_C 135.6, 132.0, 119.6, 116.3) and two non-protonated (δ_C 141.4, 139.8) carbons together with four olefinic protons at δ_H 5.57, dd ($J=5.6$, 2.4 Hz), δ_H 5.38, m, δ_H 5.23, dd ($J=15.5$, 7.2 Hz) and δ_H 5.16, dd ($J=15.5$, 6.7 Hz) suggested the existence of three double bonds in the molecule. The COSY spectrum showed a correlation from the proton at δ_H 5.16, dd ($J=15.5$, 6.7 Hz, H-22; δ_C 135.6) to the proton δ_H 5.23, dd ($J=15.5$, 7.2 Hz, H-23; δ_C 132.0), suggesting that they belong to a *trans* disubstituted double bond. In addition, the olefinic proton at δ_H 5.23, dd ($J=15.5$, 7.2 Hz, H-23) also exhibited a correlation to the methine proton at δ_H 1.85, m (H-24; δ_C 42.8). In turn, the multiplet of the methine proton at δ_H 1.85 (H-24) exhibited cross peaks to the multiplet of the methine proton at δ_H 1.47 (H-25; δ_C 33.1) and the doublet of the methyl protons at δ_H 0.92 ($J=6.6$ Hz, CH₃-28; δ_C 17.6), whereas the multiplet of the methine proton at δ_H 1.47 (H-25; δ_C 33.1) exhibited cross peaks to the two methyl doublets at δ_H 0.84 ($J=6.6$ Hz, CH₃-26; δ_C 20.0) and δ_H 0.82 ($J=6.6$ Hz, CH₃-27; δ_C 19.7). These correlations suggested the presence of the (1*E*)-3,4-dimethylpent-1-en-1-yl moiety. On the other hand, the multiplet at δ_H 1.99 (H-20; δ_C 40.4) displayed COSY correlations to the methyl doublet at δ_H 1.02 ($J=6.4$ Hz, CH₃-21; δ_C 21.1), 5.16, dd ($J=15.5$, 6.7 Hz, H-22; δ_C 135.6) as well as to the multiplet at δ_H 1.24 (H-17; δ_C 55.7), suggesting the presence of the coupling system of (3*E*)-5,6-dimethylhept-3-en-2-yl portion (Figure 17).

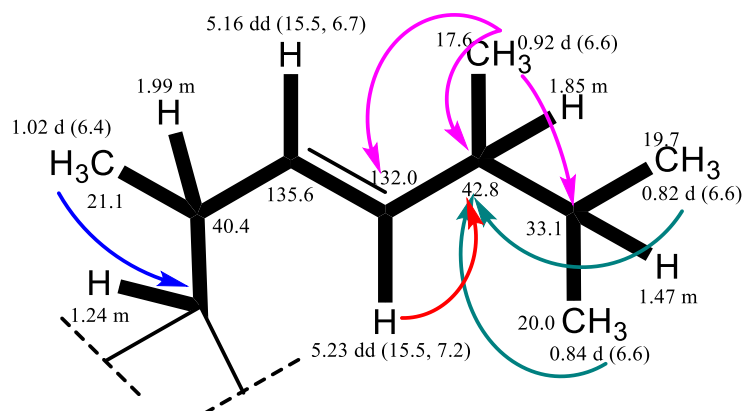


Figure 17 - Key COSY (bold line) and HMBC (arrow) correlations of (3E)-5,6-dimethylhept-3-en-2-yl portion.

The HMBC spectrum also showed correlations from CH₃-28 to the carbons at δ_c 132.0 (C-23), 42.8 (C-24) and 33.1 (C-25), from H₃-26 and H₃-27 to C-24, H-23 to C-24 and H₃-21 to the methine carbon at δ_c 55.7 (C-17), confirming the above structure (Figure 17).

Another portion of the molecule contains two tertiary methyl groups at δ_H 0.95, s/ δ_c 16.3 and δ_H 0.63, s/ δ_c 12.0, two tetrasubstituted double bonds as evidenced by the presence of two quaternary carbons at δ_c 141.4 (C-8) and δ_c 139.8 (C-5) as well as two protonated sp^2 at δ_c 119.6 (CH-6; δ_H 5.57, dd, $J=5.6$, 2.4 Hz) and δ_c 116.3 (CH-7; δ_H 5.38, m), one oxymethine carbon at δ_c 70.5 (CH-3; δ_H 3.64, m), three methine sp^3 carbons at δ_c 55.7 (C-17), 54.6 (C-14) and 46.2 (C-9), seven methylene sp^3 carbons at δ_c 40.8 (C-4), 39.1 (C-12), 38.4 (C-1), 32.0 (C-2), 31.6 (C-16), 28.3 (C-15) and 23.0 (C-11), and two quaternary sp^3 carbons at δ_c 42.8 (C-13) and 37.0 (C-10). The COSY spectrum displayed cross peak from H-6 to H-7 as well as weak cross peaks from H-6 to the multiplets δ_H 2.45 and δ_H 2.28 (H₂-4; δ_c 40.8). In turn, these methylene protons also showed correlations to the oxymethine proton at δ_H 3.64, m (H-3; δ_c 70.5) and H-6. The HMBC spectrum showed cross peaks from H-6 to C-8. All these correlations support the existence of the 5,6-disubstituted 4a-methyl-1,2,3,4,4a,5-hexahydronaphthalen-2-ol portion (Figure 18).

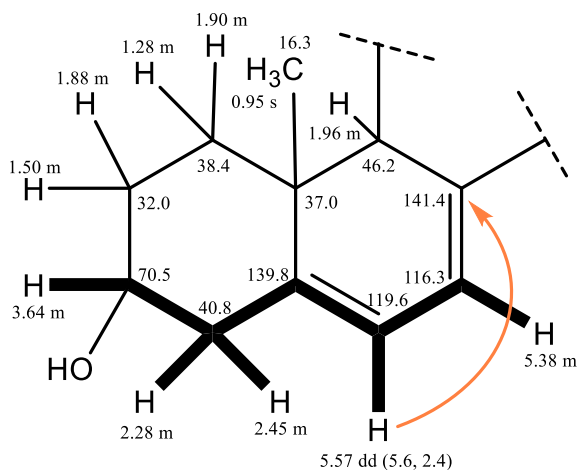


Figure 18 - Key COSY (bold line) and HMBC (arrow) correlations of 5,6-disubstituted 4a-methyl-1,2,3,4,4a,5-hexahydronaphthalen-2-ol portion.

Moreover, the HMBC spectrum also displayed correlation from the methyl singlet at δ_{H} 0.63 (CH_3 -18; δ_{C} 12.0) to the carbon at δ_{C} 39.1 (C-12), 54.6 (C-14) and 55.7 (C-17), the multiplet at δ_{H} 1.87 (δ_{C} 54.6) to the carbons at δ_{C} 46.2 (C-9), 55.7 (C-17) and 12.0 (C-18), and the multiplet at δ_{H} 1.24 (H-17; δ_{C} 55.7) to the carbons at δ_{C} 39.1 (C-12), 42.8 (C-13) and 21.1 (C-21). Consequently, the hexahydronaphthalene moiety was fused with the octahydro-1H-indene moiety through C-8 and C-9, forming thus a tetracyclic skeleton of androstane (Figure 19).

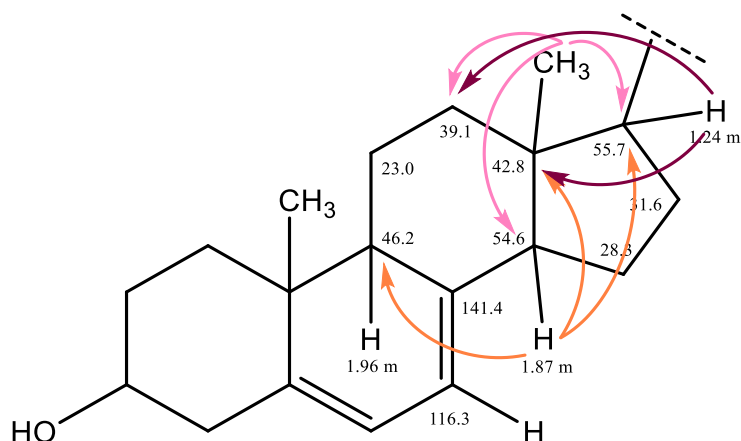


Figure 19 - Key HMBC (arrow) correlations of the tetracyclic skeleton of androstane.

Since the COSY spectrum exhibited a correlation from the multiplet at δ_{H} 1.99 (H-20; δ_{C} 40.4) of first fragment (Figure 18) H-17 of second fragment (Figure 19), it was clear that these two fragments were connected through the methine sp^3 carbons at δ_{C} 40.4 (C-20) of the former and at δ_{C} 55.7 (C-17) of the latter. This hypothesis was reinforced by the

HMBC correlation from the methyl doublet at δ_{H} 1.02 ($J=6.4$; δ_{C} 21.1) to C-17, as well as from H-17 to C-20, and CH₃-21 (Figure 20). This gives the final structure of the compound.

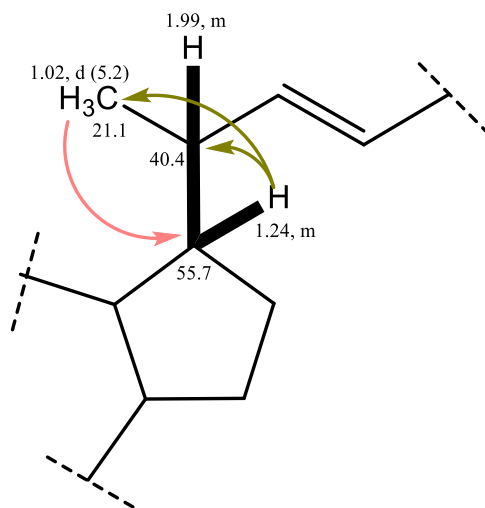
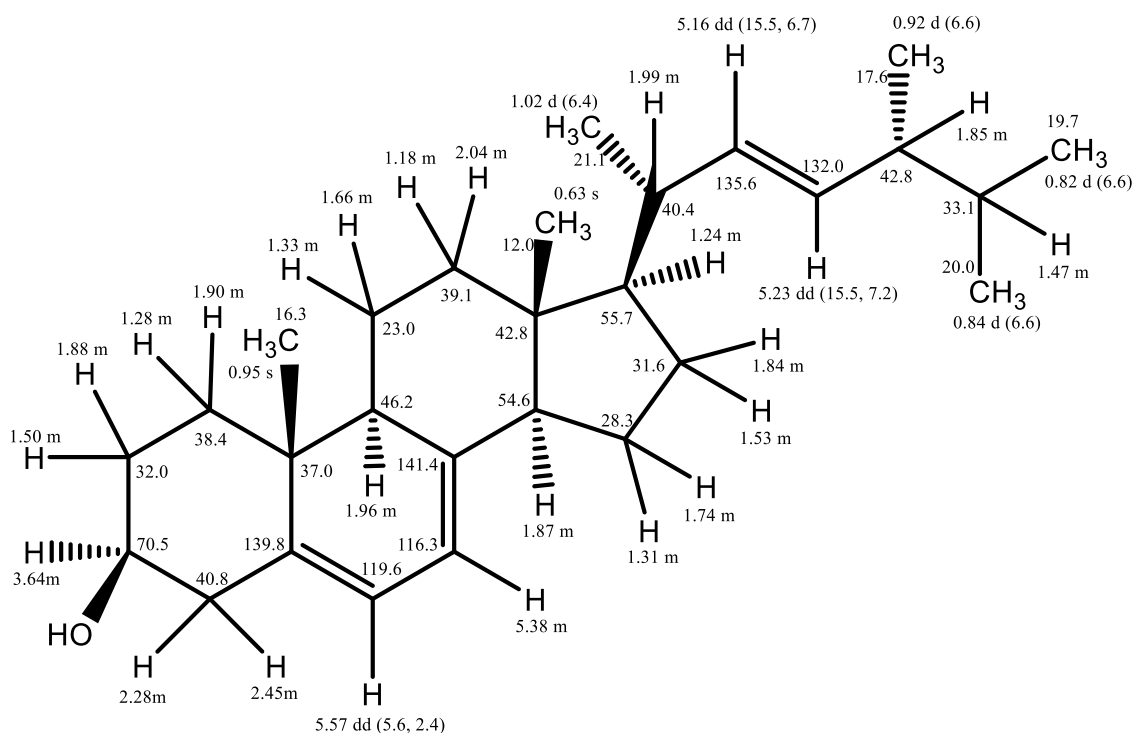


Figure 20 - Key COSY (bold line) and HMBC (arrow) correlations for **I**

Taking into account the molecular formula, the chemical shift values of the ^1H and ^{13}C and the COSY and HMBC correlations, the structure of **I** was established as ergosterol. The assignment of its ^1H and ^{13}C chemical shift values is shown in Figure 21.



Ergosterol is widely found in many species of fungi, accounting for approximately 70% of the sterols present in fungi ¹⁸³.

In 2015, Bravo *et al.* reported the isolation of this compound from *Laetiporus sp.*, a species of mushrooms growing in *Eucalyptus sp.* in the city of Piribebuy, Cordillera department, Paraguay ¹⁸⁴.

4.1.2 Ergosterol-5,8-endoperoxide (II)

Compound **II** was isolated as colorless crystal (mp. 180-182 °C). The ¹³C NMR spectrum (Table 2, Supplementary materials - Figure 40) exhibited twenty-eight carbon signals which, through DEPTs and HSQC spectra, can be categorized as four methine sp² (δ_C 135.4, 135.2, 132.3 and 130.7), two oxyquaternary sp³ (δ_C 82.2, 79.4), two quaternary sp³ (δ_C 44.6, 30.7), seven methine sp³ (δ_C 66.5, 56.2, 51.7, 51.1, 42.8, 39.7 and 33.1), seven methylene sp³ (δ_C 39.3, 36.9, 34.7, 30.1, 28.7, 23.4 and 20.6), four secondary methyl (δ_C 20.9, 19.9, 19.6 and 17.6) and two tertiary methyl (δ_C 18.2 and 12.9) carbons. The ¹H NMR spectrum (Table 2, Supplementary materials - Figure 41), in conjunction with the COSY spectrum, showed a pair of doublets of a *cis* double bond at δ_H 6.50, d (*J*=8.5 Hz; δ_C 130.7)/ δ_H 6.24, d (*J*=8.5 Hz; δ_C 135.4), a pair of double doublets of a *trans* double bond at δ_H 5.23, dd (*J*=15.3, 7.1 Hz; δ_C 132.3)/ δ_H 5.14, dd (*J*=15.3, 7.8 Hz; δ_C 135.2), two methyl doublets at δ_H 0.88 (*J*=6.9; Hz δ_C 18.2) and δ_H 0.82 (*J*=6.7; δ_C 12.9), and four methyl doublets at δ_H 0.99, d (*J*=6.6 Hz; δ_C 20.9), δ_H 0.91, d (*J*=6.8 Hz; δ_C 17.6), δ_H 0.82, d (*J*=6.8 Hz; δ_C 19.6) and δ_H 0.83, d (*J*=6.8 Hz; δ_C 19.9).

Table 2 - ¹H and ¹³C NMR (CDCl₃, 300.13 MHz and 75.48 MHz) and HMBC assignment for Ergosterol-5,8-endoperoxide (**II**)

Position	δ _C , type	δ _H , mult. (J in Hz)	COSY	HMBC
1	34.7, CH ₂	1.94, m	---	---
		1.68, m		
2	30.1, CH ₂	1.84, m	---	---
		1.55, m		
3	66.5, CH	3.97, sept (3.6)	H - 4	---
4	36.9, CH	2.11, ddd (13.5, 5.4, 1.9)	---	C - 3, 5
		1.90, m		---
5	82.2, C	---	---	---

Position	δ_C , type	δ_H , mult. (J in Hz)	COSY	HMBC
6	135.4, CH	6.24, d (8.5)	H - 7	C - 4, 5, 8, 10, 23
7	130.7, CH	6.50, d (8.5)	H - 6	C - 5, 6, 8, 9
8	79.4, C	---	---	---
9	51.1, CH	1.57, m	---	---
10	30.7, C	---	---	---
11	23.4, CH ₂	1.50, m	---	---
		1.22, m		
12	39.3, CH ₂	1.95, m	---	---
		1.23, m		
13	44.6, C	---	---	---
14	51.7, CH	1.50, m	---	---
15	28.7, CH ₂	1.73, m	---	---
		1.39, m		
16	20.6, CH ₂	1.59, m	---	---
		1.39, m		
17	56.2, CH	1.23, m	---	---
18	12.9, CH ₃	0.82, d (6.7)	---	C - 13, 14, 17
19	17.6, CH ₃	0.91, d (6.8)	---	C - 23, 24, 25
20	39.7, CH	2.00, m	---	---
21	20.9, CH ₃	0.99, d (6.6)	---	C - 17, 20, 22
22	135.2, CH	5.14, dd (15.3, 7.8)	H - 20, 23	C - 20, 24
23	132.3, CH	5.23, dd (15.3, 7.1)	H - 22, 24	C - 20, 24
24	42.8, CH	1.84, m	H - 24	---
25	33.1, CH	1.48, m	---	---
26	19.9, CH ₃	0.83, d (6.8)	---	C - 24, 25, 27
27	19.6, CH ₃	0.82, d (6.8)	---	C - 24, 25, 26
28	18.2, CH ₃	0.88, d (6.9)	---	C - 23

The general features of the ^1H and ^{13}C NMR spectra of **II** resembled those of ergosterol. Like ergosterol, **II** also contained the (3*E*)-5,6-dimethyl-3-en-2-yl side chain which was confirmed by COSY and HMBC correlations (Figure 22). The COSY spectrum displayed correlations from the double doublet at δ_H 5.23 ($J=15.3$, 7.1, H-23; δ_C 132.3) to the double doublet at multiplet at δ_H 5.14 ($J=15.3$, 7.8 Hz, H-22; δ_C 135.2) and δ_H 1.84 (H-24; δ_C 42.8)

and from the double doublet at δ_{H} 5.14 ($J=15.3, 7.8$ Hz, H-22; δ_{C} 135.2) to the multiplet at δ_{H} 2.00 (H-20; δ_{C} 39.7). This structure was supported by the HMBC correlations from a methyl doublet at δ_{H} 0.99 ($J=6.6$ Hz, CH₃-21; δ_{C} 20.9) to C-17 (δ_{C} 56.2), C-20 (δ_{C} 39.7) and C-22 (δ_{C} 135.2), from H-22 to C-24 (δ_{C} 42.8) and C-20 (δ_{C} 39.7), from H-23 to C-20 and C-24, from a methyl doublet at δ_{H} 0.83, d ($J=6.8$ Hz, CH₃-26; δ_{C} 19.9) to C-24, C-25 (δ_{C} 33.1) and C-27 (δ_{C} 19.6), from another methyl doublet at δ_{H} 0.82, d ($J=6.8$ Hz, CH₃-27; δ_{C} 19.6) to C-24, C-25 and C-26 (δ_{C} 19.9) and from a methyl doublet at δ_{H} 0.88 ($J=6.9$ Hz, CH₃-28; δ_{C} 18.2) to C-23 (Figure 22).

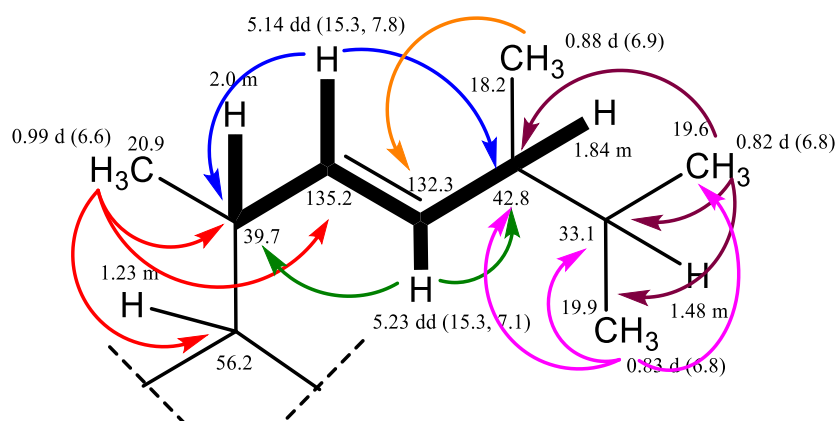


Figure 22 - Key COSY (bold line) and HMBC (arrow) correlations of (3E)-5,6-dimethylhept-3-en-2-yl side chain.

