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Bioactive Secondary Metabolites from the Culture of the Marine Sponge-Associated Fungus *Neosartorya fennelliae* KUFA 0811

Tin Shine Aung

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INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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BIOACTIVE SECONDARY METABOLITES FROM THE CULTURE OF THE MARINE SPONGE-ASSOCIATED FUNGUS *NEOSARTORYA FENNELLIAE* KUFA 0811

Dissertation for applying to the degree of Master in Marine Sciences – Marine Resources, as submitted to the institute of Biomedical Sciences Abel Salazar of the University of Porto.

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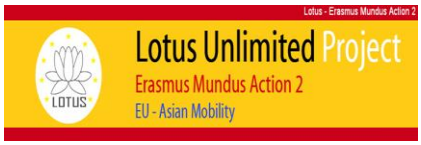


ABSTRACT

This thesis reports the chemical study of the marine-derived fungus *Neosartorya fennelliae* KUFA 0811, isolated from the marine sponge, *Clathria reinwardtii,* which was collected from Samaesan Island in the Gulf of Thailand at Amphur Sattahip, Chonburi province, Thailand. The ethyl acetate extract of the culture of this fungus supplied β-sitostenone (**NF1**), ergosta-4,6,8(14),22-tetraen-3-one (**NF2**), dehydromevalonic lactone (**NF3**), byssochlamic acid (**NF4**), cyathisterone (**NF5**), chevalone B (**NF6**), aszonalenin (**NF7**), secalonic acid A (**NF8**), helvolic acid (**NF9**) and fellutanine A (**NF10**). The structures of the isolated compounds were established based on extensive analysis of 1D and 2D NMR spectral data, HRMS, as well as by comparison of their NMR data to those reported in the literature. Moreover, the stereochemistry of secalonic acid A was also confirmed by X-ray analysis.

Keywords: Marine-derived fungus, *Neosartorya fennelliae*, Trichocomaceae, indole-alkaloids, meroditerpene, ergosterol derivatives, secalonic acid A

RESUMO

A presente tese reporta o estudo químico do fungo marinho, *Neosartorya fennelliae* KUFA 0811, isolado da esponja marinha, *Clathria reinwardtii* que foi colhida da ilha de Samaesan no Golfo da Tailândia na província de Chonburi. Do extrato de acetato de etilo da cultura deste fungo, foram isolados β-sitostenona (**NF1**), ergosta-4,6,8(14),22-tetraen-3-ona (**NF2**), lactona de desidromevalônico (**NF3**), ciathisterona (**NF5**), chevalona B (**NF6**), aszonalenina (**NF7**), ácido secalónico A (**NF8**), ácido helvólico (**NF9**) e fellutanina A (**NF10**). As estruturas dos compostos foram estabelecidas pela análise dos dados de RMN (uni- e bidimensional), dos espectros de Massa da alta resolução, bem como pela comparação dos seus dados espectrais com aqueles encontrados na literatura. Além disso, a estereoquímica do ácido secalônico A foi também confirmada pelos dados de cristalografia de raios-X.

Palavras-chave: Fungo marinho, *Neosartorya fennelliae,* Trichocomaceae, alcalóides indólicos, meroditerpeno, derivados de ergosterol, ácido secalônico A

LIST OF ABBREVIATIONS

$[M+H]^+$	Pseudo-molecular ion (Positive ion mode)
$[\alpha]_D^T$	Specific optical rotation at Temperature ºC for D (sodium) line
®	Registered Trademark
TM	Trademark
©	Copyright
AcO	Acetoxy group
$CDCl_3$	Deuterated chloroform
COSY	Correlated Spectroscopy
brs	Broad singlet
dd	Double doublet
ddd	Double double doublet
dt	Double triplet
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO-d_6	Deuterated dimethylsulfoxide
EU	European Union
FDA	Food and Drug Administration
g	Gram
mg	milligram
µg	microgram
TLC	Thin Layer Chromatography
GI_{50}	Half maximal growth inhibitory concentration
HMBC	Heteronuclear Multiple Bond Correlation
HRMS	High Resolution Mass Spectrometry

HSQC	Heteronuclear Single Quantum Coherence
Hz	Hertz
IC_{50}	Half maximal inhibitory concentration
J	Coupling constant in Hz
KUFA	Kasetsart University Fungal Agriculture
m	Multiplet
m/z	Mass per charge
Me_2CO	Acetone
MeOH	Methanol
MHz	Mega hertz
MIC	Minimum Inhibitory Concentration
mm	Millimeter
mL	Mililiter
mp	Melting point in ºC
MS	Mass Spectrometry
NCI	National Cancer Institute
NMR	Nuclear Magnetic Resonance
ºC	Celsius degrees
ORTEP	Oak Ridge Thermal Ellipsoid Plot
PTLC	Preparative Thin Layer Chromatography
q	Quartet
s	Singlet
sp.	Species (singular)
spp.	Species (plural)
t	Triplet

UV	Ultraviolet
δ	Chemical shift value in ppm
ε	Molar absorptivity (molar extinction coefficient)
Å	Ångström
λ	Lambda
1D	One-Dimension
2D	Two-Dimension
HIV	Human Immunodeficiency Virus
AIDS	Acquired Immune Deficiency Syndrome
HRESIMS	High-Resolution Electrospray Ionization Mass Spectrometry
OSMAC	One Strain-Many Compounds

Objectives of the Thesis

The main objective of the present study are

- To investigate the secondary metabolites produced by *Neosartorya fennelliae* KUFA 0811.

- To discover novel and bioactive products from *Neosartorya fennelliae* KUFA 0811.

In chapter I, pertaining to background literature of respective areas concerning this thesis and in chapter II, pertaining to selected species from *Aspergillus* section *Fumigati* based on three criteria, (i) heterothallic species in *Aspergillus* section *Fumigati*, similar species with investigated fungus based on (ii) genetic and (iii) morphology.

CHAPTER I
INTRODUCTION

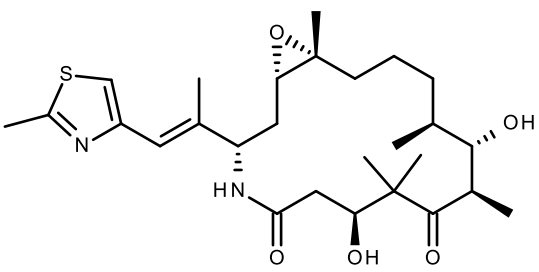
1.1. General Introduction

Nature is an invaluable present for human beings due to all living things and their survivals are related to natural phenomena. Nature provides all the essential things we need such as water, food and natural remedies for all organisms. Before the evolution of modern medicine, Mankind was using raw plants to cure and sustain its health for both daily and emergency purposes. Such kinds of traditional ways were more developed in tropical and subtropical countries since there is a more favorable environment for the growth of plants and other inhabitants (Grime, 1977). Because of the effectiveness of medicinal plants in the health care system, modern scientists are still focusing on natural products to find the solution for new-age challenges: diseases such as cancer and all other infectious diseases. According to the estimation of Saura-Calixto (2010), higher plants cover 25% of modern medicine either directly or indirectly. Moreover, Patwardhan *et al*. (2004) mentioned that 80% of inhabitants in developing countries remain focused on traditional medicine. Since these plants represent 25% of modern medicine and 80% of people in developing countries are still relying on natural products, medicinal plants are intricately related to world health. Even though natural products have long been used in traditional medicine, scientific research focused on the identification and characterization of compounds from natural sources only started in 20th century. On the one hand, studying medicinal plants from terrestrial sources is quite limited due to several factors such as global warming, a food supplement for the growing human population and others. One of the more important critical reasons is that most of the known medicinal plants have already been investigated in the past (Cordell, 2000). On the other hand, challenges of major harmful diseases are increasing daily (Morens *et al.*, 2004). To cover needs, scientists are seeking new trends. The challenge lies precisely in which is the better alternative medicine for these requirements. Aquatic sources are strong contenders to fulfill the need of natural products since water covers 70% of our planet and offers a rich source of marine biodiversity. Based on the deduction “A large part of scaffolds of marine natural products are novel in comparison with those of terrestrial sources”, marine natural products are potential sources for the natural products-based drug discovery (Kong *et al*., 2010). In a comparison of marine and terrestrial samples, 1% of the tested marine samples showed anti-tumor potential while the tested terrestrial samples accounted for only 0.1% in the national cancer preclinical cytotoxicity screen. Moreover, the first marine-derived drug was successfully imported into the market and it became extremely remarkable in marine natural product history (Mayer *et al*., 2010). Nowadays, some of the products derived from marine sources are Food and Drug Administration certified and many others are in clinical

trial stages (Montaser and Luesch, 2011). Based on the development of marine natural products research, it can be assumed that MNPs will be one of the most effective sources of alternative medicine for major disease challenges in the coming decades.

1.2. Development of Natural Products-NPs in Drug Discovery

Natural products have been studied in terms of finding new drugs for many decades. Because of many successful studies, more than 100 new products have been used in the clinical stage, particularly as anticancer agents and antifungals. Therefore, it has become one of the most productive sources for the development of drug discovery (Cragg and Newman, 2013). Moreover, natural products are not only productive sources of modern medicine but also the primitive source of traditional medicine due to its usefulness in traditional cure and prevention (Harvey, 2008). It is very intriguing why natural products have become both productive and primitive sources of drug discovery. Since natural products have been the source of most of the active ingredients of medicines, the role of natural products in drug discovery is unquestionable. Furthermore, more than 80% of drug substances were either natural products or inspired by a natural compound until the development of high-throughput screening and modern technology (Sneader, 1996). In a three-year period of the past decade, from 2005 to 2007, up to 13 natural products derived drugs were approved for marketing worldwide. In spite of 13 drugs approved for marketing, only five of these drugs represented as the first members of new human drug classes: Ixabepilone (**1**), retapamulin (**2**), trabectedin (**3**), exenatide (**4**) and ziconotide (**5**) are shown in Figure 1 (Butler, 2008). Among them, ziconotide (PRIALT®) is useful in neuroactive peptide treatment for severe chronic pain and another candidates to cure severe chronic pain sufferers for whom opiate therapy was not enough. Besides this, there is no report of intolerance to ziconotide and these patients show relief (Miljanich, 2004).



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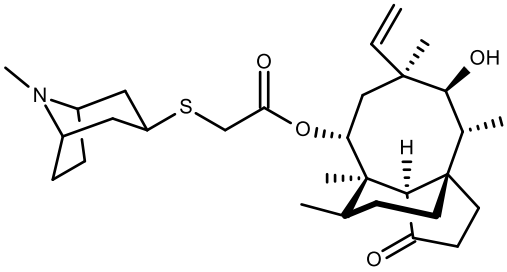

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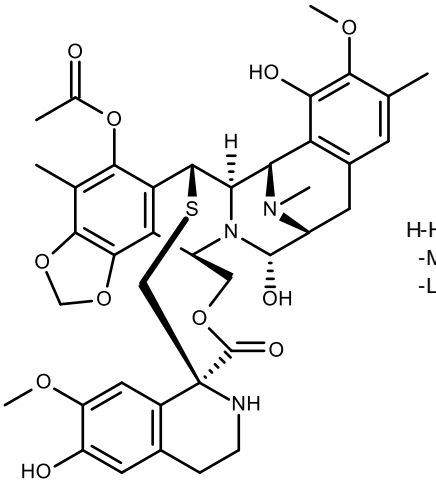

3

H-His-Gly-Glu-Gly-Thr-Phe-Thr-Ser-Asp-Leu-Ser-Lys-Gln-
-Met-Glu-Glu-Glu-Ala-Val-Arg-Leu-Phe-Ile-Glu-Trp-Leu-
-Lys-Asn-Gly-Gly-Pro-Ser-Ser-Gly-Ala--Pro-Pro-Pro-Ser-NH_2

4

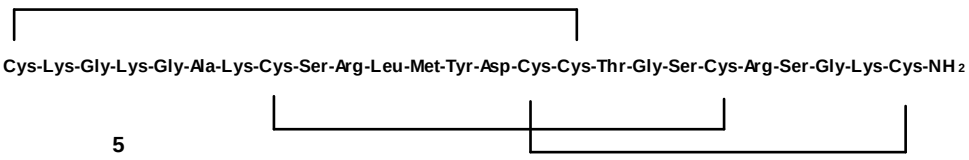

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Figure 1. Structures of ixabepilone (**1**), retapamulin (**2**), trabectedin (**3**), exenatide (**4**) and ziconotide (**5**).

From January 2008 to December 2013, a total of 25 NP and NP-derived drugs shown in Table 1 were additionally approved for marketing. Among them, 12 new drugs are human drug classes, which are brentuximab vedotin (**6**), dolatsatin 10 (**7**), ado-trastuzumab emtansine (**8**), maytansine (**9**), fingolimod (**10**), lead: myricocin (**11**), romidepsin (**12**), carfilzomib (**13**), epoxomicin (**14**), ingenol mebutate (**15**), mifamurtide (**16**), muramyl dipeptide (**17**), spinosyn A (**18**) and B (**19**), dapagliflozin (**20**), phlorizin (**21**), eribulin (**22**), halichondrin B (**23**), fidaxomicin (**24**) and omacetaxine mepesuccinate (**25**) shown in Figure 2a, 2b and 2c (Butler *et al*., 2014). Due to slow progress in developing durable therapies for parasitic diseases, the discovery of the two drugs avermectin B1a (**26**) and B1b (**27**), ivermectin B1a (**28**) and B1b (**29**) complexes and artemisinin (**30**) shown in Figure 3 came from natural product origin and became the major options for parasitic diseases. This is one of the great achievements in natural product history in latter years and the scientists who found these complexes were awarded the Nobel prize in Physiology or Medicine for 2015 (Crump and Omura, 2011; Tambo *et al*., 2015; Tu., 2011). Nowadays, Ivermectin B1a and B1b (28 and 29) has been used free of charge as the major medicine against onchocerciasis and lymphatic filariasis globally (Crump and Omura, 2011; Tambo *et al*., 2015). However, for certain types of diseases, there is still a lack proper drugs so new innovative drugs are still needed to supply the pharmaceutical industries, because only one third of all diseases can be treated efficiently, after more than 100 years of research in the pharmaceutical industry (Müller *et al*., 2000). Therefore, natural products research in terms of developing new drugs still plays an essential role. As pointed out by Ganesan (2008), drugs discovery from natural products can be improved in the future. There will be an increase due to efficacy and selectivity for the target or achieving optimal pharmacokinetic and pharmacodynamic properties. This idea was mentioned in the example, “the opium alkaloid morphine is an important drug that is obtained solely from nature and continues to be used in both extract and pure form”. In addition, the morphine (**31**) shown in Figure 3 (Rishton, 2008) was the main reason that encourages the further discovery of many semisynthetic and fully synthetic compounds due to the same pharmacophore that are successful second generation opioid drugs. In not only all empirical observations but also folklore, natural product extracts were the first medicines available to Mankind for a long period of time in the past, with no other sources.

Table 1. New natural products (NPs) derived drugs from 2008 to 2013 [adapted from Butler *et al*., 2014 and the copyright license © from Royal Society of Chemistry, shown in Appendix].

Year	Generic name (trade name)	Lead compound (source)	Disease area
2008	Ceftobiprole medocaril (Zeftera®, Zevtera™)	Cephalosporin C (fungus)	Antibacterial
2008	Umirolimus (Biomatrix™)	Sirolimus (actinomycetes)	Cardiovascular
2008	Methylnaltrexone (Relistor®)	Morphine-plant	Opioid-induced constipation
2009	Tebipenem pivoxil (Orapenem®)	Thienamycin (actinomycetes)	Antibacterial
2009	Telavancin (Vibativ®)	Vancomycin (actinomycetes)	Antibacterial
2009	Romidepsin (Istodax®)	Romidepsin (bacteria)	Cancer
2009	Vinflunine (Javlor®)	Vinorelbine (vinblastine) (plant)	Cancer
2009	Nalfurafine (Remitch®)	Morphine (plant)	Pruritus
2010	Cabazitaxal (Jevtana®)	Paclitaxel (plant)	Cancer
2010	Fingolimod (Gilenya®)	Myricocin (fungus)	Multiple sclerosis
2010	Ceftaroline fosamil (Teflaro®)	Cephalosporin C (fungus)	Antibacterial
2010	Eribulin (Halaven®)	Halichondrin B (sponge)	Cancer
2010	Mifamurtide (Mepact®)	Muramyl dipeptide (bacteria)	Cancer
2010	Zucapasaicin (Zuacta®)	Capsaicin (plant)	Pain
2011	Fidaxomicin (Dificid®)	Fidaxomicin (actinomycetes)	Antibacterial
2011	Spinosad (Natroba™)	Spinosyn A: D 5: 1 (actinomycetes)	Antiparasitic
2011	Brentuximab vedotin	Dolastatin 10 (sea hare-cyanobacteria)	Cancer
2012	Ingenol mebutate (Picato®)	Ingenol mebutate (plant)	Actinic keratosis
2012	Dapagliflozin (Forxiga®)	Phlorizin (plant)	Type 2 diabetes
2012	Omacetaxine mepesuccinate (Synribo®)	Omacetaxine mepesuccinate (plant)	Oncology
2012	Carfilzomib (Kyprolis®)	Epoxomicin (actinomycetes)	Oncology
2012	Arteroloane/ piperaquine (Synriam™)	Artemisinin (plant)	Antiparasitic
2012	Novolimus (DESyne™)	Sirolimus (actinomycetes)	Cardiovascular
2013	Canagliflozin (Invokana®)	Phlorizin (plant)	Type 2 dibetes
2013	Ado-trastuzuma emtansine (Kadcycla®),	Maytansine (bacteria/plant)	Cancer

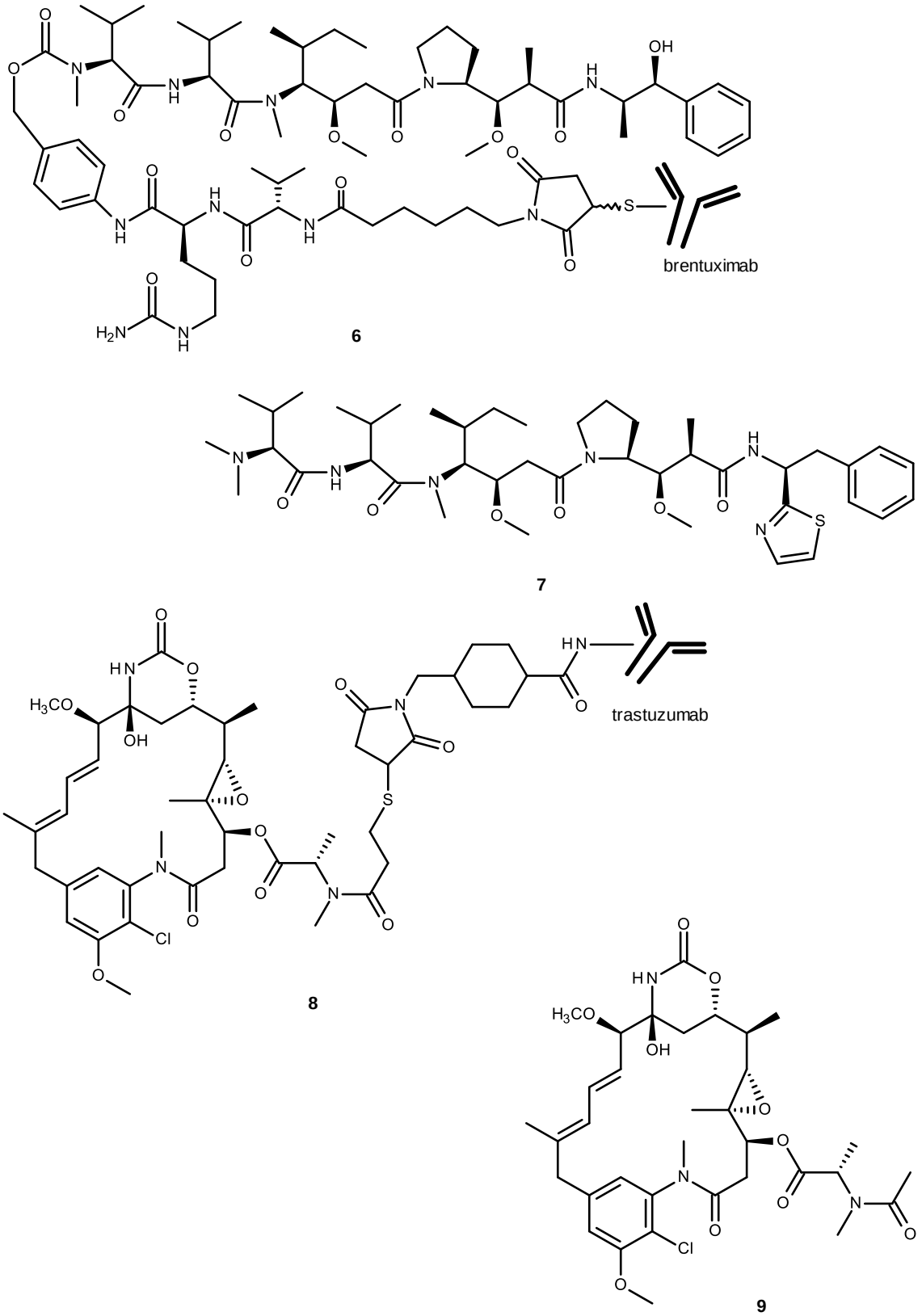

Figure 2a. Structures of vedotin (**6**), dolatsatin 10 (**7**), ado-trastuzumab emtansine (**8**) and maytansine (**9**).

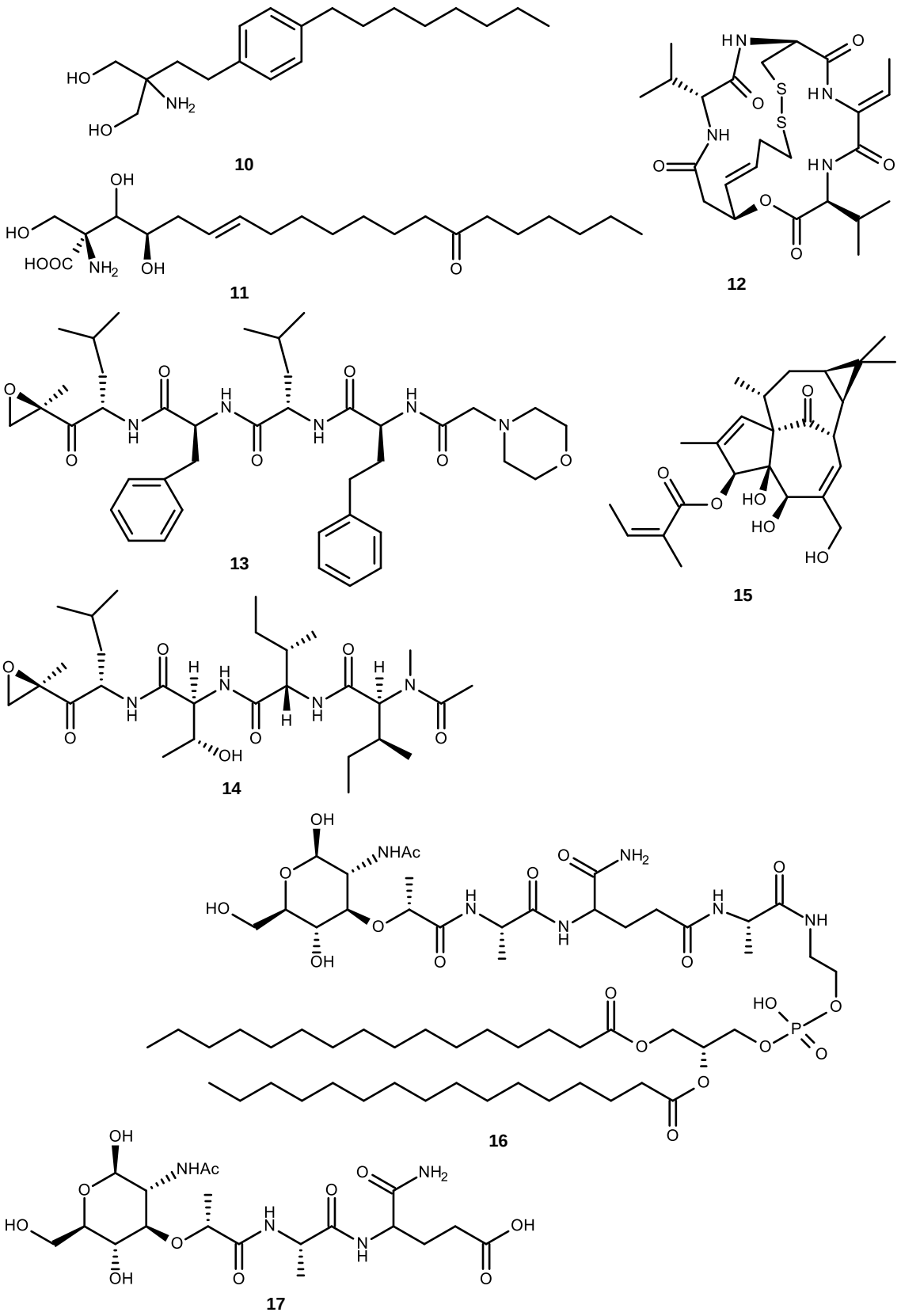

Figure 2b. Structures of fingolimod (**10**), myricocin (**11**), romidepsin (**12**), carfilzomib (**13**), epoxomicin (**14**), ingenol mebutate (**15**), mifamurtide (**16**) and muramyl dipeptide (**17**).

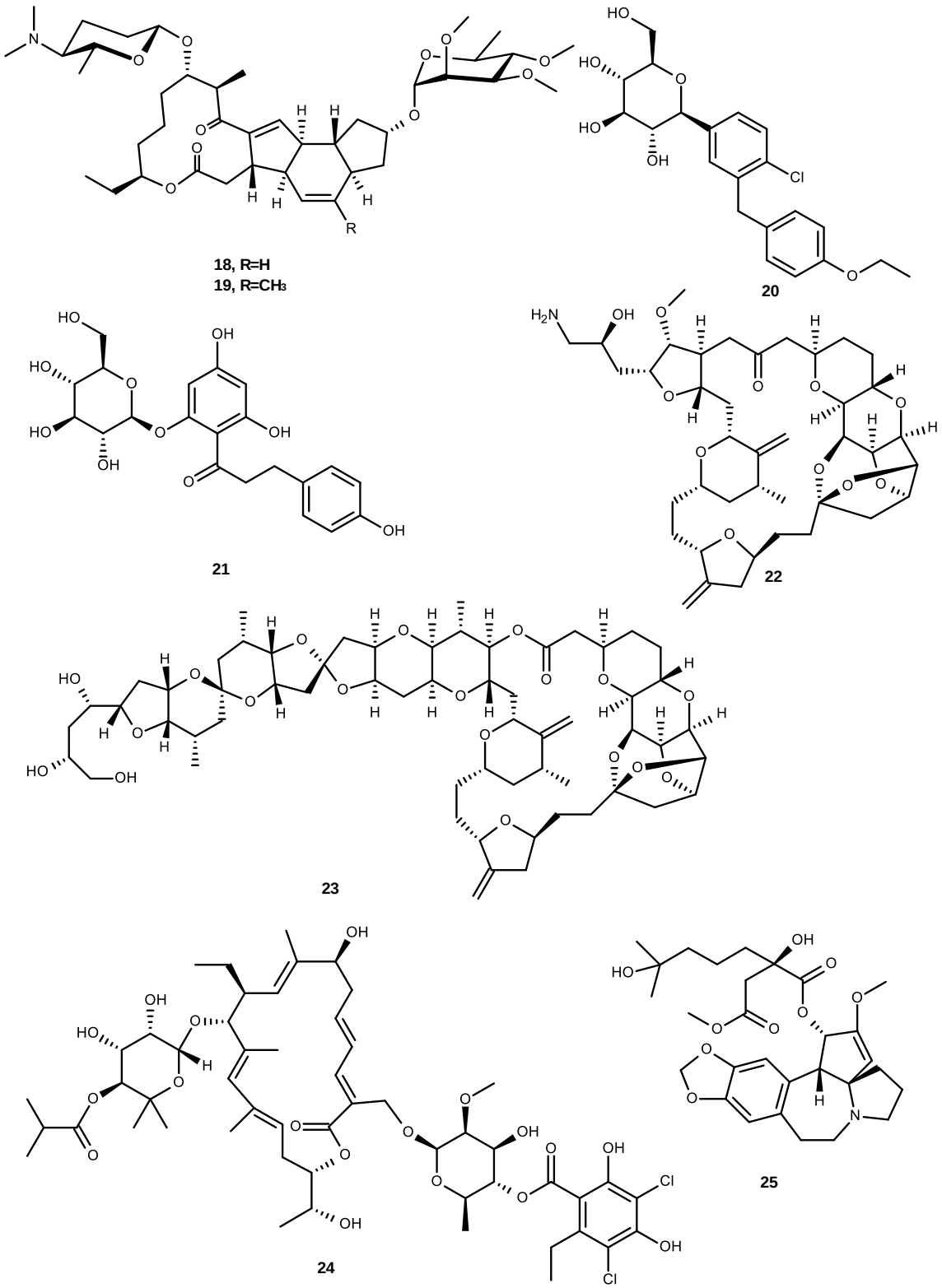

Figure 2c. Structures of spinosyn A (**18**) and B (**19**), dapagliflozin (**20**), phlorizin (**21**), eribulin (**22**), halichondrin B (**23**), fidaxomicin (**24**) and omacetaxine mepesuccinate (**25**).

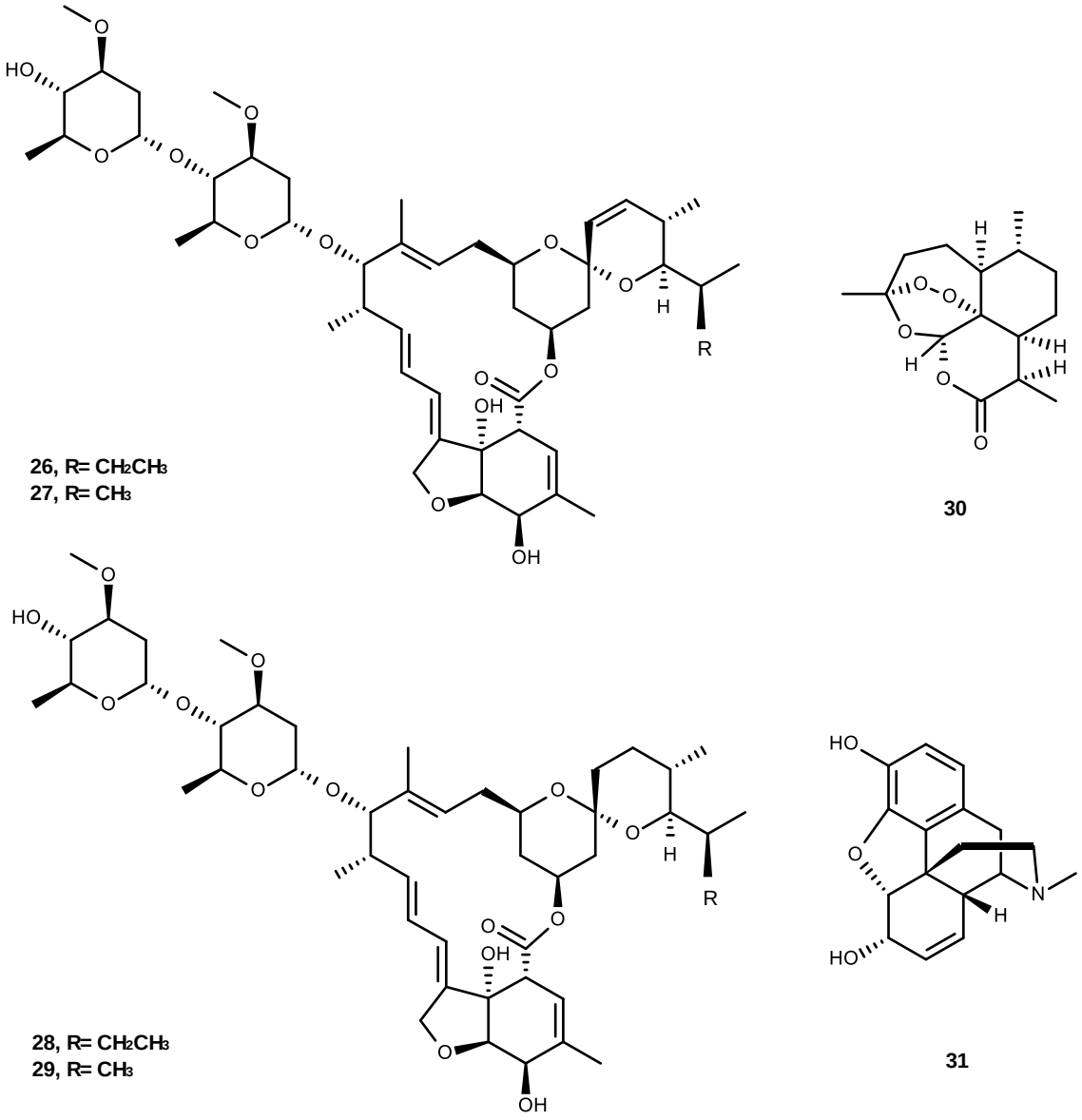

Figure 3. Structures of avermectin B1a (**26**) and B1b (**27**), ivermectin B1a (**28**) and B1b (**29**) complexes, artemisinin (**30**) and morphine (**31**).

1.3. Marine Natural Products-MNPs: Future Trend of Drug Discovery

Based on empirical knowledge, water covers about 71% of the world, of which around 97% is salty marine water (Shiklomanov and Rodda, 2004). Marine natural products means both primary and secondary metabolites, from marine sources and they are biologically active products in general. Some scientific literature refers only secondary

metabolites even though some marine primary metabolites like lipids, enzymes and complex hetero-polysaccharides are also in this category (Carte, 1996, Von Döhren and Gräfe, 1997). Marine natural products research was started by the study of intertidal and subtidal inhabitant organisms since 60 years ago. This type of study made more advances in recent decades due to the development of scuba diving technology. This development initiated a new trend of drug discovery to explore the marine environment and significantly improved research with marine natural products (Carte, 1996). Natural product scientists are very impressed with marine organisms because of their low molecular metabolite production (Bugni and Ireland, 2004). Scientific reports of novel compounds from marine metabolites increased daily and indicate that marine natural products might be a prolific source of new natural products (Faulkner, 1984).

Moreover, the marine ecosystem is surprisingly diverse and ranges from the smallest like microscopic bacteria and viruses to the largest animals such as whales, which means larger than the animals found on land (Munn, 2006). In addition, some of them, especially microorganisms, rely more on chemical weapons known as venoms than their physical strength for survival (Kaplan *et al*., 1982). This indicates that these organisms have not only richer chemical properties but also more potential for biologically active natural products than the terrestrial counterparts. Living organisms that live in aquatic or terrestrial environments can produce secondary metabolites known as natural products during their metabolism and this can be used for multiple purposes such as medicine, food, cosmetics, insecticides and animals (Carte, 1996). In the past, most of the medicinally useful natural products usually came from plant sources (Verma and Singh, 2008). However, extracts obtained from various marine sources including marine organisms, whether by culture or direct collection from a sample, continue to yield an array of novel compounds during the recent years (Rateb and Ebel, 2011; Samson and Varga, 2009; Zin *et al*., 2016a; Wang *et al*., 2016; Solecka *et al*., 2012; Stonik, 2016). By estimation of Hu *et al*. (2011), 75% of the isolated compounds came from marine invertebrates. Moreover, there are certain marine natural products which have been isolated from various marine organisms from 1985 to 2012. In addition, they also have different chemical compositions; such as terpenoids, steroids (including steroidal saponins), alkaloids, ethers (including ketels), phenols (including quinones), strigolactones, peptides and others (these cannot be classified into the above seven classes) shown in Figure 4 (Hu *et al*., 2015).

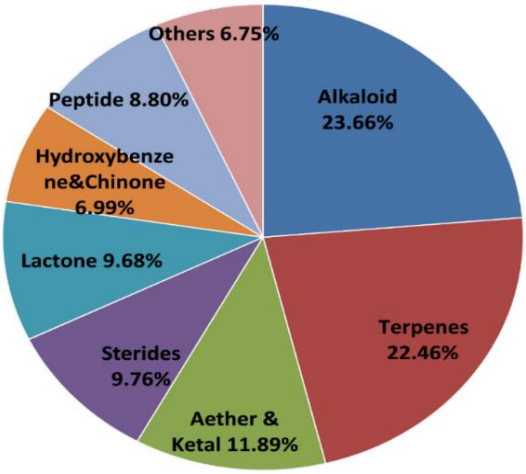

Figure 4. The proportion of bioactive compounds from marine organism (Hu *et al*., 2015) [The permission of the author; shown in Appendix].

In the marine world, sponges are extremely distinct from other species with 7000 to 9000 described species and other unidentified species (McClintock and Baker, 2001)**.** The total number of new isolated compounds from different types of marine sources from 2001 to 2010 is shown in Figure 5.

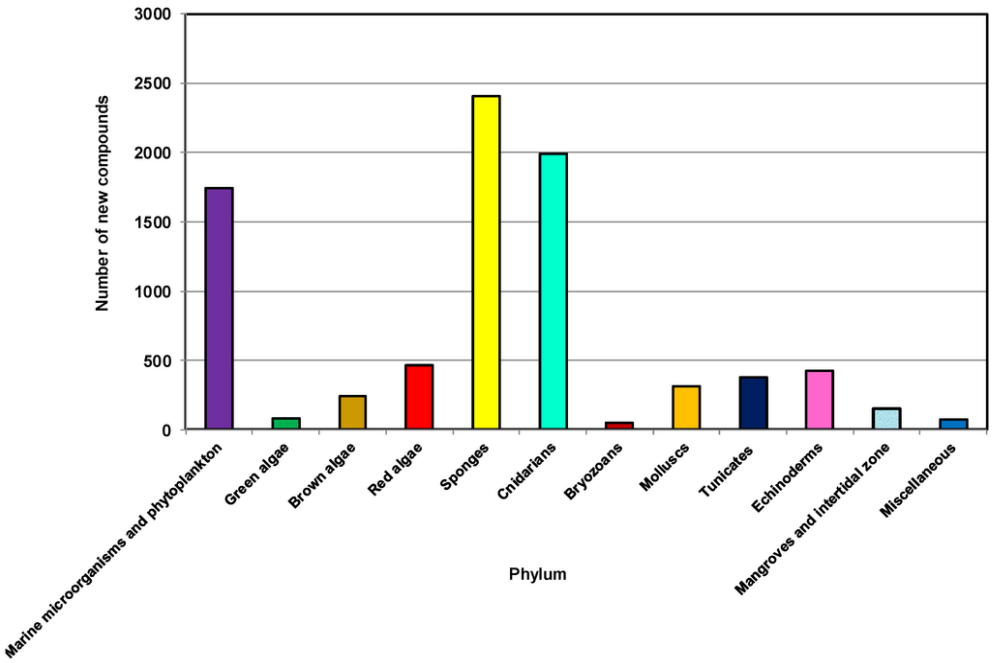

Figure 5. Total number of new compounds isolated from different types of marine sources from 2001–2010 (Mehbub *et al*., 2014) [The permission of the author; shown in Appendix].

About 7000 marine natural products, 25% from algae, 33% from sponges, 18% from coelenterates and 24% from representatives of other invertebrate phyla like tunicates, sea hares, starfish and sea cucumbers had already been isolated by 2004 and this number increased to more than 16000 marine natural products in 2015 (Kijjoa and Sawangwong, 2004; Horta *et al*., 2015). According to these two results, compound isolation from marine natural products increased more than two fold within eleven years and compound isolations only in the previous decade is higher than the total of the five decades started in the 1960s according to the comparison of data from Kijjoa and Sawangwong (2004) and Horta *et al*. (2015). This is proof of the marine diversity richness and development of marine natural products study in the last decades.

Additionally, many secondary metabolites isolated from marine natural products show bioactivities such as anticancer, antibacterial or antimicrobial, antifungal, antiviral and some other activities (Carte, 1996, Von Döhren and Gräfe, 1997). Bioactivities of new isolated marine natural products from 1985 to 2012 are shown in Figure 6. According to the results in Figure 6, one can assume that some isolated compounds may be used as marine drugs in therapeutic, pharmacology, cosmetic and nutraceutical areas. Based on the literature reported, cytarabine (**32**), vidarabine (**33**) and eribulin mesylate (**34**), all of which are from marine drug derivatives, are shown in Figure 7 and ziconotide (**5**) shown in Figure 1 are already used in clinical and therapeutic stages (Chhikara *et al*., 2010; Gerwick and Moore, 2012; Horta *et al*., 2015; Melvin *et al*., 2013; Sagar *et al*., 2010). These developments are a major success in marine natural product milestones. Moreover, the Food and Drug Administration (FDA) certified marine natural compound numbers have increased to seven and three of them were approved in the last decade (Horta *et al*., 2015). This means that studying marine natural products has developed significantly in the last decade on a long journey with many bottlenecks, which has been surpassed and solved.

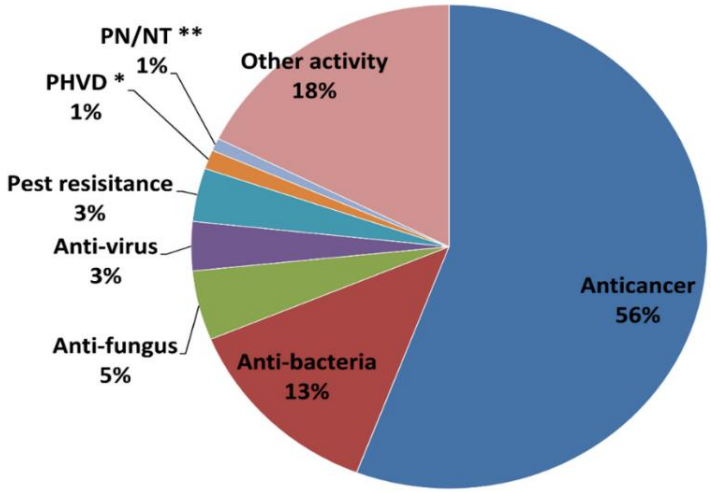

Figure 6. Bioactivities of new marine natural products (* PHVD: Prevention of heart and vascular disease, ** PN/NT: Protection of neurons/neurotoxicity) (Hu *et al*., 2015) [The permission of the author; shown in Appendix].

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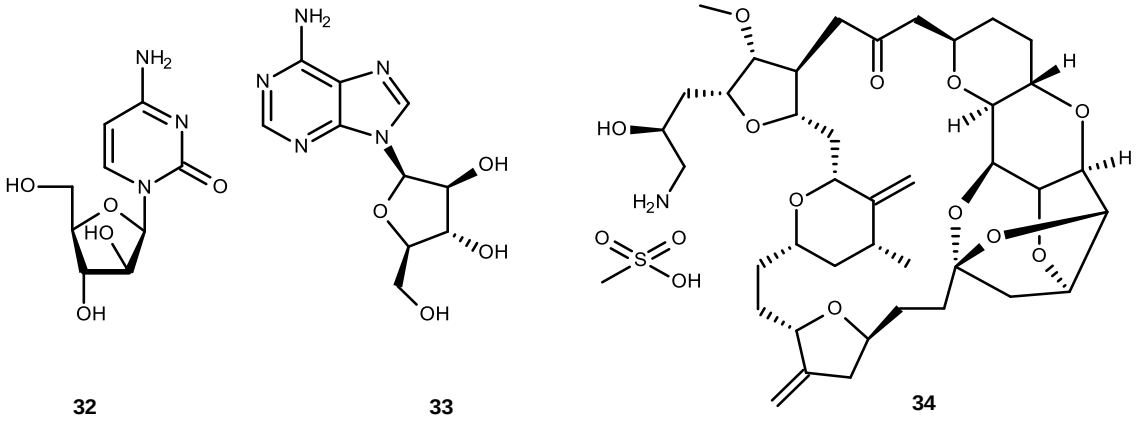

Figure 7. Structures of cytarabine (**32**), vidarabine (**33**) and eribulin mesylate (**34**).

According to all the above, the developments representing the current situation of all marine-derived in pharmaceutical industries is intriguing. Until 2014, 840 trials from all sources of marine-derived are in clinical trials in the list of NIH “National Institutes of Health, Bethesda, MD, USA” (Newman and Cragg, 2014). Especially, research deals with marine-derived drugs are mainly targeted at cancer since this disease is responsible for 8.8 million deaths in 2015 alone according to World Health Organization data. The name of compounds, marine organism source, structure, activity as inhibitor or inducer of

autophagy, and disease in which they are involved is shown in Table 2. Unfortunately, the average proportion of new bioactive compounds have decreased even though a larger number of new compounds have been explored in recent years. Total numbers of new compounds isolated from 1985 to 2012 including bioactive and inactive compounds are shown in Figure 8. According to results in Figure 8, searching for new highly active marine natural compounds increased every year and continued in many research laboratories all over the world. Consequently, research papers and reports on marine natural products are published more and more every year. Since many research works and projects focused on marine natural products and 56% of new isolated marine natural products from 1985 to 2012 have anticancer activity which represents 8.8 million deaths in 2015, MNPs could be one of the future trends of drugs discovery (Hu *et al*., 2015).

