Biologically Active Secondary Metabolites Investigation of Marine Organism and Microorganisms

Dissertation towards a MPhil Degree
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Submitted by
Cheng Cheng
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Declaration

This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

Signed: 

Date: 23.10.2013

Praise the Lord

Abstract

Secondary metabolites from the marine sponge and an associated marine microorganism were investigated using state of the art high resolution mass spectrometry and nuclear magnetic resonance spectroscopy to conduct metabolomic dereplication and structure elucidation of biologically active metabolites.

The isolation and purification of bioactive compounds from the marine sponge *Callyspongia aff. implexa* was undertaken using a bioassay-guided purification scheme. Structure elucidation of the purified compounds was accomplished through spectroscopic and spectromitric methods including NMR and MS. Dereplication was conducted on the bioactive fractions corroborating the structures of previously isolated compounds. Three compounds were isolated; namely β-sitosterol, A37-2-B1, a 3, 7-hyroxyl-sterol with an acetylene side chain as well as A45-3-3, an polyacetylene which was also proven to be present in the bioactive fractions.

Metabolomics tools were applied to dereplicate extracts of the marine-associated bacterium *Actinokineospora* EG49 which led to the isolation of an anti-trypanosomal quinone, which resembles angucycline metabolites. Structure elucidation of quinone metabolites, namely EG49D and EG49F4B, from *Actinokineospora* EG49 was conducted. Supervised Orthogonal Partial Least Square-Discriminant Analysis (OPLS-DA) was used to choose the best culture method to optimise the production of the bioactive metabolite.

The production of quorum sensing (QS) signalling molecules, Acyl Homoserine Lactones (AHLs) by a panel of Gram-negative bioluminescent bacteria was investigated using high resolution tandem mass spectrometry. This was used to complement and validate results obtained from a traditional TLC screening approach in which the long acyl-chain AHLs are difficult to visualise. Preliminarily investigation of the possible relationships between the quorum sensing signals and cryptic secondary metabolites was also carried out. A group of "candidate" compounds that may be involved in the QS regulation process was created using supervised Orthogonal Partial Least Square-Discriminant Analysis for use in further investigations for antibiotics.

Contents

Acknowledgement

I would like to express my deepest gratitude to all those who helped me to make this thesis possible.

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Lastly, I would like to offer my best regards and blessings to all of those who supported me in any respect during the completion of the project.

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List of Laboratory chemicals

Solvent	Purchase from
Acetone	Sigma-Aldrich Chemical Co. (St. Louis, MO, USA)
n-Hexane	Sigma-Aldrich Chemical Co. (St. Louis, MO, USA)
Ethyl acetate	Sigma-Aldrich Chemical Co. (St. Louis, MO, USA)
Methanol	VWR International Ltd (Leighton Buzzard, UK)
Dichloromethane	Fisher Scientific UK Ltd (Leics, Loughborough, UK)
Dimethyl sulfoxide	Sigma-Aldrich Chemical Co. (St. Louis, MO, USA)
Isopropanol	Sigma-Aldrich Chemical Co. (St. Louis, MO, USA)
Deuterated chloroform	Sigma-Aldrich Chemical Co. (St. Louis, MO, USA)
Deuterated dimethyl sulfoxide	Sigma-Aldrich Chemical Co. (St. Louis, MO, USA)
Glacial acetic acid	Fisher Scientific UK Ltd (Leics, Loughborough, UK)
Concentrated sulphuric acid	Fisher Scientific UK Ltd (Leics, Loughborough, UK)
Anisaldehyde	Fisher Scientific UK Ltd (Leics, Loughborough, UK)

Abbreviations

Thin Layer Chromatography-TLC

Medium Pressure Liquid Chromatography-MPLC

High Pressure Liquid Chromatography-HPLC

Nuclear Magnetic Resonance-NMR

Mass Spectrometry-MS

Electrospray Ionisation-ESI

Higher-energy Collisional Dissociation-HCD

High Resolution Fourier Transform Mass Spectrometry-HRFTMS

Dictionary of Natural Products-DNP

Dichloromethane-DCM

Methanol-MeOH

Ethyl Acetate-EtOAc

Formic Acid-FA

Dimethyl sulfoxide-DMSO

Correlation Spectroscopy-COSY

Distortionless Enhancement by Polarization Transfer-DEPT

Heteronuclear Multiple Bond Correlation -HMBC

Heteronuclear Multiple Quantum Correlation-HMQC

Total Proton Correlation Spectroscopy-TOCSY

Acyl-homoserine Lactones-AHLs

Hexanoyl homoserine lactone-6HHL

3-oxohexanoyl homoserine lactone-6OHHL

3-oxo-Heptanoyl-homoserine lactone-7OHHL

Octanoyl homoserine lactone-OHL

3-oxoctanoyl homoserine lactone-OOHL

N-Decanoyl homoserine lactone-DHL

N-Decanoyl homoserine lactone-DHL

Tetradecanoyl homoserine lactone-THL

Principal component analysis-PCA

Orthogonal partial least squares-Discriminant Analysis-OPLS-DA

Chapter 1 Introduction

Antibiotic resistance is resistance of pathogenic bacteria to an antibiotic to which it was previously responsive. The resistant pathogenic bacteria often cause prolonged illness and even higher risk of death. In the past 20 years, antibiotic resistance has become increasingly perceived as a global health threat and is a challenging knot in the treatment of infections in hospitals and even increasingly in communities (Stubbings *et al*., 2009). The current situation of finding novel antibiotics or other treatment approaches against the exacerbating antibiotic-resistant infections remains severe and unoptimistic. Since the upsurge of research in terrestrial plants and microorganisms in the last two decades, marine sources have been afresh brought into focus in recent years for the search of potential antibiotics. In this introduction, the currently reported secondary metabolites from marine sponge *Callyspongia* sp. and its associated or symbiotic microorganisms were investigated. Additionally, a brief introduction to bacterial "Quorum Sensing" (QS) is presented to provide some context for the third part of the work which involved a survey of QS molecules.

1.1 Bioactive Secondary Metabolites from the Marine Sponge *Callyspongia* sp. and its Associated Microorganisms

Compared to terrestrial organisms, the search for novel natural chemical structures from marine sources antagonizing drug resistance is even today a poorly studied but promising area. The significant distinction between terrestrial and marine environments could be one of the reasons that people found a remarkable number of structurally unique and highly active metabolites from marine sources, especially numerous unprecedented chemical skeletons from marine-derived microorganisms. In addition to the broad sea area around the earth and the exceedingly rich biodiversity, marine animals, plants and marine microorganisms do provide a capacious platform for seeking novel potential drugs. Hence, marine sponges which are sessile and irremovable but interestingly predator-defensive have attracted the attention of researchers assuming that this invertebrate could be an efficient producer, and an abundant source, of anti-cancer, anti-parasitic and antibiotic secondary metabolites (Proksch *et al*., 2006).

With the increasing studies of marine sponges nowadays, current research also proved the therapeutic potency of marine sponge in various bioactivity assays, which make it inarguably another promising source of discovering novel bioactive compounds with therapeutic purpose. KRN7000 (α-Galactosylceramide) was one of the cases of potential new drugs discovered from

the marine sponges and is currently undergoing clinical trials (Proksch *et al*., 2006). However, based on the fact that there are few cases (only four updated in 2004) (Proksch *et al*., 2006) compared to the studies of terrestrial plants, to-date research for screening bioactive compounds from marine sponges still remain to be stepped up.

Since Faulkner's study in 1978 (Thomas *et al*., 2010), and combined with other ecological and physiological studies of marine sponges (Taylor *et al*., 2007), a hypothesis was proposed that the true origin of some bioactive secondary metabolites was possibly the associated microorganisms themselves by the fact that a great number of symbiotic or associated marine microorganisms were found to produce the same or highly similar compounds with those isolated from the extracts of their host sponges (Vacelet *et al*.,1978). This discovery undoubtedly provides a prospect of achieving large-scale production of the bioactive secondary metabolites through fermentation and/or gene modification rather than the conventionally artificial synthesis or mariculture of the sponges. The marine microorganisms associated with the host sponges were initially studied from an ecological angle that their secondary metabolites were considered as chemical defence agents to deter predators (Rooney *et al*., 1998) and compete for space with other sessile species (Tsukamoto *et al*., 1997) or even used as nutrition (Wilkinson *et al*., 1978). With the development of pharmaceutical science, a number of them were also found to display pharmacological properties, such as antibiotic activity against pathogenic bacteria and fungi (Wang *et al*., 1996), cytotoxicity towards particular tumour cell lines (Zheng *et al*., 2006) and immunosuppressive activities of enzymes (Höller *et al*., 1999).

We are currently interested in marine sponges of the genus *Callyspongia* and their symbiotic or associated microorganisms. This sponge genus currently comprises of 182 species that appear to harbour pharmacologically active compounds. According to a report summarised in 2009 only 15 species have been chemically studied, which indicates a great developing area for screening biomedical compounds (Ibrahim *et al*., 2010). In this section, we attempt to survey the literature and research on sponges of this genus and their associated microorganisms in the aspects of currently discovered pharmacologically active secondary metabolites.

1.1.1 Secondary metabolites isolated from sponge *Callyspongia* sp. and their biological activities

In this part, the secondary metabolites discovered in the organic extracts of sponge *Callysongia* sp. and their biological activities are summarised. A review of literature until 2013 identified

seven types of secondary metabolites that were isolated from the marine sponge *Callyspongia* sp. : polyacetylenes, peptides, alkaloids, lipids, terpenoids, steroids and polyketones.

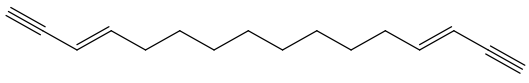
1.1.1.1 Polyacetylenes

Polyacetylenes are distinctively associated with marine sponges, especially those of the order *Haplosclerida*. Hence, different acyl-chain lengths, number and position of unsaturation, functionality and oxidation patterns of these compounds were used to assist sponge taxomony. C2l~C22 polyacetylenes were always considered to be characteristic of sponge *Callyspongia* sp. Additionally, in *Callyspongia* sp. C23 polyacetylenic sulphate and C22~C23 hydroxypolyacetylenes were pretty common compared to other species sponges (Liu *et al*. 2008.)

Normal polyacetylenes

The first polyacetylene isolated from sponge *Callispongia sp.* was a symmetrical diacetylenic hydrocarbon callydiyne (1). It was discovered early in 1991 by Miao from the methanolic extracts of the marine sponge *Callyspongia flammea* which was collected off reefs of Madang (New Guinea). However, no biological activity was reported for this compound (Miao *et al*. 1991). Another structurally similar polyacetylene was found by Braekman in 2003 from the *Callyspongia pseudoreticulata* collected off Ujung Pandang (Indonesia). It was a C-20 polyacetylene (2) isolated from the CH_2Cl_2-soluble fraction from methanolic extract. This symmetrical diacetylene showed potent toxicity against nauplii of the brine shrimp *Artemia salina* with the LD_{50} value of 2 µg/mL. The absolute configuration at C-3 and C-18 were both determined to be S-configuration , but the compound mixture also had trace stereoisomers (3S, 18S), (3R, 18R) and/or (3S, 18R) present (Braekman *et al*. 2003). Research groups from Japan have conducted in-depth study on sponge *Callyspongia* sp. Fusetani's group discovered seven novel polyacetylene derivatives in 1997 including callytetrayne (3), callypentayne (4), and five triols callytriols A –E (5-9) from sponge *Callyspongia truncata* collected off Sagami Bay (Japan). An ecological study of these compounds along with the isolated compounds siphonodiol (10), callyspongins A (11) and B (12) was carried out. callytriol C (7), callytriol E (9) and callyspongin A (11) were found to be the most potent among the ten in inducing metamorphosis in the ascidian *Halocynthia roretzi* larvae with the same low ED_{100} value of 0.13 µg/mL. Additionally, five triols (callytriols A-E) (5-9), siphonodiol (10), callyspongins A (11) and B (12) were also found to have antifouling activity against the barnacle *Balanus amphitrite* larvae but with ED_{50} values that range from 0.24 to 4.5 µg/mL. Stereochemical determination of these molecules was hampered by the small amounts isolated (Tsukamoto *et al*. 1997). Almost at the same time, in Umeyama's study on the same sponge *Callysongia* sourced off the coast of

Tokushima prefecture (Japan), callypentayne (4) and callytetrayne (3) were also reported and given the alternative names of callyberyne A (3) and callyberyne C (4) respectively. However, the species of this sponge was not identified although a detailed taxonomic description was performed. Besides those two compounds, another novel C-21 polyacetylene was discovered in the same sponge and given the name Callyberyne B (13) (Umeyama *et al*., 1997). Until now, there has been no other pharmacological assay used to test these compounds except for Fusetani's ecological study in which callyberynes A (3) and C (4) were found to have potent metamorphosis-inducing activity towards the *ascidian Halocynthia roretzi* with ED_{100} values of 0.25 µg/mL (Tsukamoto *et al*. 1997). From a new paragraph biosynthetic, the rare non-oxygen associated polyacetylenes were considered the most likely to be decarboxylated from a C-22 fatty acid precursor in the sponges, which may contribute to understanding of biosynthetic pathway of these compounds (Umeyama *et al*., 1997). In 2010, on the same sponge, Umeyama's group discovered a new polyacetylene diol, callyspongidiol (14) from the EtOAc extract with anti-proliferative activity against human promyelocytic leukemia cells (HL-60) with IC_{50} values of 6.5 µg/mL. Another two known polyaceylene diols, siphonodiol (10) and 14, 15-dihydrosiphonodiol (15) were also isolated from the same extract and showed anti-proliferative activity with IC_{50} values of 2.8 and 6.5 µg/mL respectively (Umeyama *et al*., 2010). These three compounds were also reported to activate human dendritic cells by phenotypic and functional maturation and altered cytokine production which to some extent exhibited antitumour properties (Umeyama et al., 2010; Takei *et al*., 2010). In addition, siphonodiol (10) was also reported as an H,K-ATPase inhibitor in 1987 (Fusetani *et al*. 1987).



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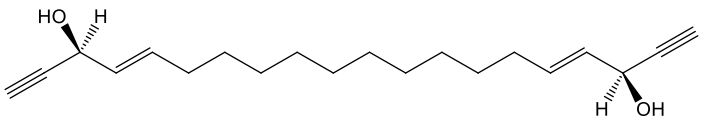

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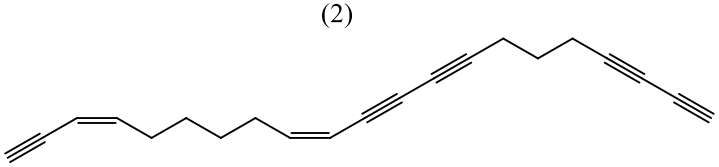

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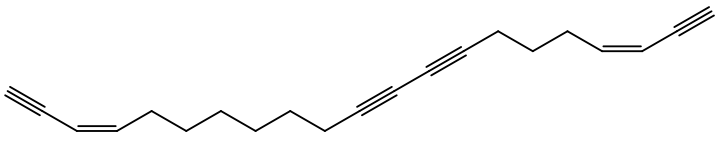

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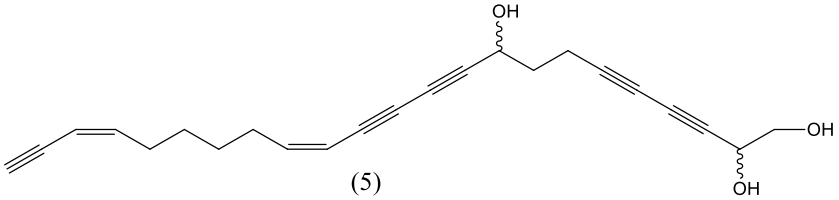

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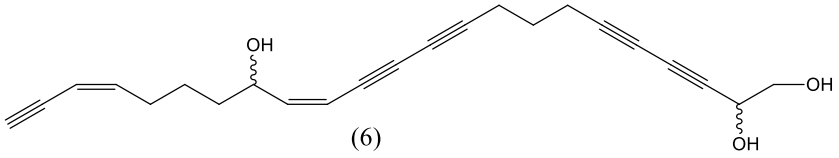

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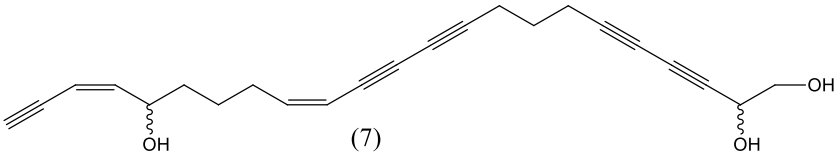

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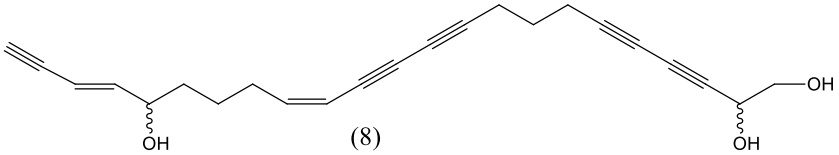

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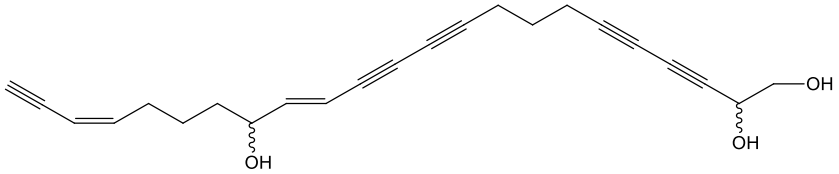

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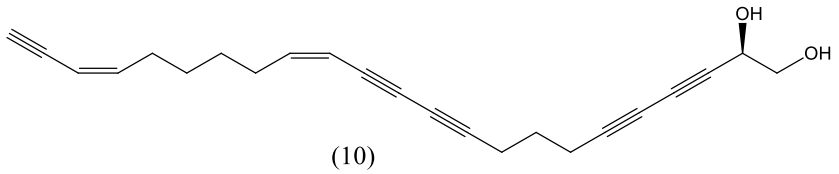

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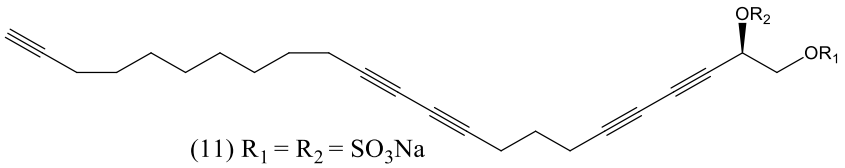

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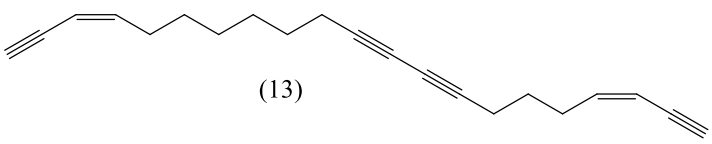
(12) $R_1 = SO_3Na$, $R_2 = H$



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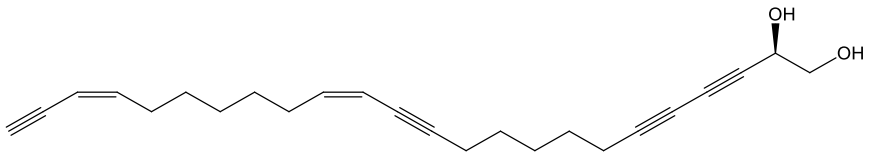

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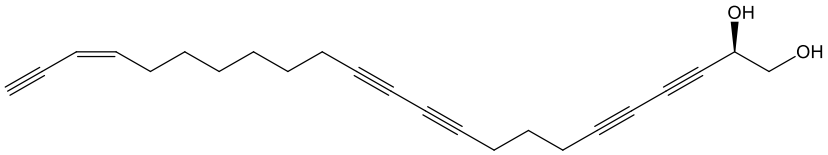

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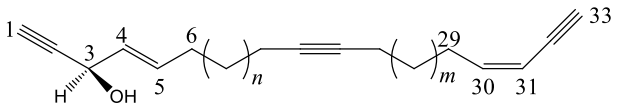
Callyspongynes A (**Error! Bookmark not defined.**) and B (**Error! Bookmark not defined.**), reported in 1998 by Rooney, were another two polyacetylenes isolated from *Callyspongia* sp. The sponge studied by Rooney was sourced off Barwon Heads in South Australia. Its species was not stated. These two compounds both exhibited a unique chemical structure containing two terminal acetylenes and an uncommon "central" acetylenic functionality. The absolute configuration at C-3 was assigned as R-configuration in both. They were speculated to serve as chemical defence agents against both competitive sponges and predators (Rooney *et al*. 1998).

From Youssef's study on the Red Sea sponge *Callyspongia* sp., seven straight-chain polyacetylenes aikupikanynes A–F (18-23) and octahydro-siphonochalyne (24) were isolated in 2000 from the CH_3Cl fraction of the methanolic extract. In his study, aikupikanynes E (22) and F (23) of sufficient amount were selected for biological assays on mouse lymphoma (P-388, ATCC: CCL 46), human lung carcinoma (A-549, ATCC: CL 8), and human colon carcinoma (HT-29, ATCC: HTB 38) cancer cell lines and showed moderate antitumor activity with IC_{50} values of 5 and 10 µg/mL respectively. Unfortunately, aikupikanynes A-D (18-21) were not tested on these assays due to their limited quantities. Again, the species remains to be identified (Youssef *et al*. 2000).



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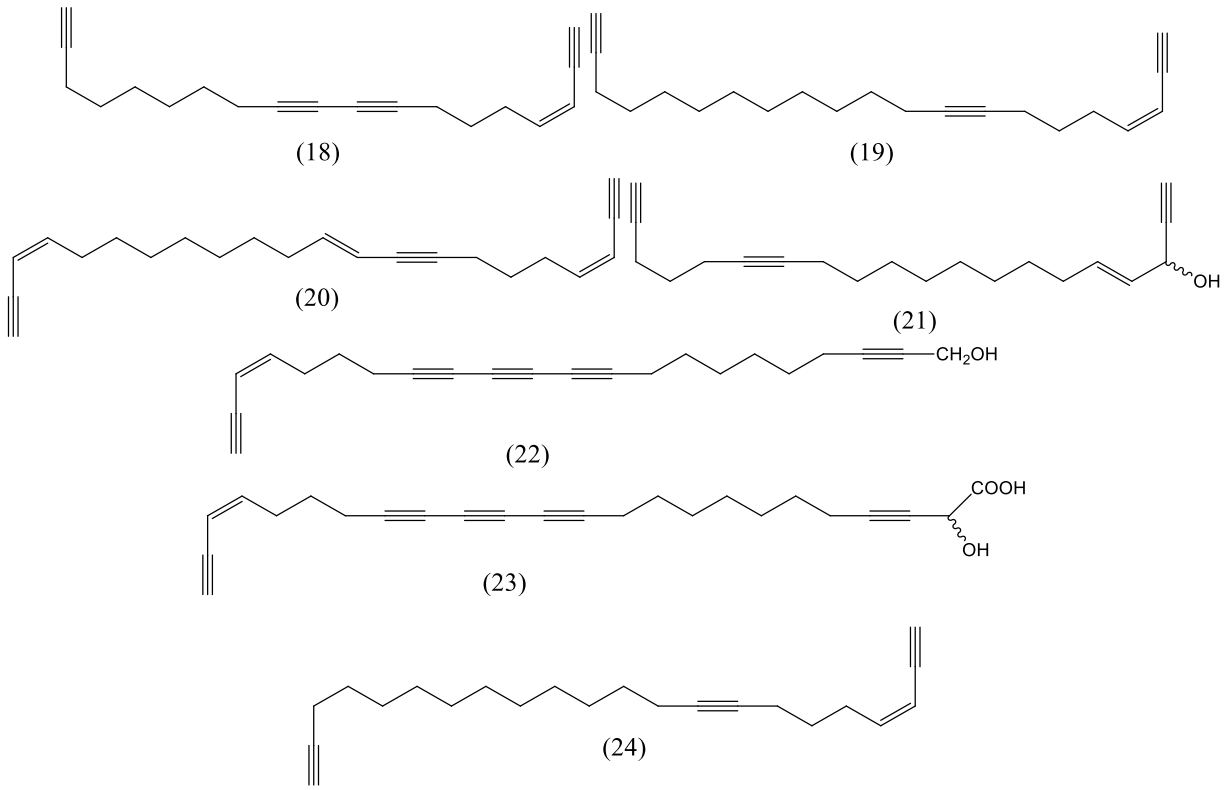
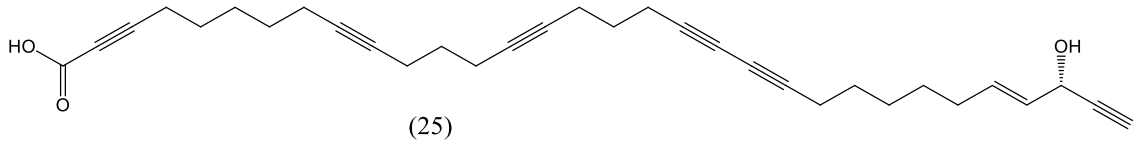
(17) n+m = 20



Polyacetylenic derivatives

In 1996 the first two polyacetylene sulphates, Callyspongin A (11) and Callyspongin B (12), were reported by Ikegami. They were isolated from the methanolic extract of *Callyspongia trunata* which was collected off the coast of Sada-misaki (Japan). These two compounds were found to inhibit the fertilisation process of starfish, of which callyspongin A (11) with the IC_{50} value of 6.3 µm/mL showed greatly higher potency than callyspongin B (12) (IC_{50}=50 µm/mL). Interestingly, embryonic cell division was not affected during the whole inhibitory process, which is extremely rare in the known fertilisation inhibitors that achieve infertility through inhibiting embryonic cell division. The absolute stereochemistry of callyspongin A (11) at C-2 was determined to be the *R*-configuration. There was no study on the stereochemistry of callyspongin B (12). But *R*-configuration was most likely considered due to the same origin of these two compounds. Still, it may deserve to be investigated (Uno *et al*. 1996). Two research groups from the University of Amsterdam and University of Tokyo have continued the study of the lipophilic and hydrophilic extractions of *Callyspongia truncata* which was collected off the Kii Peninsula (Japan). A C-32 polyacetylenic acid, callyspongynic acid (25), was found in the lipophilic extract with an appreciable activity for inhibiting α- glucosidase (IC_{50} = 0.25 µg/mL) in an anti-diabetes study. The inhibition of α- glucosidase was supposed to be ascribed to the unique structure that the carboxylic acid and the allylic hydroxyl attach to the end acetylenes.

However, it failed to inhibit β- glucosidase, β-galactosidase, thrombin or trypsin even at the concentration of 100 µl/mL. The absolute configuration at C-30 was determined a *R*-configuration (Nakao *et al*., 2003). Additionally, in their ongoing study of this species another polyacetylenic acid which contains a sulphate group was discovered in 2003. It was the first case of a sulphate containing acetylenic acid being isolated from marine organisms and given the name of callysponginol sulphate A (26). The absolute configuration at C-2 which links to the sulphate was determined to be *R*-configuration. This compound inhibited membrane type 1 matrix metalloproteinase (MT1-MMP), a key enzyme contributing to tumour growth, with the IC_{50} value of 15.0 µg/mL. Since the same test was also applied to the desulphated callysponginol and some structurally unrelated sulphate compounds with no inhibitory activity detected, the sulphate functional group was speculated to contribute to the inhibitory activity (Fujita *et al*. 2003). In the hydrophilic extract reported in the same study, halistanol sulphates (27) which were initially isolated from the marine sponge *Halichondria* cf. *moorei* Bergquist (Fusetani *et al*.,1981) were also found in *Callyspongia truncate* exhibiting potent inhibition of MT1-MMP with an IC_{50} value of 19 µg/mL (Fujita *et al*., 2003). In addition, HIV-inhibition was also found earlier in 1994 in this type of sterol (Bifulco *et al*., 1994) In the same year of 2003, callyspongamide A (28) isolated from the Red Sea sponge *Callyspongia fidtularis* was reported by Youssef's group. This polyacetylenic amide was found to be moderately cytotoxic against HeLa cells with an IC_{50} value of 4.1 µg/mL. The metabolic origin of this compound was also discussed in this study since callyspongamide A (28) was structurally similar with a secondary metabolite hermitamide A (29) (Tan *et al*., 2000) which was isolated from a marine microbe. This actually provides a support of the previous hypothesis that some of the secondary metabolites isolated from sponges, such as callyspongamide A (28), are probably of a microbial origin. The absolute configuration remains to be established (Youssef *et al*., 2003).



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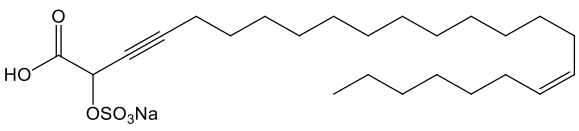

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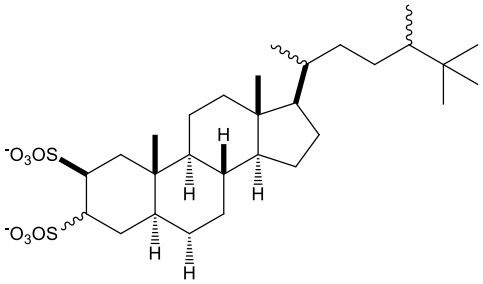

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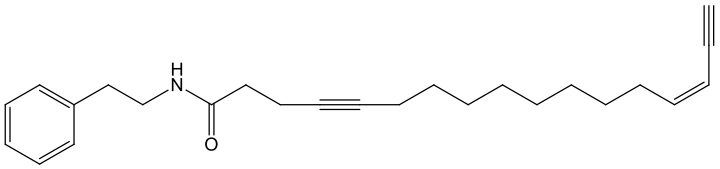

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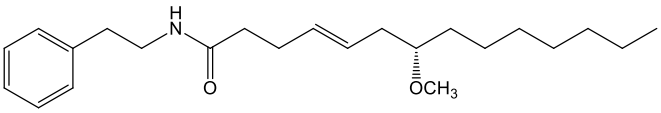


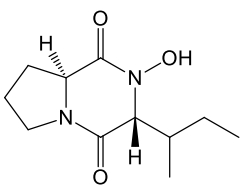
(29)

1.1.1.2 Peptides

Peptides currently discovered in sponge *Callyspongia* sp. belong to two types: dipeptides and cyclic peptides. The amino acids contained in the marine sponge peptides were usually of D-configuration which is very rare in animals, implying that these secondary metabolites were most likely to be produced by symbiotic microorganisms [**Error! Bookmark not defined.**].

Dipeptides

Callyspongidipeptide A (30) was a proline-containing dipeptide discovered by Yang in 2009 from a Chinese sponge *Callyspongia* sp. with another two compounds (31) (32). These three compounds were tested for their cytotoxic activity against SGC-7901 (human stomach cancer), HepG2 (human liver cancer), and HeLa cell lines but were devoid of any activities (Yang *et al*., 2009).



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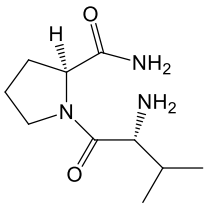

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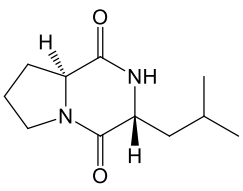
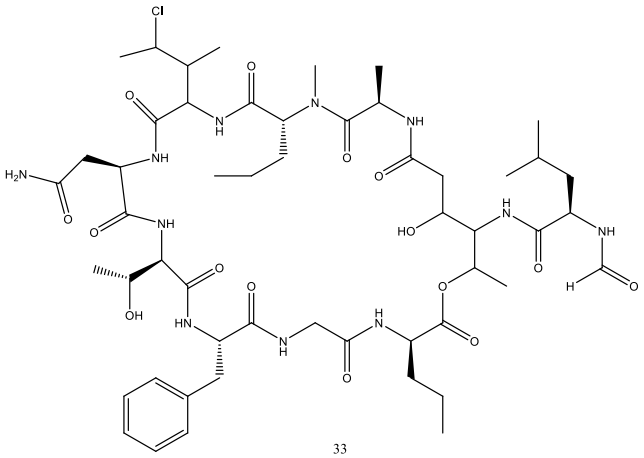


(32)

Cyclic peptides

Compared to the rare dipeptides isolated from the sponge *Callyspongia*, cyclic peptides are much more common. From an Australian sponge *Callyspongia bilamellata*, Capon in 2002 reported two cyclic depsipeptides, phoriospongins A (33) and B (34). They were isolated from the ethanolic extract of the sponge and showed considerable nematocidal activity against livestock parasite *Haemonchus contortus* with LD_{99} value of 8.3 µg/mL (Capon *et al*., 2002). Callynormine A (35) was a unique heterodetic peptide isolated from a Kenyan marine sponge *Callyspongia abnormis* (2004). This compound also brought about a novel class of heterodetic peptide in which an α-amido-β-aminoacrylamide cyclisation functionality links the ring and all the amino acids were L-configuration (Berer *et al*., 2004). From Ibrahim's recent study on sponge *Callyspongia aerizusa* which was sourced from Ambon, Indonesia, a series of cytotoxic cyclic peptides were isolated from the methanolic extract. They were determined to be analogues of callynormine A (35) and proposed the names of callyaerins A-H (36-43). Callyaerin G (42) was isolated in considerable quantity and firstly reported to exhibit strong cytotoxicity towards the mouse lymphoma cell line (L5178Y) (ED_{50} = 0.41 µg/mL) and moderately against HeLa cells (ED_{50} = 4.43 µg/mL). It was also tested on rat brain tumour (PC12) cell lines but showed no activity (Ibrahim *et al*., 2008). Callyaerin E (40) seems to be the most active among the 8 analogues due to its potent and wide bioactivity in all the trials tested in this study including brine shrimp mortality assay, cytotoxicity test, and antibiotic assay. Notably, it exhibited the most potent activity against the L5178Y cell line with an ED 50 value of 0.39 µg/mL and was also strong towards the rat brain tumour (PC12) cell lines in which the other 7 were invalid. Callyaerin H (43), however, also displayed considerable anti-tumour property against L5178Y cell lines with the ED50 value of 0.48 µg/mL. Towards the antimicrobial activity, aside from callyaerin E (40), callyaerin A (36) and B (37) inhibited the growth of most of the trial bacteria including *Staphylococcus aureus*, *Candida albicans* and *Escherichia coli* although the potency was still not very high. Callyaerin A (36) showed the best activity against *Candida albicans* and *Escherichia coli*. Callyaerin F (41), however, almost showed only moderate anti-tumour or antimicrobial activities against the trial cancer cell lines or bacterial strains. In addition, their analogues with different numbers of proline residues were tested in a number of assays and showed different biological activities. Therefore, the varying cytotoxicities also suggests a fact that increasing number of proline residues may contribute to the cytotoxic potency (Ibrahim *et al*., 2010).



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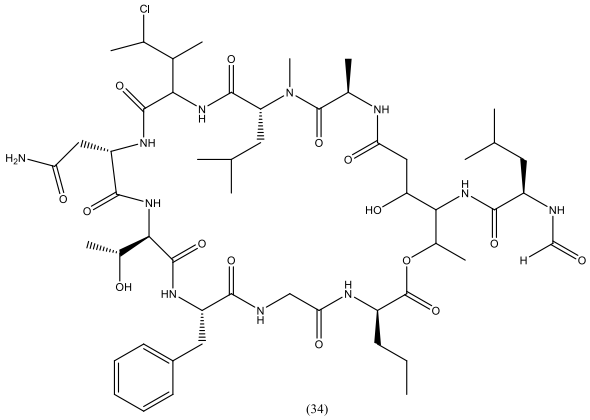

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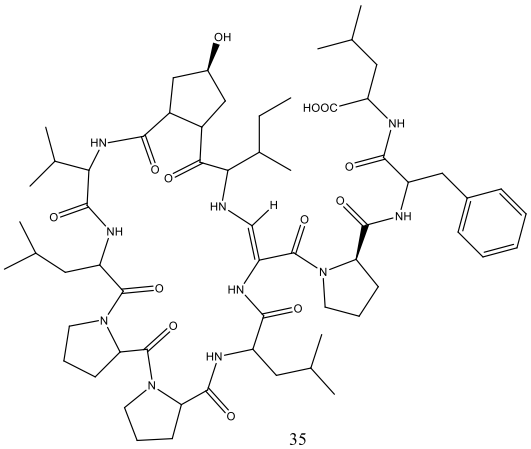
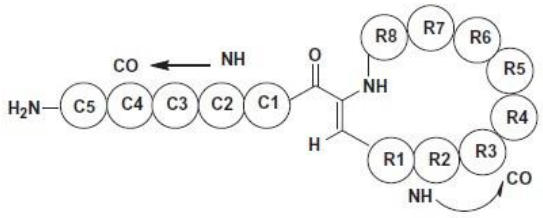


35

		Ring	Sidechain
		R1 R2 R3 R4 R5 R6 R7 R8	C1 C2 C3 C4 C5
1	A	Ile Hyp Val Ile Leu Pro Pro Leu	Pro Ile Phe Gly
2	B	Ile Hyp Ile Ile Leu Pro Pro Leu	Pro Ile Ile
3	C	His Hyp Leu Leu Pro Pro Val	Pro Leu Phe Gly
4	D	Ile Ile Phe Pro Hyp Pro Leu	Pro Ile Asn Ala Ile
5	E	Leu Pro Phe Phe Pro Pro Val	Pro Ile Ile Gly
6	F	Val Pro Val Phe Pro	Pro Leu Phe Ile
7	G	Leu Pro Pro Pro Pro Leu	Pro Phe Phe Phe
8	H	Val Pro Val Phe Pro Pro Leu	Pro Ile

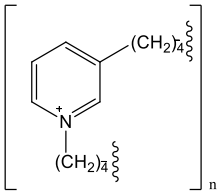
Footnote: Hyp is γ-hydroxyproline.