The remaining 19 carbons that belong to the β -hydroxyandrostane skeleton are similar to that of ergosterol. Since the ^1H and ^{13}C NMR spectra of **II** revealed the presence two olefinic protons of a *cis* double bond (δ_{C} 135.4/ δ_{H} 6.24, d, $J=8.5$ Hz) and (δ_{C} 130.7/ δ_{H} 6.50, d, $J=8.5$ Hz), and two oxygenated quaternary sp^3 carbons at δ_{C} 82.2 (C-5) and 79.4 (C-8), instead of two conjugated double bonds, in the androstane moiety, it was suggested that **II** is ergosterol-5,8-endoperoxide (Figure 23). This hypothesis was supported by the HMBC correlations from the methylene proton at δ_{H} 2.11, ddd ($J=13.5, 5.4, 1.9$ Hz; H-4; δ_{C} 36.9) to C-3 (δ_{C} 66.5) and C-5 (δ_{C} 82.2). Additionally, the olefinic proton at δ_{H} 6.24, d ($J=8.5$ Hz, H-6; δ_{C} 135.4) displayed HMBC cross peaks to the carbons at δ_{C} 36.9 (C-4), 30.7 (C-10), 79.4 (C-8), and 82.2 (C-5), while the signal of the olefinic proton at δ_{H} 6.50, d ($J=8.5$ Hz, H-7; δ_{C} 130.7) exhibited HMBC cross peaks to the carbons at δ_{C} 135.4 (C-6), C-5, C-8 and 51.1 (C-9), suggesting that the double bond was located on C-6/C-7, whereas C-5 and C-8 were oxygenated (Figure 23).

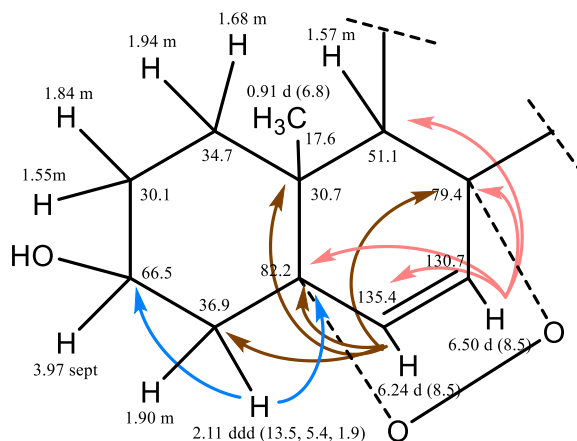


Figure 23 - Key HMBC (arrow) correlations of portion β -hydroxyandrostane.

The values of the chemical shift of ^1H and ^{13}C NMR, as well as the COSY and HMBC correlations of **II**, when compared to the data reported in the literature, confirmed the identity of **II** as ergosterol-5,8-endoperoxide (Figure 24) ¹⁸⁵⁻¹⁸⁶.

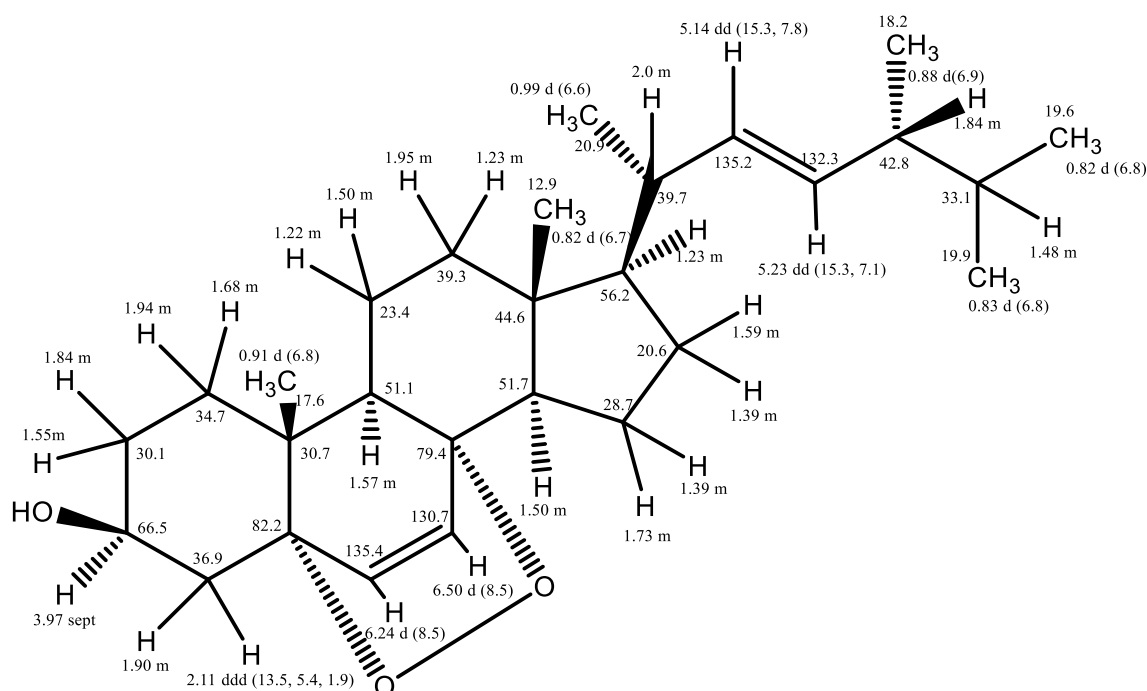


Figure 24 - ^1H and ^{13}C assignments for ergosterol-5,8-endoperoxide (**II**)

Literature search revealed that ergosterol-5,8-endoperoxide was isolated from alcoholic extracts of *Ganoderma lucidum* which presented an anti-melanogenic activity. To study the activity, an analogue of ergosterol-5,8-endoperoxide was examined for the anti-melanogenic activity towards B1610F7 cells. Ergosterol-5,8-endoperoxide at 2 mg/mL inhibited the melanin synthesis of B16 10F7 cells by 9.2% when compared to untreated

cells; however, ergosterol did not inhibit the melanogenesis of B1610F7 cells. The results suggested that the peroxy moiety between C-5 and C-8 of ergosterol-5,8-endoperoxide plays an important role in the anti-melanogenic effect. The levels of TRP-1 in B1610F7 cells stimulated with ergosterol-5,8-endoperoxide were also examined. Fluorescence microscopic observation revealed that the level of TRP-1 in B1610F7 cells was decreased by ergosterol-5,8-endoperoxide. It was suggested that three melanogenic enzymes, i. e. tyrosinase, TRP-1 and TRP-2 were regulated by the same melanocyte inducing transcription factor (MITF). The anti-melanogenic effect of ergosterol-5,8-endoperoxide might occur via the suppression of melanogenic enzymes in B1610F7 cells ¹⁸⁷.

4.1.3 Acremine S [(3S, 5R, 10S)-3,10-dihydroxy-2,2,8-trimethyl-1-oxospiro[4.5]dec-8-en-7-one] (III)

Compound **III** was isolated as a yellowish viscous liquid, and its molecular formula $C_{12}H_{18}O_4$ was established based on the (+)-HRESIMS m/z 227.1293 $[M+H]^+$ (calculated 227.1283) (Supplementary materials - Figure 42), indicating four degree of unsaturation.

Table 3 - ^1H and ^{13}C NMR ($CDCl_3$, 300.13 MHz and 75.48 MHz) and HMBC assignment for [(3S, 5R, 10S)-3,10-dihydroxy-2,2,8-trimethyl-1-oxospiro[4.5]dec-8-en-7-one] (**III**)

Position	δ_C , type	δ_H , mult. (J in Hz)	COSY	HMBC
2	85.8, C	---	---	---
3	77.3, CH	3.90, dd (4.3, 1.9)	H - 4	---
4	39.4, CH_2	2.20, m	H - 3	C - 2, 3, 5, 6, 10
5	87.1, C	---	---	---
6	53.0, CH_2	β - 2.67, d (15.5)	H - 6 α	C - 4, 5, 7, 8, 10
		α - 2.77, d (15.5)	H - 6 β	
7	197.8, CO	---	---	---
8	135.6, C	---	---	---
9	147.2, CH	6.58, t (1.6)	H - 10, 13	C - 5, 7, 13
10	73.6, CH	4.56, t (2.1)	H - 9, 13	---
11	27.8, CH_3	1.20, s	---	C - 2, 3, 12
12	22.6, CH_3	1.31, s	---	C - 2, 3, 11
13	15.0, CH_3	1.78, dd (2.1, 1.6)	H - 9, 10	C - 7, 8, 9

The ^{13}C NMR spectrum of **III** exhibited twelve carbon signals which, in combination with DEPT and HSQC spectra (Table 3, Supplementary materials - Figure 43 and 45), can be categorized as one conjugated ketone carbonyl (δ_{C} 197.8), one quaternary sp^2 (δ_{C} 135.6), one methine sp^2 (δ_{C} 147.2), two oxyquaternary sp^3 (δ_{C} 87.1 and 85.8), two oxymethine sp^3 (δ_{C} 77.3 and 73.6), two methylene sp^3 (δ_{C} 53.0 and 39.4) and three methyl (δ_{C} 27.8, 22.6 and 15.0) carbons. The ^1H NMR spectrum, in combination with the HSQC spectrum (Table 3, Supplementary materials - Figure 44 and 45), revealed the presence of a triplet of the olefinic proton at δ_{H} 6.58 ($J=1.6$ Hz; δ_{C} 147.2), a triplet of an oxymethine proton at δ_{H} 4.56 ($J=2.1$ Hz; δ_{C} 73.6), a double doublet of another oxymethine proton at δ_{H} 3.90 ($J=4.3, 1.9$ Hz; δ_{C} 77.3), two doublets of geminally coupled methylene protons at δ_{H} 2.67 ($J=15.5$ Hz; δ_{C} 53.0) and δ_{H} 2.77 ($J=15.5$ Hz; δ_{C} 53.0), a multiplet of two methylene protons at δ_{H} 2.20 (δ_{C} 39.4), a double doublet of methyl protons at δ_{H} 1.78 ($J=2.1, 1.6$ Hz; δ_{C} 15.0) and two methyl singlets at δ_{H} 1.31 (δ_{C} 22.6) and δ_{H} 1.20 (δ_{C} 27.8), respectively. The COSY spectrum (Table 3, Supplementary materials - Figure 47) displayed correlations from the olefinic proton at δ_{H} 6.58 ($J=1.6$ Hz; H-9) to the triplet at δ_{H} 4.56 ($J=2.1$ Hz; H-10) and the methyl double doublet at δ_{H} 1.78 ($J=2.1, 1.6$ Hz; Me-13), while the HMBC spectrum (Table 3, Supplementary materials - Figure 46) showed correlations from H₃-13 to the carbonyl carbon at δ_{C} 197.8 (C-7), the olefinic carbons at δ_{C} 147.2 (C-9) and δ_{C} 135.6 (C-8), H-9 to the oxyquaternary sp^3 at δ_{C} 87.1 (C-5), C-7 and the methyl carbon at δ_{C} 15.0 (Me-13), the doublets at δ_{H} 2.67 and 2.77 (H₂-6) to the C-5, C-7, C-8 and the carbon at δ_{C} 73.6 (C-10). Taking together the ^1H and ^{13}C chemical values and the COSY and HMBC correlations, the partial structure of the molecule was elucidated as 4-hydroxy-2-methylcyclohex-2-en-1-one (Figure 25).

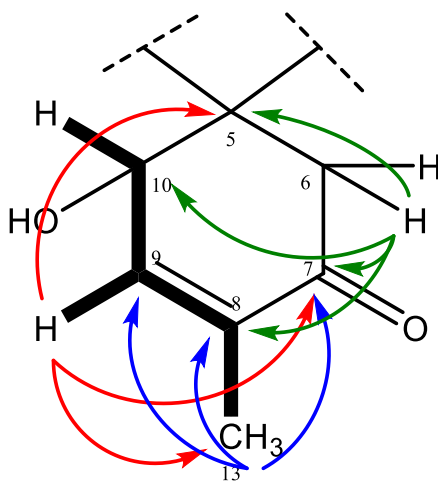


Figure 25 - Key COSY (bold line) and HMBC (arrow) correlations of 4-hydroxy-2-methylcyclohex-2-en-1-one portion

That another portion of the molecule was 2,2-dimethyloxolan-3-ol (Figure 26) was evidenced by the COSY correlations from the multiplet at δ_{H} 2.20 ($\text{H}_2\text{-4}$) to the double doublet at δ_{H} 3.90 (H-3), as well as the HMBC correlation from $\text{H}_2\text{-4}$ to the carbon signals at δ_{C} 85.8 (C-2), δ_{C} 77.3 (C-3) and C-5 , the methyl singlet at δ_{H} 1.20 ($\text{H}_3\text{-11}$) to C-2 , C-3 and the methyl carbon at δ_{C} 22.6 (Me-12), the methyl singlet at δ_{H} 1.31 ($\text{H}_3\text{-12}$) to C-2 , C-3 and the methyl carbon at δ_{C} 27.8 (Me-11).

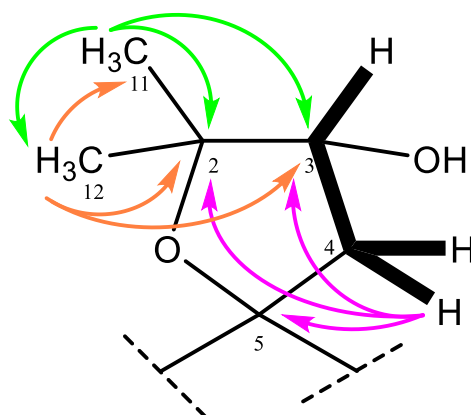


Figure 26 - Key COSY (bold line) and HMBC (arrow) correlations of 2,2-dimethyloxolan-3-ol portion

That the 2,2-dimethyloxolan-3-ol (Figure 26) was *spiro*-fused with the 4-hydroxy-2-methylcyclohex-2-en-1-one (Figure 25) moiety at C-5 was corroborated by the HMBC correlation from $\text{H}_2\text{-4}$ to C-5 , C-6 and C-10 , as well as from $\text{H}_2\text{-6}$ to the carbon at δ_{C} 39.4 (C-4) and C-5 . Taken together the two partial structures and HMBC correlations, the planar structure of **III** was established as 3,10-dihydroxy-2,2,8-trimethyl-1-oxospiro[4.5]dec-8-en-7-one (Figure 27).

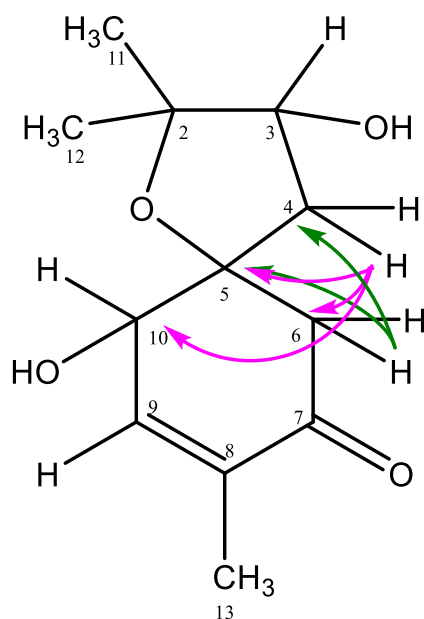


Figure 27 – Key HMBC (arrow) correlations of 3,10-dihydroxy-2,2,8-trimethyl-1-oxospiro[4.5]dec-8-en-7-one (III)

The COSY and HMBC correlations allow the assignment of the ^1H and ^{13}C chemical shift values for III as shown in Figure 28.

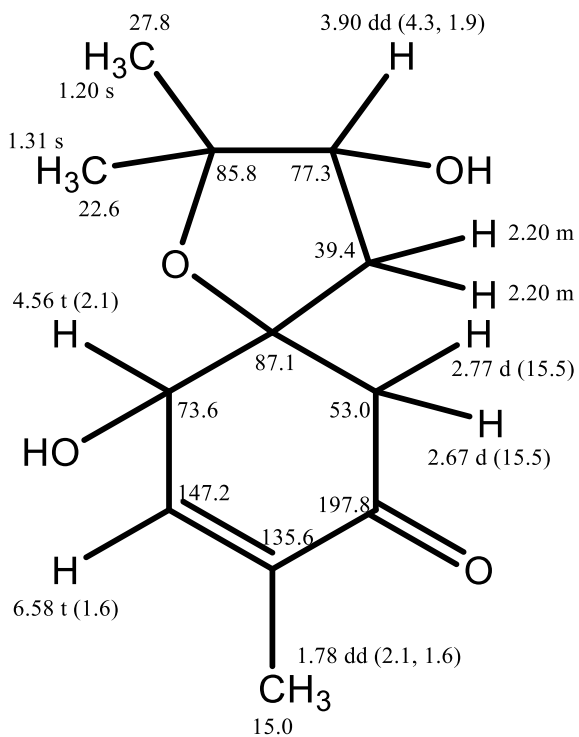


Figure 28 - ^1H and ^{13}C assignments for (3S, 5R, 10S)-3,10-dihydroxy-2,2,8-trimethyl-1-oxospiro[4.5]dec-8-en-7-one (III)

Structurally, **III** possesses three stereogenic carbons, i.e. C-3, C-5 and C-10. Since **III** was obtained as viscous oil and was not possible to obtain a suitable crystal for a determination of the absolute configurations of its stereogenic carbons by X-ray analysis. However, since **III** has an enone chromophore, the absolute configurations of its stereogenic carbons were established by comparison of its calculated and experimental ECD spectra. Compound **III** has three stereogenic-centres and, therefore, eight possible configurations. Conformational analysis revealed 36 molecular mechanics (MM) conformational energy minima for each configuration, by combining two conformations for each ring and three conformations for each of the two hydroxyl groups. To find the most significant conformations of each configuration, MM models were re-minimized using a quantum mechanical DFT method coupled with a small basis set. Then, the most populated (lowest energy) conformations were further refined using the same DFT method but with a large basis set. The first 50 ECD transitions were then calculated (TD-DFT) for each model. Comparison of the experimental ECD spectrum with the eight final calculated spectra, one for each configuration, revealed a better match with the spectrum obtained from the (3*S*, 5*R*, 10*S*) model (Figures 29 and 30).

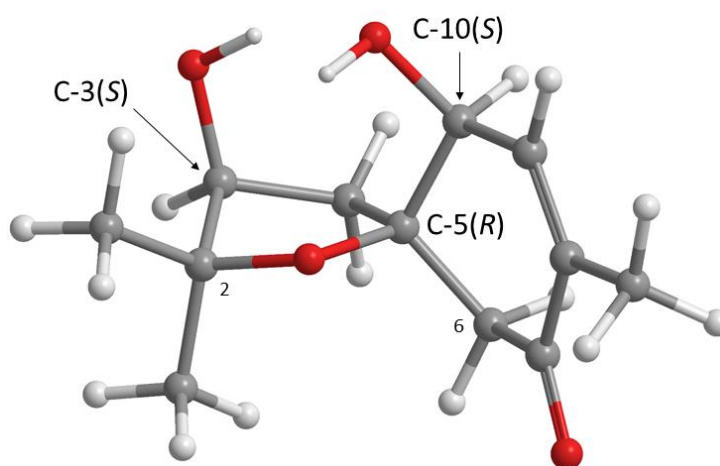


Figure 29 - Most abundant conformation of **III** (APFD/6-311+G(2d,p) lowest energy conformer) in its (3*S*, 5*R*, 10*S*) configurations.

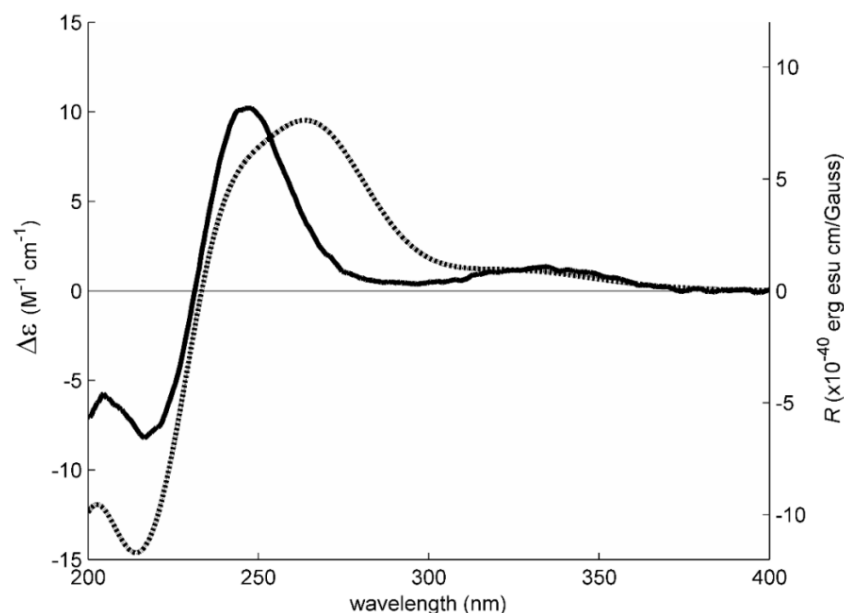


Figure 30 - Experimental ECD spectrum (solid line, left axis) of **III** in methanol and theoretical ECD spectrum (dotted line, right axis) of its (3*S*, 5*R*, 10*S*) configuration.