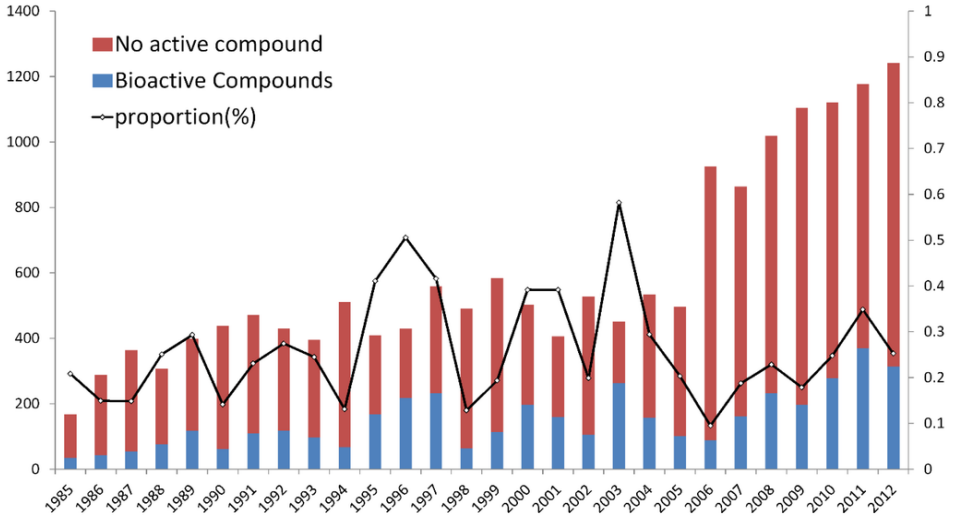

Figure 8. Variation in number of new marine natural products for 1985–2012 (Hu *et al*., 2015) [The permission of the author; shown in Appendix].

Table 2. Name of compounds, marine organism source, structure, activity as inhibitor or inducer of autophagy, and disease in which they are involved [adapted from Ruocco *et al*., 2016].

Compound	Source	Structure	Autophagy Print	Disease
Monanchocidin A	sponge	alkaloid	Inducer	Leukemia, Prostate Cancer
Clionamines	sponge	aminosteroid	Inducer	Breast cancer
Papuamine	sponge	alkaloid	Inducer	Breast cancer
Rhabdastrellic acid A	sponge	triterpenoid	Inducer	Lung cancer
Stellettin A	sponge	triterpene	Inducer	Melanoma
Xestospongin B	sponge	alkaloid	Inducer	Neuroblastoma
Araguspongine C	sponge	alkaloid	Inducer	Breast cancer
Ilamaquinone	sponge	quinone	Inducer	Colon cancer
Ovothiol A	sea urchin	thiol	Inducer	Liver cancer
Hirsutanol	fungus	sesquiterpene	Inducer	Breast cancer
Xanthocillin X	fungus	diphenol	Inducer	Liver cancer
Salinosporamide A	bacteria	lactone	Inducer	Prostate Cancer
Chromomycin A2	bacteria	polyketide	Inducer	Melanoma
Coibamide A	cyanobacterium	cyclopeptide	Inducer	Glioblastoma
EPA and DHA	Algae	fatty acids	Inducer	Lung cancer
Fucoxanthin	Algae	carotenoid	Inducer	Uterine Cancer
Methanolic extract	Algae	phenol	Inducer	Uterine Cancer
Manzamine A	sponge	alkaloid	Inhibitor	Pancreatic cancer
Petrosaspongiolide	sponge	terpenoid	Inhibitor	Chronic inflammation
Bafilomycin	algae	macrolide antibiotic	Inhibitor	Retinal disease
Polyphenols	algae	phenol	Inhibitor	Pancreatic cancer

1.4. Fungi Biodiversity in Aquatic Habitats

Fungi can be found in marine environments from the deep sea to polar ice territory (Imhoff, 2016). Fungi comprise microorganisms such as yeast, mushroom, molds and belong to the group of eukaryotic organisms. These organisms are in the kingdom of Fungi (Petersen, 2013). Based on the hypothesis of (Hawksworth and Rossman, 1997), 1.5 million fungal species can be found on Earth. Among them, only approximately 70,000 have

been described, thus about 1.43 million remain undescribed. According to Shearer *et al*. (2007), fungi species from aquatic habitats have lived in water throughout their whole life cycles. This is important for further investigation on how many species can be recognized in aquatic habitats and represented as aquatic fungi. According to literature, a certain type of fungi could be adapted for survival in fresh water. However, it is difficult to characterize where species fall along this gradient of adaptation to aquatic habitats (Shearer *et al*., 2007). More than 3000 fungi species of ascomycetes are adapted to survive in fresh water as saprobic and they could enhance their growth and sporulation in this environment (Kirk *et al*., 2008; Raja *et al*., 2010). Moreover, other flagellated fungi such as Chytridiomycota, Blastocladiomycota, and Monoblepharomycota can be found in aquatic environments as well (James *et al*., 2006). Not only they but also members of Neocallimastigomycota also live in an aquatic environment. On the other hand, only a few fresh water basidiomycetes can be found in aquatic habitats. More precisely, only 10 basidiomycetes are included in aquatic habitats among 465 species of fresh water aquatic fungi, as all basidiomycetes have been rejected in aquatic habitats. In addition, zygomycetes are almost absent in the aquatic environment (Shearer *et al*., 2007). This could be the great interrogation in the future, on the difference between fresh water and marine water bodies for the adaption of fungi.

Some specialized fungi could survive in marine water (Kohlmeyer and Volkmann-Kohlmeyer, 1991). More precisely, more than 1500 species of marine fungi occur in aquatic (Hyde and Goh, 1998). Water covers most parts of the world with five oceans including the Arctic, Atlantic, Indian, Pacific and Southern Oceans and estuaries. There are many organic substrates in these marine worlds and they are the home for filamentous marine ascomycetes (Shearer *et al*., 2007). Moreover, marine fungi are also distinctly found in mangrove forests. According to literature, 625 fungal species have been found only from mangrove environments (Schmit and Shearer, 2003). Mangrove fungi are quite impressive due to their extremely wide distribution (Kohlmeyer, 1986). According to Schmit and Shearer (2003), South East Asia is the major place for mangrove associated marine fungi, more than any other part of the globe. In addition, some species of fungi could be grown in both fresh water and marine water. For example, *Aniptodera,* which is classified as a primary fresh water fungus, can be found in both fresh water and marine environments (Shearer and Miller, 1977). Since the marine world is more diverse and unique than terrestrial counterpart, there is no doubt about how fungi biodiversity in aquatic is unique and interesting for natural products scientists.

1.5. Fungi as a Reservoir of Marine Natural Products

Nowadays, the search for novel and bioactive molecules by the term of drug discovery, marine-derived natural resources is becoming an important research area. In the past, fungi from terrestrial sources produced many therapeutically significant molecules (Saleem *et al*., 2007). In the late 1920s, Fleming discovered penicillin G (**35**), shown in Figure 9, from the filamentous fungus *Penicillium notatum*, from a terrestrial source as the major discovery of drugs from fungi and the observation of these therapeutic uses was a great achievement of 20th century in medicine (Walsh, 2003). Unfortunately, the potential of marine fungi has only been investigated to a limited extent due to the late development of marine natural products investigation (Richards *et al*., 2012). There are several barriers interfering with the development in the area of marine pharmaceuticals. The main challenges are harvesting of a marine organism, culturing of a marine organism in the laboratory, difficulties of sustaining and insufficiency of material for a complete study (Bhadury *et al*., 2006). Presently, ocean resources contain various types of living organisms such as fish, shellfish, other animals, vegetation, algae, bacteria, and fungi. However, these natural resources have been explored only to a limited extent; many of them are biologically active and potentially useful (Thorne-Miller, 1999). The environment of marine organisms is significantly different from terrestrial organisms, therefore the secondary metabolites produced from different organisms will differ considerably (Saleem *et al*., 2007).

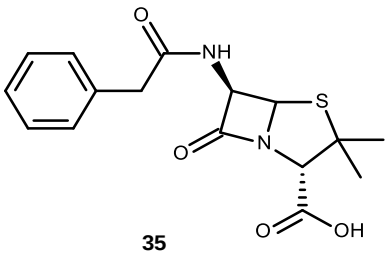

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Figure 9. Structure of penicillin G (**35**).

Based on the Staniek *et al*. (2008) suggestion, fungi are the most frequently encountered endophytes. The existence of fungi which are mainly referred to as endophytes are found inside the organs of asymptomatic organisms (Guérin, 1898). Marine associated fungi from diverse environments contain elevated salt levels, typical of the marine situation and in association with invertebrate condition, they are very sensitive to culture media (Abrell, *et al*., 1996). It can be assumed that marine fungi could produce

bioactive metabolites and consequently can be the reservoir of marine natural products (Saleem *et al*., 2007). Marine fungi have proven a rich source of bioactive natural products (Altomare *et al*., 2000; Bhadury *et al*., 2006). Generally, the growth of most marine micro-organisms is not only unique but also in extreme habitats and consequently they have the capability to produce unique and unusual secondary metabolites. It can be chemical defense or adaptation of fungi for competing substrate (Bhadury *et al*., 2006; Fenical and Jensen, 1993). Bhadury *et al*. (2006) deduced that the production of marine fungi unique secondary metabolites is possible due to variable environmental pressure.

According to Xu *et al*. (2015), 105 marine fungal strains were isolated from 12 different classes of marine materials and had been used for the further isolation of antibacterial or antifungal compounds in 5 years from 2010 to 2015. These 12 classes are shown in Figure 10.

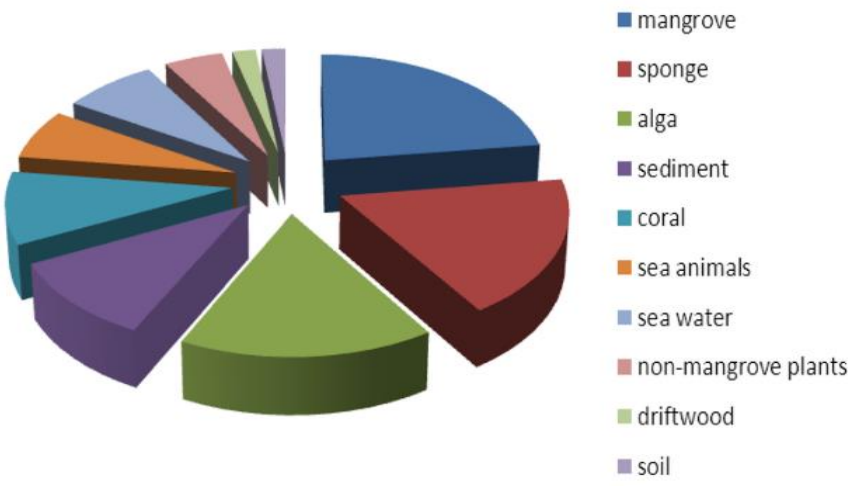


Figure 10. Numbers of fungal strains from different isolation materials (Xu *et al*., 2015) [The permission of the author; shown in Appendix].

Among 12 classes, fungal strain isolation that can produce antibacterial or antifungal compounds mostly come from algae, sponges, mangroves and these are the most common sources of fungi. Moreover, 20 new compounds have been isolated from the 11 fungal strains cultured from marine sediments shown antifungal or antibacterial activities in a total of 116 new compounds cultured from all other marine sources shown antifungal

or antibacterial activities. This means that soil fungi are also a potential for the bioactive secondary metabolites isolation. Moreover, several compounds including indole alkaloids were isolated from *Neosartorya siamensis* which have been isolated from soil fungi in recent years (Buttachon *et al*., 2012). However, fungi isolated from sediments seem to be underestimated and fungi from sponges may be overestimated due to 21 new compounds isolated from the 19 fungal strains cultured from marine sponges showing antifungal or antibacterial activities (Xu *et al*., 2015). Moreover, unexplored habitats with their unknown fungi are also a potential in searching for novel compounds in terms of biological importance or for their pharmaceutical attributes (Hawksworth *et al*., 1997). During the recent years, research papers which have studied marine organisms were increasingly published and many kinds of species, their activities and many of bioactive molecules have been discovered (Hu *et al*., 2015). The growth of marine natural products chemistry has led to the identification of larger numbers of novel compounds from marine fungi source. Consequently, this will be useful for many further investigations concerning marine natural products pharmaceutical pipelines.

1.6. Discovery of Marine Fungi and Applications for Pharmaceutical Purposes

In general, both fungi collecting and culturing from the environment are still essential not only to identify specimens and taxonomy but also to study their roles in the environment. Moreover, it needs to provide strains for biological assay, pharmaceutical study and all other processes (Blackwell, 2011). Based on empirical knowledge, some diseases are still lacking in efficient treatment such as cancer, HIV-AIDS, Alzheimer's disease, Parkinson's disease, arthritis and other major diseases. All these diseases entice one to find the effective solution from both sources, natural and synthetic products. Currently, identification of marine fungi is growing day by day and these growing numbers become the sources of the novel bioactive compounds or potentially life-saving secondary metabolites (Bhadury *et al*., 2006). Penicillin G (**35**) (Figure 9), cephalosporin C (**36**) and ergotamine (**37**) are well-known fungal secondary metabolites shown in Figure 11 (Bhadury *et al*., 2006; Keller *et al*., 2005; Kupka *et al*., 1981; Zajdel *et al*., 2015). Therefore, marine-derived fungi also have potential to isolate bioactive secondary metabolites since fungi from terrestrial sources have produced many useful secondary metabolites. Nowadays, many marine fungal-derived compounds show several bioactivities. Among them, sargassamide, halimide and avrainvillamide have shown selective inhibition of cancer cell lines. Moreover, it is shown in *vivo* activity in preclinical models (P-388 lymphocytic leukemia) and two of the above potential drugs have been processed in the pharmaceutical industry and are in preclinical development (Bhadury *et al*., 2006). There are many marine drug candidates including from fungal sources proposed for the screening phase and preclinical studies (Keller *et al*., 2005).

There is no confusion that marine compounds have the potential to treat not only cancer but also other types of diseases. For example, varixanthone (**38**) isolated from *Emericella variecolor* (strain M75-2) which was taken from a sponge (*Porifera*) collected in the Caribbean waters of the Mochima Bay (Mochima National Park, Sucre State, Venezuela was active against *Escherichia coli, Proteus sp*., *Bacillus subtilis*, *Staphylococus aureus*, showing a minimal inhibitory concentration (MIC) of 12.5 µg/mL (Malmstrøm *et al*., 2002; Saleem *et al*., 2007). Moreover, lunatin (**39**) isolated from marine fungus *Curyularia lunata* which was collected from the sponge, *Niphates olemda* showed activity against *Staphylococus aureus*, with the MICs of 8.5 mm in 5 and 10 µg/disk and active against *Escherichia coli* with the MICs of 9.0 mm zone inhibition in 5 µg/disk (Agusta *et al*., 2006; McDonald *et al*., 1999). In addition, compounds isolated from marine-derived fungi also

show antiviral activities. For example, equisetin (**40**) and phomasetin (**41**) are isolated from marine-derived fungi *Fusarium heterosporum* and a *Phoma sp*. inhibited *in vitro* recombinant integrase enzyme with the IC_{50} value between 2.6–8.3 µg/mL (Singh *et al*., 1998; Sugie *et al*., 2002). Speradine A (**42**) isolated from the marine fungus, *Aspergillus tamarii* (M143) which was separated from driftwood, collected at Seragaki Beach, Okinawa Island showed antibacterial activity against *Mycrococcus luteus* with the MIC of 16.7 µg/mL and also exhibited inhibitory activity against Ca^{2+}-ATPase (IC_{50} 2.93 µg/mL), inhibitory activity against histone deacetylase (IC_{50} 100 µg/mL), respectively (Tsuda *et al*., 2003). According to all the results mentioned above, there is no more doubt that the discovery of marine fungi and their applications for pharmaceutical purposes play a vital role in the fight against major diseases.

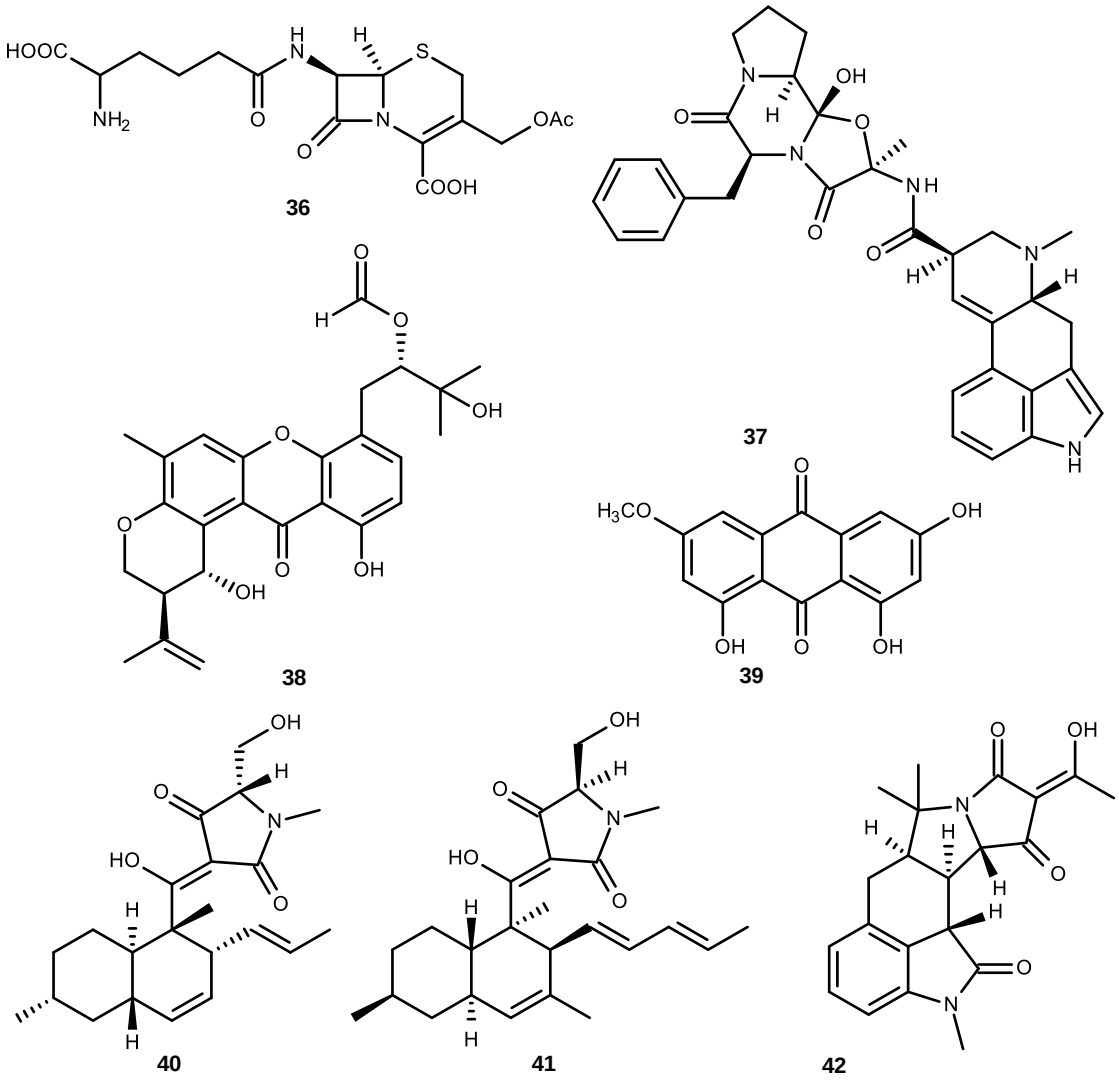


Figure 11. Structures of cephalosporin C (**36**), ergotamine (**37**) varixanthone (**38**), lunatin (**39**), equisetin (**40**), phomasetin (**41**) and speradine A (**42**).

1.7. The Genus *Neosartorya* (Mollach & Cain, 1972)

Kingdom	⇒	**Fungi**
Division	⇒	**Ascomycota**
Phylum/sub-division	⇒	**Pezizomycotina**
Class	⇒	**Eurotiomycetes**
Sub-class	⇒	**Eurotiomycetidae**
Order	⇒	**Eurotiales**
Family	⇒	***Trichocomaceae***
Genus	⇒	***Neosartorya***

(http://www.mycobank.org/Biolomics.aspx?Table=Mycobank&MycoBankNr_=318629)

In 1972, Mollach and Cain identified a new genus of fungi as "N*eosartorya"* which refers to a member of the *Aspergillus fischeri* series under a series of the *Aspergillus fumigatus* group which are defined by Rapper and Fennell in 1965 (Rapper and Fennell, 1965; Samson *et al*., 1990). One of the most important kingdoms concerning the botanical naming system are fungi. More specifically, this is *Neosartorya,* a teleomorph of *Aspergillus* genus. In the past, *Aspergillus* had a dual nomenclature in the naming system. This means that the naming system of *Aspergillus* genus didn't follow the Rules of the International Botanical Code. The anamorphic stage of genus *Neosartorya* belongs to *Aspergillus* section *Fumigati* characterized by uniseriate, columnar conidial head, flask-shaped vesicle in shades of blue-green to dark-green (Rapper and Fennel, 1965). Based on the current naming system, the Melbourne code, *Neosartorya* and *Aspergillus* overlap each other in some species (Hawksworth, 2004; 2011; McNeill, 2011). Literature survey showed that *Aspergillus* genus has a larger impact on many areas and is one of the most impressive fungal genera to scientists (Pitt and Samson, 2007). Moreover, *Aspergillus* section *Fumigati* is more sensitive in sexual reproduction than others (Hong *et al*., 2010).

The other important fact of note concerning the *Neosartorya* genus is heterothallism. Heterothallism was first reported in the genus *Aspergillus* by Kwon, Fennell, and Raper in 1964 (Kwon-Chung and Kim, 1974). Even though most of the species in the *Trichocomaceae* family are homothallic, some species in this family are heterothallic and

seven species of *Neosartorya* genus which include the *Aspergillus* section *Fumigati* are recognized as heterothallic species. Based on previous literature and our best knowledge, there are ten heterothallic species in the *Trichocomaceae* family which were: *Emericella heterothallica*, *Neosartorya fennelliae, Neosartorya otanii*, *Neosartorya spathulata*, *Neosartorya udagawae*, *Neosartorya nishimurae, Neosartorya tsurutae, Neosartorya indohii*, *Talaromyces derxii*, *Byssochlamys spectabilis* (Kwon-Chung and Sugui, 2009; Samson and Varga, 2009). Among them, *Emericella hererothallica, Talaromyces derxii*, *Byssochlamys spectabilis* are not included in the *Neosartorya* genus nor do they belong to the *Aspergillus* section *Fumigati* (Horn *et al*., 2009; Houbraken *et al*., 2008; Samson and Varga, 2009; Samson *et al*., 2007 and 2011). Heterothallic species are able to interbreed freely under natural conditions and these kind of species are most popular among evolutionists (Dobzhansky, 1937; Samson and Varga, 2009).

As for distinguishing species, species in the *Aspergilus* section *Fumigati* is difficult to differentiate from other closely related species because of variation between macro and micromorphology (Samson *et al.,* 2006). Major morphological character of the ascomycete genus, *Neosartorya* is ascospore ornamentation (Kozakiewicz, 1989). Basically, *Aspergillus* section F*umigati* could be found around 50 ℃ but do not survive at 10 ℃. Based on genetics, they are not only unique but also a very homogeneous taxon. Moreover, they are distinctly differentiated from related species in terms of molecular characters including β-tubulin, actin and calmodulin gene sequences (Samson *et al.,* 2006). After replacement of the International Code of Botanical Nomenclature with International Code of Nomenclature for algae, fungi and plants, one genus name *Aspergillus* refers to both teleomorph and anamorph. Even though, the term of *Neosartorya* is still allowed for the fungi species which were classified before the enforcement of new naming system, the Melbourne Code (Hawksworth, 2004; 2011; McNeill, 2011).

Generally, fungi can live as endophytes which means they live within the living host organism (eg. plant) for a certain amount of time without any harmful threat to the host (Tan and Zou, 2001; Schulz *et al*., 2002). Since species under *Neosartorya* genus are heat-resistant, species under *Neosartorya* genus in terms of food are also important to focus on. Species under *Neosartorya* genus can cause the spoiling of heat-processed acidic foods by the formation of heat-resistant ascosporesin. On the other hand, *Aspergillus fumigatus* have never been reported as a spoiling agent in heat-processed food products even though anamorphs of *Aspergillus fumigatus* are phylogenetically and morphologically related with *Neosartorya* species. Due to the above difference, it is critical to differentiate between the genus of *Neosartorya* and *Aspergillus* in terms of the food industry (Yaguchi *et al.,* 2012).

In addition, species in *Neosartorya genus* have potential in terms of drug discovery due to bioactive secondary metabolite production from species in this genus (Hong *et al*., 2008).

1.8. Distribution of Fungi Species in *Aspergillus* Section *Fumigati*

Fungi can be found in almost all habitats on Earth (Hawksworth and Rossman, 1997; Raspor and Zupan, 2006). Basically, species in *Aspergillus* section *Fumigati* are also found in almost all parts of the world. Samson *et al*. (2007) described the distribution of numerous species in their review including species which are overlapped between *Aspergillus* and *Neosartorya* genus under *Aspergillus* section *Fumigati*. Species under this section have been found in Australia, Argentina, Belgium, Brazil, Brunei, Canada, Denmark, the Dominican Republic, Ecuador, Fiji, Ghana, India, Japan, Kenya, South Korea, Liberia, Morocco, Netherlands, Nicaragua, Pakistan, Russia, Spain, Sri Lanka Sudan, Suriname, Taiwan, the United Kingdom, the United States of America, Venezuela and Zambia shown in Figure 12. Besides this, new species of *Aspergillus* section *Fumigati*, have been found in China and Thailand (Eamvijarn *et al*., 2013a; Yaguchi *et al*., 2010). Therefore, it can be assumed that many species of fungi in this section have been found in many parts of the world and more species will continue to be found in future.

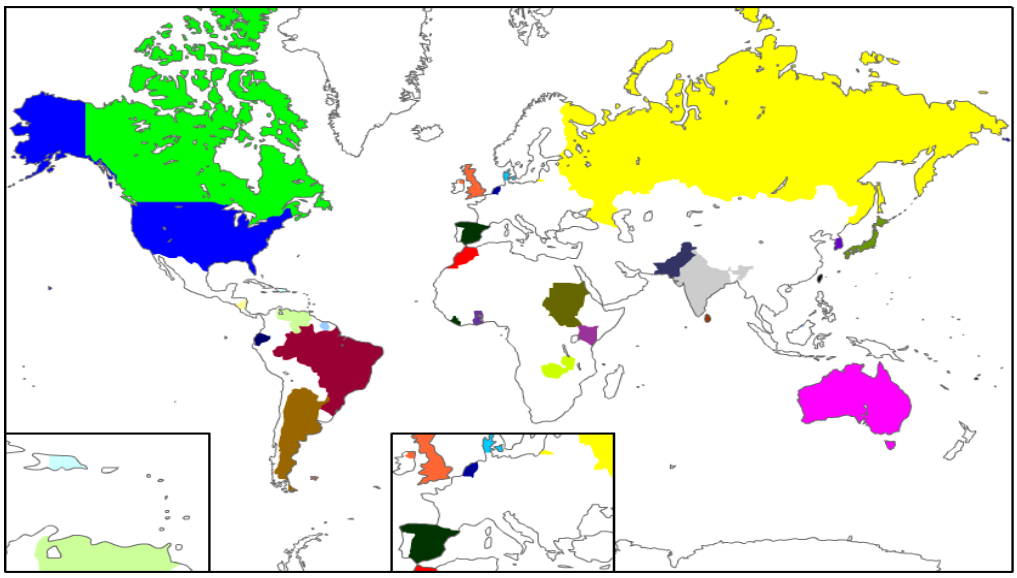
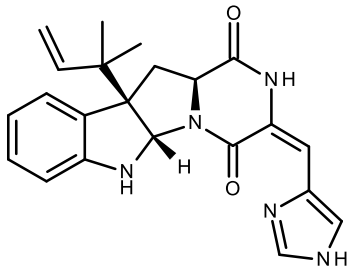


Figure 12. Distribution of fungi Species in *Aspergillus* section *Fumigati* (Data obtained from Samson *et al*, 2007; 2014).

CHAPTER II
SECONDARY METABOLITES FROM SIMILAR SPECIES IN *ASPERGILLUS* SECTION *FUMIGATI* WITH *N. FENNELLIAE*

2.1. Nature of Secondary Metabolites

Generally, secondary metabolites can be released during normal cellular metabolism (Campbell, 1985). Secondary metabolites are known as differentiation products due to their functions. They act as chemical signals between organisms and species (Ciegler *et al*., 1973). The identical secondary metabolite could be produced by unrelated species and sometimes can be released by species which are from a different order or kingdom (Frisvad *et al*., 1998). Not only in species from a different order or kingdom but also the same secondary metabolites can be produced from a completely different biosynthetic route. For instance, 3-nitropropionic acid can be produced in fungi from L-aspartate and in the plant *Indigofera spincata* from malonate (Baxter *et al*., 1994). However, some secondary metabolites are being produced within related species only and this means that they have a close phylogenetic relationship such as roquefortine C (**43**) (Figure 13) (Shangguan and Joullié, 2009) which have the amino acid and terpene containing the secondary metabolite, which has been found only within related species of *Penicillium* (Frisvad and Filtenborg, 1989). Some secondary metabolites are present in all species of the same genus because of their specific value, consequently they come together under the same genus or species specific like anthraquinone derivatives, physcion (**44**) and erythroglaucin (**45**) (Figure 13) are presented in all species of the genus *Eurotium* due to their value in protection against physical stress such as radiation (Anke *et al*., 1980; Frisvad *et al*., 1998; Gould and Raistrick, 1934; Mueller *et al*., 1999). Therefore, the profile of secondary metabolites is critical in characterizing fungal species since the individual secondary metabolites are of value in chemotaxonomy (Frisvad *et al*., 1998).



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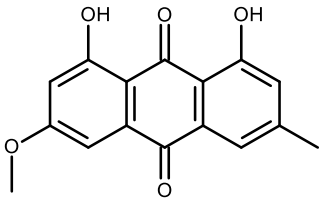

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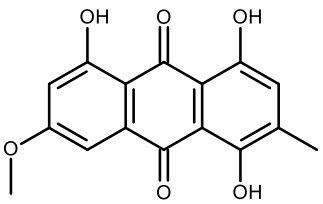

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Figure 13. Structures of roquefortine C (**43**), physcion (**44**) and erythroglaucin (**45**).

2.2. Secondary Metabolites from Species in *Aspergillus* Section *Fumigati*

For drug discovery, bioactive secondary metabolites from certain kinds of species could be considered as having potential (Hong *et al*., 2008). Species under section *Fumigati* produce many different secondary metabolites such as mycotoxins which are known as having potential in pharmacology and biotechnology fields (Wong *et al*. 1993; Tomoda *et al*. 1994; Larsen *et al*. 2007; Samson *et al*. 2007). Many secondary metabolites including bioactive peptides/proteins, lectins, enzymes, hydrophobins and aegerolysins have been produced from the genus *Aspergillus* (Frisvad and Larsen, 2015). Secondary metabolites isolated from the *Aspergillus* section *Fumigati* showed anticancer activity not only *in vitro* but also *in vivo* (Bladt *et al*., 2013). Some structures of the most important extrolites from this section such as aszonapyrone A (**46**), chevalone A (**47**), verruculogen (**48**), helvolic acid (**49**), gliotoxin (**50**), fumigaclavine A (**51**), 8-O-methyl asterric acid (**52**), fumiquinazoline F (**53**), tryptoquivaline A (**54**), sulochrin (**55**), aszonalenin (**56**), trypacidin (**57**) and fmagilin (**58**), fumitremorgin C (**59**), pseurotin A (**60**), pyripyropene A (**61**), were shown in Figure 14a and 14b (Eamvijarn *et al*., 2013b; Frisvad and Larsen, 2016; Latif *et al*., 2009; Maiya *et al*., 2007; Ohshiro *et al*., 2011).

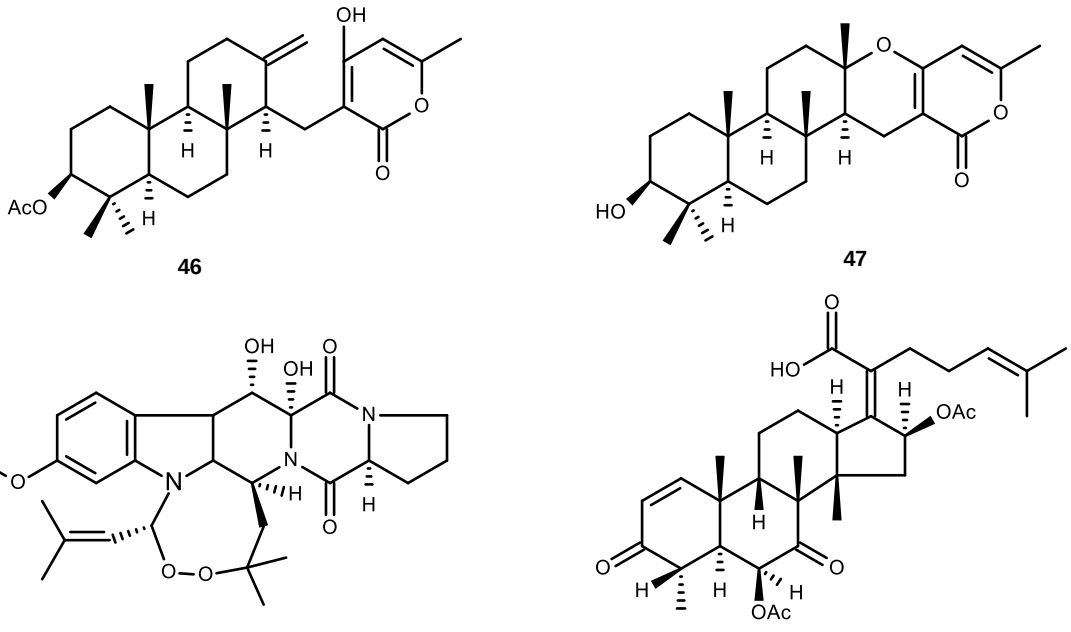


Figure 14a. Structures of aszonapyrone A (**46**), chevalone A (**47**), verruculogen (**48**) and helvolic acid (**49**).

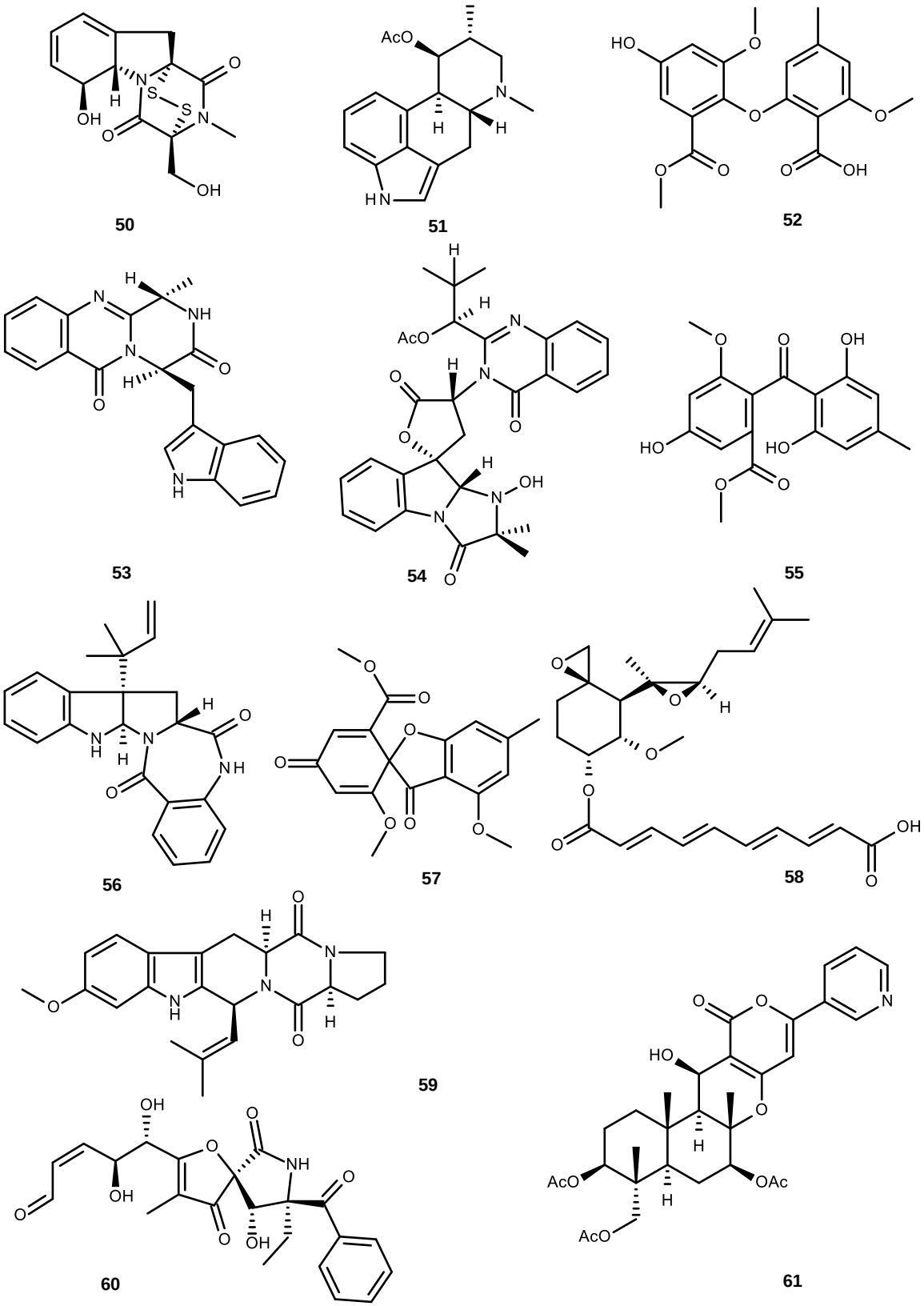


Figure 14b. Structures of gliotoxin (**50**), fumigaclavine A (**51**), 8-O-methyl asterric acid (**52**),

fumiquinazoline F (**53**), tryptoquivaline A (**54**), sulochrin (**55**), aszonalenin (**56**), trypacidin (**57**) and fmagilin (**58**), fumitremorgin C (**59**), pseurotin A (**60**) and pyripyropene A (**61**).

Therefore, *Neosartorya* genus which are a teleomorph of *Aspergillus* genus also have potential to isolate secondary metabolites which may have several biological activities (Matsuzawa, 2014). There are several secondary metabolites already isolated from various species under *Neosartorya* genus in section *Fumigati* shown in Table 3. Among them, some are bioactive including both newly identified and known compounds, may contribute to infection in human and other animals. For example, gliotoxin (**50**) which is known as important for the infection process has been reported from all pathogenic species such *as Neosartorya denticulata, N. ferenczii* and *N. pseudofischeri* (Frisvad and Larsen, 2016).

Table 3. Isolated secondary metabolites from *Neosartorya* genus in *Aspergillus* section *Fumigati* [adapted from Samson *et al*., 1990 and 2007].

Species	Secondary Metabolites
N. assulata	indole alkaloids and apolar metabolites
N. aurata	helvolic acid, yellow unidentified compounds
N. aureola	fumagillin, tryptoquivaline, tryptoquivalone, pseurotin A and viriditoxin
N. australensis	wortmannin-like, aszonalenin-like
N. coreana	aszonalenins
N. denticulata	gliotoxin, viriditoxin
N. fennelliae	asperfuran, aszonalenin, fumigaclavine, viridicatumtoxin, trypacidin
N. ferenczii	asperfuran, aszonalenin, fumigaclavine, viridicatumtoxin, gliotoxin-like, fumigatins, aszonalenin-like
N. fischeri	terrein, fumitremorgins A & C, tryptoquivaline A, trypacidin, TR-2, verruculogen, sarcin, aszonalenins, fischerin, neosartorin, fiscalins, helvolic acid
N. galapagensis	gregatins
N. glabra	asperpentyn, avenaciolide, wortmannin-like compound
N. hiratsukae	avenaciolide
N. laciniosa	aszonalenins, tryptoquivaline, tryptoquivalone
N. multiplicata	helvolic acid
N. papuensis	wortmannin-like
N. pseudofischeri	asperfuran, cytochalasin-like compound, fiscalin-like compound, pyripyropens, gliotoxin
N. quadricincta	quinolactacin, aszonalenins
N. spinosa	aszonalenins, 2-pyrovoylaminobenzamide, pseurotin
N. spathulata	xanthocillins, aszonalenins
N. stramenia	quinolactacin, avenaciolide
N. tatenoi	aszonalenins
N. udagawae	fumigatin, fumagillin, tryptoquivaline, tryptoquivalone
N. warcupii	wortmannin-like, aszonalenin-like, chromanols-like, tryptoquivaline-like and tryptoquivalone-like

2.3. Secondary metabolites from Similar Species in *Aspergillus* Section Fumigati with *Neosartorya fennelliae*

Marine-derived fungus, *Neosartorya fennelliae* for performing chemical investigation in this thesis belongs to *Aspergillus* section *Fumigati* and the phenomenon of cross-mating between two adjacent colonies occurred. Moreover, the ascogonial coil of *N. fennelliae* resembles *N. fischeri* but it has smaller, mature cleistothecia than *N. fescheri*. *Neosartorya fennelliae* which is not only the second heterothallic species in the *Trichocomaceae* family but can also be deduced as the first heterothallic species in section *Fumigati* since identification of this fungi is prior to all heterothallic species in section *Fumigati* (Kwon-Chung and Kim, 1974). As I mentioned in Chapter 1, some species whether *Aspergillus* or *Neosartorya* genus in *Aspergillus* section *Fumigati* are pathogenic and cause food spoiling. Moreover, certain species from *Aspergillus* section *Fumigati* which have been studied in the past about secondary metabolite production have potential for drug development (Hong *et al*., 2008). The two similar species with *N. fennelliae* based on β-tubulin, calmodulin, actin sequence and ITS sequence are *N. denticulata* and *N. ferenczii* and isolated secondary metabolites from these two species were described in this chapter II (Samson *et al*., 2007) and secondary metabolites of all heterothallic species in section *Fumigati* were also mentioned in this chapter II. Besides this, the secondary metabolites of *N. fischeri* which resemble conidial (columnar) and ascosporic states (convex surface) with *N. fennelliae* and pivotal species in *Aspergillus* section *Fumigati* were also reported in this section 2.3 (Kwon *et al*., 1964; Samson *et al*., 2007). Therefore, isolation of secondary metabolites from the above species in this section is the main idea of this study. All the species described in this chapter were categorized in Figure 15.

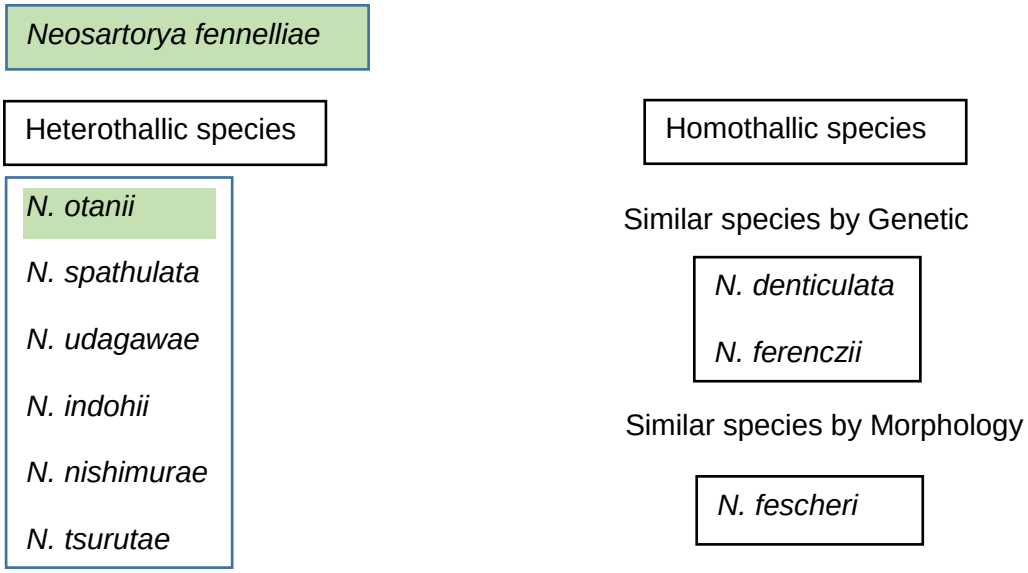


Figure 15. All the described species in chapter II from *Aspergillus* section *Fumigati*.

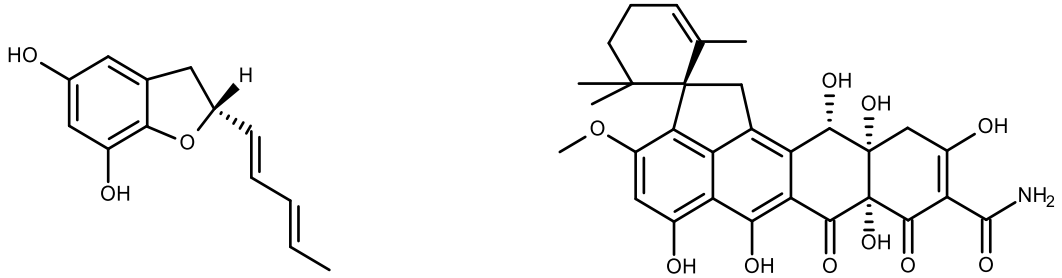
2.3.1. *Neosartorya fennelliae* Kwon-Chung and Kim

This fungus species, *Neosartorya fennelliae* in either *Neosartorya* or *Aspergillus* genus was isolated from eyeballs of laboratory rabbits by Kwon-Chung and S.J. Kim in 1974. Sexual state or teleomorph of this fungus is defined as *Neosartorya*; asexual or anamorph state of this species is defined as *Aspergillus* (Kwon-Chung and Kim, 1974). The conidial and ascosporic states of *N. fennelliae* resemble *N. fischeri* also known as *Aspergillus fischeri* (Kim and Kwon-Chung, 1974; Kwon-Chung and Kim, 1974). Thermophilic fungi play essential roles in terms of potential commercial enzyme production and degradation (Langarica-Fuentes *et al*., 2014). Therefore, the secondary metabolites from these fungi which are thermophilic is also interesting. There are five secondary metabolites already reported from *N. fennelliae*, fumigaclavine A (**51**), aszonalenin (**56**), trypacidin (**57**) shown in Figure 14b and asperfuran (**62**) (Pfefferle *et al*., 1990), viridicatumtoxin (**63**) (Chooi *et al*., 2010) were shown in Figure 16 respectively (Samson *et al*., 1990 and 2007).