（36-43）[**Error! Bookmark not defined.**]

1.1.1.3 Alkaloids

The alkaloids isolated from *Callyspongia* sp. were mostly pyridinium alkaloids. In 1993, Davies-Coleman reported an epidermal growth factor (EGF)-receptor inhibitor which was isolated from the methanolic extract of a Micronesian sponge *Callyspongia fibrosa*. It was demonstrated to be a polymeric pyridinium alkaloid (44) but the chemical structure has get to be verified (Daviescoleman *et al*., 1993). Untenines A (45), B(46), C(47) are three 3-pyridine derivatives containing a terminal nitroalkyl group found by Sheng in 1996 from an Okinawan Marine Sponge *Callyspongia* sp.. Hence, no sponge species were mentioned in this study. All of them were found to have potent anti-microfouling activity (IC_{100} = 3.0, 6.1 and 5.8 mg/mL respectively). Additionally, it was the first example of nitroalkyl compounds isolated from marine organisms and untenine A was successfully synthesised in this study (Wang *et al*., 1996). From the sponge *Callyspongia ridleyi* sourced from Papua New Guinea, halitoxins (48) were isolated and used for an electrophysiological actions study by Gordon's group in 2000. The compounds were discovered to assist other molecules in entering the target cells without destroying the cell membrane (Scott *et al*., 2000). Other studies on these compounds also showed a group of bioactivities including selective activity against seawater bacteria, protection of the host sponge from microbial pathogenesis, toxicity to mice and sea urchin eggs, general cytotoxicity as well as hemolytic and neurotoxic properties (Kelman *et al*., 2009; Berlinck *et al*., 1996). Voogd did research on the Indonesian sponge *Callyspongia biru*. In this sponge amphitoxin (49) was isolated as the major constituent from the crude methanolic extraction which showed favourable bioactivities (Voogd *et al*., 2005). Potent antimicrobial activity against specific marine bacteria was reported by Kelman in 2001 indicating a defensive role against microbial pathogenesis within the sponge (Kelman *et al*., 2001). It was also considered to be used as a potential insecticide (Thompson *et al*., 2010). An important point to note is that this compound and other 3-alkylpyridines were also speculated the genuine chemotaxonomic markers of marine sponges in the family of Callyspongiidae and Niphatidae. However, *Callyspongia truncata* was an exception, since no amphitoxins or 3-alkylpyridines of any kind were traced in this species up to our current review (Becking *et al*., 2007). Niphatoxin C (50) was isolated recently in 2007 from an Australian sponge *Callyspongia* sp. by Quinn's group. It showed $P2X_7$ receptor antagonism and was also found to be cytotoxic for THP-1 cells (Buchanan *et al*., 2007).



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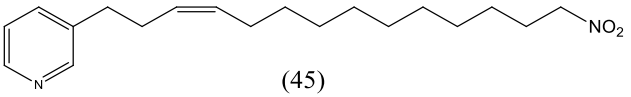

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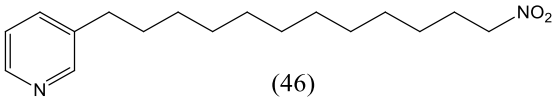

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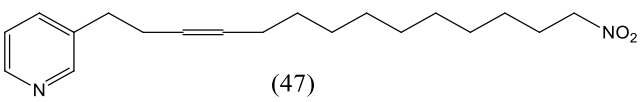

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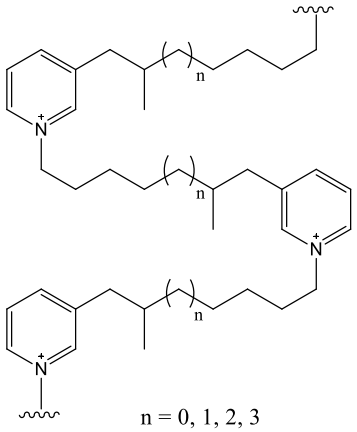
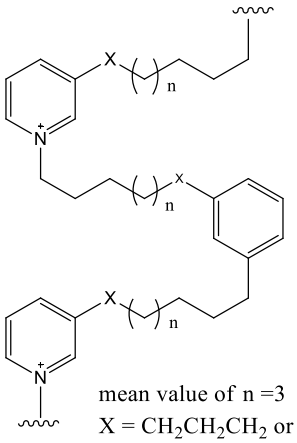


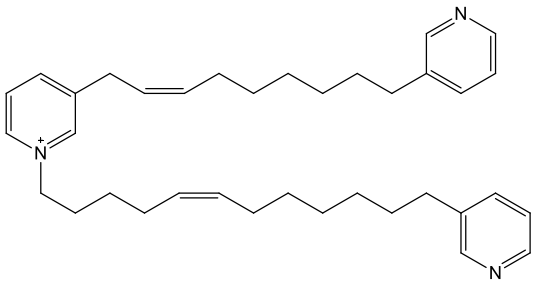
n = 0, 1, 2, 3

(48)



mean value of n =3
X = $CH_2CH_2CH_2$ or $CH_2CH{=}CH$
ratio 1:1

(49)

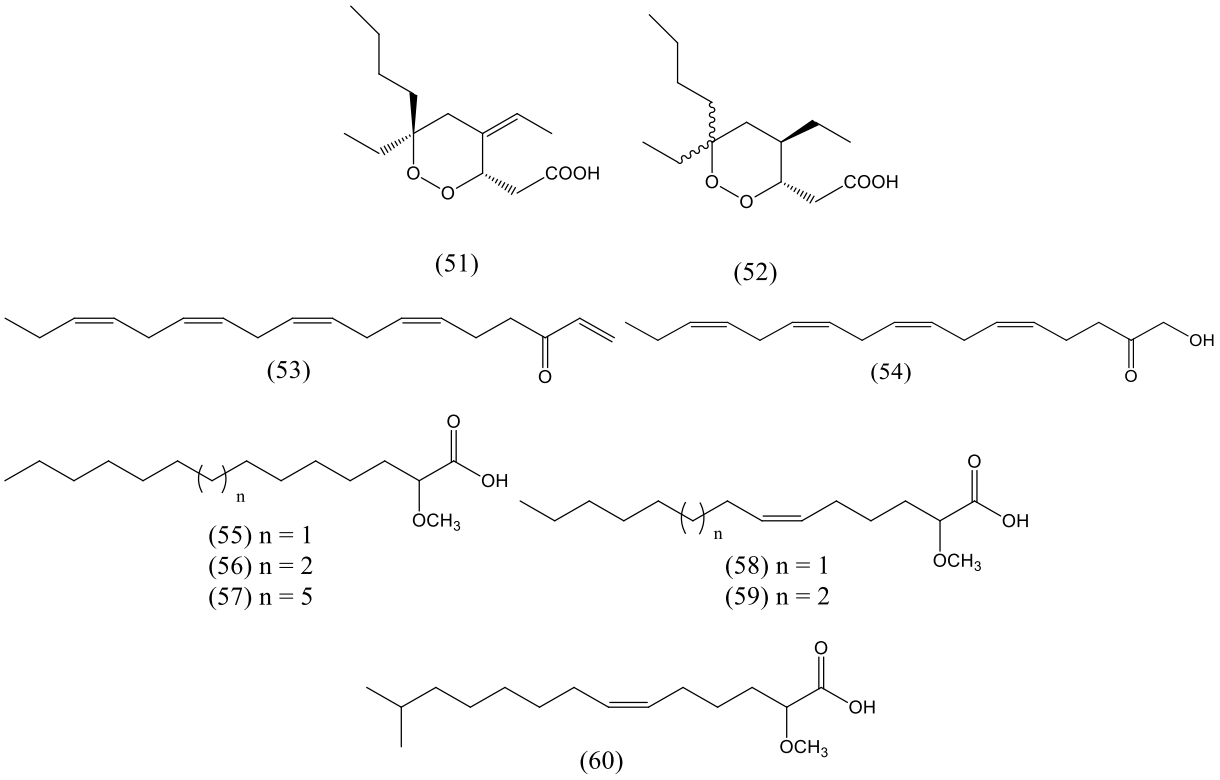


(50)

1.1.1.4 Lipids/Fatty acids

Lipids found in sponge *Callyspongia* sp. were mostly fatty acids. Two cyclic-peroxide-containing acids (51) (52) were found by Toth in 1994 from a New Guinea species of *Callspongia* sp.. The sponge species was not mentioned. These two compounds have not been named but showed murine leukemia cell growth inhibition with ED_{50} values of 5.5 and 2.6 µg/mL respectively (Toth *et al.*, 1994). In 1997, Urban isolated a novel lipid (53) with a terminal olefin and a previously known polyunsaturated fatty acid (54) from an Australian sponge *Callyspongia* sp. (Urban *et al.*, 1997) From Caribbean sponge *Callyspongia fallax,* a group of 2-methoxylated fatty acids including three saturated fatty acids (55-57) and three monoenoic methoxylated fatty acids (58-60) were isolated by Carballeira in 2001. 2-methoxylated C14-C16

fatty acids were suggested to originate from the phospholipid membrane of an associated microorganism within the sponge due to its limited quantity and the strange double bond position (at C-6) which is different from that found in sponges (at C-5 or C-9) (Carballeira et al., 2001).



(51) (52)

(53) (54)

(55) n = 1
(56) n = 2
(57) n = 5

(58) n = 1
(59) n = 2

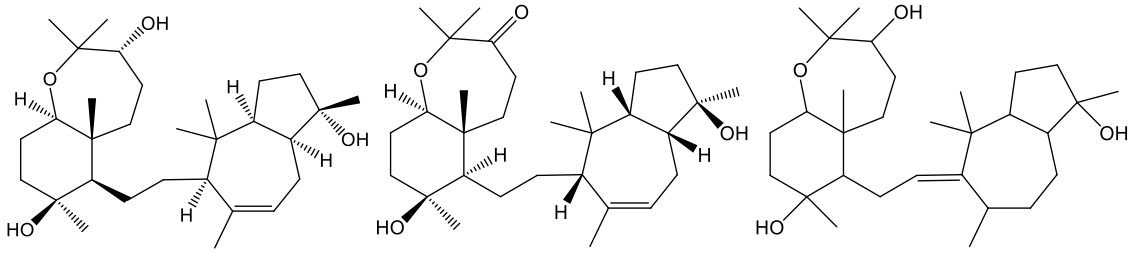
(60)

1.1.1.5 Terpenes

Triterpenoids

The terpenoids are the most abundant non-steroidal secondary metabolites isolated form marine sponges. In *Callyspongia* sp. triterpenoids seem to be the most common. From our current review, there were 31 triterpenoids discovered in the sponge Callspongia sp. since Carmely's study in 1983. These triterpenoids were classified according to their unique and different skeletons, namely the sipholanes, siphonellane, neviotane and dahabane. 21 sipholanes were found in Red Sea sponge *Callyspongia siphonella* including sipholenol A-M (61-73), sipholenone A-E (74-78) and two glycosides derivatives sipholenoside A (79) and B (80). Compounds of this class possess two separate bicyclic systems of perhydrobenzoxepine and bicyclo-decane which are linked to each other by an ethylene bridge. Six siphonellanes including siphonellinol A-E (82-86) and a siphonellinol C-23-hydroperoxide (87), however, consist of one bicylic system and one single cyclic system linked through the ethane bridge. Only two neviotanes and one dahabanes were discovered respectively and neviotanes A (88), B (89) and

dahabane A (90) (Carmely *et al.*, 1986). In recent years, Sayed's group conducted a cytotoxic evaluation on four cancer cell lines as well as the reversal effect assessment towards the P-gp-mediated multidrug resistance of these triterpenoids. The tested compounds, including sipholenols A (61), J (70), k (71), L (72), M (73), sipholenone E (78), siphonellinol D (84) and an isolated unnamed compound which consists of octahydro-2 H-chromene (rings "A" and "B") and octahydroazulene (rings "C" and "D") systems connected through an ethylene bridge (81), were all found to be devoid of any cytotoxicity against the drug-sensitive cell line KB-3-1, the transfected MRP1 clone cells KB/MRP1, and two drug-selected MDR cell lines. However, sipholenol A (61), sipholenone E (78), sipholenol L (72), and siphonellinol D (84) exhibited potent activity in reversing P-gp-mediated multidrug resistance and were proved to act in a similar fashion in resistant KB-C2 cells in the pharmacophore modelling (Jain *et al.*, 2009; Jain *et al.*, 2007). Akaterpin (91) was another triterpene discovered by Fukami from sponge *Callyspongia* sp. in 1997. It inhibited phosphalidylinositol-specific phospholipase C (PI-PLC) considerably with an IC_{50} value of 0.5 µg/mL and neutral sphingomyelinase weakly with an IC_{50} of 30 µg/mL (Fukami *et al*., 1997).



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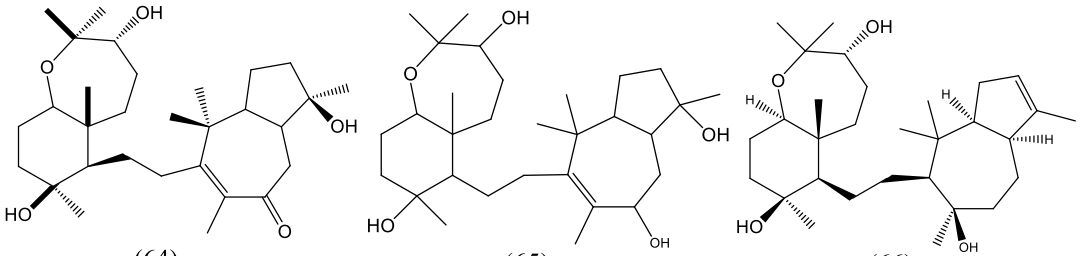

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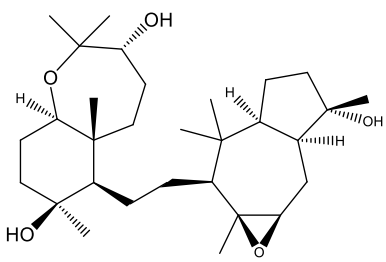

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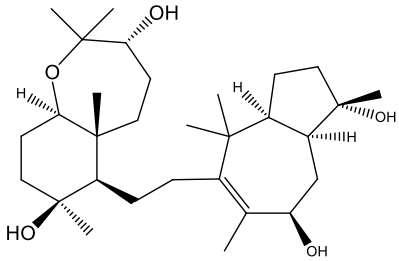

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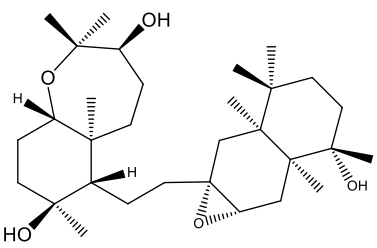

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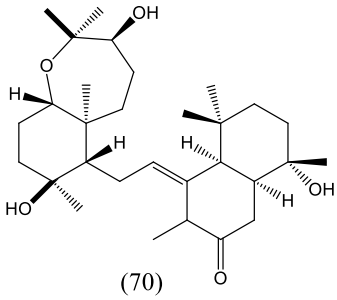

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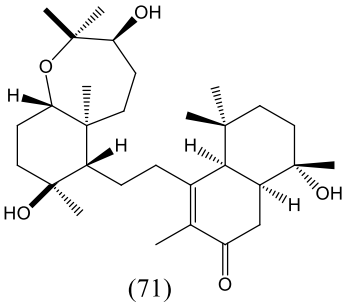

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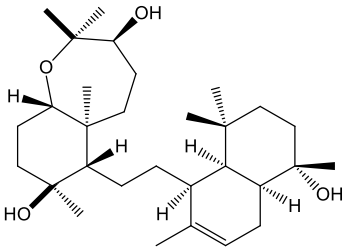

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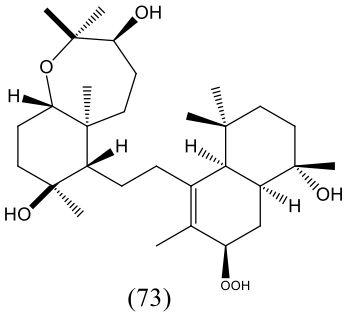

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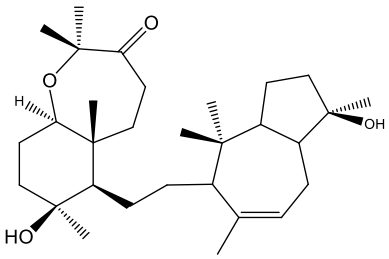

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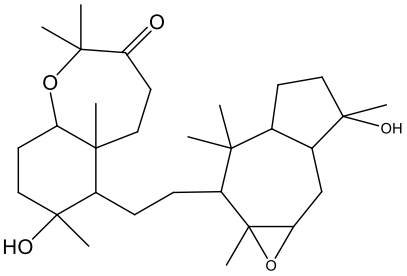

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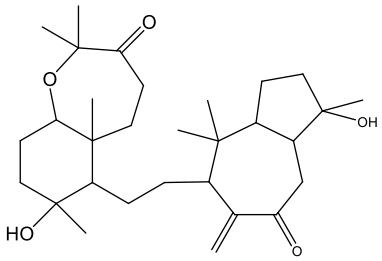

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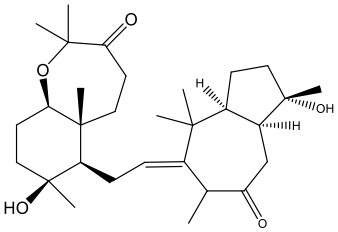

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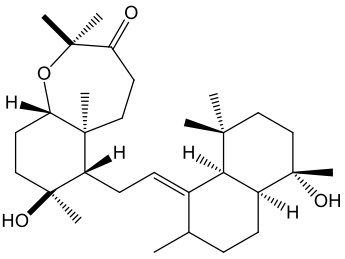

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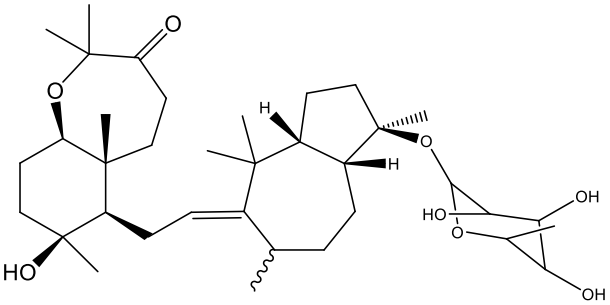

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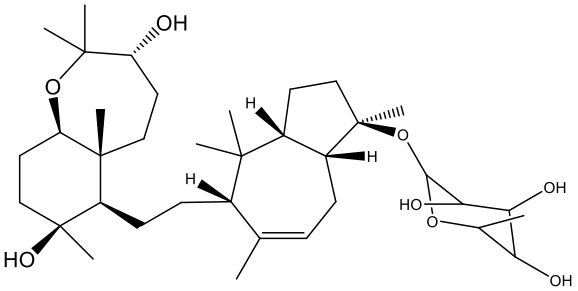

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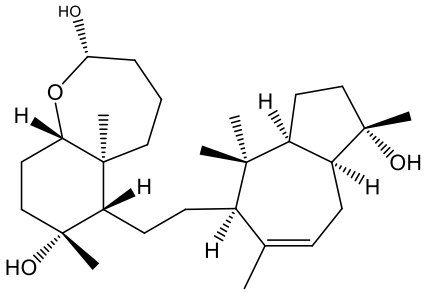

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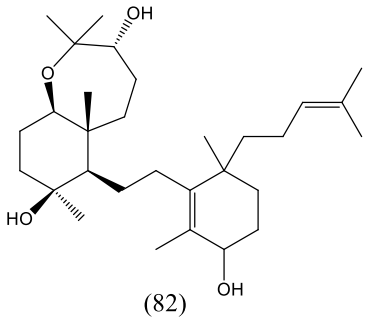

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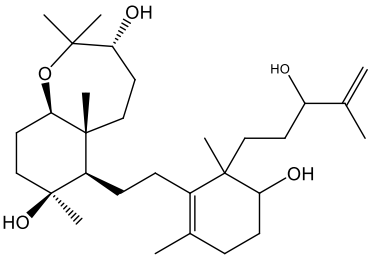

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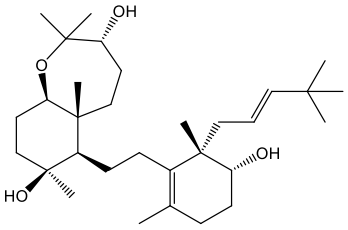

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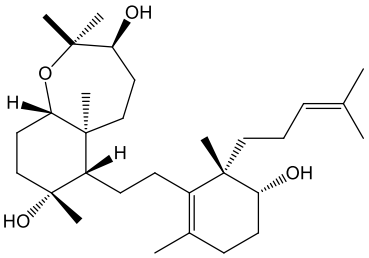

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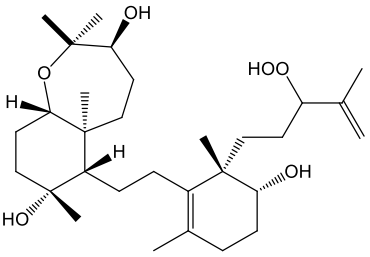

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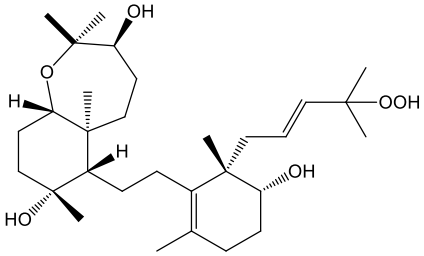

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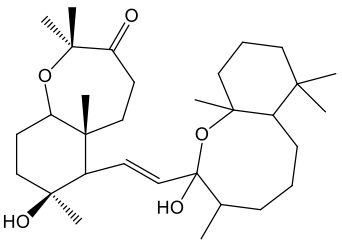

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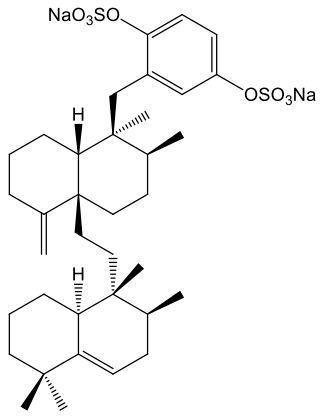

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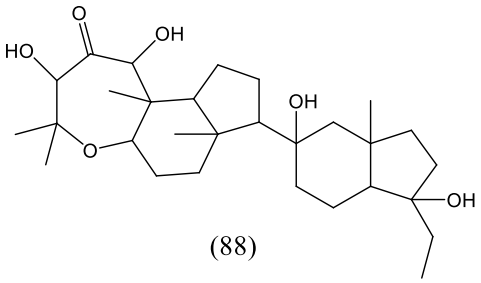

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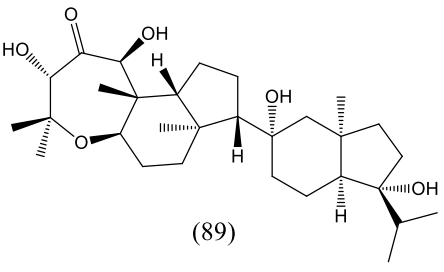
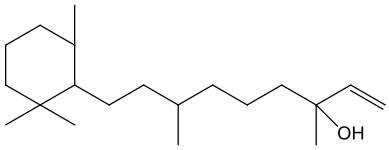


(89)

Other terpenoids

A saturated six membered cyclic diterpene, callyspinol (92) was isolated by Garg in 1995 from the antiviral active extract of the sponge *Callyspongia spinosissima* collected off the south eastern coast of India. However, callsypinol itself was deficient of any antiviral activity (Garg *et al.*, 1995). Three disulfated meroterpenoids with anti-parasitic properties were discovered by Berlinck from the methanolic extract of a Brazilian sponge *Callyspongia* sp. in 2006. The major metabolite isoakaterpin (93) showed anti-*Leishmania spp*. activity, inhibiting its adenosine phosphoribosyl transferase (APRT) with an IC_{50} value of 1.05 µg/mL. Ilhabelanol (94) and ilhabrene (95) were not able to be used for the enzyme-based assay due to the insufficient amounts. But the low IC_{50} value of 0.7 µg/mL obtained from the fraction which contained these two compounds suggested that they probably possess APRT inhibitory activities like isoakaterpin (93) (Gray *et al.*, 2007).



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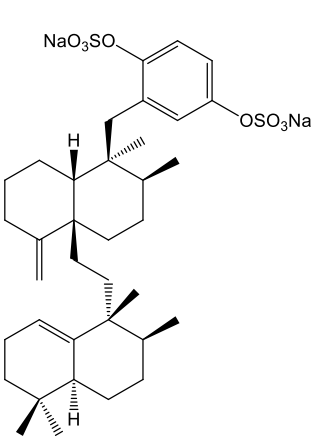

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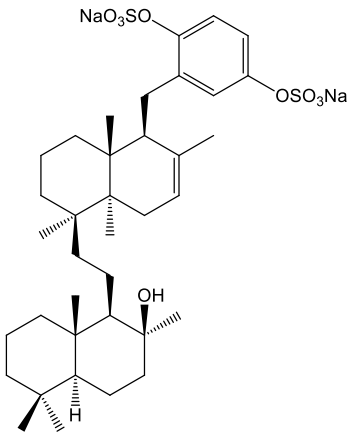

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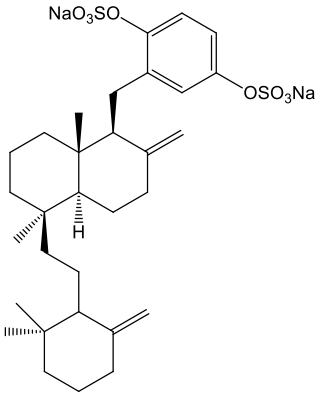
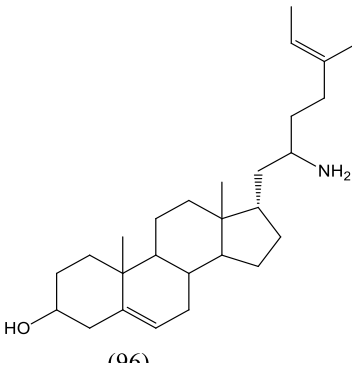


(95)

1.1.1.6 Steroids

Eight known sterols, among them the principal isofucosterol (96), were examined by Erdman and Scheuer in the sterol composition study of sponge *Callyspongia diffusa* in 1975 (Theobald, et al., 1978). In 1978, another new steroid, 24-ketocholesterol (97) was added to those described by Djerassi's group (Theobald, et al., 1978). This is a steroidal allene compound which was speculated to be degraded from isofucosterol (96). More recently in 2010, four new polyhydroxylated sterols (98-101) with two known sterols, namely 24 S-24-methylcholesterol (102) and 24S -24 methyl-cholestane-3β, 5α, 6β, 25-tetraol-25-mono acetate (103) and butyl

alcohol (104), were reported by Rao from an Indian sponge *Callyspongia fibrosa* collected from the Gulf of Mannar. The major known steroid (103) was found to have considerable activity against the chloroquine-resistant strain of the malaria parasite *Plasmodium falciparum*, even better than on the chloroquine-sensitive strain. Three new polyhydroxylated sterols (98, 100 and 101) also exhibited moderate antimalarial activity but 24 S-24-methylcholesterol (102), butyl alcohol (104) were inactive (Rao et al., 2010).



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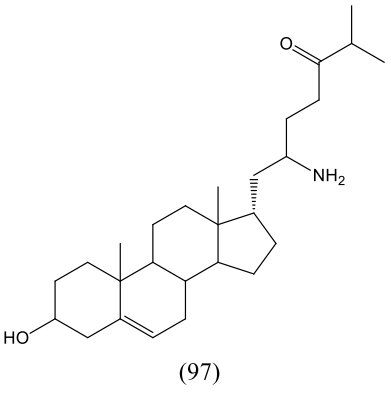

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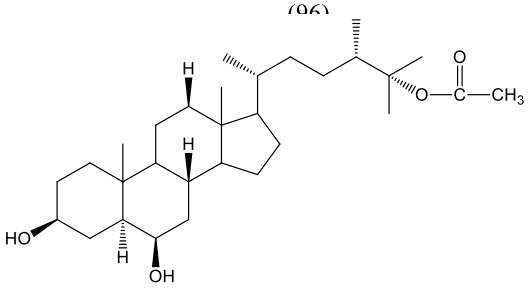

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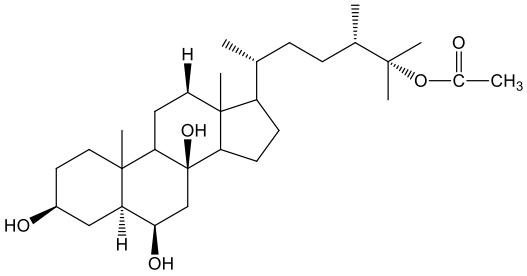

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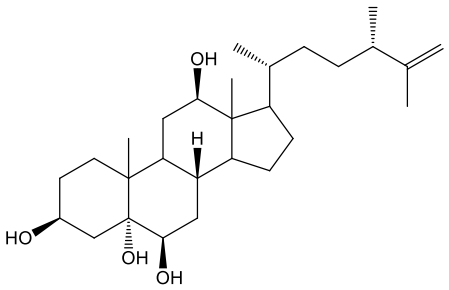

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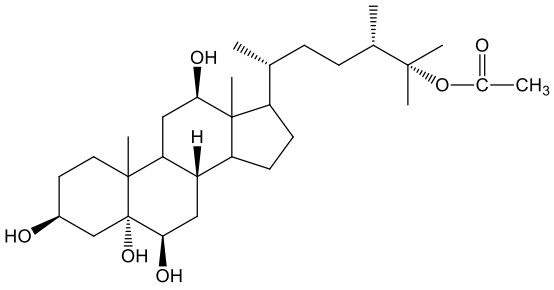

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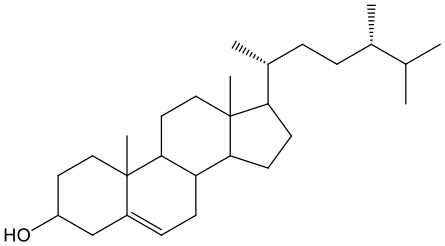

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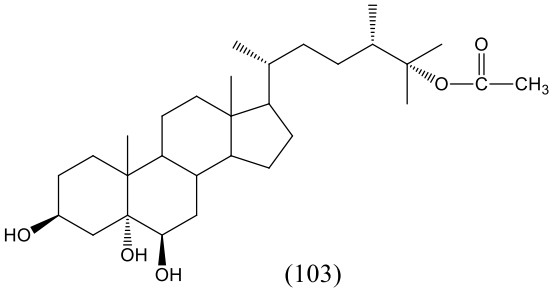

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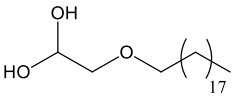
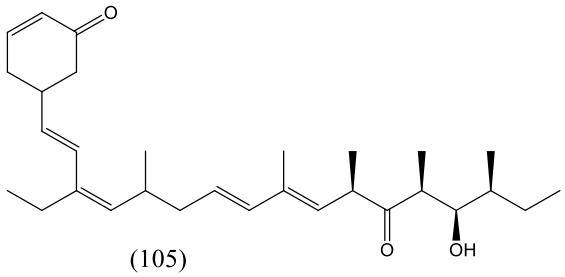


(104)

1.1.1.7 Callystatin A

From our review, callystatin A (105) was the only polyketide discovered in sponge *Callyspongia* sp. It was first isolated by Kobayashi from the acetone extract of *Callyspongia truncata* (Kobayashi *et al*., 1997). Due to its potent cytotoxicity, synthetic approaches to this compound and its fragments are being developed (Pujari *et al*., 2012; Dias *et al*., 2005).



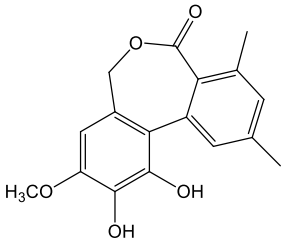
(105)

1.1.2 Secondary metabolites isolated from *Callyspongia* sp. microbial symbionts

The symbiotic microorganisms of medicinal value presently reported in sponge *Callyspongia* sp. comprise only two fungal strains and several *Streptomyces* strains.

1.1.2.1 Fungi

In 1999, Höller isolated a tyrosine kinase inhibitor ulocladol (106) and an antifungal metabolite 1-hydroxy-6-methyl-8- (hydroxymethyl) xanthone (107) from the fungus *Ulocladium botrytis* (strain no. 193A4) that was found in the Caribbean marine sponge *Callyspongia vaginalis.* 1-hydroxy-6-methyl-8- (hydroxymethyl) xanthone (107) showed inhibitory properties against *Ustilago violacea* and *Eurotium repens* (Höller *et al.*, 1999). The fungus *Cladosporium herbarum* isolated from an Indonesian sponge *Callyspongia aerizusa* was found to yield herbaric acid (108), cladospolide B (109), iso-cladospolide B (110), four macrolides namely pandangolides 1 (111), 2 (112), 3 (113), 4 (114) and two furan carboxylic acids derivatives Sumiki's acid (115) and acetyl Sumiki's acid (116). At present, only Sumiki's acid (115) and acetyl Sumiki's acid (116) have been found to have biological activities, inhibiting *Bacillus subtilis.* and *Staphylococcus aureus (concentrations)* (Jadulco *et al.*, 2001,2002; Smith *et al.*, 2000).



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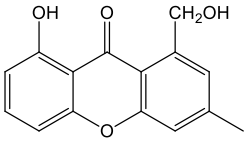

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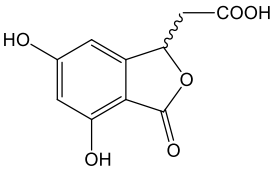

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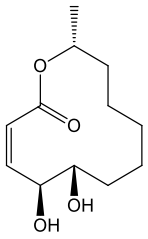

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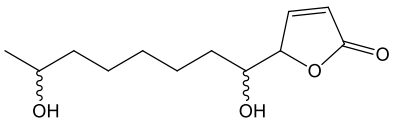

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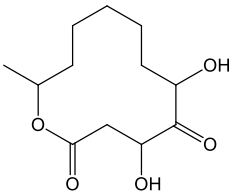

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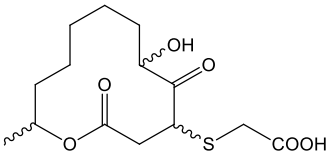

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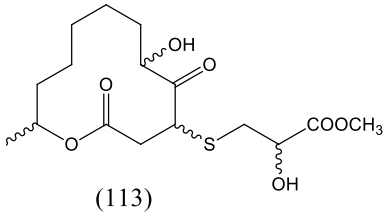

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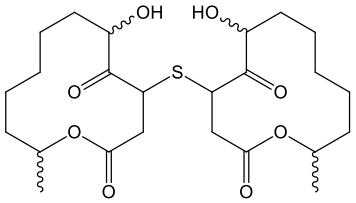

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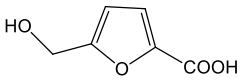


(115)

1.1.2.2. Bacteria

In the case of *Callyspongia diffusa*-associated streptomycetes, Dharmaraj has carried out effective research, especially in the field of large-scale production of their bioactive secondary metabolites through microbial fermentation. In 2009, Dharmaraj successfully fermented a *Streptomyces* strain (AQBWWS1), isolated from the sponge *Callyspongia diffusa,* under fluorescent white light to give a massive yield of carotenoids, a food-grade colour pigment which was commonly produced by streptomycetes. In his on-going study in 2011, another scale-up production was achieved for L-asparaginase, an anti-neoplastic agent used in the chemotherapy of lymphoblastic leukaemia, by submerged fermentation of *Streptomyces noursei* MTCC 10469 using Tryptone Glucose Yeast extract (TGY) broth (Dharmaraj *et al.*, 2011). Another three *Streptomyces* strains found in the sponge *Callyspongia diffusa,* AQBCD03, AQBCD11 and AQBCD24 demonstrated antagonism against fish and shellfish pathogens including *Aeromonas hydrophila*, *Serratia* sp. and *Vibrio* spp.. However, the bioactive substances responsible for these activities still remain to be isolated (Dharmaraj, 2011).

1.1.3 Production methods

The large-scale production of natural products can be achieved using several approaches including synthesis of target molecules, and the large-scale cultivation of the source organisms such as terrestrial plants, and for marine sponges, mariculture in the sea. The compounds isolated from *Callyspongia* sp. that have been prepared by chemical synthesis are summarised in **Table 1.** Regarding mariculture for the purpose of obtaining bioactive secondary metabolites, experiments using this marine sponge have also been carried out. In Voogd's study of the potential of marine sponges for mariculture, *Callyspongia (Euplacella) biru* was the only sponge that exhibited considerable survival and growth rates, which implied appreciable production of the bioactive secondary metabolite amphitoxin (Voogd, 2007). However, his on-going experiments of evaluating the ecological function of amphitoxin indicated an unexpected fact that cultured sponge tissue yields less amphitoxin than those collected from the wild (Voogd, et al., 2005). In the literature to date, only mariculture has only been attempted with *Callyspongia (Euplacella) biru.*

Based on this current review, only two strains from sponge *Callyspongia sp.* were fermented successfully. There are also fewer reports of sponge *Callyspongia* sp. associated microorganisms compared to the other species. Since *Callyspongia* sp. is a big family consisting of an abundance of potential drug sources, the symbiotic or associated microorganisms from these species are still worth investigating, especially *Streptomyces sp.* which are well known for being rich in antimicrobial secondary metabolites. But the two successful cases that were investigated as mentioned above (Dharmaraj *et al.*, 2011) have pioneered and presented an optimistic prospect for the following research on *Callyspongia sp*.