This is not in complete agreement with the findings of Suciati *et al.*⁷³ since they reported the structure of acremine O (compound **6** in Suciati *et al.*), which is an analogue of **III**, where C-4 is oxidized to a ketone carbonyl and C-5 is chlorinated (according to the numbering in Ref. 73), also isolated from the fungus *A. persicinum*, to which they assigned the absolute stereochemistry (3*R*, 5*R*). Intrigued by this discrepancy, we obtained the ROESY spectrum of **III** and compared its data to what would be expected from the energy-optimized conformations of the (3*S*, 5*R*, 10*S*) and (3*R*, 5*R*, 10*S*) DFT models of **III**. The ROESY spectrum (Supplementary materials - Figure 48) exhibited correlations from H-3 to H₂-4 and H₃-11, whereas H₃-11 exhibited correlations not only to H₂-4 but also to H-6 at δ_{H} 2.67, d ($J=15.5$ Hz). These ROESY correlations implied that H-3, Me-11 and H-6 (at δ_{H} 2.67) are on the same face. That means OH-3 and Me-12 are on the opposite face to Me-11 and H-3. On the other hand, H-10 exhibited a weak correlation to H-6 at δ_{H} 2.77 (d, $J=15.5$ Hz), suggesting that they were on the same face. The difference of the chemical shift values of H₂-6 was attributed to the anisotropic effect of the carbonyl group (C-7) of the cyclohexenone ring. One of H₂-6 (δ_{H} 2.67, d, $J=15.5$ Hz) was deshielded when compared to another (δ_{H} 2.77, d, $J=15.5$ Hz), implying that the former was in the equatorial position (H-6 β) while the latter was in the axial position (H-6 α) (Figure 31).

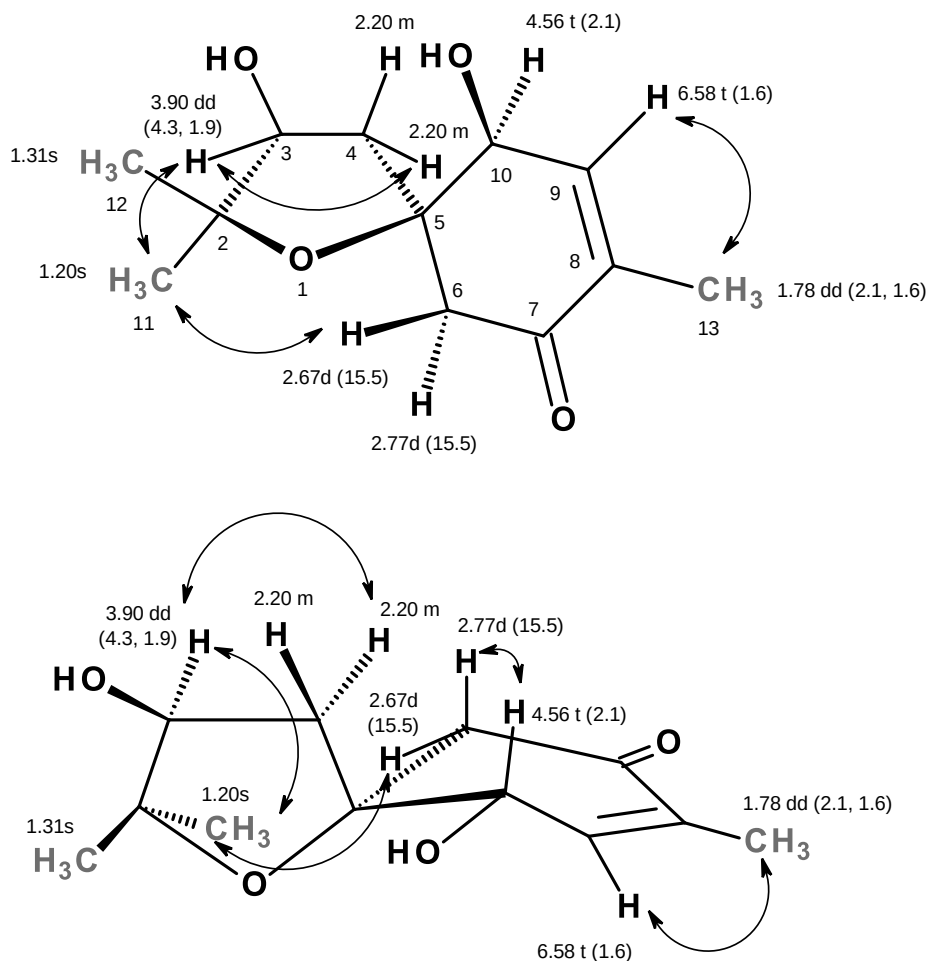


Figure 31 - Key ROESY correlations in **III**.

These ROESY correlations are compatible with the (3*S*, 5*R*, 10*S*) model because, in this model, all these nuclei are on the same side of the five-membered ring and near enough to observe NOE signals. Inversion of the configuration of C-3 from (*S*) to (*R*) puts H-3 and H-6 (δ_{H} 2.77, d, $J=15.5$ Hz) on opposite ringsides (and a bit farther from the C-2 methyl groups). Therefore, according to our C-3(*R*) configuration model, H-3 and H-6 (δ_{H} 2.67, d, $J=15.5$ Hz) are expected to show (probably weak) NOE cross peaks to both Me-11 and Me-12. Since H-3 and H-6 (δ_{H} 2.67, d, $J=15.5$ Hz) are both observed to be near Me-11 and not Me-12, as inferred from ROESY data, we propose the (3*S*, 5*R*, 10*S*) configuration for **III** (Figure 16). Since **III** has never been previously reported, it was named acremine S, following the series of the prenylated polyketides, isolated from *Acremonium* species.

4.1.4 Acremine T [4-(Hydroxymethyl)-2-methyl-1-benzofuran-6-ol] (IV)

Compound **IV** was isolated as a white amorphous solid. The ^1H NMR in $\text{DMSO-}d_6$ spectrum (Table 4, Supplementary materials - Figure 51) showed that it still contained some impurities. However, the major component displayed a broad singlet of a phenolic hydroxyl proton at δ_{H} 9.28, a singlet of two aromatic protons at δ_{H} 6.70, a broad singlet of an olefinic proton at δ_{H} 6.47, a triplet at δ_{H} 5.17 ($J=5.6$ Hz, 1H), a doublet at δ_{H} 4.58 ($J=5.6$ Hz, 2H) and a methyl doublet at δ_{H} 2.36 ($J=1.0$ Hz).

Table 4 - ^1H and ^{13}C NMR ($\text{DMSO-}d_6$, 300.13 MHz and 75.48 MHz) and HMBC assignment for [4-(hydroxymethyl)-2-methyl-1-benzofuran-6-ol] (**IV**).

Position	δ_{C} , type	δ_{H} , mult. (J in Hz)	COSY	HMBC
2	152.6, C	---	---	---
3	101.0, CH	6.47, s	H ₃ - 9	C - 2, 4, 8
4	134.7, C	---	---	---
5	109.3, CH	6.70, s	H ₂ - 10	C - 4, 6, 7, 10
6	154.4, C	---	---	---
7	95.6, CH	6.70, s	---	C - 6, 8a
8	155.0, C	---	---	---
8a	118.4, C	---	---	---
9	13.7, CH ₃	2.36, d (1.0)	---	C - 2, 3
10	61.0, CH ₂	4.58, d (5.6)	H - 5, OH - 6	C - 4, 5, 8a
	OH-6	9.28, brs	---	C - 5, 6, 7
	OH-10	5.17, t (5.6)	H ₂ - 10	C - 10

The ^{13}C NMR spectrum (Table 4, Supplementary materials - Figure 50) exhibited, for the main component, ten carbon signals which were categorized, by DEPT and HSQC spectra (Table 4, Supplementary materials - Figure 52), to five non-protonated sp^2 (δ_{C} 155.0, 154.4, 152.6, 134.7 and 118.4), three protonated sp^2 (δ_{C} 109.3, 101.0 and 95.6), one oxymethylene sp^3 (δ_{C} 61.0) and one methyl (δ_{C} 13.7) carbons. The COSY spectrum (Table 4, Supplementary materials - Figure 54) displayed a correlation from a singlet at δ_{H} 6.70 (H-5) to a doublet at δ_{H} 4.58 (H₂-10), from the hydroxyl triplet at δ_{H} 5.17 (OH-10) to H₂-10. The presence of a 2,3-substituted 5-hydroxybenzyl alcohol was substantiated by HMBC correlations (Table 4, Supplementary materials - Figure 53) from the broad singlet of the phenolic hydroxyl proton at δ_{H} 9.28, brs (OH-6) to C-5 (δ_{C} 109.3/ δ_{H} 6.70), C-7 (δ_{C}

95.6/ δ_{H} 6.70) and C-6 (δ_{C} 154.4, weak), H-5 to C-7, C-4 (δ_{C} 134.7), C-6 and C-10 (δ_{C} 61.0/ δ_{H} 4.58), H-7 to C-6 and C-8a, from H-10 to C-5, C-4, C-8a (δ_{C} 118.4) and from the OH-10 (δ_{H} 5.17, t, $J=5.6$ Hz) to C-10 (Figure 32).

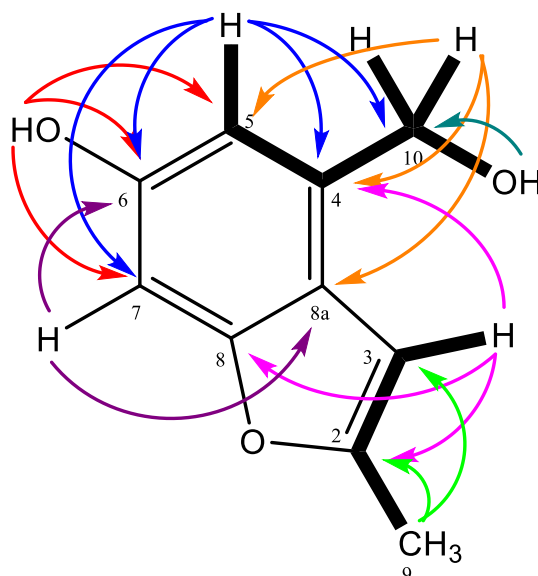


Figure 32 - Key COSY (bold line) and HMBC (arrow) correlations for **IV**

That the 2,3-substituted 5-hydroxybenzyl alcohol was fused to a 2-methylfuran ring, through C-8 (δ_{C} 155.0) and C-8a (δ_{C} 118.4) was supported by HMBC correlations (Table 4, Supplementary materials - Figure 53) from H-3 (δ_{H} 6.47, brs) to C-4, C-8 and C-2 (δ_{C} 152.6) and from the methyl doublet at δ_{H} 2.36 ($J=1.0$ Hz, H₃-9) to C-2 and C-3 (δ_{C} 101.0). The COSY and HMBC correlations allow the assignment of the ^1H and ^{13}C chemical shift values for **IV** as shown in Figure 33.

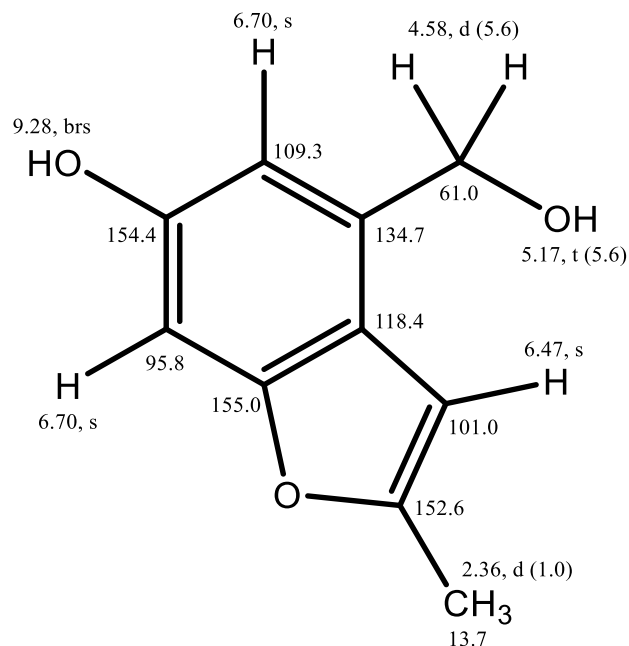


Figure 33 - ¹H and ¹³C assignments for 4-(Hydroxymethyl)-2-methyl-1-benzofuran-6-ol (**IV**)

In order to confirm the structure of **IV**, the compound was further purified by passing through a Sephadex LH-20 column and its ¹³C and ¹H NMR spectra (Supplementary materials - Figures 50 and 51) showed that the impurities were completely removed. Interestingly, the ¹H NMR spectrum of the pure compound, also in DMSO-*d*₆, did not exhibit the triplet of OH-10 (δ_{H} 5.17, $J=5.6$ Hz) and H₂-10 appeared as a singlet at δ_{H} 4.58 instead of a doublet with $J=5.6$ Hz. The HSQC and HMBC spectra (Supplementary materials - Figures 52 and 53) were also in agreement with those obtained before re-purification. Since the pure compound exhibited the (-)-HRESIMS m/z at 177.0556 (M-H)⁺ (calculated for C₁₀H₉O₃, 177.0552) (Supplementary materials - Figure 49), its molecular formula was established as C₁₀H₁₀O₃, corresponding to six degrees of unsaturation. Therefore, **IV** was elucidated as 4-(hydroxymethyl)-2-methyl-1-benzofuran-6-ol. A structure search through SciFinder displayed the structure of **IV** with the name 6-hydroxy-2-methyl-4-benzofuranmethanol and the CAS Registry Number 1083198-52-0. However, there is no reference reporting its source or its NMR data. Therefore, **IV** was named acremine T.

4.1.5 Lumichrome (V)

Compound **V** was isolated as a yellow amorphous solid, its molecular formula $C_{12}H_{10}N_4O_2$ was established based on the (+)-HRESIMS m/z 243.0888 $[M+H]^+$ (calculated 243.0882), indicating ten degree of unsaturation.

Table 5 - ^1H and ^{13}C NMR (DMSO- d_6 , 500.16 MHz and 125.78 MHz) and HMBC assignment for lumichrome (**V**)

Position	δ_C , type	δ_H , mult. (J in Hz)	COSY	HMBC
1	NH	11.69, brs*	---	---
2	150.1, C	---	---	---
3	NH	11.84, brs*	---	---
4	160.7, C	---	---	---
4a	130.2, C	---	---	---
5	---	---	---	--
5a	138.3, C	---	---	---
6	128.7, CH	7.92, s	H - 11	C - 7, 9a, 11
7	144.7, C	---	---	---
8	138.9, C	---	---	---
9	125.8, CH	7.71, s	H - 12	C - 5a, 8, 12
9a	141.6, C	---	---	---
10	---	---	---	---
10a	146.5, C	---	---	---
11	19.6, CH ₃	2.47, s	H - 6	C - 6, 7, 8
12	20.2, CH ₃	2.50, s	H - 9	C - 7, 8, 9

The ^{13}C NMR spectrum (Table 5, Supplementary materials - Figure 55) of **V** exhibited twelve carbon signals which, in combination with DEPT and HSQC spectra, can be categorized as eight quaternary sp^2 (δ_C 160.7, 150.1, 146.5, 144.7, 141.6, 138.9, 138.3 and 130.2), two protonated sp^2 (δ_C 128.7 and δ_C 125.8) and two methyl (δ_C 20.2 and δ_C 19.6) carbons. The ^1H NMR spectrum (Table 5, Supplementary materials - Figure 56) exhibited besides two aromatic singlets at δ_H 7.92 and δ_H 7.71, and two methyl singlets at δ_H 2.50 and δ_H 2.47, a broad signal of two protons at δ_H 11.84 and δ_H 11.69, characteristic of the NH protons. The COSY spectrum displayed correlations from the aromatic proton at δ_H 7.92, s (H-6) to the methyl singlet at δ_H 2.47 (Me-11) as well as from

another singlet of the aromatic proton at δ_{H} 7.71 (H-9) to the methyl singlet at δ_{H} 2.50 (Me-12). This pattern of correlations supported the existence of a 1,3,4,6-tetrasubstituted benzene ring where the two methyl substituents are on the vicinal carbons (Figure 34). This partial structure was supported by HMBC correlations from H-9 to the quaternary sp^2 carbons at δ_{C} 138.3 (C-5a), 138.9 (C-8) and the methyl carbon at δ_{C} 20.2 (CH₃-12), H-6 the quaternary sp^2 carbons at δ_{C} 144.7 (C-7), 141.6 (C-9a) and the methyl carbon at δ_{C} 19.6 (CH₃-11), the methyl singlet at δ_{H} 2.50 (Me-12) to C-7, C-8 and C-9 (δ_{C} 125.8) and from the methyl singlet at δ_{H} 2.47 (Me-11) to the protonated sp^2 carbon at δ_{C} 128.7 (C-6), C-7 and C-8 (Figure 34).

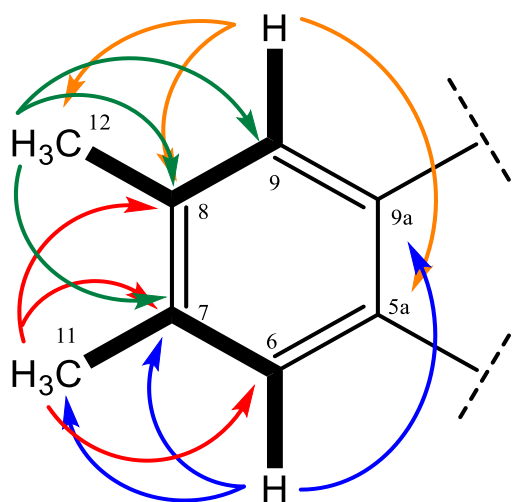


Figure 34 - Key COSY (bold line) and HMBC (arrow) correlations of 1,3,4,6-tetrasubstituted benzene ring

As this partial structure corresponds to C_8H_8 , another part of the molecule must have $\text{C}_4\text{H}_2\text{N}_4\text{O}_2$, corresponding to four protonated sp^2 quaternary carbons (δ_{C} 160.7, 150.1, 146.5 and 130.2), four nitrogen, two oxygen and two hydrogen atoms. Taking together the number of the carbon, hydrogen, nitrogen and oxygen atoms and the chemical carbon shift values, this moiety should have two amide carbonyl groups, two secondary amines (NH) and two imine ($\text{C}=\text{N}$) groups. The chemical shift values of the quaternary sp^2 carbons at δ_{C} 160.7 and 150.1 resemble those of the amide carbonyl carbons whereas the carbons at δ_{C} 146.5 and 130.2 could be assigned to the imine carbons of 6,7-dihydropteridine-2,4(1*H*,3*H*)-dione (Figure 35).

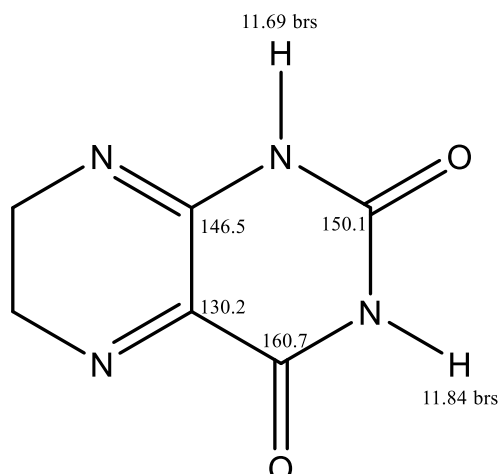


Figure 35 – Structure of the 6,7-dihydropteridine-2,4(1*H*,3*H*)-dione portion

Combination of these two portions results in the complete structure of **V** (Figure 36). Based on the finding by Silva M. *et al.*¹⁸⁸ and, taking into account the molecular formula, the chemical shift values of the ^1H and ^{13}C , the structure of **V** was elucidated as lumichrome (Figure 36).