Fumigaclavine A (**51**) isolated from *Aspergillus fumigatus* (strain no. CY018) which was isolated from the healthy stem of *Cynodon dactylon* collected, in November 2001 from Yancheng Biosphere Reserve, Jiangsu Province, China was tested for antibacterial activity against *Actinomyces israelii*, *Bacteroids distasonis, Bacteroids vulgatus, Peptostreptococcus anaerobius, Staphylococcus anaerobius* with the MIC of 128 µg/mL for

all tests and active against *Veillonella parvula* with the MIC of 64 µg/mL (Spilsbury and Wilkinson, 1961; Xu *et al*., 2014; Zhang *et al*., 2016). In addition, Indole alkolid, aszonalenin (**56**) from *N. fischeri* which was isolated from coastal forest soil at Samaersarn Island (altitude 12340 2300 N, 100570 2300 E), Chonburi Province, Thailand tested with three cell lines and showed to be inactive in all three cell lines, *in vitro* growth of MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and A375-C5 (melanoma) cell lines (Eamvijarn *et al*., 2013b). Trypacidin (**57**) was isolated from *Aspergillus fumigatus* NRRL 35693 which was obtained from Pharmacology-Toxicology Laboratory New Collection (NCPT) was tested for cytotoxicity with A549 (alveolar lung cells) and human bronchial epithelial cells and showed the effect on decreasing cell viability and triggering cell lysis at an IC_{50} close to 2.41 µg/mL on both cells. (Gauthier *et al*., 2012). According to Pfefferle *et al*. (1990), Dihydrobenzofuran derivative, asperfuran (**62**) produced by *A. oryzae*, HA 302-84 which was isolated from soil samples that were collected in China inhibited chitin synthase from *Coprinus cinereus* with IC_{50} of 65.4 µg/mL. In addition, asperfuran (**62**) induced morphological changes (reduced growth) in *Mucor miehei* and IC_{50} was 25 µg/mL and also exhibited weak cytotoxicity in HeLa S3 and L1210 (mouse lymphocytic leukemia cells) and IC_{50} was 25 µg/mL (Pfefferle *et al*., 1990). According to the experimental result of Bladt *et al*. (2013), viridicatumtoxin (**63**) was isolated from *Penicillum brasilianum* (IBT 22244) which was collected from the IBT culture collection at the Department of Systems Biology, Technical University of Denmark as one of the most cytotoxic compounds tested for chronic lymphocytic leukemia (CLL) cells in this screening campaign with LC_{50} value between 0.396 and 0.002 µg/mL.

Neosartorya otanii was characterized by Takada, Horie and Abliz from African soil in 2001 (Takada *et al*., 2001). However, the structure pattern and general dimensions of the anamorph of this fungus is supposed to be *N. fennelliae*. Samson *et al*. (2007) deduced that *N. fennelliae* and *N. otanii* are the same species. Therefore, these two species are identical and consequently there are only six heterothallic species in *Aspergillus* section *Fumigati* and only nine heterothallic species in the Trichocomaceae family.

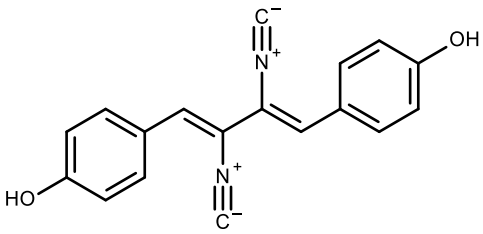


62 **63**

Figure 16. Structures of asperfuran (**62**) and viridicatumtoxin (**63**).

2.3.2. *Neosartorya spathulata* Takada and Udagawa

This species was identified in Taiwan by Takada and Udagawa in 1986 (Takada *et al*., 1986). Aszonalenins and xanthocillins were already isolated from this fungus (Samson *et al*., 2007). Samson *et al*. (2007) didn't mention specifically which compound was isolated from this species. Although, some isolated xanthocillins compounds have antimicrobial activity. For instance, xanthocillin X (**64**) shown in Figure 17 was isolated from marine fungus *Penicillium commune* SD-118 which was isolated from a deep-sea sediment sample collected from the South China Sea (at a depth of 1188 m), in September 2008 exhibited distinct antimicrobial activity in the growth of Gram positive, *Staphylococcus aureus* and Gram negative, *Escherichia coli* with MICs of 2 and 1 μg/mL, respectively (Shang *et al*., 2012).



64

Figure 17. Structure of xanthocillin X (**64**).

2.3.3. *Neosartorya udagawae* Horie, Miyaji & Nishimura

This species was isolated from Brazilian soil by Horie, Miyaji & Nishimura in 1995 (Horie *et al*., 1995). Based on Samson *et al*. (2007) and Tamiya *et al*. (2015), there are 12 compounds already isolated from this species which are helvolic acid (**49**), fumigaclavine A (**51**), tryptoquivaline A (**54**), sulochrin (**55**), trypacidin (**57**), fumigallin (**58**) and, pyripyropene A (**61**) shown in Figure 14a, 14b and tryptoquivalone (**65**) (Demain *et al*, 1976), fumiquinazoline F (**66**) (Liu, *et al*., 2005) and G (**67**) (Wang and Ganesan, 1998), pyripyropene E (**68**) (Prompanya *et al*., 2014) and fumigaclavine C (**69**) (Gomes *et al*., 2015) shown in Figure 18. Helvolic acid (**49**) was isolated from fresh healthy leaves of *Anoectochilus setaceus* which was collected from the Kanneliya forest reserve (6 09′–6 18′N & 80 19′–80 27′ E), Galle, Sri Lanka, in October 2011 and active against the Gram-

positive bacteria, *Bacillus subtilis* (MIC, 2 µg/mL) and methicillin resistant *Staphylococcus aureus* (MIC, 4 µg/mL) (Ratnaweera *et al*., 2014). Moreover, helvolic acid (**49**) was isolated from *Neosartorya spinosa* KKU-1NK1 which was isolated from soils, collected from forests surrounding Pha Nok Kao Silvicultural Station, Khon Kaen Province in Thailand, was active against acid-fast bacteria (*Mycobacterium tuberculosis* H37Ra strain) with the MIC ranging from 12.5-25.0 µg/mL (Sanmanoch *et al*., 2016). Pyripyropene A (**61**) isolated from *Aspergillus* spp. which was isolated from the marine-sediment, collected from a depth of 70 m off Gokasyo Gulf, Mie Prefecture, Japan and showed selective growth inhibition against (Human Umbilical Vein Endothelial Cells) HUVECs (IC_{50} 58.4-1051.2 µg/mL) (Hayashi *et al*., 2009; Lefkove *et al*., 2007; Zbidah *et al*., 2013). Fumigaclavine C (**69**) isolated from *Cynodon dactylon* which was collected in early November 2001 from Yancheng Biosphere Reserve, Jiangsu Province and showed to inhibit *vitro* bioactive assays against human pathogenic fungi, *Candida albicans,* with the MIC of 31.5 µg/mL. (Liu *et al*., 2004).

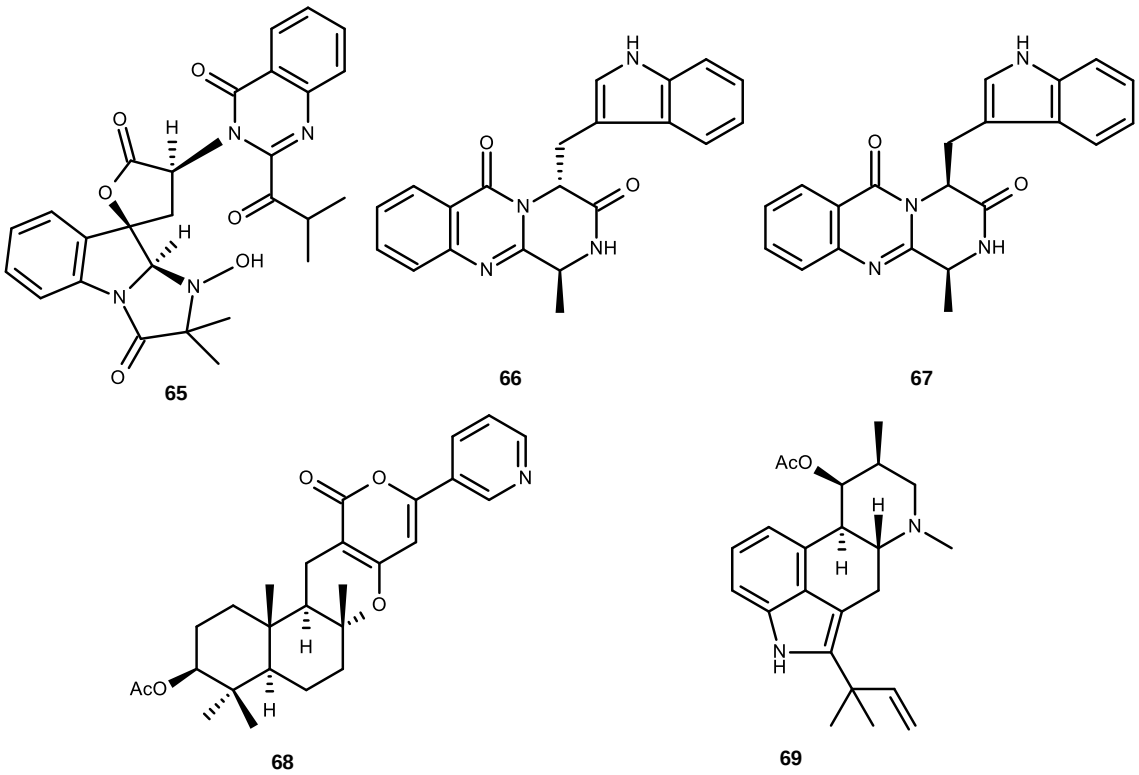


Figure 18. Structures of tryptoquivalone (**65**), fumiquinazoline F (**66**) and G (**67**), pyripyropene E (**68**) and fumigaclavine C (**69**).

2.3.4. *Neosartorya denticulata* Samson, Hong & Frisvad

Neosartorya denticulata was identified by Hong *et al*. (2008) due to its unique denticulate ascospores with a prominent equatorial furrow. These fungi produced unique secondary metabolites such as gliotoxin (**50**) shown in Figure 14b and viriditoxin (**70**) (Saeed, 2016) shown in Figure 19. This species is similar to *N. fennelliae* and *N. ferenczii* (Samson *et al*., 2007). Gliotoxin (**50**) isolated from *Aspergillus* spp. strain YL-06 which was isolated from a marine brown alga collected in Ulsan, Korea, induced pro-apoptotic in human HeLa cervix carcinoma and SW 1353 chondrosarcoma cells and also induced the activation of caspase-3, caspase-8 and caspase-9, the down-regulation of Bcl-2, the up regulation of Bax and the release of cytochrome c (cyt c). Moreover, this compound also showed a dual inhibition of FTase and GGTase by inhibiting protein isoprenylation and cell proliferation in breast cancer MCF-7 and MDA-MB-231 cell lines (Gomes *et al*., 2015; Nguyen *et al*., 2013; Vigushin *et al*., 2004). Moreover, gliotoxin (**50**) isolated from strains Y90086 inhibited the proliferation of HUVEC cells in a dose-dependent manner with IC_{50} values of 40 µg/mL and displayed weaker activity *in vitro* growth-inhibitory activity in cancer than in HUVEC cells (Evidente *et al.,* 2014). Viriditoxin (**70**) are very specific and the toxicity of this compound may preclude its further development in drug discovery (Anderson *et al*., 2012). For instance, viriditoxin (**70**) was isolated from the endophytic fungus *Paecilomyces variotii* FEL 32 which was isolated from the healthy leaves of *Laguncularia racemosa* (L.) and collected from the estuary of the Paripe River, Ilha de Itamaracá, Pernambuco, was tested against Gram positive, *Staphylococcus aureus* with MIC values between 0.5 and 2 µg/mL. (Silva *et al*., 2013). Moreover, viriditoxin (**70**) shows activity against bacterial pathogens such as methicillin resistant and methicillin sensitive strains of *Staphylococcus aureus* with MIC of 4–8 µg/mL and vancomycin resistant, *Enterococcus faecalis* and vancomycine sensitive, *Enterococcus faecium* with the MIC of 2–16 µg/mL, respectively (Wang *et al*., 2003).

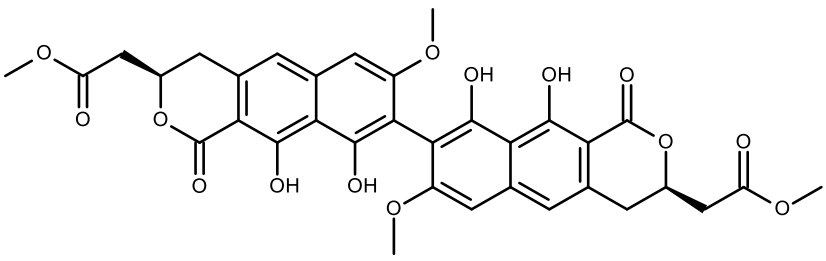


Figure 19. Structure of viriditoxin (**70**).

2.3.5. *Neosartorya ferenczii* Varga & Samson

This new species was isolated in 2007 by Samson and Varga named as *Neosartorya ferenczii*. Generally, *Neosartorya* species are characterized based mainly on ascospore morphologies. This species produced fumigaclavine A (**51**), aszonalenin (**56**), asperfuran (**62**), viridicatumtoxin (**63**) shown in Figure 14b and 16 (Samson *et al*., 2007). The bioactivity of isolated compounds from this species was not mentioned in literature but some compounds which are identical to isolated compounds from others species showed bioactivity in other assays and mentioned in section 2.2 and sub-section 2.3.1.

2.3.6. *Neosartorya fischeri* Malloch & Cain

Neosartorya fischeri, which are well known fungi due to human aspergillosis are also under section *Fumigati*. This species was first reported in 1972 by Malloch and Cain (Malloch and Cain, 1972). This fungus is a heat-resistant mold that causes spoiling in fruits and vegetables and can be found in common environments. (Girardin *et al*., 1995; Nielsen, 1991; Tan *et al*., 2012; Summerbell, *et al*., 1992). According to the literature survey of Wong *et al*. (1993), three new compounds, named fiscalins A (**71**), B (**72**), and C (**73**), were isolated from *N. fischeri* which was isolated from a plant rhizosphere collected near the WeFung Chi Cascade region of Taiwan shown in Figure 20. These compounds, fiscalins A (**71**) have *Ki* value 27, fisclains B (**72**) have Ki value 67.2 and fisclains C (**73**) have Ki values of 38.2 µg/mL, respectively to inhibit human neurokinin (NK-1) by binding with radiolabeled substance P (decapeptide) ligand to this receptor.

Moreover, two cyclopentanone derivatives, isoterrein (**74**) and terrain (**75**) were first identified from this species by Wakana *et al*., 2006 shown in Figure 20. In 2013, two new metabolites including a new aszonalenin analogue, 1-formyl-5-hydroxyaszonalenin (**76**) and a new monocyclic meroditerpene, sartorypyrone A (**77**) were isolated from the Soil-fungus *Neosartorya fischeri* (KUFC 6344), which was collected at Samaersarn Island (altitude 12° 34′ 23″ N, 100° 57′ 23″ E), Chonburi Province, Thailand, in November 2008. These two secondary metabolites were tested with three cell lines, *in vitro* growth inhibitory activity on the MCF-7 (breast adenocarcinoma), NCI-H460 (non-small cell lung cancer) and A375-C5 (melanoma) cell lines by the protein binding dye SRB method. Sartorypyrone A (**77**) displayed distinct growth inhibitory activity against all tested cell lines, with GI_{50}= 21.16 ± 3.47 µg/mL for MCF- 7, 17.0 ± 1.83 µg/mL for NCI-H460 and 9.83 ± 0.87 µg/mL, A375-C5 while 1-formyl-5-hydroxyaszonalenin (**76**) was inactive against all three cell lines at 62.7 µg/mL, the highest concentration tested (Eamvijarn *et al*., 2013b).

In addition, two new compounds fischeacid (**78**) and fischexanthone (**79**) were isolated from *N. fischeri* strain 1008F$_1$ shown in Figure 20. These two new compounds did not show cytotoxicity and antiphytoviral effect when evaluating these two assays under the concentration of 200 µg/mL (Tan *et al*., 2012). However, the two other compounds also isolated from *N. fischeri* strain 1008F$_1$, named AGI-B4 (**80**) and 3,4-dihydroxybenzoic acid (**81**) exhibited distinct inhibitory effect on the replication of tobacco mosaic virus (TMV), with IC_{50} values of 82.68 and 97.02 µg/mL, respectively (Tan *et al*., 2012). Moreover, many secondary metabolites already isolated and reported from this species are verruculogen (**48**), helvoic acid (**49**), tryptoquivaline A (**54**), aszonalenin (**56**), trypacidin (**57**), fumitremorgins C (**59**) shown in Figure 14a, 14b and *α*-sarcin A (**82**) (Wool, 1997), fumitremorgins A (**83**) (Yamazaki *et al.,* 1980), TR-2 (**84**) (Willingale *et al*., 1983) and neosartorin (**85**) (Proksa *et al*, 1998) were shown in Figure 21 (Samson *et al*., 2007).

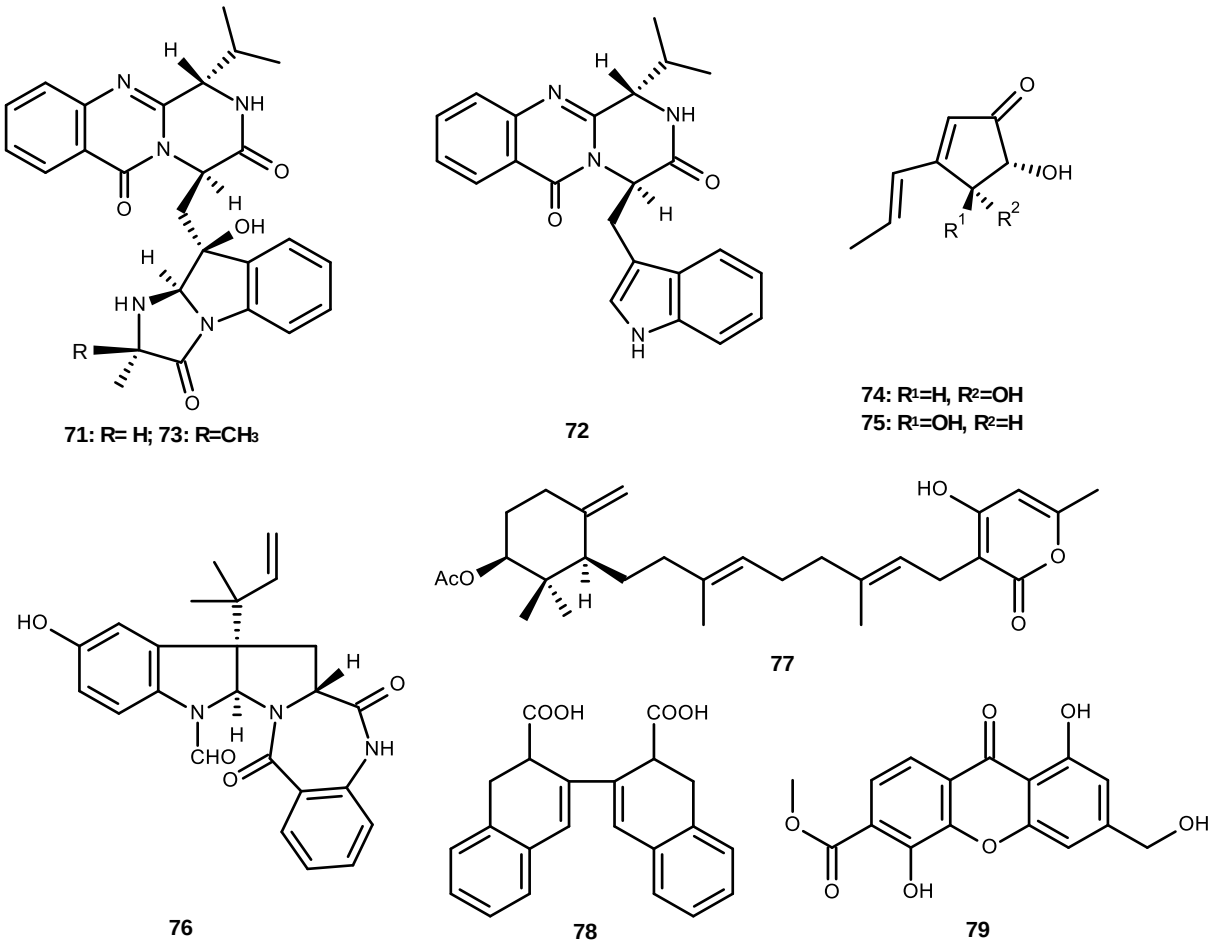


Figure 20. Structures of fiscalins A (**71**), B (**72**), C (**73**), isoterrein (**74**), terrain (**75**), 1-formyl-5-hydroxyaszonalenin (**76**), sartorypyrone A (**77**), fischeacid (**78**) and fischexanthone (**79**).

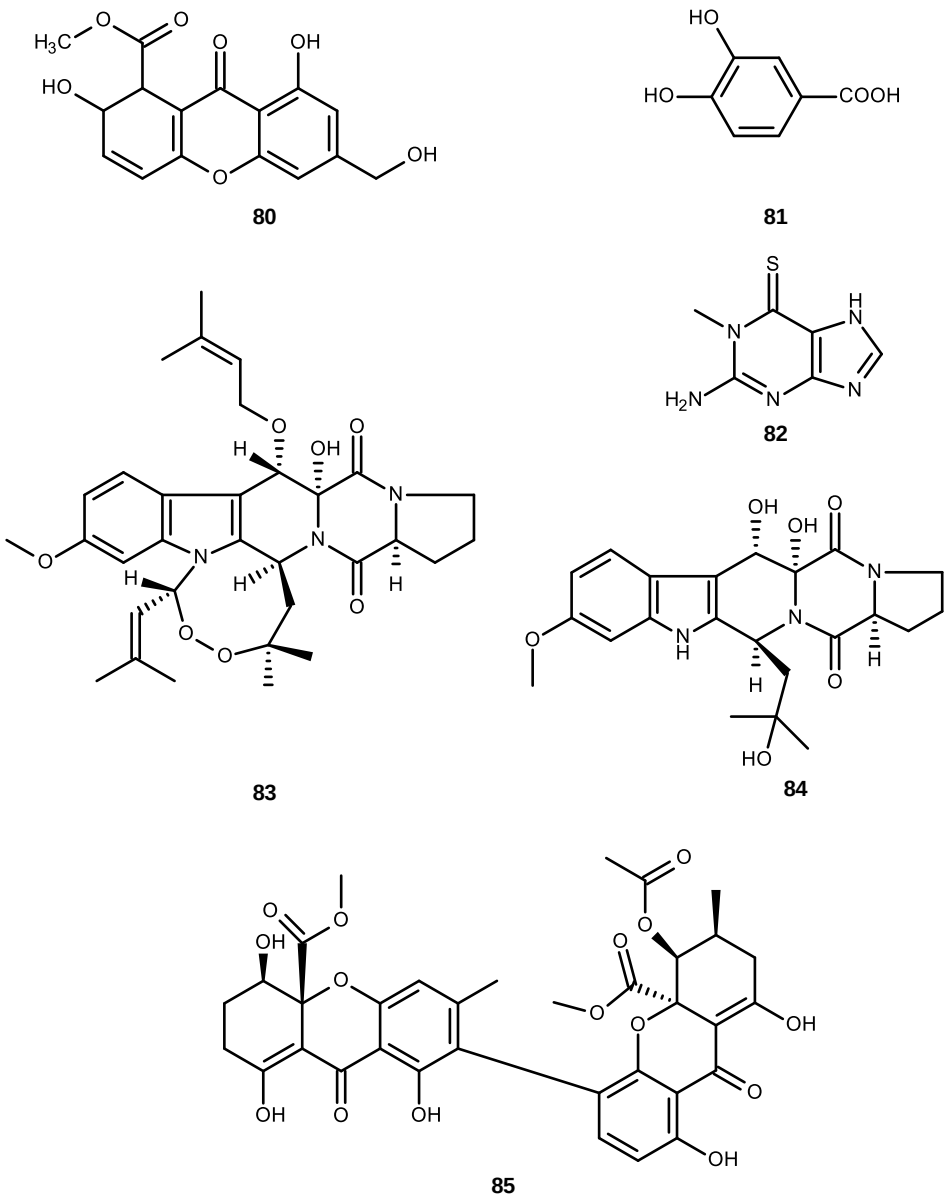


Figure 21. Structures of AGI-B4 (**80**), 3,4-dihydroxybenzoic acid (**81**), *α*-sarcin A (**82**), fumitremorgins A (**83**), TR-2 (**84**) and neosartorin (**85**).

CHAPTER III
MATERIALS AND METHODS

3. Materials and Methods

3.1. General Experimental Procedures

3.1.1. Chemical requirements

(i) Silica gel 60 0.2-0.5 mm; 70-230 mesh, Merck®

(ii) LiChroPrep silica gel 60 0.04-0.063 mm, Merck®

(iii) Sephadex™ LH-20

(iv) Silica gel 60 GF_{254}, Merck®

(v) GF_{254} (Macherey-Nagel), ALUGRAM®

(vi) Solvents in analytical reagent grade from Merck® and Fischer®

(a) Chloroform ($CHCl_3$)

(b) Acetone (Me_2CO)

(c) Methanol (CH_3OH)

(d) Ethyl acetate (EtOAc)

(e) Dichloromethane (CH_2Cl_2)

(f) Formic acid (HCO_2H)

3.1.2. Material requirements

(i) Bruker AMC instrument

(ii) Waters Xevo QToF mass spectrometer coupled to a Waters Aquity UPLC system

(iii) Shimadzu mini-1240UV-VIS spectrophotometer

(iv) ATT Mattson Genesis Series FTIR™

(v) Bock monoscope

(vi) ADP410 Polarimeter

(vii) Analytical balance AND GH-202

(viii) Büchi Heating Bath

(ix) B-49, Büchi Rotavapor R-210

(x) Büchi Vacuum Module V-801 EasyVac

(xi) Vacuum Pump V-700

(xii) Various typical chemical apparatus which are normally used in Chemistry laboratory

3.1.3. Extraction and Fractionation

The crude ethyl acetate extract obtained from Department of Plant Pathology, Faculty of Agriculture, Kasetsart University, Bangkok, Thailand and dissolved in $CHCl_3$ and washed with H_2O three times by using separating funnel. The organic layer was dried with anhydrous Na_2SO_4, filtered and evaporated under reduced pressure and the extract obtained. The extract was applied on column chromatography of silica gel 60 (0.2-0.5 mm; 70-230 mesh, Merck, LiChroPrep silica gel 60 0.04-0.063 mm, Merck) and eluted with a mixture of petrol-$CHCl_3$, $CHCl_3$-Me_2CO and MeOH 250 mL per fraction were collected. Solvents were evaporated under reduced pressure by using rotary evaporator to reuse in continuous separation of column chromatography.

3.1.4. Isolation and Purification

Collected fractions were applied on TLC plate (silica Gel 60 GF_{254}, Merck), 0.25 mm thickness (coated with silica gel sheets GF_{254} (Macherey-Nagel), ALUGRAM®, Silica G/UV_{254} on glass plate and activated at 110ºC in the oven Binder for 4 hours) and visualized by either under UV_{254nm} and UV_{365nm} or developed with iodine vapor. Measurement of the weight of each fraction was performed on the analytical balance AND GH-202. The column fractions were grouped according to their TLC profile and were purified by either crystallization or preparative TLC. The fractions containing complex mixture were further purified by small column chromatography.

3.1.5. Structure Elucidation

Isolated compounds from column chromatographic separation were detected by 1D and 2D Nuclear Magnetic Resonance spectral analysis (^1H and ^{13}C, DEPTs 90º and 135º, COSY, HSQC, HMBC, NOESY) at ambient temperature in $CDCl_3$ or DMSO-d_6 on a Bruker AMC instrument operating either at 300.13 for ^1H and 75.47 MHz for ^{13}C, NMR or 500.13 MHz for ^1H and 125.77 MHz for ^{13}C, NMR based on the purification and quantity of respective compounds. In cases when the isolated compound turned to crystal state, X-Ray crystallographic analysis was performed. Isolated compounds were measured by high resolution mass spectra with a Waters Xevo QToF mass spectrometer coupled to a Waters Aquity UPLC system to deduce the total mass of isolated compounds. Ultraviolet and infrared spectra of isolated compounds were recorded on a Shimadzu mini-1240UV-VIS

spectrophotometer using 1.000 cm quartz cells, and on an ATT Mattson Genesis Series FTIR™ using WinFIRST software to determine the functional groups of isolated compounds respectively. Determination of melting points was done by Bock monoscope and optical rotations were determined on an ADP 410 Polarimeter to confirm the isolated compounds.

3.2. Isolation and Identification of the Biological Material

The tested strain KUFA 0811 was isolated from the marine sponge, *Clathria reinwardtii* (Figure 22a) collected from Samaesan Island, Amphur Sattahip, Chonburi province, in February 2015. First, the sponge was cleaned with 1 percent sodium hypochlorite solution for 1 min and then washed with sterilized seawater for 3 times, and later dried on sterile filter paper under a laminar flow hood, then cut into small pieces (5 x 5 mm), and put on malt extract agar (MEA) plates which have 70% seawater and 300 mg/L of streptomycin sulfate. Later, the plates were incubated at 28°C for 7 days, after which the hyphal tips were transferred onto a slant MEA and maintained as pure culture for further identification. The tested fungus was identified as *Neosartorya fennelliae* Kwon-Chung and Kim, based on morphological characteristics observed under a light microscope and study of the ornamentation of ascospores was conducted using the scanning electron microscopy (SEM: JEOL JSM 6400) and was also confirmed by analysis sequence of the internal transcribed spacer (ITS) gene by following the procedure which is described in Zin *et al*. (2015). Its gene sequences were deposited in GenBank with accession number KU955859.

The pure cultures were deposited as KUFA 0811 (Figure 22b) at Kasetsart University Fungal Collection, Department of Plant Pathology, Faculty of Agriculture, Kasetsart Unversity, Bangkok, Thailand.

Figure 22a. The marine sponge, *Clathria reinwardtii*. (Photograph: Tida Dethoup, Department of Plant Pathology, Kasetsart University, Bangkok, Thailand).

Figure 22b. The isolated fungus, *Neosartorya fennelliae* KUFA 0811 Kwon-Chung and Kim. (Photograph: Tida Dethoup, Department of Plant Pathology, Kasetsart University, Bangkok, Thailand).

3.3 Extraction and Isolation of Metabolites from *Neosartorya fennelliae* Kwon-Chung and Kim KUFA 0811

The fungus was cultured for one week at 28°C in 10 Petri dishes (i.d. 90 mm) containing 25 mL of MEA. In order to obtain the mycelial suspension, the mycelia were transferred into 500 mL Erlenmeyer flasks containing 200 mL of PDB (potato dextrose broth), and incubated on a rotary shaker at 120 rpm for one week at 28°C to prepare the mycelial suspension. Fifty 1,000 mL Erlenmeyer flasks, each containing 300 g cooked rice, were autoclaved at 121°C for 15 minutes, and then inoculated with 20 mL of mycelial suspension of *N. fennelliae* and incubated at 28°C for 30 days. Then, 500 mL of ethyl acetate was added to each flask and macerated for 7 days. Filtration was done with filter paper (Whatman No.1) to give the organic solutions which were combined and then evaporated under reduced pressure to furnish the crude ethyl acetate extract (135 g).

The crude ethyl acetate (135 g) was dissolved in 1000 mL of $CHCl_3$ and then washed with H_2O (3 x 500 mL) by using separating funnel. The organic layer was combined and dried with anhydrous Na_2SO_4, filtered and evaporated under reduced pressure to give 83.5 g of the chloroform crude extract, which was applied on a column chromatography over silica gel (0.2-0.5 mm Merck, 420 g) and eluted with mixtures of petrol-$CHCl_3$, $CHCl_3$-Me_2CO and MeOH wherein 250 mL fractions were collected as follows: fractions 1-30 (petrol-$CHCl_3$, 1:1), 1-86 (petrol-$CHCl_3$, 3:7), 87-202 (petrol-$CHCl_3$, 1:9), 203-436 ($CHCl_3$), 437-579 ($CHCl_3$-Me_2CO, 9:1), 580-690 ($CHCl_3$-Me_2CO, 7:3), 691-705 ($CHCl_3$-MeOH, 95:5), 706-737 (Me_2CO) and 738-749 (MeOH), respectively.

Fractions 31-60 were combined (6.12 g) and purified by using preparative TLC (silica gel G_{254}, petrol-$CHCl_3$-HCO_2H, 1:9:0.1) to give the mixture of **NF1** and **NF2** (155.8

mg). The mixture of compounds (155.8 mg) were repurified by using preparative TLC (silica gel G_{254}, petrol-$CHCl_3$-EtOAc-HCO_2H, 1:8:1:0.1) and recrystallized in MeOH and to give 8 mg of white precipitate **NF1** and 10.5 mg of **NF2**, respectively.

Fractions 106-135 were combined (2.54 g) and purified by using preparative TLC (silica gel G_{254}, petrol-$CHCl_3$-HCO_2H, 1:9:0.1) to give 92.6 mg of yellow liquid mixture. The yellow liquid mixture (92.6 mg) was repurified by using preparative TLC (silica gel G_{254}, petrol-$CHCl_3$-EtOAc-HCO_2H, 1:8:1:0.1) to give 33.6 mg of compound mixture and the compound mixture was applied on a Sephadex LH-20 column chromatography (10 g) and eluted with MeOH and a mixture with MeOH:CH_2Cl_2 (1:1), wherein 1 mL sub-fractions (sfrs) were collected as follows: sfrs 1-20 (MeOH) and sfrs 21-30 (MeOH:CH_2Cl_2, 1:1). Sfrs 16–30 were combined (12.5 mg) to give **NF3**.

Fractions 211-225 were combined (0.20 g) and crystallized in a mixture of petrol and $CHCl_3$ to give 12.3 mg of **NF4** and mother liquor (0.19 g) which was combined altogether with fractions 136-210 (0.64 g) and fractions 226-255 (0.41 g) in total of (1.25 g), applied over a LiChroprep silica gel 60 column (0.04-0.063 mm Merck, 35 g) and eluted with mixtures of petrol-$CHCl_3$, $CHCl_3$ and MeOH wherein 250 mL sub-fractions were collected as follows: sub-fractions 1-77 (petrol-$CHCl_3$, 1:1), 78-142 (petrol-$CHCl_3$, 3:7), 143-220 (petrol-$CHCl_3$, 1:9), 221-255 ($CHCl_3$) and 256-258 (MeOH). Sub-fractions 51-63 were combined (48.6 mg) and crystallized in a mixture of petrol and $CHCl_3$ to give an additional 26 mg of **NF4**. Sub-fractions 125-220 were combined (176 mg) and recrystallized in a mixture of petrol and $CHCl_3$ to give 12.7 mg of **NF5**.

Fractions 361-420 were combined (0.31 g) and purified by using preparative TLC (silica gel G_{254}, petrol-$CHCl_3$-EtOAc-HCO_2H, 1.5:8:0.5:0.1) to give an additional 9 mg of **NF4** and mother liquor (0.21 g) which was combined altogether with fractions 256-360 (1.33 g) and fractions 421-443 (2.99 g) in total of (4.9 g)**,** applied over a LiChroprep silica gel 60 column (0.04–0.063 mm Merck, 65 g) and eluted with a mixture of petrol-$CHCl_3$, $CHCl_3$-Me_2CO 250 mL fractions were collected as follows: sub-fractions 1-250 (petrol-$CHCl_3$, 1:1), 251-386 (petrol-$CHCl_3$, 3:7), 387-605 (petrol-$CHCl_3$, 1:9), 606-858 ($CHCl_3$) and 59-865 ($CHCl_3$-Me_2CO, 9:1). Sub-fractions 316-365 were combined (176 mg) and separated by using preparative TLC (silica gel G_{254}, petrol-$CHCl_3$-EtOAc-HCO_2H, 1:8:1:0.1) to give 30.5 mg of **NF6**, which was recrystallized in MeOH. Sub-fractions 418-480 were combined (11.3 mg) and precipitated in MeOH to give 7 mg of **NF7**.

Fractions 449 were combined (0.74 g) and crystallized in Me_2CO to give yellow precipitate (40.4 mg) and applied on Sephadex LH-20 column chromatography (10 g), and eluted with mixture of MeOH: CH_2Cl_2 (1; 1) and MeOH, 1 mL sub-fractions were collected

as follows: Sfrs 1-20 (MeOH) and Sfrs 21-44 (MeOH: CH_2Cl_2). Sub-fractions 15-44 (35.2 mg) were combined and recrystallized in Me_2CO to give 34 mg of **NF8** and 0.70 g of mother liquor.

Fractions 453-457 were combined (1.49 g) and crystallized in Me_2CO to give 118.7 mg of **NF8** and mother liquor (1.37 g). The mother liquor (1.37 g) was precipitated in MeOH to give188 mg of yellow solid, 12.3 mg of white precipitate **NF9** and mother liquor (1.18 g). The yellow solid (188 mg) was applied on sephadex LH-20 column chromatography (10 g) and eluted with (MeOH-CH_2Cl_2, 1:1) wherein 20 sub-fractions (sfrs) of 10 mL were collected. Sub-fractions 10–12 were combined (10.6 mg) and crystallized in MeOH to give an additional 8.7 mg of **NF9**. Fractions 617-623 were combined (0.40 g) and precipitated in MeOH to give **NF10** (7.4 mg) and mother liquor (0.25 g).

3.4. Physical Characteristics and Spectroscopic Data

***β*-Sitostenone (NF1)**: White solid; (mp. 96-100 °C); For ^1H and ^{13}C NMR data ($CDCl_3$, 300.13 and 75.4 MHz), see **Table 4**, (+)-HRESIMS *m/z* 413.3778 $[M+H]^+$, (calculated for $C_{29}H_{49}O$, 413.3783).

Ergosta-4,6,8(14), 22-tetraen-3-one (NF2): White solid; (mp. 108-113 °C); For ^1H and ^{13}C NMR ($CDCl_3$, 300.13 and 75.4 MHz), see **Table 5**.

Dehydromevalonic lactone (NF3): Colourless oil; For ^1H and ^{13}C NMR ($CDCl_3$, 300.13 and 75.4 MHz), see **Table 8**.

Byssochlamic acid (NF4): White solid; (mp. 166-168 °C); For ^1H and ^{13}C NMR ($CDCl_3$, 300.13 and 75.4 MHz), see **Table 7**. (+)-HRESIMS *m/z* 333.1326 $[M+H]^+$ (calculated for $C_{18}H_{21}O_6$ 333.1326).

Cyathisterone (Ergosta-7,22-diene-3,6-dione) (NF5): White solid; (mp. 200-202 °C); $[\alpha]_D^{23}$ +200 (*c* 0.03, $CHCl_3$); For ^1H and ^{13}C NMR data ($CDCl_3$, 300.13 and 75.4 MHz), see **Table 6**, (+)-HRESIMS *m/z* 411.3262 $[M+H]^+$, (calculated for $C_{28}H_{43}O_2$, 411.3263).

Chevalone B (NF6): White amorphous solid, (mp 161-163 °C); $[\alpha]_D^{20}$ +11 (*c* 0.12, $CHCl_3$); For ^1H and ^{13}C NMR data ($CDCl_3$, 300.13 and 75.4 MHz), See **Table 10**, (+)-HR-ESIMS *m/z* 457.2949 $[M+H]^+$ (calculated for $C_{28}H_{41}O_5$, 457.2954).

Aszonalenin (NF7): White solid, (mp. 249-250 °C); For ^1H and ^{13}C NMR ($CDCl_3$, 300.13 and 75.4 MHz), see **Table 11**.

Secalonic acid A (NF8); Yellow crystal, (mp. 268-270 °C); For ^1H and ^{13}C NMR spectra (DMSO-d_6, 300.13 and 75.4 MHz) see **Table 12**, (+)-HRESIMS *m/z* 639.1733 $[M+H]^+$, (calculated for $C_{32}H_{31}O_{14}$, 639.1714).

Helvolic acid (NF9): White amorphous solid, (mp. 201-203 °C); For ^1H and ^{13}C NMR data ($CDCl_3$, 500.13 and 125.77 MHz), see **Table 7**, (+)- ASAP-HRMS *m/z* 509.2903 $[M+H]^+$, (calculated for $C_{33}H_{44}O_8$, 568.7070).

Fellutanine A (NF10): Yellow viscous semi-solid, (mp. 270−272 ºC); For ^1H and ^{13}C NMR (DMSO-d_6), 500.13 and 125.77 MHz), see **Table 13**, (+)-HRESIMS *m/z* 373.1675 $[M+H]^+$, (calculated for $C_{22}H_{21}N_4O_2$, 373.1665).

3.5. X-Ray Crystallographic Analysis of Secalonic acid A

Crystal detection for X-ray crystallographic analysis was given by slow evaporation of a solution in MeOH for secalonic acid A. Diffraction data were assembled at 293 K with a Gemini Ultra equipped with CuKα radiation (λ = 1.54184 Å). The structures were interpreted by direct methods operating SHELXS-97 and clarified with SHELXL-97 (Sheldrick, 2008). Atoms such as carbon and oxygen were clarified anisotropically. Hydrogen atoms were put at their appropriate positions by operating asuitable HFIX instructions in SHELXL, and taking in subsequent refinement cycles, or were directly established from difference Fourier maps and were refined freely with isotropic displacement parameters. All the obtained detail results including the data collection, bond lengths and angles, torsion angles, refinement and tables of atomic coordinates have been deposited with the Cambridge Crystallographic Data Centre.

CHAPTER IV
RESULTS AND DISCUSSION

4.1. Chemical Investigation of *Neosartorya fennelliae* KUFA 0811

Chemical investigation of the crude ethyl acetate extract of the marine sponge-associated fungus *Neosartorya fennelliae* KUFA 0811 resulted in isolation of ergosterol derivatives; β-sitostenone (**NF1**), ergosta-4,6,8(14),22-tetraen-3-one (**NF2**) and cyathisterone (ergosta-7,22-diene-3,6-dione) (**NF5**), lactone; dehydromevalonic lactone (**NF3**), nonadride; byssochlamic acid (**NF4**), merediterpene; chevalone B (**NF6**), indolylmethyl 1, 4-bezodiazepen-2,5-dione; aszonalenin (**NF7**), *bis*-xanthone; secalonic acid A (**NF8**), tetracyclictriterpenoid; helvolic acid (**NF9**) and diketopiperazine; fellutanine A (**NF10**) (Figure 23).

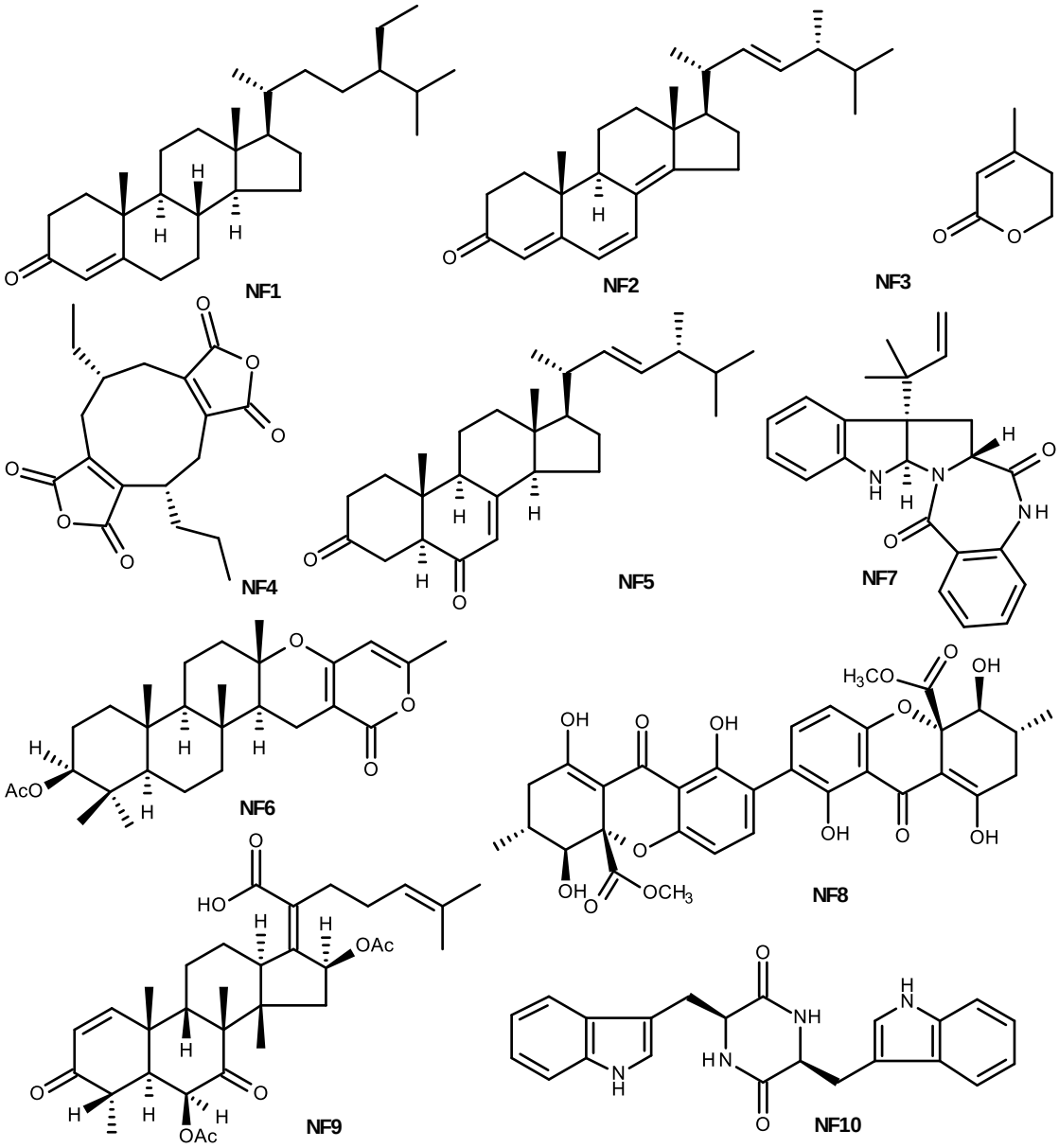


Figure 23. Constituents of *Neosartorya fennelliae* KUFA 0811.