Table 1. List of bioactive compounds isolated from sponge *Callyspongia* sp. and the symbiotic/associated microorganism

Compounds type	Name	Synthesis	Bioactivities
Polyacetylene	callydiyne		
	C20 polyacetylene		Toxic against nauplii of the brine shrimp *Artemia salina* (Braekman *et al*., 2003)
	callytriols A callytriols B callytriols C callytriols D callytriols E		Inducing metamorphosis in the ascidian *Halocynthia roretzi* larvae and antifouling against the barnacle *Balanus amphitrite* larvae (Tsukamoto *et al*., 1997)
	callyspongin A callyspongin B		Inhibiting fertilisation process of starfish (Uno *et al*., 1996) and inducing metamorphosis in the ascidian *Halocynthia roretzi* larvae and antifouling against the barnacle *Balanus amphitrite* larvae (Tsukamoto *et al*., 1997)
	callyberynes A (callypentayne) callyberynes C (callytetrayne)	Yes (Lopez *et al*., 2003; 2006) Yes (Hosoi et al., 2011; Dusza *et al*. 1960)	Inducing metamorphosis in the ascidian *Halocynthia roretzi* larvae (Tsukamoto *et al*., 1997)
	callyberynes B	Yes (Hosoi et al., 2011; Dusza *et al*. 1960)	
	callyspongidiol siphonodiol 14,15-dihydrosiphonodiol		Activating human dendritic cells (Takei *et al*., 1987) and antiproliferative against human promyelocytic leukaemia cells (HL-60) (Umeyama *et al*., 2010)

Compounds type	Name	Synthesis	Bioactivities
Polyacetylene	callyspongynes A		Speculated as chemical defence agents (Rooney *et al*., 1998)
	aikupikanynes A aikupikanynes B aikupikanynes C aikupikanynes D		
	aikupikanynes E aikupikanynes F		Moderately cytotoxic against mouse lymphoma (P-388, ATCC: CCL 46), human lung carcinoma (A-549, ATCC: CL 8), and human colon carcinoma (HT-29, ATCC: HTB 7) (Youssef *et al*., 2010)
	octahydrosiphonochalyne		
	callyspongynic Acid		Inhibiting α- glucosidase (Nakao *et al*., 2003)
	callysponginol sulphate A halistanol sulphates		Inhibiting membrane type 1 matrix metalloproteinase (Fujita *et al*., 2003)
	callyspongamide A		Moderately cytotoxic against HeLa cells (Youssef *et al*., 2003)
Peptides	callyspongidipeptide A compound 31 compound 32		
	phoriospongin A phoriospongin B		Nematocidal against livestock parasite *Haemonchus contortus* (Capon *et al*., 2002)
	callynormine A	Analogues- Yes (Pappo *et al*., 2006)	

Compounds type	Name	Synthesis	Bioactivities
Peptides	callyaerins A		Cytotoxic against tumour cell lines (Zheng *et al*., 2006)
	callyaerins B callyaerins C callyaerins D callyaerins E		
	callyaerins F		
	callyaerins G		Cytotoxic against mouse lymphoma (L5178Y), human cervix carcinoma (Hela) (Ibrahim *et al*., 2008)
	callyaerins H		Cytotoxic against tumour cell lines (Zheng *et al*., 2006)
Alkaloids	a polymeric pyridinium alkaloid		Inhibiting epidermal growth factor receptor (Daviescoleman *et al*., 1993)
	untenines A	Yes (Wang *et al*., 1996)	Anti-microfouling activity (Wang *et al*., 1996)
	untenines B untenines C		
	halitoxin		Selective activity against seawater bacteria, protecting the host sponge from microbial pathogenesis, toxic to mice and sea urchin eggs, general cytotoxicity as well as hemolytic and neurotoxic properties (Kelman *et al*., 2009) (Berlinck *et al*., 1996)
	amphitoxin		
	niphatoxin C		Antagonism against P2X7 receptor (Buchanan *et al*., 2007)

Compounds type	Name	Synthesis	Bioactivities
fatty acids	acid 1 (51) acid 2 (52)		Inhibiting murine leukaemia cell growth (Toth *et al.*, 1994)
	2-methoxytetradecanoic acid 2-methoxypentadecanoic acid 2-methoxyoctadecanoic acid (6Z)-2-methoxy-6-tetradecenoic acid (6Z)-2-methoxy-6-pentadecenoic acid (6Z)-2-methoxy-13-methyl-6-tetradecenoic acid		
Triterpenoids	sipholenol A sipholenol B sipholenol C sipholenol D sipholenol E monoacetate sipholenol F sipholenol G sipholenol H sipholenol I sipholenol J		Reversing P-glycoprotein-mediated MDR to colchicines (Jain *et al.*, 2009)

Compounds type	Name	Synthesis	Bioactivities
Triterpenoids	sipholenol K		
	sipholenol L		
	sipholenol M		
	sipholenone A		Reversing P-glycoprotein-mediated MDR to colchicines (Jain *et al*., 2009)
	sipholenone B		
	sipholenone C		
	sipholenone D		
	sipholenone E		
	compound 2		
	sipholenoside A		
	sipholenoside B		
	siphonellinol (3)		
	siphonellinol B		
	siphonellinol C		
	siphonellinol C-23-hydroperoxide		
	siphonellinol D		
	siphonellinol E		
	neviotine A		
	neviotine B		
	dahabinone A		

Compounds type	Name	Synthesis	Bioactivities
Triterpenoids	akaterpin	Yes (Hosoi *et al*., 2011)	Inhibiting phosphatidylinositol-specific phospholipase C (Fukami *et al*., 1997)
	callyspinol		
	ilhabelanol ilhabrene isoakaterpin		Anti-*Lesihmania* sp. (Gray *et al.*, 2007).
Sterols	isofucosterol	Yes (Dusza *et al*., 1960; Fagerlund *et al*., 1960)	
	24-ketocholesterol	Yes (Khripach *et al*., 2002)	
	24S-24-methylcholesterol	Yes (Li *et al*., 1983)	
	24S -24 methyl-cholestane-3β,5α,6β,25-tetraol-25-mono acetate (4a) compound 98 compound 100 compound 101		Anti-malaria parasite (Rao et al., 2010)
polyketide	callystatin A		Potent cytotoxicity (Dias *et al*., 2005)

1.1.4 Summary

For the purpose of supplying a clear guide of up-to-date research on marine sponge *Callyspongia* sp. in the aspect of its medicinal applications, a summary of published bioactive secondary metabolites was summed up into **Table 1** including their corresponding bioactivities and synthetic conditions. Two pie graphs depicting the compounds' structural distribution (**Fig. 1.1**) and the distribution of compounds isolated from studied sponges including those which species are unknown but the location from which the sponge was taken was stated (**Fig. 1.2**) were also produced to offer a potential research perspective. There were more than 105 bioactive secondary metabolites reported in sponge *Callyspongia* sp. up to 2012 that can be categorised into seven main types, of which terpenes occupy the majority with 34% followed by 27% for alkaloids. (**Fig. 1.1**) Sponge *Callyspongia siphonella* was currently one species that has been isolated the most of compounds and all the 30 compounds are triterpenes. Most of them possess certain bioactivities, such as Reversing P-glycoprotein-mediated Multi-Drug-Resistance to colchicines which was used in the treatment of rheumatic diseases, and being inhibitory towards parasite *Leishmania* spp. Other sponges were generally reported as having one or two bioactive compounds discovered (listed above), however, hinting at a promising research direction. (**Fig. 1.2**)

Additionally, regarding the sponge-derived symbiotic microorganisms, the study towards sponge *Callyspongia* sp. is only the tip of the iceberg. As what we have surveyed above, no more than 10 microorganisms possessing pharmacological bioactivities were screened. Herein, the bacteria mostly belong to the order Actinomycetales which have already shown a large metabolic capacity in the cases of other marine sponges. (Tabares *et al*., 2011; Li *et al*., 2011; Wei *et al*., 2011; Engelhardt *et al*., 2010; Motohashi *et al*., 2010) Hence, isolation and inoculation of this particular bacterial order will help researchers to narrow down their heavy screening work and continues to be one of the key strategies in potential drug investigation. Still, the rich diversity of marine sponges of genus *Callyspongia* sp. may indicate a more abundant source of the bioactivity-associated microorganisms. Therefore, further research in these aspects is of great value and is highly advocated.

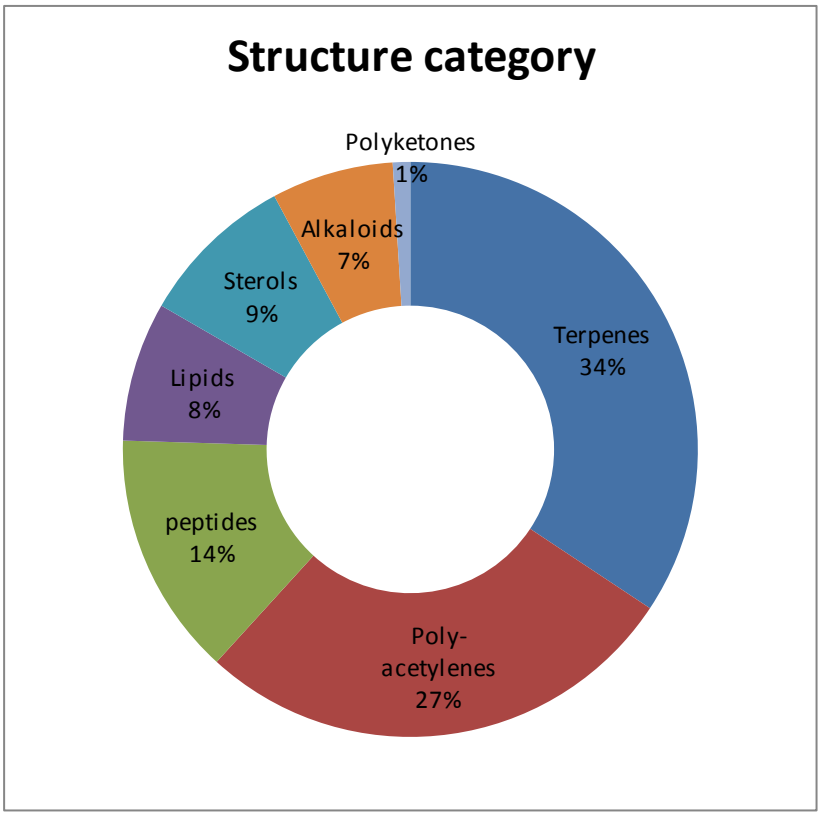


Fig. 1.1 Distribution of the structure types of isolated compounds from *Callyspongia* sp.

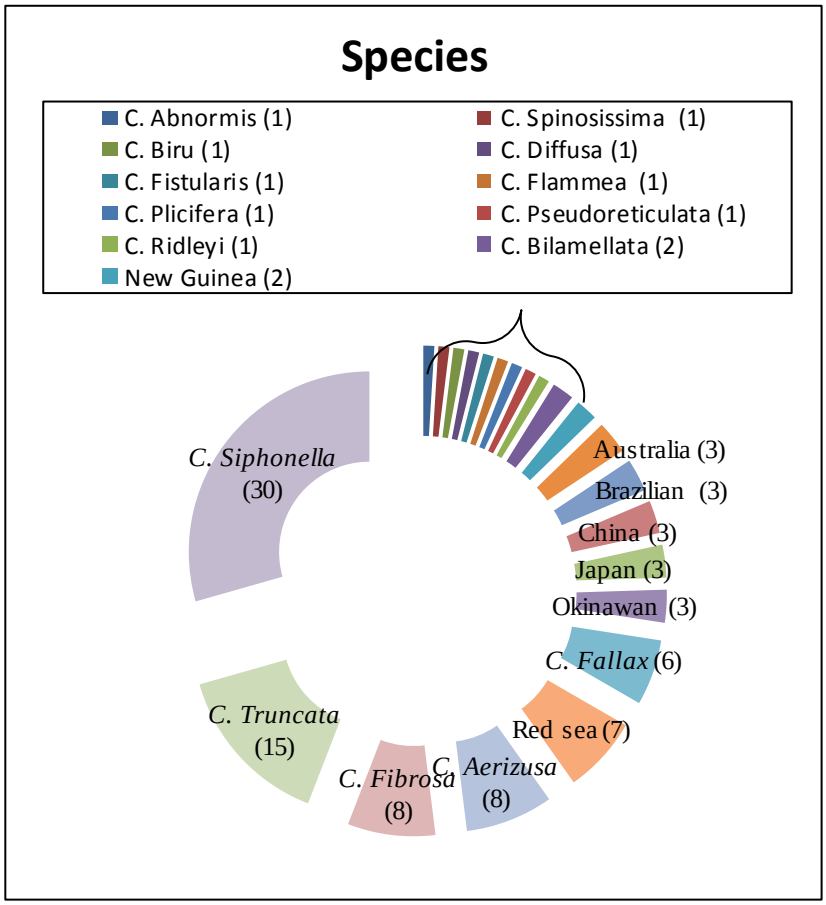
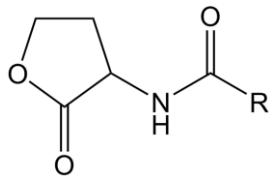


Fig. 1.2 Distribution of the numbers of the compounds isolated from studied sponge species

1.2 Quorum sensing

The process of chemical communication between bacteria by producing and detecting the accumulation of a series of small signal molecules which are secreted into their surrounding environment is known as 'quorum sensing' (QS). This cell-to-cell communication in the simplest form helps bacteria to regulate their 'population', the so called 'quorum' to survive under different environmental conditions. With increasing research in this area, the QS molecules were found to not only play a part in the cell population regulation, but also are involved in a series of bacterial physiological activities, such as biofilm formation and persistence, virulence, bioluminescence and antibiotic production. (Bassler *et al*., 2006)

One class of QS molecules are the *N*-Acyl homoserine lactones which contain different N-acyl side chain lengths varying from C4-C14. These QS molecules are typically made by Gram-negative bacteria. They are produced by the protein LuxI and can freely cross the cell membrane. If these molecules accumulate to a high concentration, the LuxR, a transcription factor, will bind them to form LuxR-AHL complexes. The complex finally binds to a particular DNA region to activate further gene expression. The best-known example in Gram-negative bacteria is in *Vibrio* (*Aliivibrio*) *fischeri* which uses 3-oxo-C6-HSL to activate bioluminescence when the population of the cells accumulate to a certain level. **Fig. 1.3** illustrates the bioluminescence mechanism of *V. fischeri*. (QS in Vibrio fischeri, http://www.nottingham.ac.uk/quorum/fischeri3.htm1)



N-Acyl homoserine lactones

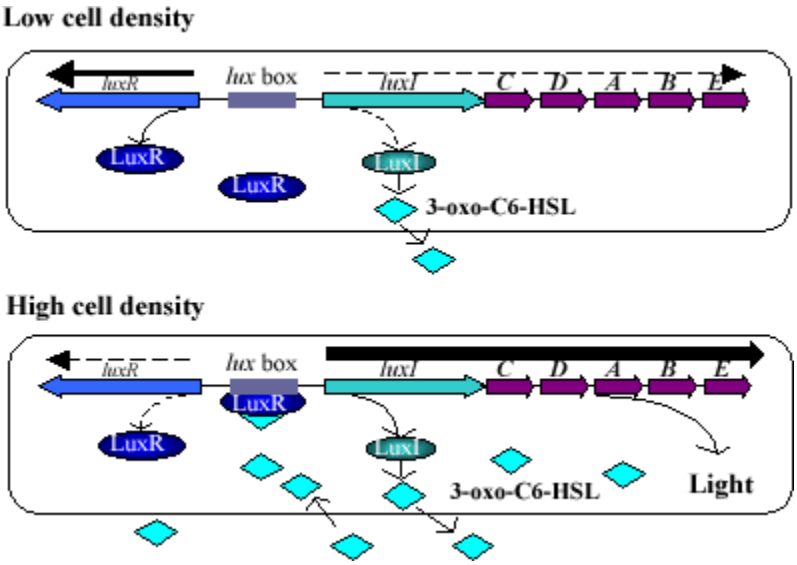


Fig.1.3 Molecular mechanism of QS in *V. fischeri*
(http://www.nottingham.ac.uk/quorum/fischeri3.htm)

Recently, AHLs were also reported to play important roles in activating the production of antimicrobial factors that inhibit the growth of other bacteria in response to interspecies competition. (Chandler *et al.*, 2010) Modifying the activity of gene expression through modulating the activity of AHLs may be of significant benefit in activating the production of antibiotics, which offers a possible approach for developing novel biotechnologically-useful products.

1.3 Aim of the work

The general purpose of the work was to investigate the secondary metabolites of marine organisms and their associated microorganisms.

The first aim (Chapter 2) was to isolate the bioactive compounds (especially with anti-tuberculosis potential) from the marine sponge *Callyspongia aff. implexa* which was collected from the Red Sea, assisted by bioassay-guided fractionation, employing high resolution NMR and MS for structure elucidation of the compounds.

The second aim (Chaper 3) was to dereplicate the secondary metabolites from a bioactive sponge-associated bacterium strain *Actinokineospora* EG49 which was isolated from the marine sponge *Spheciospongia (Callyspongia) vagabunda* using high resolution MS data. Bio-assay screening including general pathogenic bacteria and trypanosomes were carried out on *Actinokineospora* EG49 extracts.

The third aim (Chapter 4) was to use high resolution MS to investigate the AHL signalling compounds which are difficult to visualise using the TLC plate bioassay in bioluminescent *Vibrio* strains collected from the coastal waters of the UK, including some associated with bryozoans. Multivariate data analysis was carried out to highlight the possible relationships between the quorum sensing signals and the cryptic secondary metabolites in the complicated biological system.

Chapter 2 Isolation and identification of bioactive compounds from the Marine Sponge *Callyspongia aff.implexa*

2.1 Materials and Methods

2.1.1 General procedure for fractionation and isolation

The freeze-dried sponge collected from the Red Sea was extracted with equal volumes of MeOH and DCM. The crude sponge extract (65 g) was obtained from Dr Usama Ramadan Abdelomohsen (University of Würzburg).

Several chromatographic techniques were employed for the isolation and purification work, including, size exclusion chromatography, MPLC and preparative HPLC. TLC was used as a routine monitoring procedure to check for complexity and purity of the fractions.

2.1.1.1 Thin layer chromatography

This technique was used to predict the presence of compounds in the crude extracts and fractions, to determine the eluting system to be used in column chromatography, to monitor the There were two types of TLC plates used in this chapter: silica performance of further separation processes and to check the purity of isolated compounds.

gel 60F_{254} (Aluminium Sheets) and silica gel 60 RP-18 F_{254} (Aluminium Sheets) which were both purchased from Merck (Germany). Samples were dissolved in appropriate organic solvents and then spotted approximately 1 cm (1 cm above the bottom edge) of the TLC plate. The mobile phase was a binary solvent system usually with miscible solvents of different polarities mixed in varying proportions. The developing length was 5 cm. Developed plates were initially examined under short (254 nm) and long (366 nm) wavelength ultraviolet (UV) radiation to detect UV active compounds. Aromatic compounds can be visualised under short UV wavelength while long UV wavelength could be used to visualise some alkaloids. After marking the UV active compounds, the TLC plate was then sprayed with the anisaldehyde-sulphuric acid reagent and heated with a heating gun at 170 ℃ to see non-UV active compounds in various colours.

2.1.1.2 Size exclusion chromatography (SEC)

Size exclusion chromatography, also known as gel filtration, molecular sieve or gel exclusion chromatography, is a separation technique using a macroporous absorption resin as the packing material to sieve sample molecules based upon their size and shape. Diaion HP20, one of the common macroporous absorption resins, is a highly porous styrenic adsorbent resin with relatively large-sized pores and is usually used in the desalting and decolorization of natural products. Here we used diaion HP20 to remove the sea salts from the marine sponge extract.

Dry loading was applied by mixing the whole crude extract (dissolved in methanol) in a sufficient amount of diaion HP20 and leaving it to dry overnight. The mixed sample was loaded into an open column filled with diaion HP20 that had been activated with methanol. The sample was fractionated into 15 fractions by elution with a gradient of decreasing polarity. (**Fig 2.1**)

2.1.1.3 Flash chromatography

Flash chromatography, also called Medium Pressure Liquid Chromatography (MPLC) uses the same principle as open column chromatography, but applies artificial pressure to drive the solvent through the stationary phase to get a rapid or "flash" separation with relatively higher resolution. The equipment used were purchased from Buchi (Oldham, UK) and Grace (Deerfield, US). Bioassay-guided fractionation was applied for further separation and purification. The selection of the column size and solvent system depended on the amount of the sample and how compounds eluted on the TLC Plate. The compounds of interest should have an Rf value between 0.15~0.20 in the solvent system used. Columns used in this work were silica gel columns purchased from Buchi (Oldham, UK) and Grace (Deerfield, US). The solvent systems were binary with polarity increasing if gradient elution was required.

2.1.2 General procedure for NMR experiments

JEOL (JNM LA400) 400 MHz and Bruker 400 and 500 MHz instruments were used. NMR tubes for routine NMR experiments were purchased from NORELL US. Shigemi 5mm symmetrical NMR microtubes use with small amounts of samples were purchased from SIGMA-ALDRICH, US. Samples were dissolved in deuterated solvents of about 0.6 mL for the normal NMR tubes and 0.2 mL for the Shigemi NMR Tubes.

Fractions were analysed initially by one dimensional ^{1}H NMR. Pure compounds were usually analysed by 1D and 2D NMR including ^{1}H, ^{13}C, COSY (proton correlation spectroscopy), TOCSY (total proton correlation spectroscopy), HMBC (heteronuclear multiple bond correlation) and HMQC (heteronuclear multiple quantum correlation). Known metabolites and partial structures of new compounds were compared with spectral data from the literature.

2.1.3 General procedure for dereplication study using MS data

Extracts (1mg/mL of mixture usually in MeOH) were analysed on an Accela HPLC from Thermo Scientific (Bremen, Germany) combined with Exactive (Orbitrap) mass spectrometer from Thermo Fisher Scientific (Bremen Germany).The column attached to the HPLC was HiChrom, ACE (Berkshire, UK) C_{18}, 75 mm × 3.0 mm, 5 µm column. The mobile phase consisted of purified water (A) and acetonitrile (B) with 0.1% FA in each solvent. The gradient started with 10% B linearly increased to 100% B at a flow rate of 300 µL/min within 30 min and remained isocratic for the next 5 min before linearly decreasing back to 10% B for the following 1 min. The mobile phase was then equilibrated for 9 min before the next injection and the total analysis time for each sample was 45 min. The injection volume was 10 µL and the tray temperature was maintained at 12 ^{o}C with the column temperature at room temperature?

High Resolution mass spectrometry was carried out in both positive and negative ionisation modes with a spray voltage at 4.5 kV and capillary temperature at 320 ^{o}C. The mass range was set from *m/z* 150-1500 for ESI-MS.

Raw data were initially sliced into negative and positive data sets by RealOffice tool from the Xcalibur software package (Thermo Scientific, the UK). The sliced data sets were imported into MzMine2.6, a framework for differential analysis of mass spectrometry data, for data processing. The picked peaks were exported and searched in the Marinlit database which was updated in Feb 2012 for dereplication. The picked peaks shown in the following tables in results and discussion were double checked with MS raw data in Xcalibur2.1.

2.1.4 Bioassay

The bioassays, including the anti-trypanosomal and anti-bacterial assays, were all performed using the oxidation-reduction (REDOX) indicator Alamar blue to visualise the sample activity. Alamar blue, also called resazurin, is a blue dye which can be reduced into the bright pink fluorescent resorufin once the sample is active in killing the pathogen. The fluorescence value is measured under the excitation and emission wavelengths of 560 nm and 590 nm respectively and used for evaluating the potency of the sample.

For early-stage bioassay experiments for the purpose of screening for bioactive factions or compounds, the test was carried out by calculating the % difference of the fluorescence value

between the blank (DMSO) and the test sample. Gentamycin was used as positive control for the anti-bacterial test and suramin was used for the anti-trypanosomal test. The smaller the % difference is, the more active the samples are. The concentrations of the drugs (positive control) in different tests were tabulated in **Table 2.1**. DMSO was used as the solvent and the concentration for the extracts/fractions and single compounds are 10 mg/mL and 10 mM respectively.

The main biotest procedure used for the fractions from marine sponge *Callyspongia aff. implexa* was against *Mycobacterium marinum* (strain ATCC BAA-535) which infects fish with fish tuberculosis and closely resembles *Mycobacterium tuberculosis*. The reason of using *M. marinum* instead of *M. tuberculosis* in early-stage research is two-fold. The growth period of *M. marinum* is shorter than that of *M. tuberculosis*, which allows the higher throughput of samples. Additionally, *M. marinum* is less potent compared to *M. tuberculosis* in affected humans. This makes it safer to handle the bacteria in general BioSafetyLevel-2 laboratories.

In addition, for the purpose of bioactivity screening, SMRSA 106, EMRSA 16 and *Escherichia coli* were also tested on the initial fractions which were obtained through size exclusion chromatography. All the tests were carried out by Ms Carol J. Clements (University of Strathclyde).

Table 2.1 Antibiotic positive controls for anti-microbial and anti-trypanosomal bioassays

Test	Drug (Positive control)	Drug concentration
***M.marinum* (ATCC BAA535)**	Gentamycin	100 µg/ml
SMRSA 106	Gentamycin	200 ug/ml
EMRSA 16	Gentamycin	200 ug/ml
***E.coli* (ATCC 8739)**	Gentamycin	500 µg/ml
***P.aeruginosa* (ATCC 27853)**	Gentamycin	500 µg/ml
***K.pneumoniae* (ATCC 13883)**	Gentamycin	500 µg/ml
***T.b.brucei* (S427)**	Suramin	20 µg/ml

2.2 Results and discussion

2.2.1 Bioassay-guided separation

Tuberculosis (TB) is an infectious disease usually caused by the strain *Mycobacterium tuberculosis* which currently infected one third of the world's population. It is a curable disease but still give rise to a high fatality. Nearly 1.4 million deaths in 2010 were reported by the World Health Organisation (Liu *et al*., 2012). Since standard anti-TB drugs have been used for decades,

the resistance towards single drugs turned up years ago. However, because of the unappreciated treatment and incorrect use of anti-TB drugs in primary cases, multidrug-resistance towards, at least, isoniazid and rifampicin which are the two most powerful first line anti-TB drugs was growing and currently found in about 0.3 million notified TB patients in the world in 2011 (World Health Organisation 2011). Even although multidrug-resistance Tuberculosis is treatable and curable by applying second-line drugs the therapeutic schemes are still limited and second-line drugs are not always available. The discovery of novel anti-TB candidates is therefore important if we are to effectively treat TB.

The methanolic crude extract was initially fractionated by size exclusion chromatography using Diaion HP20 as the stationary phase and H_2O and methanol as the mobile phase with a step gradient from 100 % H_2O to 100% methanol followed by column flush with acetone and methanol. (**Fig. 2.1**) Fractions 10 to 15 showed potential inhibitory effect against *M. marinum* with % of control value from 0.5 to 6.5. All the fractions were inactive against SMRSA 106, EMRSA 16 and *E.coli* ATCC 8739. (**Table 2.2**) The values shown in the four assays seem to be reproducible except for those investigated in the anti-*M. marinum* bioassay. But one thing is certain, inhibitory activity was found in the relatively less polar factions from Fraction 10 to Fraction 15 in which most of the sea salts had been removed. Fraction 12 was initially picked for further separation and resulted in the separation of three compounds including β-Sitosterol, A37-2-B1, A45-3-3. However, the low recovery rate led to low output of the single compounds, which made it more difficult to generate a highly resolved NMR spectrum for structure elucidation. Therefore, Fractions 10 to 15 were pooled for further separation using HPLC and 28 secondary fractions were generated. The 28 secondary fractions were tested again against *M. marinum*. (**Table 2.3**) Since the limited practical time, further separation was paused but dereplication study was performed to investigate the potential bioactive secondary metabolites. Because of the limited amounts, the three compounds which were separated from Fractions 12 were not able to test for anti-*M. marinum* bioassay. In order to know if they have possible anti-*M. marinum* activity, these three compounds were check back on TLC by comparing the Rf values between the bioactive secondary fractions and themselves. A37-2-B1 and A45-3-3 were both found present in the bioactive fractions, which suggested that A37-2-B1 and A45-3-3would possibly anti-*M.marinum* active.

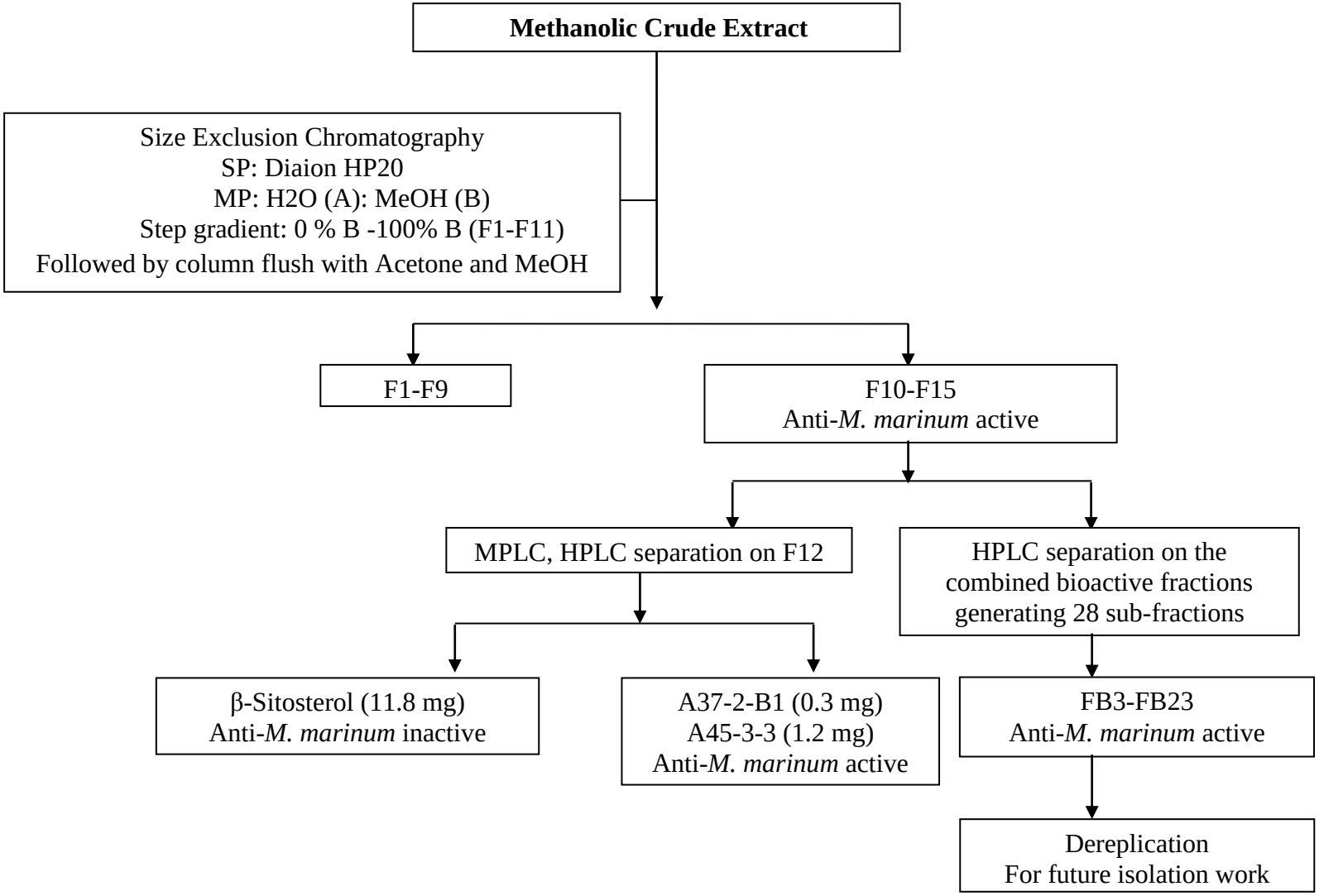


Fig. 2.1 Separation procedure of Sponge *Callyspongia aff.implexa*. extract

Table 2.2 Bioassays for the initial fractions collected from size exclusion chromatography

	M. marinum **ATCC BAA535** **% control**	**SMRSA 106** **% control**	**EMRSA 16** **% control**	***E. coli*** **ATCC 8739** **% control**
F1	102.8	101.3	103.9	110.2
F1	108.2	102.6	102.2	107.9
F2	125.2	103.2	102.5	109.4
F2	108.5	102.9	102.6	109.9
F3	105.4	105.0	99.7	108.2
F3	106.4	103.0	103.7	108.7
F4	97.7	104.7	102.5	108.1
F4	214.9	104.5	102.1	109.5
F5	116.2	105.1	101.1	109.9
F5	161.6	101.7	101.3	107.1
F6	148.7	100.5	100.3	99.0
F6	124.2	98.2	100.9	86.6
F7	135.9	102.2	101.4	88.9
F7	114.7	100.6	100.9	86.3
F8	100.5	103.8	100.3	87.3
F8	132.4	103.5	100.9	83.2
F9	80.5	103.7	101.7	81.4
F9	84.0	104.6	102.9	87.3
F10	1.1	107.3	105.4	92.6
F10	47.3	104.8	106.0	89.7
F11	1.1	109.2	108.2	94.6
F11	2.3	109.5	108.0	83.7
F12	15.4	109.0	107.7	82.1
F12	0.5	110.1	107.7	80.4
F13	83.7	106.2	105.6	82.0
F13	2.8	108.0	106.7	82.4
F14	1.2	107.0	107.1	82.4
F14	4.2	109.0	104.7	80.8
F15	17.8	107.1	96.5	103.5
F15	6.5	107.3	88.8	95.4

Table 2.3 Anti-*M. marinum* bioassay for the secondary fractions from the pooled Fraction 10-15

Secondary fractions	*M.marinum* (ATCC BAA535) % control
FB1	137.9
FB2	14.9
FB3	7.9
FB4	1.6
FB5	12.7
FB6	4.7
FB7	0.3
FB8	6.1
FB9	1.4
FB10	-1.3
FB11	-1.0
FB12	3.2
FB13	0.5
FB14	0.2
FB15	-0.5
FB16	-0.5
FB17	6.2
FB18	-0.3
FB19	-0.1
FB20	-1.2
FB21	-0.3
FB22	-0.1
FB23	-0.2
FB24	39.0
FB25	49.5
FB26	54.4
FB27	79.9
FB28	60.9

2.2.2 Dereplication study and bioassay-guided fractionation

Dereplication using MS data was carried out on the bioassay-guided secondary fractions by searching the major compounds (>E07 ion peak area) in the Marinlit database. **Table 2.4** showed the possibly present compounds in the fractions. Since there was a number of options for those compounds which have the same predicted formula, it was difficult to state all the possible compounds in this part. Compounds with reasonable isolated sources, for instance the plant or marine organisms, were preferentially tabulated in **Table 2.4**. However, there were still a great number of identified compounds sourced from various microorganisms including bacteria and fungi. These compounds were grouped into the unidentified part in **Table 2.5** with the predicted formula within 5 ppm mass tolerance for the dereplication investigation of the secondary metabolites from this sponge.

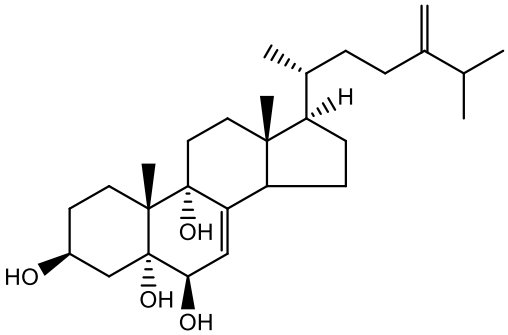
A compound which was given the molecular mass of 446.3391 was found in both negative and positive mode indicating the possible presence of a polyhydroxylated sterol "24-methylene-5a-cholest-7-ene-tetraol" which was previously isolated from marine sponge *Porifera Spongia officinalis*. (Migliuolo *et al*., 1990) It is also obvious that most of the other identified compounds are triterpenoids which reveal a fact that this sponge is in rich of terpene compounds. These type of compounds have been reported to have anti-TB activities. Recently in 2012, two new lanostane triterpenes astraodoric acids A and B which were isolated from the Thailand plant *Edible Mushroom Astraeus odoratus* were found to exhibit moderate anti-TB activities (Arpha *et al*., 2012). Another triterpene namely oleanolic acid which was isolated recently from plant *Lippia lacunose* was also found to inhibit *M. tuberculosis* but inactive against rifampicin resistant strains (Castellar *et al*., 2011). From a Papua New Guinea plant *Rhus taitensis*, the triterpnene namely tetrahydroxysqualene was isolated and shown anti-TB activity with an MIC of 10.0 µg/mL. (Noro *et al*., 2008) Two known triterpenes, 3R-hydroxylup-20(29)-en and cycloartanol from the plant *Sapium haematospermum* were also found to inhibit *M. tubeculosis* with MIC of 13.4, and 8 µg/mL respectively (Woldemichael *et al*., 2004)

By reviewing the literature, saringosterol and its isomers were found previously reported the anti-TB activity with MIC of 0.25, 1, 0.125 µg/mL respectively. This compound was also successfully synthesised (Wächter et al., 2001). Other identified compounds from Marinlit database haven't been reported any anti-TB activities. The anti-*M. marinum* activity of this

sponge may be attributed to the abundant presence of triterpenes. This also suggests a strategy to focus on the separation of triterpenes compounds.