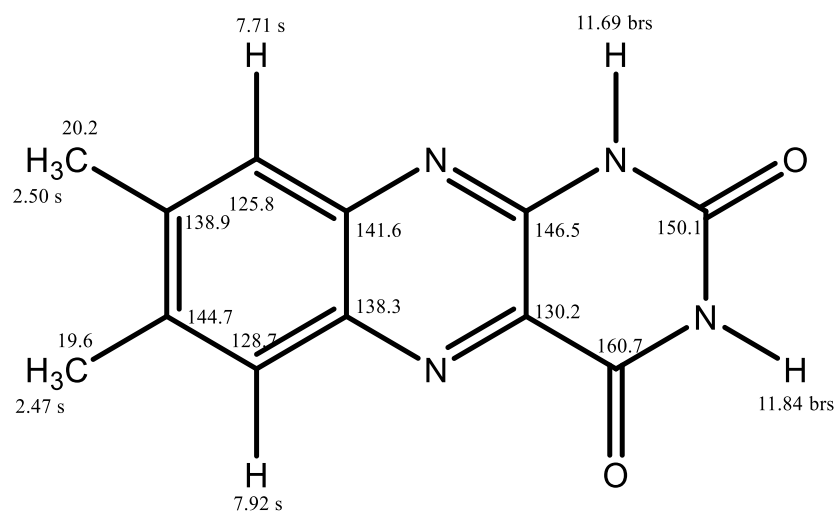


Figure 36 - ^1H and ^{13}C assignments for lumichrome (**V**)

Literature research revealed that lumichrome has been isolated from a variety of organisms. In 1972, George *et al.*, reported lumichrome as a photoconversion product of riboflavin (vitamin B2) in plant tissue¹⁸⁹. Later on, in 1974, Sasaki *et al.* obtained lumichrome from the culture of the fungus *Aspergillus oniki* 1784¹⁹⁰. On the other hand, Silva *et al.* reported the isolation of lumichrome from the marine alga *Gelidium microdon*, collected from the coast of the Azores Archipelago¹⁹¹. Recently, Kumla *et al.* isolated lumichrome from the culture extract of the marine sponge-associate fungus *Neosartorya*

fennelliae KUFA 081 ¹⁹². Interestingly, there was no report on anticholinesterase and antityrosinase activities of lumichrome.

4.2 Biological assays

4.2.1 Tyrosinase inhibitory assay

The new compounds **III** and **IV**, and lumichrome (**V**) were tested for their antityrosinase activity using kojic acid and quercetin as positive controls. All the tested compounds demonstrated their ability to inhibit the activity of the enzyme tyrosinase.

The results of the inhibitory activity of **III**, **IV** and **V** toward tyrosinase are shown in Table 6. The positive controls, kojic acid and quercetin, showed IC₅₀ values of 40.02 μ M and 30.73 μ M, respectively. At a concentration of 21.12 μ M, **IV** displayed higher inhibitory capacity than **III** and **V** and even more potent than kojic acid, the most used positive control for tyrosinase inhibitor.

Table 6 - TYR inhibitory activity of **III**, **IV** and **V**

Compounds	% inhibition at 21.12 μ M	IC ₅₀ (μ M)
Kojic acid	50.51 $\pm$ 3.07 *	40.02 $\pm$ 2.18 *
Quercetin	51.24 $\pm$ 1.30 *	30.73 $\pm$ 1.33 *
III	24.68 $\pm$ 2.19 *	n.d.
IV	55.28 $\pm$ 3.41 *	35.14 $\pm$ 1.28 *
V	8.82 $\pm$ 0.76 *	n.d.

Results are given as mean $\pm$ SEM of three independent experiments performed in triplicate; n.d.: not determined. *p < 0.05

The curves in Figure 37 showed that **IV** has a different kinetic profile from that of kojic acid. The kinetics of inhibition of the two compounds is different despite their similar inhibitory capacity at 21.12 μ M. These data suggest that **IV** might have a different mode of inhibition on L-DOPA from that of kojic acid.

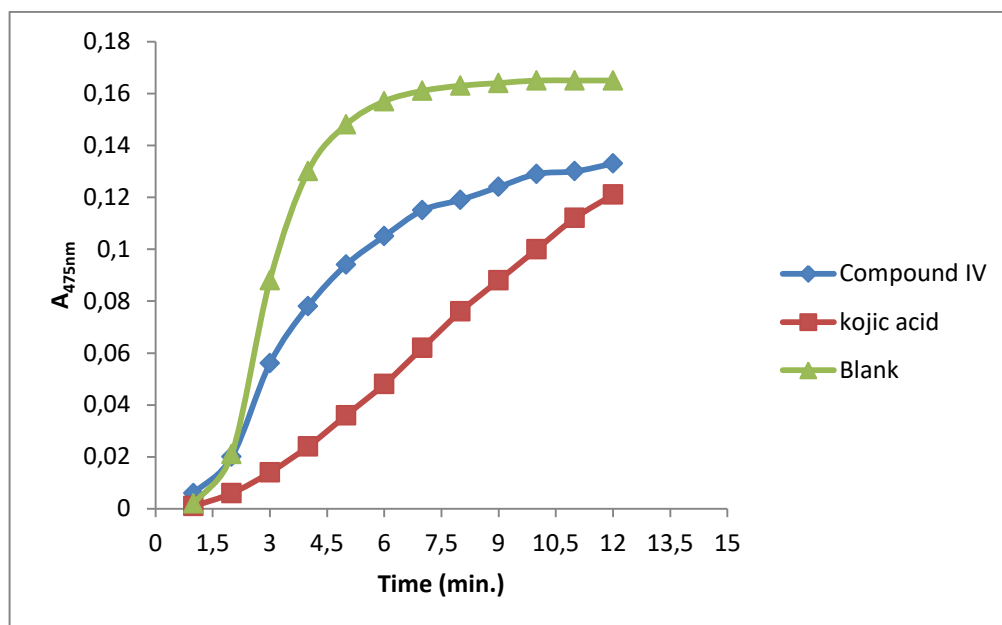


Figure 37 - Action of tyrosinase on L-DOPA alone (▲) or in the presence of kojic acid (■) or IV (◆). Spectrophotometric recordings measured at 475 nm. All measurements were performed in triplicates.

4.2.2 Acetylcholinesterase and butyrylcholinesterase inhibitory assay

Acremine S (III), acremine T (IV) and lumichrome (V) were tested for their anticholinesterase activity using galantamine as a positive control by a modified Ellman's method¹⁷⁷. All the three compounds displayed AChE and BChE inhibitory activities in this assay, and the results are shown in Table 7. The positive control, galantamine, showed an IC₅₀ value of 11.31 μM while lumichrome (V) exhibited higher AChE inhibitory activity than not only acremines S (III) and T (IV) but also the positive control, galantamine.

Table 7 - AChE inhibitory activity of III, IV and V

Compounds	% inhibition at 6.6 μM	IC ₅₀ (μM)
Galantamine	39.76 ± 2.22 *	11.31 ± 0.11 **
III	10.42 ± 0.33 *	n.d.
IV	14.08 ± 1.27 *	n.d.
V	39.23 ± 1.77 *	12.24 ± 0.12 **

Results are given as mean ± SEM of three independent experiments performed in triplicate; n.d.: not determined. *p<0.05; **p<0.01.

Regarding the inhibitory activity of the tested compounds toward butyrylcholinesterase, the final results (Table 8) showed that all the compounds exhibited BChE inhibitory

activity. Interestingly, acremine S (**III**) was found to exhibit stronger activity than galantamine while the inhibitory activity of acremine T (**IV**) was comparable to that of galantamine.

Table 8 - BChE inhibitory activity of **III**, **IV** and **V**

Compounds	% inhibition at 6.25 μ M	IC ₅₀ (μ M)
Galantamine	10.42 $\pm$ 0.21 *	n.d.
III	30.71 $\pm$ 0.39 **	n.d.
IV	10.53 $\pm$ 1.01 **	n.d.
V	3.44 $\pm$ 0.09 *	n.d.

Results are given as mean $\pm$ SEM of three independent experiments performed in triplicate; n.d.: not determined. * p <0.05; ** p <0.01.

In summary, acremine S (**III**) has higher inhibition power for BChE with a percentage inhibition of about 30% at 6.25 μ M, whereas lumichrome (**V**) displayed the greatest inhibitory power toward AChE, with about 40% inhibition at 6.6 μ M.

Chapter 5 – Conclusion

This study describes the isolation, purification, structure elucidation and anti-melanogenic and anticholinesterase activity evaluation of the secondary metabolites produced by the culture of the fungus *Acremonium persicinum* KUFA 1007, which was isolated from the marine sponge *Mycale* sp., which was collected from the Gulf of Thailand, in September 2016.

Isolated from the crude ethyl acetate extract of the culture of this fungus are ergosterol (I), ergosterol-5,8-endoperoxide (II), and the previously undescribed acremine S (III), acremine T (IV), in addition to lumichrome (V). The structures of the isolated compounds were established by extensive analysis of the 1D and 2D NMR spectra, including ^1H , ^{13}C NMR, DEPT, COSY, HSQC and HMBC as well as HRESIMS data. In the case of the previously described compounds, comparison of their ^1H and ^{13}C NMR data with those reported in the literature was used to confirm their identity. In the case of acremine S, whose structure contains three stereogenic centers, the absolute configurations of their stereogenic carbons were established by comparison of the calculated and experimental ECD spectra with the aid of ROESY correlations.

Compounds III, IV and V were evaluated for their inhibitory activities toward the enzymes tyrosinase and cholinesterases by colorimetric methods, using quercetin and kojic acid (for antityrosinase assay), and galantamine (for AChE and BChE assays) as positive controls. All the compounds tested showed activities in both assays. In the antityrosinase assay, acremine T (IV) was found to be more active, with an enzymatic inhibition of approximately 55% (21.12 μM), while acremine S (III) also displayed, although weaker, interesting inhibitory activity (25% at 21.12 μM). On the other hand, for the anticholinesterase assays lumichrome (V) was found to inactivate AChE (39.23% of inhibition at 6.6 μM) while acremine S (III) showed strong inhibitory activity toward BChE (30.71% inhibition at 6.25 μM).

Of note is that although acremine T (IV) was not the strongest acetylcholinesterase inhibitor among the tested compounds, it was the most promising compound since it has a dual activity, i.e. antityrosinase and acetylcholinesterase inhibitory activities.

Numerous natural compounds, isolated from marine organisms, have already been in use for skin-care products. It should be noted that marine-derived fungi are among the marine organisms with the greatest potential for the production of bioactive compounds which can be exploited for various commercial purposes including cosmetics and cosmeceuticals. Numerous studies have demonstrated the isolation of promising tyrosinase inhibitors,

many of which have better activity than the positive control described in the literature. Likewise, the results obtained in this study reinforce this idea that marine-derived fungi can be a promising source of compounds with potential antityrosinase and anticholinesterase activities.

Chapter 6 – Appendix

6.1 NMR spectra of isolated compounds

6.1.1 NMR spectra of ergosterol (I)

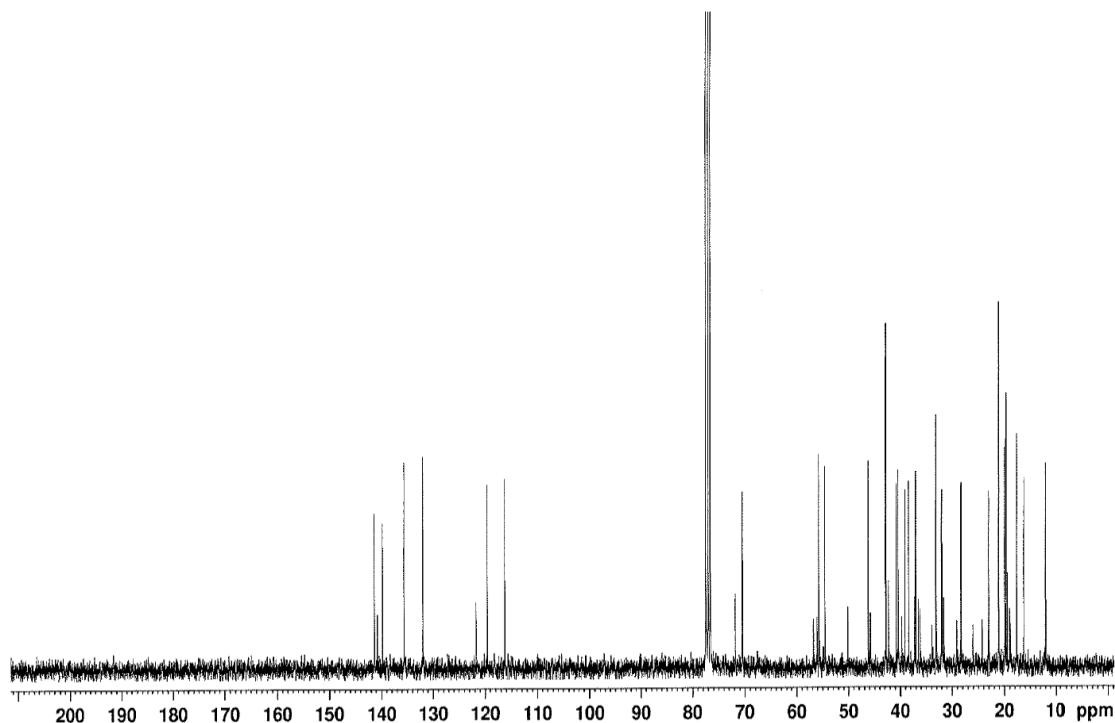


Figure 38 - ^{13}C NMR spectrum of I (CDCl_3 , 75.4 MHz)

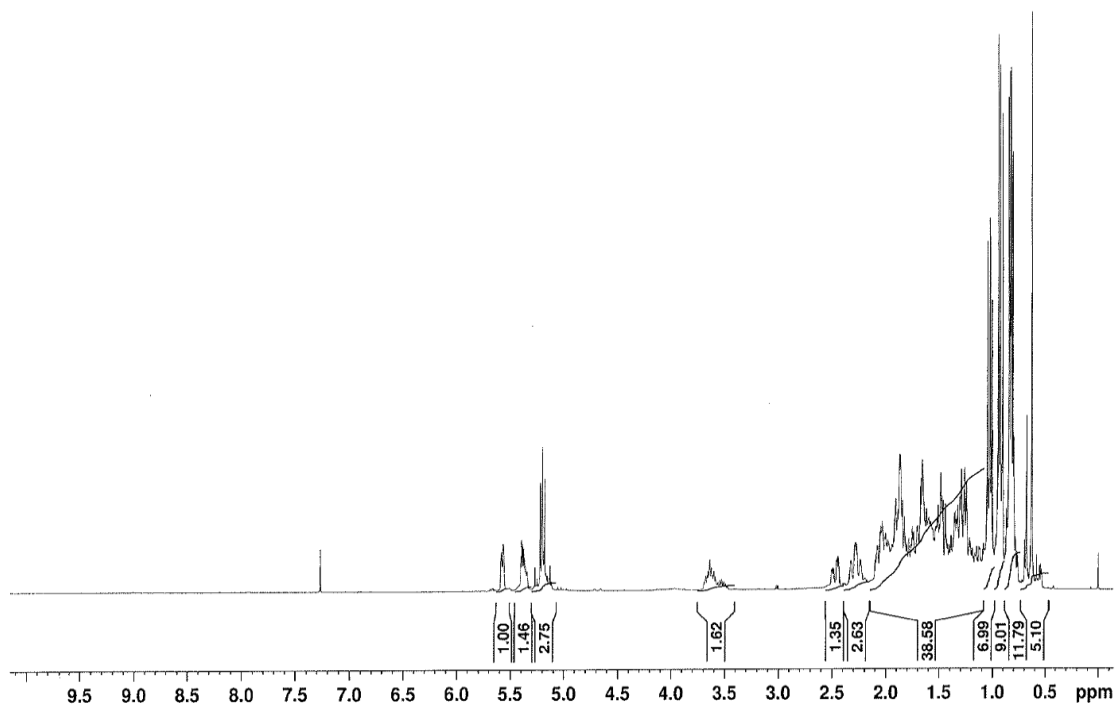


Figure 39 - ^1H NMR spectrum of I (CDCl_3 , 300.13 MHz)

6.1.2 NMR spectra of ergosterol-5,8-endoperoxide (II)

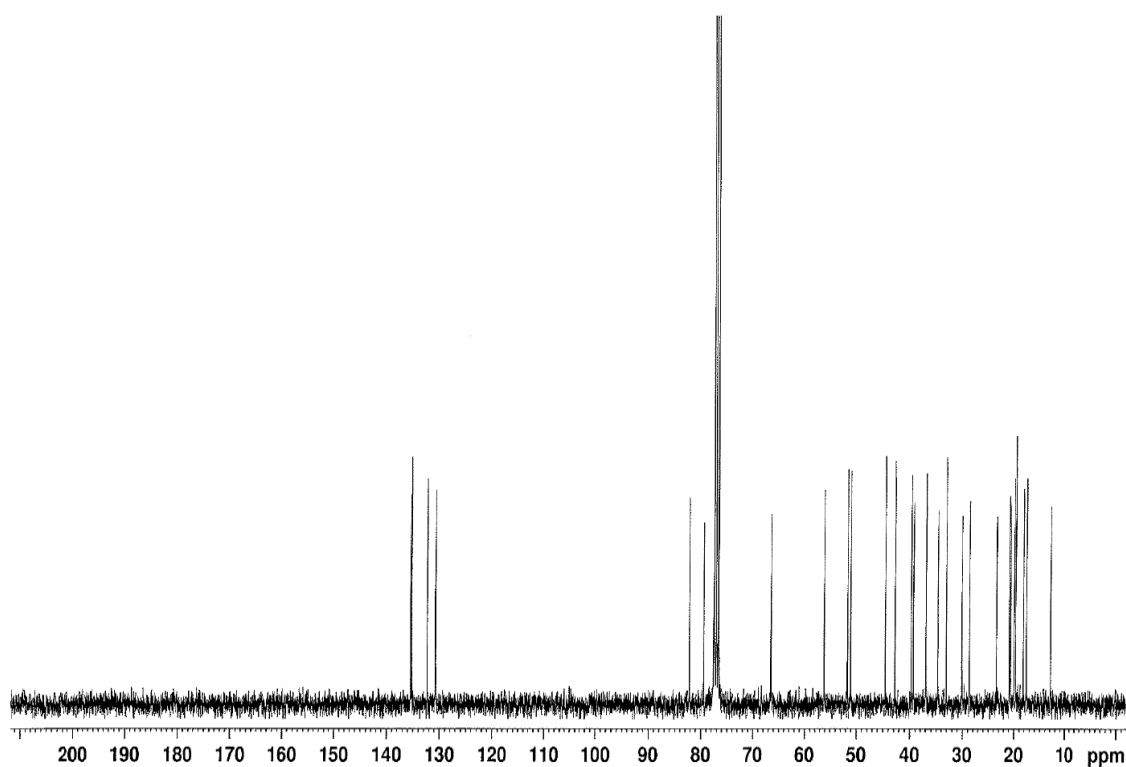


Figure 40 - ^{13}C NMR spectrum of II (CDCl_3 , 75.4 MHz)

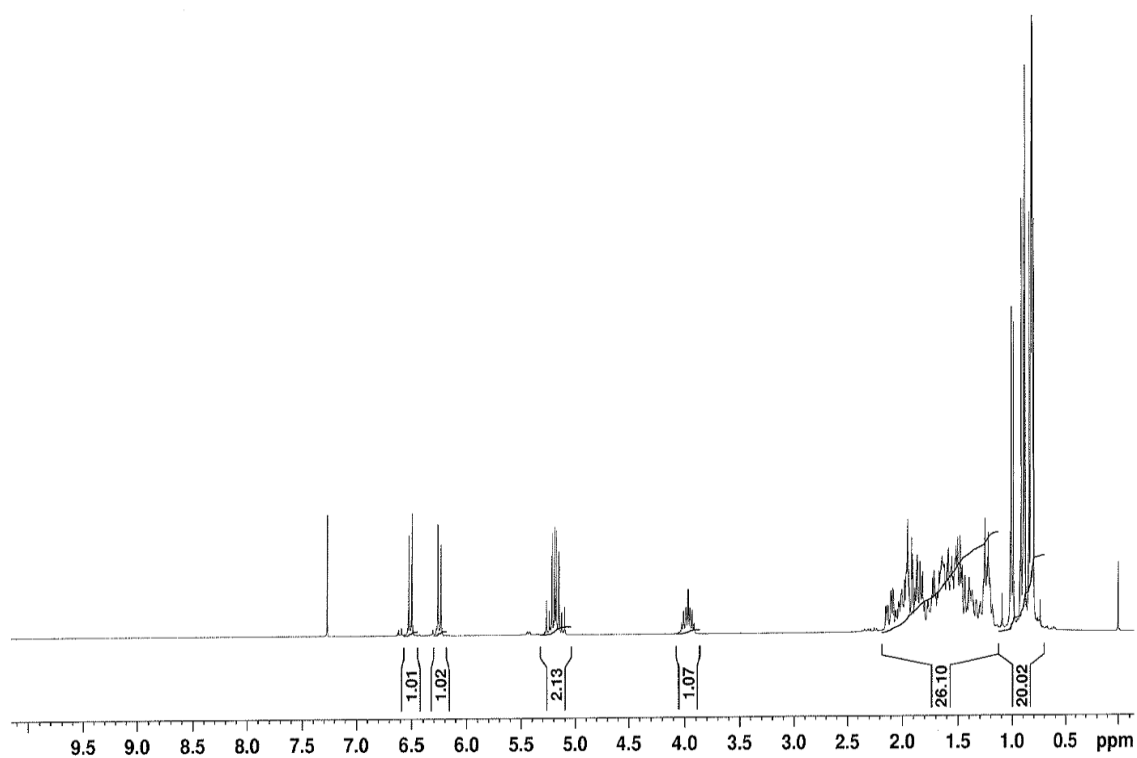


Figure 41 - ^1H NMR spectrum of II (CDCl_3 , 300.13 MHz)