4.1.1. Structure Elucidation of Ergosterol Derivatives

4.1.1.1. *β*-sitostenone (NF1)

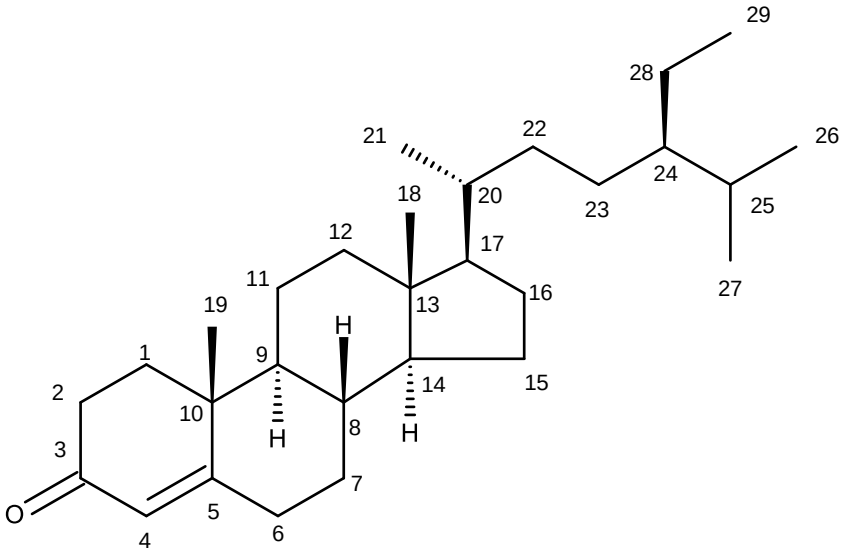


Figure 24. Structure of *β*-sitostenone (**NF1**).

NF1 was isolated as white solid (mp. 96-100 °C) and its molecular formula $C_{29}H_{48}O$ was determined based on the (+)-HRESIMS *m/z* 413.3778 $[M+H]^+$ (calculated 413.3783), indicating six degrees of unsaturation. The ^{13}C NMR, DEPTs and HSQC spectra (Table 4) indicated the presence of one conjugated ketone carbonyl (δ_C 199.8), one quaternary sp^2 (δ_C 171.8), two quaternary sp^3 carbons (δ_C 42.4 and 38.6), one methine sp^2 (δ_C 123.7), seven methine sp^3 (δ_C 56.0, 55.9, 53.8, 45.8, 36.1, 35.6 and 29.1), eleven methylene sp^3 (δ_C 39.6, 35.7, 34.0, 33.9, 33.0, 32.1, 28.2, 26.0, 24.2, 23.1 and 21.0) and six methyl groups including one primary methyl (δ_C 11.9) carbons, three secondary methyl (δ_C 19.8, 19.0 and 18.7) carbons and two tertiary methyl (δ_C 17.4 and 12.0) carbon in total of 29 signals, respectively.

The ^1H NMR spectrum, in conjunction with the HSQC spectrum (Table 4), showed the signals of eight methine protons at δ_H 5.72, *brs* (δ_C 123.7), δ_H 2.01, *m* (δ_C 35.6), δ_H 1.66, *m* (δ_C 29.1), δ_H 1.36, *m* (δ_C 36.1), δ_H 1.07, *m* (δ_C 56.0), δ_H 1.07, *m* (δ_C 55.9), δ_H 0.93, *m* (δ_C 45.8), δ_H 0.92, *m* (δ_C 53.8) and six methyl protons at δ_H 1.18, *s* (δ_C 17.4), δ_H 0.92, *d* (*J* = 6.5 Hz; δ_C 18.7), δ_H 0.85, *d* (*J* = 7.1 Hz; δ_C 11.9), δ_H 0.84, *d* (*J* = 6.5 Hz; δ_C 19.8), δ_H 0.81, *d* (*J* = 6.7 Hz; δ_C 19.0), δ_H 0.71, *s* (δ_C 12.0), respectively.

Assignments of the above-mentioned ^{13}C NMR spectrum indicates 29 carbon signals and the general features of the ^{1}H NMR spectrum, which determined that compound **NF1** is a steroid. The presence of 5-ethyl-6-methylheptan-2-yl side chain, similar to that of sitosterol, was evidenced by the COSY correlations (Table 4) from the multiplet at δ_H 1.23, (H-28; δ_C 23.1) to methyl doublet at δ_H 0.85 (*J* = 7.1 Hz, CH_3-29; δ_C 11.9), from the multiple at δ_H 1.36 (H-20; δ_C 36.1) to methyl doublet at δ_H 0.92 (*J* = 6.5 Hz, CH_3-21; δ_C 18.7), from the multiplet at δ_H 1.66 (H-25; δ_C 29.1) to methyl doublet at δ_H 0.81 (*J* = 6.7 Hz, CH_3-27; δ_C 19.0) and the HMBC correlations from H_3-21 to C-22 (δ_C 34.0), from H_3-27 to C-24 (δ_C 45.4) and C-25 (δ_C 29.1), from H_2-28 to C-25 (δ_C 29.1) and C-29 (δ_C 11.9), from H_3-29 to C-28 (δ_C 23.1) (Figure 25).

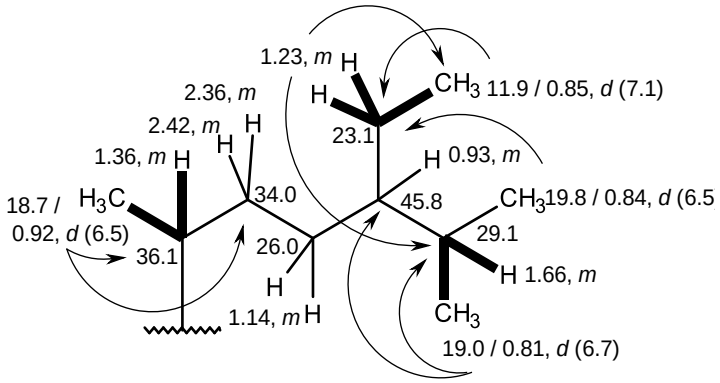


Figure 25. COSY (▬) and HMBC (⟶) correlations of 5-ethyl-6-methylheptan-2-yl side chain.

The remaining 19 carbons that belong to the four-ring steroid androstane skeleton, are similar to that of sterol. Since the ^{1}H NMR displayed the downfield vinyl proton at δ_H 5.72 *brs* (H-4) and ^{13}C NMR confirmed the presence of carbon-carbon double bond at δ_C 123.7 (C-4) and δ_C 171.8 (C-5) and the downfield chemical shift of C-5 (δ_C 171.8) also indicated the presence of the conjugate carbonyl function. On the basis of its spectroscopic data and comparison with previously reported data (Pongpuntaruk, 2010), suggested that **NF1** is stigmast-4-en-3-one (Figure 23). This hypothesis was supported by the HMBC (Table 4) correlations from H-4, broad singlet at δ_H 5.72 to C-6 (δ_C 33.0) and C-10 (δ_C 38.6), suggesting also the presence of a double bond between C-4 and C-5, H_3-19, singlet at δ_H 1.18 to C-1 (δ_C 35.7), C-5 (δ_C 171.8), C-6 (δ_C 33.0), C-8 (δ_C 35.7) and C-10 (δ_C 38.6), from H_3-18, singlet at δ_H 0.71 to C-12 (δ_C 39.6), C-14 (δ_C 55.9) and C-17 (δ_C 56.0), from H_2-11, multiplet at δ_H 1.38 to C-19 (δ_C 17.4), and from H_2-7, multiplet at δ_H 1.01 to C-14 (δ_C 55.9), (Figure 26) respectively.

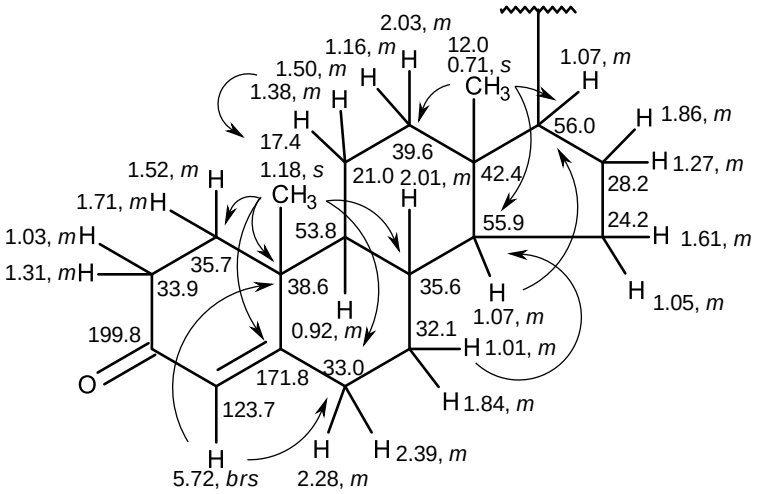


Figure 26. Key HMBC (⟶) correlations of androstane skeleton.

The 5-ethyl-6-methylheptan-2-yl linked to the androstane skeleton was shown by the HMBC correlations from H_3-21 to C-17 (δ_C 56.0), C-18 (δ_C 12.0) and from H_3-18 (δ_H 0.71, s) to C-17 (δ_C 56.0), C-21 (δ_C 18.7). Therofore, the complete structure of **NF1** was shown in Figure 27.

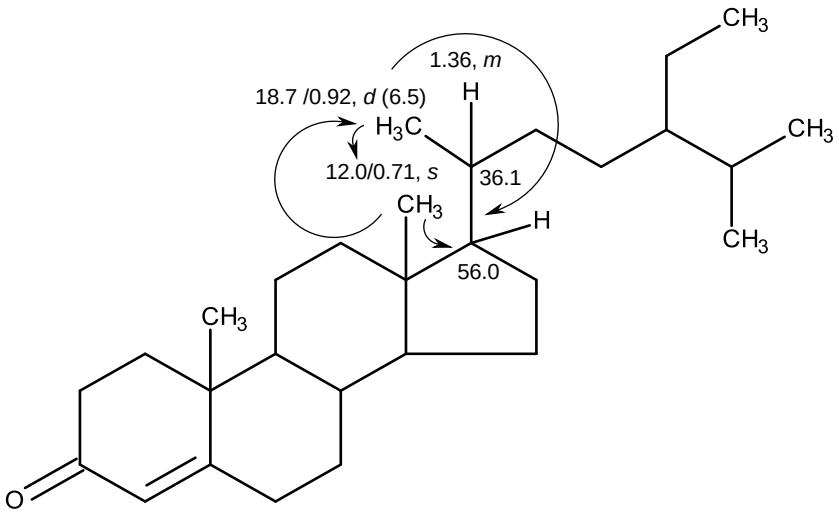


Figure 27. HMBC (⟶) correlations between androstane skeleton and 5-ethyl-6-methylheptan-2-yl side chain.

Taking into account the number of carbon atoms, the ^{1}H and ^{13}C chemical shift values and the COSY and HMBC correlations are in agreement with previous reported data from Pongpuntaruk (2010), the structure of **NF1** was identified as β-sitostenone (Figure 28).

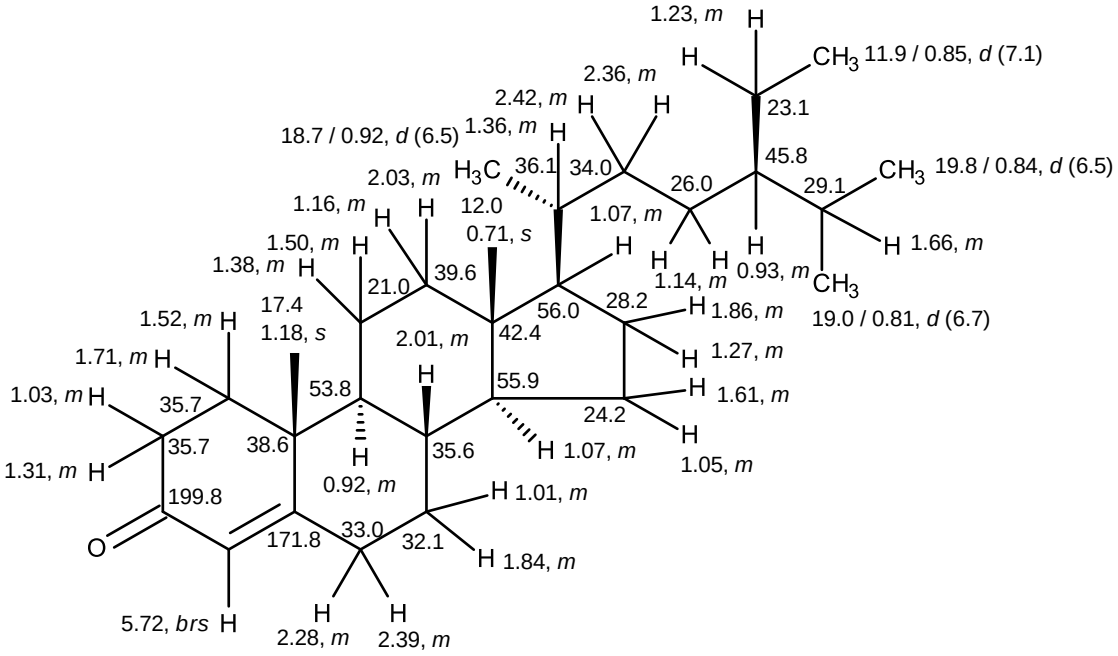


Figure 28. ^{1}H and ^{13}C assignments for **NF1**.

Literature search revealed that **NF1,** β-sitostenone was already isolated from the stem bark of *Zanthoxylum pistaciiflorum* which was collected from Lai-I, Pingtung County,Taiwan, in February 2002 and tested cytotoxicities against leukaemia (P-388) and human colon carcinoma (HT-29) cell lines *in vitro*. However, this compound was inactive in both cell lines with greater than 50, ED_{50} µg/mL (Chen *et al*., 2004). Moreover, β-sitostenone was already reported from endophytic fungus isolated from *Annulohypoxylon squamulosum* BCRC 34022, which was collected from Fu-shan Botanical Garden, I-lan County, Taiwan, in August, 2004 (Cheng *et al*., 2012).

Table 4. ^{1}H and ^{13}C NMR ($CDCl_3$, 300.13 and 75.4 MHz) and HMBC assignment for **NF1** and ^{1}H and ^{13}C NMR data of *β*-sitostenone (Pongpuntaruk, 2010).

Position	Pongpuntaruk, 2010		δ_C, type	δ_H (*J* in Hz)	COSY	HMBC
	δ_C, type	δ_H (J in Hz)				
1a	35.7, CH_2	1.54, *m*	35.7, CH_2	1.52, *m*	H-1b	-
b		1.67, *m*		1.71, *m*	H-1a	-
2a	33.9, CH_2	2.28, *m*	33.9, CH_2	1.03, *m*	-	-
b		2.50, *m*		1.31, *m*	-	-
3	198.9, CO	-	199.8, CO	-	-	-
4	123.6, CH	5.72, *brs*	123.7, CH	5.72, *brs*	H-6b	C-6,10
5	171.0, C	-	171.8, C	-	-	-
6a	32.9, CH_2	2.25, *m*	33.0, CH_2	2.28, *m*	-	-
b		2.40, *m*		2.39, *m*	H-4	-
7a	32.1, CH_2	1.10, *m*	32.1, CH_2	1.01, *m*	H-7b	C-14
b		1.85, *m*		1.84, *m*	H-7a	-
8	35.7, CH	1.71, *m*	35.6, CH	2.01, *m*	-	-
9	53.8, CH	0.92, *m*	53.8, CH	0.92, *m*	-	C-19
10	38.6, C	-	38.6, C	-	-	-
11a	21.0, CH_2	1.40, *m*	21.0, CH_2	1.38, *m*	-	C-19
b		1.50, *m*		1.50, *m*	-	-
12a	39.5, CH_2	1.15, *m*	39.6, CH_2	1.16, *m*	H-12b	-
b		2.04, *m*		2.03, *m*	H-12a	-
13	42.4, C	-	42.4, C	-	-	-
14	55.9, CH	1.00, *m*	55.9, CH	1.07, *m*	-	C-17
15a	24.1, CH_2	1.23, *m*	24.2, CH_2	1.05, *m*	-	-
b		1.29, *m*		1.61, *m*	-	-
16a	28.1, CH_2	1.27, *m*	28.2, CH_2	1.27, *m*	H-16b	-
b		1.32, *m*		1.86, *m*	H-16a	-
17	56.1, CH	1.11, *m*	56.0, CH	1.07, *m*	-	C-14
18	12.0, CH_3	0.71, *s*	12.0, CH_3	0.71, *s*	-	C-12, 14, 17, 21
19	17.4, CH_3	1.18, *s*	17.4, CH_3	1.18, *s*	-	C-1, 5, 6, 8,10
20	36.1, CH	2.01, *m*	36.1, CH	1.36, *m*	H-21	-
21	18.7, CH_3	0.92, *d* (6.3)	18.7, CH_3	0.92, *d* (6.5)	H-20	C-17, 18, 20, 22
22a	34.0, CH_2	2.39, *m*	34.0, CH_2	2.36, *m*	-	-
b		-		2.42, *m*	-	-
23	26.0, CH_2	1.17, *m*	26.0, CH_2	1.14, *m*	-	-
24	45.8, CH	0.93, *m*	45.8, CH	0.93, *m*	-	-
25	29.1, CH	1.26, *m*	29.1, CH	1.66, *m*	H-27	-
26	19.8, CH_3	0.85, *d* (6.9)	19.8, CH_3	0.84, *d* (6.5)	-	C-28
27	19.2, CH_3	0.84, *d* (6.6)	19.0, CH_3	0.81, d (6.7)	H-25	C-24, 25
28	23.1, CH_2	1.29, *m*	23.1, CH_2	1.23, *m*	H-29	C-25, 29
29	11.4, CH_3	0.83, *d* (6.6)	11.9, CH_3	0.85, *d* (7.1)	H-28	C-28

4.1.1.2. Ergosta-4,6,8(14),22-tetraen-3-one (NF2)

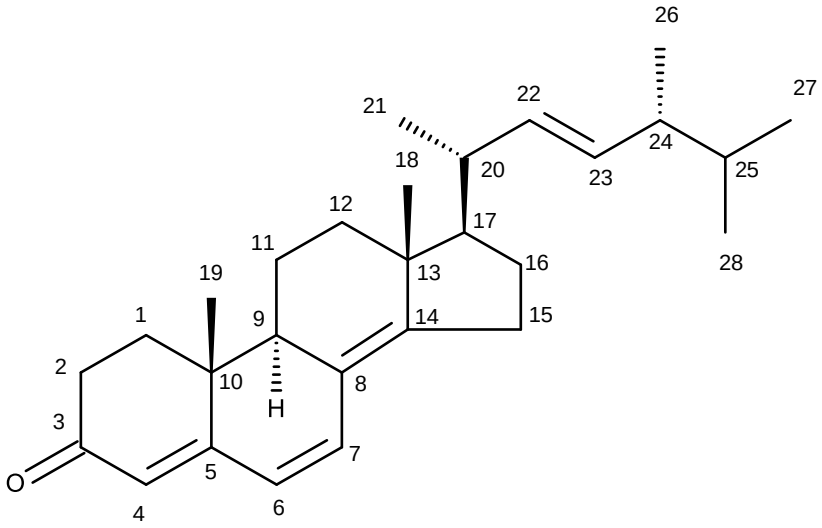


Figure 29. Structure of ergosta-4,6,8(14),22-tetraen-3-one (**NF2**).

NF2 was isolated as white solid (mp. 108-113 °C) and the ^{13}C NMR, DEPTs and HSQC spectra (Table 5) indicated the presence of one conjugated ketone carbonyl (δ_C 200.0), three quaternary sp^2 carbon (δ_C 164.5, 156.0 and 124.4), five methine sp^2 (δ_C 135.0, 134.1, 132.5, 124.5 and 123.0), five methine sp^3 (δ_C 55.7, 44. 3, 42.9 , 39.3 and 33.1), two quaternary sp^3 (δ_C 44.0 and 36.8), six methylene sp^3 (δ_C 35.6, 34.1 (2C), 27.7, 25.4 and 19.0) and six methyl groups including four secondary methyl (δ_C 21.2, 20.0, 19.7 and 17.7) carbons and two tertiary methyl (δ_C 19.0 and 16.7) carbons in total of 28 signals, respectively.

The ^{1}H NMR spectrum, in conjunction with the HSQC spectrum (Table 5), showed the signals of ten methine protons at δ_H 6.61, *d* (*J* = 9.4 Hz; δ_C 134.1), δ_H 6.03, *d* (*J* = 9.5 Hz; δ_C 124.5), δ_H 5.73, *s* (δ_C 123.0), δ_H 5.27, *dd* (*J* = 15.2, 7.6 Hz; δ_C 132.5), δ_H 5.20, *dd* (*J* = 15.2, 7.6 Hz; δ_C 135.0), δ_H 2.13, *m* (δ_C 39.3), δ_H 2.11, *m* (δ_C 44.3), δ_H 1.85, *m* (δ_C 42.9), δ_H 1.47, *m* (δ_C 33.1) and δ_H 1.25, *m* (δ_C 55.7) and one singlet of a trisubstituted double bond at δ_H 5.73, *s* (δ_C 123.0) and two methyl singlets at δ_H 0.99, *s* (δ_C 16.7) and δ_H 0.96, *s* (δ_C 19.0) and four methyl doublets at δ_H 1.06, *d* (*J* = 6.7 Hz; δ_C 21.2), δ_H 0.93, *d* (*J* = 6.8 Hz; δ_C 17.7), δ_H 0.85, *d* (*J* = 6.7 Hz; δ_C 20.0) and δ_H 0.83, *d* (*J* = 6.7 Hz; δ_C 19.7). The ^{1}H NMR spectrum indicated that there are a pair of doublets of a *cis* double bond at δ_H 6.61 (*J* = 9.4 Hz; δ_C 134.1) / δ_H 6.03 (*J* = 9.5 Hz; δ_C 124.5), and a pair of double doublets of a *trans* double

bond at δ_H 5.27 (*J* = 14.6, 6.5 Hz; δ_C 132.5) /δ_H 5.20 (*J* = 15.2, 7.6 Hz; δ_C 135.0), and one singlet of a trisubstituted double bond at δ_H 5.73 (δ_C 123.0) and two methyl singlets at δ_H 0.99 (δ_C 16.7) and δ_H 0.96 (δ_C 19.0) and four methyl doublets at δ_H 1.06 (*J* = 6.7 Hz, δ_C 21.2), δ_H 0.93 (*J* = 6.8 Hz, δ_C 17.7), δ_H 0.85 (*J* = 6.7 Hz, δ_C 20.0) and δ_H 0.83 (*J* = 6.7 Hz, δ_C 19.7).

The presence of the *(3E)*-5-6-dimethylhept-3-en-2yl side chain, similar to that of ergosterol was evidenced by the COSY correlations from the double doublet at δ_H 5.20 (*J* = 15.2, 7.6 Hz, H-22) to the double doublet at δ_H 5.27 (*J* = 14.6, 6.5 Hz, H-23; δ_C 132.5) and a multiple at δ_H 2.13 (H-20; δ_C 39.3), from H-23 to multiple at δ_H 1.85 (H-24; δ_C 42.9), from the methyl doublet at δ_H 1.06 (*J* = 6.7 Hz, H-21; δ_C 21.2) to H-20, from the methyl doublet at δ_H 0.93 (*J* = 6.8 Hz, H-26; δ_C 17.7) to H-24, as well as from the multiple at δ_H 1.47 (H-25; δ_C 33.1) to H-24, the methyl doublet at δ_H 0.83 (*J* = 6.7 Hz, H-28; δ_C 19.7) and the methyl doublet at δ_H 0.85 (*J* = 6.7 Hz, H-27; δ_C 20.0), respectively and shown in Figure 30.

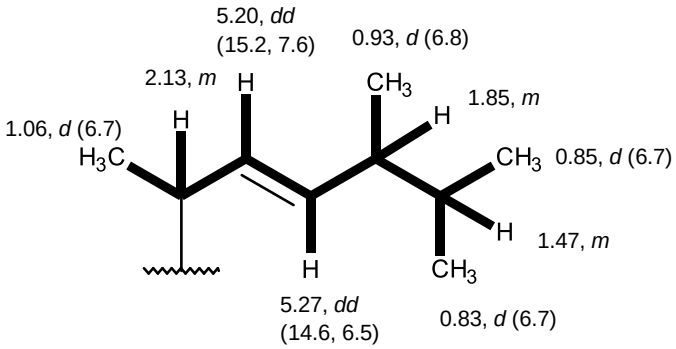


Figure 30. COSY (▬) correlations of the protons of the *(3E)*-5,6-dimethylhept-3-en-2-yl side chain of **NF2**.

This was supported by the HMBC correlations from H_3-21 to C-20 and C-22, from H-22 to C-20 and C-24, from H-23 to C-24, from H-24 to C-25, 26, 27 and 28, from H-25 to C-23, 24, 26, 27 and 28, from H_3-26 to C-24, 25 and 27, from H_3-27 to C-24, C-25 and C-28 and from H_3-28 to C-23, 24 and 25. HMBC correlations of the *(3E)*-5,6-dimethylhept-3-en-2-yl side chain is shown in Figure 31.

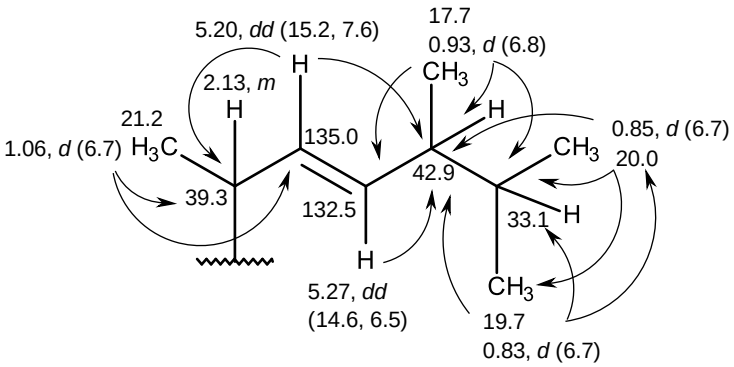


Figure 31. Key HMBC (⟶) correlations of the *(3E)*-5,6-dimethylhept-3-en-2-yl side chain.

Taking into account the presence of the *trans* double bond and four secondary groups in the side chain, the *cis* double bond, the trisubstututed double bond and the two tertiary methyl groups have to be located in the cyclopentanoperhydrophenanthrene nucleus of **NF2**.

Since the HMBC spectrum exhibited correlations to each other from H_3-21 to methine sp^3 multiplet at δ_c 55.7 and these methine sp^3 multiplet at δ_H 1.25 (δ_C 55.7) and another the tertiary methyl singlet at δ_H 0.96 (δ_C 19.0) have HMBC correlations to the quaternary sp^3 carbon at δ_C 44.0, the tertiary methyl singlet at δ_H 0.96 (δ_C 19.0), methine sp^3 carbon at δ_C 55.7 and the quaternary sp^3 carbon at δ_C 44.0 were assigned to CH_3-18 and CH-17 and 13, respectively (Figure 32). Therefore, another tertiary methyl singlet δ_H 0.99 (δ_C 16.7) was assigned to CH_3-19. Moreover, both the singlet at δ_H 0.99 (H_3-19; δ_C 16.7) and the singlet at δ_H 5.73 (δ_C 123.0) exhibited HMBC cross peaks to the quaternary sp^3 carbon at δ_C 36.8 and methine sp^3 carbon at both δ_C 34.1 (C-1 and 2) while the H_3-19 also showed HMBC cross peak to methine sp^3 at δ_C 44.3 (C-9), the quaternary sp^2 carbon at δ_C 164.5 (C-5), the trisubstituted double bond was placed between C-4 and C-5. Therefore, the singlet at δ_H 5.73 was assigned to H-4, whereas the carbons at δ_C 44.3, δ_C 36.8, δ_C 123.0 and δ_C 164.5 were assigned to C-9, C-10, C-4 and C-5, respectively (Figure 32). Moreover, the HMBC spectrum (Table 5) also exhibited correlations from the proton of the *cis* double bond at δ_H 6.03 (J = 9.5 Hz; δ_C 124.5) to C-5, C-10, and the quaternary sp^2 carbon at δ_C 124.4 (C-8) as well as from another proton of the *cis* double bond at δ_H 6.61 (J = 9.4 Hz; δ_C 134.1) to C-5, the quaternary sp^2 carbon at δ_C 156.0 (C-14), methine sp^2 at δ_C 124.5 (C-6) and methine sp^3 carbon at δ_C 44.3 (C-9) (Figure 32). Therefore, the *cis* double bond was placed between C-6 (δ_C 124.5) and C-7 (δ_C 134.1), while the tetrasubstituted double bond was located between C-8 (δ_C 124.4) and C-14 (δ_C 156.0). This

was supported by the HMBC correlations from H_3-19 to C-9 and from H_3-18 to C-14 (Figure 32).

The *(3E)*-5-6-dimethylhept-3-en-2yl side chain linked to the androstane skeleton was shown by the COSY correlation from H-17, multiplet at δ_H 1.25 (δ_C 55.7) to H-20, multiplet at δ_H 2.13 (δ_C 39.3). This was confirmed by HMBC correlations from H-17 to C-21 (δ_C 21.2) and from H_3-21 to C-17 (δ_C 55.7). Hence the complete structure of **NF2** (Figure 32).

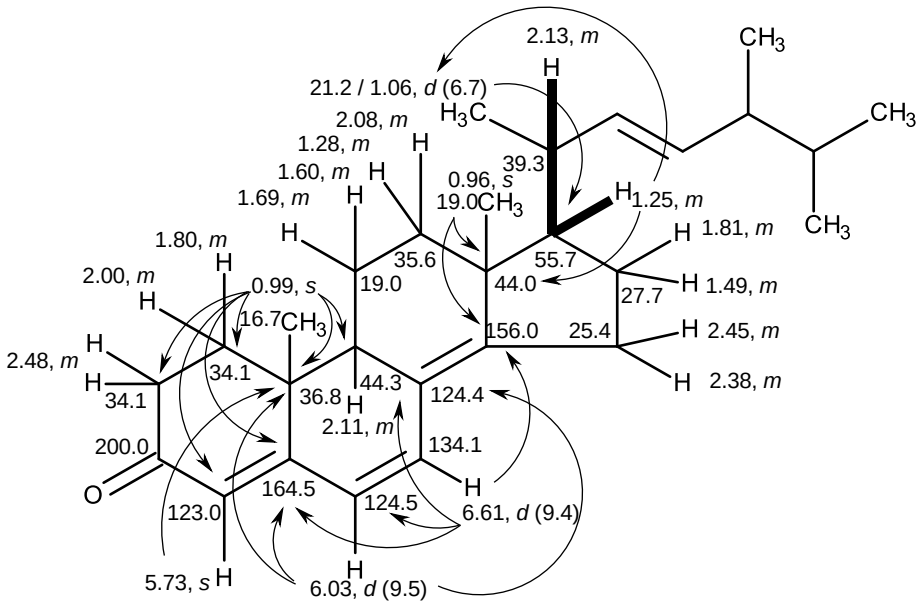


Figure 32. Key COSY (▬) and HMBC (⟶) correlations of androstane skeleton linked with *(3E)*-5-6-dimethylhept-3-en-2yl side chain.

Taking into account the number of carbon atoms, the ^{1}H and ^{13}C chemical shift values, the COSY, HMBC correlations from the structure of **NF2** was identified as ergosta-4,6,8(14),22-tetraen-3-one (Figure 33). This compound was already isolated from the mushroom (*Polyporus umbellatus*), the sclerotia of *P. umbellatus* were collected from the market at Seoul and identified by Dr. K. H. Ka (Korea Forest Research Institute, Korea) and tested cytotoxic activities with four human cancer cell lines such as HT-29 (colon cancer), HeLa 229 (cervix cancer), Hep3B (liver cancer), and AGS (stomach cancer) showed IC_{50} value of 7.2 ± 0.17 µg/mL, 26.3 ± 0.93 µg/mL, 5.0 ± 0.41 µg/mL and 22 ± 0.16 µg/mL, respectively (Lee *et al*., 2005) and also isolated from methanolic extract of fresh

fruit bodies of *Xylaria sp*. which was collected from Ninh Binh province, Vietnam in July 2004 and showed inhibitory activity of nitric oxide production in RAW 264.7 cells stimulated by lipopolysaccharide with the IC_{50} value of 11381.3 µg/mL (Ngoc *et al*., 2008) and recent isolation also reported from the fungus *Apiospora montagnei* was isolated from the leaf surface of *Vanilla siamensis*, collected from Pathum Thani Province, Thailand and displayed very weak cytotoxicity against human epidermoid carcinoma (KB) cell lines and human small cell lung cancer (NCI-H187) cell lines with IC_{50} values of 18.90 and 23.10 µg/mL, respectively (Arthan *et al*., 2017). The compound **NF2** was recently isolated from marine sponge-assoiciated fungus*Talaromyces stipitatus* KUFA 0207 which was isolated from the marine sponge *Stylissa flabelliformis* and collected by scuba diving at a depth of 10-15 m, from the coral reef at Samaesarn Island (12°34′36.64″ N 100°56′59.69″ E) in the Gulf of Thailand, Chonburi Province, in April 2014 (Noinart *et al*., 2017).

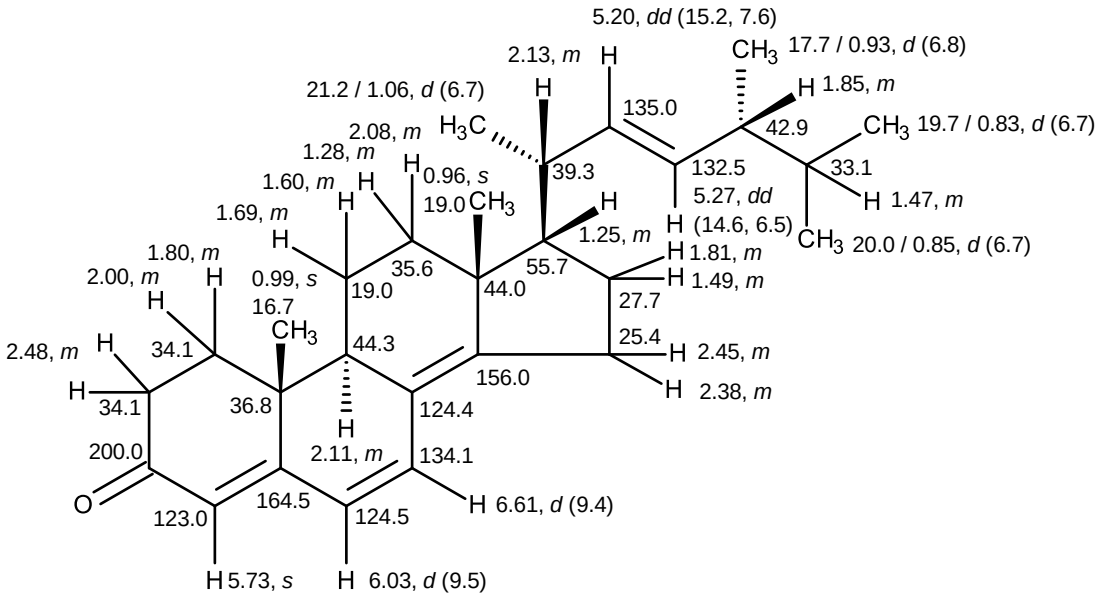


Figure 33. ^{1}H and ^{13}C assignments for **NF2**.

Table 5. ^{1}H and ^{13}C NMR ($CDCl_3$, 300.13 and 75.4 MHz) and HMBC assignment for **NF2**.

Position	δ_C, type	δ_H (*J* in Hz)	COSY	HMBC
1a	34.1, CH_2	1.80, *m*	H-2	C-10, 19
b		2.00, *m*	H-2	C-2, 10
2	34.1, CH_2	2.48, *m*	-	CO-3
3	200.0, C	-	-	-
4	123.0, CH	5.73, *s*	-	C-1, 2, 6, 10
5	164.5, C	-	-	-
6	124.5, CH	6.03, *d* (9.5)	H-7	C-5, 8, 10
7	134.1, CH	6.61, *d* (9.4)	H-6	C-5, 6, 9, 14
8	124.4, C	-	-	-
9	44.3, CH	2.11, *m*	H-12a	-
10	36.8, C	-	-	-
11a	19.0, CH_2	1.60, *m*	H-12a	-
b		1.69, *m*	H-12a	-
12a	35.6, CH_2	1.28, *m*	-	-
b		2.08, *m*	H-12a	C-11, 13, 18
13	44.0, C	-	-	-
14	156.0, C	-	-	-
15a	25.4, CH_2	2.38, *m*	H-16a	C-14
b		2.45, *m*	H-16a	-
16a	27.7, CH_2	1.49, *m*	H-17	-
b		1.81, *m*	-	-
17	55.7, CH	1.25, *m*	H-16a, 20	C-13, 16, 21
18	19.0, CH_3	0.96, *s*	-	C-12, 13, 14
19	16.7, CH_3	0.99, *s*	-	C-1, 2, 5, 9, 10
20	39.3, CH	2.13, *m*	H-21, 22	-
21	21.2, CH_3	1.06, *d* (6.7)	H-20	C-17, 20, 22
22	135.0, CH	5.20, *dd* (15.2, 7.6)	H-20, 23	C-20, 24
23	132.5, CH	5.27, *dd* (14.6, 6.5)	H-24	C-24
24	42.9, CH	1.85, *m*	H-28	C-25, 26, 27, 28
25	33.1, CH	1.47, *m*	H-24, 26, 27	C-23, 24, 26, 27, 28
26	19.7, CH_3	0.83, *d* (6.7)	H-25, 27	C-24, 25, 27
27	20.0, CH_3	0.85, *d* (6.7)	H-25, 26	C-24, 25, 28
28	17.7, CH_3	0.93, *d* (6.8)	H-24	C-23, 24, 25

4.1.1.3. Cyathisterone (NF5)

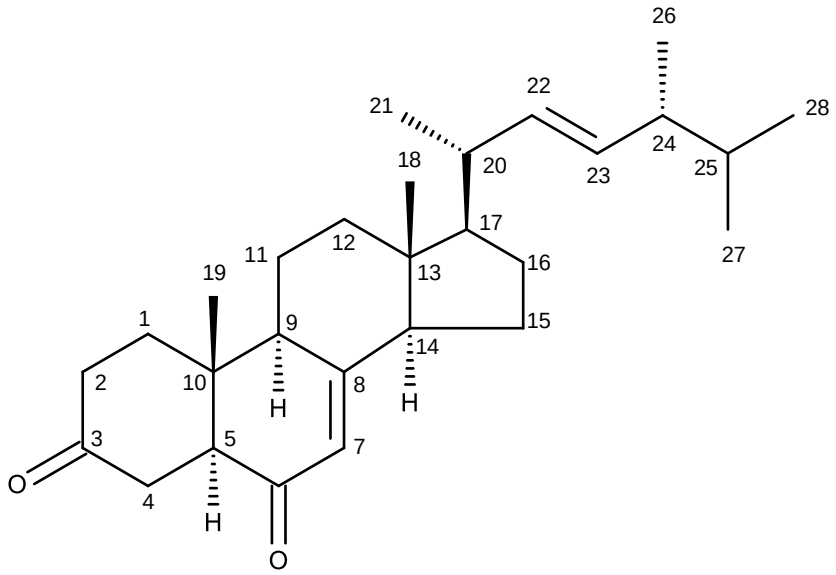


Figure 34. Structure of cyathisterone (**NF5**).

NF5 was isolated as white solid (mp. 200-202 ºC), $[\alpha]_D^{23}$ + 200 (*c* 0.03, $CHCl_3$) and its molecular formula $C_{28}H_{42}O_2$ was based on the (+)-HRESIMS *m/z* 411.3262 $[M+H]^+$ (calculated 411.3263), indicating eight degees of unsaturation. The general features of ^{13}C NMR, DEPTs and HSQC spectra (Table 6) indicated the presence of one ketone carbonyl (δ_C 211.0) one conjugated ketone carbonyl (δ_C 198.3), one quaternary sp^2 (δ_C 163.8), three methine sp^2 (δ_C 132.6, 134.9 and 122.8), seven methine sp^3 (δ_C 56.1, 55.7, 54.6, 49.7, 40.3, 42.8 and 33.1), two quaternary sp^3 (δ_C 44.5 and 38.3), seven methylene sp^3 (δ_C 38.6, 38.2, 37.3, 37.0, 27.9, 22.6 and 22.0) and six methyl groups including four secondary methyl (δ_C 21.1, 20.0, 19.7 and 17.6) carbon and two tertiary methyl (δ_C 12.8 and 12.7) carbon in total of 28 signals, respectively.

The ^1H NMR spectrum in conjunction with the HSQC spectrum (Table 6), showed a triplet of the olefinic proton of the trisubstituted double bond at δ_H 5.78 (*J* = 2.1 Hz), two doublets of the *trans* double bond at δ_H 5.25 (*J* = 15.2, 7.2 Hz) and δ_H 5.16 (*J* = 15.3, 7.8 Hz), two singlets of tertiary methyls at δ_H 1.08 and δ_H 0.65, and four doublets of secondary methyls at δ_H 1.05 (*J* = 6.6 Hz), δ_H 0.92 (*J* = 6.8 Hz), δ_H 0.83 (*J* = 6.8 Hz) and δ_H 0.84 (*J* = 6.7 Hz). This general skeleton obtained from ^1H and ^{13}C NMR resembles ergosterol (Gao *et al*., 2003). The **NF5** also contained the (*3E*)-5-6-dimethylhept-3-en-2yl moiety which was confirmed by the COSY and HMBC correlations, shown in Table 6.

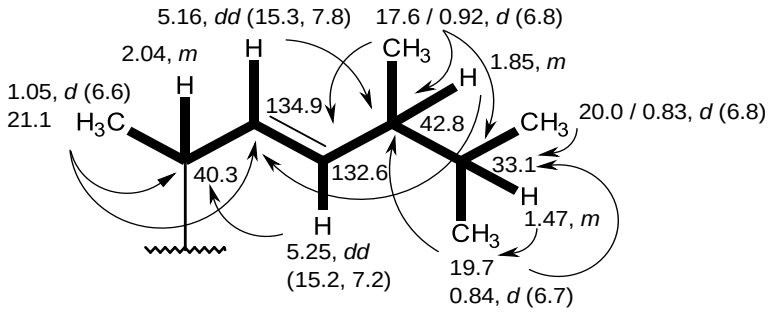


Figure 35. Key COSY (▬) and HMBC (⟶) correlations of the protons of the *(3E)*-5,6-dimethylhept-3-en-2-yl side chain of **NF5**.

Contrary to **NF5**, the ^{1}H and ^{13}C NMR spectra revealed the presence of the trisubstituted double bond was located between C-7 and C-8, and the conjugated ketone carbonyl was on C-6 of the cyclopentanoperhydrophenanthrene moiety, which was supported by the COSY correlations from H-7, triplet at δ_H 5.78 (J = 2.1; δ_C 122.8) to H-9, multiplet (δ_H 2.26; δ_C 49.7) and H-14, multiplet at δ_H 2.09 (δ_C 55.7), as well as by the HMBC correlations from H-7 to C-9 (δ_C 49.7), from H_3-19 to C-1 (δ_C 38.2), C-5 (δ_C 54.6), C-9 and C-10 (δ_C 38.3), from H-5, double doublet at δ_H 2.65 (J = 12.8, 4.4 Hz; δ_C 54.6) to C-6 (δ_C 198.3). Since H-2, multiplet δ_H 2.38 (δ_C 37.3) and H-4, multiplet at δ_H 2.58 (δ_C 37.0) exhibited cross peaks to the carbonyl carbon at δ_C 211.0, another ketone function was placed on C-3. Moreover, H-14, multiplet at δ_H 2.09 (δ_C 55.7) exhibited COSY cross peak to H-15b, multiplet at δ_H 1.60 (δ_C 22.6) and H-17, multiplet at δ_H 1.36 (δ_C 56.1) to H-16b, multiplet at δ_H 1.37 (δ_C 27.9), respectively.