Table 2.4 Identified compounds from the bioassay-guided fractions from the database Marinlit

	Polarity	MS *m/z*	MW	Chemical Formula	Name	SOURCE
2.1	N/P	447.3467	446.3391	C28H46O4	24-methylene-5a-cholest-7-ene-tetraol	*Porifera Spongia officinalis*
	N/P	445.3327	446.3391	C28H46O4	24-methylene-5a-cholest-7-ene-tetraol	*Porifera Spongia officinalis*
2.2	N	295.2282	296.2346	C18H32O3	(13R,5E,9E)-13,14-Dihydroxy-6,10, 14-trimethylpentadeca-5,9-dien-2-one	[A] Marine brown algae *Cystoseiraceae*
2.3					Moniliforminol A	[A] marine brown alga *Cystophora moniliformis*
2.4					Moniliforminol B	[A] marine brown alga *Cystophora moniliformis*
2.5	P	399.3259	398.3179	C27H42O2	3beta-Hydroxy-cholesta-5,25-dien-24-one	Sponge *Haliclona oculata*
2.6					5beta-Cholest-3-ene-7,11-dione	[A] red alga *Hypnea musciformis*
2.7					6alpha-Hydroxy-cholesta-4,22-diene-3-one	[A] marine red alga *Hypnea musciformis*
2.8	N	407.3535	408.3598	C26H48O3	Ficulinic acid A	Sponge *Ficulina ficus*
2.9	P	429.3731	428.3649	C29H48O2	Isodecortinol	[A] marine green alga *Codium arabicum*
2.10					Saringosterol	[A] *Cystoseira barbata*
2.11					Varninasterol	[A] brown alga *Spatoglossum variabile*
2.12					24S-Stigmasta-4,25-diene-3beta,6beta-diol	[A] alga *Laurencia majuscula*
2.13					Decortinol	[A] marine algae *Codiaceae*
2.14					Spatosterol	[A] *Spatoglossum variabile*
2.15					Stigmastadiendiol deriv.	[A] alga *Laurencia majuscula*
2.16					24R,28R-Epoxy-24-ethylcholesterol	[A] brown alga *Sargassum carpophyllum*
2.17					3beta-28x-Dihydroxy-24-ethylcholesta-5,23Z-diene	[A] marine brown alga *Ishige okamurae*
2.18	N	457.2967	458.3027	C28H42O5	1-Demethyl-zosterdiol A	[A] Marine brown alga *Cystoseira* sp.
2.19	P	473.3632	472.3547	C30H48O4	7-Acetoxy-24-methylenecholest-5-ene-3,7,16-triole	[An] soft coral *Sinularia dissecta*



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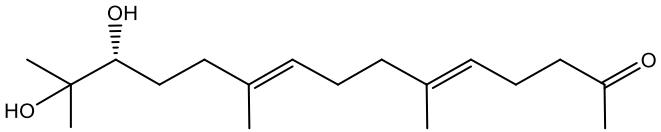

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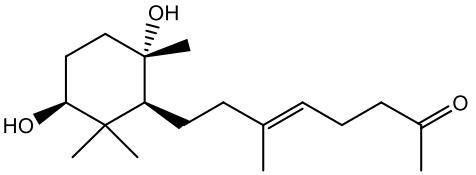

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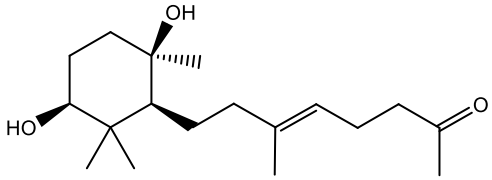

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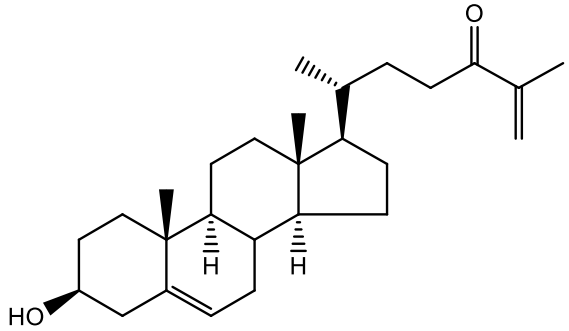

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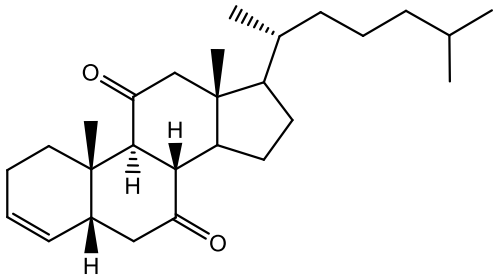
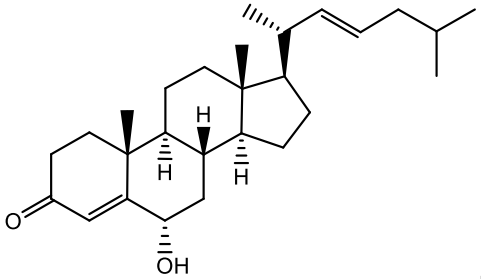


2.6



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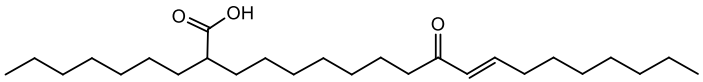

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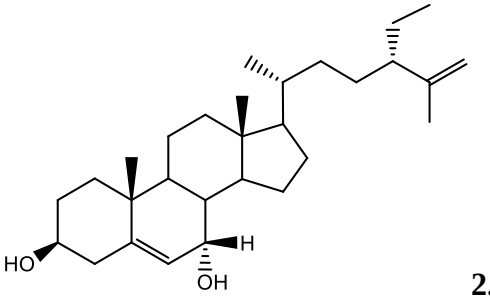

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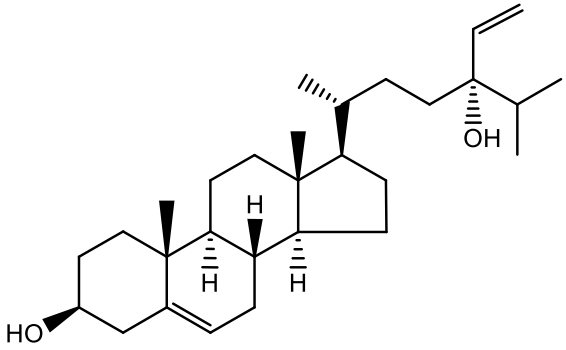

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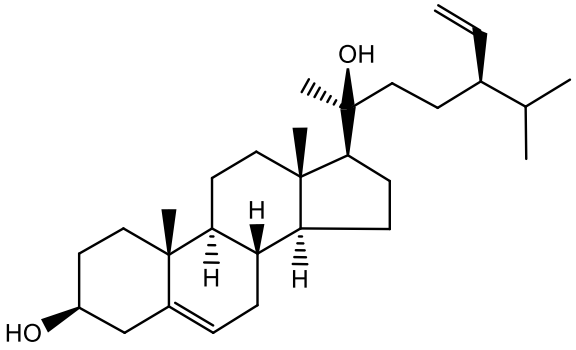

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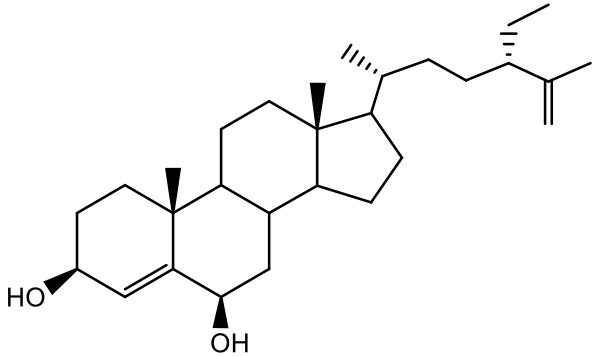

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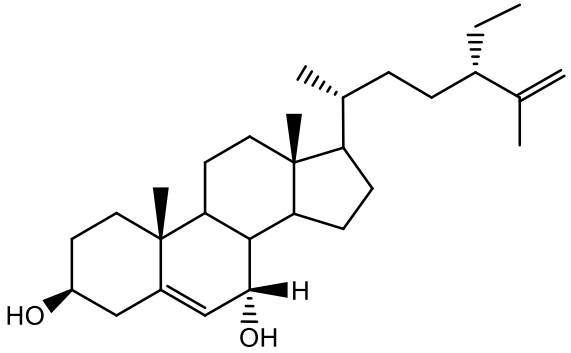

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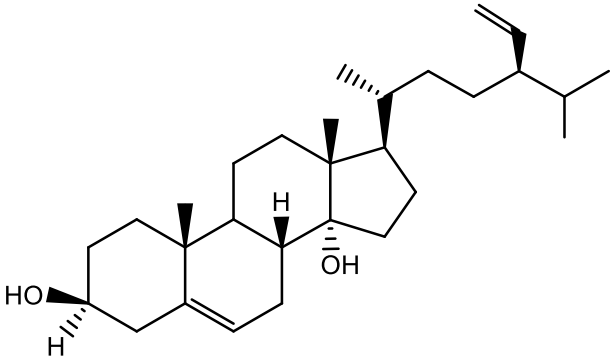

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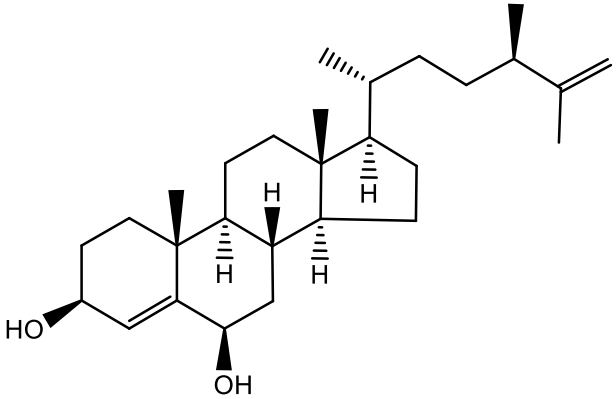

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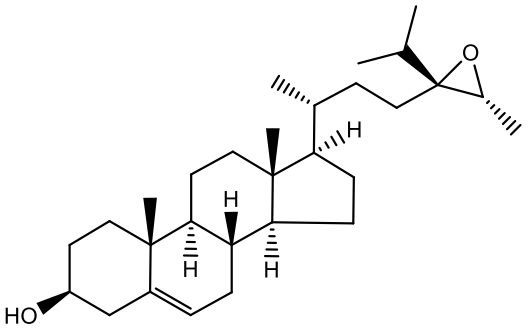

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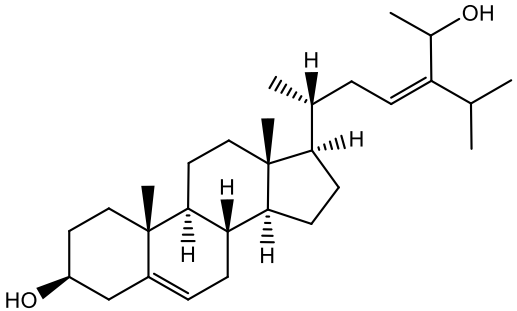

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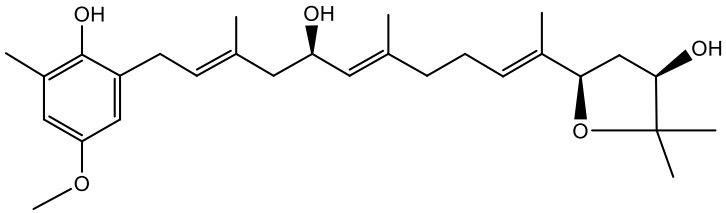

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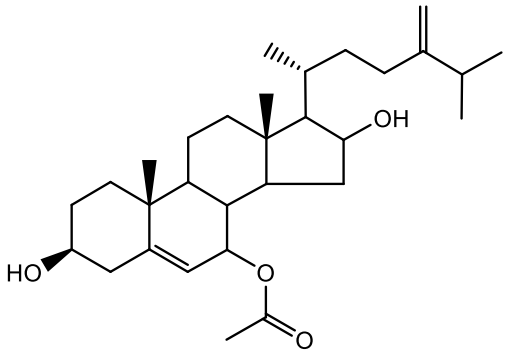


2.19

Table 2.5 Unidentified compounds of the bioassay-guided fractions.

Negative *m/z*	Rt (min)	Peak Area	Predicted Formula	Tolerance (ppm)
229.1447	7.34	6.42E+08	C12H22O4	0.513
295.2282	11.94	7.51E+08	C18H32O3	0.447
339.1606	8.44	4.32E+07	C21H24O4	1.732
339.2909	14.63	8.34E+08	C21H40O3	0.624
351.197	10.54	4.61E+07	C23H28O3	1.287
353.3064	16.27	8.53E+08	C22H42O3	1.287
395.2807	12.11	6.63E+08	C23H40O5	1.018
407.3536	25.11	1.54E+08	C26H48O3	1.329
415.286	18.75	1.56E+08	C26H40O4	1.51
443.281	11.58	1.26E+09	C27H40O5	0.005
445.3327	19.83	3.47E+08	C28H46O4	2.328
457.2967	12.69	1.94E+09	C28H42O5	0.967
Positive *m/z*	**Rt (min)**	**EG49 AE**	**Predicted Formula**	**Tolerance (ppm)**
199.1326	7.83	7.18E+08	C11H18O3	-0.227
238.1439	5.3	6.56E+07	C13H19NO3	-4.228
242.2477	14.48	3.34E+08	C15H31NO	0.473
277.1798	7.84	5.67E+07	C17H24O3	-3.889
292.1906	7.19	1.78E+08	C17H25NO3	-4.131
304.264	11.24	2.25E+08	C20H33ON	1.804
308.1234	12.14	6.08E+08	C14H17N3O5	-2.035
313.201	7.84	1.17E+08	C17H28O5	0.03
318.2793	12.29	1.10E+08	C21H35NO	0.593
346.2169	12.14	9.39E+07	C24H27ON	0.921
347.2585	9.45	4.42E+08	C22H34O3	1.09
349.2008	7.52	5.24E+08	C20H28O5	1.602
358.238	8.39	1.05E+08	C22H31NO3	1.032
364.2638	12.18	5.10E+08	C25H33NO	2.495
366.3369	16.24	3.32E+08	C23H43NO2	0.611
371.3266	7.31	2.54E+08	C21H42O3N2	-0.511
372.2904	11.29	4.12E+08	C24H37O2N	1.784
379.2844	12.08	7.33E+07	C23H38O4	0.511
380.259	10.29	6.60E+07	C25H33NO2	1.563
385.2741	8.78	3.38E+07	C25H36O3	1.008
406.3106	14.81	3.75E+08	C28H39NO	0.267
410.3051	10.79	3.04E+07	C27H39NO2	0.229
413.3159	13.89	1.85E+08	C26H40N2O2	-0.448
426.3006	8.7	1.63E+08	C27H39NO3	0.632
427.2134	8.77	4.55E+07	C26H26N4O2	1.656
447.3467	19.79	8.44E+07	C28H46O4	-0.461
471.3474	8.97	5.92E+07	C30H46O4	1.302
517.375	11.49	3.39E+07	C29H49O4N4	0.324
597.4341	23.09	1.29E+08	C34H60O8	3.678

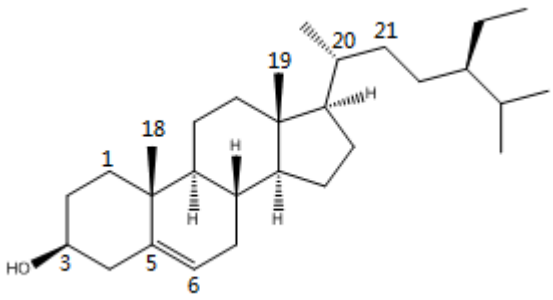
2.2.3 Structure elucidation

Three compounds isolated from the primary bioactive Fraction 12 were checked back using TLC plate to be present in the secondary bioactive fractions, indicating a possibility that these isolated compounds may contribute to the anti-*M.marinum* activity except β-sitosterol which is commonly known inactive sterol.

2.2.3.1 Sitosterol

The molecular ion peak was afforded by GC-EIMS as *m/z* 414.34 with the predicted formula of $C_{29}H_{50}O$ from the online NIST database. In the ^{1}H NMR spectrum (**Fig. 2.2**), the signals from δ 0.6 to 2.4 are quite busy, which indicated the sterol structure. Therefore, the doublet at δ 5.34 and the multiplet at δ 3.52 with ^{1}H integration of one proton each were assigned to H-6 and H-3, respectively. Since the integration of the signal at δ 5.14 was only half compared of H-6, this signal was considered to be an impurity or belonging to another compound rather than an olefinic proton which may be present in the side chain of a sterol structure. The methyl doublet-like signal at 0.68 was integrating for four protons. This actually represents two methyl singlet resonances from two compounds as it did not show the typical COSY correlation. The doublet-like signals gave a coupling constant of 5.9 Hz instead of the expected 6.5 Hz signal. This methyl singlet at 0.68 is characteristic for CH_3-18.

In the ^{13}C NMR spectrum (**Fig. 2.3**), the carbons at δ 140.84 and 121.81 were respectively assigned to C-5 and C-6. C-3 was attributed to the signal at δ 71.90. Other proton signals without any overlap were assigned accordingly by HMQC. By comparing the ^{1}H and ^{13}C spectra of β-sitosterol (**Table 2.6**) which gave similar data assignments, this compound was identified as β-sitosterol.



β-sitosterol

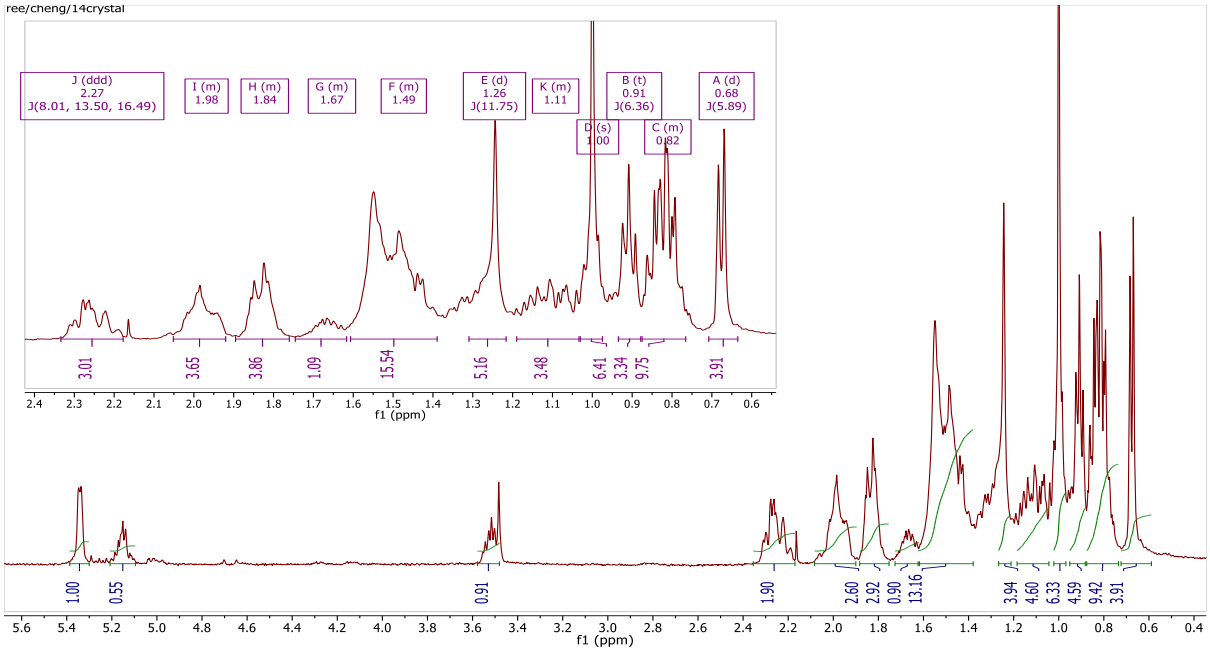


Fig. 2.2 ^{1}H NMR spectrum of isolated β-sitosterol

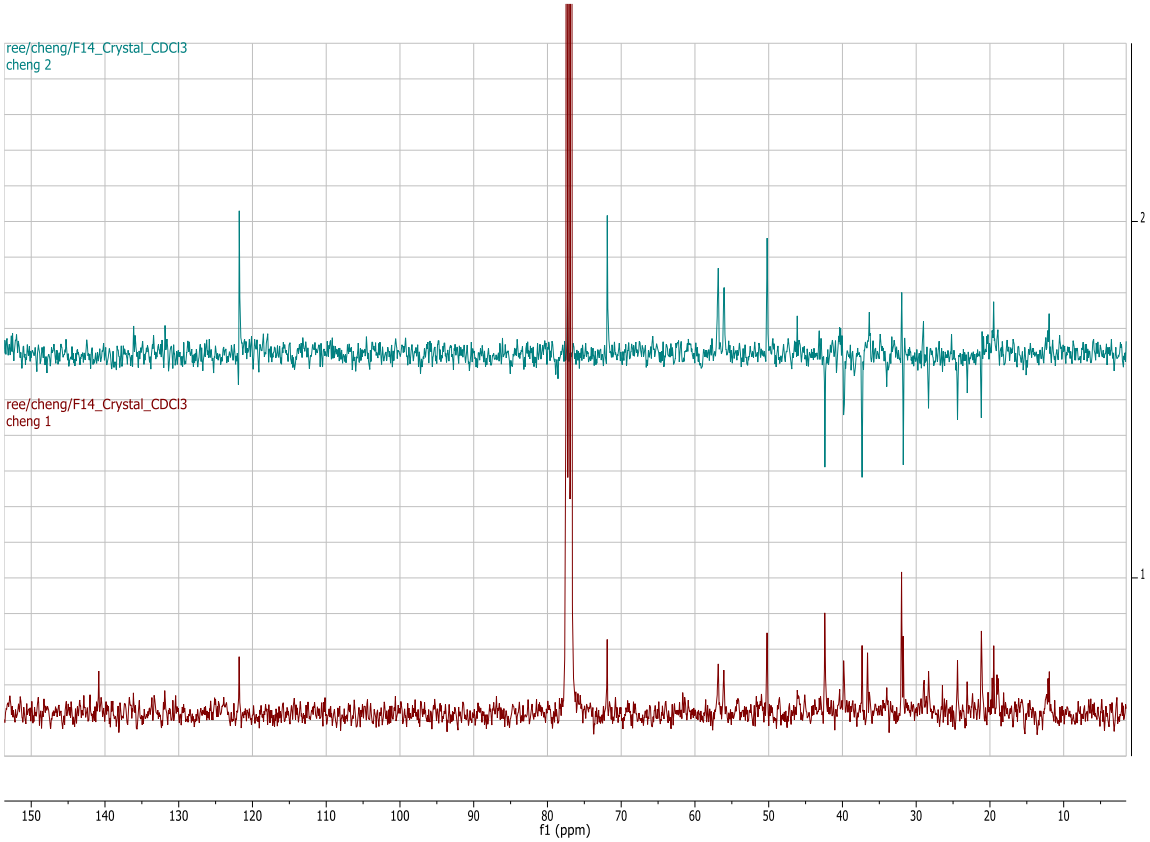


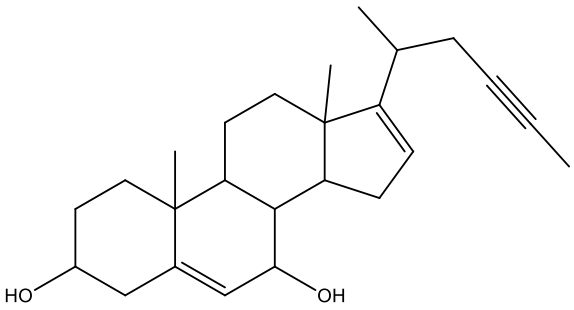
Fig. 2.3 ^{13}C and 135º DEPT NMR spectrum of the isolated β-sitosterol

Table 2.6 NMR spectrum of the isolate sitosterol and reference β-sitosterol (Pateh *et al*., 2009)

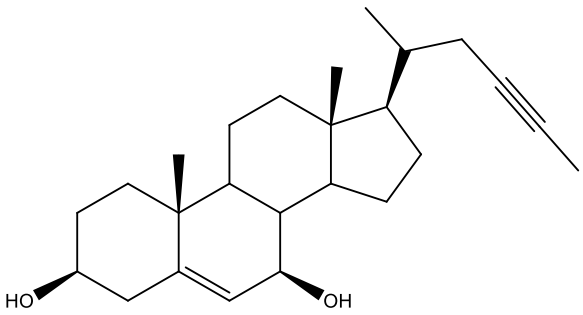
C/H No.	Isolated sitosterol				Reference β-sitosterol	
	^{1}H	COSY	^{13}C	135° DEPT	^{1}H	^{13}C
1	1.86, t 1.11, t		37.34	CH_2		37.3
2	1.49, t 1.84, t	H-3	31.75	CH_2		31.6
3	3.51, m	H-2, H-4	71.89	CH	3.52 m	71.8
4	2.29, d	H-3	42.85	CH_2		42.2
5	-		141.14			140.8
6	5.34, brd	H-7	121.81	CH	5.34 br s	121.7
7	1.96, m	H-6	31.98	CH_2		31.9
8	1.49, m		31.98	CH		31.9
9	0.91, t		50.22	CH		51.2
10			37.11			36.5
11	1.49, m		21.16	CH_2		21.1
12	1.84, m		39.84	CH_2		39.8
13	-		42.4			42.3
14	1.08, m		56.84	CH		56.8
15	1.55, m		24.4	CH_2		24.3
16	1.24, m		28.32	CH_2		28.3
17	1.11, m		56.09	CH		56.0
18	0.67, s		11.95	CH_3	0.68 s	11.9
19	1.00, s		19.48	CH_3	1.01 s	19.4
20			36.59	CH		36.2
21	1.00, s		18.91	CH_3	0.92 (d, 6.4)	18.8
22			33.98	CH_2		33.9
23			26.44	CH_2		26.1
24	0.91, m		46.13	CH		45.9
25	1.65, m		29	CH		29.2
26	0.81, d		19.7	CH_3	0.814 (d, 6.5)	19.8
27	0.83, d		19.52	CH_3	0.833 (d, 6.5)	19.3
28	1.00, m		23.08	CH_2		23.1
29	0.84, t		12.13	CH_3	0.845 (t, 7.5)	12.2

2.2.3.2 A37-2-B1

This compound was isolated as white crystals and the molecular ion was given at *m/z* 367.2648 $[M-H]^-$ in the negative mode with the predicted formula of $C_{25}H_{35}O_2$. (**Fig. 2.4)** Upon comparison of the ^1H NMR spectrum with that of the isolated β-sitosterol, this compound was also assumed to be a sterol with the oxymethine proton at δ 3.59 for C-3 (**Fig. 2.5a**) and similar busy signals between δ 0.6 to δ 2.4 ppm (**Fig. 2.5b)**. The proton spectra of both compounds also indicated the presence of a similar impurity. There was one more oxymethine proton observed at δ 3.85 which integrated for one proton indicating the presence of the other hydroxyl group. (**Fig. 2.6**) The broad doublet at δ 5.60 which shifted downfield compared to β-sitosterol and the correlation between this proton and the proton at δ 3.85 in the COSY spectrum (**Fig. 2.7a**) revealed the olefinic proton at C-6 and the other hydroxyl group at C-7. The multiplet at δ 5.17 also integrating for one proton was assumed to be an olefinic proton C-16 as deduced from the spin system of its proton revealed in the COSY spectrum. However, since the molecular formula revealed eight degrees of unsaturation, by subtracting five rings and two double bonds in the parent structure there were still one more unsaturated position left to be assigned. In the ^1H NMR spectrum, there were no other olefinic protons observed. Therefore, an alkynyl group was assumed to be present instead of two olefins. However, due to the limited quantity of the compound, this was not detected by HMBC (**Fig. 2.8**) except for signals already observed for sitosterol like the correlation of the CH_3-19 at δ 1.00 ppm to C-5 at δ 146.7 ppm. (**Fig. 2.7b**) The downfield shift of C-5 can be explained by the presence of the hydroxyl group on C-7. The 1D and 2D spectral data were comparable to those of gelliusterol A (Gallimore *et al.,* 2001) except for the undetected carbon shift signal at δ 3.3 ppm expected for the acetylenic methyl group.



A37-2-B1
Chemical Formula: $C_{25}H_{36}O_2$
Exact Mass: 368.2715



Gelliusterol A
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Exact Mass: 370.2872

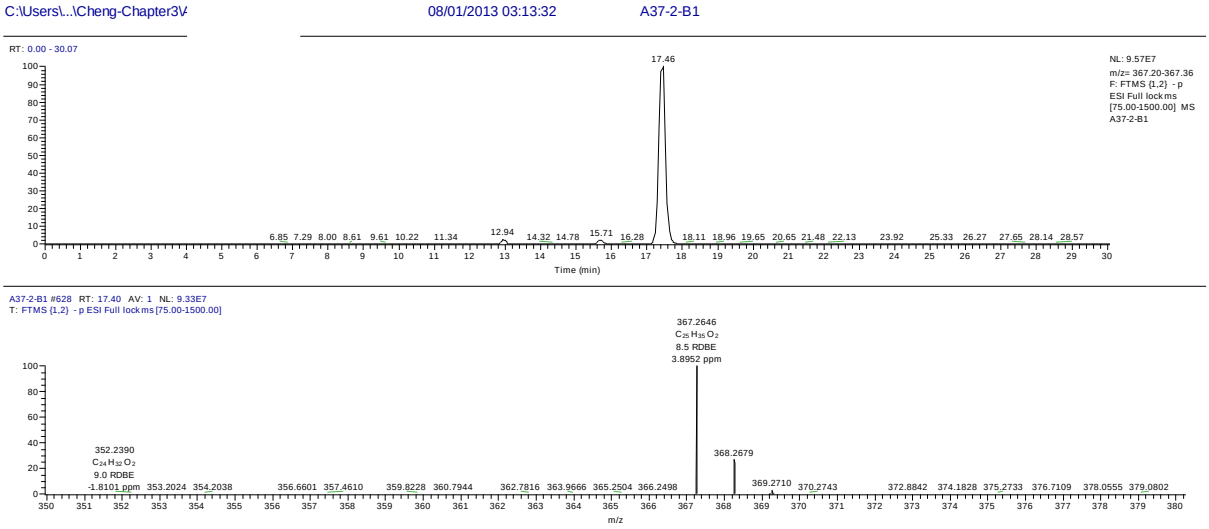


Fig 2.4 Extracted ion chromatogram (negative mode) and mass spectral data 17.46 min

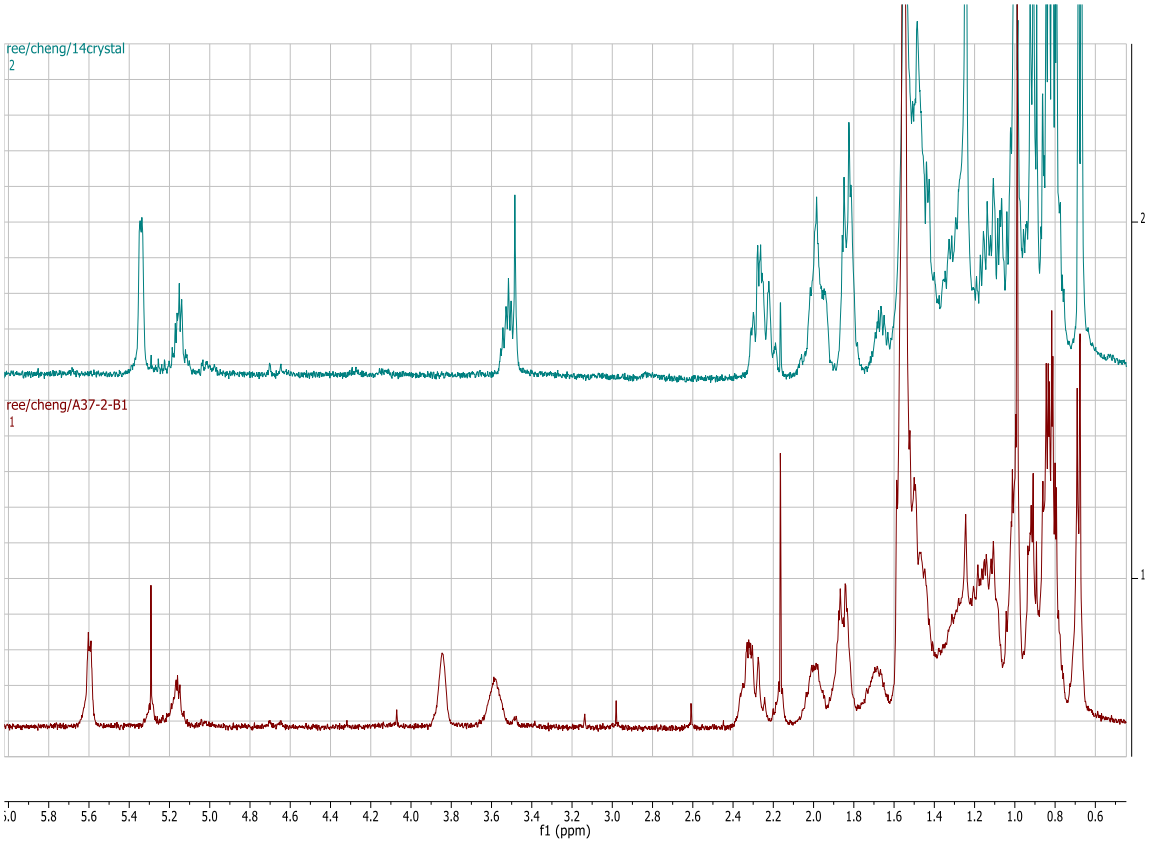


Fig. 2.5a ^{1}H NMR spectrum of β-sitosterol (top) and A37-2-B1 (bottom)

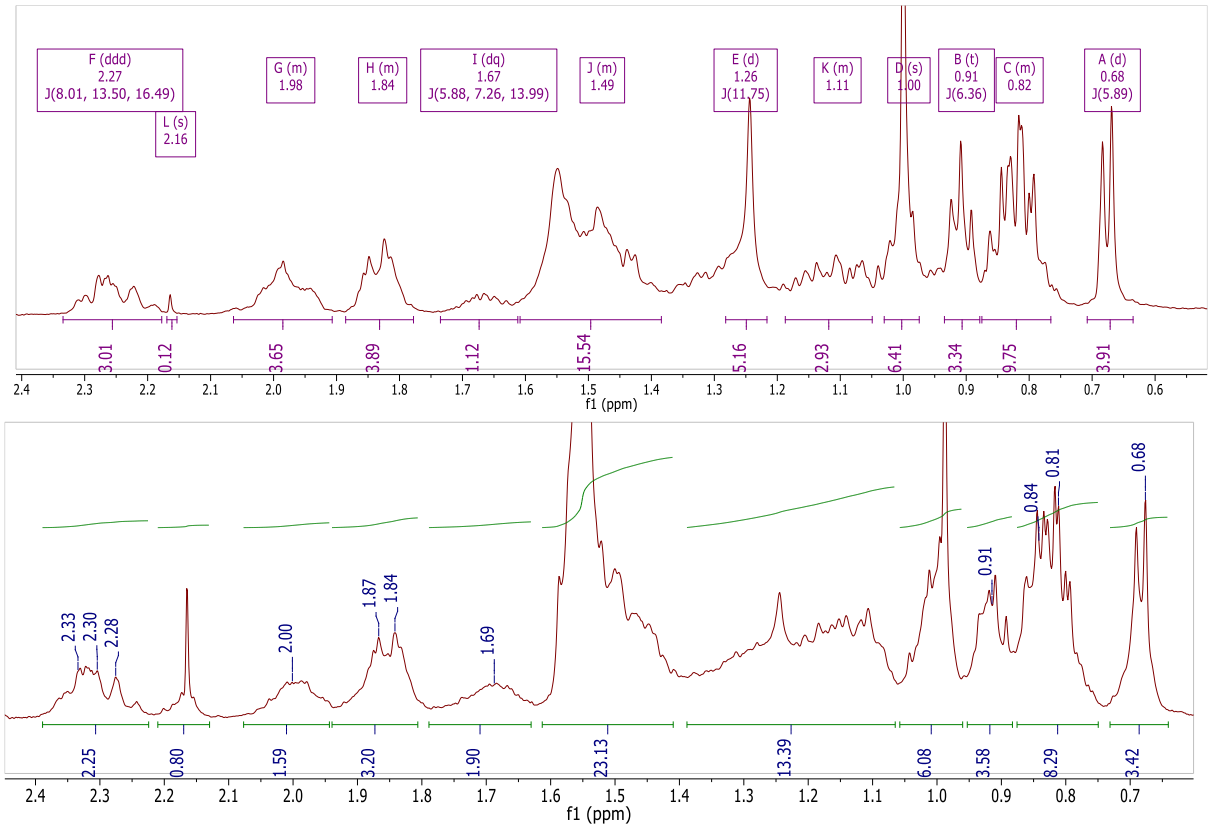


Fig. 2.5b Up field region of the^{1}H NMR spectra of β-sitosterol (top) and A37-2-B1 (bottom)