6.1.3 NMR spectra of acremine S (III)

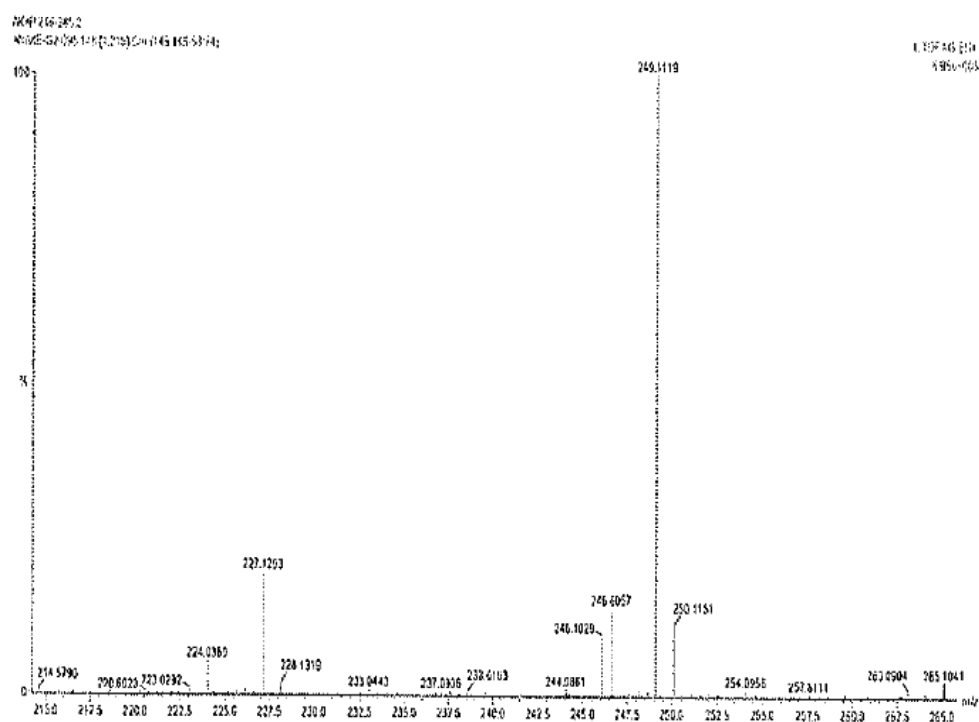


Figure 42 - (+)-HRESIMS spectrum of III

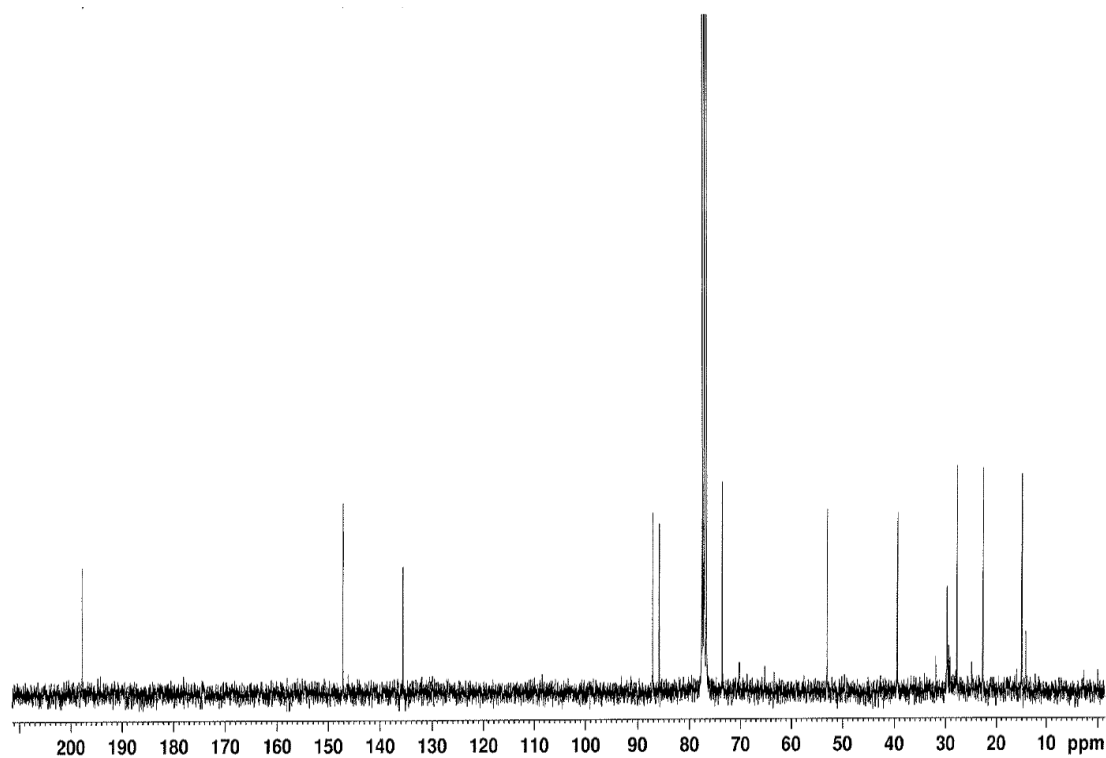


Figure 43 - ¹³C NMR spectrum of III (CDCl₃, 75.4 MHz)

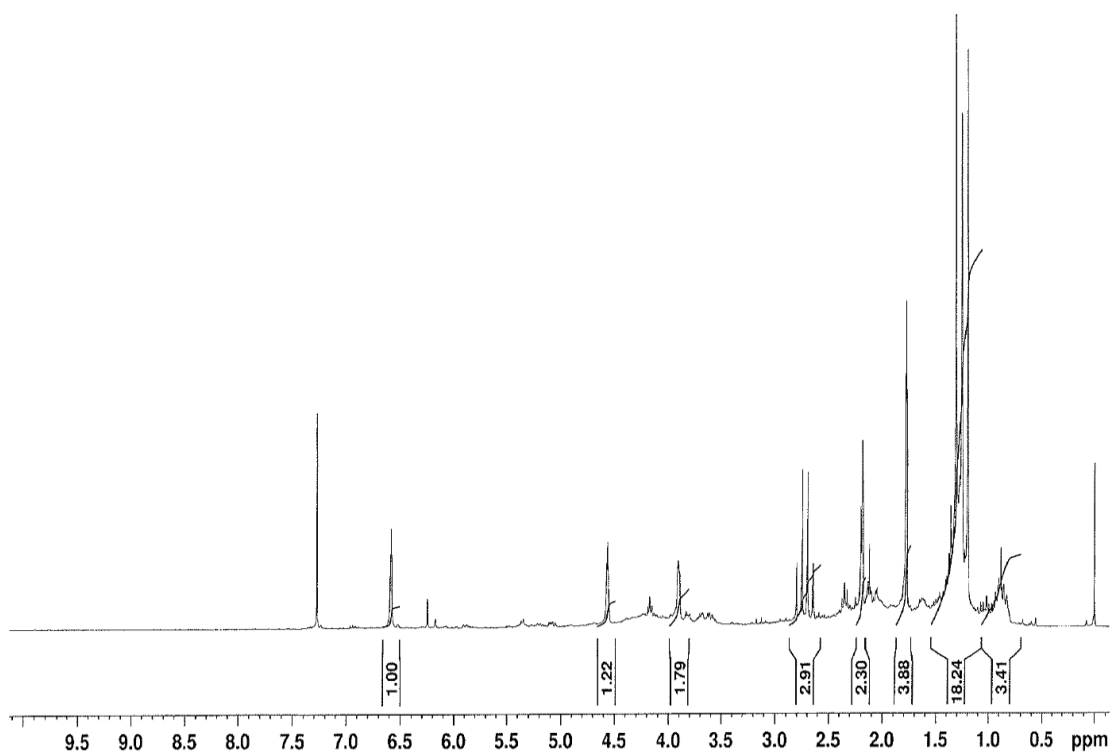


Figure 44 - ^1H NMR spectrum of **III** (CDCl_3 , 300.13 MHz)

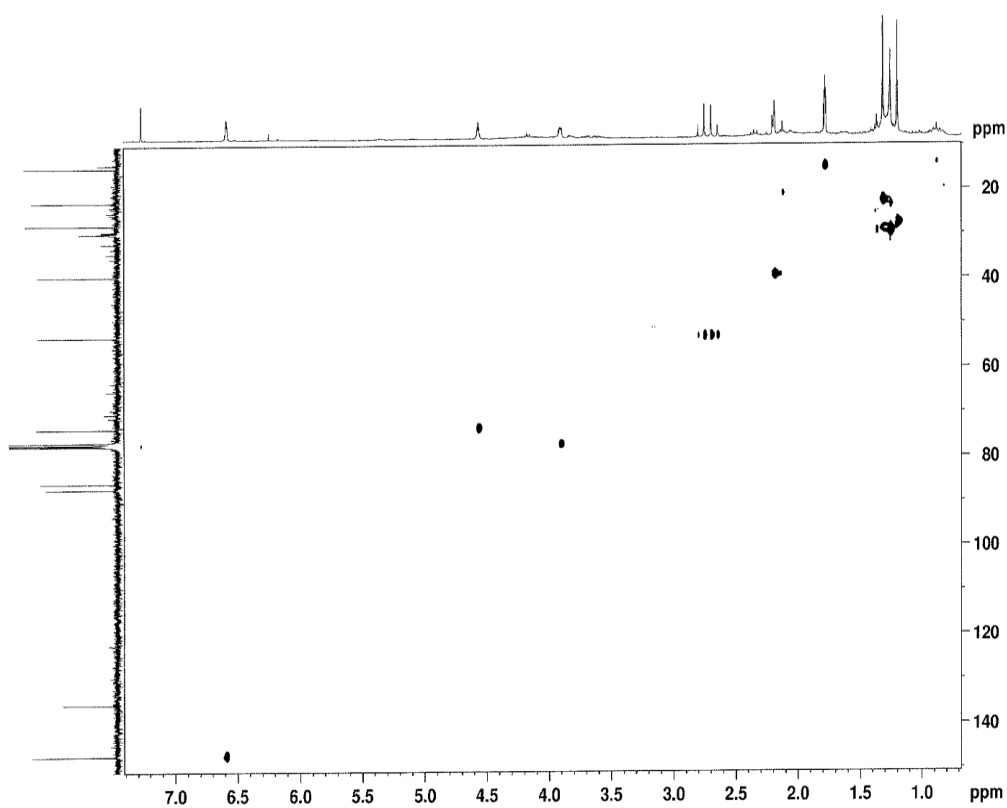


Figure 45 - HSQC spectrum of **III** (CDCl_3 , 300.13 MHz)

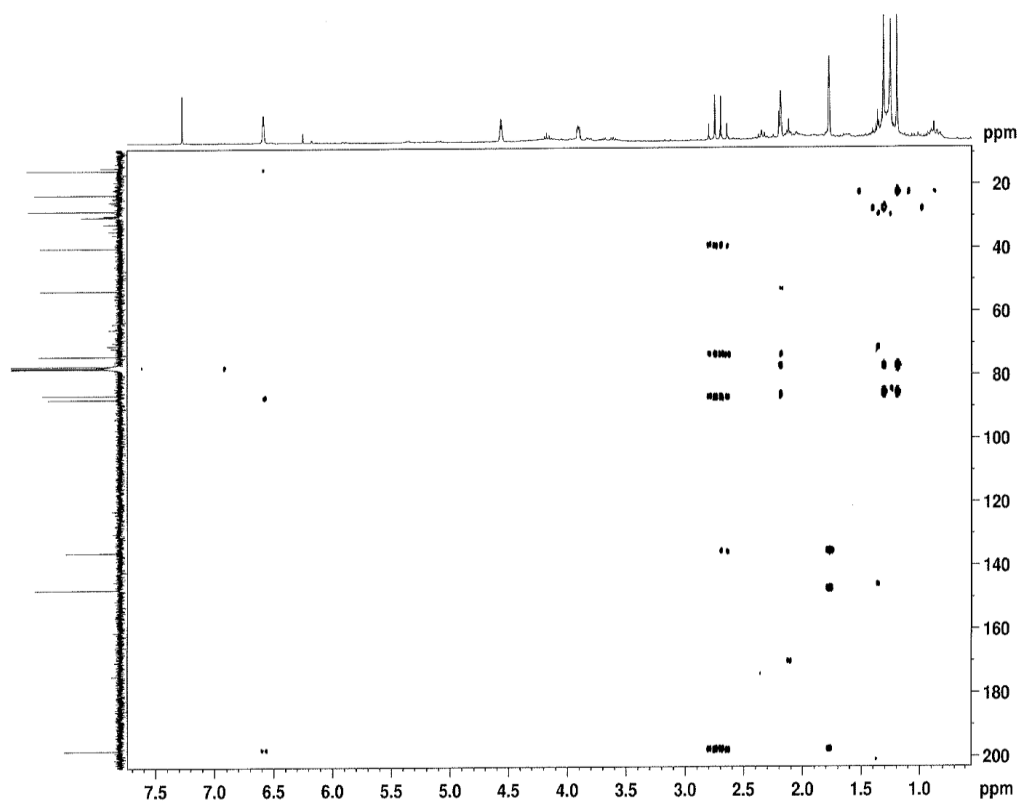


Figure 46 - HMBC spectrum of III (CDCl_3 , 300.13 MHz)

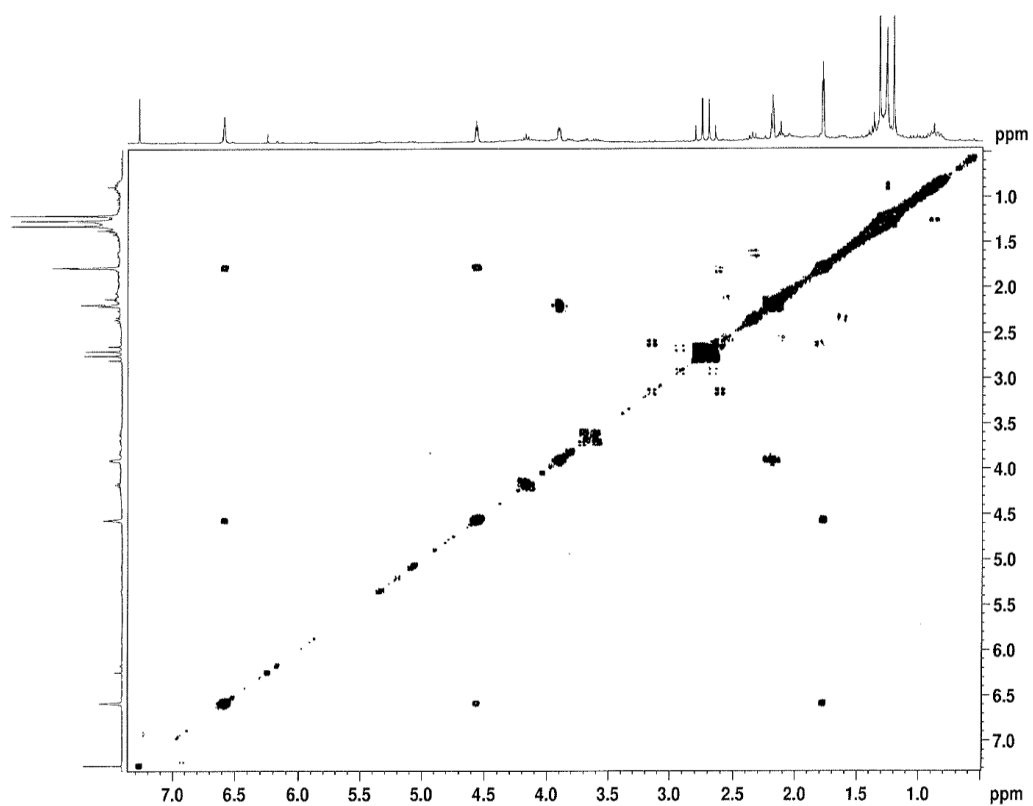


Figure 47 - COSY spectrum of III (CDCl_3 , 300.13 MHz)

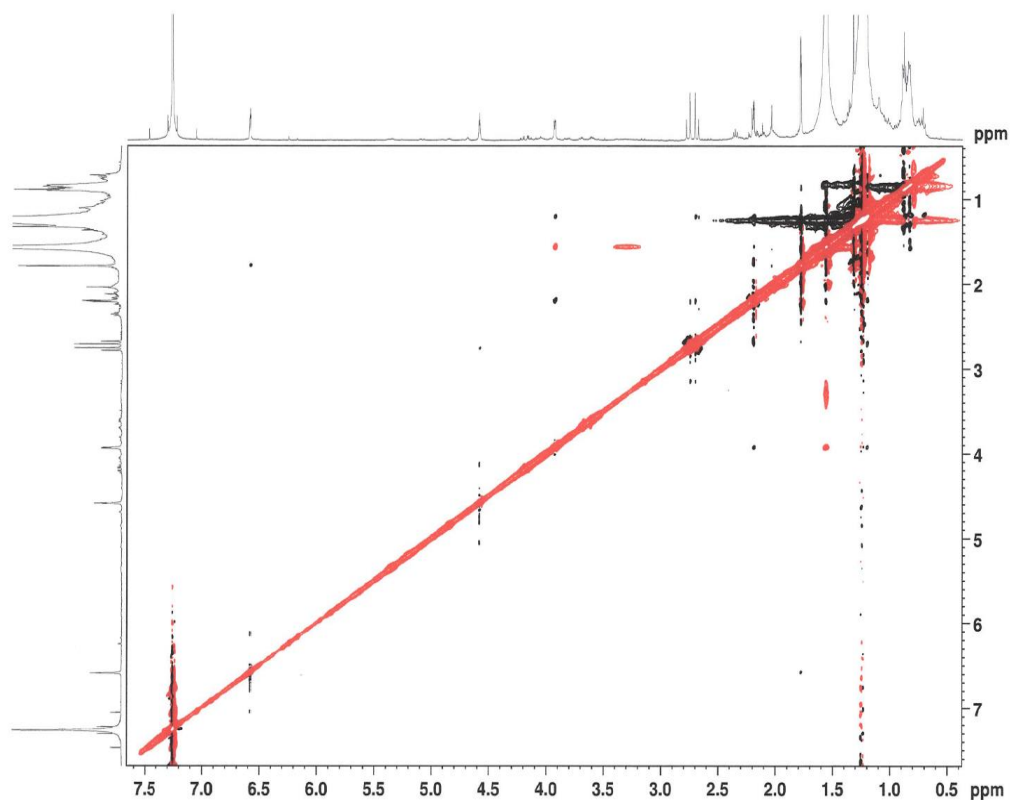


Figure 48 - ROESY spectrum of **III** (CDCl_3 , 500.16 MHz)