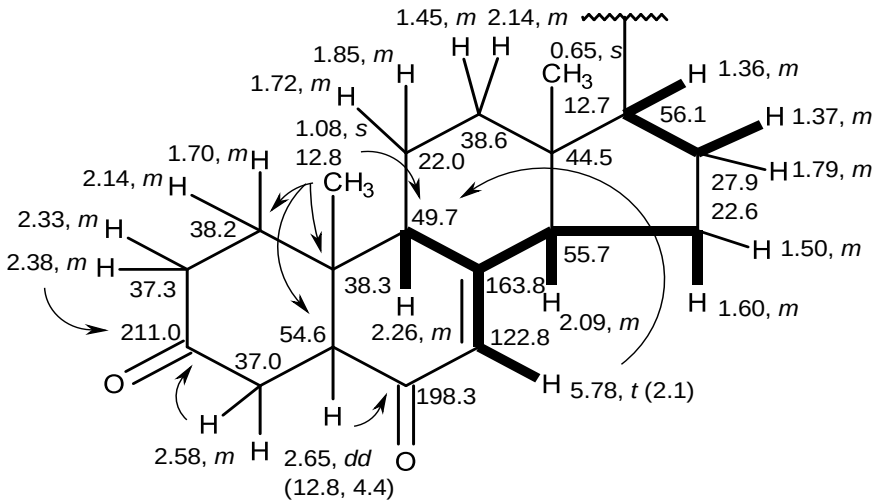


Figure 36. Key COSY (▬) and HMBC (⟶) correlations of cyclopentanoperhydrophenanthrene.

The *(3E)*-5-6-dimethylhept-3-en-2yl side chain linked to the cyclopentanoperhydrophenanthrene moiety was displayed by the COSY correlation from H-17, multiplet at δ_H 1.36 (δ_C 56.1) to H-20, multiplet at δ_H 2.04 (δ_C 40.3) and the HMBC correlations from H_3-21 to C-17 (δ_C 56.1), respectively. Therofore, the complete structure of **NF5** is shown below.

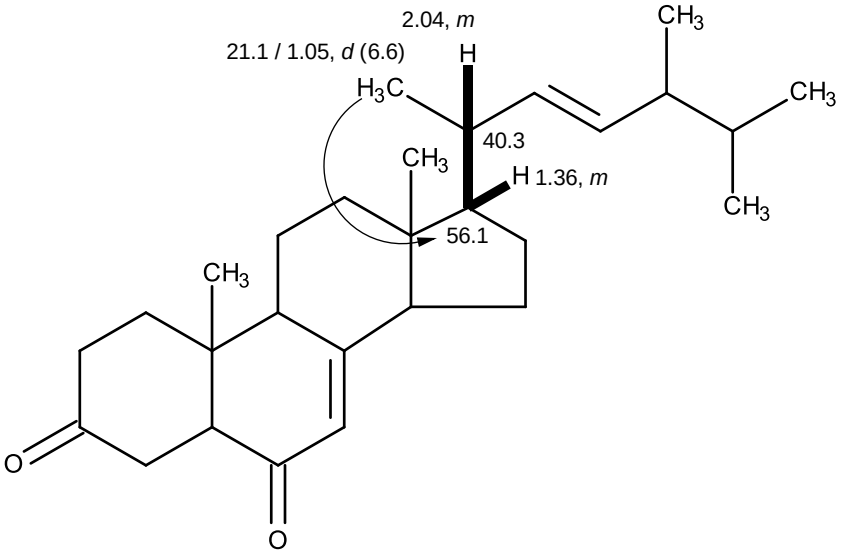


Figure 37. Key COSY (▬) and HMBC (⟶) correlations between cyclopentanohydrophenathrene moiety and *(3E)*-5-6-dimethylhept-3-en-2yl side chain.

Taking into account the number of carbon atoms, the ^{1}H and ^{13}C chemical shift values and the COSY and HMBC correlations are in agreement with previous reported data from Noinart *et al*. (2017). The structure of **NF5** was identified as ergosterol derivative, ergosta-7,22-diene-3,6-dione (cyathisterone) shown in Figure 38. This compound was first isolated from dichloromethane extract of fruiting body of *Calvatia cyathiformis* (Bosc.) which was collected from China (Kawahara *et al*., 1994) and recently isolated from the fungus *Apiospora montagnei* which was isolated from the leaf surface of *Vanilla siamensis*, collected from Pathum Thani Province, Thailand and which exhibited weak cytotoxicity against the NCI-H187 lung cancer cell cell line with an IC_{50} value of 6.08 µg/mL (Arthan *et al*., 2017). Moreover, cyathisterone was also recently isolated from marine sponge-associated fungus *Talaromyces stipitatus* KUFA 0207 which was isolated from the marine sponge *Stylissa flabelliformis* and collected by scuba diving at a depth of between 10 and 15 m, from the coral reef at Samaesarn Island (12°34′36.64″ N 100°56′59.69″ E) in the Gulf of Thailand, Chonburi Province, in April 2014 (Noinart *et al*., 2017).

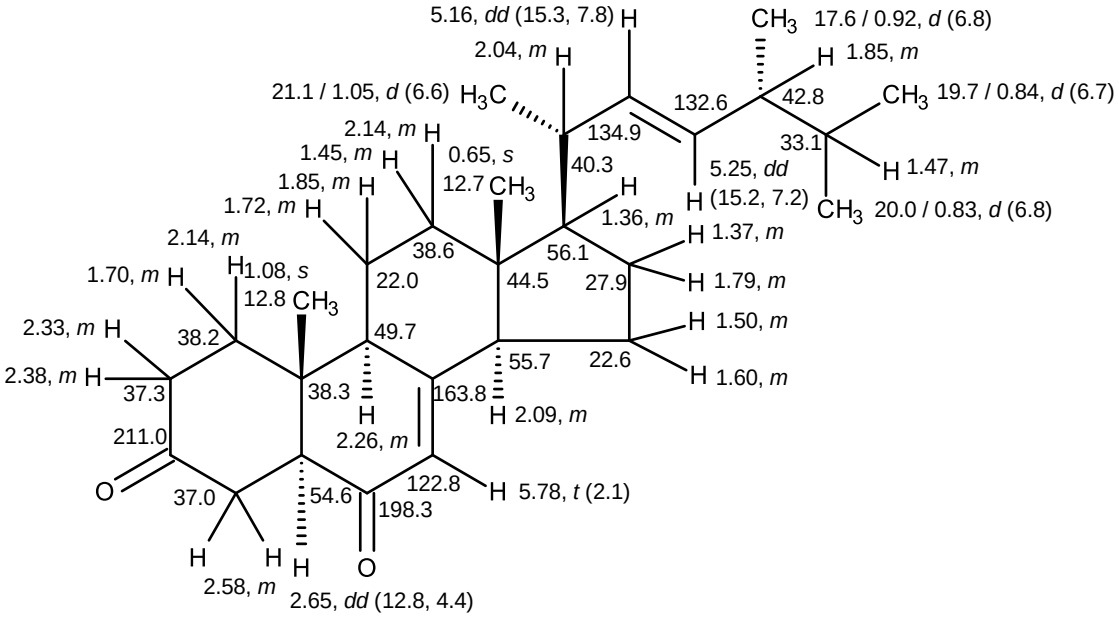


Figure 38. ^{1}H and ^{13}C assignments for **NF5**.

Table 6. ^{1}H and ^{13}C NMR ($CDCl_3$, 300.13 and 75.4 MHz) and HMBC assignment for **NF5**.

Position	δ_C, type	δ_H, (*J* in Hz)	COSY	HMBC
1a	38.2, CH_2	1.70, *m*	H-1b, 2a	C-19
b		2.14, *m*	H-1a, 2b	-
2a	37.3, CH_2	2.33, *m*	H-1a, 1b, 2b	-
b		2.38, *m*	H-1a, 1b, 2a	CO-3
3	211.0, CO	-	-	-
4	37.0, CH_2	2.58, *m*	H-5	C-5, 10, CO-3
5	54.6, CH	2.65, *dd* (12.8, 4.4)	H-4	C-4, CO-6
6	198.3, CO	-	-	-
7	122.8, CH	5.78, *t* (2.1)	H-9, 14	C-9
8	163.8, C	-	-	-
9	49.7, CH	2.26, *m*	H-7, 11a	-
10	38.3, C	-	-	-
11a	22.0, CH_2	1.72, *m*	-	-
b		1.85, *m*	-	-
12a	38.6, CH_2	1.45, *m*	-	C-18
b		2.14, *m*	-	-
13	44.5, C	-	-	-
14	55.7, CH	2.09, *m*	H-7, 15b	C-7, 8
15a	22.6, CH_2	1.50, *m*	-	-
b		1.60 *m*	-	-
16a	27.9, CH_2	1.37, *m*	-	-
b		1.79 *m*	-	-
17	56.1, CH	1.36, *m*	H-16a, 20	-
18	12.7, CH_3	0.65, *s*	-	C-19
19	12.8, CH_3	1.08, *s*	-	C-1, 5, 9, 10
20	40.3, CH	2.04, *m*	H-21, 22	-
21	21.1, CH_3	1.05, *d* (6.6)	H-20	C-17, 20, 22
22	134.9, CH	5.16, *dd* (15.3, 7.8)	H-20, 23	C-24
23	132.6, CH	5.25, *dd* (15.2, 7.2)	H-22, 24	C-20
24	42.8, CH	1.85, *m*	H-23, 25, 26	C-22, 23, 25, 28
25	33.1, CH	1.47, *m*	H-24, 27, 28	C-23, 28
26	17.6, CH_3	0.92, *d* (6.8)	H-24	C-23, 24, 25
27	20.0, CH_3	0.83, *d* (6.8)	H-25	C-24, 25, 28
28	19.7, CH_3	0.84, *d* (6.7)	H-25	C-24, 25, 27

4.1.2. Structure Elucidation of Lactone, Dehydromevalonic Lactone (NF3)

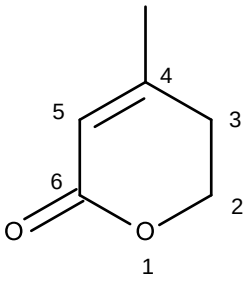


Figure 39. Structure of dehydromevalonic lactone (**NF3**).

NF3 was isolated as colourless oil and the ^{13}C NMR, DEPTs and HSQC spectra (Table 7) indicated the presence of one conjugated ketone carbonyl at δ_C 164.7, one quaternary sp^2 (δ_C 157.8), one methine sp^2 (δ_C 116.8), two methylene sp^3 (δ_C 65.9 and δ_C 29.2) and one tertiary methyl carbon (δ_C 23.0) in total of 6 signals, respectively. The ^1H NMR spectrum, in conjunction with the HSQC spectrum (Table 7), showed the signals of one methine sp^2 proton at δ_H 5.82, *ddd* (*J* = 2.8, 2.8, 1.4 Hz, δ_C 116.8) and one methyl proton at δ_H 2.01, *t* (*J* = 0.6 Hz, δ_C 23.0).

The COSY spectrum (Table 7) displayed correlations between the double doublet at δ_H 4.38 (*J* = 6.3, 6.3 Hz, H-2; δ_C 65.9) and double doublet at δ_H 2.38 (*J* = 6.5, 6.5 Hz, H-3; δ_C 29.2). In turn, the olefinic proton of double double doublet at δ_H 5.82 (*J* = 2.8, 2.8, 1.4 Hz, H-5; δ_C 116.8) gave cross peaks to tertiary methyl carbon of triplet at δ_H 2.01 (*J* = 0.6 Hz; δ_C 23.0) and double doublet at δ_H 2.38 (*J* = 6.5, 6.5 Hz; δ_C 29.2) whereas the double doublet at δ_H 2.38 (*J* = 6.5, 6.5 Hz, H-3; δ_C 29.2) also gave cross peaks to double doublet at δ_H 4.38 (*J* = 6.3, 6.3 Hz, H-2; δ_C 65.9), the olefinic proton of double double doublet at δ_H 5.82 (*J* = 2.8, 2.8, 1.4 Hz, H-5; δ_C 116.8) and methyl carbon of triplet at δ_H 2.01 (*J* = 0.6 Hz; δ_C 23.0), suggesting the structure of **NF3** as 4-methyl-2,3-dihydropyran-6-one (Figure 40).

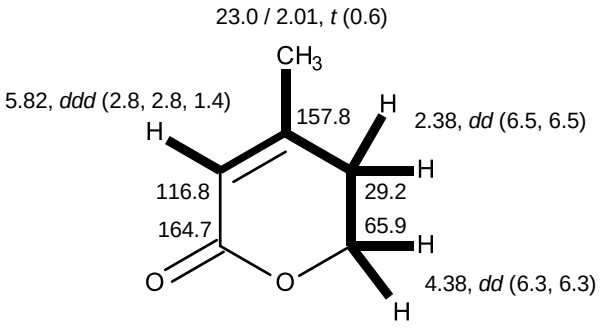


Figure 40. COSY (▬) correlations of **NF3**.

This was confirmed by the HMBC spectrum (Table 7) cross peaks of the tertiary methyl carbon of triplet at δ_H 2.01 (*J* = 0.6 Hz; δ_C 23.0) to the carbons at δ_C 65.8 (C-2), δ_C 116.8 (C-5) and δ_C 157.8 (C-4), of the double doublet at δ_H 2.38 (*J* = 6.5, 6.5 Hz; H-3; δ_C 29.2) to the carbons at δ_C 65.8 (C-2), δ_C 116.8 (C-5) and δ_C 157.8 (C-4) also, of the double doublet at δ_H 4.38 (*J* = 6.3, 6.3 Hz, H-2; δ_C 65.9) to the carbons at δ_C 29.2 (C-3), δ_C 157.8 (C-4) and δ_C 164.7 (C-6) (Figure 41).

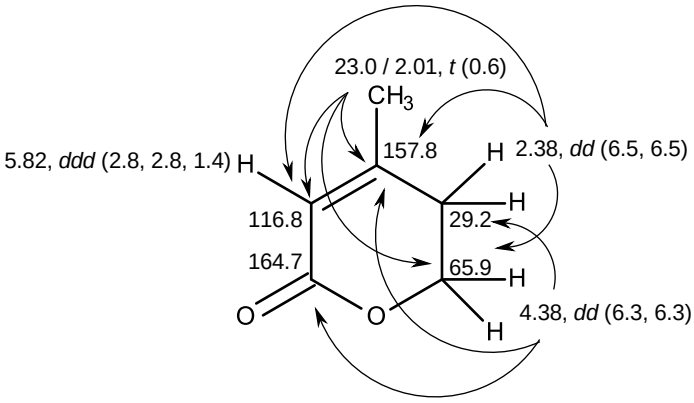


Figure 41. HMBC (⟶) correlations of **NF3**.

Taking into account the number of carbon atoms, the ^{1}H and ^{13}C chemical shift values and the COSY, HMBC correlations, the structure of **NF3** was identified as dehydromevalonic lactone also known as 4-methyl-2,3-dihydropyran-6-one (Figure 42). This compound was already isolated from *Cornus mas* fruits (var. *cultivar*) which were collected from trees of the settlement Kypseli in Imathia, Greece in September 2008 (Bakirtzi *et al*., 2013). Moreover, this compound was also isolated from the endophytic fungus, *Peyronellaea sp*. XW-12 strain, cultures from *Huperzia serrata* which was collected from Xishuangbanna Tropical Plant Garden, Chinese Academy of Science, Yunnan Province, China (Ying *et al*., 2014) and already reported by the synthesis (Bonadies *et al*., 1984).

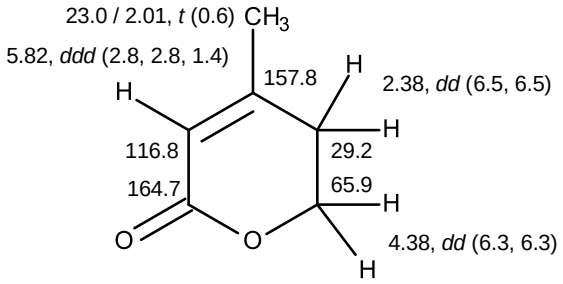


Figure 42. ^{1}H and ^{13}C assignments for **NF3**.

Table 7. ^{1}H and ^{13}C NMR ($CDCl_3$, 300.13 and 75.4 MHz) and HMBC assignment for **NF3**.

Position	δ_C, type	δ_H (*J* in Hz)	COSY	HMBC
2	65.9, CH_2	4.38, *dd* (6.3, 6.3)	H-3	C-3, 4, 6
3	29.2, CH_2	2.38, *dd* (6.5, 6.5)	H-2, 5, CH_3-4	C-2, 4, 5
4	157.8, C	-	-	-
5	116.8, CH	5.82, *ddd* (2.8, 2.8, 1.4)	H-3, CH_3-4	-
6	164.7, CO	-	-	-
CH_3-4	23.0, CH_3	2.01, *t* (0.6)	H-3, 5	C-3, 4, 5

4.1.3. Structure Elucidation of Nonadride, Byssochlamic Acid (NF4)

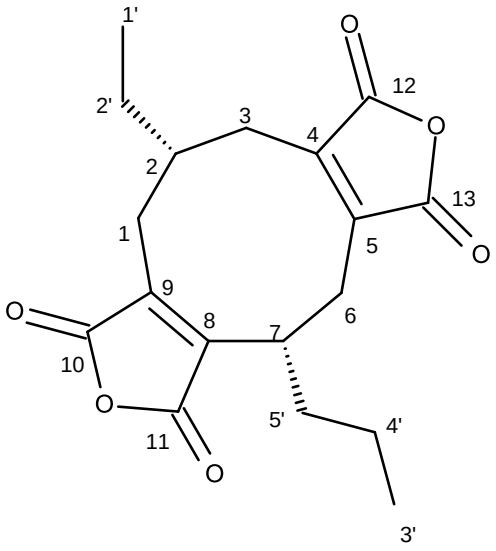


Figure 43. Structure of byssochlamic acid (**NF4**).

NF4 was isolated as white solid (mp. 167-168 ºC) and its molecular formula $C_{18}H_{20}O_6$ was based on the (+)-HRESIMS *m/z* 333.1326 $[M+H]^+$ (calculated for $C_{18}H_{21}O_6$ 333.1338) indicating nine degrees of unsaturation. The ^{13}C NMR, DEPTs and HSQC spectra (Table 9) indicated the presence of four conjugated ketone carbonyl (δ_C 165.3, 165.2, 164.9 and 164.1), four quaternary sp^2 (δ_C 146.2, 146.0, 145.0 and 144.1), two

methine sp^3 (δ_C 47.9 and 40.8), six methylene sp^3 (δ_C 31.5, 28.0, 23.4, 22.0, 21.8 and 21.4) and two primary methyl (δ_C 13.9 and 12.9) carbons in total of 18 signals respectively.

The ^1H NMR spectrum, in conjunction with the HSQC spectrum (Table 8), showed the signals of two methine sp^3 of multiplet at δ_H 2.11 (δ_C 47.9), δ_H 2.44 (δ_C 40.8) and two methyl groups of triplet at δ_H 0.87 (J = 7.1 Hz, δ_C 13.9), δ_H 1.08 (J = 7.0 Hz, δ_C 12.9).

The primary methyl triplet at δ_H 1.08 (J = 7.0 Hz; δ_C 12.9, C-1′) have COSY correlation (Table 9) to methylene sp^3 multiplet at δ_H 2.10 (δ_C 23.4, C-2′b) and this primary methyl triplet at δ_H 1.08 (J = 7.0 Hz, δ_C 12.9; C-1′) also has HMBC correlations to δ_C 47.9 (C-2) and δ_C 23.4 (C-2′). Besides this, methine sp^3 multiplet at δ_H 2.11 (δ_C 47.9, C-2) also has HMBC correlations to methylene sp^3 carbon at δ_C 28.0 (C-3) and δ_C 12.9 (C-1′). In addition, methylene sp^3 triplet at δ_H 1.76 (J = 12.6 Hz; δ_C 28.0, C-3a) also has HMBC correlations to quaternary carbon at δ_C 144.1 (C-5) and methine sp^3 carbon at δ_C 47.9 (C-2) and methylene sp^3 double doublet at δ_H 2.89 (J = 12.9, 2.9 Hz; δ_C 28.0, C-3a) also have cross peak to ketone carbonyl at δ_C 165.2 (C-13) and δ_C 164.9 (C-12). Moreover, the methylene sp^3 multiplet at δ_H 3.15 (δ_C 22.0; C-6) and methylene sp^3 double doublet at δ_H 2.89 (J = 12.9, 2.9 Hz; δ_C 28.0, C-3a) have HMBC correlation with quaternary carbon at δ_C 145.0 (C-4), δ_C 164.9 (C-12) and δ_C 165.2 (C-13) respectively and obtained fragment A.

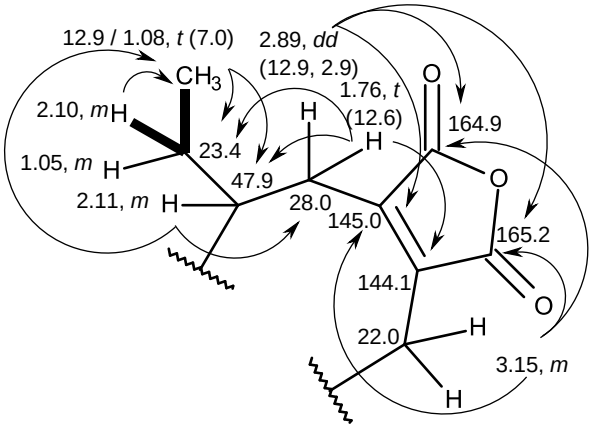


Figure 44. Key COSY (▬) and HMBC (⟶) correlations of fragment A.

In turn, the primary methyl triplet at δ_H 0.87 (J = 7.1 Hz, δ_C 13.9; C-3′) has COSY cross peak to methylene sp^3 multiplet at δ_H 1.15 (δ_C 21.8; C-4′) and HMBC cross peak to δ_C 21.8 (C-4′) and δ_C 31.5 (C-5′) and methylene sp^3 multiplet at δ_H 1.54 (δ_C 31.5) has COSY

correlation to methylene sp^3 multiplet at δ_H 1.15 (δ_C 21.8) and HMBC correlations to quaternary sp^2 carbon at δ_C 146.0. Moreover, the methine sp^3 multiplet at δ_H 2.44 (δ_C 40.8) has HMBC correlations to quaternary carbon at δ_C 146.2, ketone carbonyl at δ_C 164.1, methylene sp^3 carbon at δ_C 31.5 and methylene sp^3 multiplet at δ_H 2.24 (δ_C 21.4) to carbonyl ketone at δ_C 165.3 and obtained fragment B below.

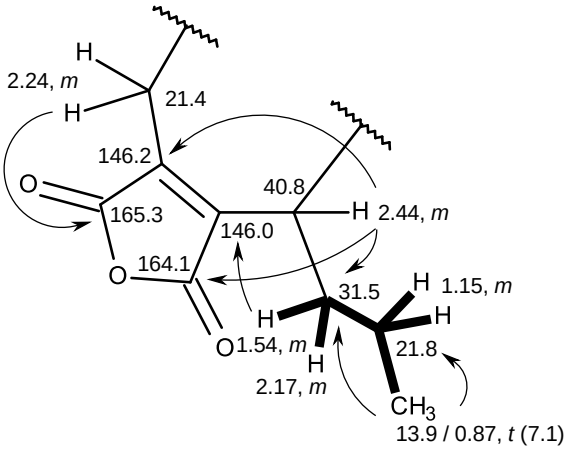


Figure 45. Key COSY (▬) and HMBC (⟶) correlations of fragment B.

To complete the structure, this fragment is connected with the fragment A, above by HMBC correlation; from the methine sp^3 multiplet at δ_H 2.44 (δ_C 40.8) to methylene sp^3 carbon at δ_C 22.0 and methylene sp^3 multiplet at δ_H 2.24 (δ_C 21.4) to quaternary sp^2 at δ_C 145.0, respectively (Figure 46). That the dihydrofuran-2, 5-dione was on C-4 and C-5, and the furan-2, 5-dione was on C-8 and C-9, was substantiated by the HMBC cross peaks of H_2-6 (δ_H 3.15, *m*; δ_C 22.0) to C-12 (δ_C 164.9), of H_2-3 (δ_H 2.89, *dd*, *J* = 12.9. 2.9; δ_C 28.0) to C-13 (δ_C 165.2), as well as of H-7 (δ_H 2.44, *m*; δ_C 40.8) to C-11 (δ_C 164.1) and H_2-1 (δ_H 2.24, *m*; δ_C 21.4) to C-10 (δ_C 165.3).

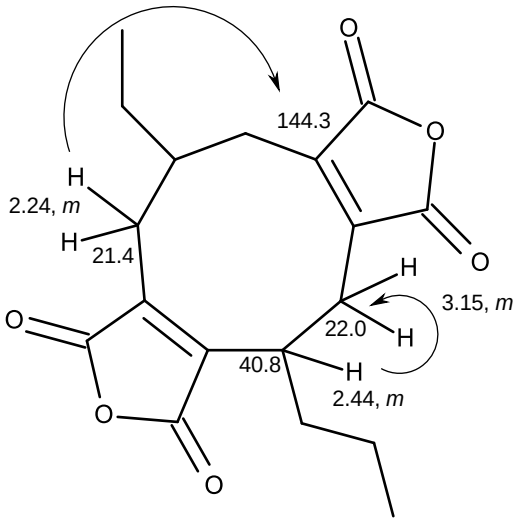


Figure 46. HMBC (⟶) correlations between fragment A and fragment B.

Taking into account the number of carbon atoms, the ^{1}H and ^{13}C chemical shift values and the COSY, HMBC correlations and comparison with the reported data (Table 8) (Szwalbe *et al*., 2015), the structure of **NF4** was identified as byssochlamic acid (Figiure 45). This compound was already isolated from common food contaminant *Byssochlamys fulva*. (Raistrick and Smith, 1933; White *et al*., 2000) and being isolated for the first time from nature in 2006, from the mangrove fungus (No. k38 strain) which was collected from the South China Sea coast (Li *et al*., 2006).

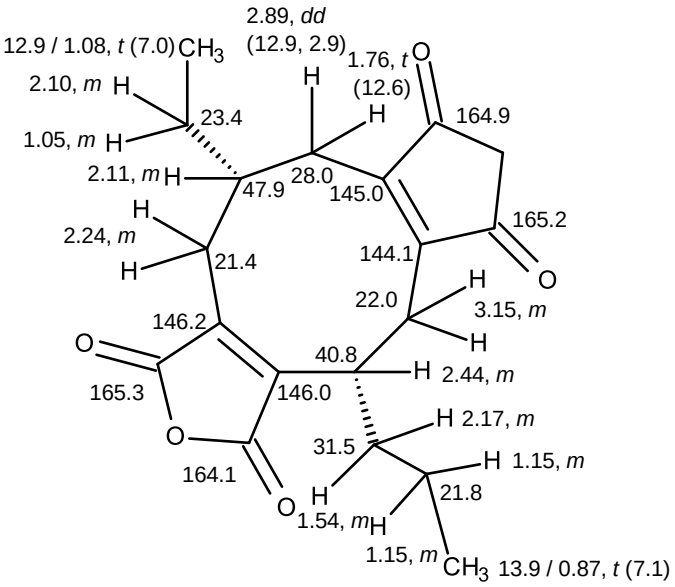


Figure 47. ^{1}H and ^{13}C assignments for **NF4**.

Table 8. ^{1}H and ^{13}C NMR ($CDCl_3$, 300.13 and 75.4 MHz) and HMBC assignment for **NF4** and reported data from Szwalbe *et al*., 2015.

	Szwalbe *et al*., 2015		**NF4**		
Position	δ_C, type	δ_H, (*J* in Hz)	δ_C, type	δ_H, (*J* in Hz)	HMBC
1	30.2, CH_2	2.29, *d* 2.72, *m*	21.4, CH_2	2.24, *m*	C-4, 10
2	40.4, CH	1.83-1.98, *brs*	47.9, CH	2.11, *m*	C-3, 1′
3a	29.5, CH_2	2.63, *m*	28.0, CH_2	1.76, *t* (12.6)	C-2, 5, 2′
b		2.35, *m*		2.89, *dd* (12.9, 2.9)	C-4, 12, 13
4	144.3, C	-	145, 0, C	-	-
5	143.3, C	-	144.1, C	-	-
6	28.2, CH_2	2.85, *o* 2.90, *m*	22.0, CH_2	3.15, *m*	C-4, 12, 13
7	34.9, CH	3.40, *m*	40.8, CH	2.44, *m*	C-6, 8, 11, 5′
8	144.7, C	-	146.0, C	-	-
9	143.6, C	-	146.2, C	-	-
10	165.6, CO	-	165.3, CO	-	-
11	165.4, CO	-	164.1, CO	-	-
12	165.4, CO	-	164.9, CO	-	-
13	165.0, CO	-	165.2, CO	-	-
1′	11.7, CH_3	1.12, *t* (7.3)	12.9, CH_3	1.08, *t* (7.0)	C-2, 2′
2′a	30.2, CH_2	1.64, *m*	23.4, CH_2	1.05, *m*	-
b		1.55, *m*		2.10, *m*	C-1′
3′	13.9, CH_3	0.95, *t* (7.3)	13.9, CH_3	0.87, *t* (7.1)	C-4′, 5′
4′	20.8, CH_2	1.43, *m* 1.35, *m*	21.8, CH_2	1.15, *m* -	- -
5′a	36.3, CH_2	1.69, *m*	31.5, CH_2	1.54, *m*	C-9
b		-		2.17, *m*	-

4.1.4. Structure Elucidation of Meroditerpene, Chevalone B (NF6)

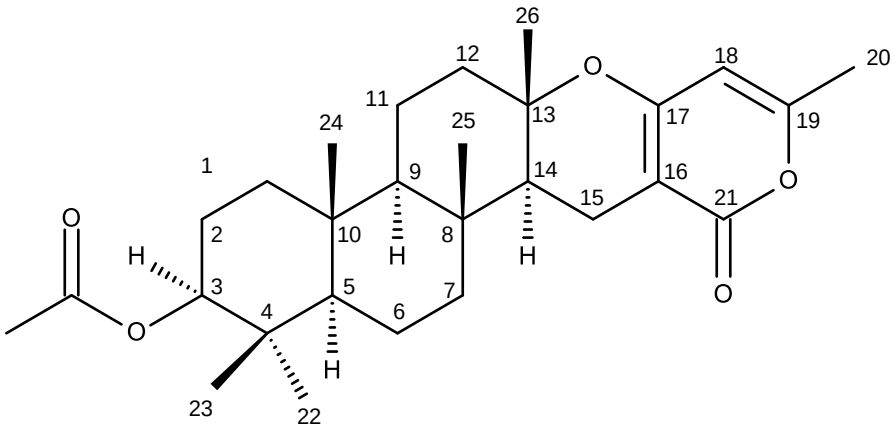


Figure 48. Strucuture of chevalone B (**NF6**).

NF6 was isolated as white amorphorus solid (mp. 161-163 °C), $[\alpha]_D^{20}$ + 11 (*c* 0.12, $CHCl_3$) and its molecular formula ($C_{28}H_{40}O_5$) was determined based on (+)-HR-ESIMS *m/z* 457.2949 $[M+H]^+$ (calculated for $C_{28}H_{41}O_5$, 457.2954) and indicated nine degrees of unsaturation. The ^{13}C NMR, DEPTs and HSQC spectra (Table 9) indicated the presence of one ester carbonyl (δ_C 171.0), one conjugated ester carbonyl (δ_C 165.4), three quaternary sp^2 (δ_C 163.3, 159.8 and 97.8), one methine sp^2 (δ_C 100.7), one oxymethine sp^3 (δ_C 80.7), three methine sp^3 (δ_C 60.2, 55.5 and 51.9), one oxyquaternary sp^3 (δ_C 80.5), three quaternary sp^3 (δ_C 37.8, 37.2 and 37.0), seven methylene sp^3 (δ_C 40.9, 40.2, 38.0, 23.5, 18.7, 17.8 and 16.8) and seven methyl (δ_C 27.9, 21.3, 20.5, 19.7, 16.5, 16.4 and 16.1) carbons in total of 28 signals respectively.

The ^1H NMR spectrum, in conjunction with the HSQC spectrum (Table 9), showed the signals of one olefinic proton at δ_H 5.69, *s* (δ_C 100.7), one oxymethine proton at δ_H 4.46, *dd* (*J* = 10.9, 5.1 Hz, δ_C 80.7), three methine sp^3 protons at δ_H 1.46, *dd* (*J* = 12.9, 4.8 Hz, δ_C 51.9), 0.93, *dd* (*J* = 12.4, 2.0 Hz, δ_C 60.2) and 0.87, *m* (δ_C 55.5) and seven methyl groups at δ_H 2.18, *s* (δ_C 19.7), 2.05, *s* (δ_C 21.3), 1.20, *s* (δ_C 20.5), 0.89, *s* (δ_C 16.1), 0.87, *s* (δ_C 16.4), 0.86, *s* (δ_C 16.5) and 0.85, *s* (δ_C 27.9).

The COSY spectrum (Table 9) exhibited the cross peak to each other between the oxymethine proton signal of double doublet at δ_H 4.46 (*J* = 10.9, 5.1 Hz; δ_C 80.7) and methylene sp^3 protons of multiplet at δ_H 1.69 (δ_C 23.5), between the methylene sp^3 protons

of multiplet at δ_H 1.73 (δ_C 38.0) and δ_H 1.06 (δ_C 38.0) and methylene sp^3 protons at δ_H 1.06, *m* (δ_C 38.0) also have COSY cross peak to methylene sp^3 protons of multiplet at δ_H 1.69 (δ_C 23.5), respectively. The HMBC spectrum (Table 9) showed correlations from the methylene sp^3 protons at δ_H 1.06, *m* (δ_C 38.0) to δ_C 16.4, from oxymethine protons signal at δ_H 4.46, *dd* (*J* = 10.9, 5.1 Hz, δ_C 80.7) to the ester carbons at δ_C 171.0 and methyl carbon at δ_C 16.1 and also from methyl proton at δ_H 2.05, *s* (δ_C 21.3) to ester carbonyl at δ_C 171.0, from methyl proton of singlet at δ_H 0.89 (δ_C 16.1) to δ_C 80.7 and δ_C 37.8, δ_C 55.5 and methyl proton of singlet at δ_H 0.85 (δ_C 27.9) to δ_C 80.7, δ_C 37.8, δ_C 55.5 and δ_C 16.1, respectively. Furthermore, methyl proton at δ_H 0.87, *s* (δ_C 16.4) also exhibited HMBC cross peak to δ_C 38.0 and δ_C 60.2, respectively. The HMBC correlations also displayed from methyl proton at δ_H 0.86, *s* (δ_C 16.5) to δ_C 37.0, δ_C 60.2 and δ_C 37.2, respectively. Moreover, COSY correlations also displayed from methine sp^3 at δ_H 0.87, *m* (δ_C 55.5) to methine sp^3 at δ_H 1.46, *m* (δ_C 17.8), from methine sp^3 at δ_H 1.46, *m* (δ_C 17.8) to methine sp^3 at δ_H 1.60, *m* (δ_C 17.8), from methine sp^3 at δ_H 1.60, *m* (δ_C 17.8) to methylene sp^3 at δ_H 1.03, *m* (δ_C 40.9), from methylene sp^3 at δ_H 1.03, *m* (δ_C 40.9) to methylene sp^3 at δ_H 1.86, *dt* (δ_C 40.9), respectively. The above COSY and HMBC correlations suggested the presence of 2-acetoxy-5,6-disubstituted-1,1,4a,6-tetramethyldecalin fragment.

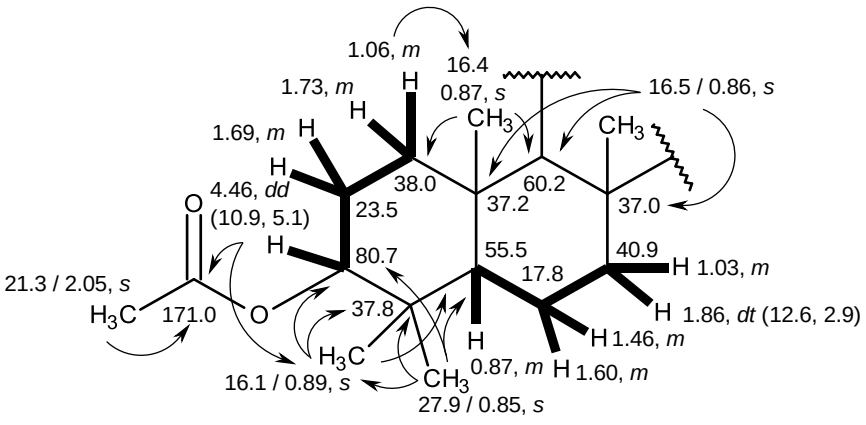


Figure 49. COSY (▬) and HMBC (⟶) correlations of 2-acetoxy-5,6-disubstituted-1,1,4a,6-tetramethyldecalin fragment.

Moreover, proton signals from 2-acetoxy-5,6-disubstituted-1,1,4a,6-tetramethyldecalin fragment continued to be connected by COSY and HMBC correlations;

from methine sp^3 proton at δ_H 0.93, *dd* (*J* = 12.7, 2.0; δ_C 16.1) to δ_H 1.35, *dd* (*J* = 12.9, 3.3; δ_C 18.7) in COSY correlations and HMBC correlations also displayed from methyl proton at δ_H 0.86, s (δ_C 16.5) to δ_C 80.5 respectively. In addition, methyl singlet at δ_H 1.20 (δ_C 20.5) also exhibited HMBC cross peaks to the methine sp^3 carbon at δ_C 51.9 and the methylene sp^3 carbon at δ_C 40.2 and quaternary sp^3 carbon at δ_C 80.5, the perhydrophenanthrene skeleton was suggested. Moreover, the COSY spectrum showed correlations of the methylene sp^3 double doublet at δ_H 2.43 (*J* = 16.9, 4.8 Hz; δ_C 16.8) and 2.16 (*J* = 10.5, 4.1 Hz; δ_C 16.8) to the methine double doublet at δ_H 1.46 (*J* = 12.9, 4.8 Hz; δ_C 51.9), consequently the methylene group (δ_C 16.8) was linked to the methine carbon at δ_C 51.9 and confirmed the presence of the perhydrophenanthrene portion.

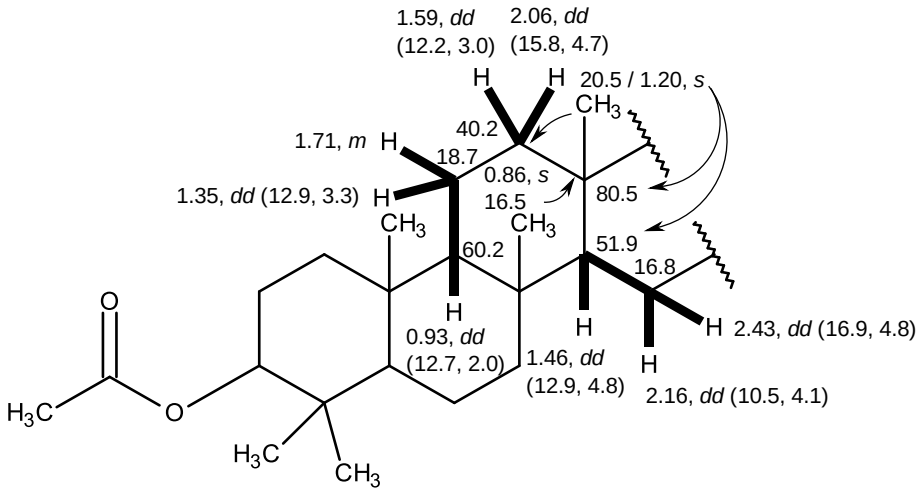


Figure 50. Key COSY (▬) and HMBC (⟶) correlations of **NF6**.

Other parts of the molecule contain the olefinic proton of singlet at δ_H 5.69 (δ_C 100.7) and the methyl protons signal of singlet at δ_H 2.18 (δ_C 19.7), both indicated cross peak to each other in the COSY correlations, supporting an allylic coupling. This suggestion was confirmed by the HMBC correlations from the singlet at δ_H 2.18 (δ_C 19.7) to the methyl carbon at δ_C 100.7. In addition, the olefinic proton of singlet at δ_H 5.69 (δ_C 100.7) showed HBMC correlations to the quaternary sp^2 carbons at δ_C 97.8. Therefore, this portion might include two double bonds. Moreover, HMBC spectrum also indicated correlations of the olefinic proton of singlet at δ_H 5.69 (δ_C 100.7) and the methyl singlet at δ_H 2.18 (δ_C 19.7) to the quaternary sp^2 carbon at δ_C 159.8. Based on these correlations and the existence of a

quaternary sp^2 carbon at δ_C 165.4 and δ_C 163.3, indicated that this portion of the molecule is supposed to be 6-methyl- 2*H*-pyran-1-one.

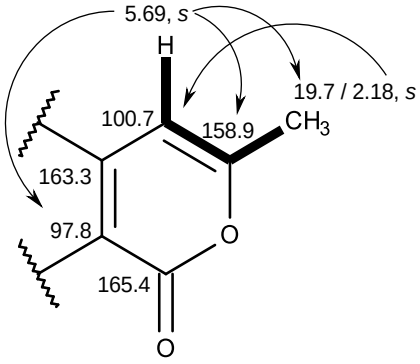


Figure 51. COSY (▬) and HMBC (⟶) correlations of 6-methyl- 2*H*-pyran-1-one.

Based on the NMR spectra, the perhydrophenanthrene portion was linked to the 6-methyl-2*H*-pyran-1-one portion by HMBC correlations from the methylene protons of double doublet at δ_H 2.43 (*J* = 16.9, 4.8 Hz; δ_C 16.8) to the quaternary sp^2 carbons at δ_C 97.8 and δ_C 163.3 and the carbonyl carbon at δ_C 165.4 of the 6-methyl-2*H*-pyran-1-one ring. Therefore, the structure of **NF6** corresponded as described below (Figure 52). The position of the acetoxyl group on C-3 (δ_C 80.7) was deduced as *β*-oriented due to the presence of oxymethine proton as a double doublet at δ_H 4.46 (*J* = 10.9, 5.1 Hz) (Kojima and Hogura, 1989; Gomes *et al*., 2014b).

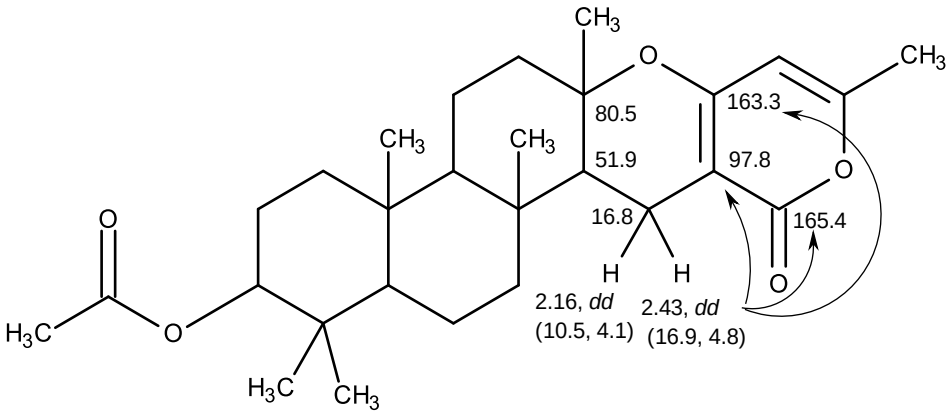


Figure 52. HMBC (⟶) correlations between perhydrophenanthrene portion and 6-methyl- 2*H*-pyran-1-one.

According to the previous reported data, **NF6** is supposed to be chevalone B (Figure 53). Based on Kanokmedhakul *et al*. (2011), chevalone B, a meroditerpene was first isolated from the fungus *Eurotium chevalieri* which is collected from rhizosphere soil of para rubber tree, 40 cm soil depth at Surathani Province, Thailand, in 2007 and displayed distinct cytotoxicity against human epidermoid carcinoma (KB) and human small cell lung cancer (NCI-H187) cell lines with the IC_{50} values of 2.9 and 3.9 µg/mL, respectively. Moreover, this compound was also isolated from *Neosartorya paulistensis* KUFC 7897 which was isolated from the marine sponge *Chondrilla australiensis* and collected from Mu Kho Lan Beach, Chonburi Province, Thailand in May 2010 and tested antibacterial activity against four respective strains such as *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa*, which revealed to be inactive in all cell lines (Gomes *et al*., 2014b). In 2014, chevalone B was also isolated from sponge-associated marine fungus, *Aspergillus similanensis* KUFA 0013, which was isolated from the marine sponge *Rhabdermia sp*., collected from the coral reef of the Similan Islands, Phang Nga Province, Thailand, by scuba diving at 10 m depth, in April 2010 (Prompanya *et al*., 2014). In 2015, chevalone B was also reported from *Neosartorya takakii* KUFC 7898 which was isolated from the alga *Amphiroa sp*. and collected from Samaesarn Island in the Gulf of Thailand, Chonburi Province, in September 2011 (Zin *et al*., 2015).

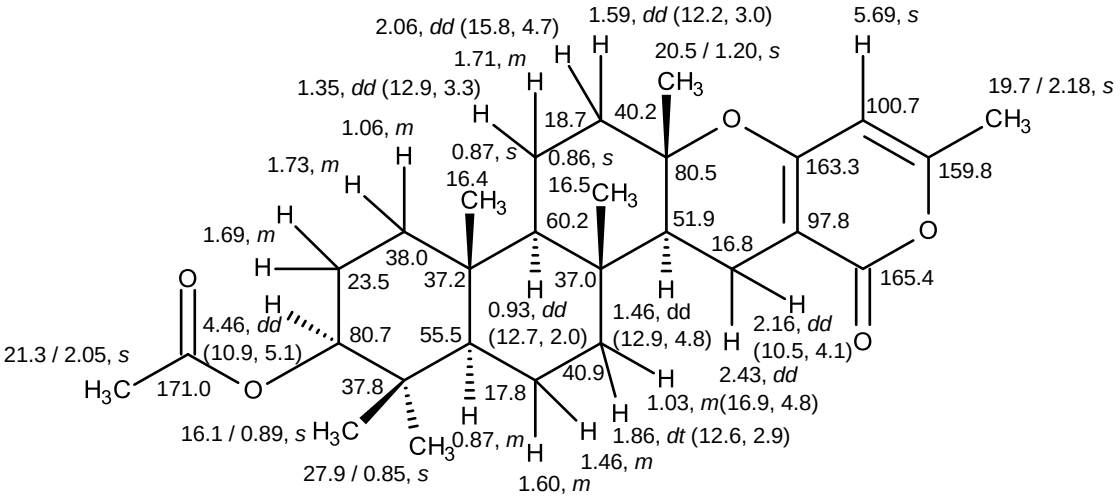


Figure 53. ^{1}H and ^{13}C assignments for **NF6**.