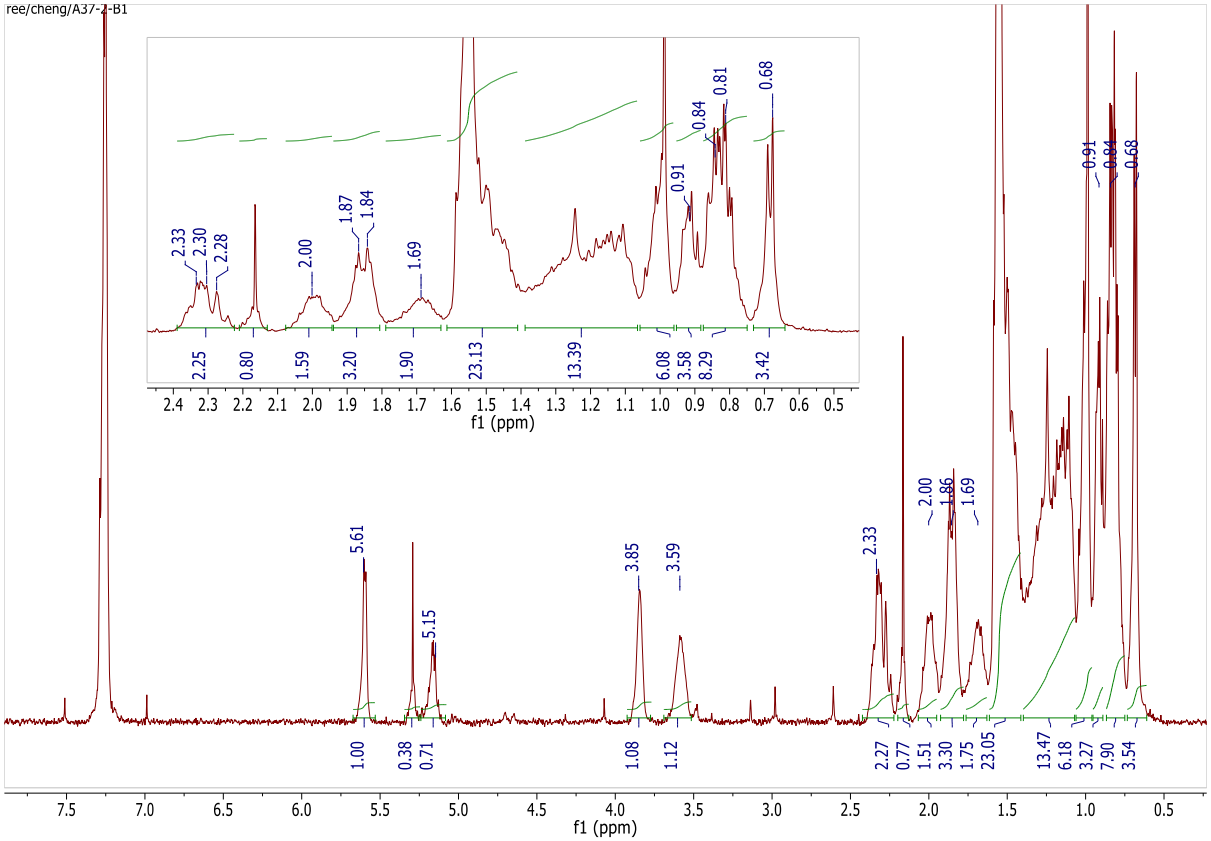


Fig. 2.6 ^{1}H NMR spectrum of A37-2-B1

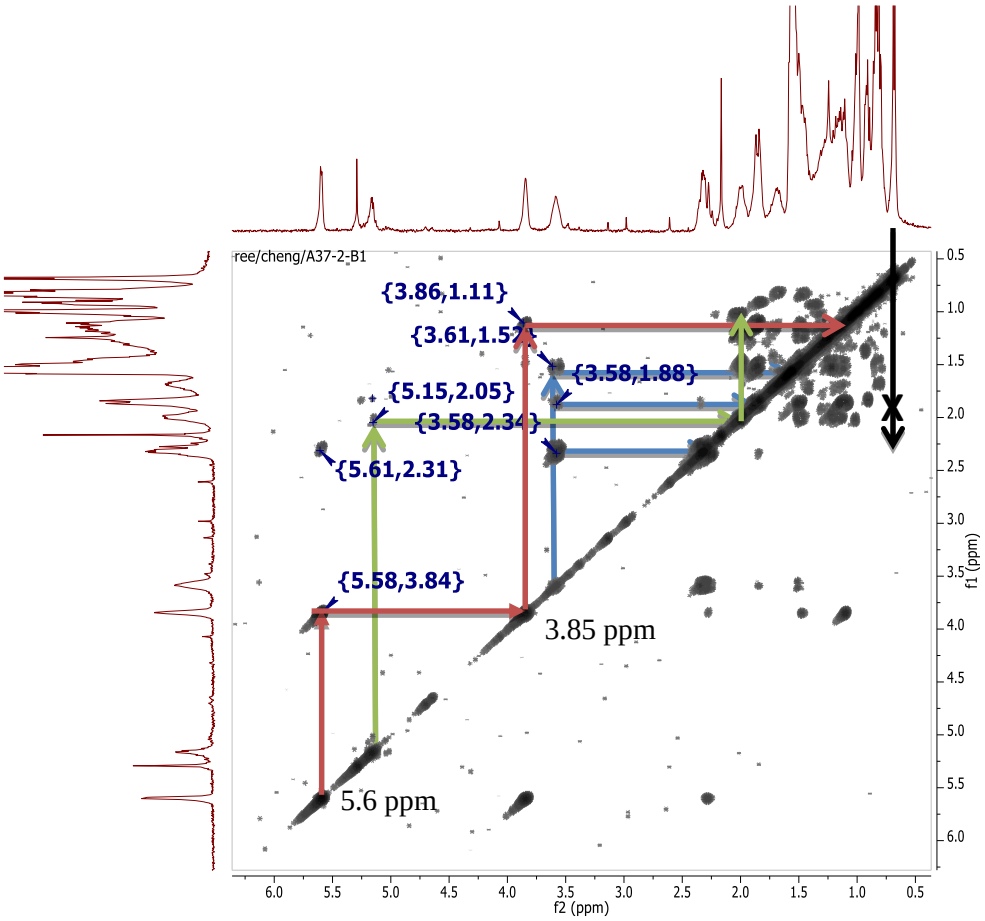


Fig. 2.7a COSY spectrum of A37-2-B1

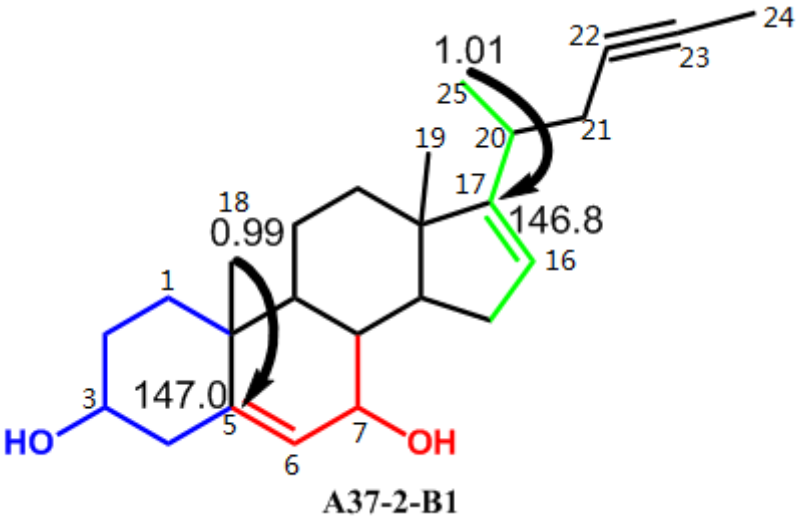


Fig. 2.7b ^{1}H-^{1}H COSY correlation (coloured bonds) of A37-2-B1 (black arrows show key HMBC correlations)

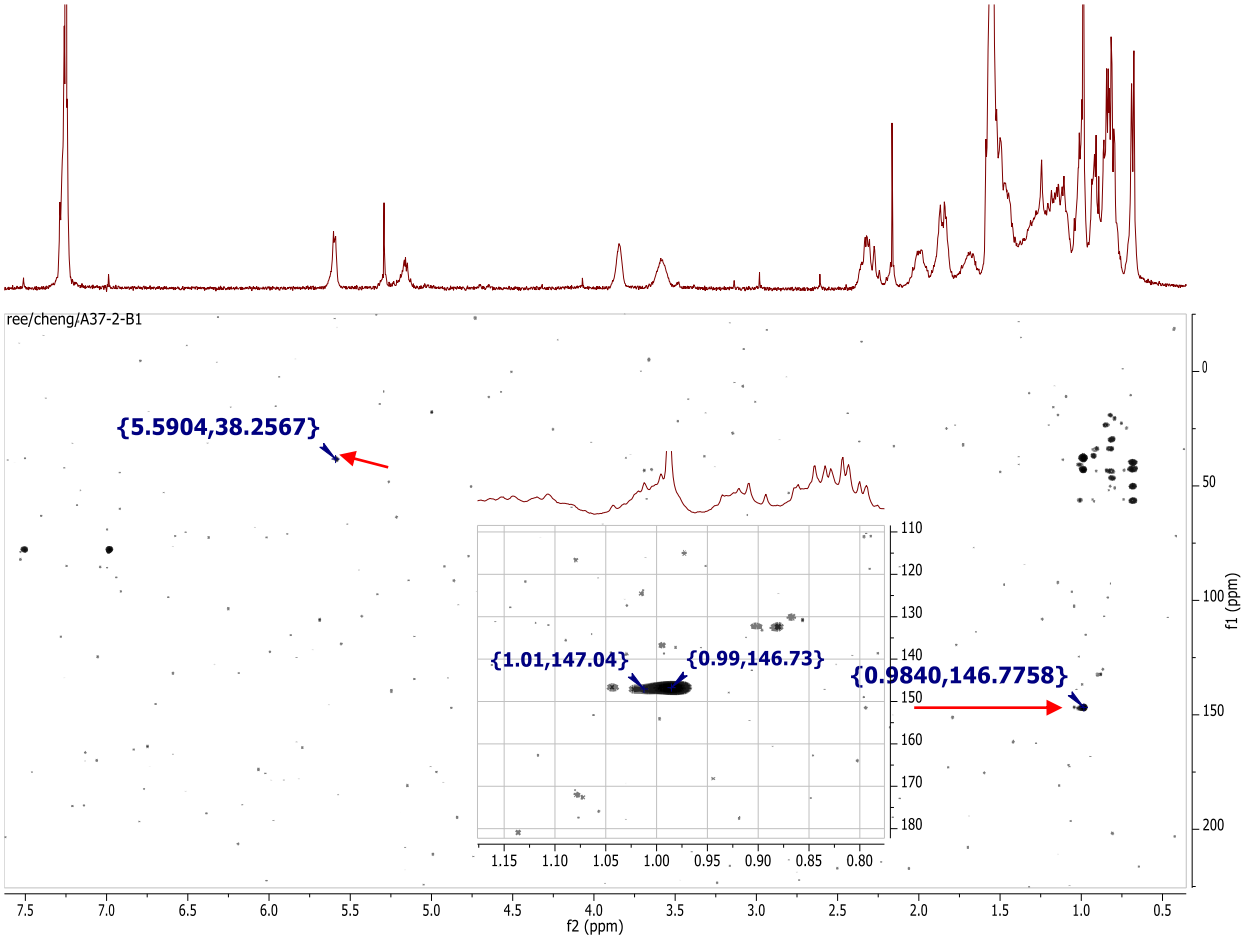


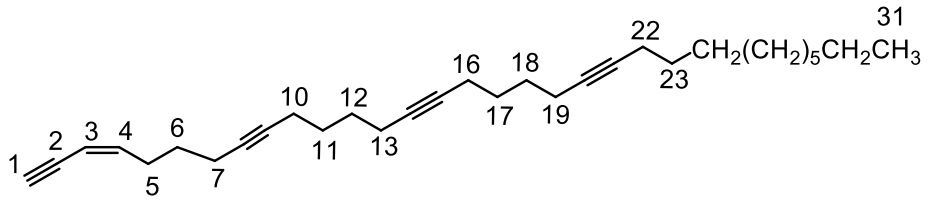
Fig. 2.8 HMBC spectrum of A37-2-B1

Table 2.7 NMR spectra of compound A37-2-B1 and gelliusterol (Gallimore *et al*., 2001)

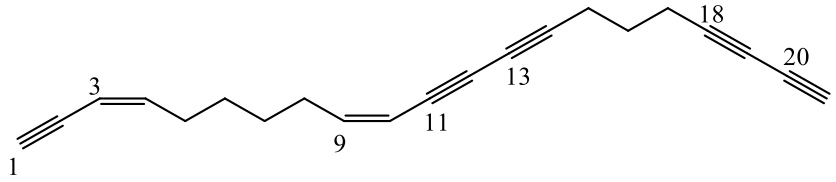
C/H No.	A37-2-B1 Measured in $CDCl_3$ (^1H: 600 MHz; ^{13}C: 150 MHz)				Gelliusterol	
	^1H	COSY	^{13}C	HMBC	^1H	^{13}C
1	1.11, m				1.13, m	38.1
	1.87, m				1.84, m	32.1
2	1.49, m				1.47, m	72.1
	1.84, m				1.78, m	42.9
3	3.58, m	H-2, H-4			3.47, m	146.7
4	2.34, m				2.25, m	125
5			146.7			65.9
6	5.61, d	H-7			5.53, d	39
7	3.85, br s	H-6			3.75, br s	43.4
8	1.49, m	H-7			1.44, m	38.5
9	0.91, t				1.30, m	21.8
10						38.5
11	1.49, m				1.53, m	21.8
12	1.11, m				1.15, m	40.5
	2.00, m				2.00, dt	
13						43.2
14	1.08, m	H-15			1.45, m	50.7
15	2.05, m	H-16			1.10, m	25.1
					1.78, m	
16	5.15, d	H-15			1.26, m	29.1
					1.85, m	
17			146.8		1.27, m	56.3
18	0.68, d			C-5	0.71, s	12.2
19	1.00, s				1.00, s	18.6
20					1.50, m	37
21	1.01, s			C-17	1.06, d	18.6
22	2.33, d				1.95, m	26.7
					2.14, dt	
23						78.2
24						77
25	1.69, m				1.73, t	3.3

2.2.3.3 A45-3-3

This compound was isolated as a yellow oil. The ^{1}H NMR spectrum revealed the presence of 46 protons, the multiplet at δ 5.97 and the doublet at δ 5.47 indicated two adjacent olefinic protons, and this was confirmed by the COSY spectrum in which a correlation between them was observed (**Fig. 2.9 and 2.10**). The proton at δ 3.09 ppm with singlet and 1H integration was assigned to the terminal alkynyl proton combined with the observation of the linked carbon at δ 81.74. Since a weak correlation was observed between protons at δ 5.47 and 3.09, this compound was assumed to have a chain structure in which an alkenyl group links to the terminal alkynene. The multiplet at δ 2.17 and 2.33 was assigned to the alkyl protons next to olefinic group. The signals in the alkyl group region were broad although the sample was dried in the desiccator under vacuum for 15 min to remove any water. Therefore, it was assumed that this compound was still mixed with other alkyl compounds (UV inactive) even though only one spot was visualised on the TLC plate. Because of the broadness of the peaks, the integration of the protons in this region cannot be applied to count the alkyl protons properly. In addition, weak correlations were shown in the HMBC spectrum due to the limited amount of the sample even though 75 scans were applied for 12 hours running. This made it impossible to elucidate the alkyl part therefore the structure was only proposed below. However, it could be concluded that this compound is possibly a polyacetylene by comparing the ^{1}H and ^{13}C NMR spectra of callypentayne. (**Table 2.8**)



A45-3-3
(*Z*)-hentriaconta-3-en-1,8,14,20-tetrayne
Chemical Formula: $C_{31}H_{46}$
Exact Mass: 418.3600



callypentayne

Table 2.8 NMR spectra of compound A45-3-3 and reference Callypentayne (Umeyama *et al*., 1997)

A45-3-3 Measured in $CDCl_3$ (^1H: 600 MHz; ^{13}C: 150 MHz)					**Callypentayne**		
C/H No.	**^1H (ppm)**	**COSY**	**^{13}C (ppm)**	**HMBC**	**C/H No.**	**^1H (ppm)**	**^{13}C (ppm)**
1	3.01, s	H-3	81.74		**1**	3.09	81.3
2	-	-	80.42		**2**	-	80.4
3	5.47, d	H-1, H-4, H-5	108.82		**3**	5.46	108.3
4	5.97, m	H-3, H-5	145.2		**4**	6.00	145.7
5	2.15, m	H-3, H-4, H-6	32.03	C-3	**5**	2.36	30.0
6	1.54, m	H-5	19.27		**6**	1.46	28.1
7	2.33, m		32.03		**7**	1.46	28.2
8			80.42		**8**	2.36	30.5
9			80.42		**9**	6.05	147.7
10	2.33, m		32.03		**10**	5.48	108.2
11	1.54, m		19.27		**11**	-	72.5
12	1.54, m		19.27		**12**	-	77.9
13	2.33, m		32.03		**13**	-	65.5
14			80.42		**14**	-	82.9
15			80.42		**15**	2.48	18.7
16	2.33, m		32.03		**16**	1.79	26.7
17	1.54, m		19.27		**17**	2.42	18.2
18	1.54, m		19.27		**18**		76.8
19	2.33, m		32.03		**19**		82.9
20			80.42		**20**		68.2
21			80.42		**21**	1.99	66.2
22	2.33, m		32.03				
23	1.54, m		19.27				
24-30	1.25,m						
31	0.87,m						

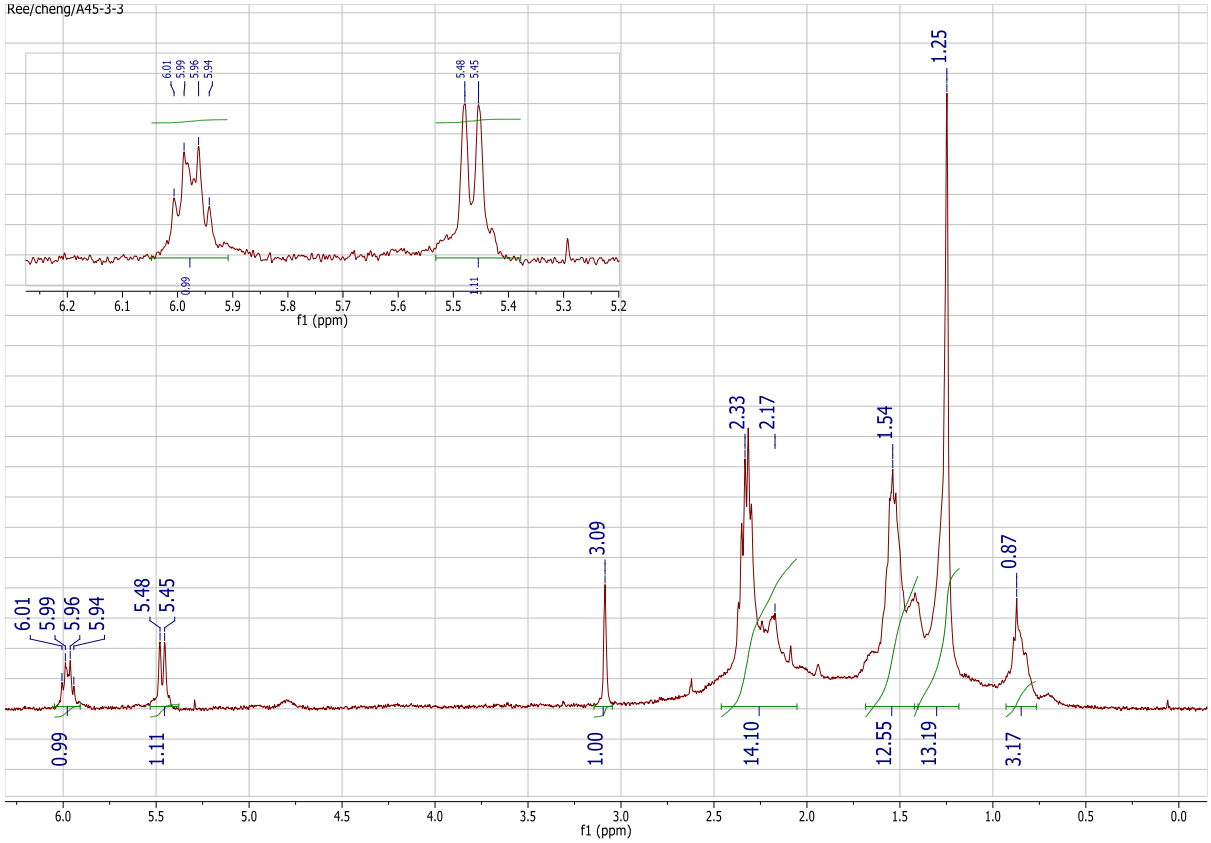


Fig. 2.9 ^{1}H NMR spectrum of A45-3-3

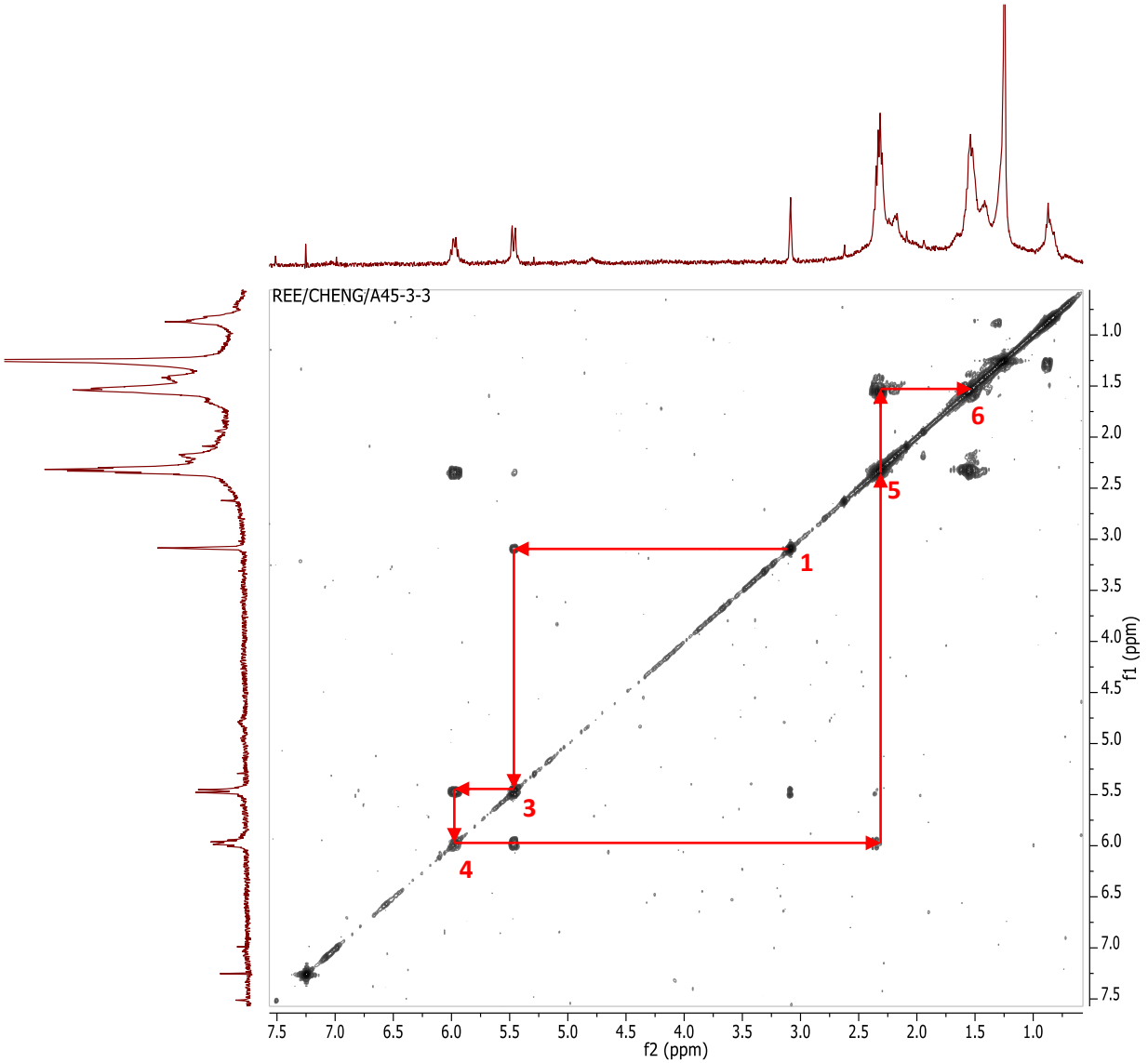


Fig. 2.10 COSY spectrum of A45-3-3

Chapter 3 Dereplication of the Marine Sponge *Spheciospongia (Callyspongia) vagabunda* associated-bacterium *Actinokineospora* EG49

3.1 Materials and Methods

3.1.1 Cultivation of Microorganisms

3.1.1.1 Solid fermentation

Actinokineospora (Tang, *et al*., 2012; Lisdiyanti *et al.,* 2010) EG49 which was isolated from sponge *Spheciospongia (Callyspongia) vagabunda* was obtained from Dr Usama Ramadan Abdelomohsen (University of Würzburg) for metabolite extraction and chemical analysis. Further re-inoculation was carried out and the appearances of the colonies on the plates as well as under the microscope (120×10) are shown in ISP2 agar (**Fig. 3.1 and Fig. 3.2)**. The recipe of ISP2 agar and artificial seawater are shown in **Table 3.1 and Table 3.2**). Broth cultures of this bacterium were grown at 27 ℃. It took 4 weeks to grow EG49 on agar and 6 weeks in broth until a change in medium colour could be observed.

Table 3.1 ISP2 agar menu for *Actinokineospora* EG 49

Yeast Extract	4 g/L
Malt Extract	10 g/L
Glucose	4 g/L
Agar	18 g L
Artificial Seawater	1 L
pH	7.3

Table 3.2 Artificial Seawater recipe

NaCl	23.470 g	**KCl**	0.664 g
Na_2SO_4	3.920 g	**KBr**	0.096 g
$MgCl_2•6H_2O$	10.640 g	**H_3BO_3**	0.026 g
$CaCl_2$	1.100 g	**$SrCl_2$**	0.024 g
$NaHCO_3$	0.192 g	**NaF**	0.003 g
H_2O	1.000 L		

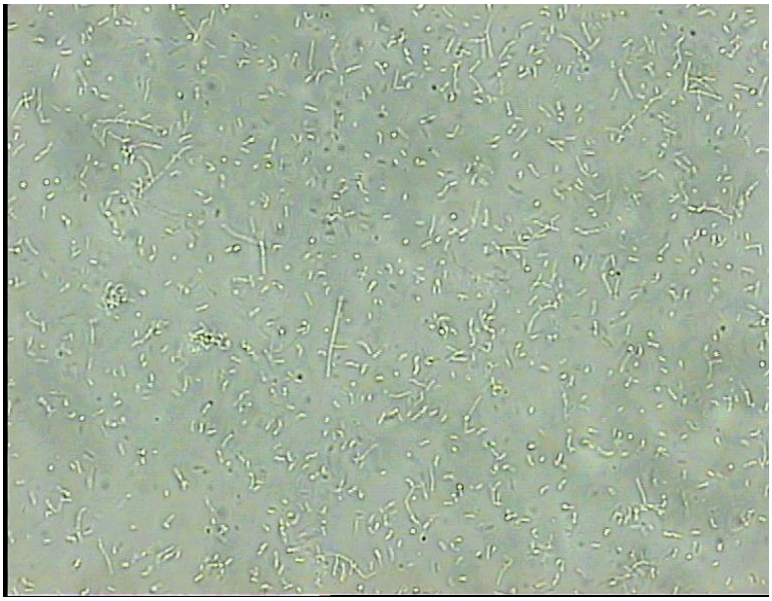


Fig. 3.1 Digital microscope image (120×10) of EG49 bacteria

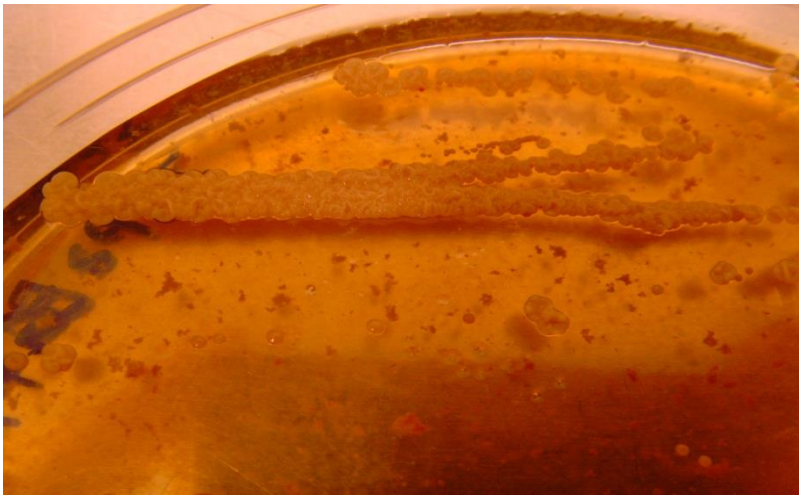


Fig. 3.2 *Actinokineospora* EG49 grown on the Petri dish

(Picture was taken on the 24th day after re-inoculation)

The following fermentation experiments were conducted by Dr Usama Ramadan Abdelomohsen in the University of Würzburg to investigate the yield of target compounds from different culture methods. OPLS-DA analysis was performed using the MS data from these samples.

3.1.1.2 Liquid cultivation

One 2 L Erlenmeyer flask containing 1.5 L of ISP2 medium was inoculated with 1 mL of well grown culture of *Actinokineospora* strain EG49 with shaking at 150 rpm at 30 °C for 7

days. After cultivation, filtration was done and the supernatant was extracted with ethyl acetate (2 x 750 mL). The cells were macerated in double volume of methanol with shaking for 3 h (150 rpm) then filtered. The extracts were concentrated under vacuum and stored at 4 °C for chromatographic analysis (Liquid ethyl acetate and Liquid methanolic).

3.1.1.3 Liquid cultivation with XAD

One 2 L Erlenmeyer flask containing 1.5 L of ISP2 medium amended with 20g/L XAD-16 was inoculated with 1 mL of well grown culture of *Actinokineospora* strain EG49 with shaking at 150 rpm at 30 °C for 7 days. XAD-16 is highly absorbent resin which was used to concentrate organic compounds in crude culture broth to try to improve the output of the interesting secondary metabolites. After cultivation, filtration was carried out, the supernatant was extracted with ethyl acetate (2 x 750 mL). The cells and XAD were macerated in double volume of methanol with shaking for 3 h then filtered. The extracts were concentrated under vacuum rotary evaporator and stored at 4 °C for chromatographic analysis.

3.1.1.4 Cultivation in calcium alginate beads

Cells culture of *Actinokineospora* strain EG49 (500 mL) were collected by centrifugation at 8000 rpm for 10 min and the pellet was then mixed with 4 % (w/v) sodium alginate, containing HEPES buffer (1 %). Sodium alginate mixture was then slowly dropped with a 5 mL syringe into 1.5 % (w/v) $CaCl_2$ at 4 °C, stirred until solidification, and the beads were then washed three times with sterile distilled water. 300 mL of calcium alginate beads with immobilized bacteria were then transferred to a 2 L Erlenmeyer flask containing 1.2 L of ISP2 medium and incubated at 30 °C with shaking for 7 days. After filtration, beads were extracted by shaking with methanol for 3 h then filtered. Supernatant was extracted with ethyl acetate (2 x 750 mL). The extracts were concentrated under vacuum and stored at 4 °C for analysis (Beads ethyl acetate and Beads methanolic). Calcium alginate beads were used as slow-release system for delivering nutrients from medium to culture cell.

Control experiments were also carried out using the same approaches but without bacteria. The control liquid culture was extracted only with ethyl acetate because there were no cells.

3.1.2 Extraction of *Actinokineospora* EG49

The inoculated agar (24 days inoculation) was cut into pieces and left overnight in 250mL of EtOAc to kill the bacteria. Homogenisation using motor stand assembled homogeniser was conducted for 10 min afterwards. The agar-solvent mixture was further macerated overnight in EtOAc then filtered. The binary solvents were separated using a separating funnel and the EtOAc layer was then condensed to obtain the EtOAc extract. The aqueous layer was partitioned with EtOAC for a total of three times. For the broth medium, the volume of EtOAC used for each partition was equal to the broth volume. The whole extraction procedure was the same as that of the agar.

3.1.3 NMR Experiments

A JEOL (JNM LA400) 400 MHz instrument was used. 1D ^{1}H, ^{13}C and ^{13}C DEPT NMR experiments in deuterated DMSO were carried out on the total agar extract of *Actinokineospora* EG49. 1D ^{1}H NMR in $CDCl_3$was done on the broth extracts.

3.1.4 Dereplication using MS data

Analyses were performed on an Accela HPLC from Thermo Scientific (Bremen, Germany) coupled with Exactive (Orbitrap) mass spectrometer from Thermo Fisher Scientific (Bremen Germany) as described in Chapter 2.

3.1.5 Bioassay

Actinokineospora EG49 was tested on a panel of bioassays including Typanosoma *brucei* (strain S427), *Escherichia coli* (strain ATCC 8739), *Pseudomonas aeruginosa* (strain ATCC 27853), *Klebsiella pneumoniae* (strain ATCC 13883) and *Klebsiella pneumoniae* (strain ATCC BAA-2146 (NDM-1 positive)). All the tests were carried out by Ms Carol J. Clements at the University of Strathclyde using the same protocol described in Chapter 2.

3.2 Results and discussion

3.2.1 Actinokineospora EG49

3.2.1.1 Bioassay screening for antitrypanosomal activity

The agar extract of *Actinokineospora* EG49 showed potential anti-trypanosomal activity (**Table 3.4**)

.

Table 3.4 Bioassay results for Actinomycete EG49 agar extracts

	E.coli **ATCC 8739 % control**	***P.aeruginosa*** **ATCC 27853 % control**	***K.pneumoniae*** **ATCC 13883 % control**	***K.pneumoniae*** **ATCC BAA-2146 (NDM-1 positive) % control**	***T.b.brucei*** **S427 % control**
EG49-Agar Extact	84.8	45.2	72.5	68.9	-0.1
EG49-Agar Extact	82.1	46.5	69.1	64.2	-0.2

Human African trypanosomiasis, also known as sleeping sickness, is a parasitic disease caused by the protozoan species *Trypanosoma brucei* which are transmitted to humans through the bite of an infected tsetse fly (*Glossina* genus) with a high incidence in rural Africa. About 7000 new cases were reported by World Health Organisation (WHO) in 2010 with a decreasing trend compared to the previous 50 years. There are two stages of infection; the parasites multiply in blood, lymph and subcutaneous tissues in the first and infect the central nervous system by crossing the blood-brain barrier in the second. In the first stage, the patient experiences fever, headaches, joint pain and itching. However, more obvious signs can be observed in the second stage such as altered consciousness, daytime slumber but insomnia at night. It is fatal if left untreated. Regarding the treatment, pentamidine and suramin are the two approved drugs that have been used for about half a century for the treatment of first stage *T.b. gambiense* and *T.b. rhodesiense* infections respectively. For the second stage treatment, melarsoprol is applied but with various undesirable side effects and even some fatalities. An increasing resistance towards melarsoprol has also been observed. Since these drugs are not profitable compared to the others such as anti-cancer drugs, the pharmaceutical industry usually halt production periodically. Therefore, the discovery and development of new anti-trypanosomal drugs which could be cheaply and efficiently manufactured are highly sought after for the improvement of heath care in rural Africa. [The World Health Organisation] EG49 appears to be a good potential source of anti-trypanosomal compounds since the extract showed potent activity against *T. brucei* and could be inoculated in lab environment.

3.2.1.2 NMR spectroscopy

The ^{1}H multiplets at δ 6.5-9.5 ppm combined with the ^{13}C at δ 125-130 ppm indicated the presence of aromatic structures, possibly N-containing rings, in the EG49 agar extract. ^{1}H multiplets at δ 4.0-4.5 ppm combined with the ^{13}C at δ 50-70 ppm implied the existence of RCH_3-O- and RCH_2-O- structures, possibly saccharide structures. In addition, ^{1}H signals at δ 5.0- 5.5 ppm may indicate anomeric protons, but this would need to be confirmed by a 2D ^{1}H COSY spectrum. ^{1}H singlet at δ 10.75 ppm and δ 11 ppm suggested the possible presence of aromatic-OH/-NH_2 structures. ^{13}C 135° DEPT NMR spectrum was added showing better peak intensity **(Fig. 3.3 and Fig. 3.4**).