6.1.4 NMR spectra of acremine T (**IV**)

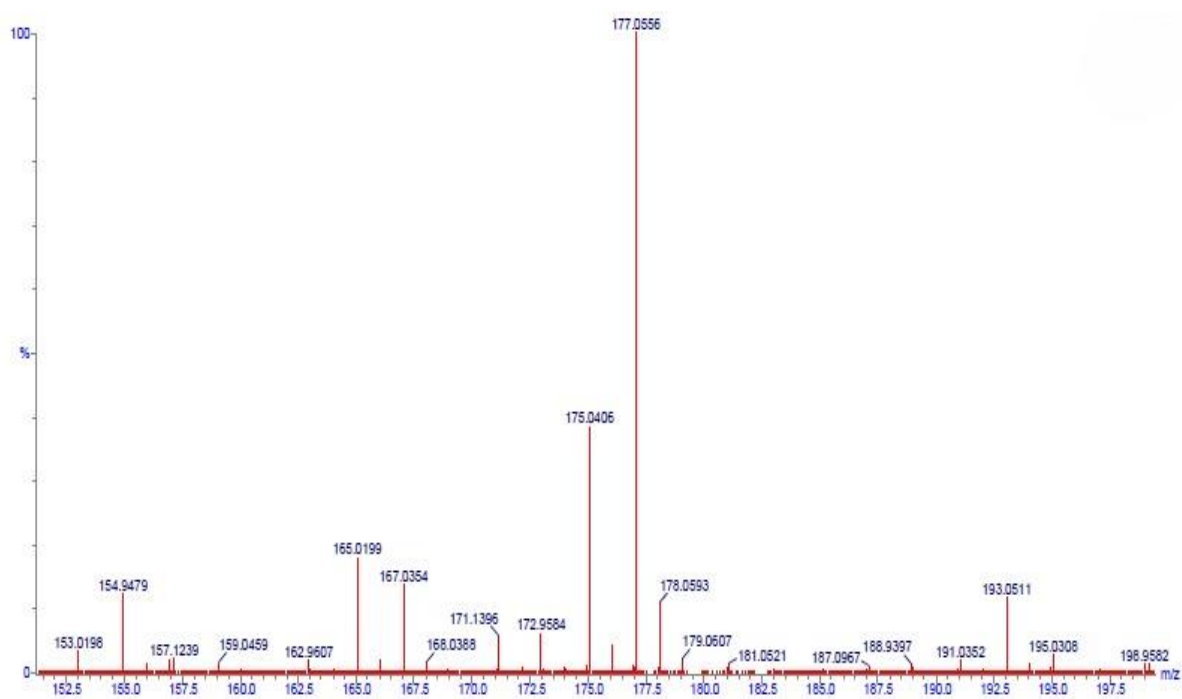


Figure 49 - (-)-HRESIMS spectrum of **IV**

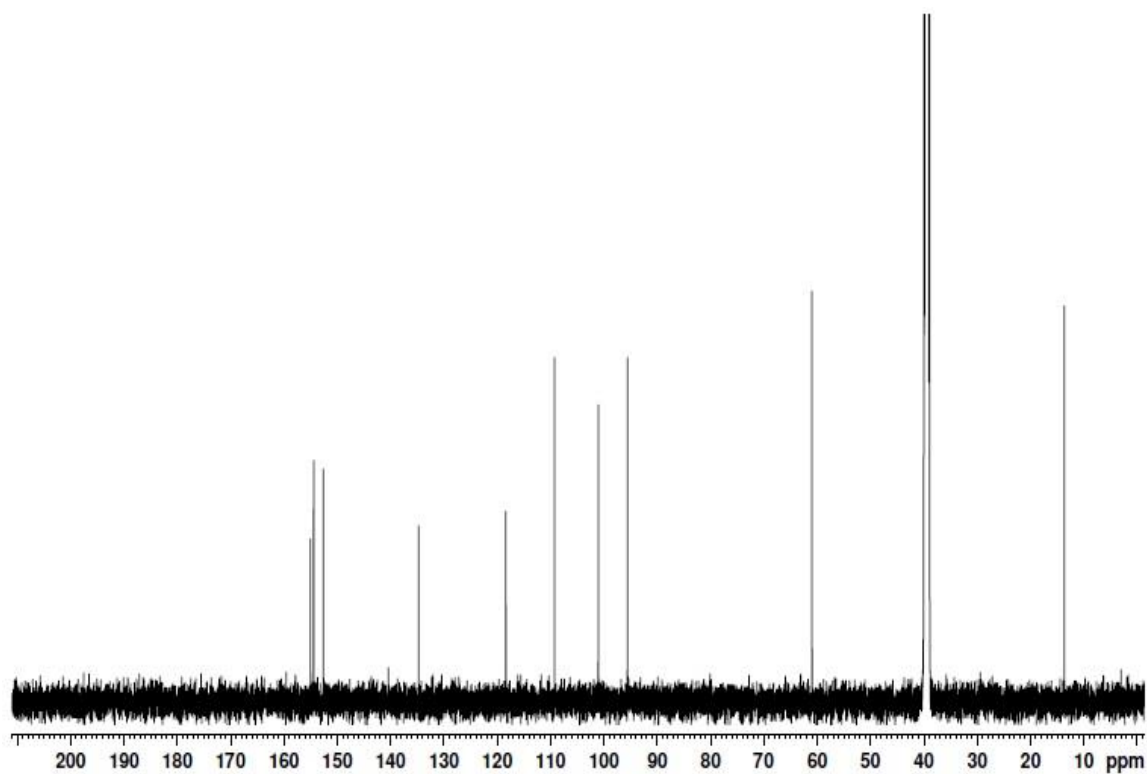


Figure 50 - ^{13}C NMR spectrum of IV (DMSO- d_6 , 75.4 MHz)

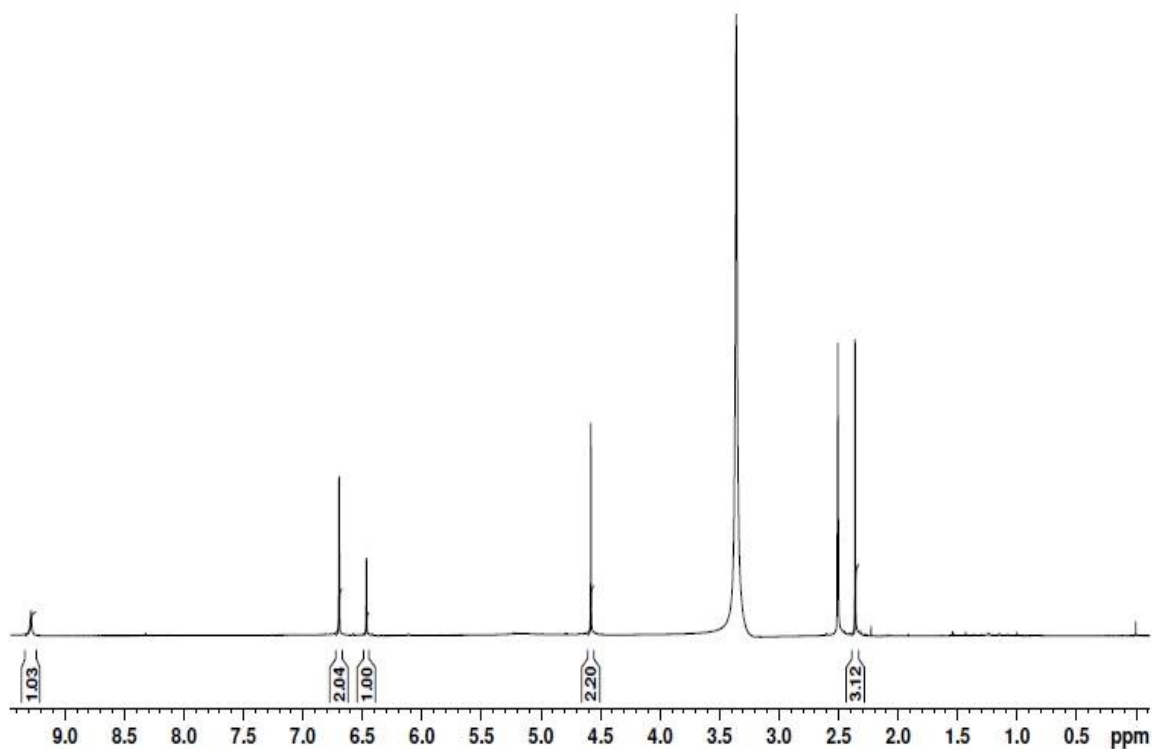


Figure 51 - ^1H NMR spectrum of IV (DMSO- d_6 , 300.13 MHz)

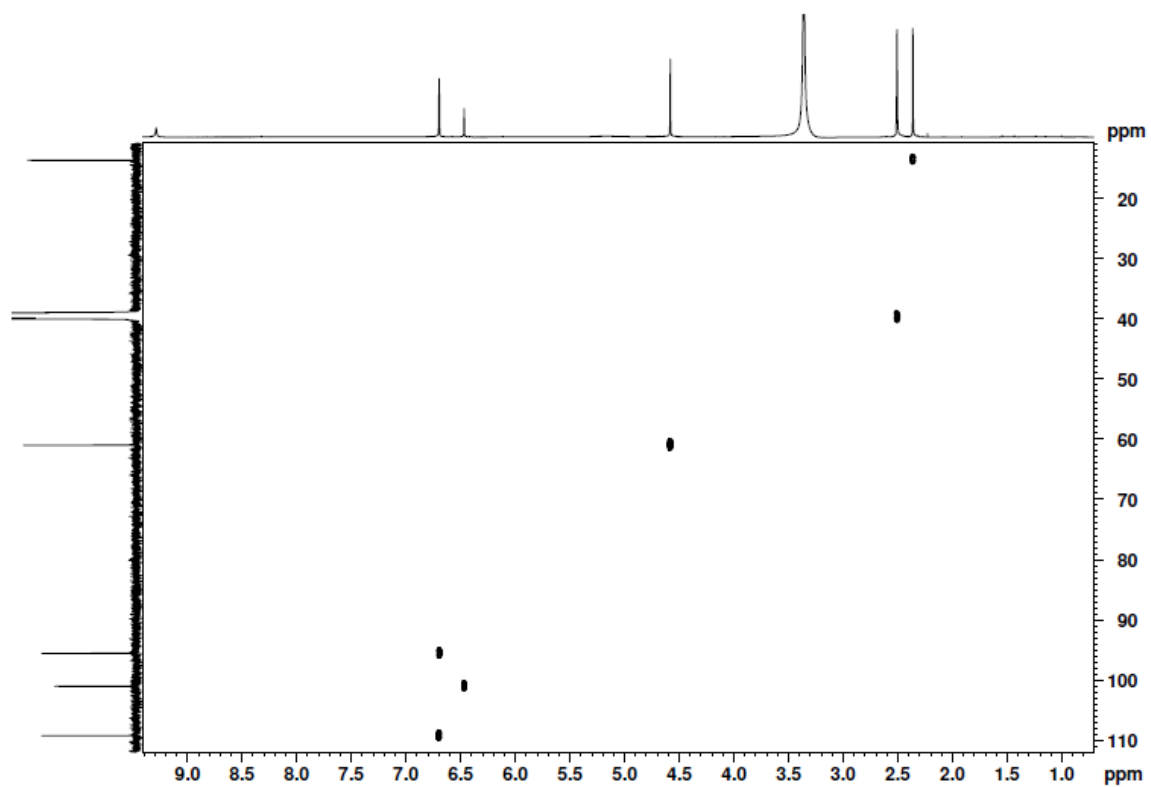


Figure 52 - HSQC spectrum of IV (DMSO-*d*₆, 300.13 MHz)

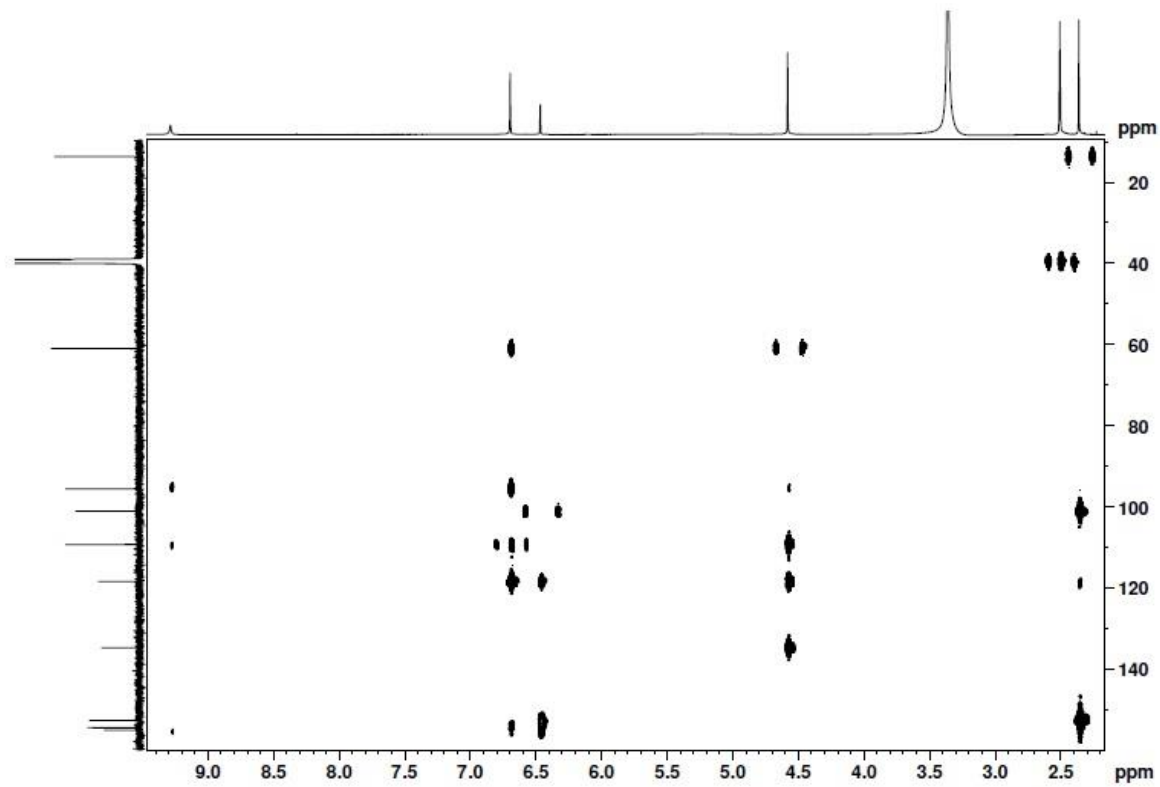


Figure 53 - HMBC spectrum of IV (DMSO-*d*₆, 300.13 MHz)

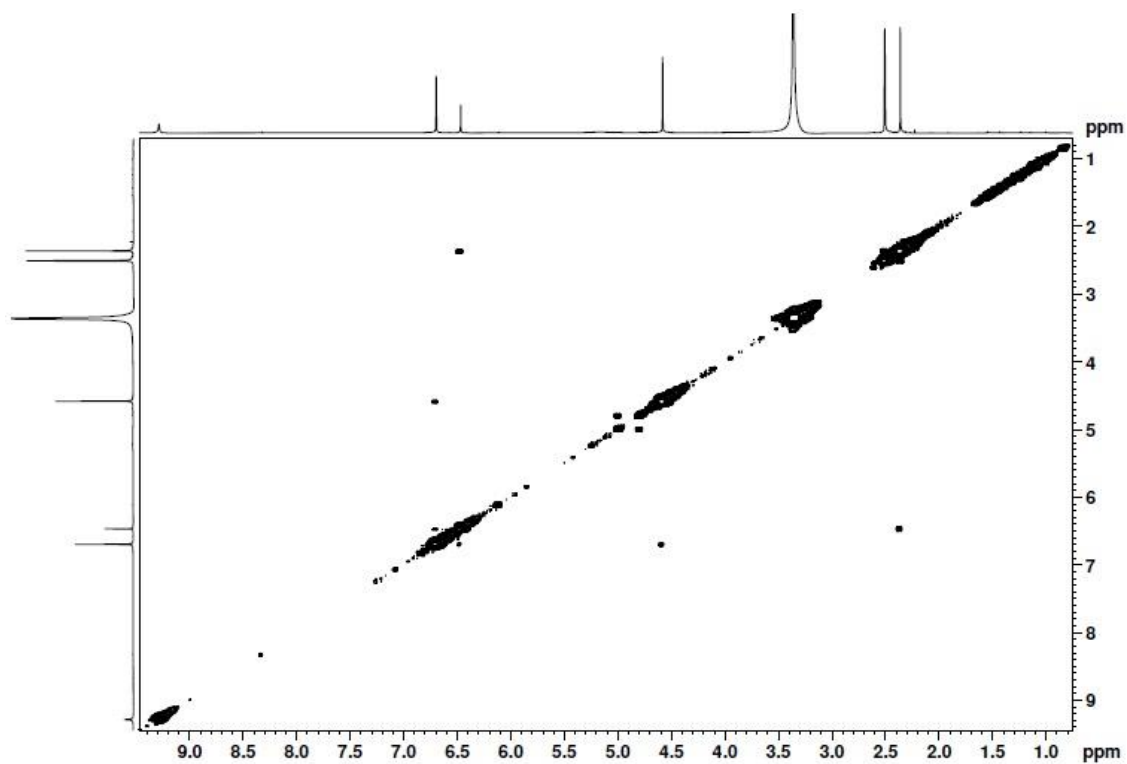


Figure 54 - COSY spectrum of **IV** (DMSO-*d*₆, 300.13 MHz)

6.1.5 NMR spectra of lumichrome (**V**)

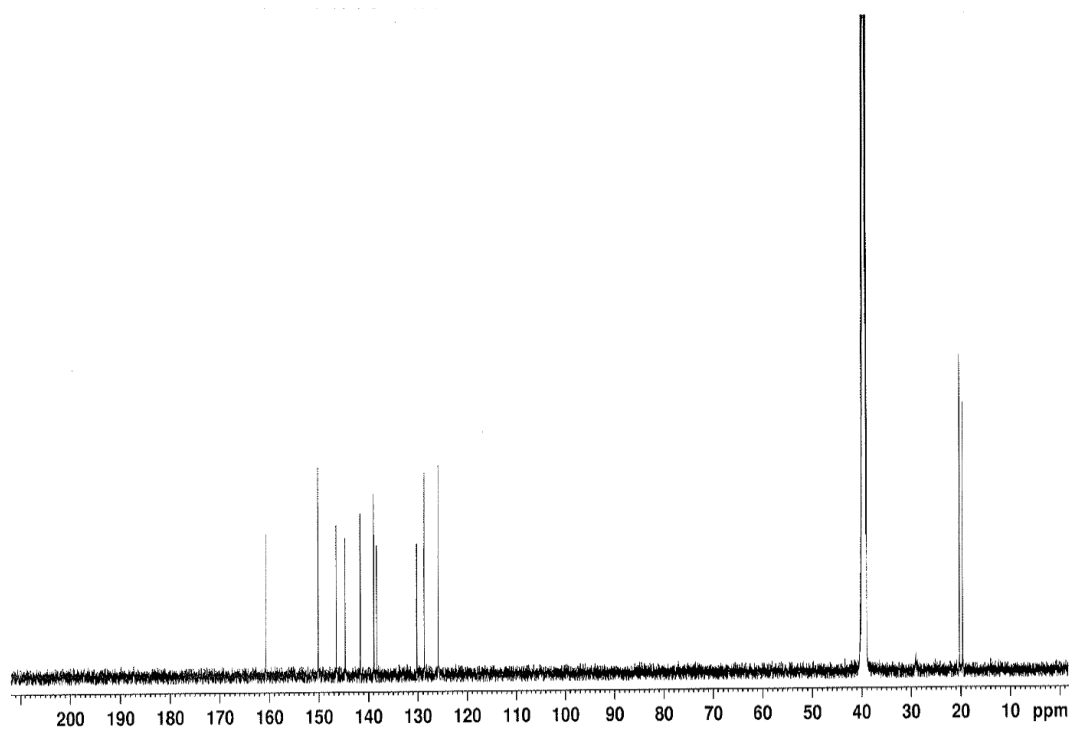


Figure 55 - ¹³C NMR spectrum of **V** (DMSO-*d*₆, 125.7 MHz)

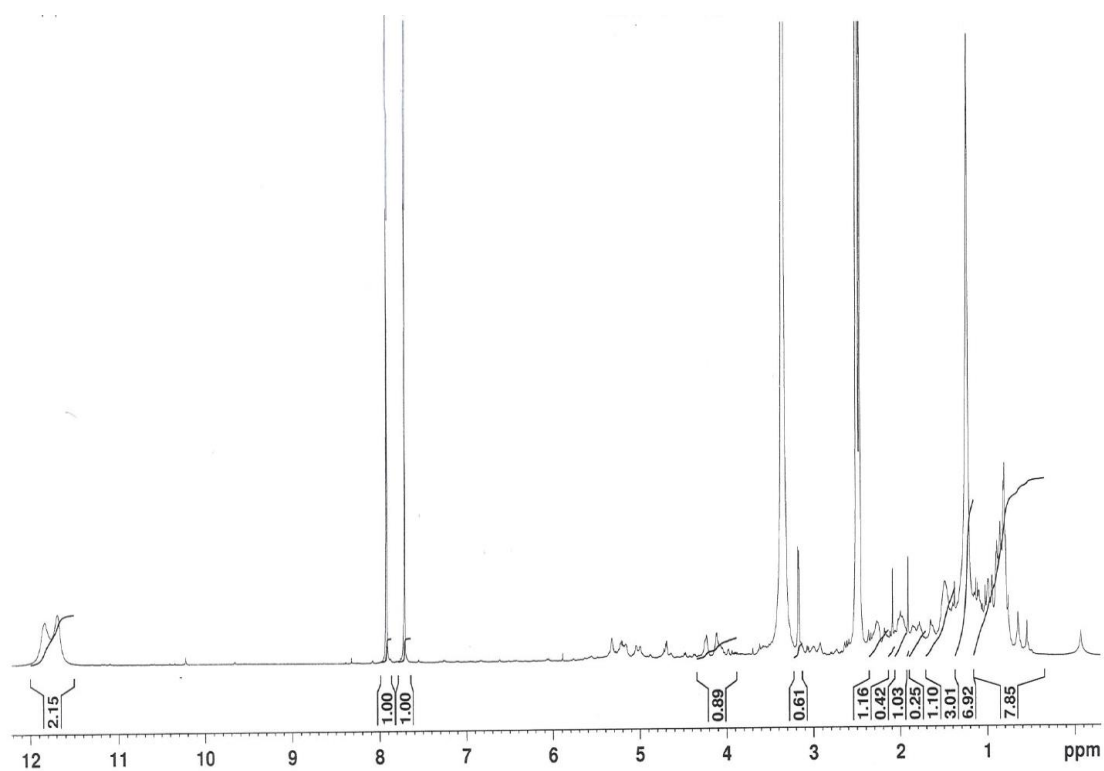


Figure 56 - ^1H NMR spectrum of **V** ($\text{DMSO-}d_6$, 500.16 MHz)

6.2 Published article



Article

A New Meroterpene, A New Benzofuran Derivative and Other Constituents from Cultures of the Marine Sponge-Associated Fungus *Acremonium persicinum* KUFA 1007 and Their Anticholinesterase Activities

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Abstract: Previously unreported meroterpene, acremine S (**1**), and benzopyran derivative, acremine T (**2**), were isolated, together with lumichrome (**3**), ergosterol (**4**) and ergosterol 5,8-endoperoxide, from cultures of the marine sponge-associated fungus *Acremonium persicinum* KUF1007. The structure of the previously unreported compounds was established based on an extensive analysis of 1D and 2D NMR spectra as well as HRMS data. The absolute configurations of the stereogenic centers of **1** were established, unambiguously, based on NOESY correlations and comparison of calculated and experimental electronic circular dichroism (ECD) spectra. Compounds **1–3** were tested for their *in vitro* acetylcholinesterase and butyrylcholinesterase inhibitory activities.