Table 9. ^{1}H and ^{13}C NMR ($CDCl_3$, 300.13 and 75.4 MHz) and HMBC assignment for **NF6**.

Position	δ_C, type	δ_H, (*J* in Hz)	COSY	HMBC
1a	38.0, CH_2	1.73, *m*	H-1b	-
B		1.06, *m*	H-1a, 2	C-24
2	23.5, CH_2	1.69, *m*	H-3	-
3	80.7, CH	4.46, *dd* (10.9, 5.1)	H-2	Ac-3, 23
4	37.8, C	-	-	-
5	55.5, CH	0.87, *m*	H-6b	-
6a	17.8, CH_2	1.60, *m*	H-6b, 7b	-
b		1.46, *m*	H-5, 6a	-
7a	40.9, CH_2	1.86, *dt* (12.6, 2.9)	H-7b	
b		1.03, *m*	H-7a	-
8	37.0, C	-	-	-
9	60.2, CH	0.93, *dd* (12.7, 2.0)	H-11b	-
10	37.2, C	-	-	-
11a	18.7, CH_2	1.71, *m*	H-9	-
b		1.35, *dd* (12.9, 3.3)	H-9	-
12a	40.2, CH_2	2.06, *dd* (15.8, 4.7)	H-12b	-
b		1.59, *dd* (12.2, 3.0)	H-12a	-
13	80.5, C	-	-	-
14	51.9, CH	1.46, *dd* (12.9, 4.8)	H-15a, 15b	C-25
15a	16.8, CH_2	2.43, *dd* (16.9, 4.8)	H-14, 15b	C-16, 17, 21
b		2.16, *dd* (10.5, 4.1)	H-14, 15a	-
16	97.8, C	-	-	-
17	163.3, C	-	-	-
18	100.7, CH	5.69, *s*	H-20	C-16, 19
19	159.8, C	-	-	-
20	19.7, CH_3	2.18, *s*	H-18	C-18, 19
21	165.4, C	-	-	-
22	27.9, CH_3	0.85, *s*	-	C-3, 4, 5, 23
23	16.1, CH_3	0.89, *s*	-	C-3, 4, 5
24	16.4, CH_3	0.87, *s*	-	C-1, 9
25	16.5, CH_3	0.86, *s*	-	C- 8, 9, 10,13
26	20.5, CH_3	1.20, *s*	-	C-12, 13, 14
Ac-3	171.0, CO	-	-	-
	21.3, CH_3	2.05, *s*	-	CO (Ac-3)

4.1.5 Structure Elucidation of Indolymethyl 1,4-bezodiazepen-2,5-dione, Aszonalenin (NF7)

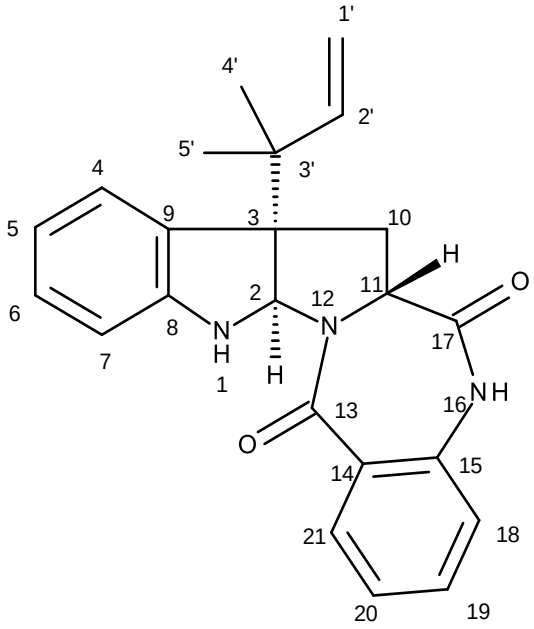


Figure 54. Structure of aszonalenin (**NF7**).

NF7 was isolated as white solid (mp. 249-250 °C) and the ^{13}C NMR, DEPTs and HSQC spectra (Table 10) indicated the presence of two amide carbonyls (δ_C 170.0 and 166.8), four quaternary sp^2 (δ_C 149.1, 134.1, 131.2 and 126.9), nine methine sp^2 (δ_C 143.8, 132.6, 131.2, 128.6, 125.3, 125.0, 120.6, 118.4 and 109.2), one methylene sp^2 (δ_C 114.3), two quaternary sp^3 (δ_C 60.7 and 41.5), two methine sp^3 (δ_C 81.7 and 57.0), one methylene sp^3 (δ_C 33.4) and two tertiary methyl (δ_C 22.7 and 22.5) carbons in total of 23 signals respectively.

The ^{1}H NMR spectrum, in conjunction with the HSQC spectrum (Table 10), showed the presence of eight aromatic protons at δ_H 7.83, *dd* (*J* = 7.9 Hz, 1.5; δ_C 131.2), δ_H 7.44, *ddd* (*J* = 7.7, 7.7, 1.4 Hz; δ_C 132.6), δ_H 7.22, *ddd* (*J* = 7.6, 7.6, 0.8 Hz; δ_C 125.3), δ_H 7.15, *d* (*J* = 7.4 Hz; δ_C 125.0), δ_H 7.08, *ddd* (*J* = 7.6, 7.6, 1.1 Hz; δ_C 128.6), δ_H 6.92, *d* (*J* = 7.8 Hz; δ_C 120.6), δ_H 6.72, *ddd* (*J* = 7.2, 7.2, 0.8 Hz; δ_C 118.4), δ_H 6.62, *dd* (*J* = 7.7 Hz; δ_C 109.2), one doublet at δ_H 5.10, *d* (*J* = 7.8; δ_C 114.3), four double doublets at δ_H 6.11 (*J* = 17.2, 10.8 Hz; δ_C 143.8), δ_H 3.99 (*J* = 8.9, 7.7; δ_C 57.0), δ_H 3.47 (*J* = 14.0, 7.6; δ_C 33.4) and δ_H 2.41 (*J*

= 13.9, 9.0, δ_C 33.4), one double double doublet at δ_H 5.12 (*J* = 11.4, 11.4, 1.2; δ_C 114.3), one singlet at δ_H 5.58, s (δ_C 81.7), two methyl singlet at δ_H 1.13 (δ_C 22.7) and δ_H 1.05 (δ_C 22.5) and one singlet of NH group at δ_H 8.12 and one doublet of NH group at δ_H 6.15, d (*J* = 2.1), respectively.

The COSY correlations displayed the cross peak from H-18, doublet at δ_H 6.92 (*J* = 7.8 Hz; δ_C 120.6) to H-19, double double doublet at δ_H 7.44 (*J* = 7.7, 7.7, 1.4 Hz; δ_C 132.6), from H-19, double double doublet at δ_H 7.44 (*J* = 7.7, 7.7, 1.4 Hz; δ_C 132.6) to H-18, doublet at δ_H 6.92 (*J* = 7.8 Hz; δ_C 120.6) and H-20, double double doublet at δ_H 7.22 (*J* = 7.6, 7.6, 0.8 Hz; δ_C 125.3), from H-20, double double doublet at δ_H 7.22 (*J* = 7.6, 7.6, 0.8 Hz; δ_C 125.3) to H-19, double double doublet at δ_H 7.44 (*J* = 7.7, 7.7, 1.4 Hz; δ_C 132.6) and H-21, double doublet at δ_H 7.83, (*J* = 7.9 Hz, 1.5; δ_C 131.2), from H-21, double doublet at δ_H 7.83 (*J* = 7.9 Hz, 1.5; δ_C 131.2) to H-20, double double doublet at δ_H 7.22 (*J* = 7.6, 7.6, 0.8 Hz; δ_C 125.3), respectively. The correlations indicated the the presence of 1,2-disubstituted benzene ring and this benzene ring was fused to the 1,4-bezodiazepen-2,5-dione ring by the HMBC correlations and formed 3,4-dihydro-1*H*-1,4-benzodiazepine-2,5-dione portion. This was confirmed by the HMBC correlations from H-18 to C-14 (δ_C 126.9) and C-20, from H-19 to C-21, from H-20 to C-14 and C-18, from H-21 to C-13 and C-19, from singlet of NH group at δ_H 8.12 to C-11 and C-14, respectively.

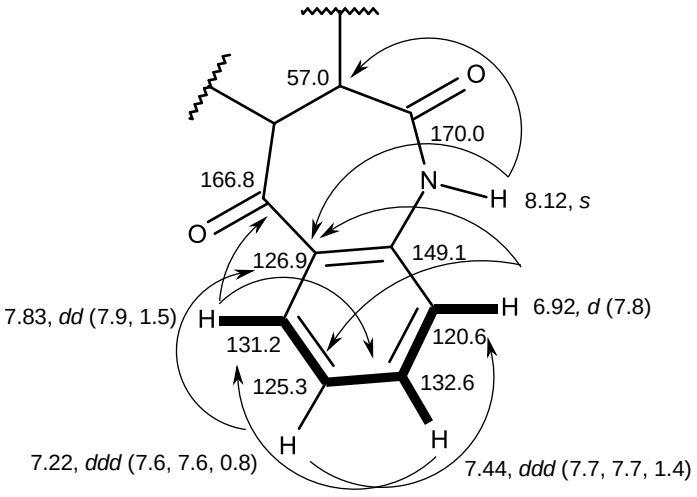


Figure 55. COSY (▬) and HMBC (⟶) correlations of 3,4-dihydro-1*H*-1,4-benzodiazepine-2,5-dione portion.

The COSY spectrum also exhibited the correlations from H-4, double at δ_H 7.15 (*J* = 7.4 Hz; δ_C 125.0) to H-6, double double doublet at δ_H 7.08 (*J* = 7.6, 7.6, 1.1 Hz; δ_C 128.6) and H-5, double double doublet at δ_H 6.72 (*J* = 7.2, 7.2, 0.8 Hz; δ_C 118.4), from H-5, double double doublet at δ_H 6.72 (*J* = 7.2, 7.2, 0.8 Hz; δ_c 118.4) to H-4, doublet at δ_H 7.15 (*J* = 7.4 Hz; δ_C 125.0) and H-6, double double doublet at δ_H 7.08 (*J* = 7.6, 7.6, 1.1 Hz; δ_C 128.6), from H-6, double double doublet at δ_H 7.08 (*J* = 7.6, 7.6, 1.1 Hz; δ_C 128.6) to H-4, doublet at δ_H 7.15 (*J* = 7.4 Hz; δ_C 125.0), H-5, double double doublet at δ_H 6.72 (*J* = 7.2, 7.2, 0.8 Hz; δ_C 118.4) and H-7, double doublet at δ_H 6.62 (*J* = 7.7 Hz; δ_C 109.2), from H-7, double doublet at δ_H 6.62 (*J* = 7.7 Hz; δ_C 109.2) to H-4, doublet at δ_H 7.15 (*J* = 7.4 Hz; δ_C 125.0) and δ_H H-6, double double doublet at δ_H 7.08 (*J* = 7.6, 7.6, 1.1 Hz; δ_C 128.6), respectively. The correlations indicated the presence of another 1,2-disubstituted benzene ring and this 1,2-disubstituted benzene ring was part of the 2,3-dihydro-1*H*-indole moiety by the HMBC correlations. This was confirmed by the HMBC correlations from H-4 to C-6, from H-5 to C-7 and C-9 (δ_C 131.2), from H-6 to C-4 and from H-7 to C-5 and C-9, from H-2, δ_H 5.58, *s* (δ_C 81.7) to C-9, respectively. The proton signal of singlet at δ_H 8.12 had already shown HMBC correlations to the signal of C-11 (δ_C 57.0) and C-14 (δ_C 126.9) so the other formyl group δ_H 6.15, d (*J* = 2.1) was placed on N-1.

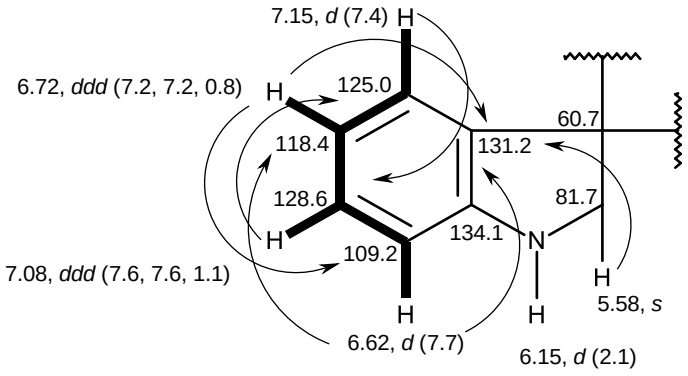


Figure 56. COSY (▬) and HMBC (⟶) correlations of 2,3-dihydro-1*H*-indole moiety.

The coupling system of the aromatic protons observed in the COSY and HMBC spectrum (Table 11) revealed the presence of two, 1,2-disubstituted benzene rings. In addition, HMBC spectrum indicated that one of the 1,2-disubstituted benzene rings was part of a 2,3-dihydro-1*H*-indole moiety while another of the 1,2-disubstituted benzene rings was part of a 3,4-dihydro-1*H*-1,4-benzodiazepine-2,5-dione portion. The 2,3-dihydro-1*H*-

indole and the 3,4-dihydro-1*H*-1,4-benzodiazepine-2,5-dione portions were linked together through a pyrrolidine ring which was confirmed by the HMBC cross peaks of H-10a (δ_H 2.41, *dd*, *J* = 13.9, 9.0 Hz; δ_C 33.4) to C-3 (δ_C 60.7), C-9 (δ_C 131.2), from H-10b (δ_H 3.47, *dd*, *J* = 14.0, 7.6 Hz; δ_C 33.4) to C-3 (δ_C 60.7), C-9 (δ_C 131.2), C-11 (δ_C 57.0), C-17 (δ_C 170.0) and from H-11 (δ_H 3.99, *dd*, *J* = 8.9, 7.7 Hz; δ_C 57.0) to C-10 (δ_C 33.4), respectively.

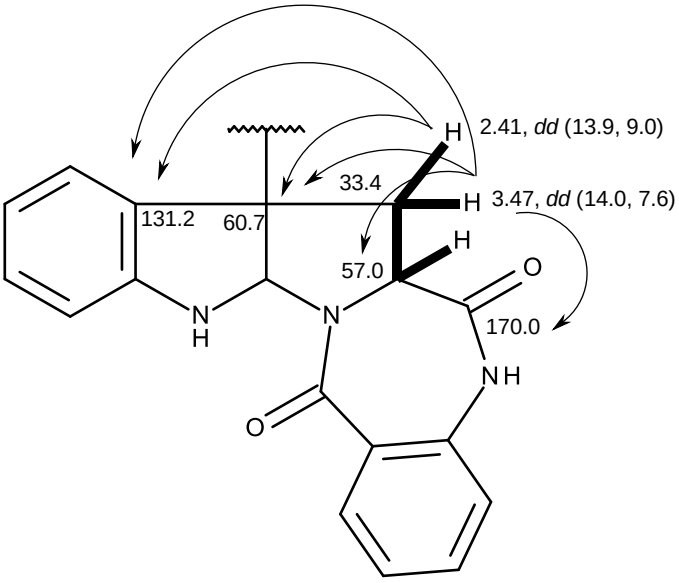


Figure 57. COSY (▬) and HMBC (⟶) correlations between 2,3-dihydro-1*H*-indole and the 3,4-dihydro-1*H*-1,4-benzodiazepine-2,5-dione portions via pyrrolidine ring.

The 2-methylbut-3-en-2-yl substituent was placed on C-3 which was substituted by the HMBC correlations from the H-2 (δ_H 5.58, *s*; δ_C 81.7) to the C-3′ (δ_C 41.5) and from H_3-5′ (δ_H 1.05, *s*; δ_C 22.5) to C-3 (δ_C 60.7), C-2′ (δ_C 143.8), C-3′ (δ_C 41.5) and H-4′ (δ_H 1.13, *s*; δ_C 22.7), from H-4′ (δ_H 1.13, *s*; δ_C 22.7) to C-3 (δ_C 60.7), C-2′ (δ_C 143.8), C-3′ (δ_C 41.5) and C-5′ (δ_C 22.5), from H-2′ (δ_H 6.11, *dd*, *J* = 17.2, 10.8 Hz; δ_C 143.8) to C-3′ (δ_C 41.5), C-4′ (δ_C 22.7) and C-5′ (δ_C 22.5), from H-1a (δ_H 5.12, *ddd*, *J* = 11.4, 11.4, 1.2), and H-1b (δ_H 5.10, *d*, *J* = 7.8) (δ_C 114.3) to C-2′ (δ_C 143.8), C-3′ (δ_C 41.5), respectively.

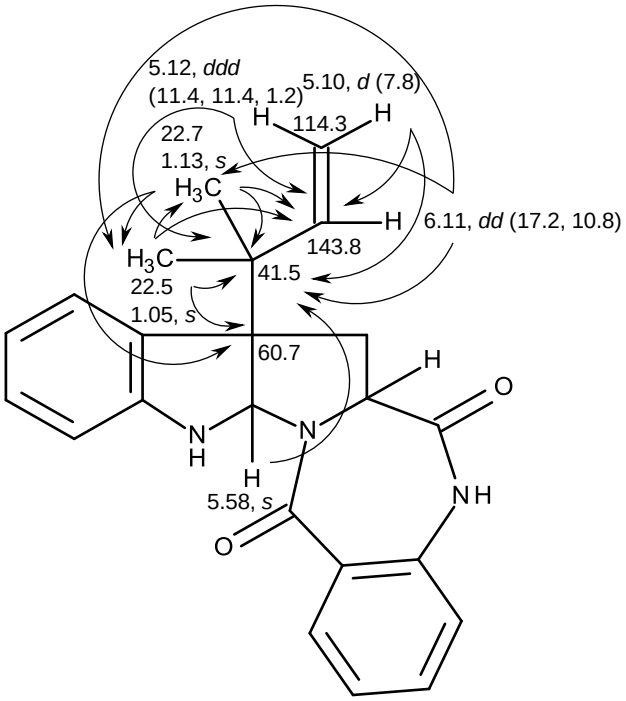


Figure 58. HMBC (⟶) correlations 2-methylbut-3-en-2-yl substituent of **NF7**.

Taking into account the number of carbon atoms, the ^{1}H and ^{13}C chemical shift values and the COSY, HMBC correlations and comparison with the reported data (Yin *et al*., 2009), the structure of **NF7** was identified as aszonalenin.

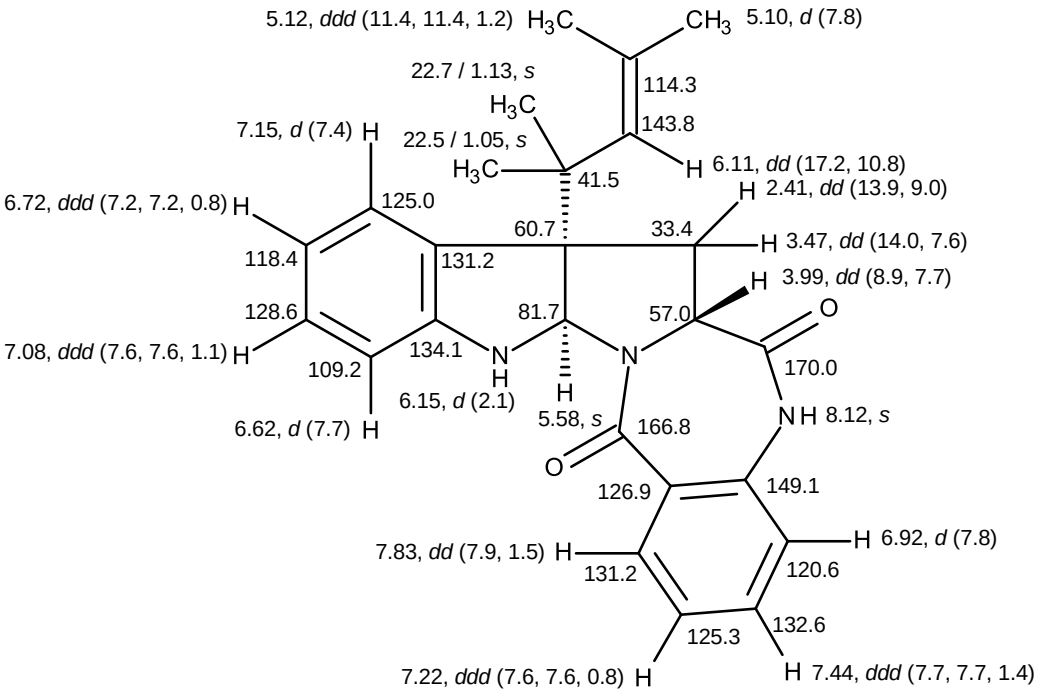


Figure 59. ^{1}H and ^{13}C assignments for **NF7**.

Aszonalenin isolated from the soil fungus, *Neosartorya fischeri* which was collected from coastal forest soil at Samaersarn Island, Chonburi Province, Thailand in November 2008, which was tested for cytotoxicity with three cell lines; breast carcinoma (MCF-7), non-small cell lung cancer (NCI-H460), and melanoma (A375-C5). However, aszonalenin proved to be inactive against all three cell lines even in the highest concentration at 56 µg/mL (Eamvijarn, *et al*., 2013b). In recent years, aszonalenin was also reported from algae associated fungus, *Neosartorya takakii* KUFC 7898 which was isolated from the alga *Amphiroa sp*. and collected from Samaesarn Island in the Gulf of Thailand, Chonburi Province, in September 2011 (Zin *et al*., 2015).

Table 10. ^{1}H and ^{13}C NMR ($CDCl_3$, 300.13 and 75.4 MHz) and HMBC assignment for **NF7**.

Position	δ_C, type	δ_H, (*J* in Hz)	COSY	HMBC
2	81.7, CH	5.58, *s*	-	C-3′, 9
3	60.7, C	-	-	-
4	125.0, CH	7.15, *d* (7.4)	H-5, 6	C-6
5	118.4, CH	6.72, *ddd* (7.2, 7.2, 0.8)	H-4, 6	C-7, 9
6	128.6, CH	7.08, *ddd* (7.6, 7.6, 1.1)	H-4, 5, 7	C-4
7	109.2, CH	6.62, *d* (7.7)	H-4, 6	C-5, 9
8	134.1, C	-	-	-
9	131.2, C	-	-	-
10a	33.4, CH_2	2.41, *dd* (13.9, 9.0)	H-10b, 11	C-2, 3, 9
b		3.47, *dd* (14.0, 7.6)	H-10a, 11	C-3, 3′, 9, 11, 17
11	57.0, CH	3.99, *dd* (8.9, 7.7)	H-10a, 10 b	C-10
13	166.8, C	-	-	-
14	126.9, C	-	-	-
15	149.1, C	-	-	-
17	170.0, C	-	-	-
18	120.6, CH	6.92, *d* (7.8)	H-19	C-14, 20
19	132.6, CH	7.44, *ddd* (7.7, 7.7, 1.4)	H-18, 20	C-21
20	125.3, CH	7.22, *ddd* (7.6, 7.6, 0.8)	H-19, 21	C-14, 18
21	131.2, CH	7.83, *dd* (7.9, 1.5)	H-20	C-13, 19
1′a	114.3, CH_2	5.12, *ddd* (11.4, 11.4, 1.2)	H-2′	C-2′, 3′
b		5.10, *d* (7.8)	H-2′	C-2′, 3′
2′	143.8, CH	6.11, *dd* (17.2, 10.8)	H-1′a, H-1′b	C-3′, 4′, 5′
3′	41.5, C	-	-	-
4′	22.7, CH_3	1.13, *s*	-	C-2′, 3, 3′, 5′
5′	22.5, CH_3	1.05, *s*	-	C-2′, 3, 3′, 4′
H-1		6.15, *d* (2.1)	-	-
H-16		8.12, *s*	-	C-11, 14

4.1.6. Structure Elucidation of *Bis*-xanthone, Secalonic Acid A (NF8)

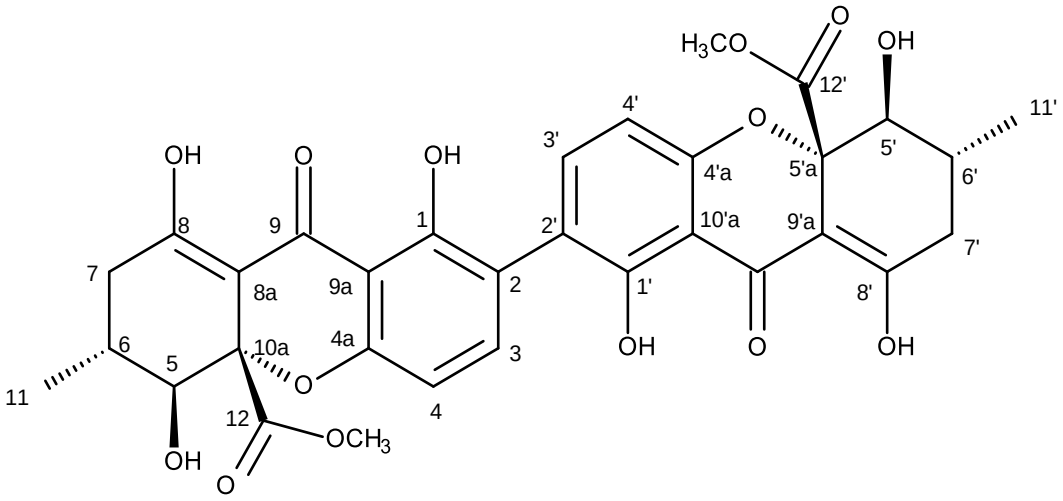


Figure 60. Structure of secalonic acid A (**NF8**).

NF8 was isolated as yellow crystal (mp. 268-270 °C) and its molecular formula was determined based on (+)-HRESIMS *m/z* 639.1733 $[M+H]^+$ (calculated for $C_{32}H_{31}O_{14}$, 639.1714). The ^{13}C NMR, DEPTs and HSQC spectra (Table 11) indicated the presence of one conjugated ketone carbonyl (δ_C 186.5), one ketone or enolic (δ_C 178.2), one ester carbonyl (δ_C 170.0), two oxyquaternary sp^2 (δ_C 158.9 and 158.5), three quaternary sp^2 (δ_C 117.3, 106.3 and 101.7), two methine sp^2 (δ_C 140.2 and 107.5), one oxyquaternary sp^3 (δ_C 85.2), one oxymethine sp^3 (δ_C 75.2), one methine sp^3 (δ_C 29.9), one methylene sp^3 (δ_C 35.8) and one secondary methyl (δ_C 17.7) carbons and one methoxy (δ_C 52.9) in total of 16 signals respectively.

The ^1H NMR spectrum, in conjunction with the HSQC spectrum (Table 11), showed the signals of two methine sp^2 protons (*ortho*-coupled aromatic protons) at δ_H 7.47, *d* (*J* = 8.5 Hz, δ_C 140.2), δ_H 6.63, *d* (*J* = 8.5 Hz, δ_C 107.5), two methine sp^3 proton at δ_H 2.31, *m* (δ_C 29.9), δ_H 3.82, *dd* (*J* = 11.0, 5.7 Hz, δ_C 75.2) and one methyl proton at δ_H 1.04, *d* (*J* = 6.4 Hz, δ_C 17.7), one methoxy proton at δ_H 3.61, *s* (δ_C 52.9) and three hydroxyl groups; at δ_H 6.05, *dd* (*J* = 5.8, 2.4), one hydrogen-bonded phenolic hydroxyl at δ_H 11.63, *brs* and one enolic hydroxyl at 13.67, brs respectively.

The *ortho*-coupled aromatic protons fused to the 2,3,5-trisubstituted phenol were exhibited by the HMBC (Table 11) correlations from the hydrogen-bonded phenolic

hydroxyl group at δ_H 11.63 *brs* to the oxyquaternary sp^2 carbon at δ_C 158.5 (C-1) and the quaternary sp^2 carbons at δ_C 117.3 (C- 2) and δ_C 106.3 (C-9a), from the doublet at δ_H 7.47 (J = 8.5 Hz) to C-1 (δ_C 158.5), C-2 (δ_C 117.3) and the other oxyquaternary sp^2 carbon at δ_C 158.9 was assigned to C-4a and from the doublet at δ_H 6.63 (J = 8.5 Hz) to C-2 (δ_C 117.3), C-4a (δ_C 158.9) and C-9a (δ_C 106.3) and described below (Figure 61).

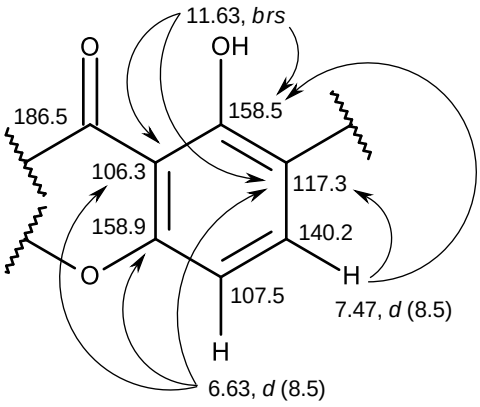


Figure 61. HMBC (⟶) correlations of **NF8**.

In COSY correlations (Table 11), double doublet methine sp^3 at δ_H 3.82 (J = 11.0, 5.7 Hz, H-5) displayed cross peak to the hydroxyl double doublet at δ_H 6.05 (J = 5.8, 2.4 Hz, OH-5) and another methine sp^3 multiplet proton at δ_H 2.31 (δ_C 29.9, H-6) and methine sp^3 proton of multiplet at δ_H 2.31 (δ_C 29.9, H-6) also exhibited COSY correlations to oxymethine sp^3 double doublet at δ_H 3.82 (δ_C 75.2, H-5) and methyl doublet at δ_H 1.04 (J = 6.4 Hz, δ_C 17.7, H-11) while methyl doublet at δ_H 1.04 (J = 6.4 Hz, H-11) also has cross peak to methine sp^3 multiplet at δ_H 2.31 (δ_C 29.9, H-6). Moreover, methylene sp^3 multiplet at δ_H 2.47 (δ_C 35.8, H-7a) and methylene sp^3 multiplet at δ_H 2.64 (δ_C 35.8, H-7b) also have COSY cross peak to each other and suggesting the presence of the following coupling system:

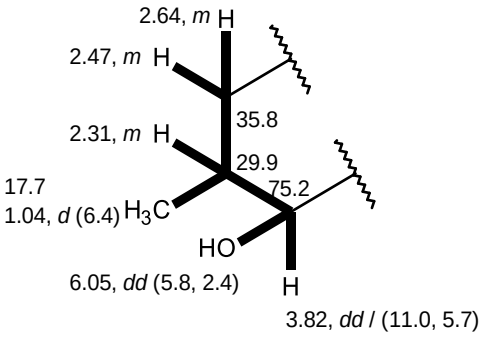


Figure 62. COSY (▬) correlations of **NF8**.

This was supported by HMBC correlations from the proton signal of the hydroxyl double doublet of proton at δ_H 6.05 (*J* = 5.8, 2.4 Hz, OH-5) to the oxymethine sp^3 at δ_C 75.2 (C-5) and the methine sp^3 carbon at δ_C 29.9 (C-6) and also from the signal of the double doublet of the methine proton at δ_H 3.82 (*J* = 11.0, 5.7 Hz, H-5) have cross peaks to the oxyquaternary sp^3 carbon at δ_C 85.2 (C-10a) and the ester carbonyl at δ_C 170.0 (C-12). This coupling supported that C-10a is connected to the ester group and this was confirmed by the correlations from methoxyl singlet at δ_H 3.61 (δ_C 52.9) to this ester carbonyl group. Therefore, substituents on the oxyquaternary sp^3 carbon at δ_C 85.2 was an acetyl group. Additionally, the HMBC spectrum also displayed cross peaks from the multiplets at δ_H 2.47 (δ_C 35.8) and δ_H 2.64 (δ_C 35.8) to the enolic carbon at δ_C 178.2 and multiplet at δ_H 2.64 (δ_C 35.8) also showed cross peak to the methine sp^3 carbon at δ_C 75.2 and the doublet of methyl proton at δ_H 1.04 (*J* = 6.4 Hz; δ_C 17.7) also exhibited cross peak to δ_C 75.2, δ_C 35.8 and δ_C 29.9 as well, supporting the presence of the following moiety.

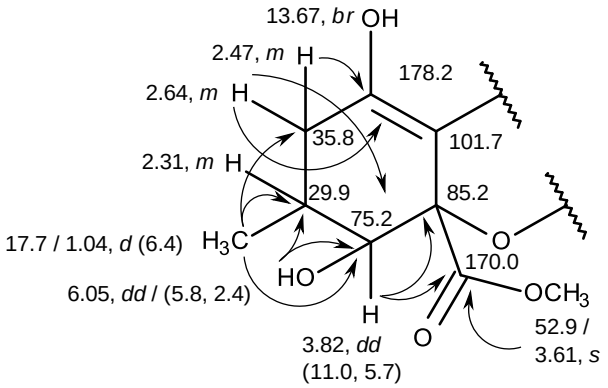


Figure 63. HMBC (⟶) correlations of **NF8**.

Taking account ^{1}H and ^{13}C NMR data, this compound could be deduced as methyl 1,4-dihydroxy-3-methyl-9-oxo-2,3,4,9-tetrahydro-4a*H*-xanthene-4a-carboxylate.

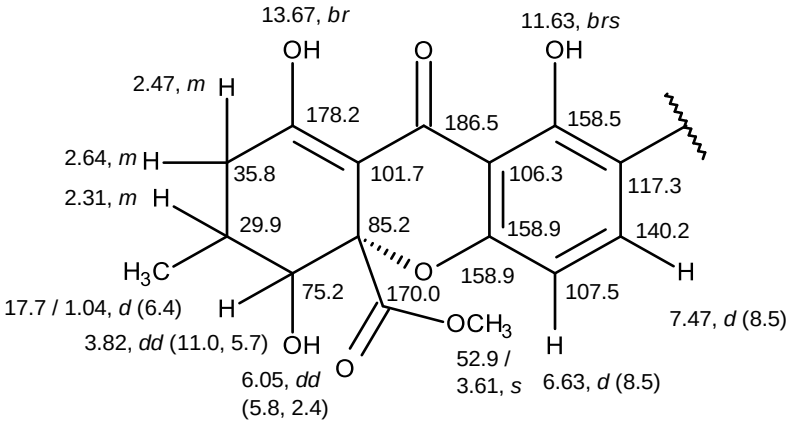


Figure 64. ^{1}H and ^{13}C chemical shift values of **NF8** partial structure.

This structure lacks a substituent on the carbon at δ_C 117.3 of the phenolic moiety. At any rate, this structure also accounts for $C_{16}H_{15}O_7$ which is only half of its molecular formula $C_{32}H_{30}O_{14}$, a result obtained from (+)-HRESIMS *m/z* 639.1714 for its $[M+H]^+$. According to its molecula formula, **NF8** must correspond to dimer of the proposed structure, linked through its carbon at δ_c 117.3. This was confirmed by X-ray analysis due to this compound being crystallized. The Ortep view shown in Figure 65 also determines the absolute configurations of C-5/C-5′, C-6/C-6′ and C-10a/C-10′a, respectively as C-5*S*/C-5′*S*, C-6*R*/C-6′*R*, and C-10a*S*/C-10′a*S*.

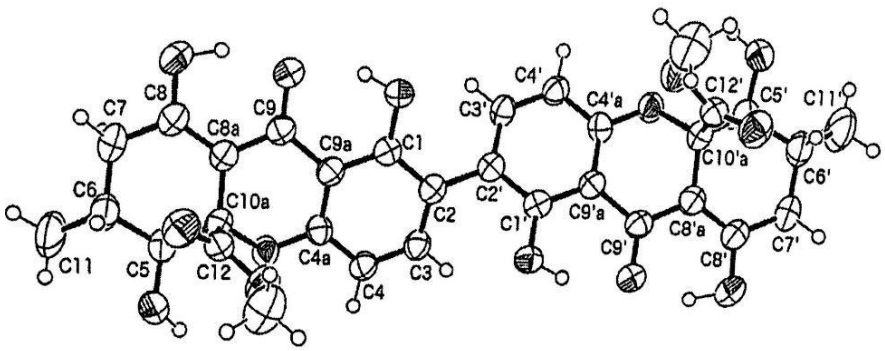


Figure 65. Ortep view of **NF8**.

NF8 (Figure 66) was already isolated from food borne toxigenic fungi, *Aspergillus ochraceus* which was isolated from moldy rice collected in Chiba and Miyagi in 1970 (Yamazaki *et al*., 1971) and this compound was already isolated also from the lichens *Pseudoparmelia sphaerospora* which was collected on bark in Vila Piraputanga, Mato Grosso do Sul, Brazil, in August, 1989 and recent investigation was performed from marine sponge-associated fungus, *Talaromyces stipitatus* KUFA 0207 which was isolated from marine sponge *Stylissa flabelliformis* collected by scuba diving at a depth of 10–15 m, from the coral reef at Samaesarn Island (12°34′36.64″ N 100°56′59.69″ E) in the Gulf of Thailand, Chonburi Province, in April 2014. This isolated secalonic acid A was tested for anti-obesity by using the zebrafish Nile red assay and indicated death result (toxicity) for all tested zebrafish larvae after a 24-hour exposure (Noinart *et al*., 2017).

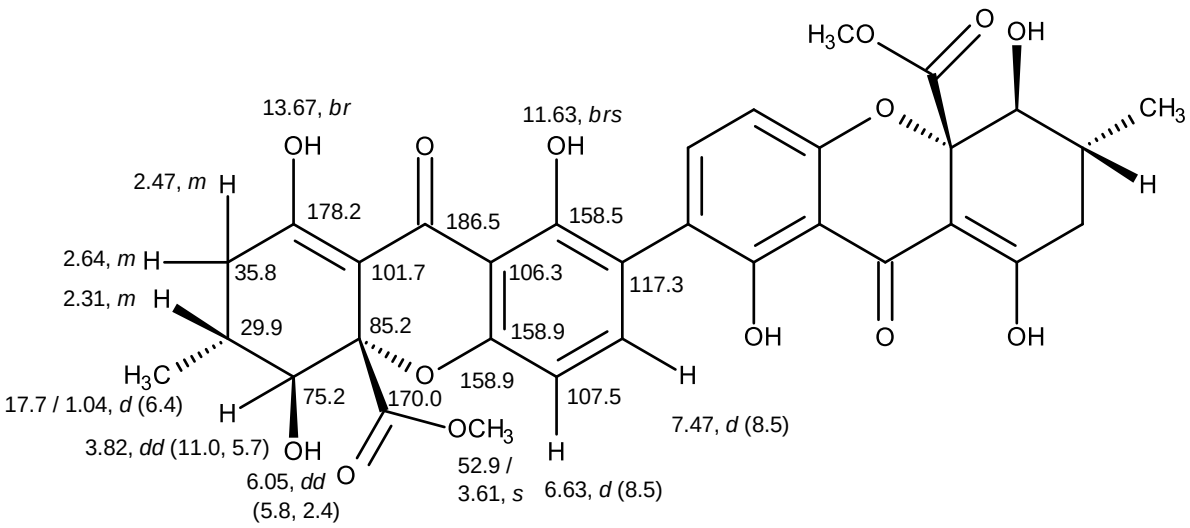


Figure 66. ^{1}H and ^{13}C assignments for **NF8**.

Table 11. ^{1}H and ^{13}C NMR (DMSO-d_6, 300.13 and 75.4 MHz) and HMBC assignment for **NF8**.

Position	δ_C, type	δ_H, (*J* in Hz)	COSY	HMBC
1 (1′)	158.5, C	-	-	-
2 (2′)	117.3, C	-	-	-
3 (3′)	140.2, CH	7.47, *d* (8.5)	H-4 (4′)	C-1 (1′), 2 (2′)
4 (4′)	107.5, CH	6.63, *d* (8.5)	H-3 (3′)	C-2 (2′), 4a (4a′), 9a (9a′)
4a (4a′)	158.9, C	-	-	
5 (5a′)	75.2, CH	3.82, *dd* (11.0, 5.7)	OH-5 (5′), H-6 (6′)	C-10a (10a′), (12′)
6 (6a′)	29.9, CH	2.31, *m*	H-5 (5′), 7 (7′), 11 (11′)	-
7a (7a′)	35.8, CH_2	2.47, *m*	H-7b (7b′)	C-8 (8′)
b (b′)		2.64, *m*	H-7a (7a′)	C-5 (5′), 8 (8′)
8 (8′)	178.2, C	-	-	-
8a (8a′)	101.7, C	-	-	-
9 (9′)	186.5, CO	-	-	-
9a (9a′)	106.3, C	-	-	-
10a (10a′)	85.2, C	-	-	-
11 (11′)	17.7, CH_3	1.04, *d* (6.4)	H-6 (6′)	C-5 (5′), 6 (6′), 7 (7′)
12 (12′)	170.0, C	-	-	-
OCH_3-12	52.9, CH_3	3.61, *s*	-	C-12 (12′)
OH-1 (1′)	-	11.63, *brs*	-	C-1 (1′), 2 (2′), 9a (9a′)
OH-5 (5′)	-	6.05, *dd* (5.8, 2.4)	H-5 (5′)	C-5 (5′), 6 (6′)
OH-8 (8′)	-	13.67, *br*	-	-

4.1.7. Structure Eluidation of Tetracyclictriterpenoid: Helvolic Acid (NF9)

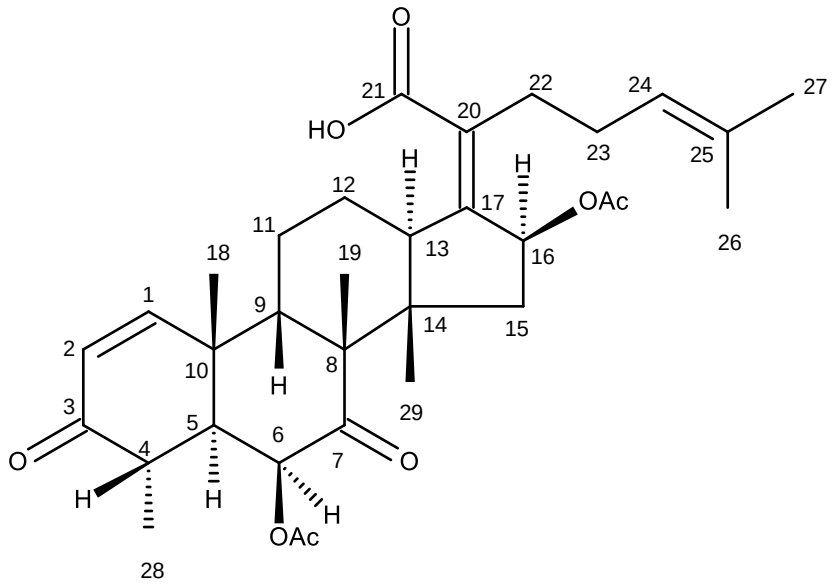


Figure 67. Structure of helvolic acid (**NF9**).

NF9 was isolated as white amorphous solid (mp. 201-203 ºC) and its molecular formula $C_{33}H_{44}O_8$ was based on the (+)-ASAP-HRESIMS *m/z* 509.2903 $[M+H]^+$ (calculated for $C_{33}H_{44}O_8$, 568.7070), indicating twelve degrees of unsaturation. The ^{13}C NMR, DEPTs and HSQC spectra (Table 12) indicated the presence of two conjugated ketone carbonyl (δ_C 201.4 and 173.8), one ketone carbonyl (δ_C 208.8), two carbonyl groups which belong to two acetate (δ_C 170.3 and 168.9), three quaternary sp^2 (δ_C 147.8, 132.9 and 130.6), three quaternary sp^3 (δ_C 52.7, 46.6 and 38.2), three methine sp^2 (δ_C 157.2, 127.8 and 122.8), six methine sp^3 (δ_C 73.8, 73.4, 49.4, 47.2, 41.7 and 40.4), five methylene sp^3 (δ_C 40.6, 28.6, 28.3, 25.9 and 23.9) and two methyl (δ_C 20.8 and 20.5) carbons, three secondary methyl (δ_C 25.8, 17.8 and 13.1) carbons and three tertiary methyl (δ_C 27.5, 18.3 and 17.9) carbons in total of 33 signals respectively.

The ^1H NMR spectrum, in conjunction with the HSQC spectrum (Table 12), showed the signals of three methine sp^2 proton at δ_H 7.31, *d* (*J* = 10.1 Hz; δ_C 157.2), δ_H 5.87, *d* (*J* = 10.0 Hz, δ_C 127.8) and δ_H 5.11, *dd* (*J* = 7.2, 7.2 Hz; δ_C 122.8), six methine sp^3 proton at δ_H 5.23, *s* (δ_C 73.8), δ_H 5.90, *d* (*J* = 8.5 Hz, δ_C 73.4), δ_H 2.58, *d* (*J* = 10.7 Hz, δ_C 49.4), δ_H 2.27, *d* (*J* = 11.6 Hz, δ_C 47.2), δ_H 2.62, *dd* (*J* = 13.2, 2.8 Hz, δ_C 41.7) and δ_H 2.78, *dq* (*J* = 6.8 Hz, δ_C 40.4), and two methyl at δ_H 2.12, *s* (δ_C 20.8) and δ_H 1.95, *s* (δ_C 20.5) carbons, three secondary methyl at δ_H 1.69, *s* (δ_C 25.8), δ_H 1.61, *s* (δ_C 17.8) and δ_H 1.28, *d* (*J* = 6.8 Hz, δ_C

13.1) carbons and three tertiary methyl at δ_H 1.45, *s* (δ_C 27.5), δ_H 1.18, *s* (δ_C 18.3) and δ_H 0.93, *s* (δ_C 17.9) respectively.