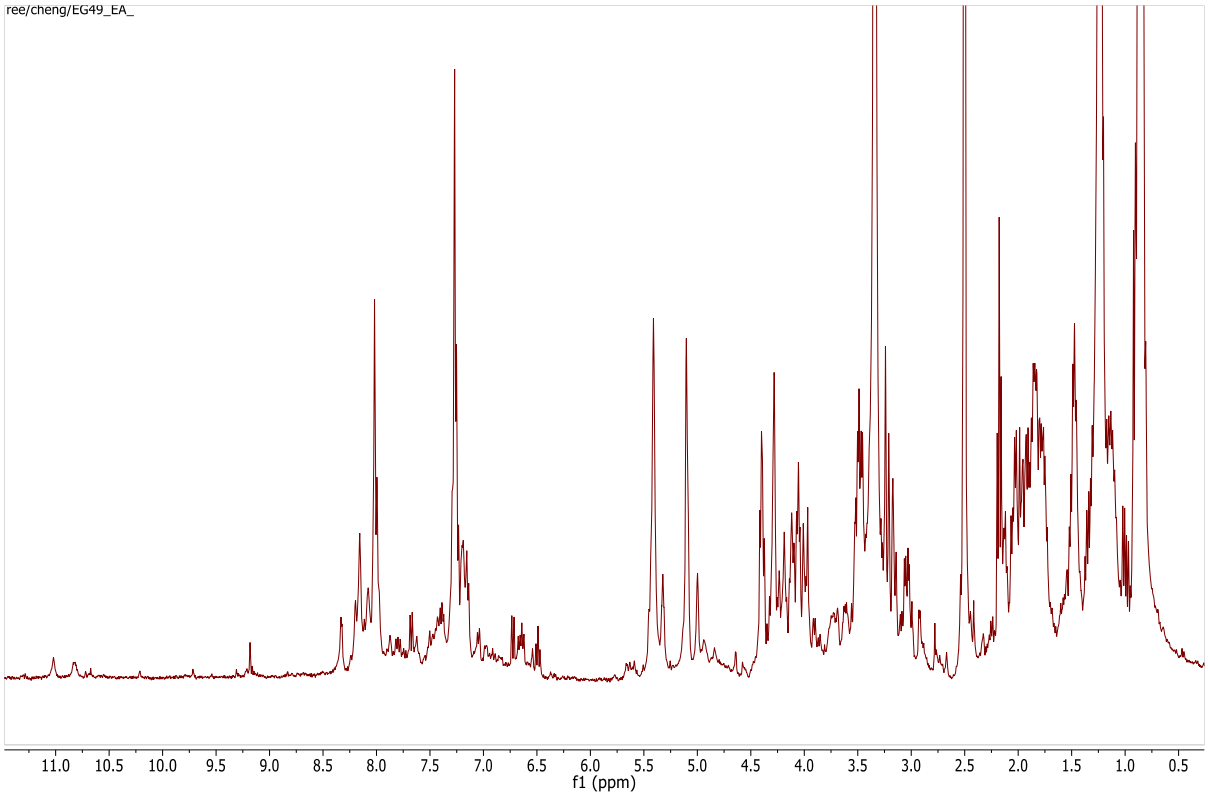


Fig. 3.3 1D ^{1}H NMR spectrum of *Actinokineospora* EG49 agar extract in DMSO

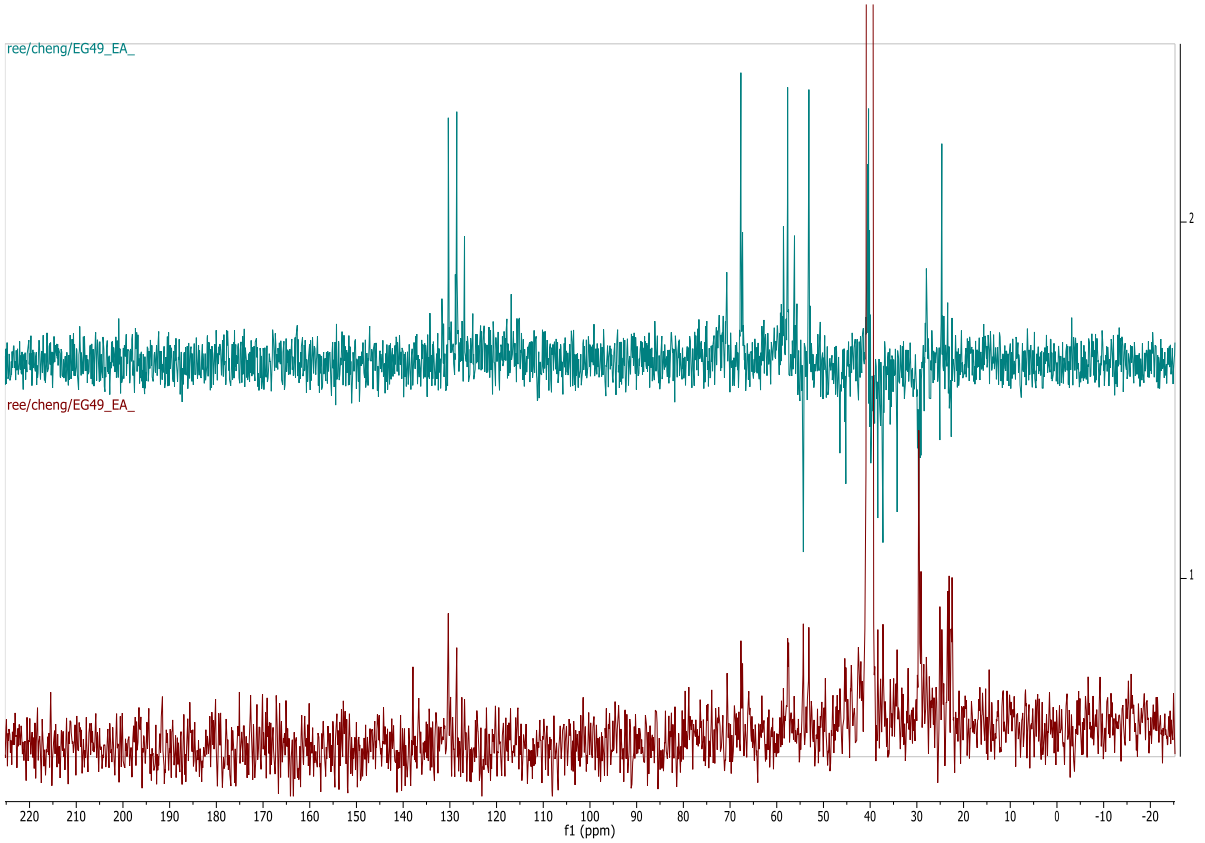


Fig. 3.4 1D ^{13}C and 135^{o} DEPT NMR spectrum of *Actinokineospora* EG49 agar extract

3.2.1.3 Dereplication of Actinokineospora EG49 agar extracts

Dereplication studies which include identification and quantification of secondary metabolites in natural product extracts can be a colossal job (Ebada *et al*., 2008; Kjer *et al*., 2010; Yuliana *et al*., 2011). Secondary metabolites in wide range of concentrations have diverse atomic arrangements which results in variations in chemical and physical properties which will require variable isolation techniques to identify them. Reliable, robust, selective and high resolution analytical methods are therefore required in identifying and quantifying multiple chemical groups of natural products. Mass spectrometry and NMR spectroscopy are complementary analytical methods and are commonly employed in tandem as metabolomics tools. Mass spectrometry is sensitive even at femtogram levels but may not be reproducible between instrument types and ionization capabilities of the metabolites. While NMR data is reproducible, it may not be sensitive enough to detect metabolites at lower concentrations.

The relatively new science of metabolomics can be used as a tool to enhance identification and dereplication, as in bioassay-guided isolation work (Yuliana *et al*., 2011) Through metabolomics approach, the link between chemical profile and bioactivity pattern of the secondary metabolites can be statistically correlated. To date, metabolomics is not yet widely

applied in bioactive screening of natural products although it has several advantages over the reductionist approach. Metabolomics can be applied to dereplicate the biosynthesis of the natural product at different development stages of their biological source as well as simultaneously screen for the bioactivity. By using combinations of different analytical methods, the bioassay-guided isolation route is shortened and rapid dereplication of known activities is rapidly delivered (Ebada *et al*., 2008; Kjer *et al*., 2010).

Dereplication of secondary metabolites from the promising anti-trypanosomal active isolate *Actinokineospora* strain EG49 was achieved by HRFTMS using the LTQ-Orbitrap and high resolution NMR. Secondary metabolites were identified with the aid of existing high resolution MS and NMR records from online and in-house databases like Dictionary of Natural Products (DNP); MarinLit, a database for marine natural products; and AntiMarin, a database of microbial secondary metabolites. MZmine (Pluskal *et al*., 2010) and SIMCA P+ 13.0 were utilized to perform differential analysis of sample populations to find significant expressed features of complex biomarkers between parameter variables.

Utilising the AntiMarin Database, known compounds were identified at a MW tolerance within 5 ppm as shown in **Table 3.5**. Most of the identified compounds have been previously isolated from *Streptomyces.* The identified compounds were also highlighted in the MS chromatogram showing the distribution of the known and unknown compounds. (**Fig. 3.5**) It is clear from the chromatograms that more than half of the peaks have not been identified by the database, especially for some of the major compounds showing strong peak intensity and good resolution; for example, peaks at 24.74 min and 26.75 min as observed in the negative mode. The *m/z* value and the predicted formula of these unidentified compounds were tabulated in **Table 3.6**. In addition, nearly two-thirds of the identified peaks in the data base have been described to be from other sources, including fungi, sponges, ciliates and algae. These compounds were not listed because they were not of bacterial origin and the metabolites were limited to those considered to be as bacterial metabolites particularly those of the actinomycetes.

Atramycin B (highlighted in **Table 3.5**) seems to be present in the agar extract with a high confidence level, since it was found in both positive and negative modes with peak areas above E+07 at the same retention time. This compound is an isotetracenone-type antitumour antibiotic isolated from *Streptomyces atratus* BY90 in 1991 (Fujioka *et al*., 1991) with production through fermentation achieved by the Cfm Oskar Tropitzsch

company. Additionally, a common feature found among most of the identified compounds was the quinone structure. Quinone has been reported to have antitrypanosomal activity, the inhibitory mechanism of which is assumed to be the induction of oxidative stress against trypanothione reductase, a key enzyme involved in the trypanosomal antioxidant thiol metabolism. (Hoet *et al.*, 2004) Therefore, it is assumed that the quinone compounds may contribute to the antitrypanosomal activity of EG49 agar extract.

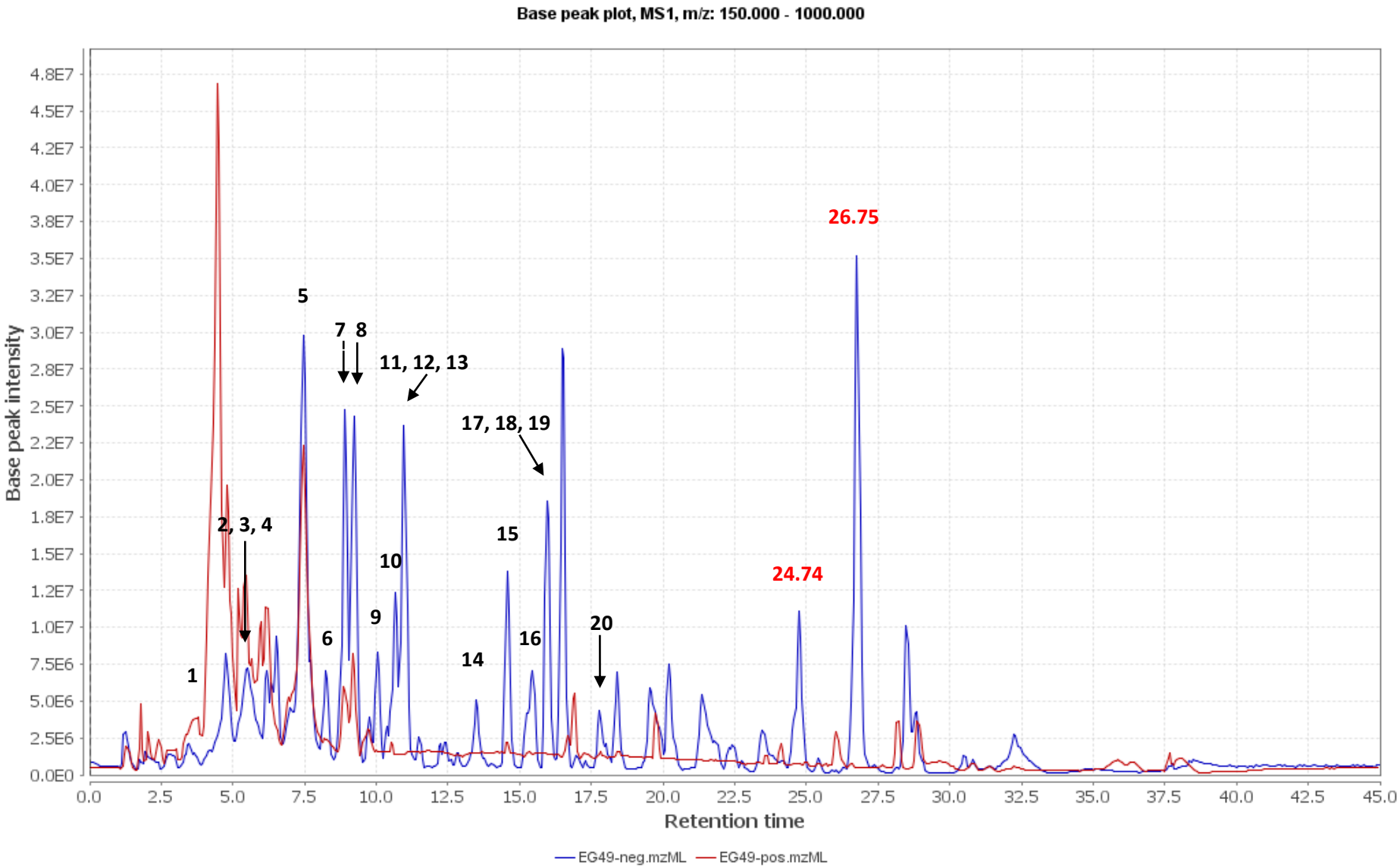
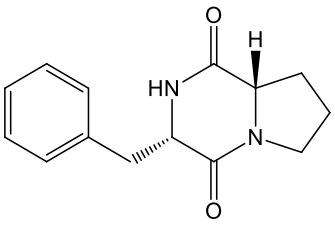


Fig. 3.5 Total ion chromatogram of EG49 agar extract

Table 3.5 Identified compounds of *Actinokineospora* EG49 extract from the database Marinlit

	Ionisation Mode	Rt(min)	MS *m/z*	Molecular Weight	Chemical Formula	Name	Tolerance (ppm)	Source	Peak Area
3.1	P	4.03	245.1282196	244.1206	C14H16N2O2	cis-cyclo(L-Phe,L-Pro)	1.25	[B] arctic ice bacterium	1.35E+07
3.2	N	5.15	281.1241455	282.1323	C12H18N4O4	8R-3[(1R,2S,3R,4S)-2,3-Dihydroxy-4-(hydroxymethyl)-cyclopentyl]-3,6,7,8-tetrahydroimidazo-[4,5-d][1,3]diazepin-8-ol	-3	[B] *Saccharothrix*	3.25E+07
3.3	P	5.17	227.1391296	226.1312	C11H18N2O3	cyclo[L-(4-Hydroxyprolinyl)-D-leucine]	2.86	[B] marine bacterium A108	4.43E+08
3.4						cis-4-(D)-Hydroxyprolyl diketopiperazine II		[B] marine bacterium A108	
3.5	N	7.72	387.0724182	388.0789	C19H16O9	6a,12a-Dihydroxy-4a,12b-epoxy-(2R)-PD-116198	2.07	[B] *Streptomyces phaeochromogenes*	1.55E+08
3.6	N	8.22	657.1825562	658.1892	C32H34O15	Elloramycin E	0.92	[B] *Streptomyces olivaceus*	1.27E+08
3.7	N	8.84	216.03017	217.037	C11H7NO4	2-Amino-3-carboxy-1,4-naphthoquinone	2.22	[B] *Propionibacterium freudenreichii*	3.41E+06
3.8	P	8.87	453.1546021	452.1466	C25H24O8	Atramycin B	1.65	[B] *Streptomyces atratus*	1.71E+07
3.8	N	8.9	451.1399231	452.1466	C25H24O8	Atramycin B	1.38	[B] *Streptomyces atratus*	7.67E+07
3.9	N	10.03	501.140213	502.147	C25H26O11	F 840020	1.03	0	1.11E+08
3.10	N	10.61	522.140564	523.1473	C27H25NO10	(3aS)-Jadomycin S	1.02	[B] *Streptomyces venezuelae*	1.90E+07
3.11	N	10.96	341.0666809	342.0734	C18H14O7	Fuchurmycin B	1.6	[B] *Streptomyces sp.*	4.19E+08
3.12						PK-8		[B] *Streptomyces roseofulvus* mutant	
3.13						Momofulvenone-A		[B] *Streptomyces diastatochromogenes*	
3.14	N	13.47	297.0404358	298.0472	C16H10O6	3,8-Dihydroxy-1-methylanthraquinone-2-carboxylic acid; 671-F; DMAC	1.73	[B] *Streptomyces sp.*	8.07E+07
3.15	N	14.57	514.1351318	515.1416	C17H29N3O13S	Bulgecin B	1.63	[B] *Streptomyces sp.*	1.86E+07
3.16	N	15.46	495.1296387	496.1364	C26H24O10	Gilvocarcin A	1.03	[B] *Streptomyces gilvotanareus;*	1.39E+08
3.17	N	15.94	253.050705	254.0574	C15H10O4	Daidzein	2.42	[B] *Streptomyces sp.*	4.29E+07
3.18	N	15.94	355.082489	356.0891	C19H16O7	Komodoquinone B	1.98	[B] *Streptomyces sp.*	2.36E+08
3.19						beta1-Rhodomycinone		[B] *Streptomyces purpurascens*	
3.20	P	17.81	370.0922546	369.0843	C19H15NO7	Protetrone	1.8	[B] *Streptomyces aureofaciens*	1.85E+07



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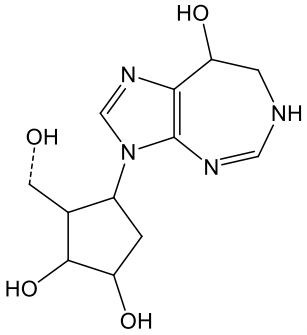

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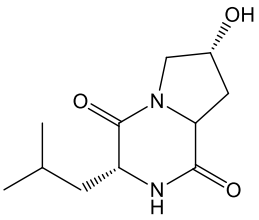

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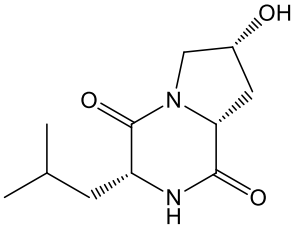

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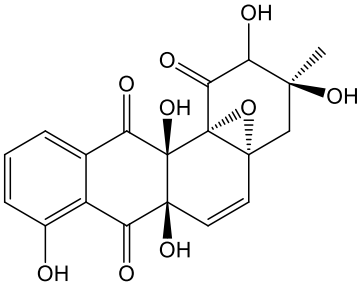

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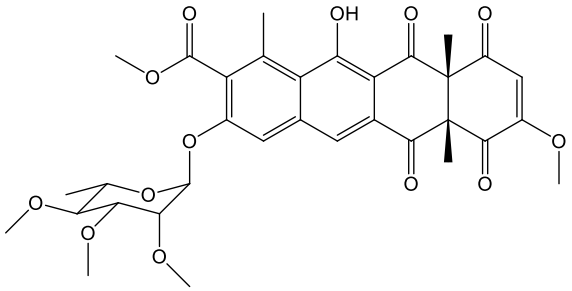

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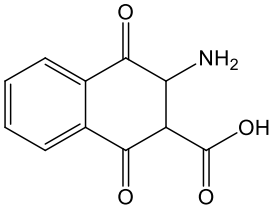

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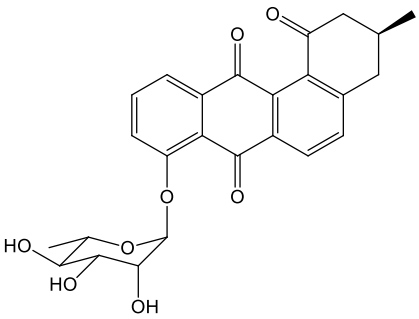

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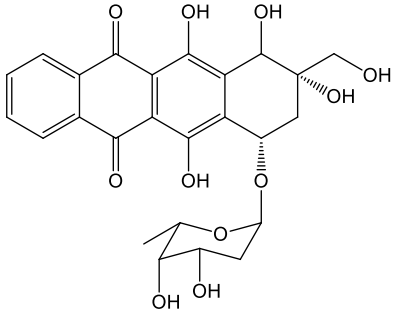

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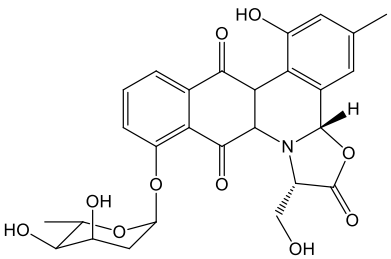

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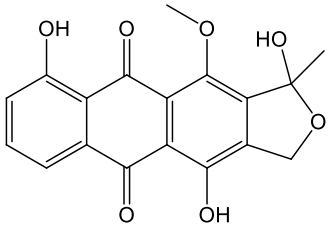
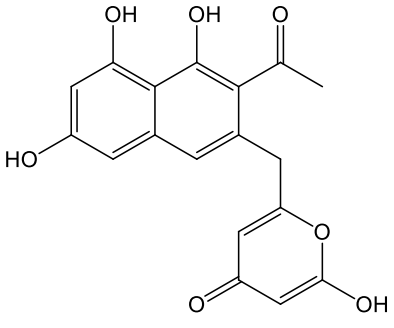


3.11



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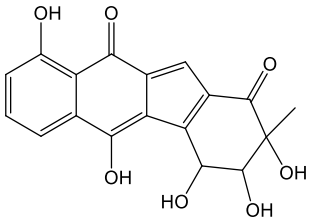

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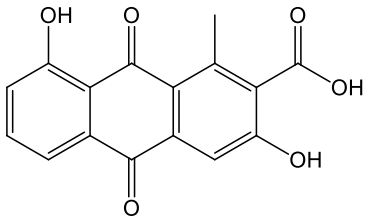

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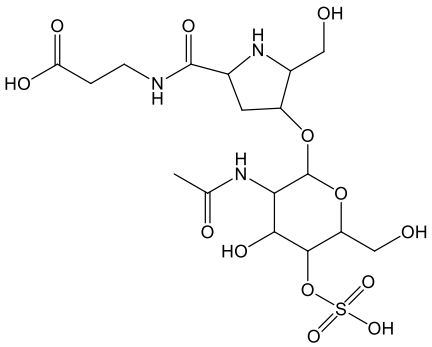

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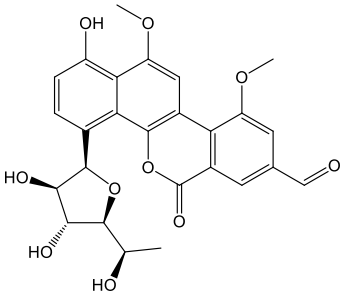

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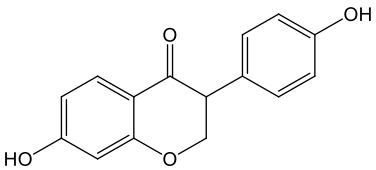

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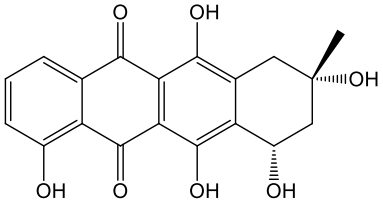

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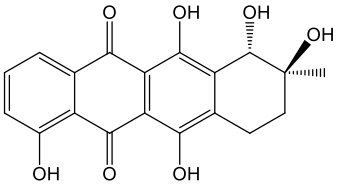

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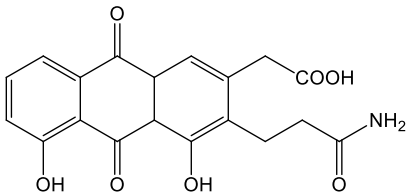


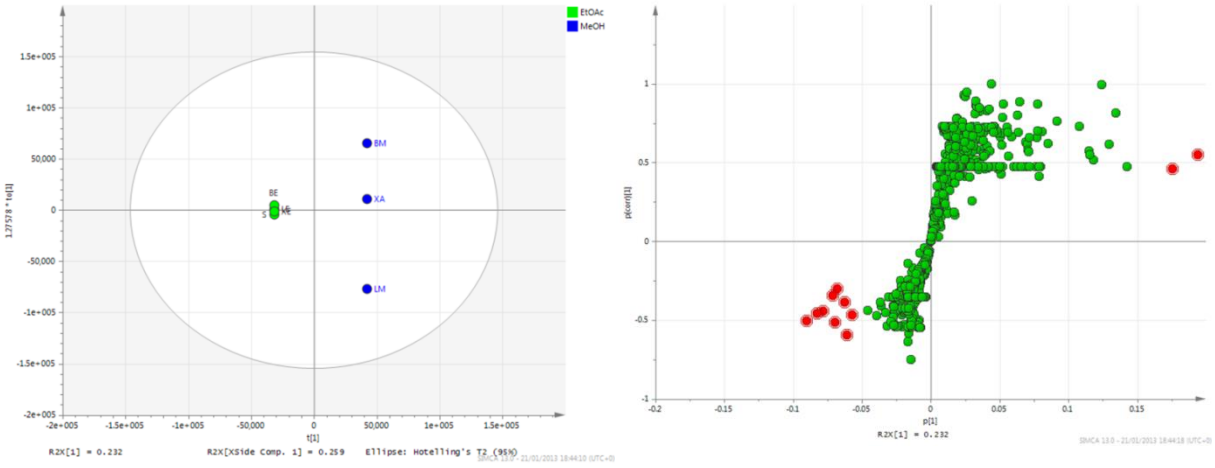
3.20

Table 3.6 Unidentified compounds of *Actinokineospora* EG49 extract from the database Marinlit

Negative *m/z*	Rt (min)	EG49 AE	Predicted Formula	Tolerance (ppm)
225.06174	1.25	4.86E+07	C7H13O8	0.664
287.08850	1.93	1.87E+07	C11H15N2O7	-0.119
298.09308	4.76	1.94E+08	C13H16O7N	-1.929
647.15375	8.22	3.70E+07	C30H25O12N5	5.178
243.17139	8.27	3.86E+07	C12H23O3N2	0.181
289.17697	8.27	9.57E+07	C13H25N2O5	0.466
216.03017	8.84	3.41E+06	C11H6O4N	-0.282
633.17438	8.90	6.08E+07	C21H29O15N8	-1.746
643.20306	8.90	3.64E+08	C30H33O13N3	1.545
357.06175	9.17	1.58E+07	C18H13O8	0.349
611.17676	9.74	1.17E+07	C31H31O13	2.076
370.09338	10.55	7.49E+07	C19H16O7N	0.932
687.17505	10.84	4.16E+07	C26H27O13N10	-2.218
401.08789	10.96	3.69E+07	C17H9O7N6	0.489
509.10866	11.26	1.86E+07	C25H15O6N7	-0.686
317.11424	11.50	3.82E+07	C16H17O5N2	-0.015
515.11932	12.19	9129900.063	C25H23O12	-0.465
497.14540	14.57	1.75E+08	C26H25O10	0.141
514.13513	14.57	1.86E+07	C25H24O11N	-0.688
565.13263	14.57	1.67E+07	C24H25O14N2	2.660
449.12424	15.29	7.67E+07	C25H21O8	0.043
371.07724	16.47	3.50E+08	C19H15O8	-0.487
737.16619	17.78	2.97E+06	C30H27O14N9	-8.390
324.08777	17.78	5.84E+07	C18H14O5N	-0.203
265.14798	18.38	6.90E+06	C18H19ON	3.121
311.16870	19.54	5.87E+06	C9H23O6N6	0.785
279.16348	19.54	1.38E+07	C19H21NO	2.534
353.20047	21.30	2.69E+07	C22H27O3N	2.259
325.18439	21.36	5.44E+06	C10H25O6N6	0.874
293.17932	22.29	2.96E+07	C20H23NO	2.651
257.21234	24.74	1.89E+08	C15H29O3	0.124
271.22800	26.75	5.05E+08	C16H31O3	0.597
285.24356	28.48	1.52E+08	C17H33O3	0.252
269.21237	28.84	9.34E+07	C16H29O3	0.899
360.25473	30.49	1.78E+07	C22H34NO3	1.035
Positive *m/z*	**Rt (min)**	**EG49 AE-neg**	**Predicted Formula**	**Tolerance (ppm)**
245.12822	4.03	1.35E+07	C14H17O2N2	1.557
227.13913	5.17	4.43E+08	C9H17O2N5	1.307
230.24791	16.91	6.78E+07	C12H30N4	1.497
370.09225	17.81	1.85E+07	C16H18O10	4.607
258.27921	19.75	5.22E+07	C16H36ON	1.063

Positive *m/z*	Rt (min)	EG49 AE-neg	Predicted Formula	Tolerance (ppm)
420.33209	28.21	4.54E+07	C20H44O5N4	1.026
282.27917	28.87	6.08E+07	C18H36ON	1.114

A dereplication study was also done to provide a metabolomic profile for monitoring and exploring the relationship between the different culture methods, and bioactivity to metabolome evolution in the sponge associated bacterium *Actinokineospora* strain EG49. Metabolomic profiling was first performed to choose the best extraction solvent either with methanol or ethyl acetate. As shown in **Figure 3.6**, a set of different metabolites are being extracted by the respective solvents with more of the anthaquinone derivatives going to the ethyl acetate fractions (**Table 3.7**).The anti-trypanosomal active compound **EG49D** was also only found in the ethyl acetate extract. From the OPLS-DA scores plot there was no significant difference between the media from which the ethyl acetate extracts were prepared in comparison to the more separated features observed for the methanolic extracts.

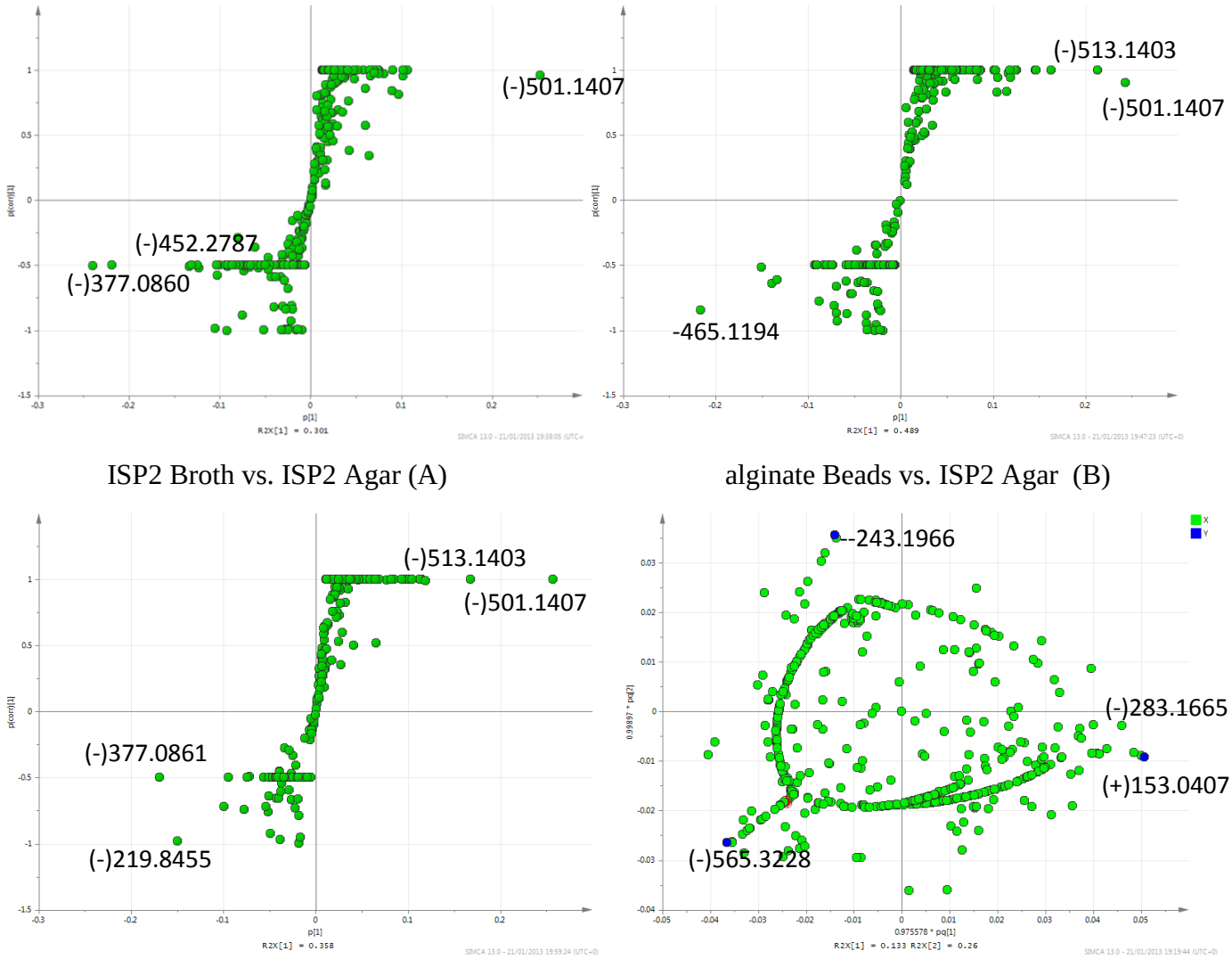


(A) (B)

Fig. 3.6 OPLS-DA Scores plot (A) and S-plot (B) by the presence of different secondary metabolites in the methanolic and ethyl acetate extracts

Table 3.7 The "end point" compounds as shown on the S-plot for **Fig. 3.5**

Solvent	Ionisation Mode	MS *m/z*	Rt(min)	Chemical Formula	Name
EtOAc	P/N	467.1350	8.27	C25H24O9	Atramycin A
EtOAc	P/N	469.1494	8.28	C25H24O9	Atramycin A
EtOAc	N	513.1404	8.28	C26H25O11	Unknown
EtOAc	N	611.1774	8.98	C31H32O13 (new)	EG49D
EtOAc	N	657.1828	10.50	C32H34O15	Elloramycin E
EtOAc	N	501.1407	10.76	C25H26O11 (new)	EG49F4B
EtOAc	N	467.1350	15.13	C25H24O9	BE-12406-A
EtOAc	N	449.1247	15.97	C25H22O8	Galtamycinone
MeOH	N	377.0860	1.38	C15H9O3N10	Unknown
MeOH	N	452.2787	21.50	C25H40O7	Unknown



ISP2 Broth vs. ISP2 Agar (A)

alginate Beads vs. ISP2 Agar (B)

ISP2 -XAD vs. ISP2 Agar (C)

ISP2 Broth vs. ISP2-XAD vs. alginate beads (D)

Fig. 3.7 S-plot and PCA analysis of the dereplicated mass spectral data between four different growth media for EG49 ethyl acetate extracts

The metabolomics profiling was also performed to choose best culture medium either using the normal ISP2 agar or the modified media. The OPLS-DA analysis was conducted respectively between the normal ISP2 agar and the individual modified medium. The S-plots of this experiment (**Fig. 3.7 A, B, C)** indicated a high output of EG49F4B and an unknown secondary metabolite with the negative *m/s* value of 513.1404[M-H]$^-$ in the normal ISP2 agar medium. This unknown secondary metabolite has also been marked in the solvent optimization experiment (**Table 3.7**), which provides a hit for structure elucidation of new isolated compounds. However, EG49D haven't been found in this experiment. Therefore, a scatter plot was performed to investigate the yield of this compound in the four different media. (**Fig. 3.8**)

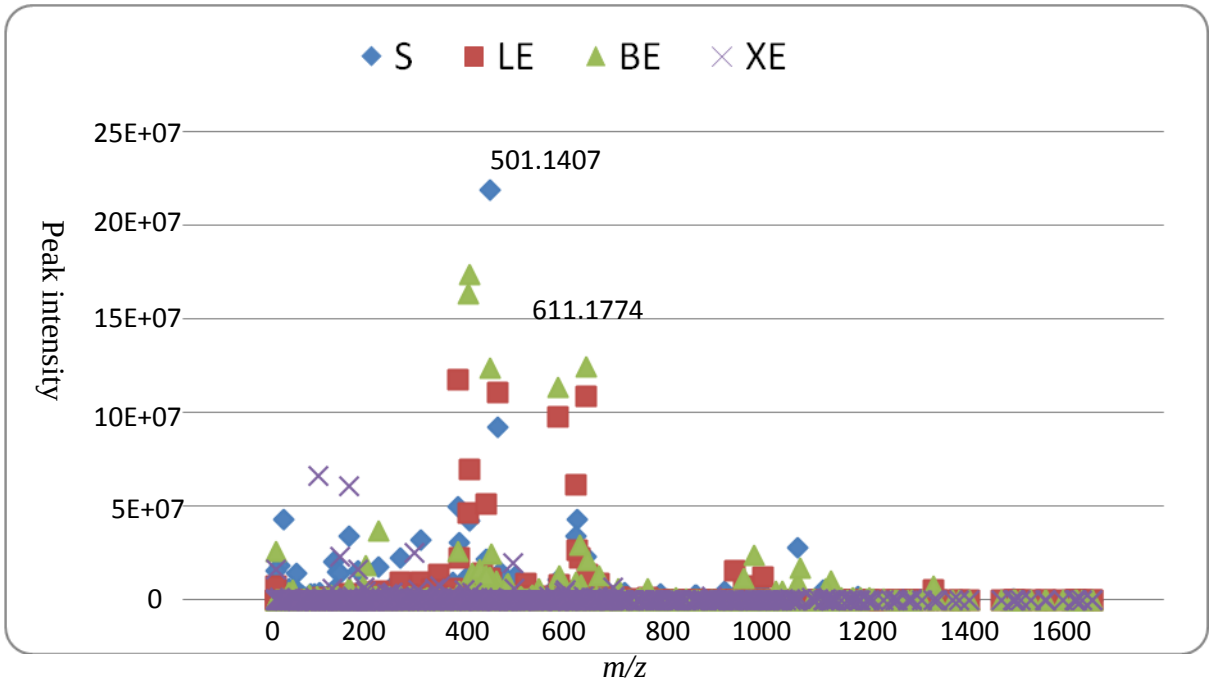


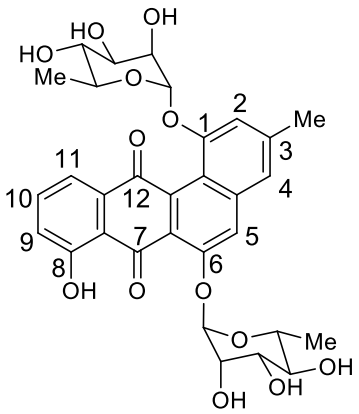
Fig. 3.8 Scatter plot of the dereplicated mass spectral data of EG49 in four different growth media: S = ISP2 agar, LE = ISP2 broth, XE = ISP2 broth with XAD, and BE = calcium alginate beads

In this scatter plot, the production of EF49F4B (*m/z* 501.1407 $[M-H]^-$) was only present in the solid extract at the highest concentration while the active anti-trypanosomal compound EG49D (*m/z* 611.1774 $[M-H]^-$) was produced at highest yield in liquid broth and calcium alginate beads media. (**Fig. 3.8**) From the peak intensities, there seems to be no big differences using the ISP2 broth and calcium alginate beads medium to achieve the production of EG49D. EG49D was also one of the highest amounts of the secondary metabolites produced in ISP2 broth. From the point of scaling up the cultivation, normal ISP2 liquid broth culture is the most ideal medium to achieve high production of the bioactibe EG49D. In addition, ethyl acetate is the right solvent for the extraction of these target compounds.