Keywords: *Acremonium persicinum*; Hypocreaceae; marine sponge-associated fungus; meroterpene; acremine S; acremine T; benzofuran; anticholinesterase activities

1. Introduction

The genus *Acremonium* (Hypocreaceae) includes approximately 100 species and are known as saprobic on dead plants or soil dwellers [1]. Many species of this genus have been identified as producers of structurally and biologically diverse secondary metabolites [2] including hydroquinone inhibitors of neutral sphingomyelinase (N-SMase) [3], anti-phytopathogenic meroterpenoids [4–6], as well as the enzyme cellulase [7]. Marine-derived species of *Acremonium* have also been investigated for their secondary metabolites as exemplified by a sponge-derived *Acremonium* sp. which produced anti-inflammatory sesquiterpenoids [8], and a mangrove-derived *Acremonium* sp. which produced phthalide and isocoumarin derivatives [9]. Concerning *Acremonium persicinum*, both marine-derived and soil-derived strains have shown to be interesting sources of structurally variable secondary

metabolites. Suciati et al. [10] reported the isolation of several previously described acremines and spiroacremines as well as new acremines and chloroacremines from *A. persicinum*, isolated from the marine sponge *Anomoianthella rubra*. Recently, Nakamura et al. [11] reported a novel antifungal hexapeptide, ASP2397, from *A. persicinum* MF-347833, isolated from leaf litter, collected at Endau Rompin National Park in Malaysia, while Wang et al. [12] described the isolation of four peptaibiotics, acremotins A–D, from the culture of the soil-derived fungus *A. persicinum* SC0105.

Recently, much attention has been paid to marine-derived compounds with potential for treatment of neurological disorders such as Alzheimer's diseases (AD) [13]. Acetylcholinesterase (AChE) and butyrylcholine esterase (BuChE), which break down acetylcholine and butyrylcholine, are considered as promising targets in the management of AD. Surprisingly, only a few novel AChE inhibitors were reported from marine-derived fungi. Examples of these are the oxylipin, (8E, 12Z)-10,11-dihydroxyoctadeca-8,12-dienoic acid, and a steroid, 3 β ,4 α -dihydroxy-26-methoxyergosta-7,24(28)-diene-6-one, obtained from the cultures of an endophytic fungus, *Aspergillus flavus*, which possessed low activity to inhibit AChE [14]. On the other hand, Wu et al. [15] have found that the oxaphenalenone dimers, talaromycesones A and B, as well as the isopentenyl xanthenone, talaroxanthenone, displayed potent acetylcholinesterase inhibitory activities. Liu et al. [16] have found that the ethyl acetate extract of the cultures of *Aspergillus ochraceus* SH0701, isolated from the sea sediment in China, potentially inhibited acetylcholinesterase; however, they did not isolate the compounds from this active extract.

Inspired by these few and recent findings, thus, in our ongoing search for new bioactive compounds from marine-derived fungi, from the Gulf of Thailand, we investigated secondary metabolites from the cultures of *A. persicinum* KUFA1007, which was isolated from the marine sponge *Mycale* sp., collected from the coral reef at Samaesarn Island, Chonburi Province, in the Gulf of Thailand. The isolated compounds were assayed for their capacity to inhibit the enzymes AChE and BuChE.

Fractionation of the ethyl acetate crude extract of the cultures of *A. persicinum* KUFA007 by column chromatography, followed by purification by crystallization, preparative TLC and Sephadex LH-20 column, furnished previously undescribed spiroacremines (1) and benzofuran derivative (2), in addition to the previously reported lumichrome (3), ergosterol (4) and ergosterol 5,8-endoperoxide (5) (Figure 1). The structures of the previously undescribed compounds were established based on extensive analysis of their 1D and 2D NMR as well as HRMS data while the identity of the known compounds were elucidated by comparison of their ^1H and ^{13}C NMR data with those of the authentic samples as well as from the literature. Compounds 1–3 were tested for their anticholinesterase activity against AChE and BuChE by the modified Ellman's method.

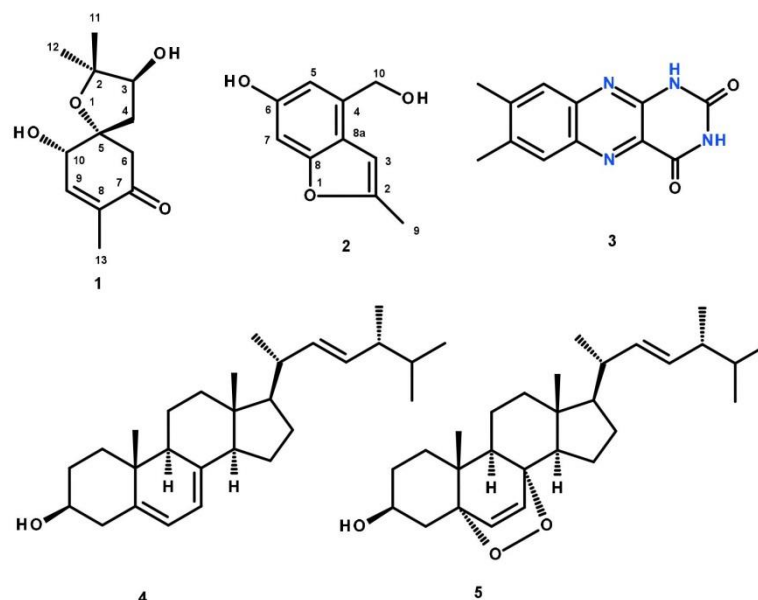


Figure 1. Structures of secondary metabolites isolated from the cultures of the marine sponge-associated fungus *Acremonium persicinum* KUFA1007.

2. Results and Discussion

The structures of lumichrome (3) [17–19], ergosterol (4) [20], and ergosterol 5,8-endoperoxide (5) [21] (Figure 1) were elucidated by analysis of their 1D and 2D NMR spectra as well as HRMS data, and also by comparison of their spectral data (Figures S23–S33, Table S1) with those reported in the literature.

Compound 1 was isolated as a yellowish viscous oil, and its molecular formula $C_{12}H_{18}O_4$ was established based on the (+)-HRESIMS m/z 227.1293 $[M + H]^+$ (calculated 227.1283) (Figure S10), indicating four degrees of unsaturation. The ^{13}C NMR spectrum, in combination with DEPT and HSQC spectra (Table 1, Figures S2–S4 and S6), exhibited twelve carbon signals, which can be categorized as one conjugated ketone carbonyl (δ_C 197.8), one quaternary sp^2 (δ_C 135.6), one methine sp^2 (δ_C 147.2), two oxyquaternary sp^3 (δ_C 87.1 and 85.8), two oxymethine sp^3 (δ_C 77.3 and 73.6), two methylene sp^3 (δ_C 53.0 and 39.4) and three methyl (δ_C 27.8, 22.6 and 15.0) carbons. The ^1H NMR spectrum (Table 1, Figure S1), in combination with the HSQC spectrum (Table 1, Figure S6), revealed the presence of a triplet of an olefinic proton at δ_H 6.58 ($J = 1.6$ Hz; δ_C 147.2), a triplet of an oxymethine proton at δ_H 4.56 ($J = 2.1$ Hz; δ_C 73.6), a double doublet of another oxymethine proton at δ_H 3.90 ($J = 4.3, 1.9$ Hz; δ_C 77.3), two doublets of geminally coupled methylene protons at δ_H 2.67 ($J = 15.5$ Hz; δ_C 53.0) and δ_H 2.77 ($J = 15.5$ Hz; δ_C 53.0), a multiplet of two methylene protons at δ_H 2.20 (δ_C 39.4), a double doublet of methyl protons at δ_H 1.78 ($J = 2.1, 1.6$ Hz, δ_C 39.4), and two methyl singlets at δ_H 1.31 (δ_C 22.6) and δ_H 1.20 (δ_C 27.8), respectively. The COSY spectrum (Table 1, Figure S5, Figure 2) displayed correlations from the olefinic proton at δ_H 6.58 ($J = 1.6$ Hz, H-9) to the triplet at δ_H 4.56 ($J = 2.1$ Hz; H-10) and the methyl double doublet at δ_H 1.78 ($J = 2.0, 1.6$ Hz, Me-13), while the HMBC spectrum (Table 1, Figure S7, Figure 2) showed correlations from H₃-13 to the carbonyl carbon at δ_C 197.8 (C-7) and the olefinic carbons at δ_C 147.2 (C-9) and 135.6 (C-8), H-9 to C-7, the oxyquaternary sp^3 carbon at δ_C 87.1 (C-5) and the methyl carbon at δ_C 15.0 (Me-13), the doublets at δ_H 2.67 and 2.77 (H₂-6)

to the carbon at δ_C 73.6 (C-10), C-8, C-7 and C-5. Taking together the ^1H and ^{13}C chemical shift values and the COSY and HMBC correlations, the partial structure of the molecule was established as 4-hydroxy-2-methylcyclohex-2-en-1-one. Another portion of the molecule was evidenced to be 2,2-dimethyloxolan-3-ol by the COSY correlation (Table 1, Figure 2 and Figure S5) from the multiplet at δ_H 2.20 (H₂-4) to the double doublet at δ_H 3.90 (H-3), as well as the HMBC correlations (Table 1, Figure 2 and Figure S7) from H₂-4 to the carbon signals at δ_C 77.3 (C-3), 85.8 (C-2), C-5, the methyl singlet at δ_H 1.20 (H₃-11) to C-2, C-3 and the methyl carbon at δ_C 22.6 (Me-12), the methyl singlet at δ_H 1.31 (H₃-12) to C-2, C-3 and the methyl carbon at δ_C 27.8 (Me-11). That the 2,2-dimethyloxolan-3-ol was *spiro*-fused with the 4-hydroxy-2-methylcyclohex-2-en-1-one moiety at C-5 was corroborated by the HMBC correlation (Table 1, Figure S7, Figure 2) from H₂-4 to C-5, C-6 and C-10 as well as from H₂-6 to C-4 and C-5. Taken together the two partial structures and the HMBC correlations, the planar structure of **1** was established as 3,10-dihydroxy-2,2,8-trimethyl-1-oxospiro[4.5]dec-8-en-7-one.

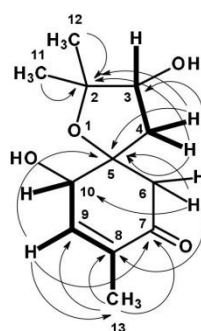


Figure 2. Key COSY (bold line) and HMBC (arrow) correlations in **1**.

Table 1. ^1H and ^{13}C NMR (CDCl₃, 300.13 and 75.4 MHz) and HMBC assignment for **1**.

Position	δ_C , Type	δ_H , (J in Hz)	COSY	HMBC
2	85.8, C	-	-	
3	77.3, CH	3.90, dd (4.3,1.9)	H-4	
4	39.4, CH ₂	2.20, m	H-3	C-2,3,5,6,10
5	87.1, C	-	-	
6a	53.0, CH ₂	2.67, d (15.5)	H-6b	
6b		2.77, d (15.5)	H-6a	C-4,5,7,8,10
7	197.8, CO	-	-	
8	135.6, C	-	-	
9	147.2, CH	6.58, t (1.6)	H-10, 13	C-5,7,13
10	73.6, CH	4.56, t (2.1)	H-9, 13	
11	27.8, CH ₃	1.20, s	-	C-2,3,12
12	22.6, CH ₃	1.31, s	-	C-2,8,11
13	15.0, CH ₃	1.78, dd (2.1,1.6)	H-9, 10	C-7,8,9

Structurally, **1** possesses three stereogenic carbons, i.e., C-3, C-5 and C-10. Since **1** was obtained as a viscous oil, it was not possible to obtain a suitable crystal to determine the absolute configurations of its stereogenic carbons by X-ray analysis. On the other hand, since **1** has an enone chromophore, the absolute configurations of its stereogenic carbons were established by comparison of the calculated and experimental electronic circular dichroism (ECD) spectra. Compound **1** has three stereogenic centers

and, therefore, eight possible configurations. Conformational analysis revealed 36 molecular mechanics (MM) conformational energy minima for each configuration, by combining two conformations for each ring and three conformations for each of the two hydroxyl groups. To find the most significant conformations of each configuration, MM models were re-minimized using a quantum mechanical density functional theory (DFT) method coupled with a small basis set. Then, the most populated (lowest energy) conformations, spanning a window of 2 kcal/mol, were further refined using the same DFT method but with a large basis set. The first 50 ECD transitions were then calculated (TD-DFT) for each model. Comparison of the experimental ECD spectrum with the eight final calculated spectra, one for each configuration, revealed a better match with the spectrum obtained from the (3*S*, 5*R*, 10*S*) model (Figures 3 and 4).

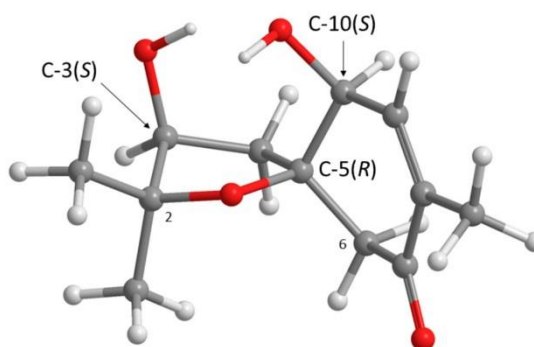


Figure 3. Most abundant conformation of **1** (APFD/6-311+G(2d,p) lowest energy conformer) in its (3*S*, 5*R*, 10*S*) configuration.

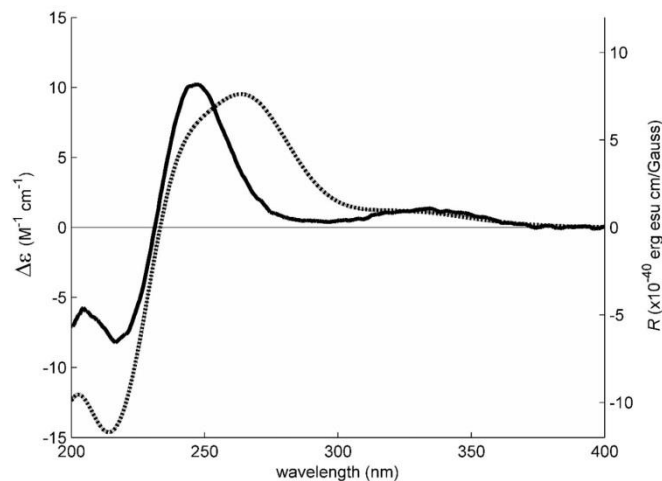


Figure 4. Experimental electronic circular dichroism (ECD) spectrum (solid line, left axis) of **1** in methanol and theoretical ECD spectrum (dotted line, right axis) of its (3*S*, 5*R*, 10*S*) configuration.

The structure of **1** can be considered as an analogue of acremine O (compound **6** in Ref. [10]), where C-4 is oxidized to a ketone carbonyl and C-5 is chlorinated (according to the numbering in

Ref. [10]). Although the absolute configuration of C-5 in **1** is the same as that of acremine O (C-3 in Ref. [10]), the absolute configuration of C-3 in **1** is opposite to that of acremine O (C-8 in Ref. [10]). Intrigued by this discrepancy, we obtained the ROESY spectrum of **1** and compared its data to what would be expected from the energy-optimized conformations of the (3*S*, 5*R*, 10*S*) and (3*R*, 5*R*, 10*S*) DFT models of **1**. The ROESY spectrum (Figures S8 and S9) exhibited correlations from H-3 to H₂-4 and H₃-11, whereas H₃-11 exhibited correlations not only to H₂-4 but also to H-6 at δ_H 2.67 (d, J = 15.5 Hz). These ROESY correlations implied that H-3, Me-11 and H-6 (at δ_H 2.67) are on the same face. That means OH-3 and Me-12 are on the opposite face to Me-11 and H-3. On the other hand, H-10 exhibited a weak correlation to H-6 at δ_H 2.77 (d, J = 15.5 Hz), suggesting that they were on the same face. The difference in the chemical shift values of H₂-6 was attributed to the anisotropic effect of the carbonyl group (C-7) of the cyclohexanone ring. One of H₂-6 (δ_H 2.67, d, J = 15.5 Hz) was deshielded when compared to another (δ_H 2.77, d, J = 15.5 Hz), implying that the former was in the equatorial position (H-6 β) while the latter was in the axial position (H-6 α) (Figure 5).

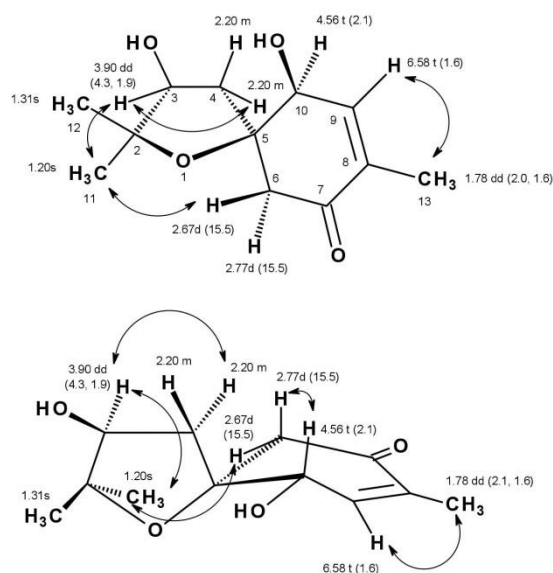


Figure 5. Key ROESY correlations in **1**.

These ROESY correlations are compatible with the (3*S*, 5*R*, 10*S*) model because, in this model, all these nuclei are on the same side of the five-membered ring and near enough to observe NOE signals (Figure 3). Inversion of the configuration of C-3 from (*S*) to (*R*) puts H-3 and H-6 (δ_H 2.77, d, J = 15.5 Hz) on opposite ringsides (and a bit further from the C-2 methyl groups). Therefore, according to our C-3(*R*) configuration model, H-3 and H-6 (δ_H 2.67, d, J = 15.5 Hz) are expected to show (probably weak) NOE cross peaks to both Me-11 and Me-12. Since H-3 and H-6 (δ_H 2.67, d, J = 15.5 Hz) are both observed to be near Me-11 and not Me-12, as inferred from ROESY data, we propose the (3*S*, 5*R*, 10*S*) configurations for **1**. Since **1** has never been previously reported, it was named acremine S, following the series of the prenylated polyketides, isolated from *Acremonium* species.

Compound **2** was isolated as a yellow amorphous solid. The ^1H NMR in DMSO- d_6 (Table 1, Figure S11) spectrum showed that it still contained some impurities. However, the major component displayed a broad singlet of a phenolic hydroxyl proton at δ_H 9.28, a singlet of two aromatic proton at δ_H 6.70, a broad singlet of an olefinic proton at δ_H 6.47, a triplet at δ_H 5.17 (J = 5.6 Hz, 1H), a doublet at

δ_H 4.58 ($J = 5.6$ Hz, 2H), a methyl doublet at δ_H 2.36 ($J = 1.0$ Hz). The ^{13}C NMR (Table 2, Figure S12) exhibited ten carbon signals which were categorized, by DEPT and HSQC spectra (Table 2, Figures S13, S14 and S16), as five non-protonated sp^2 (δ_C 155.0, 154.4, 152.6, 134.7 and 118.4), three protonated sp^2 (δ_C 109.3, 101.0 and 95.6), one oxymethylene sp^3 (δ_C 61.0) and one methyl (δ_C 13.7) carbons. The COSY spectrum (Table 2, Figure 6, Figure S15) displayed correlations from the singlet at δ_H 6.70 (H-5) to the doublet at δ_H 4.58 (H₂-10), from the hydroxyl triplet at δ_H 5.17 (OH-10) to H₂-10. The presence of a 2,3-substituted 5-hydroxybenzyl alcohol was substantiated by HMBC correlations (Table 2, Figure 6, Figure S17) from a broad singlet of the phenolic hydroxyl proton (OH-6) to C-5 (δ_C 109.3/ δ_H 6.70), C-7 (δ_C 95.6/ δ_H 6.70), C-6 (δ_C 154.4, weak), H-5 to C-7, C-8a (δ_C 118.4), C-6, and C-10 (δ_C 61.0/ δ_H 4.58), H-10 to C-5, C-8a, C-4 (δ_C 134.7), and OH-10 (δ_H 5.17, t , $J = 5.6$ Hz) to C-10.