The COSY spectrum (Table 12) displayed the correlation of the double doublet of the olefinic proton at δ_H 5.11 (*J* = 7.2, 7.2 Hz; δ_C 122.8) to the two methyl singlets at δ_H 1.61 (δ_C 17.8) and δ_H 1.69 (δ_C 25.8), as well as to the multiplet at δ_H 2.11 (δ_C 28.3). In turn, the multiplet at δ_H 2.11 (δ_C 28.3) showed crosspeaks with the multiplet at δ_H 2.48 (δ_C 28.6). Consequently, there was a coupling system of:

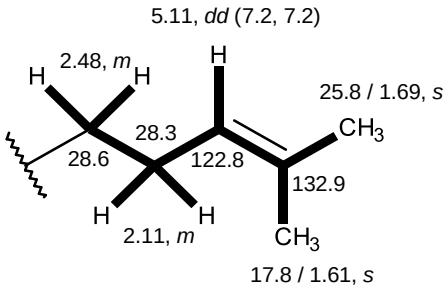


Figure 68. COSY (▬) correlations of the fragment from **NF9**.

The HMBC spactrum (Table 12) showed that the two methyl groups were correlated to each other and to the olefinic carbons at δ_C 122.8 and 132.9, from the multiplet at δ_H 2.48 (δ_C 28.6) to carboxyl carbon at δ_C 173.8 and δ_C 130.6, these correlations suggested the existence of the 6-methylnept-5-enoic acid.

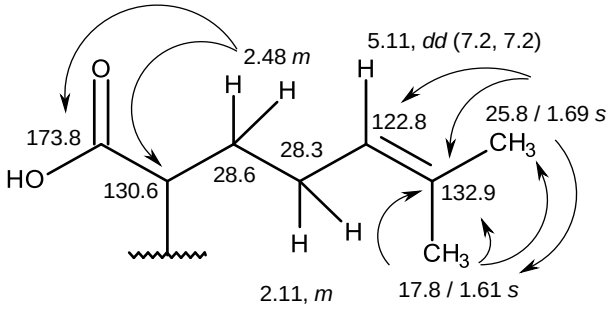


Figure 69. Key HMBC (⟶) correlations of the 6-methylnept-5-enoic acid side chain of **NF9**.

Another portion of the molecule, the COSY spectrum also showed the coupling of the two olefinic doublets at δ_H 5.87 (*J* = 10.0 Hz; δ_C 127.8) and δ_H 7.31 (*J* = 10.1 Hz; δ_C 157.2). The coupling constant of 10.0 Hz revealed that this double bond was in *cis* configuration (Figure 70). The COSY spectrum also showed that the methyl doublet at δ_H 1.28 (*J* = 6.8 Hz; δ_C 13.1) was correlated to the one portion at δ_H 2.78, *dq* (*J* = 6.8 Hz; δ_C 40.4) (Figure 70). In turn, the latter was correlated to the portion signal of double doublet at δ_H 2.27 (*J* = 11.6 Hz; δ_C 47.2). Moreover, the COSY spectrum also showed the coupling between the methine proton of double doublet at δ_H 2.27 (*J* = 11.6 Hz; δ_C 47.2) and the singlet at δ_H 5.23 (δ_C 73.8) (Figure 70). The HMBC spectrum revealed that the proton signal of double doublet at δ_H 2.27 (*J* = 11.6 Hz; δ_C 47.2) was correlated to the methine carbon at δ_C 40.4, the quaternary carbon at δ_C 38.2 and the methyl carbon at δ_C 27.5 (Figure 70). In turn, the signal of methyl protons of singlet at δ_H 1.45 (δ_C 27.5) gave cross peaks with the quaternary carbon at δ_C 38.2, the methine carbons at δ_C 41.7, 47.2 and 157.2 (Figure 70), respectively. The HMBC spectrum also showed that the olefinic proton of doublet at δ_H 7.31 (*J* = 10.1; δ_C 157.2) gave cross peaks with quaternary carbon at δ_C 38.2 and a methine carbon at δ_C 47.2 while the olefenic proton of doublet at δ_H 5.87 (*J* = 10.0; δ_C 127.8) gave a cross peak only to the quaternary carbon at δ_C 38.2. In turn, the methyl proton signal of singlet at δ_H 1.45 (δ_C 27.5) gave a cross peak with the olefenic carbon at δ_C 157.2 (Figure 70). Thus, this olefenic unit was connected to the quaternary carbon at δ_C 38.2 (Figure 70). The proton signal of double doublet at δ_H 2.27 (*J* = 11.6 Hz; δ_C 47.2) also gave cross peaks with the carbon signals at δ_C 157.2 and δ_C 201.4 while the methine proton signal at δ_H 2.78, *dq* (*J* = 6.8 Hz; δ_C 40.4) and the methyl proton signal at δ_H 1.28, *d* (*J* = 6.8 Hz; δ_C 13.1) also gave cross peaks to the carbonyl at δ_C 201.4 (Figure 70). This suggested that the carbonyl carbon at δ_C 201.4 should be connected to the methine carbon at δ_C 40.4 (Figure 70). Anyhow, there was no cross peak detected between the signal of the proton at δ_H 5.23, *s* and the carbonyl carbon at δ_C 201.4 (Figure 70). Basically, there was a strong correlation between the olefenic proton of double doublet at δ_H 7.31 (*J* = 10.1; δ_C 157.2) and the carbonyl carbon at δ_C 201.4. The proton signal of singlet at δ_H 5.23 also gave a cross peak with the carbonyl carbon at δ_C 168.9 and also has a cross peak with the quaternary carbon at δ_C 52.7. Moreover, one of the acetate groups was connected with this oxymethine carbon at δ_C 73.8 also in HMBC correlation (Figure 70). Moreover, the COSY spectrum displayed the coupling between the proton signal of double doublet at δ_H 2.62 (*J* = 13.2, 2.8 Hz; δ_C 41.7) and the proton signals of multiplet at δ_H 1.58 (δ_C 23.9) and δ_H 1.97 (δ_C 23.9) (Figure 70). In addition, the oxymethine proton signal of singlet at δ_H 5.23 (δ_C 73.8) also showed HMBC correlation to the ketone carbonyl at δ_C 208.8.

Furthermore, the HMBC spectrum revealed that the ketone carbonyl at δ_C 208.8 was correlated from the oxymethine proton of singlet at δ_H 5.23 (δ_C 73.8) and the methyl protons of singlet at δ_H 1.18 (δ_C 18.3) (Figure 71). Besides, the signal of these methyl protons at δ_H 1.18, *s* (δ_C 18.3) also exhibited cross peaks with the methine sp^3 carbon at δ_C 41.7 and the quaternary sp^3 carbons at δ_C 46.6 and 52.7. Moreover, the signal of the methyl protons at δ_H 0.93, *s* (δ_C 17.9) displayed crosspeaks not only with the quaternary carbons at δ_C 52.7 and δ_C 46.6, but also with the methine carbon at δ_C 49.4 and the methylene carbon at δ_C 40.6. Thus, this methyl group was on the quaternary carbon at δ_C 46.6 of cyclopentanoperhydro-phenanthrene moiety (Figure 71). In addition, the COSY spectrum also showed correlations between the proton signal at δ_H 2.42, *m* (δ_C 25.9) and δ_H 2.58, *d* (*J* = 10.7; δ_C 49.4), thus, the assignment of the ring was complete (Figure 70). In addition, the HMBC spectrum also showed that the proton signal of doublet at δ_H 1.91 (*J* = 14.9; δ_C 40.6), was correlated to the signal of another oxymethine carbon at δ_C 73.4, the quaternary sp^2 carbon at δ_C 147.4, besides the signals of methyl carbon at δ_C 17.9, methine sp^3 carbon at δ_C 49.4 and the quaternary sp^3 carbon at δ_C 46.6. Thus, the fourth ring of the compound was completed with the exocyclic double bond. Moreover, the proton signal of doublet at δ_H 5.90 (*J* = 8.5; δ_C 73.4) also gave crosspeak with the carbonyl carbon at δ_C 170.3, quaternary sp^2 carbon at δ_C 147.7 and the quaternary sp^3 carbon at δ_C 46.6 in the HMBC spectrum, one of the acetate group was thus oxymethine carbon (δ_C 73.4).

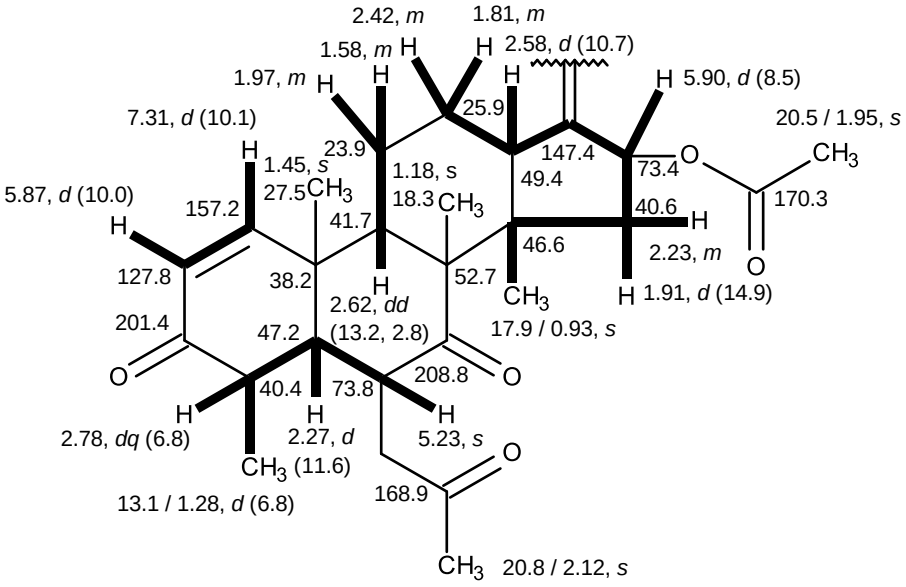


Figure 70. COSY (▬) correlations of the cyclopentanoperhydrophenanthrene moiety.

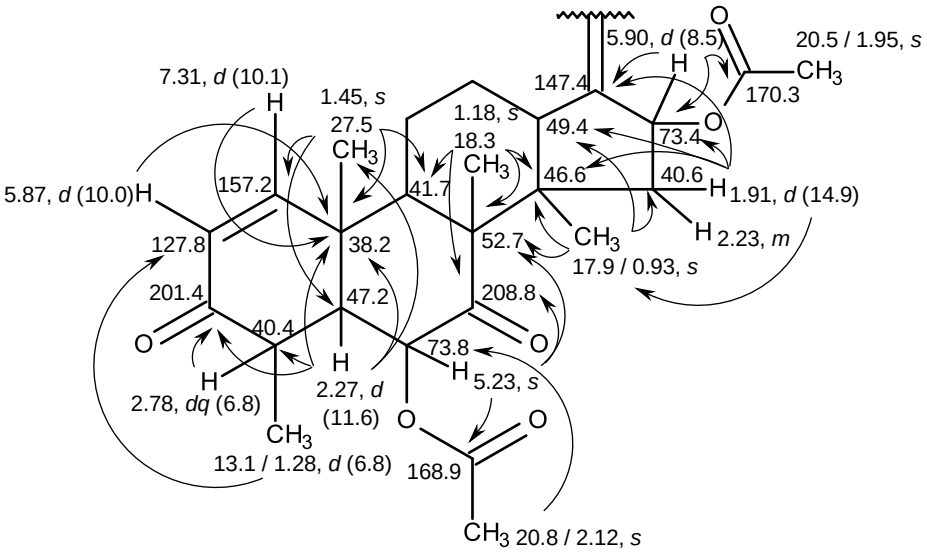


Figure 71. HMBC (⟶) correlations correlations of the cyclopentanoperhydrophenanthrene moiety.

The 6-methylnept-5-enoic acid and cyclopentanoperhydrophenanthrene moiety were connected through the quaternary sp^2 carbons at δ_C 130.6 of the former and δ_C 147.4 of the latter. This was supported by HMBC correlations from multiplet at δ_H 2.48 (δ_C 28.6) to δ_C 147.4, from the methine sp^2 proton of doublet at δ_H 2.58 (J = 10.7 Hz; δ_C 49.4) to δ_C 130.6 and also from the methine sp^3 proton at δ_H 5.90, *d* (J = 8.5 Hz; δ_C 73.4) to δ_C 130.6, respectively. Therefore, the complete structure of **NF9** was:

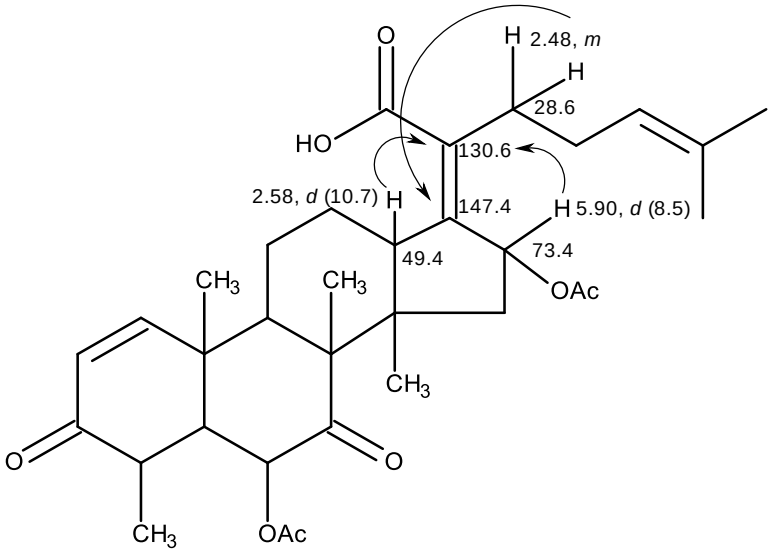


Figure 72. HMBC (⟶) correlations between cyclopentanohydrophenathrene moiety and 6-methylnept-5-enoic acid.

According to ^{1}H and ^{13}C NMR and comparison with previous reported literature from (Fulimoto *et al*, 1996), one could deduce that NMR spectrum of **NF9** corresponded to helvolic acid shown in Figure 73. The helvolic acid was already isolated from fungal endophyte, *Xylaria sp*. of orchid *Anoectochilus setaceus* which was collected from the Kanneliya forest reserve (6 09′–6 18′N & 80 19′–80 27′ E), Galle, Sri Lanka, in October 2011 and active against the Gram-positive bacteria, *Bacillus subtilis* with the MIC of 2 µg/mL and methicillin resistant *Staphylococcus aureus* with the MIC of 4 µg/mL and helvolic acid could be deduced as an antibacterial compound (Ratnaweera *et al*., 2014). Moreover, helvolic acid was already isolated from marine sponge-associated fungus *Emericellopsis minima* which was isolated from the marine sponge *Hyrtios erecta*, collected from the coral reef of the Similan Islands, Phag Nga Province, Thailand by scuba diving at 10 m depth, in April 2010 (Pinheiro *et al*., 2012). In addition, helvolic acid was recently isolated from the marine sponge-associated fungus *Neosartorya glabra* KUFA 0702 which was isolated from the marine sponge *Mycale sp*., and collected by scuba diving at a depth of 15–20 m, from the coral reef at Samaesarn Island (12°34′36.64″ N 100°56′59.69″ E) in the Gulf of Thailand, Chonburi Province, in February 2015 (Zin *et al*., 2016).

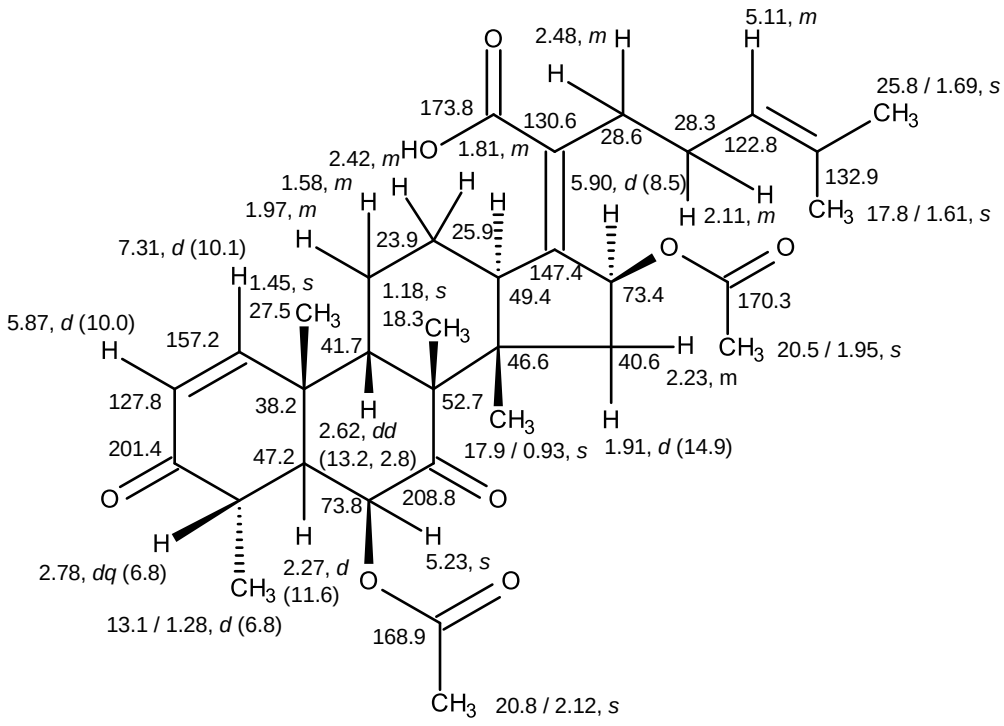


Figure 73. ^{1}H and ^{13}C assignments for **NF9**.

Table 12. ^{1}H and ^{13}C NMR ($CDCl_3$, 500.13 and 125.77 MHz) and HMBC assignment for **NF9**.

Position	δ_C, type	δ_H, (*J* in Hz)	COSY	HMBC
1	157.2, CH-1	7.31, *d* (10.1)	H-2	C-5, 6, 7, 9, 10
2	127.8, CH-2	5.87, *d* (10.0)	H-1	C-10
3	201.4, CO-3	-	-	-
4	40.4, CH	2.78, *dq* (6.8)	H-5, 28	C-3, 5, 6, 10, 28
5	47.2, CH	2.27, *d* (11.6)	H-4, 6	C-1, 3, 4, 7, 10, 18, 28
6	73.8, CH	5.23, *s*	H-5	C-5, 7, 8, 10, Ac-CO-6
7	208.8, CO-7	-	-	-
8	52.7, C-8	-	-	-
9	41.7, CH	2.62, *dd* (13.2, 2.8)	H-11a	C-1, 8, 10, 11, 18, 19
10	38.2, C	-	-	-
11a	23.9, CH_2	1.58, *m*	H-9, 11b	C-12, 18
b		1.97, *m*	H-9, 11a	C-12
12a	25.9, CH_2	1.81, *m*	H-12b, 13	C-12, Ac-CO-16
b		2.42, *m*	H-12a, 13	C-13
13	49.4, CH	2.58, *d* (10.7)	H-12a, 16	C-12, 14, 17, 20
14	46.6, C	-	-	-
15a	40.6, CH_2	1.91, *d* (14.9)	H-15b, 16	C-13, 14, 16, 17, 29
b		2.23, *m*	H-15a, 16, 29	C-8, 14, 29
16	73.4, CH-16	5.90, *d* (8.5)	-	C-14, 17, 20, Ac-CO-16
17	147.4, C	-	-	-
18	27.5, CH_3	1.45, *s*	-	C-1, 5, 9, 10
19	18.3, CH_3	1.18, *s*	-	C-7, 8, 9, 14
20	130.6, C	-	-	-
21	173.8, CO-21	-	-	-
22	28.6, CH_2	2.48, *m*	H-23	C-17, 20, 21, 23, 24
23	28.3, CH_2	2.11, *m*	H-22, 24	C-22, 24, 25
24	122.8, CH	5.11, *dd* (7.2, 7.2)	H-26, 27	C-22, 27
25	132.9, C	-	-	-
26	25.8, CH_3	1.69, *s*	H-24	C-23, 24, 25, 27
27	17.8, CH_3	1.61, *s*	H-24	C-24, 25, 26
28	13.1, CH_3	1.28, *d* (6.8)	H-4	C-3, 4, 5
29	17.9, CH_3	0.93, *s*	H-15	C-8, 13, 14, 15
Ac-CO-6	168.9, CO	-	-	-
	20.8, CH_3	2.12, *s*	-	Ac-CO-6
Ac-CO-16	170.3, CO	-	-	-
	20.5, CH_3	1.95, *s*	-	C-16, Ac-CO-16

4.1.8. Structure Elucidation of Diketopepiperazine, Fellutanine A (NF10)

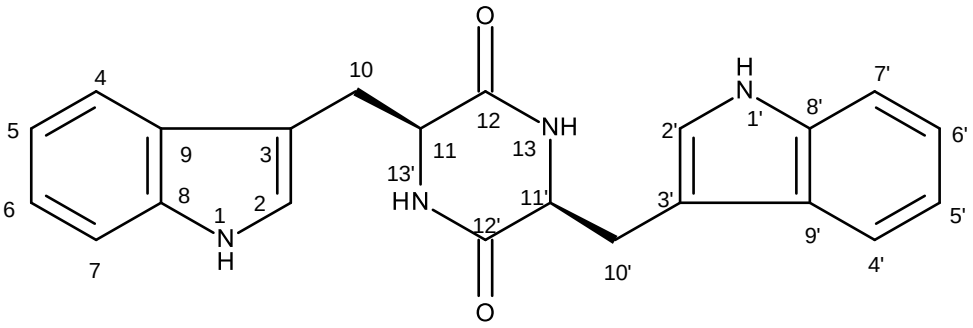


Figure 74. Structure of fellutanine A (**NF10**).

NF10 was isolated as a yellow viscous semi-solid (mp. 270−272 ºC) and its molecular formula $C_{22}H_{20}N_4O_2$ was determined based on the (+)-HRESIMS *m/z* 373.1675 $[M+H]^+$ (calculated for $C_{22}H_{21}N_4O_2$, 373.1665) and indicated fifteen degrees of unsaturation. The ^{13}C NMR, DEPTs and HSQC spectra (Table 13) indicated the presence one carbonyl (δ_C 166.8), three quaternary sp^2 (δ_C 136.1, 127.4 and 108.8), five methine sp^2 (δ_C 124.4, 120.8, 118.6, 118.4 and 111.3), one methine sp^3 (δ_C 55.3) and one methylene sp^3 (δ_C 30.0) and two methyl (δ_C 20.8 and 20.5) carbons, three secondary methyl (δ_C 25.8, 17.8 and 13.1) carbons in total of 11 signals respectively.

The ^1H NMR spectrum, in conjunction with HSQC spectrum (Table 13), displayed the signals of five methine sp^2 proton at δ_H 6.59, *d* (*J* = 2.2 Hz; δ_C 124.4), δ_H 7.04, *ddd* (*J* = 7.5, 7.5, 1.0 Hz; δ_C 120.8), δ_H 7.35, *d* (*J* = 7.9 Hz; δ_C 118.6), δ_H 6.95, *ddd* (*J* = 7.5, 7.5, 1.0 Hz; δ_C 118.4), δ_H 7.29, *d* (*J* = 8.0; δ_C 111.3), one methine sp^3 proton at δ_H 3.87, *q* (*J* = 3.8 Hz; δ_C 55.3) and two amine protons at δ_H 10.85, *brs* and δ_H 7.72, *d* (*J* = 2.5 Hz), respectively.

In COSY spectrum, the proton signal of methine sp^2 doublet at δ_H 7.35 (*J* = 7.9 Hz, H-4; δ_C 118.6) exhibited cross peak to double double doublet at δ_H 6.95 (*J* = 7.5, 7.5, 1.0 Hz, H-5; δ_C 118.4) and double double doublet at δ_H 6.95 (*J* = 7.5, 7.5, 1.0 Hz, H-5; δ_C 118.4), also showed cross peak to the proton signal of methine sp^2 double double doublet at δ_H 7.04, (*J* = 7.5, 7.5, 1.0 Hz, H-5; δ_C 120.8), the proton signal of methine sp^2 double double doublet at δ_H 7.04, (*J* = 7.5, 7.5, 1.0 Hz, H-5; δ_C 120.8) also displayed cross peak to methine sp^2 doublet at δ_H 7.29 (*J* = 8.0 Hz, δ_C 111.3), suggested the presence of 1, 2-disubstituted

ring. That 1,2-disubstitited benzene ring was linked to the indole ring, which was supported by the HMBC correlation of the methine sp^2 doublet at H-4, δ_H 7.35 (*J* = 7.9 Hz; δ_C 118.6) to the quaternary sp^2 carbon at δ_C 108.8 (C-3), δ_C 136.1 (C-8) and methine sp^2 carbon at δ_C 120.8 (C-6) and also from methine sp^2 double doublet at H-5, δ_H 6.95 (*J* = 7.5, 7.5, 1.0 Hz, H-5; δ_C 118.4) showed cross peak to quaternary sp^2 carbon at δ_C 127.4 (C-9) and methine sp^2 carbon at δ_C 111.3 (C-7), In addition, from methine sp^2 of double doublet at H-6, δ_H 7.04 (*J* = 7.5, 7.5, 1.0 Hz; δ_C 120.8) have cross peak to quaternary sp^2 carbon at δ_C 136.1 (C-8), methine sp^2 carbon at δ_C 118.6 (C-4) and δ_C 118.4 (C-5) and also from the methine sp^2 doublet at H-7, δ_H 7.29 (*J* = 8.0 Hz; δ_C 111.3) have cross peak to methylene sp^2 carbon at δ_C 118.4 (C-5) and quaternary sp^2 carbon at δ_C 127.4 (C-9) and broad singlet of amine proton at δ_H 10.85 (NH-1) exhibited HMBC cross peak to methine sp^2 carbon at δ_C 124.4 (C-2), quaternary sp^2 carbon at δ_C 108.8 (C-3), δ_C 136.1 (C-8) and δ_C 127.4 (C-9). Moreover, this was confirmed by HMBC correlations from doublet at H-2, δ_H 6.59 (*J* = 2.2; δ_C 124.4) to quarternary sp^2 carbon at δ_C 108.8 (C-3), quaternary sp^2 carbon at δ_C 136.1 (C-8) and δ_C 127.4 (C-9) respectively.

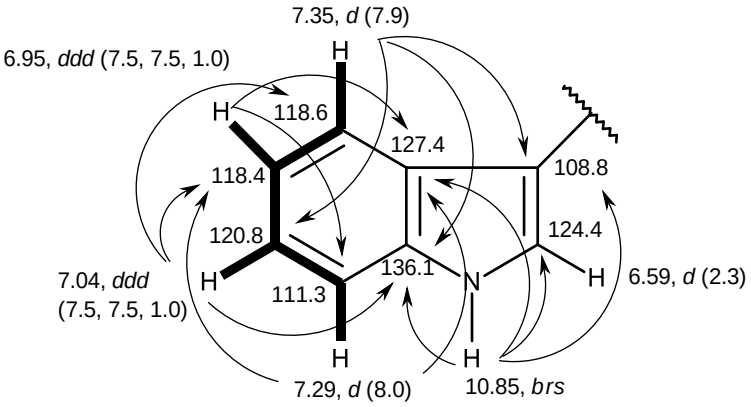


Figure 75. Key COSY (▬) and HMBC (⟶) correlations of 1,2 disubstituted 1*H*-indole ring.

The 1,2 disubstituted 1*H*-indole ring was linked to the diketopiperazine ring by the methylene bridge, which was corroborated by the HMBC cross peaks of the double doublet at δ_H 2.17 (*J* = 14.3, 6.7 Hz, H-10a; δ_C 30.0) and double doublet at δ_H 2.70 (*J* = 14.3, 4.2 Hz, H-10b; δ_C 30.0) to the methine sp^2 carbon at C-2 (δ_C 124.4), quaternary sp^2 carbon at C-3 (δ_C 108.8), C-9 (δ_C 127.4) and H-10a also to methine sp^3 carbon at C-11 (δ_C 55.3) and

H-10b also to carbonyl carbon at C-12 (δ_C 166.8). Moreover, methine sp^3 quartet at δ_H 3.87 (*J* = 3.8 Hz; δ_C 55.3) also displayed COSY correlations to double doublet at δ_H 2.17 (*J* = 14.3, 6.7 Hz, H-10a; δ_C 30.0), δ_H 2.70 (*J* = 14.3, 4.2 Hz, H-10b; δ_C 30.0) and doublet of amine proton at δ_H 7.72 (*J* = 2.5 Hz, NH-13). In addition, the doublet of amine proton at δ_H 7.72 (*J* = 2.5 Hz, NH-13) has COSY and HMBC cross peak to methine sp^3 carbon at C-11 (δ_C 55.3) and HMBC cross peak to carbonyl carbon at C-12 (δ_C 166.8). The above correlations suggested the presence of the fragment below (Figure 76).

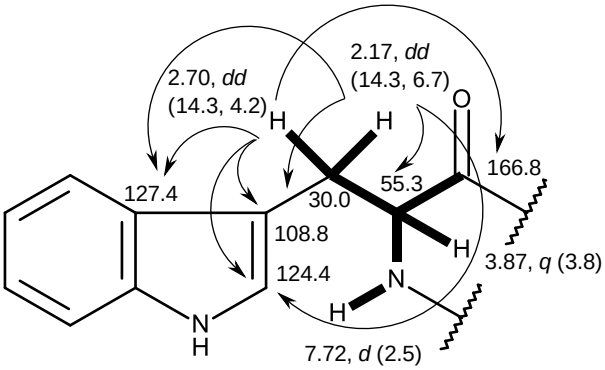


Figure 76. Key COSY (▬) and HMBC (⟶) correlations between 1,2 disubstituted 1*H*-indole ring and diketopiperazine ring.

Based on the molecular formula $C_{22}H_{20}N_4O_2$, determined based on the (+)-HRESIMS *m/z* 373.1675 $[M+H]^+$ (calculated for $C_{22}H_{21}N_4O_2$, 373.1665) which is double of the proposed structure established by the NMR data and this indicated that each carbon signal must correspond to two identical carbon atoms and the structure of **NF10** (Figure 77) must contain two identical units of fragment above. This was also confirmed by the comparision with the ^1H and ^{13}C NMR, COSY and HSQC spectra from previous reported fellutanine A, which was isolated from *Penicillum fellutanum* VKM F-3020 obtained from the All-Russian Culture Collection (VKM) Pushchino (Kozlovsky *et al*., 2000).

Fellutanine A was already isolated also from *Streptomyces* species KH29 which was isolated from a soil sample collected in Yesan, Korea and showed antimicrobial acitivity against *Candida albicans* IFO 6258, *Bacillus subtilis* IAM 1069, *Micrococcus luteus* JCM 1464, *Saccharomyces cerevisiae* IFO 1008, *Aspergillus niger* ATCC 9642, and *Staphylococcus aureus* TK 784 with the MICs of 50, 50, 12.5, 25, 25, and 0.8 µg/ml,

respectively (Lee *et al*., 2010). Moreover, fellutanine A was recently isolated from marine sponge-associated fungus *Neosartorya glabra* KUFA 0702 which was isolated from the marine sponge *Mycale sp*., and collected, by scuba diving at a depth of between 15 and 20 m, from the coral reef at Samaesarn Island (12° 34′ 36.64′′ N 100° 56′ 59.69′′ E) in the Gulf of Thailand, Chonburi Province, in February 2015 and tested for antibacterial activity against Gram-positive, *Escherichia coli* ATCC 25922 and Gram-negative, *Staphyllococus aureus* ATCC 25923 bacteria and antifungal activity against filamentous, *Aspergillus fumigatus* ATCC 46645, dermatophyte, *Trichophyton rubrum* ATCC FF5 and yeast, *Candida albicans* ATCC 10231 antibacterial activities with the MICs > 256 µg/mL and antifungal activities with the MIC > 512 µg/mL, respectively (Zin *et al*., 2016b).

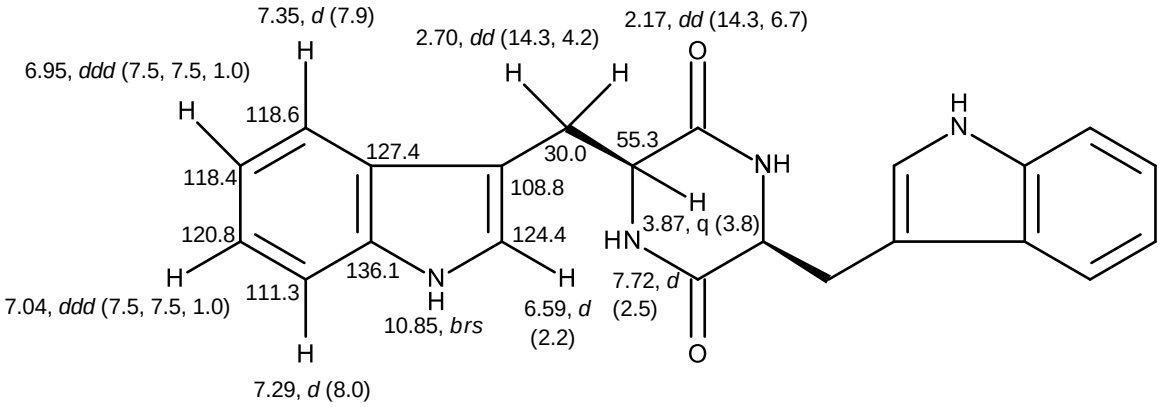


Figure 77. ^{1}H and ^{13}C assignments for **NF10**.

Table 13. ^{1}H and ^{13}C NMR (DMSO-d_6, 500.13 and 125.77 MHz) and HMBC assignment for **NF10**.

Position	δ_C, type	δ_H, (*J* in Hz)	COSY	HMBC
2	124.4, CH	6.59, *d* (2.2)	-	C-3, 8, 9
3	108.8, C		-	-
4	118.6, CH	7.35, *d* (7.9)	H-5	C-3, 6, 8
5	118.4, CH	6.95, *ddd* (7.5, 7.5, 1.0)	H-4, 6	C-7, 9
6	120.8, CH	7.04, *ddd* (7.5, 7.5, 1.0)	H-5, 7	C-4, 5, 8
7	111.3, CH	7.29, *d* (8.0)	-	C-5, 9
8	136.1, C	-	-	-
9	127.4, C	-	-	-
10a	30.0, CH_2	2.17, *dd* (14.3, 6.7)	-	C-2, 3, 9, 11
b		2.70, *dd* (14.3, 4.2)	-	C-2, 3, 9, 12
11	55.3, CH	3.87, *q* (3.8)	H-10a, 10b, NH-13	-
12	166.8, CO	-	-	-
NH-1	-	10.85, *brs*	-	C-2, 3, 8, 9
NH-13	-	7.72, *d* (2.5)	H-11	C-11, 12

CHAPTER V
CONCLUSION

5. Conclusion

The main goals of this study, ideally aimed to investigate the secondary metabolites produced by the fungus, *Neosartorya fennelliae* KUFA 0811, which was isolated from the marine sponge, *Clathria reinwardtii* collected from Samaesan Island, Amphur Sattahip, Chonburi province, Thailand in February 2015. The ethyl acetate extract of the culture of this fungus furnished β-sitostenone (**NF1**), egosta-4,6,8(14),22-tetraen-3-one (**NF2**), dehydromevalonic lactone (**NF3**), byssochlamic acid (**NF4**), cyathisterone (ergosta-7,22-diene-3,6-dione) (**NF5**), chevalone B (**NF6**), aszonalenin (**NF7**), secalonic acid A (**NF8**), helvolic acid (**NF9**) and fellutanine A (**NF10**). The structures of all the compounds isolated were established based on an extensive 1D and 2D NMR spectral, HRMS analysis, and by comparison of their NMR data to those reported in previous literature.

To the best of our knowledge, except for aszonalenin, this is the first report of β-sitostenone, ergosta-4,6,8(14),22-tetraen-3-one, dehydromevalonic lactone, byssochlamic acid, cyathisterone, chevalone B, aszonalenin, secalonic acid A, helvolic acid, fellutanine A from *N. fennelliae*. Consequently, study cannot contribute fully to the discovery of new chemical entities from natural sources which can have potential application for drug discovery, biochemical tools as well as some ecological roles.

Although some of these compounds have been used in cytotoxicity assay in previous reported literature (some were found to be active), it does not mean that they are void of other interesting biological or ecological activities. Even though the compounds isolated so far from this marine fungus, *N. fennelliae,* have different chemical scaffolds and different biological activities, it is possible to isolate other different types of compounds by using other techniques of culture such as OSMAC or co-culture with bacteria or other fungi. Anyhow, this thesis is another manifest to exhibit a richness of marine fungi and their critical role in drug discovery as marine drug potential.

According to the results presented in this study and literature backgrounds, we can conclude that the marine environment is a reservoir of natural products and critical source of bioactive metabolites, which are extremely invaluable for the development of the drug industry. In addition, this can assume that marine fungi will no longer be an underrepresented resource in future and will take part in one of the most prolific sources of drug discovery. Therefore, isolation of secondary metabolites from marine-derived fungus, *Neosartorya fennelliae*, which is the main objective of this thesis, is the pivotal and an additional enrichment to the marine science knowledge, which is the principal goal of the Master program in marine sciences-marine resources.

CHAPTER VI
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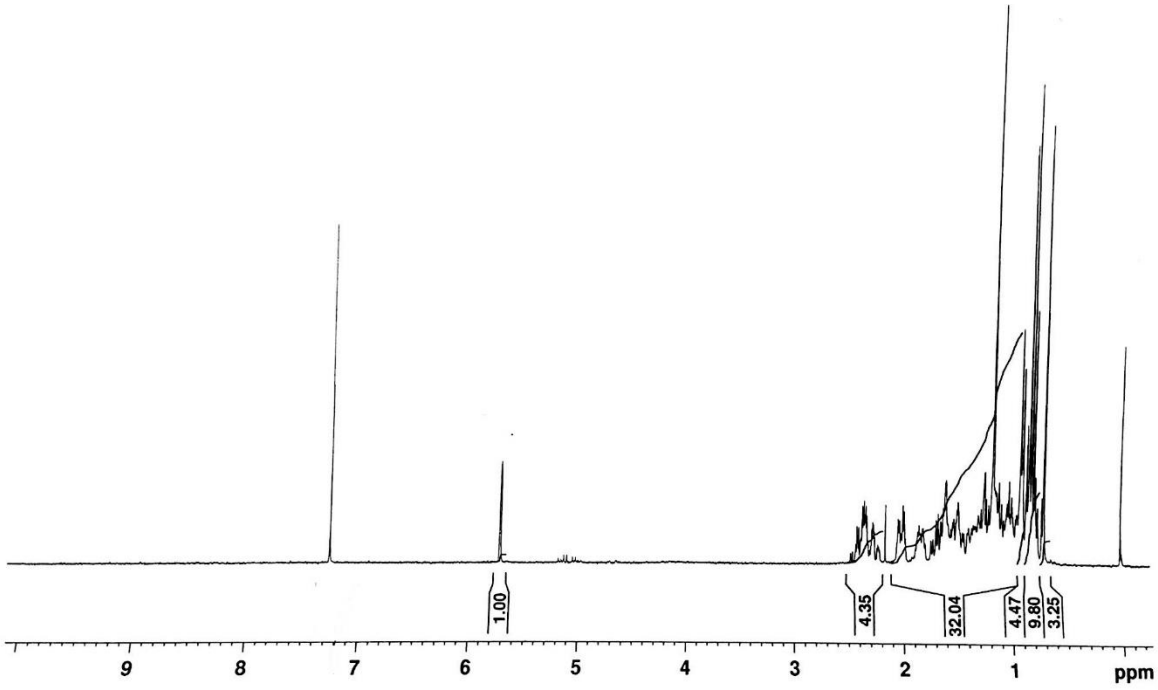
CHAPTER VII
APPENDICES

7. Appendices

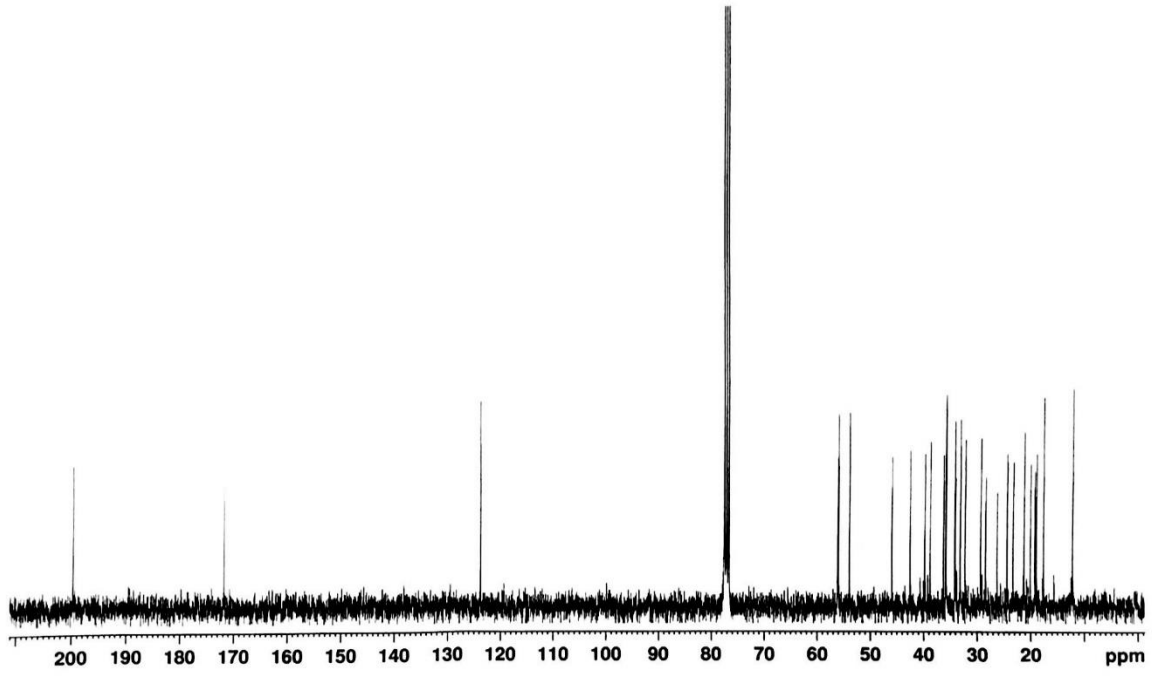
7.1. NMR Spectra of Isolated Compounds from *Neosartorya fennelliae*

7.1.1. NMR Spectrum of β-sitostenone (NF1)

^{1}H NMR Spectrum (300.13 MHz, $CDCl_3$)

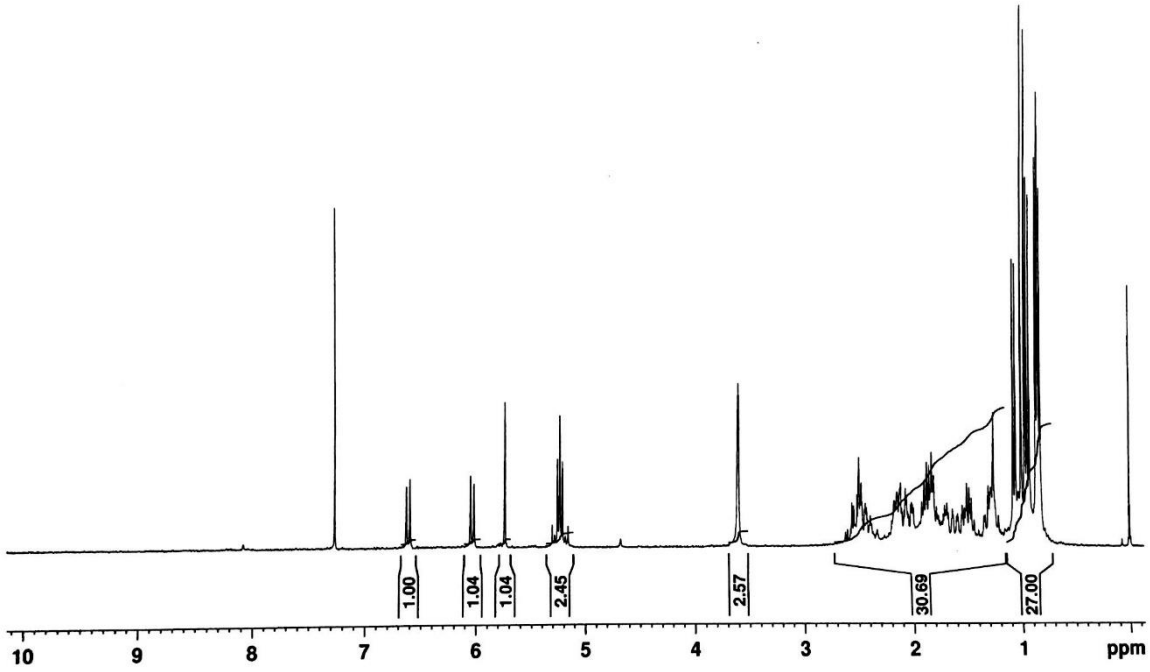


^{13}C NMR Spectrum (75.47 MHz, $CDCl_3$)



7.1.2. NMR Spectrum of Ergosta-4,6,8(14),22-tetraen-3-one (NF2)

^{1}H NMR Spectrum (300.13 MHz, $CDCl_3$)