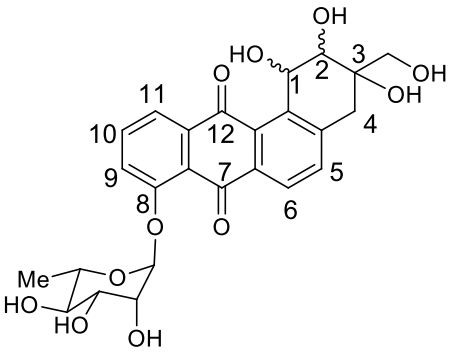
In conclusion, dereplication using metabolomics demonstrated its power and effectiveness in screening for target bioactive compounds and seeking for the ideal culture method.

3.2.2 Structure elucidation

3.2.2.1 EG49D structure elucidation



(EG49D)



(EG49F4B)

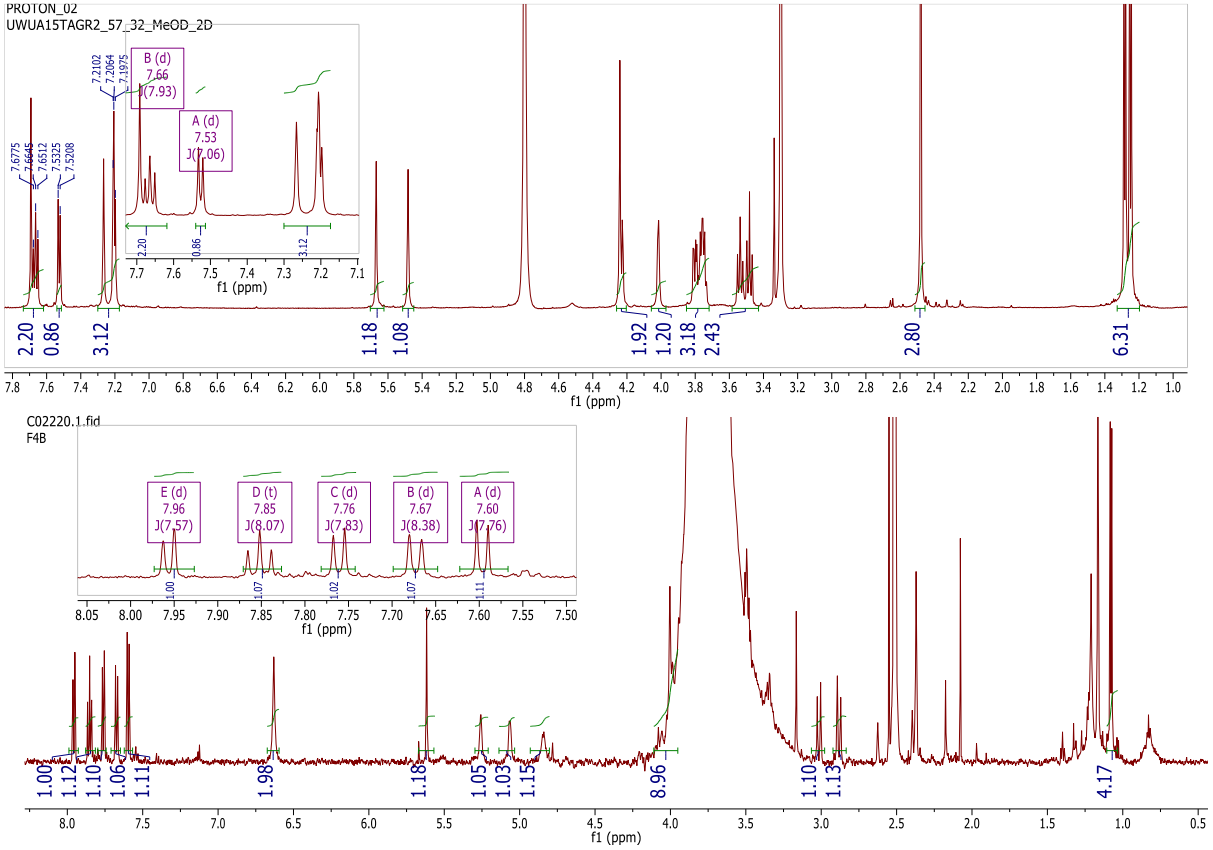


Fig. 3.9 ^{1}H NMR spectrum of EG49D (top) and EG49F4B (bottom)

3.2.2.1 EG49D structure elucidation

EG49D was obtained from Dr U.R. Abdelomohsen (University of Würzburg) as an orange oil: $[\alpha]_D$ 25 ^oC = +22 (*c* = 0.05, MeOH). This compound was purified by HPLC from the ethyl acetate extract of the liquid bacteria culture. The UV spectrum showed the maxima absorption at 238 nm which is one of the characteristic peaks of anthraquinone; 310 nm which belong to the benzenoid structures and 417 nm ascribable to the ketone groups embedded in the quinone. HR-MS/MS showed the molecular $(M-H)^-$ ion peak at *m/z* 611.1768 ($C_{31}H_{31}O_{13}$) and fragment ion peaks at *m/z* 465.12 ($C_{25}H_{21}O_9$) and 319.06 ($C_{19}H_{11}O_5$) revealed the successive breakup of two saccharide units since the subtracted molecular formula was $C_6H_{11}O_4$. This ion mass was also found in the above list of unidentified compounds (**Table 3.6**).

The ^1H NMR spectrum exhibited peaks from δ 7.24 ppm to δ 7.75 ppm suggesting the presence of aromatic structures. (**Fig. 3.9**) The doublet of doublets at δ 7.66 ppm (*J* = 7.4, 8.4 Hz) and two doublets at δ 7.52 ppm (*J* = 8.4 Hz) and δ 7.21 ppm (*J* = 7.4 Hz) which all integrated for one proton suggested an ABX aromatic spin system. Two broad singlets at δ 7.24 ppm and δ 7.32 ppm integrating for one proton each indicated an AB aromatic spin system which was further confirmed by their COSY correlations (**Table 3.8**). The COSY spectrum also indicated the existence of saccharides where two anomeric protons at *circa* δ 5.50 ppm were found to further correlate with proton resonances within the classical sugar region between 3 to δ 4 ppm. The presence of two rhamnose units were implied by the methyl doublets at δ δ1.25 ppm with coupling constants of 6.0 Hz and gave cross peaks to signals at the δ 3.50 ppm region.

Three oxygen-bearing carbons in the aromatic system were found in the ^{13}C spectrum where a carbon at δ 161.2 ppm indicated a phenol group while those shielded signals at δ 153.9 ppm and δ 152.3 ppm suggested the occurrence of saccharide substituents. Two carbon resonances at δ 180 ppm region indicated the presence of quinones.

In the HMBC spectrum, the proton doublet at δ 7.52 ppm showed a ^3J correlation with a carbon at δ 187.5 ppm which was attributed to one of the carbonyl carbons in the anthraquinone substructure. Another ^3J correlation of this proton occurred with an aromatic carbon δ 122.6 ppm which was then confirmed in the HMQC spectrum to have a direct correlation with its meta proton at δ 7.21 ppm. Since the proton at δ 7.21 ppm did not show

any correlation with carbonyl carbons but only a ^{3}J correlation with the carbon at δ 117.1 ppm, it was then attributed to H-9. Therefore, protons at δ 7.66 ppm δ 7.52 ppm were unambiguously assigned to H-10 and H-11, respectively. The quaternary carbon C-8 at δ 161.2 ppm and C-11a δ 135.5 ppm were then detected by the ^{3}J correlations with H-10 (δ 7.66 ppm). C-7a at δ 115.7 ppm was assigned through its ^{3}J correlation with H-11 at δ 7.52 ppm. Since the proton at δ 7.69 ppm showed a correlation with the other carbonyl carbon, it was attributed to be either C-5 or C-6. But the correlation was very weak which indicted a ^{4}J correlation in a "W" pattern, therefore H-5 was assigned to this proton. Since weak ^{4}J correlation between H-5 and the proton at δ δ 7.26 ppm was also observed in the COSY spectrum, H-4 was then assigned at δ 7.26 ppm. This was doubly confirmed by the ^{3}J correlation in the HMBC spectrum between H-5 and an aromatic carbon at δ 119.6 ppm which was found to be directly connected to the proton at δ 7.26 ppm (H-4) in the HMQC spectrum. The last singlet proton at δ 7.19 ppm was eventually assigned to H-2. There was one more correlation between H-5 and a carbon at δ 152.3 ppm, hence C-6 was designated to the oxygen-bearing carbon. C-2 and C-5 were assigned by HMQC δ 112.2 and δ 116.9 ppm, respectively. In the HMBC spectrum, C-4 (δ 119.6 ppm) was also ^{3}J correlated to an acyl proton at δ 2.47 ppm. This singlet integrating for three protons indicated a methyl group. Therefore, C-3 can be concluded to bear a methyl substituent. C-1 was then assigned as the last oxygen-bearing carbon in the aromatic system and the chemical shift at δ 153.9 ppm was also confirmed by its ^{2}J correlation with H-2 at δ 7.19 ppm. The structure of EG49D was elucidated as 8-hydroxy-3-methylbenz[α]anthracene-7,12-dione-1-O-α-L-rhamnopyranose-6-O-α-L-rhamnopyranoside and given the trivial name actinosporin A.

Table 3.8 NMR data of actinosporin A (**EG49D**) and actinosporin B (**EG49F4B**)

EG49D Measured in CD_3OD-d_4 (^1H: 600 MHz; ^{13}C: 150 MHz)						**EG49F4B** Measured in DMSO-d_6 (^1H: 600 MHz)	
Position C/H No.	**δ_H, mult (*J* in Hz)**	**COSY**	**δ_C**	**HMBC (δ_H to δ_C)**	**ROESY**	**δ_H, mult (*J* in Hz)**	**COSY**
1			153.9			5.64, d (4.3)	4.00
2	7.19, s	2.47	112.2	C-1, C-3, C-3-CH_3, C-4, C-12a, C-12b	H-3-CH_3, H-1A	4.00, m	5.64
3			142.0			3A - 4.83, d (11.6) 3B - 4.00, m	4.00 4.83
3-CH_3	2.47, s	7.19,7.26	20.8	C-2, C-3, C-4			
4	7.26, s	2.47	119.6	C-2, C-3-CH_3, C- 4a, C-12b	H-3-CH_3, H-5	4A - 3.01, d (13.2) 4B - 2.90, d (13.2)	2.90 3.01
4a			138.9				
5	7.69, s		116.9	C-4, C-6, C-6a, C-12b	H-4, H-1B, H-5B	7.80, d (7.8)	7.62
6			152.3			7.62, d (7.8)	7.80
6a			124.0				
7			186.2				
7a			115.7				
8			161.2				
9	7.21, dd (7.4, 1.4)	7.52,7.66	122.6	C-7a, C-11, C-10		7.71, d (8.2)	7.86, 7.96
10	7.66, dd (7.4, 8.4)	7.52,7.21	136.1	C-8, C-11, C-11a		7.86, dd (7.4, 8.2)	7.71, 7.96
11	7.52, dd (8.4, 1.4)	7.21,7.66	117.1	C-7, C-7a, C-9, C-10, C-12		7.96, d (7.4)	7.71, 7.86
11a			135.5				
12			187.5				
12a			140.0				
12b			116.1				
1'	5.48, brs	4.05	99.6	C-1, C-2', C-5'	H-2	6.55, brs	3.35
2'	4.05, s	3.80, 5.48	70.4	C-3', C-4'		3.35, m	4.93, 6.55
3'	3.80, dd (9.4, 3.4)	3.48, 4.05	70.9	C-4', C-5'		4.93, m	3.35, 3.33
4'	3.48, t (9.4)	3.80, 3.76	72.4	C-3', C-5', C-6'	H-6'	3.33, m	3.53, 4.93
5'	3.76, m	1.28	69.6	C-6'	H-3'	3.53, m	3.33, 1.10
6'	1.25, d (6.3)	3.76	16.5	C-5', C-4'		1.10, d (6.3)	3.53
1"	5.66, brs	4.22	99.0	C-8, C-5", C-4"	H-5		
2"	4.22, d (3.4)	4.25, 5.66	70.6	C-3", C-4"			
3"	4.25, s	4.22, 3.53	70.7	C-4"	H-5"		
4"	3.53, t (9.2)	4.25, 3.74	72.5	C-2", C-3", C-6"			
5"	3.74, m	3.53,1.27	69.9	C-3", C-4", C-6"			
6"	1.27, d (6.2)	3.74	16.6	C-4", C-5"			

3.2.2.2 EGF4B structure elucidation

EG49F4B was also obtained from Dr U. R. Abdelomohsen (University of Würzburg) as orange oil: $[\alpha]_D$ 25 ^oC = +18 (*c* = 0.05, MeOH). This compound was purified by HPLC from the ethyl acetate extract of the liquid bacteria culture. The mass spectrum revealed the occurrence of a mixture with a major peak of *m/z* 501.1402 in negative mode and *m/z* 503.1554 in positive mode ($C_{25}H_{26}O_{11}$). This molecular weight was found and highlighted in the identified compounds list (**Table 3.5**) and the structure was studied by comparing with the known compound found in the Marinlit database which proved that the isolated compound was different from that in the literature. Since the amount of the sample was only a mg, only ^1H and COSY NMR spectra were performed. HRMS/MS was performed obtaining a fragment ion at *m/z* 399.1079 [M-$C_4H_6O_3$] which implied a cross-ring cleavage comparable to a glycan unit and confirmed the presence of a highly hydroxylated saturated ring A. High resolution mass spectral data of the fragment ions verified the presence of four hydroxyl substituents on ring A as shown in **Fig. 3.9**. Further fragmentation of the ion at *m/z* 399.1079 afforded an ion peak at *m/z* 253.0502 indicating the loss of a saccharide unit as in **EG49D.**

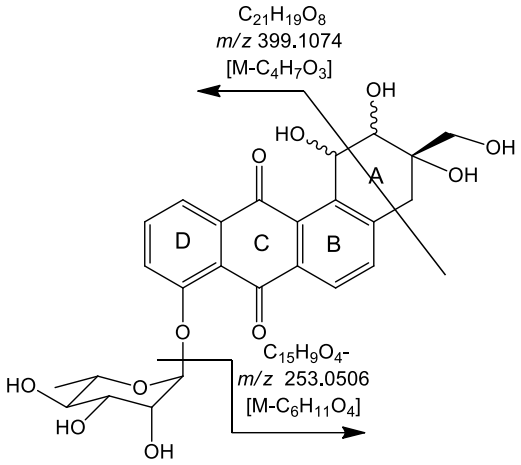


Fig. 3.10 Fragmentation of EG49F4B

The UV spectrum was very similar to that of EG49D exhibiting the maxima absorption at 254 nm which is one of the characteristic peaks of anthraquinone, shift of the bands from 310 to 379 nm indicates changes occurring at the benzenoid structure or Ring A (**Fig. 3.10)**. The ^1H NMR spectrum showed the presence of aromatic protons with peaks from δ 7.62 ppm to δ 7.96 ppm which all integrated for one proton each. (**Fig. 3.9**) The COSY spectrum revealed two spin systems in the aromatic region. Correlation between the "pseudo-triplet" at δ 7.86

ppm and two doublets at δ 7.96 and δ 7.71 ppm indicated an ABX spin system. While two ortho doublets at δ 7.80 and δ 7.62 ppm correlated with each other suggesting an AB aromatic spin system. The parent structure was determined to be a quinone with one aromatic ABX system (Ring D) and another AB system (Ring B). The presence of a rhamnose unit was also identified from the COSY spectrum (**Fig. 3.12**) with the characteristic methyl 6 Hz doublet at δ 1.10 ppm which correlated with a proton at δ 3.53 ppm as well as the broad singlet at 6.55 affording a cross peak with a signal at δ 3.35 ppm. Attachment of the rhamnose was deduced to be on C-8 as the proton doublet at C-9 was highly deshielded as observed with similarly substituted landomycin and atramycin analogues (Fujioka *et al*., 1991; Shaaban *et al*., 2011)

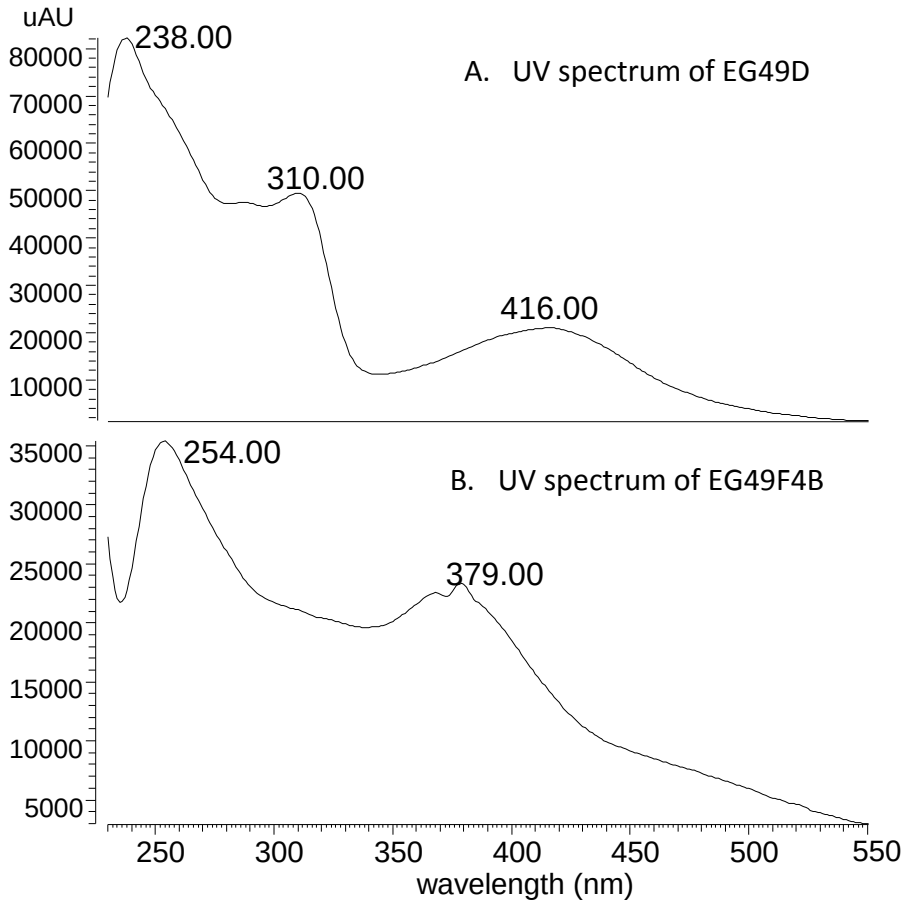


Fig. 3.11 UV spectra of ED49D andEG49F4B

Two well-resolved geminal methylene doublets at δ 3.01 ppm and δ 2.90 ppm with ^{2}J of 13.42 Hz were assigned at position 4 due to the benzylic nature of the deshielded chemical shifts as well as their being adjacent to a chiral centre at C-3. A broad doublet at 4.83 ppm

with a geminal coupling constant of 11.6 Hz was assigned to one of the hydroxyl methylene protons attached to C-3 while the other geminal proton was found to be overlapping with another proton signal at δ 4.01 ppm. The hydroxylated methine doublet at δ 5.64 ppm, with a small coupling constant of 4.3 Hz correlated with the peak at δ 4.01 ppm and the proton resonances were assigned to H-1 and H-2, respectively. The structure EG49F4B as determined as 1,2,3-trihydroxy-3-(hydroxymethyl)-8-rhamnosyl-1,2,3,4-tetrahydrotetraphene-7,12-dione and assigned the trivial name actinosporin B.

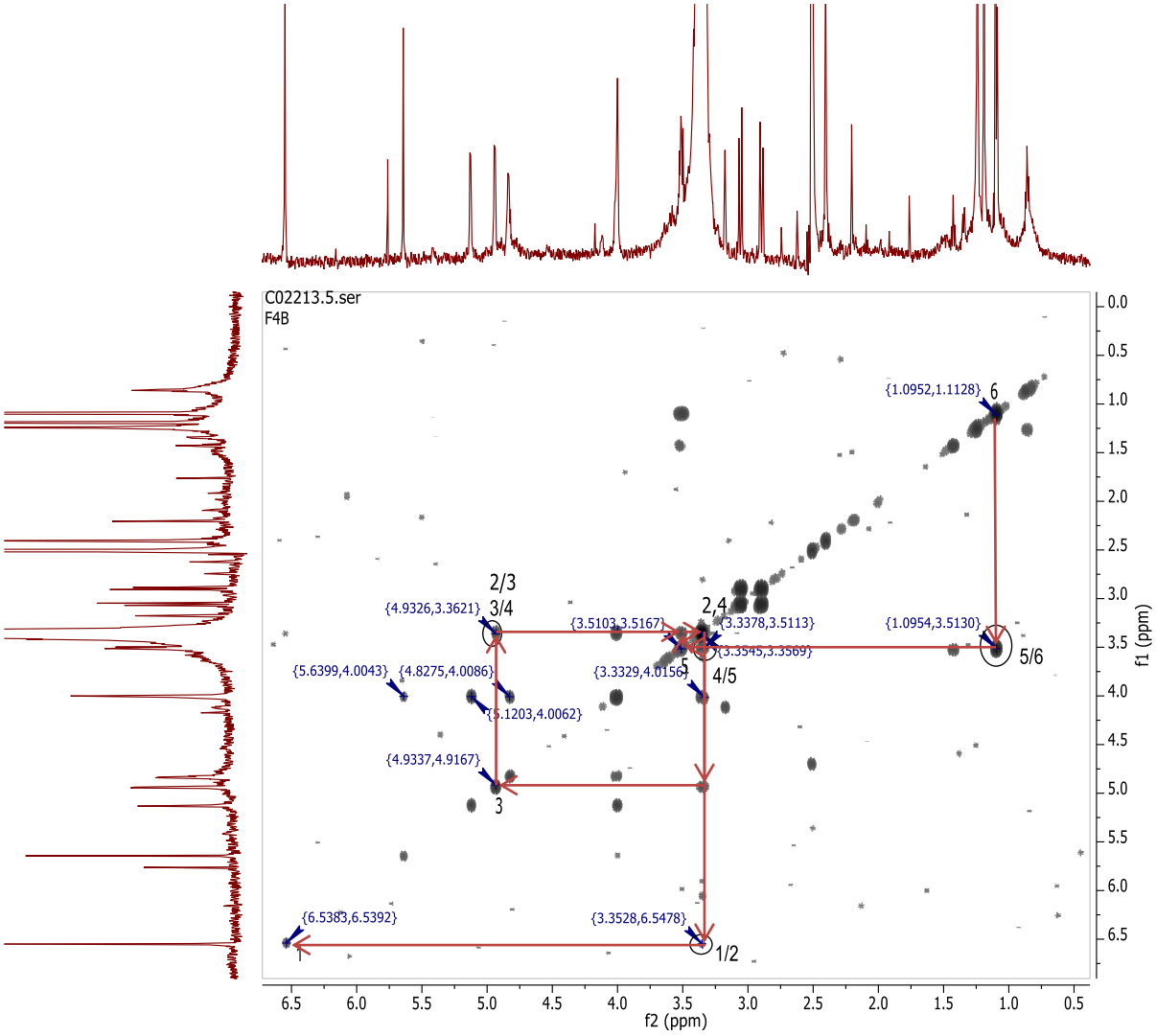


Fig. 3.12 COSY spectrum of the sugar unit in EG49F4B

3.2.3 Bioassays using EG49D and EG49F4B

EG49D and EG49F4B were screened for anti-*T.b.brucei* and anti-*M. marinum* activity. EG49F4B was tested once only since the amount was not enough for duplication. However, it

appeared to have no activity against either *T.b.brucei* or *M. marinum*. EG49D exhibited potential inhibition towards the *T.b.brucei* strain. (**Table 3.9**) In addition, the quinone structure of EG49D was also assumed to contribute to the anti-trypanosomal activity as discussed above.

Table 3.9 Anti-*T.b.brucei* and anti-*M. marinum* bioassays for EG49D and EG49F4B

Sample	***T.b.brucei*** **% control**	***M.marinum*** **% control**
EG49D	**2.9**	69.3
EG49D	**4.9**	87.9
EG49F4B	**89.2**	56.4

Chapter 4 Analysis of the Spectrum of Acyl-homoserine Lactone production by Gram-negative Bioluminescent Bacteria

4 .1 Materials and Methods

4.1.1 Cultivation of Microorganism

The cultivation was carried out by Dr Michael Winson (Bryoactives Ltd.), in marine broth (23 hours incubation at 21 ^{o}C).

4.1.2 Extraction and TLC analysis

The liquid cultures and blank medium were extracted three times with 22.5 ml of DCM equal to that of the volume of the broth. The DCM layer was collected and dried for MS analysis. Evidence for the presence of certain detectable AHLs was provided by a TLC based bioassay on a C18R matrix developed with MeOH 60:40 water and detectable stimulatory activity was revealed using a *Chromobacterium violaceum* CVBlu biosensor overlay.

4.1.3 LC-MS identification of AHLs

Analyses were performed on an Accela HPLC from Thermo Scientific (Bremen, Germany) coupled with Exactive (Orbitrap) mass spectrometer from Thermo Fisher Scientific (Bremen Germany) as described above.

Mass spectrometry was carried out only in positive ionisation modes with a spray voltage at 4.5 kV, capillary temperature at 320 ^{o}C and an in-source HCD voltage at 25 eV. The mass range was set from *m/z* 150-1500 for ESI-MS and *m/z* 50-1000 for MS/MS.

4.2 Results and Discussion

4.2.1 HPLC-MS

AHL production is not a consistent feature even in bacteria from the same species or location. Therefore pre-selection of potential AHL producers would contribute to the investigation for control of other important QS controlled activities with biotechnological benefits. Since bioluminescence is a readily-observable phenotype with high possibility of producing AHL molecules, the bioluminescent bacteria were selected in this study.

One of the purposes of MS detection of QS signalling components is to validate the results obtained from the traditional TLC approach while investigating the presence of long acyl-

chain AHLs which are difficult to visualise on a TLC plate. The detection of AHLs (**Table 1**) was conducted by comparing their individual molecular ion *m/z* value to their corresponding positive molecular ion weight and further confirmed by investigating the common lactone ion fragment peak at *m/z* 102.05 in the MS/MS ion chromatogram.

Two potentially novel 'AHL-like' signals apparently not previously reported in bioluminescent species were detected at *m/z* values of 228.123 and 272.186 respectively (based on the presence of the *m/z* 102.05 molecular ion). The *m/z* 228.123 peak was only found in strains BL112 and BL115 with two peaks shown in the ion chromatogram (**Fig. 4.1**). The predicted formula is $C_{11}H_{18}NO_4$ with the acceptant difference value at δ 0.765 ppm. An unknown AHL compound was previously visualised in BL114 on TLC plate which may correspond to these unknown mass ion peaks. More detailed MS/MS analysis of this mole ion peak at *m/z* 228.123 will be required to confirm the existence of this potentially novel AHL. The component identified at *m/z* 272.186 was found to have the same lactone fragment, with a symmetrically-shaped peak of considerable intensity in the mass spectrum (**Fig. 4.2**). The predicted formula of this compound is $C_{14}H_{26}NO_4$ with a very low acceptance difference value at δ 0.68 ppm (and has two more protons than ODHL ($C_{14}H_{24}NO_4$). It is possible that this compound is HODHL a reduced version of DHL with a hydroxyl replacing the H at the C3 position. Since there are two unsaturated positions on the acyl-chain of DHL and MS/MS analysis of this ion will be required to confirm the precise reducing position. The role of this molecule is not known, but it is possible that it may be involved in the biosynthetic pathway. It seems that this molecule would not activate the widely used CVblu biosensor used in the TLC separation bioassay. This MS-based approach is therefore the most optimal for finding these types of signal molecules.

Table 4.1 AHLs found in BL samples

	Short acyl-chain AHLs				Long acyl-chain AHLs							
m/z	200.128	214.107	228.123	228.159	242.139	256.191	270.170	272.186	298.201	284.222	312.2534	326.2326
Rt STD	8.09	4.5		13.33	9.89	17.26		13.27	18.19	20.96	24.75	
Name	6HHL	6OHHL	7OHHL	OHL	OOHL	DHL	ODHL		OdDhL	dDHL	THL	OTHL
No. of AHLs	0	13	2	20	3	1	1	13	0	0	0	1
BL7	0	√	0	√	0	0	0	0	0	0	0	0
BL9	0	√	0	√	0	0	0	0	0	0	0	0
BL11	0	0	0	0	0	0	0	0	0	0	0	0
BL13	0	0	0	√	0	0	0	0	0	0	0	0
BL17	0	√	0	√	√	0	0	0	0	0	0	0
BL18	0	√	0	√	0	0	0	0	0	0	0	0
BL20	0	√	0	√	0	0	0	0	0	0	0	0
BL27	0	√	0	√	0	0	0	0	0	0	0	0
BL28	0	√	0	√	0	0	0	0	0	0	0	0
BL29	0	0	0	0	0	0	0	√	0	0	0	0
BL30	0	0	0	√	0	0	0	√	0	0	0	0
BL37	0	0	0	0	0	0	0	√	0	0	0	0
BL38	0	0	0	0	0	0	0	√	0	0	0	0
BL54	0	√	0	√	0	0	0	√	0	0	0	0
BL55	0	0	0	0	0	0	0	0	0	0	0	0
BL57	0	0	0	0	0	0	0	0	0	0	0	0
BL58	0	0	0	0	0	0	0	0	0	0	0	0
BL61D	0	0	0	√	0	0	0	0	0	0	0	0
BL65Batch2	0	0	0	√	0	0	0	0	0	0	0	0
BL73	0	0	0	√	0	0	0	0	0	0	0	0
BL74	0	0	0	0	0	0	0	√	0	0	0	0
BL76	0	√	0	√	0	0	0	0	0	0	0	0
BL77	0	√	0	√	0	0	0	0	0	0	0	0
BL78	0	0	0	√	0	0	0	0	0	0	0	0

	Short acyl-chain AHLs				Long acyl-chain AHLs							
m/z	200.128	214.107	228.123	228.159	242.139	256.191	270.170	272.186	298.201	284.222	312.2534	326.2326
Rt STD	8.09	4.5		13.33	9.89	17.26		13.27	18.19	20.96	24.75	
Name	6HHL	6OHHL	7OHHL	OHL	OOHL	DHL	ODHL		OdDhL	dDHL	THL	OTHL
BL80	0	0	0	√	0	0	0	0	0	0	0	0
BL81SN	0	0	0	√	0	0	0	√	0	0	0	0
BL83	0	0	0	0	0	0	0	√	0	0	0	0
BL84	0	0	0	0	0	0	0	0	0	0	0	0
BL85	0	0	0	0	0	√	0	0	0	0	0	0
BL88	0	0	0	0	0	0	0	√	0	0	0	0
BL89	0	0	0	0	0	0	0	√	0	0	0	0
BL90	0	0	0	0	0	0	0	√	0	0	0	0
BL91	0	0	0	0	0	0	0	√	0	0	0	0
BL104	0	0	0	0	0	0	0	0	0	0	0	0
BL106	0	0	0	0	0	0	0	0	0	0	0	0
BL112	0	√	√	√	√	0	0	0	0	0	0	0
BL115	0	√	√	0	√	0	√	√	0	0	0	0

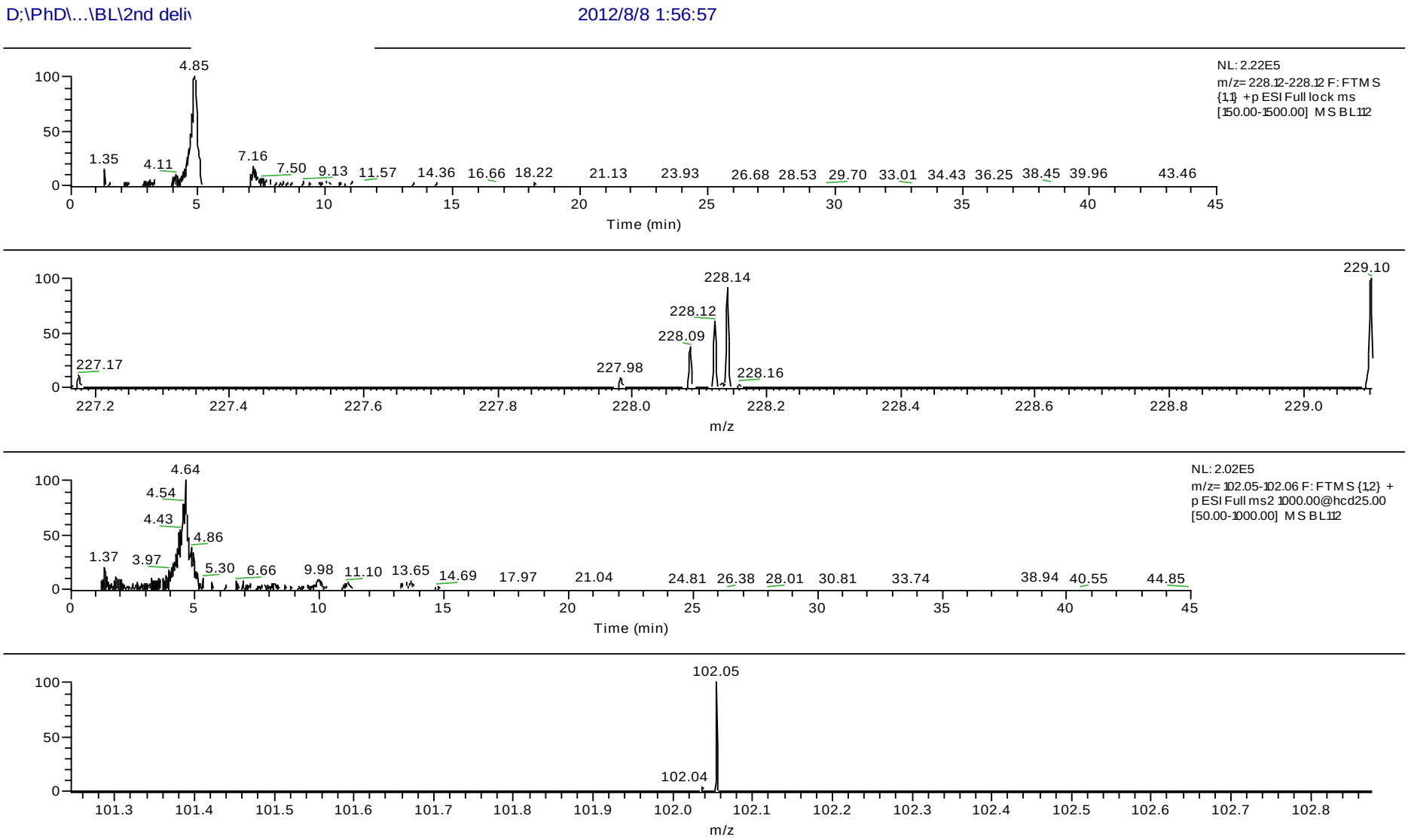


Fig. 4.1 Extracted ion chromatogram and MS spectra of BL112 indicating the presence of *m/z* 228.123

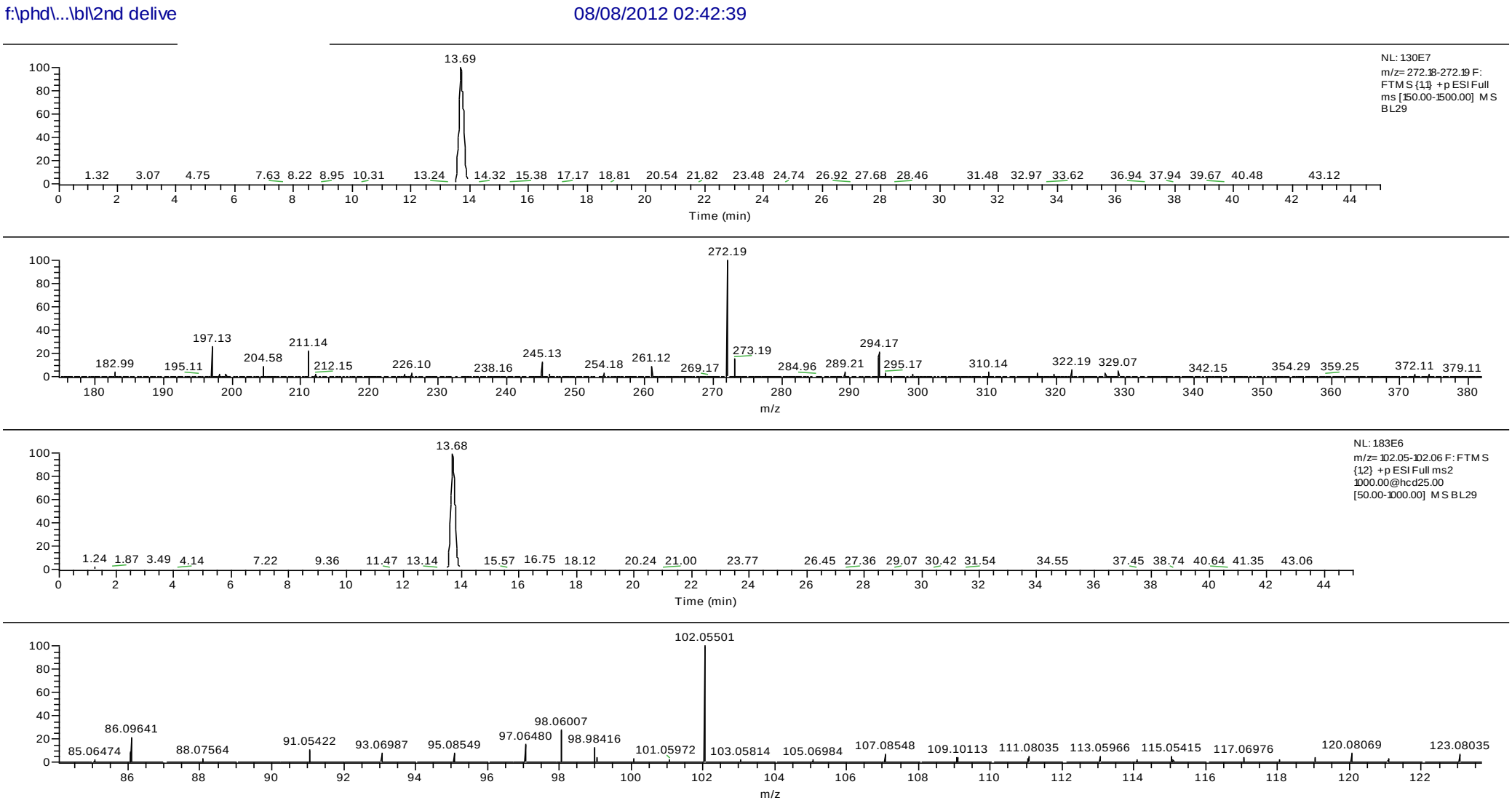


Fig. 4.2 Extracted ion chromatogram and MS spectra of BL29 indicating the presence of *m/z* 272.186

The efficiency of extracting AHLs was also investigated by comparing the AHL peak intensities obtained with homogenisation and centrifugation methods. Homogenisation was carried out by homogenising the broth culture for 15 min and filtering the suspension. Centrifugation was performed by centrifuging an equal volume of broth at 3000 rpm for 15 min. The supernatants were both extracted 3 times with equal volumes of DCM. The dried extracts were re-dissolved in 150 µL MeOH for MS detection. Data shown in **Table 4.2** indicated that for detection of AHLs the most efficient strategy was centrifugation to remove cells, followed by extraction of the supernatant, increasing the peak intensity approximately 3-fold when compared to the homogenisation method which involved a filteration step.