Table 2. ^1H and ^{13}C NMR (DMSO- d_6 , 300.13 and 75.4 MHz) and HMBC assignment for **2**.

Position	δ_C , Type	δ_H (J in Hz)	COSY	HMBC
2	152.6, C	-		
3	101.0, CH	6.47, brs	CH ₃ -9	C-2,4,8
4	134.7, C			
5	109.3, CH	6.70, s	H ₂ -10	C-6,7,8a,10
6	154.4, C	-		
7	95.6, CH	6.70, s		C-6,8a
8	155.0, C	-		
8a	118.4, C	-		
9	13.7, CH ₃	2.36, d (1.0)		C-2,3
10	61.0, CH ₂	4.58, d (5.6)	H-5, OH-6	C-4,5,8a
OH-6	-	9.28, s		C-5,6,7
OH-10	-	5.17, t (5.6)	H ₂ -10	C-10

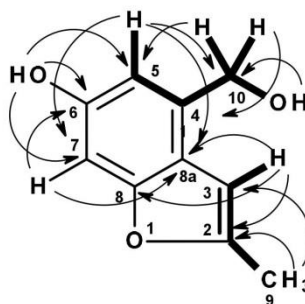


Figure 6. Key COSY (bold line) and HMBC (arrow) correlations in **2**.

That the 2,3-substituted 5-hydroxybenzyl alcohol was fused to a 2-methylfuran ring, through C-8 (δ_C 155.0) and C-8a was supported by HMBC correlations (Table 2, Figure S16, Figure 6) from H-3 (δ_H 6.47, brs) to C-8a, C-8, C-2 (δ_C 152.6), and from the methyl doublet at δ_H 2.36 (CH₃-9; δ_C 13.7) to C-2 and C-3 (δ_C 101.0).

In order to confirm the structure of **2**, the compound was further purified by passing through a Sephadex LH-20 column, and its ^1H and ^{13}C NMR spectra (Figures S18 and S19) showed that the impurities were completely removed. Interestingly, the ^1H NMR spectrum of the pure compound, also in DMSO- d_6 , did not exhibit the triplet of OH-10 (δ_H 5.17, $J = 5.6$ Hz) while H₂-10 appeared as a singlet

at δ_H 4.58 instead of as a doublet with $J = 5.6$ Hz. The HSQC and HMBC spectra (Figures S20 and S21) were also in agreement with those obtained before re-purification. Since the pure compound exhibited the (–)-HRESIMS m/z at 177.0556 ($M - H$)⁺ (calculated for $C_{10}H_9O_3$, 177.0552) (Figure S22), its molecular formula was established as $C_{10}H_9O_3$, corresponding to six degrees of unsaturation. Therefore, **2** was elucidated as 4-(hydroxymethyl)-2-methyl-1-benzofuran-6-ol. A structure search through SciFinder displayed the structure of **2** with the name 6-hydroxy-2-methyl-4-benzofuranmethanol and the CAS Registry Number 1083198-52-0. However, there is no reference reporting its source or its NMR data. Therefore, **2** was named acremine T.

Compounds **1–3** were assayed for their *in vitro* inhibitory activities against AChE and BuChE, and the results are shown in Tables 3 and 4. Lumichrome (**3**) was found to exhibit AChE inhibitory capacity ($IC_{50} = 12.24 \pm 0.12$) comparable to that of galantamine ($IC_{50} = 11.31 \pm 0.11$), while acremines S (**1**) and T (**2**), at a concentration of 6.6 μ M, showed a much weaker inhibition than lumichrome (**3**). On the contrary, acremine S (**1**) showed inhibitory activity against BuChE three folds higher than that of galantamine, whereas acremine T (**2**) exhibited comparable activity to that of galantamine, and lumichrome (**3**) showed weak activity against BuChE at a concentration of 6.25 μ M.

Table 3. Acetylcholinesterase (AChE) inhibitory activity of **1–3**.

Compound	% inhibition at 6.6 μ M	IC_{50} (μ M)
1	10.42 $\pm$ 0.33 *	n.d.
2	14.08 $\pm$ 1.27 *	n.d.
3	39.23 $\pm$ 1.77 *	12.24 $\pm$ 0.12 **
Galantamine	39.76 $\pm$ 2.22 *	11.31 $\pm$ 0.11 **

Results are given as mean $\pm$ SEM of three independent experiments performed in triplicate; n.d. = not determined.
* $p < 0.05$; ** $p < 0.01$.

Table 4. Butyrylcholine esterase (BuChE) inhibitory activity of **1–3**.

Compound	% inhibition at 6.25 μ M	IC_{50} (μ M)
1	30.71 $\pm$ 0.21 **	n.d.
2	10.53 $\pm$ 1.01 **	n.d.
3	3.44 $\pm$ 0.09 *	n.d.
Galantamine	10.34 $\pm$ 0.21 *	n.d.

Results are given as mean $\pm$ SEM of three independent experiments performed in triplicate; n.d. = not determined.
* $p < 0.05$; ** $p < 0.01$.

3. Experimental Section

3.1. General Experimental Procedures

The melting points were determined on a Stuart Melting Point Apparatus SMP3 (Bibby Sterilin, Stone, Staffordshire, UK) and were uncorrected. Optical rotations were measured on an ADP410 Polarimeter (Bellingham + Stanley Ltd., Tunbridge Wells, Kent, UK). ^1H and ^{13}C NMR spectra were recorded at ambient temperature on a Bruker AMC instrument (Bruker Biosciences Corporation, Billerica, MA, USA) operating at 300 or 500 and 75 or 125 MHz, respectively. High resolution mass spectra were measured with a Waters Xevo QToF mass spectrometer (Waters Corporations, Milford, MA, USA) coupled to a Waters Aquity UPLC system. A Merck (Darmstadt, Germany) silica gel GF₂₅₄ was used for preparative TLC, and a Merck Si gel 60 (0.2–0.5 mm) was used for column chromatography.

3.2. Fungal Material

The fungus was isolated from the marine sponge *Mycale* sp. which was collected, by scuba diving at a depth of 5–10 m, from the coral reef at Samaesan Island (12°34′36.64″ N, 100°56′59.69″ E), in

the Gulf of Thailand, in September 2016. The sponge was washed with 0.01% sodium hypochlorite solution for 1 min, followed by sterilized seawater three times, and then dried on sterile filter paper under sterile aseptic conditions. The sponge was cut into small pieces (5 mm × 5 mm) and placed on Petri dish plates containing 15 mL potato dextrose agar (PDA) medium mixed with 300 mg/L of streptomycin sulfate and incubated at 28 °C for 7 days. The hyphal tips emerging from sponge pieces were individually transferred onto PDA slants and maintained as pure cultures at Kasetsart University Fungal Collection, Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand, for further identification. The fungal strain KUFA 1007 was identified as *Acremonium persicinum*, based on morphological characteristics such as colony growth rate and growth pattern on standard media, namely Czapek's agar, Czapek yeast autolysate agar and malt extract agar. Microscopic characteristics including size, shape and ornamentation of conidiophores and spores were examined under light and scanning electron microscopes. This identification was confirmed by molecular techniques using internal transcribed spacer (ITS) primers. DNA was extracted from young mycelia following a modified Murray and Thompson method [22]. Primer pairs ITS1 and ITS4 [23] were used for ITS gene amplification. PCR reactions were conducted on Thermal Cycler and the amplification process consisted of the initial denaturation at 95 °C for 5 min, 34 cycles at 95 °C for 1 min (denaturation), at 55 °C for 1 min (annealing) and at 72 °C for 1.5 min (extension), followed by final extension at 72 °C for 10 min. The PCR products were examined by agarose gel electrophoresis (1% agarose with 1 × Tris-Borate-EDTA (TBE) buffer) and visualized under UV light after staining with ethidium bromide. DNA sequencing analyses were performed using the dideoxyribonucleotide chain termination method [24] by Macrogen Inc. (Seoul, Korea). The DNA sequences were edited using the FinchTV software (version 1.4, Geospiza Inc, Seattle, WA, USA) and submitted into the BLAST program for alignment and compared to fungal species in the NCBI database (<http://www.ncbi.nlm.nih.gov/>). Its gene sequences were deposited in GenBank with accession number MG755248.

3.3. Extraction and Isolation

The fungus was cultured for one week at 28 °C in five Petri dishes (i.d. 90 mm) containing 20 mL of potato dextrose agar per dish. The mycelial plugs (5 mm in diameter) were transferred to two 500 mL Erlenmeyer flasks containing 200 mL of potato dextrose broth and incubated on a rotary shaker at 120 rpm at 28 °C for one week. Fifty 1000 mL Erlenmeyer flasks, each containing 300 g of cooked rice, were autoclaved at 121 °C for 15 min. After cooling to room temperature, 20 mL of a mycelial suspension of the fungus was inoculated per flask and incubated at 28 °C for 30 days, after which 500 mL of EtOAc was added to each flask of the moldy rice and macerated for 7 days, and then filtered with Whatman No. 1 filter paper (GE Healthcare UK Limited, Buckinghamshire, UK). The EtOAc solutions were combined and concentrated under reduced pressure to yield 59.8 g of crude EtOAc extract which was dissolved in 500 mL of EtOAc and then filtered with Whatman No. 1 filter paper. The EtOAc solution was then washed with H₂O (3 × 500 mL) and dried with anhydrous Na₂SO₄, filtered and evaporated under reduced pressure, to give 46.3 g of crude EtOAc extract. The crude EtOAc extract was applied on a column chromatography of silica gel (390 g), and eluted with mixtures of petrol–CHCl₃ and CHCl₃–Me₂CO, wherein 250 mL fractions (Frs) were collected as follows: Frs 1–23 (petrol–CHCl₃, 1:1), 24–49 (petrol–CHCl₃, 3:7), 50–177 (petrol–CHCl₃, 1:9), 178–207 (CHCl₃), 208–453 (CHCl₃–Me₂CO, 9:1), 454–546 (CHCl₃–Me₂CO, 7:3). Frs 54–75 were combined (296.5 mg) and recrystallized in MeOH to give **4** (125.3 mg). Fr 213 (264.3 mg) was crystallized in MeOH to give **5** (48.0 mg). Frs 214–246 were combined (2.10 g) and applied on a column chromatography of silica gel (40 g) and eluted with mixtures of petrol–CHCl₃ and CHCl₃–Me₂CO, wherein 100 mL sub-fractions (sfrs) were collected as follows: sfrs 1–52 (petrol–CHCl₃, 3:7), 53–74 (petrol–CHCl₃, 1:9), 75–158 (CHCl₃), 159–218 (CHCl₃: Me₂CO, 19:1), 219–254 (CHCl₃: Me₂CO, 1:9). Sfrs 122–160 were combined (314.4 mg) and recrystallized in MeOH to give **5** (60.2 mg). Frs 246–255 were combined (174.7 mg) and purified by TLC (silica gel G₂₅₄, CHCl₃:Me₂CO:HCO₂H, 8:2:0.1) to give 10.4 mg of **2** (with impurities), which was further applied on a Sephadex LH-20 column (5 g) and eluted with MeOH, wherein 67 sfrs of

1 mL were collected. Sfrs 40–50 were combined and, after evaporation of solvent, gave 2.4 mg of **2** as a pure compound. The other part of the TLC separation of frs 246–255 was combined with frs 256–285 (318.1 mg) and applied on a column chromatography of silica gel (30 g) and eluted with mixtures of petrol-CHCl₃ and CHCl₃-Me₂CO, wherein 100 mL sfrs were collected as follows: Sfrs 1–63 (petrol-CHCl₃, 3:7), 64–127 (CHCl₃: Me₂CO, 9:1) and 128–161 (CHCl₃: Me₂CO, 7:3). Sfrs 71–75 were combined (12.0 mg) and purified by TLC (silica gel G₂₅₄, CHCl₃:Me₂CO:HCO₂H, 8:2:0.1) to give 30.9 mg of a compound which was applied on a Sephadex LH-20 column (5 g) and eluted with a 1:1 mixture of CH₂Cl₂:MeOH, wherein 62 sfrs of 1 mL were collected. By using TLC to monitor the profile of eluted compounds, these sub-fractions were divided in two groups: Group I (sfrs 8–18) and group II (sfrs 19–30). Sfrs 8–18 (group I) were combined (19.7 mg), and applied on a Sephadex LH-20 column (5 g) and eluted with a 1:1 mixture of CH₂Cl₂: MeOH, wherein 40 sub-sfrs (ssfrs) of 1 mL were collected. Sfrs 15–36 were combined and, after evaporation of solvent, gave 15.0 mg of **1**. Sfrs 19–30 (group II) were combined (5.1 mg), and applied on a Sephadex LH-20 column (5 g) and eluted with MeOH, wherein 20 ssfrs of 1 mL were collected. Sfrs 5–15 were combined and, after evaporation of solvent, gave 2.3 mg of **2**. Frs 286–315 were combined (189.3 mg) and crystallized in Me₂CO to give 6.0 mg of **3**. The mother liquor was combined with frs 316–365 (291.9 mg) and applied on a column chromatography of silica gel (10 g), and eluted with mixtures of petrol-CHCl₃ and CHCl₃-Me₂CO, wherein 100 mL sfrs were collected as follows: sfrs 1–22 (petrol:CHCl₃, 1:1), 23–41 (petrol:CHCl₃, 7:3), 54–73 (CHCl₃), 74–162 (CHCl₃: Me₂CO, 19:1), 163–178 (CHCl₃: Me₂CO, 9:1). Sfrs 79–90 were combined (98.8 mg) and recrystallized in MeOH to give an additional 20.1 mg of **3**.

3.3.1. Acremine S [(3S, 5R, 10S)-3,10-dihydroxy-2,2,8-trimethyl-1-oxospiro[4.5]dec-8-en-7-one] (**1**)

Yellowish viscous oil; [α]_D²⁰ +44 (c 0.5, CHCl₃). For ¹H and ¹³C NMR spectroscopic data (CDCl₃, 300 and 75 MHz), see Table 1; (+)-HRESIMS *m/z* 227.1293 [M + H]⁺ (calculated for C₁₂H₁₉O₄, 227.1283).

3.3.2. Acremine T [4-(hydroxymethyl)-2-methyl-1-benzofuran-6-ol] (**2**)

White amorphous solid. For ¹H and ¹³C NMR spectroscopic data (CDCl₃, 300 and 75 MHz), see Table 2; (–)-HRESIMS *m/z* 177.0556 [M – H]⁺ (calculated for C₁₀H₁₉O₃, 177.0552).

3.4. Electronic Circular Dichroism (ECD) of **1**

The ECD spectrum of **1** (7 mM in methanol) was obtained in a Jasco J-815 CD spectropolarimeter (Jasco, Mary's Court, Easton, MD, USA) with a 0.1 mm cuvette and eight accumulations. A conformational search, including dihedral driver and molecular mechanics MM2 minimizations, was done in Chem3D Ultra (Perkin-Elmer Inc., Waltham, MA, USA). Quantum chemical DFT minimizations were performed with Gaussian 16W (Gaussian Inc., Wallingford, CT, USA) using the APFD method with a 6-31G basis set, for a pre-selection of the most stable conformations, and a 6-311+G(2d,p) basis set for final minimizations and ECD spectral calculations (TD-DFT, 50 transitions). All DFT calculations included an IEFPCM solvation model of methanol. The spectrum of each relevant configuration/conformation was constructed by applying a Gaussian line broadening of 0.3 eV to each computed transition; no UV-shift was applied. The final theoretical spectrum of each possible configuration of **1** was obtained by a Boltzmann-weighted sum of the ECD spectra of its most abundant conformations, accounting for at least 90% of the theoretical conformer population [25].

3.5. Acetylcholinesterase (AChE) and Butyrylcholinesterase (BuChE) Assays

Acetylcholinesterase inhibitory activity was determined spectroscopically using the Ellman's microplate method, with modification [26]. Briefly, 20 μ L of 0.22 U/mL AChE of *Electrophorus electricus* (EC 3.1.1.7, Sigma-Aldrich, Saint Louis, MO, USA) was added to each well containing 10 μ L of tested compound (200, 100, 50, 25, 12.5, 6.25, 3.125 and 1.563 μ M in MeOH or DMSO), 100 μ L of 3 mM of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and 20 μ L of 15 mM acetylthiocholine iodide. Absorbance of the colored-end product was measured during 5 min, with 30 s intervals, at 412

nm, controlled by a BioTek Synergy™ HT Microplate Reader (Winooski, Vermont, USA). Controls containing 10 µL of compound vehicles (MeOH or DMSO) instead of tested compounds and blanks containing 20 µL of buffer [0.1% (*w/v*) bovine serum albumin in 50 mM Tris-HCl] instead of the enzyme were prepared. Percentage of enzyme inhibition was calculated as: % of inhibition = $100 - \left[\frac{(\text{Abs}_{\text{sample}} - \text{Abs}_{\text{sample's blank}})}{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}})} \times 100 \right]$ [27].

Each experiment was performed in triplicate, and galantamine was used as a positive control. The inhibitory activities of the compounds towards AChE were expressed as IC₅₀, which was determined as the effective concentration at which AChE was inhibited by 50%. The IC₅₀ was obtained by interpolation from linear regression analysis. When the solubilization of compounds was compromised, the IC₅₀ could not be determined and, in this case, the percentage of inhibition for the maximum concentration tested was used.

For the butyrylcholinesterase inhibition assay, BuChE from lyophilized horse serum (EC 3.1.1.8, Merck, Darmstadt, Germany) and butyrylthiochlorine iodide as a substrate for BuChE were used. All other reagents and conditions were the same except for 20 µL of the tested compounds were added per well.

3.6. Statistical Analysis

The assays were conducted in triplicate and all tabulated results were expressed as the mean ± SEM. Statistical analysis of the results was performed with GraphPad Prism (GraphPad Software, San Diego, CA, USA). An unpaired *t*-test was carried out to test for any significant difference between the means. Differences at the 5% confidence level were considered significant.

4. Conclusions

Marine-derived fungi are important sources of structurally diverse and biologically relevant secondary metabolites. Our group have investigated secondary metabolites from the marine-derived species of *Aspergillus*, *Neosartorya*, *Penicillium* and *Talaromyces* to evaluate their potential as antibiotics to combat multidrug-resistant pathogenic bacteria. Recently, we have focused our attention on naturally occurring compounds with potential for treatment of neurological disorders. Since only a few compounds from marine-derived fungi have been tested for neuroprotective activities, we have isolated secondary metabolites produced by marine-derived fungi to investigate their capacity as cholinesterase inhibitors. In this work, we described the isolation and structure elucidation of two previously undescribed secondary metabolites: acremines S (1) and T (2), in addition to three known compounds, including lumichrome (3), ergosterol (4) and ergosterol 5,8-endoperoxide (5), from the cultures of the fungus *Acremonium persicinum* strain KUFA1007, which was isolated from the marine sponge *Mycale* sp., collected in the Gulf of Thailand. The acetylcholinesterase and butyrylcholinesterase inhibitory activities of 1–3 were also evaluated. The results showed that lumichrome (3) possesses the inhibitory activity toward the enzyme acetylcholinesterase comparable to that of the standard drug galantamine, while acremine S (1) showed more potent antibutyrylcholinesterase activity than that of galantamine. The results of this study can pave the way to the exploitation of the secondary metabolites produced by cultures of marine-derived fungi for a development of the therapeutic arsenals for neurological disorders such as Alzheimer's disease.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1660-3397/17/6/379/s1>. Figures S1–S9, S11–S21, S23–S28 and S30–S33: 1D and 2D NMR spectra of compounds 1–5. Figures S10, S22 and S29: HRMS data for compounds 1–3. Table S1: ¹H and ¹³C NMR (DMSO-*d*₆, 500 and 125 MHz) for 3.

Author Contributions: A.K. and M.M.M.P. conceived, designed the experiment and elaborated the manuscript; A.J.S.A. performed the isolation, purification and part of the structure elucidation of the compounds; T.D. collected, isolated, identified and cultured the fungus; J.A.P. performed calculations and measurement of ECD spectra and interpretation of the results. S.C. assisted in anticholinesterase assays. S.M. provided HRMS; A.M.S.S. provided NMR spectra.

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Conflicts of Interest: The authors declare no conflict of interest.

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