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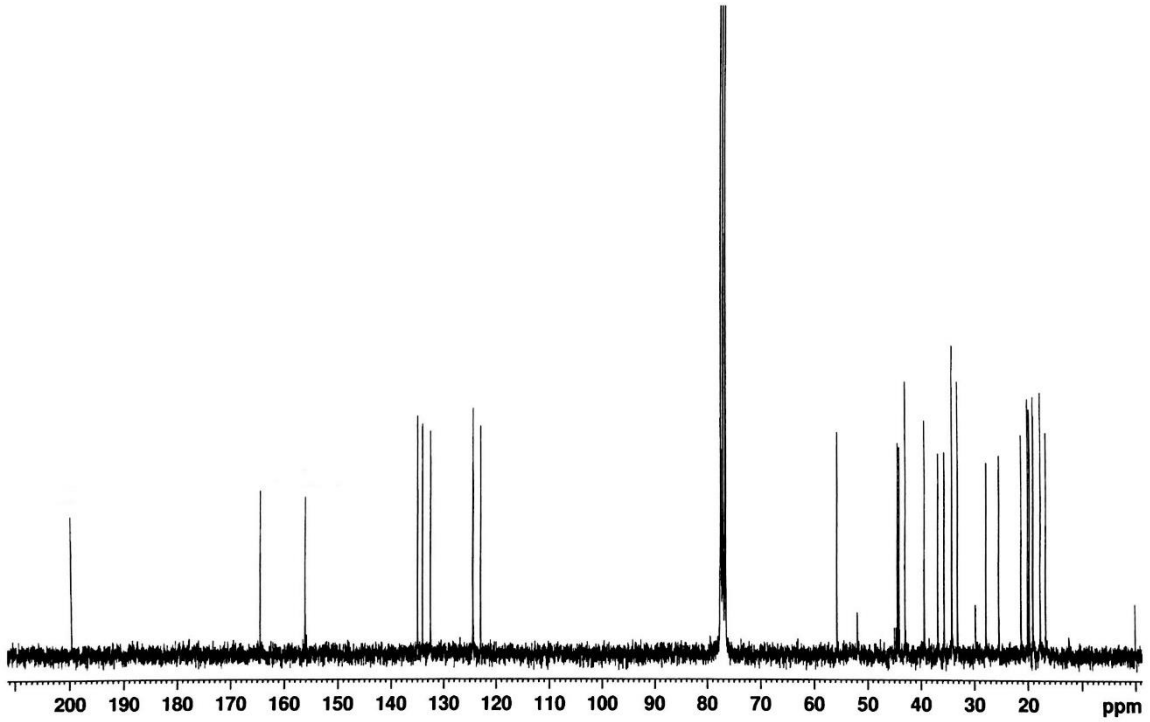
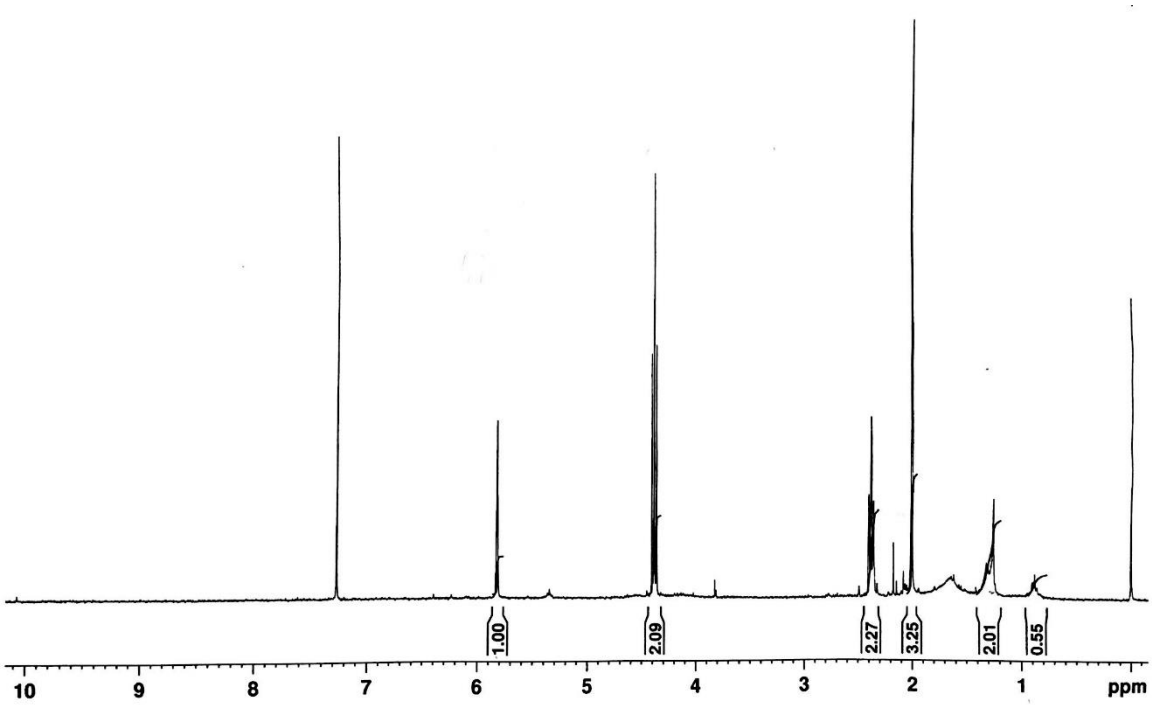


7.1.3. NMR Spectrum of Dehydromevalonic Lactone (NF3)

^{1}H NMR Spectrum (300.13 MHz, $CDCl_3$)



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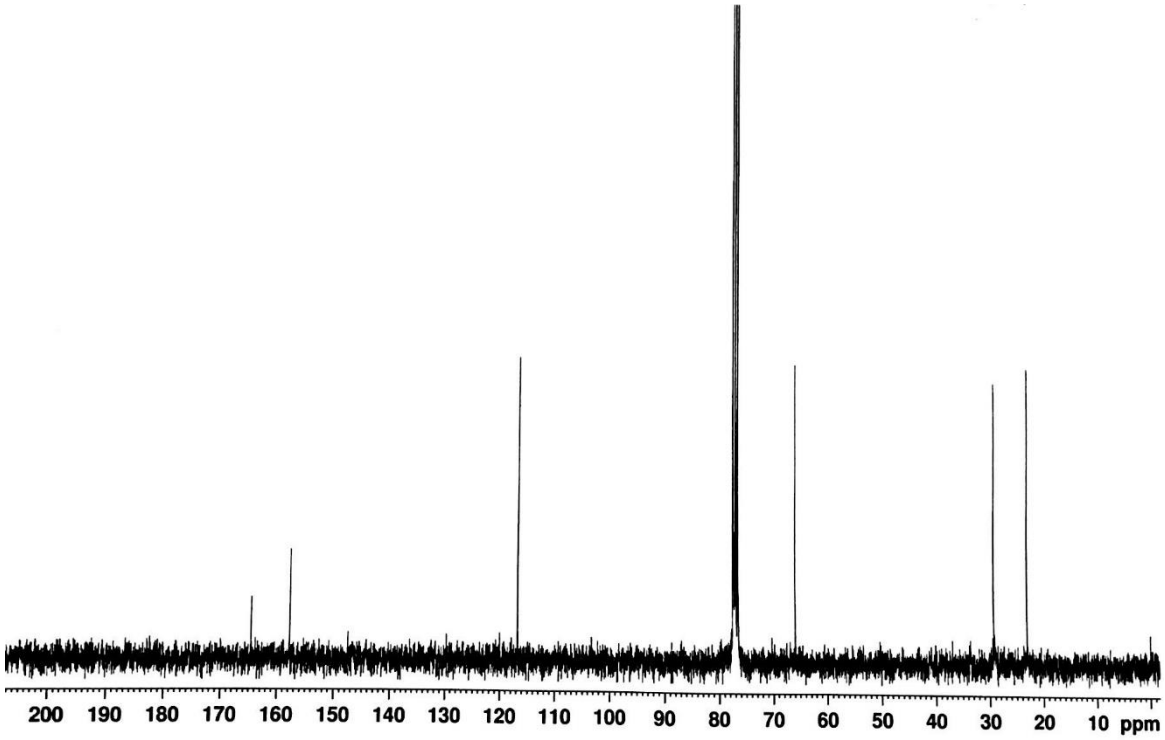
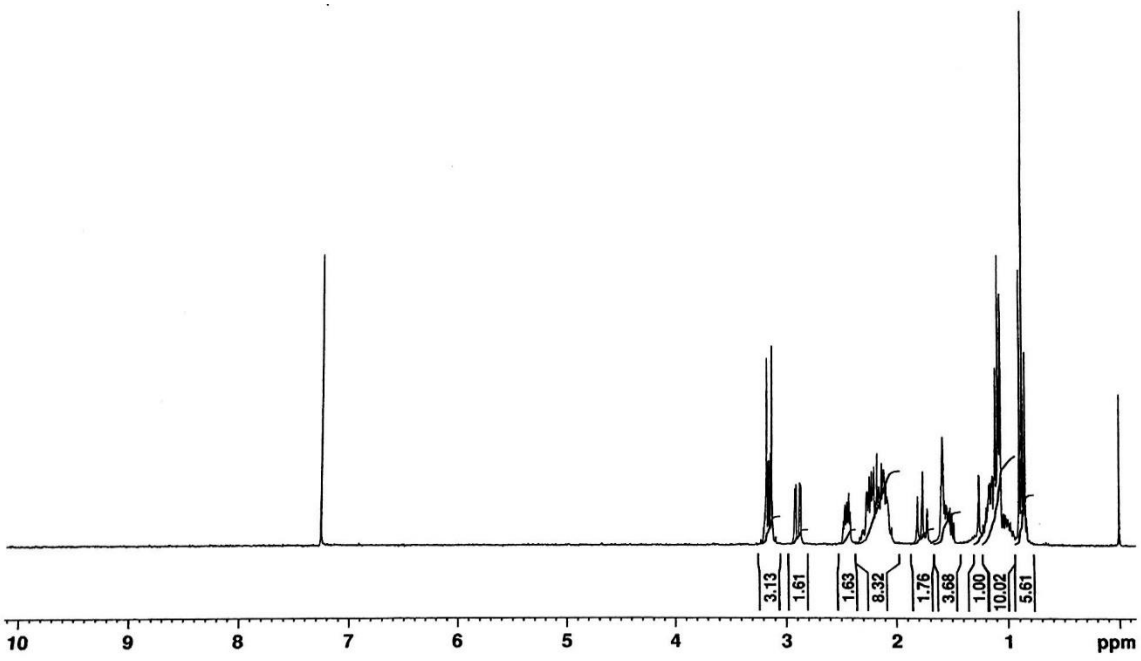
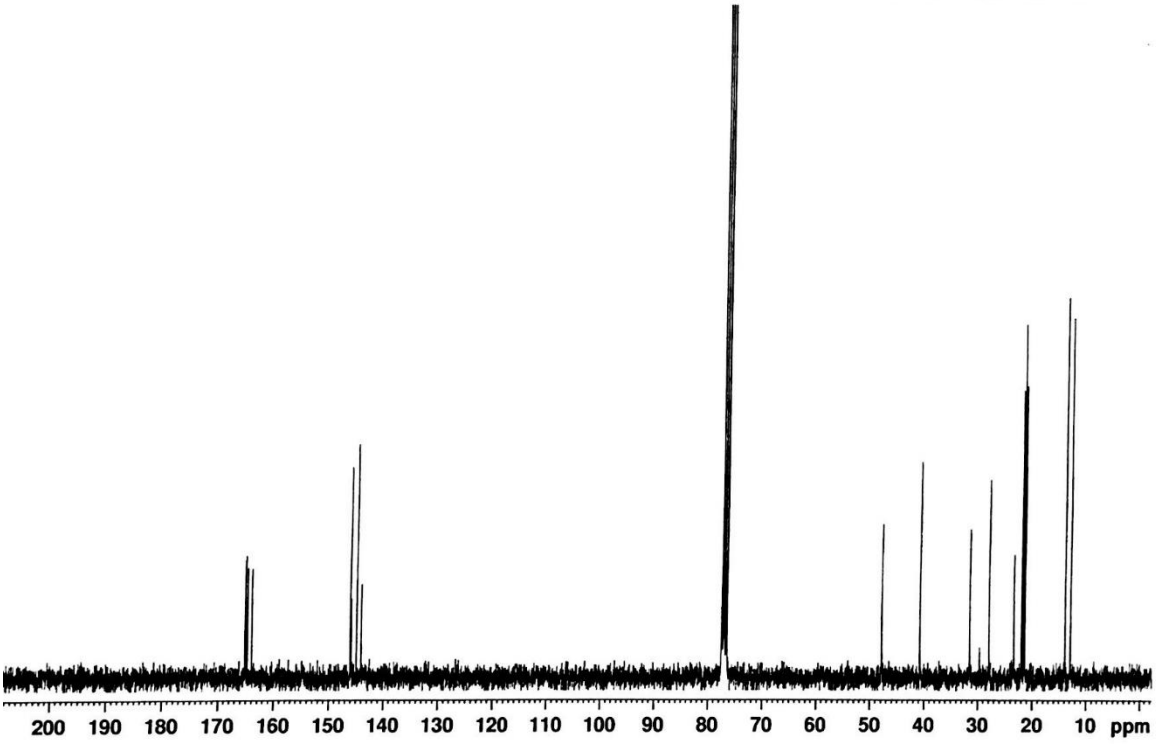


7.1.4 NMR Spectrum of Byssochlamic Acid (NF4)

^{1}H NMR Spectrum (300.13 MHz, $CDCl_3$)

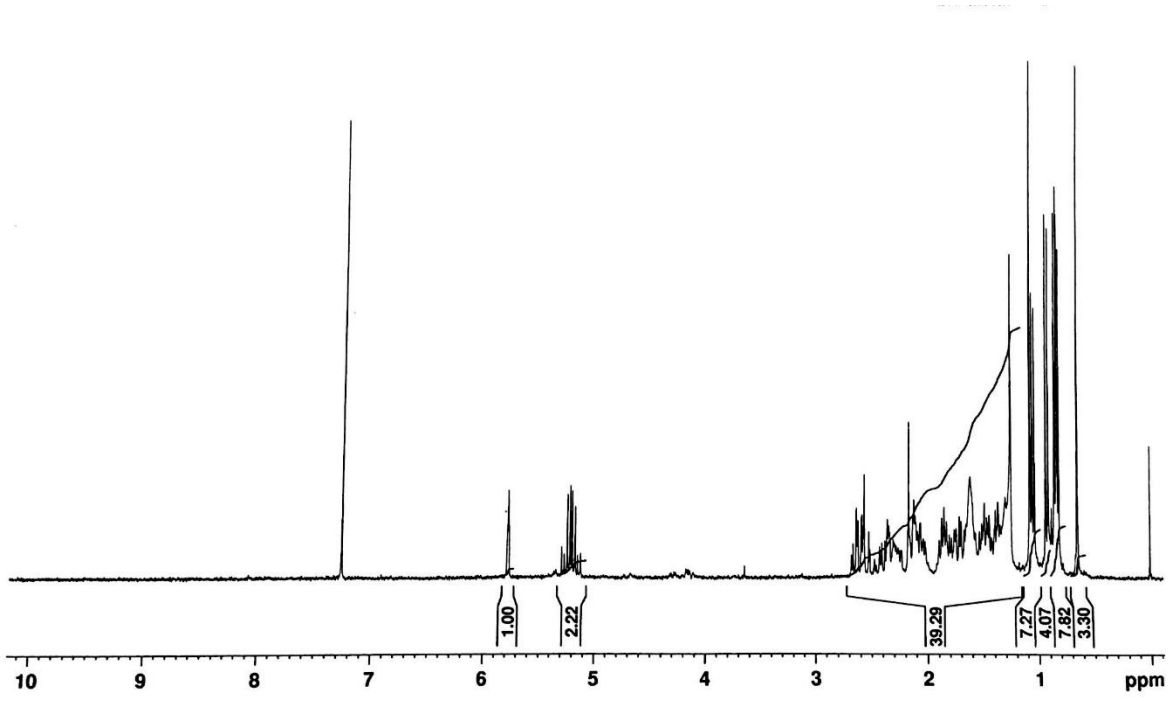


^{13}C NMR Spectrum (75.47 MHz, $CDCl_3$)

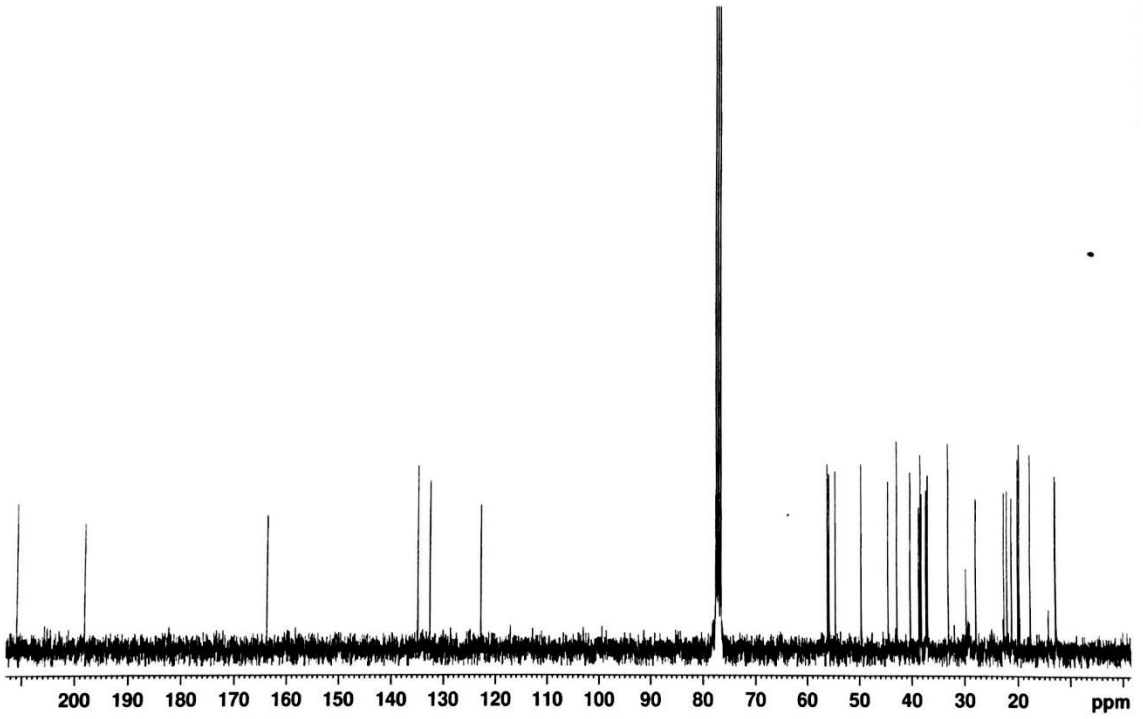


7.1.5 NMR Spectrum of Cyathisterone (NF5)

^{1}H NMR Spectrum (300.13 MHz, $CDCl_3$)

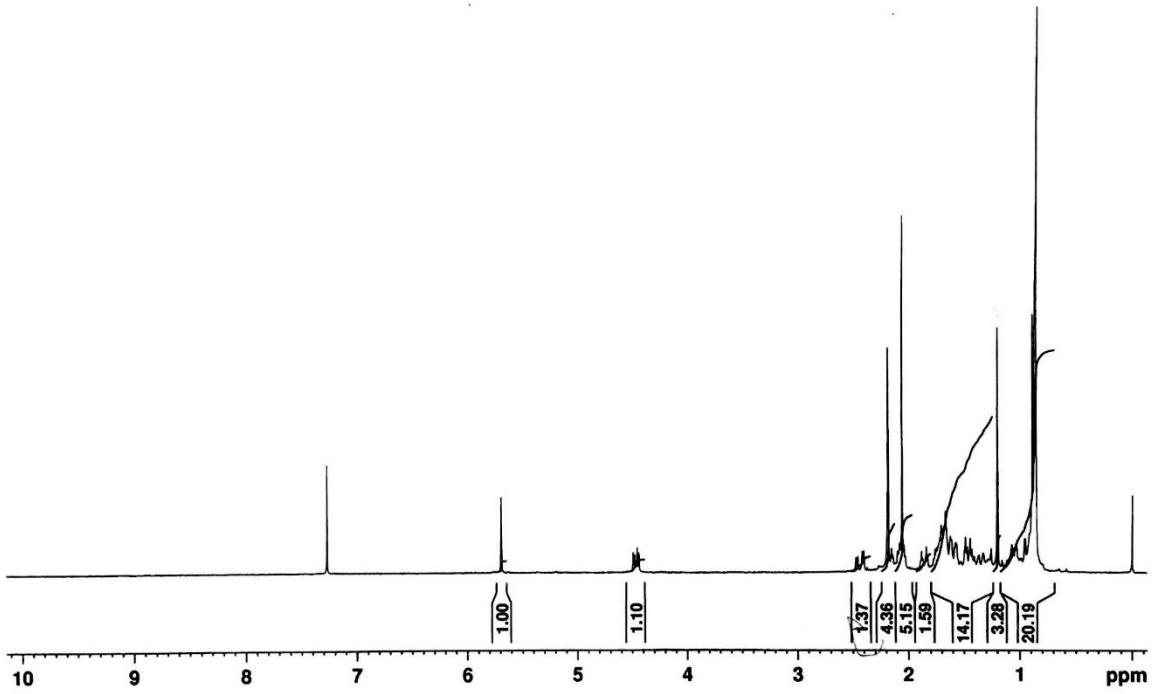


^{13}C NMR Spectrum (75.47 MHz, $CDCl_3$)

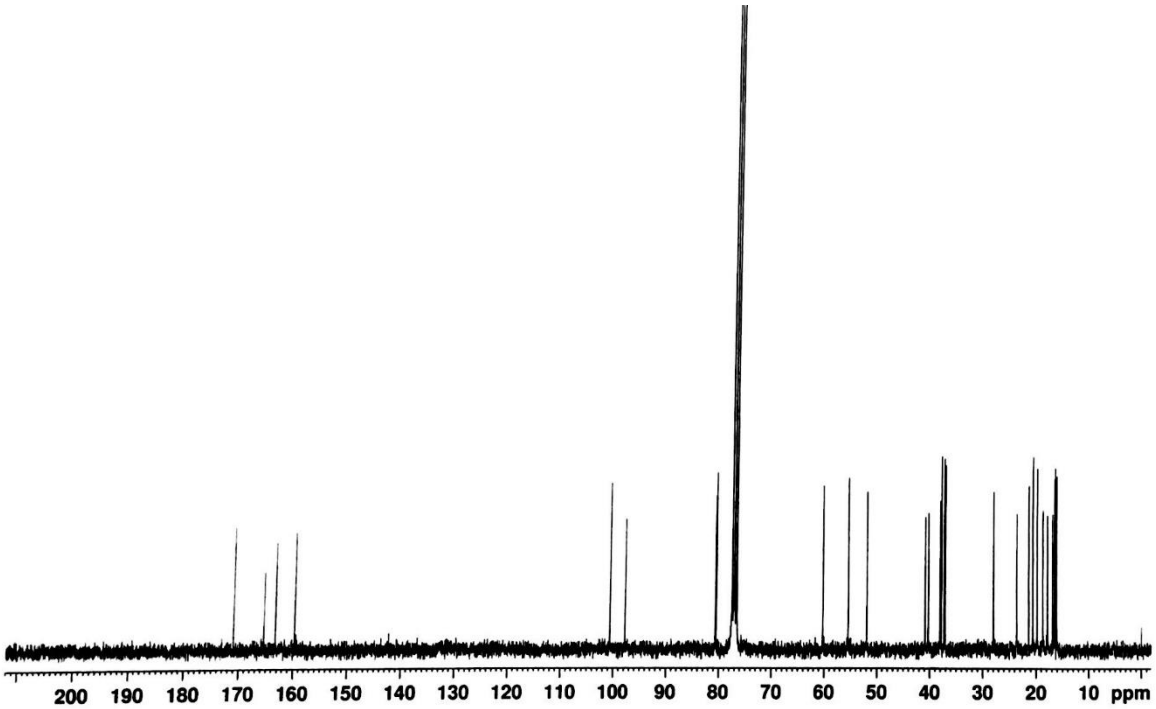


7.1.6 NMR Spectrum of Chevalone B (NF6)

^{1}H NMR Spectrum (300.13 MHz, $CDCl_3$)

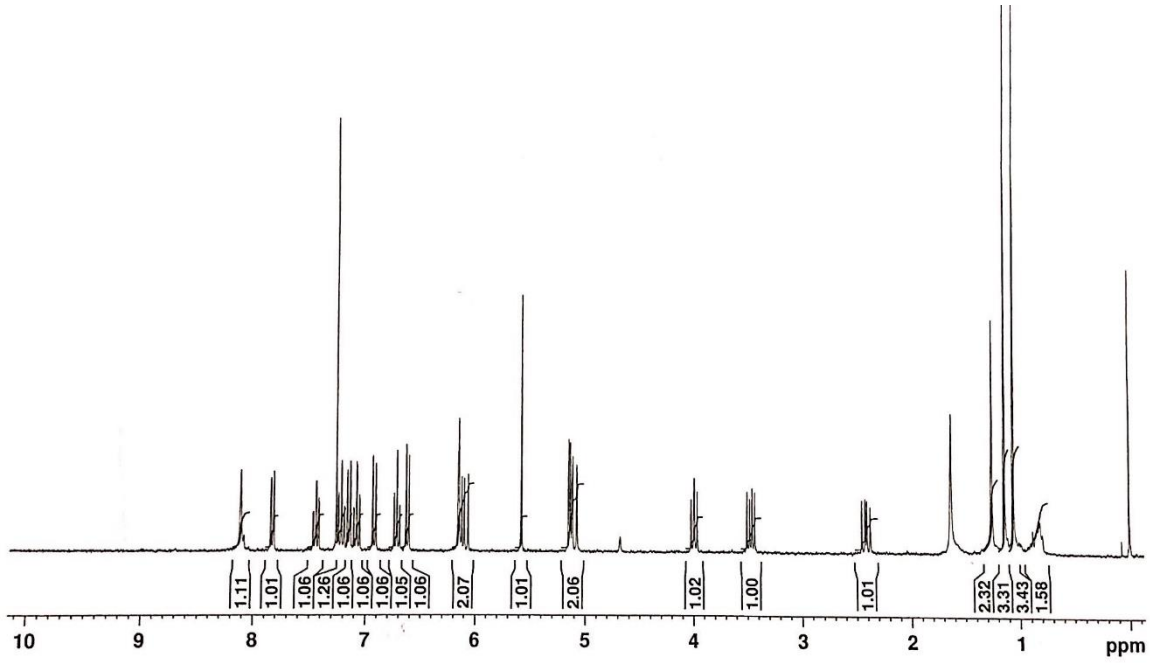


^{13}C NMR Spectrum (75.47 MHz, $CDCl_3$)

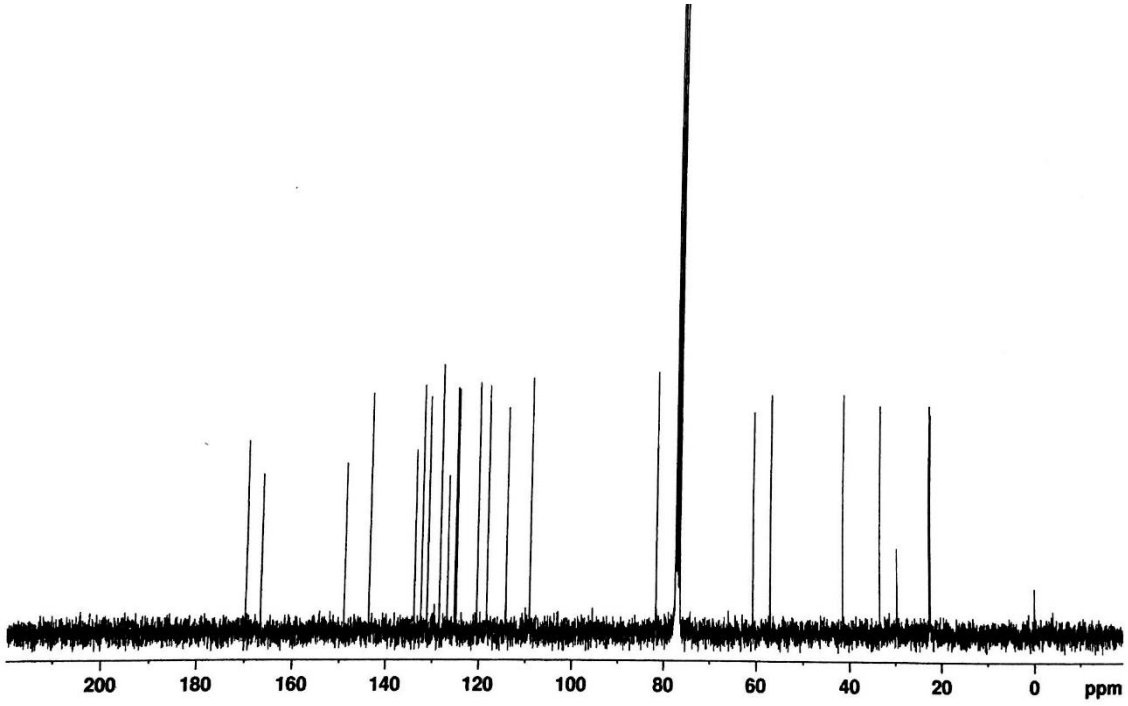


7.1.7 NMR Spectrum of Aszonalenin (NF7)

^{1}H NMR Spectrum (300.13 MHz, $CDCl_3$)

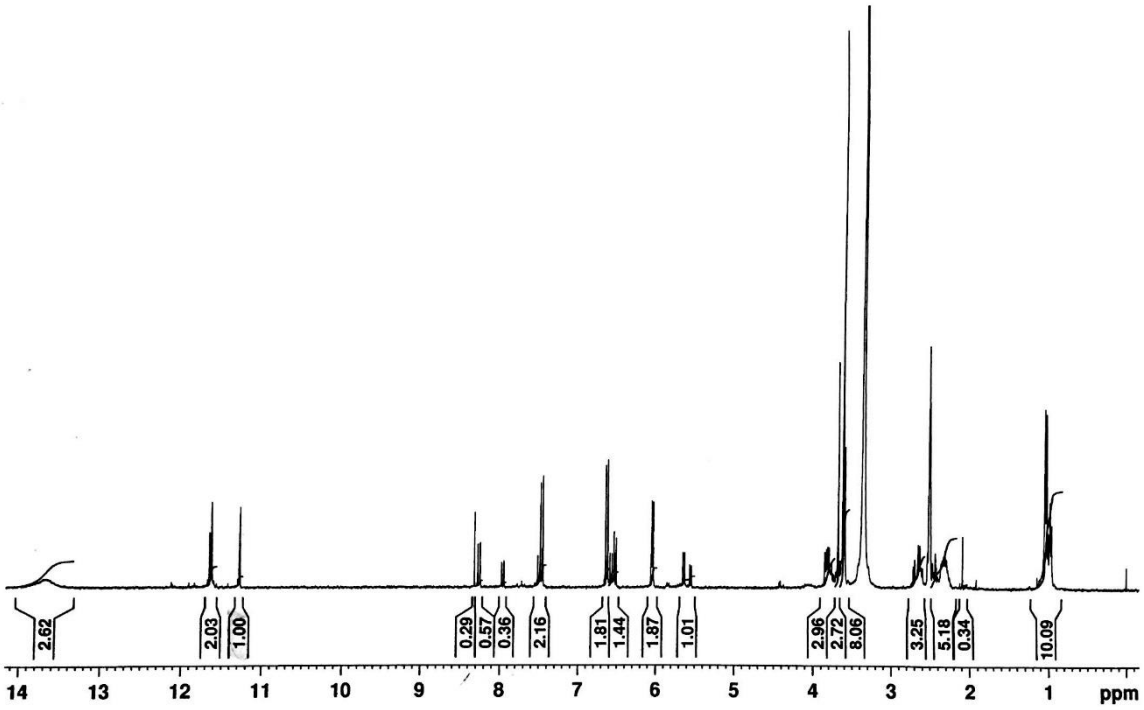


^{13}C NMR Spectrum (75.47 MHz, $CDCl_3$)

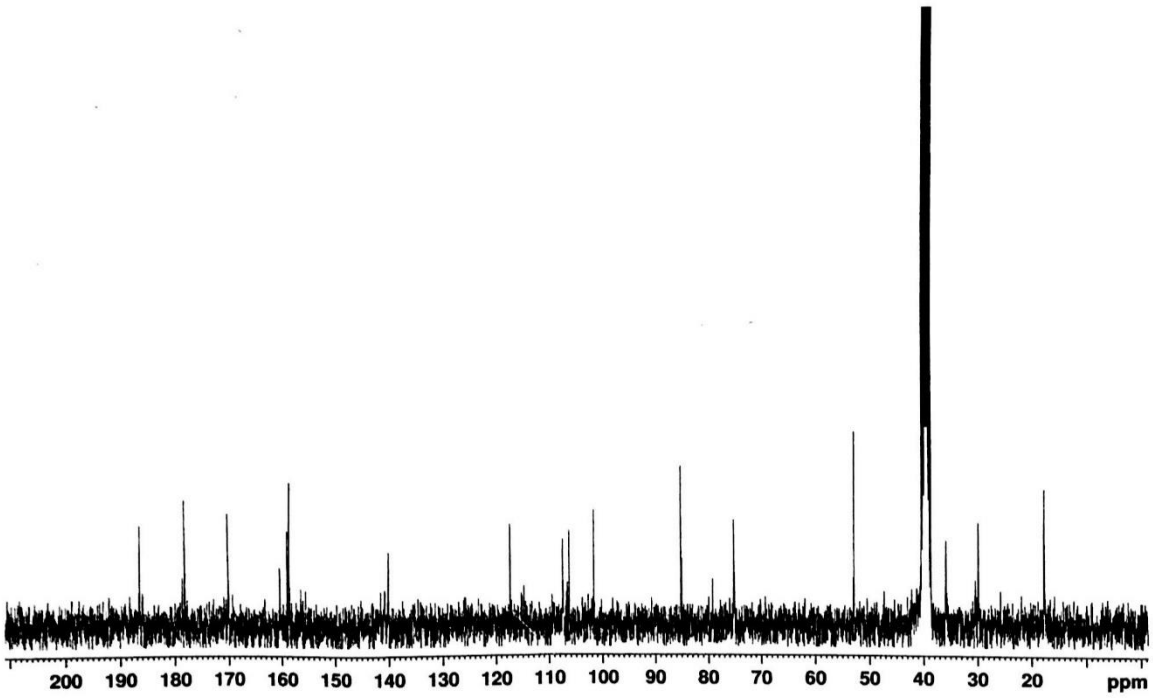


7.1.8 NMR Spectrum of Secalonic Acid A (NF8)

^{1}H NMR Spectrum (300.13 MHz, DMSO-d_6)

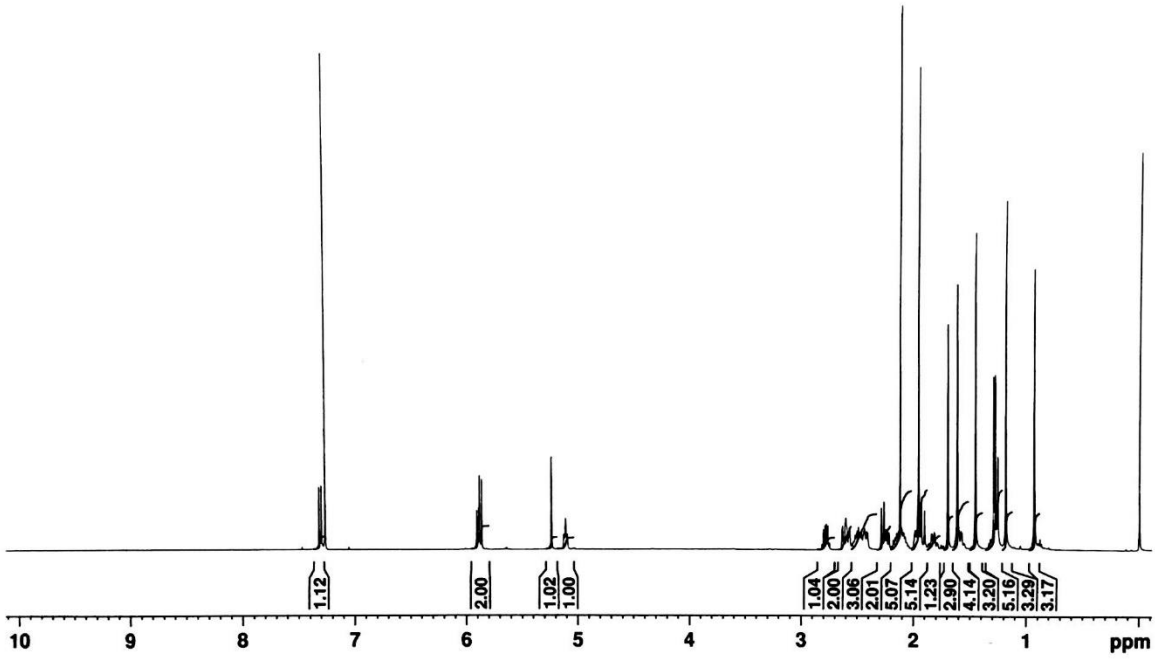


^{13}C NMR Spectrum (75.47 MHz, DMSO-d_6)

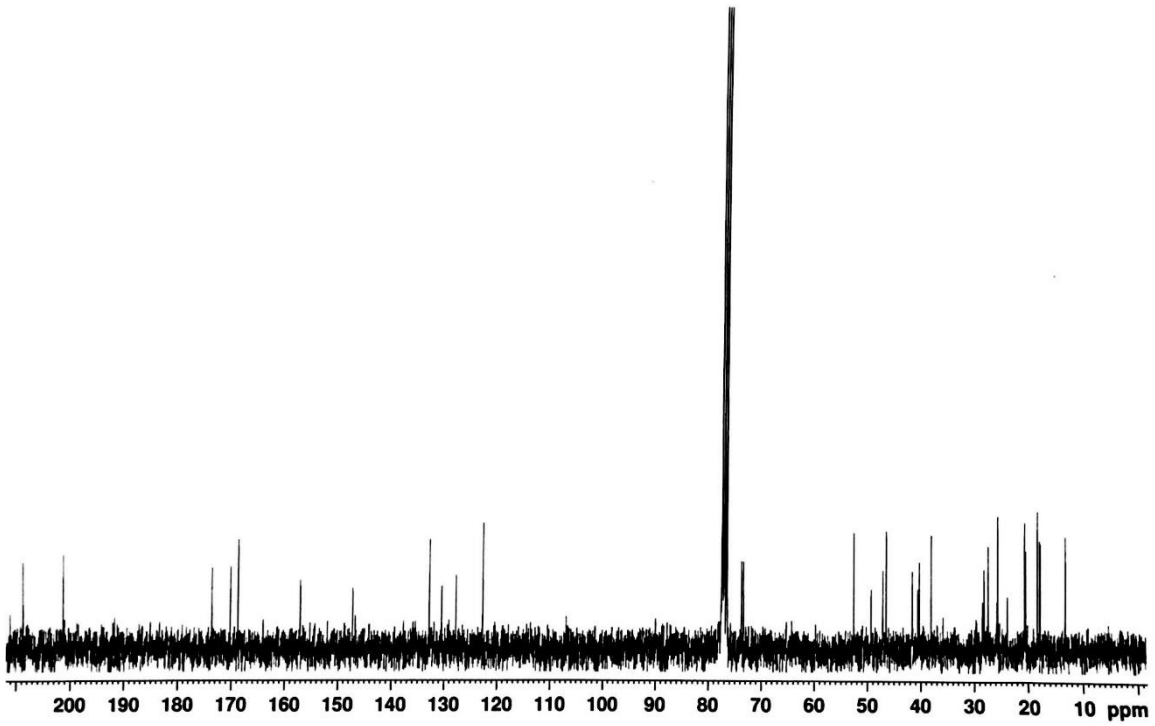


7.1.9 NMR Spectrum of Helvolic Acid (NF9)

^{1}H NMR Spectrum (500.13 MHz, $CDCl_3$)

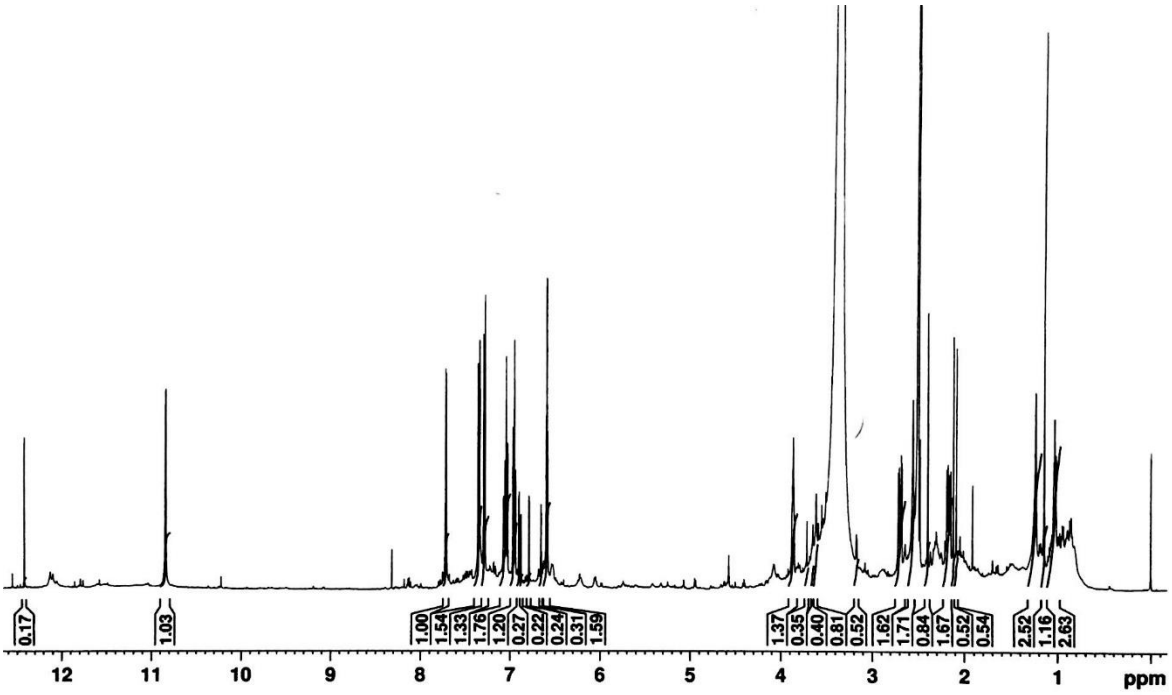


^{13}C NMR Spectrum (125.77 MHz, $CDCl_3$)

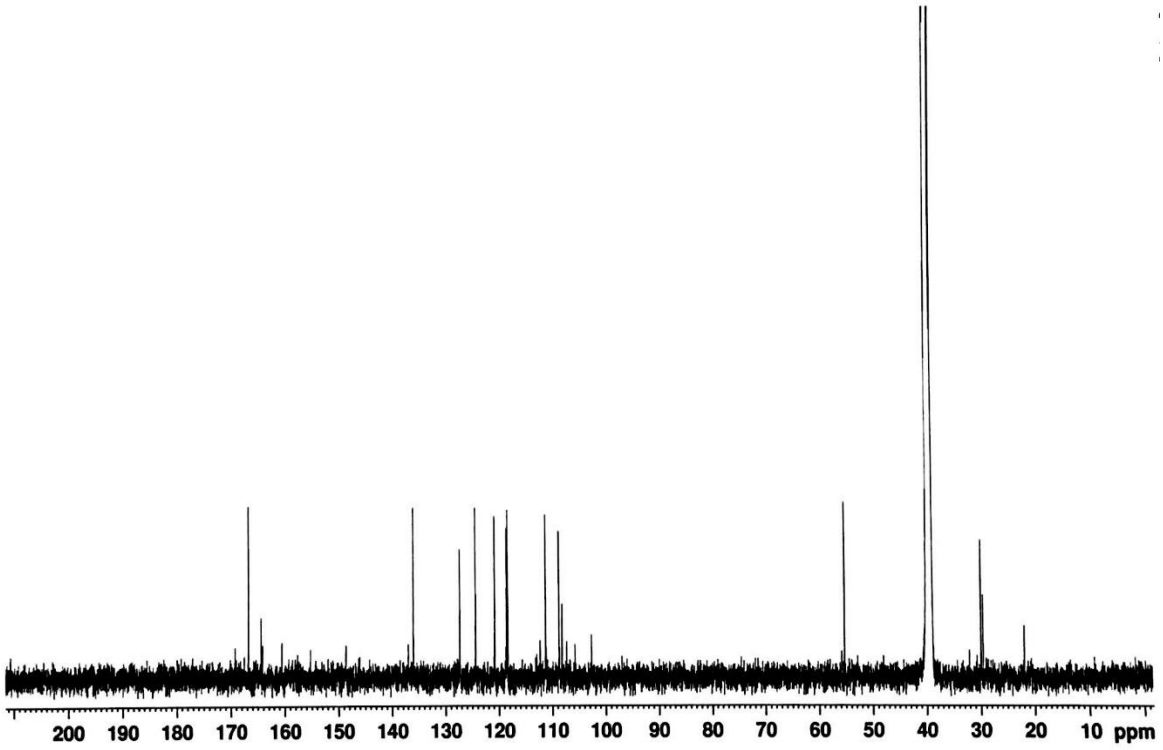


7.1.10 NMR Spectrum of Fellutanine A (NF10)

^{1}H NMR Spectrum (500.13 MHz, DMSO-d_6)



^{13}C NMR Spectrum (125.77 MHz, DMSO-d_6)



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