Table 4.2 OHL peak areas in different extraction methods

	Extraction method	**AHL intensity**	
		OHL	*m/z* 272.186
BL81	Homogenisation	12908143	18512281
BL81SN	Centrifugation	30792480	31453167

4.1.2 OPLS-DA analysis

Although the primary AHL-controlled phenotypic characteristic is expected to be bioluminescence, these signals may also be involved in stimulating (or even down-regulating the expression of) secondary metabolic activities of biotechnological interest. To preliminarily investigate the possible relationships between the quorum sensing signals and the cryptic secondary metabolites in the complicated biological system, we performed Supervised Orthogonal Partial Least Square-Discriminant Analysis (OPLS-DA) on the metabolites of these bioluminescent strains. The AHL molecules from bioluminescent bacteria we detected in this work were with the acyl-chain lengths of C6-C14, including each known variation of substitution (O, OH, H) at the C3 position. In some cases no AHL molecules were detected. In order to obtain a good scale mode in OPLS-DA analysis, those samples with no AHLs detected were not included in this analysis. Amongst these bioluminescent bacteria the MS analysis revealed that they could be subdivided into three groups in relation to AHL production, those making short acyl-chain AHLs C4-C8, those producing long acyl-chain AHLs (C10-C14) and those secreting both short and long acyl-chain AHLs. It was envisaged that these three groups of strains might differ in their control of other phenotypic characteristics.

The OPLS-DA score plot (**Fig. 4.3**) shows good separation between the short acyl-chain AHLs- and long acyl-chain AHL-producing bacteria. However, there was still an overlap

between the long acyl-chain AHLs group and the "both" group. By checking the contribution plot of BL74 and its clustering sample, the BL115 ion peak at *m/z* 294.168 ($C_{13}H_{26}O_7$/$C_{12}H_{20}O_2N_7$) appeared to be the factor responsible for the overlap between these two groups. This ion peak is only found in sample BL74 and BL115. It is not clear whether this molecule is specifically responsible for cell-cell signaling in these strains or a common metabolite specific to the strain types.

Ion peaks with relatively high p (corr) values shown in the S-plot indicate the level of contribution to the group separation in the OPLS-DA scores plot, thus providing information about the relationships of secondary metabolites within groups and differences between groups. (**Fig. 4.2**) Obviously, the presence of ion peak at *m/z* 272.186 at the end of the S-plot with high p (corr) value confirmed its role in representing the long acyl-chain AHLs group. The S-plot also revealed the secondary metabolites common to this group. (**Table 4.3**) Although the short acyl-chain AHL signals of *m/z* 214.107 and 228.159 *m/z* were shown within the lower part of the reliable region in the S-plot, they are still positioned in the region representing the short acyl-chain AHL group. One of the reasons giving rise to a false distribution was suspected to be related to the lower ion peak area of these "marker" ions compared to the "end-plot" ions.

The "end-plot" ions for short acyl-chain AHLs group were also tabulated in **Table 4.3**. As mentioned above, the possible presence of secondary metabolites such as antibiotics in the "end-plot" region should also be taken into consideration. Further MS/MS identification of the metabolites in the "end-plot" regions still needed further investigation. At this stage, only the MS positive ion mode was analysed as a preliminary investigation into the feasibility of this approach for providing meaningful correlations. It would be instructive to test this hypothesis by repeating the analysis using data from the negative ion analysis mode to confirm the presence of acidic metabolites.

The interpretation of the data suggest that a metabolomics study combined with multivariate data analysis has the potential for providing an informative and credible approach to highlighting relationships between quorum sensing induction and the secondary metabolites in complicated biological systems.

Table 4.3 The identification of "end-plot" ions by the database Marinlit

Groups	pos *m/z*	Formula	RDB	ppm	Identification
Long acyl-chain AHLs group	169.061	$C_{10}H_7N_3$	9	-3.601	
	183.076	$C_6H_9O_2N_5$	5	3.244	
	213.141	$C_3H_{17}O_3N_8$	-0.5	-3.486	
	284.114	$C_{14}H_{14}O_2N_5$	10.5	0.312	
		$C_{14}H_{16}O_3N_2$	10	-4.413	
		$C_8H_{11}O_3N_2$	4.5	-4.09	(Hydroxnitrophenyl) ethylamine (Oxpyridinyl) alanine 2,Furandiacetic acid; Diamide Aminhydroxpyridinepropanoic acid
	489.207	$C_{19}H_{31}O_{10}N_5$	7	-0.007	
		$C_{18}H_{35}O_{14}N$	2	2.727	
		$C_{17}H_{29}O_9N_8$	7.5	2.737	
		$C_{20}H_{27}O_6N_9$	12	-2.741	
		$C_{21}H_{33}O_{11}N_2$	6.5	-2.752	
Short acyl-AHLs Group	204.125	$C_{10}H_{14}N_5$	6.5	1.901	Adenine Triacanthine Zeatin
		$C_{12}H_{16}ON_2$	6	-4.677	
	243.088	$C_{12}H_{11}O_2N_4$	9.5	1.184	
		$C_{14}H_{13}O_3N$	9	-4.339	
	284.139	$C_{16}H_{18}O_2N_3$	9.5	-0.047	Cyclo (prolyltryptophyl) Ethyliden (1indoylmethylmethy2, piperazinedione) Lysergic acid Paspalic acid
		$C_{14}H_{16}ON_6$	10	4.678	
		$C_{18}H_{20}O_3$	9	-4.772	
	284.139 (186.162+H_3PO_4)				

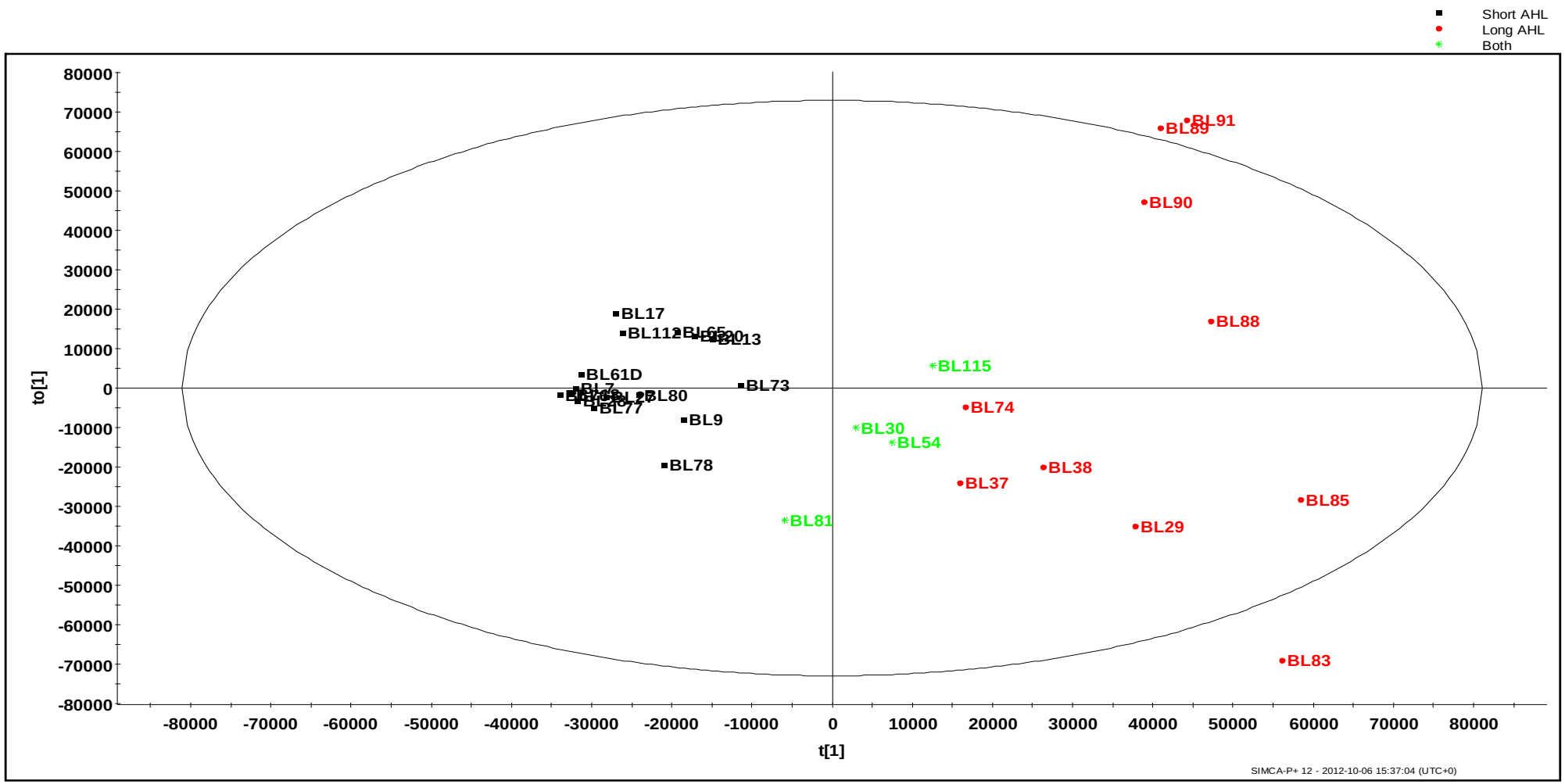


Fig. 4.3 OPLS-DA Scores plot by the presence of different AHLs in the BL samples. Black: BL samples with the presence of the short acyl-chain AHLs; green: BL samples with the presence of both short and long acyl-chain AHLs; red: Bl samples with the presence of the long acyl-chain AHLs.

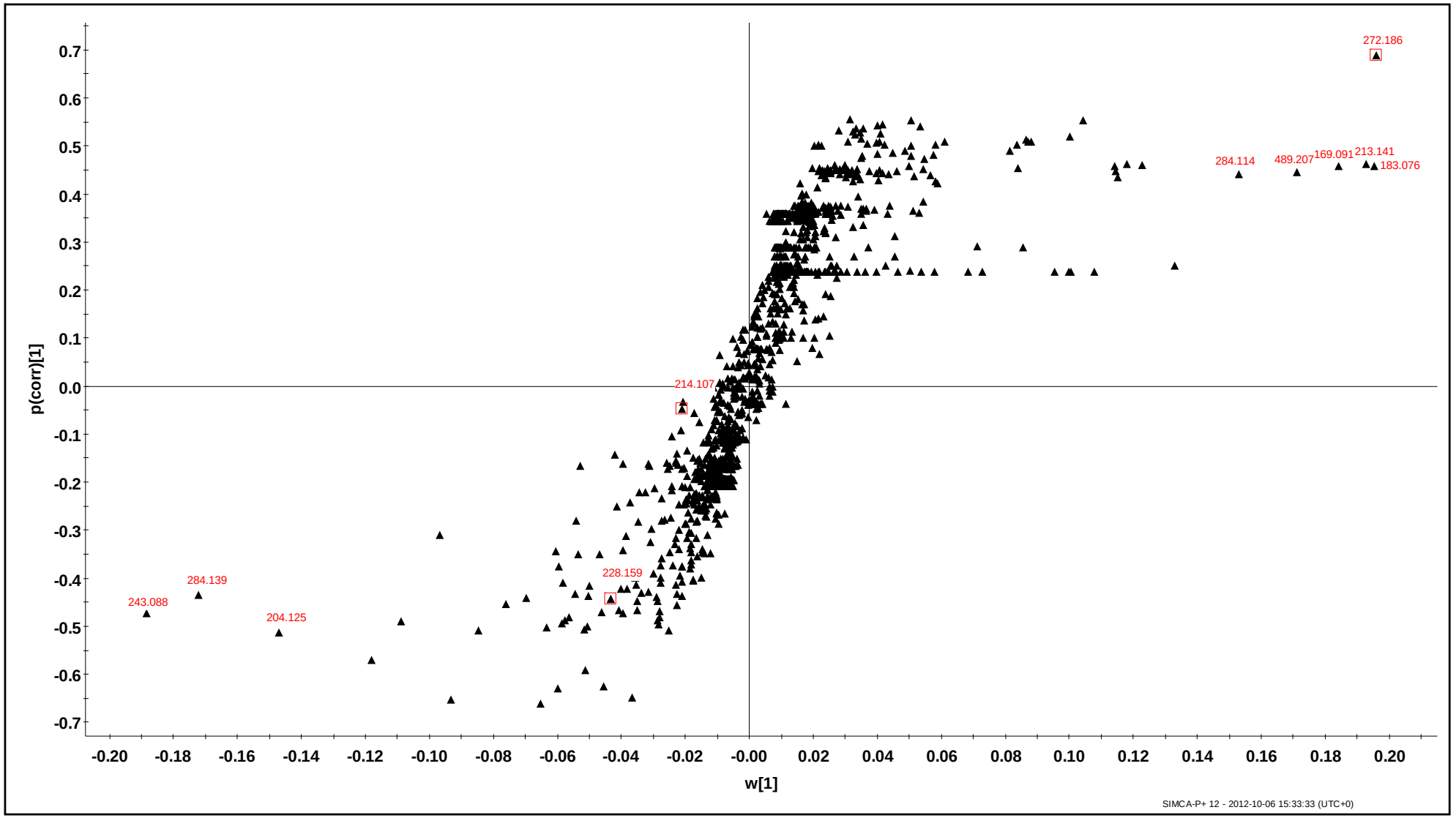


Fig. 4.4 S-Plot generated by OPLS-DA for the secondary metabolites in the BL samples

Chapter 5 Conclusion

5.1 Marine sponge *Callyspongia aff. implexa*.

The marine sponge *Callyspongia aff. implexa* was found to possess potent anti-*M.marinum* activity. Three compounds, β-sitosterol, A37-2-B1 (a triterpene) and A45-3-3 (an acetylene) were isolated and proven to be present in the bioassay-guided fractions. Due to the limited amounts isolated, the elucidation of A37-2-B1 and A45-3-3 using NMR was only partially achieved. MS analysis of A37-2-B1 showed one more degree of unsaturation on the side chain of the sterol which was speculated to be an acetylenic structure since no more olefinic protons were observed in the ^{1}H NMR spectrum. However, due to the small quantity, ^{13}C NMR was not measured. A45-3-3 showed the junction between an alkenyl and terminal acetylene while the length of the chain was determined by the ^{1}H NMR integration. EIMS is recommended to obtain the molecular mass of the acetylenes. Dereplication was conducted on the bioassay-guided fractions indicating the abundant presence of sterol compounds which also corresponded to the current isolation results. In addition, 24-methylene-5a-cholest-7-ene-tetraol which was previously isolated from marine sponge *Spongia officinalis* appears to be present in *Callyspongia aff.implexa* as evidenced by the MS signals in both positive and negative mode during the dereplication investigation.

5.2 *Actinokineospora* EG49

There are a great many secondary metabolites of *Actinokineospora* EG49 identified from the Marinlit database, most of which have been previously isolated from *Streptomyces* sp. providing a high confidence level of the presence of these compounds. Atramycin B was found in both positive and negative mode MS with relatively high peak intensity. Together with other identified compounds, compounds not been previously reported for anti-trypanosomal activity were also identified. Novel anthraquinone compounds, EG49F4B (*m/z* 501) and EG49D (*m/z* 611), (which were respectively listed among the dereplicated and unidentified metabolites in **Tables 3.5** and **3.6**) were isolated from this strain. Only EG49D showed considerable activity against trypanosomes. The minimum inhibitory concentration (MIC) against trypanosomes is yet to be determined for this compound. In this case, dereplication using metabolomics demonstrated its power and effectiveness in screening for target bioactive compounds. However, it is still worth performing further bioassays on the identified compounds for future isolation work.

5.3 Acyl-homoserine Lactone production by Gram-negative Bioluminescent Bacteria

Two potentially novel 'AHL-like' signals in which one of the unsaturated positions on the acyl-chain was surmised to be reduced into a hydroxyl group were detected at *m/z* values of 228.123 and 272.186 respectively in bioluminescent strains based on the presence of the *m/z* 102.05 molecular ion at the same retention time. HR/MS/MS analysis of these ions is expected to confirm the precise reducing position. In the OPLS-DA study of the whole secondary metabolites of the bioluminescent strains in which AHLs signals were detected, a group of "candidate" compounds that may be involved in the QS regulation process were summarised for further investigations for antibiotics.

Reference

Arpha K., Phosri C., Suwannasai N., Mongkolthanaruk W. and Sodngam S., Astraodoric acids A–D: new lanostane triterpenes from edible mushroom astraeus odoratus and their anti-*Mycobacterium tuberculosis* H37Ra and cytotoxic activity, Journal of Agriculture and Food Chemistry, 2012, 60, 9834-9841.

Bassler B.L., Losick R., Bacterially speaking, Cell, 2006, 125, 237-46.

Becking L. E., Nakao Y., Voogd de N. J., R. W. M. van Soest, N. Fusetani and S. Matsunaga, Perplexing distribution of 3-alkylpyridines in haplosclerid sponges, Porifera Research - Biodiversity, Innovation and Sustainability, 2007, 28, 173-178.

Berer N., Rudi A., Goldberg I., Benayahu Y. and Kashman Y., Callynormine A, a new marine cyclic peptide of a novel class, Organic Letters, 2004, 6, 2543-2545.

Berlinck R.G., Ogawa C.A., Almeida A.M., Sanchez M.A., Malpezzi E.L., Costa L.V., Hajdu E., de Freitas J.C., Chemical and pharmacological characterization of halitoxin from Amphimedon viridis (Porifera) from the southeastern Brazilian coast, Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology, 1996, 115, 155-163.

Bifulco G., Bruno I., Minale L., Riccio R., Novel HIV-inhibitory halistanol sulfates F-H from a marine sponge, *Pseudoaxinissa Digitata*, Natural Product Research, 1994, 57, 164–167.

Buchanan M. S., Carroll A. R., Addepalli R., Avery V. M., Hooper J. N. A. and Quinn R. J., Niphatoxin C, a cytotoxic tripyridine alkaloid from *Callyspongia* sp., Journal of Natural Products, 2007, 70, 2040-2041.

Braekman J. C., Daloze D., Devijver C., Dubut D. and R. W. M. van Soest, A new C-20 polyacetylene from the sponge *Callyspongia pseudoreticulata*, Journal of Natural Products, 2003, 66, 871-872

Chandler J.R., Heilmann S., Mittler J.E. and Greenberg E.P., Acyl-homoserine lactone-dependent eavesdropping promotes competition in a laboratory co-culture model, The ISME Journal. 2012, 6, 2219.

Capon R. J., Ford J., Lacey E., Gill J. H., Heiland K. and Friedel T., Phoriospongin A and B: two new nematocidal depsipeptides from the australian marine sponges *Phoriospongia* sp. and *Callyspongia bilamellata*, Journal of Natural Products, 2002, 65, 358-363.

Carballeira N. M. and Pagan M., New methoxylated fatty acids from the caribbean sponge *Callyspongia fallax*, Journal of Natural Products, 2001, 64, 620-623.

Carmely S. and Kashman Y., Neviotine-A, a new triterpene from the red sea sponge *Siphonochalina siphonella*, Journal of Organic Chemistry, 1986, 51, 784-788.

Castellar A., Coelho T.S., Silva P.E.A, Ramos D.F., Lourenço M.C.S., Lage C.L.S., Julião L.S., Barbosa Y.G. and Leitão S.G, The activity of flavones and oleanolic acid from Lippia lacunosa against susceptible and resistant *Mycobacterium tuberculosis* strains, Brazilian Journal of Pharmacognosy, 2011, 21, 835-840.

Daviescoleman M. T., Faulkner D. J., Dubowchik G. M., Roth G. P., Polson C. and Fairchild C., A new EGF-active polymeric pyridinium alkaloid from the sponge *Callyspongia fibrosa*, Journal of Organic Chemistry, 1993, 58, 5925-5930.

Dharmaraj S., Study of L-asparaginase production by *Streptomyces noursei* MTCC 10469, isolated from marine sponge *Callyspongia diffusa*, Iranian Journal of Biotechnology, 2011, 9, 102-108.

Dharmaraj S., Antagonistic potential of marine Actinobacteria against fish and shellfish pathogens, Turkish Journal of Biology, 2011, 35, 303-311.

Dias L. C. and Meira P. R. R., Total synthesis of the potent antitumor polyketide (−)-callystatin A, Journal of Organic Chemistry, 2005, 70, 4762-4773.

Dusza J. P., Contributions to the study of marine products. XLIX. synthesis of 29-isofucosterol, Journal of Organic Chemistry, 1960, 25, 93-96.

Ebada S. S., Edrada-Ebel R. A., Lin W. H. and Proksch P. Methods for isolation, purification and structural elucidation of bioactive secondary metabolites from marine invertebrates, Nature Protocol, 2008, 3, 1820–1831.

Engelhardt K., Degnes K. F., Kemmler M., Bredholt H., Fjaervik E., Klinkenberg G., Sletta H., Ellingsen T.E., Zotchev S.B., Production of a New thiopeptide antibiotic, TP-1161, by a marine *Nocardiopsis* species, Applied and Environmental Microbiology,2010, 76, 4969-4976.

Fagerlund U. H. M. and Idler D. R., Marine sterols. VII. the synthesis of 29-isofucosterol and the attempted synthesis of 17-dehydrocholesterol, Journal of the Fisheries Research Board of Canada, 1960, 17, 597-599.

Fujioka K., Furihata K., Shimazu A., Hayakawa Z., Seto H., Journal of Antibiotics, Isolation and characterization of atramycin A and atramycin B, new isotetracenone type antitumor antibiotics, 1991, 44, 1025-1102.

Fujita M., Nakao Y., Matsunaga S., R. W. M. van Soest, Y. Itoh, M. Seiki and N. Fusetani, Callysponginol sulfate A, an MT1-MMP inhibitor isolated from the marine sponge *Callyspongia truncate*, Journal of Natural Products, 2003, 66, 569-571.

Fusetani N., Matsunaga S. and Konosu S., Bioactive marine metabolites II. halistanol sulfate, an antimicrobial novel steroid sulfate from the marine sponge *Halichondria cf. moorei* bergquist, Tetrahedron Letters, 1981, 22,1985–1988.

Fusetani N., Sugano M., Matsunaga S. and Hashimoto K., H,K-ATPase inhibitors from the marine sponge *Siphonochalina truncata*: absolute configuration of siphonodiol and two related metabolites, Tetrahedron Letters, 1987, 28, 4311-4312.

Fukami A., Ikeda Y., Kondo S., Naganawa H., Takeuchi T., Furuya S., Hirabayashi Y., Shimoike K., Hosaka S., Watanabe Y. and Umezawa K., Akaterpin, a novel bioactive triterpene from the marine sponge *Callyspongia* sp., Tetrahedron Letters, 1997, 38, 1201-1202.

Gallimore W.A., Kelly M., Scheuer P.J., Gelliusterols A−D, new acetylenic sterols from a sponge, *Gellius* Species, Journal of Natural Products, 2001, 64, 741-744.

Garg H. S. and Agrawal S., Callyapinol, a new diterpene from sponge *callysponqia spinosissina*, Tetrahedron Letters, 1995, 36, 9035-9038.

Gray C. A., Lira de S. P., Silva M., Pimenta E. F., Thiemann O. H., Oliva G., Hajdu E., Andersen R. J. and Berlinck R. G. S., Sulfated meroterpenoids from the Brazilian sponge *Callyspongia* sp. are inhibitors of the antileishmaniasis target adenosine phosphoribosyl transferase, Journal of Organic Chemistry, 2007, 72, 1062-1062.

Höller U., König G.M. and Wright A. D., A new tyrosine kinase inhibitor from a marine isolate of *ulocladium botrytis* and new metabolites from the marine fungi *asteromyces cruciatus* and *Varicosporina ramulosa*, European Journal of Organic Chemistry, 1999, 11, 2949-2955.

Hoet S., Opperdoes F., Brun R., Quetin-Leclercq J., Natural products active against African trypanosomes: a step towards new drugs, Natural Product Reports, 2004,21, 353-364

Hosoi H., Kawai N., Hagiwara H., Suzuki T., Nakazaki A., Takao K. I., Umezawa K. and Kobayashi S., Synthesis and determination of the relative structure of akaterpin, a potent inhibitor of PI-PLC, Tetrahedron Letters, 2011, 52, 4961-4964.

Ibrahim S. R. M., Min C. C., Teuscher F., Ebel R., Kakoschke C., W. Lin, V. Wray, R. Edrada-Ebel and P. Proksch, Callyaerins A–F and H, new cytotoxic cyclic peptides from the Indonesian marine sponge *Callyspongia aerizusa*, Bioorganic & Medicinal Chemistry, 2010, 18, 4947-4956.

Ibrahim S. R. M., Edrada-Ebel R., Mohamed G. A., Youssef D. T. A., Wray V. and Proksch P., Callyaerin G, a new cytotoxic cyclic peptide from the marine sponge *Callyspongia aerizusa*, Arkivoc, 2008, 164-171.

Jadulco R., Proksch P., Wray V., Sudarsono, Berg A.T. and Gräfe U., New macrolides and furan carboxylic acid derivative from the sponge-derived fungus *Cladosporium herbarum*, Journal of Natural Product, 2001, 64, 527-530.

Jain S., Abraham I., Carvalho P., Kuang Y. H., Shaala L. A., Youssef D. T. A., Avery M. A., Chen Z. S. and Sayed K. A. E., Sipholane triterpenoids: chemistry, reversal of ABCB1/P-glycoprotein-mediated multidrug resistance, and pharmacophore modelling, Journal of Natural Products, 2009, 72, 1291-1298.

Jain S., Laphookhieo S., Shi Z., Fu L.W., Akiyama S.I., Chen Z.S., Youssef D. T. A., R. W. M. van Soest and Sayed K. A. E., Reversal of P-glycoprotein-mediated multidrug resistance by sipholane triterpenoids, Journal of Natural Products, 2007, 70, 928-931.

Kelman D., Kashman Y., Hill R. T., Rosenberg E. and Loya Y., Chemical warfare in the sea: the search for antibiotics from Red Sea corals and sponges, Pure and Applied Chemistry, 2009, 81, 1113-1121.

Kelman D., Kashman Y., Rosenberg E., Ilan M., Ifrach I. and Loya Y., Antimicrobial activity of the reef sponge *Amphimedon viridis* from the Red Sea: evidence for selective toxicity, Aquatic Microbial Ecology, 2001, 24, 9-16.

Khripach V. A., Zhabinskii V. N., Konstantinova O. V., Khripach N. B. and Antonchick A. P., Synthesis of 24-functionalized oxysterols, Russian Journal of Bioorganic Chemistry, 2002, 28, 284-288.

Kjer J., Debbab A., Aly A. H. and Proksch P. Methods for isolation of marine-derived endophytic fungi and their bioactive secondary products, Nature Protocol, 2010, 5, 479–490.

Kobayashi M., Higuchi K., Murakami N., Tajima H. and Aoki S., Callystatin A, a potent cytotoxic polyketide from the marine sponge, *Callyspongia truncate*, Tetrahedron Letters, 1997, 38, 2859-2862.

Li K., Li Q.L., Ji N. Y., Liu B., Zhang W., Cao X. P., Deoxyuridines from the marine aponge associated actinomycete *Streptomyces microflavus*, Marine Drugs, 2011, 9, 690-695.

Li X. and Djerassi C., Minor and trace sterols in marine invertebrates 40. Structure and synthesis of axinyssasterol, 25-methylfucosterol and 24-ethyl-24-methylcholesterol -- novel sponge sterols with highly branched side chains, Tetrahedron Letters, 1983, 24, 665-668.

Liu X., Chen C., He W., Huang P., Liu M., Wang Q., Guo H., Bolla K., Lu Z., Song F., Dai H., Liu M., ZhangL., Exploring anti-TB leads from natural products library originated from marine microbes and medicinal plants, Antonie Van Leeuwenhoek, 2012, 102, 447-61.

Liu Y.H., Yang B. and Dong J.D., Secondary metabolites and their bioactivities of marine sponge *Callyspongia* sp., Journal of Tropical Oceanography, 2008, 27, 73-80.

Lopez S., Fernandez-Trillo F., Midon P., Castedo L. and Saa C., Synthesis of marine polyacetylenes callyberynes A−C by transition-metal-catalyzed cross-coupling reactions to sp Centers, Journal of Organic Chemistry, 2006, 71, 2802-2810.

Lopez S., Fernandez-Trillo F, Castedo L. and Saa C., Synthesis of callyberynes A and B, polyacetylenic hydrocarbons from marine sponges, Organic Letters, 2003, 5, 3725-3728.

Lisdiyanti P., Otoguro M., Ratnakomala S., Lestari Y., Hastuti R. D., Triana E., Katsuhiko A., Widyastuti Y., Actinokineospora baliensis sp. nov., Actinokineospora cibodasensis sp. nov. and Actinokineospora cianjurensis sp. nov., isolated from soil and plant litter, International Journal of Systematic and Evolutionary Microbiology, 2010, 60, 2331-2335.

Miao S. C. and Andersen R. J., Callydiyne, a new diacetylenic hydrocarbon from the sponge *Callyspongia flammea*, Journal of Natural Products, 1991, 54, 1433-1434.

Migliuolo A., Notaro G., Piccialli V. and Sica D., New Tetrahydroxylated sterols from the marine sponge *Spongia officinalis*, Journal of Natural Products, 1990, 53, 1414-1424.

Motohashi K., Takagi M., Shin-ya K., Tetrapeptides possessing a unique skeleton, JBIR-34 and JBIR-35, isolated from a sponge-derived actinomycete, *Streptomyces* sp. Sp080513GE-23, Journal of Natural Products, 2010, 73, 226-228.

Noro J.C., Barrows L.R., Gideon O.G., Ireland C.M., Koch M., Matainaho T., Piskaut P.,

Nakao Y., Uehara T., Matunaga S., Fusetani N. and R. W. M. van Soest, Callyspongynic acid, a polyacetylenic acid which inhibits α-glucosidase, from the marine sponge *Callyspongia truncata*., Journal of Natural Products, 2003, 66, 156-156.

Pappo D. and Kashman Y., β-turn mimetic: synthesis of cyclic thioenamino peptides, Organic Letters, 2006, 8, 1177-1179.

Pateh U.U., Haruna A.K., Garba M., Iliya I., Sule I.M., Abubakar M.S., and Ambi A.A., Isolation of stigmas- terol, ß-sitosterol and 2-hydroxyhexadecanoic acid methyl ester from the rhizomes of *Stylochiton Lancifolius* Pyer and Kotchy (Aeaceae), Nigerian Journal of Pharmaceutical Sciences, 2009, 7, 19-25.

Pluskal, T., Castillo, S., Villar-Briones, A. and Orešič, M. MZmine 2: Modular framework for processing, visualizing, and analyzing mass spectrometry-based molecular profile data, BMC Bioinformatics, 2010, 11:395.

Pond C.D., and Bugni T.S., Tetrahdroxysqualene from *Rhus taitensis* shows antimycobacterial activity against *Mycobacterium tuberculosis*, Journal of Natural Products, 2008, 71, 1623-1634.

Proksch P. and Muller W.E.G., Frontiers in Marine Biotechnology, P1-10.

Pujari S. A. and Kaliappan K. P., An iterative Shimizu non-aldol approach for the stereoselective synthesis of C13-C22 fragment of callystatin A, Organic & Biomolecular Chemistry, 2012, 10, 1750-1753.

Rao T. S. P., Sarma N. S., Murthy Y. L. N., Kantamreddi V. S. S. N., Wright C. W. and Parameswaran P. S., New polyhydroxy sterols from the marine sponge *Callyspongia fibrosa* (Ridley & Dendly), Tetrahedron Letters, 2010, 51, 3583-3586.

Rooney F. and Capon R. J., Callyspongynes A and B: new polyacetylenic lipids from a southern Australian marine sponge, *Callyspongia* sp Lipids, 1998, 33, 639-642.

Scott R. H., Whyment A. D., Foster A., Gordon K. H., Milne B. F. and Jaspars M., Analysis of the structure and electrophysiological actions of halitoxins: 1,3 alkyl-pyridinium salts from *Callyspongia ridleyi*, Journal of Membrane Biology, 2000, 176, 119-131.

Shaaban K. A., Srinivasan S., Kumar R., Damodaran C., Rohr J., Landomycins P−W, cytotoxic angucyclines from *Streptomyces cyanogenus* S-136, Journal of Natural Products, 2011, 74, 2-11.

Smith C. J., Abbanat D., Bernan V. S., Maiese W. M., Greenstein M., Jompa J., Tahir A. and Ireland C. M., Novel polyketide metabolites from a species of marine fungi, Journal of Natural Products, 2000, 63, 142-145.

Stubbings w. and Labischinski H., New antibiotics for antibiotic-resistant bacteria, F1000 biology reports, 2009, 1, 40-42.

Takei M., Umeyama A., Shoji N. and Hashimoto T., Polyacetylenediols regulate the function of human monocyte-derived dendritic cells, International Immunopharmacology, 2010, 10, 913-921. Tan L. T., Okino T., and Gerwick W. H., Hermitamides A and B, toxic malyngamide-type natural products from the marine cyanobacterium *Lyngbya majuscule*, Journal of Natural Products, 2000, 63, 952–955.

Tang X.;, Zhou Y., Zhang J., Ming H., Nie G. X., Yang L. L., Tang S. K., Li W. J, Actinokineospora soli sp. nov., a thermotolerant actinomycete isolated from soil, and

emended description of the genus Actinokineospora, International Journal of Systematic and Evolutionary Microbiology, 2012, 62, 1845-1849.

Taylor M.W., Radax R., Steger D. and Wagner M., Sponge-associated microorganisms: evolution, ecology, and biotechnological potential, Microbiology and Molecular Biology Reviews, 2007, 71, 295-347.

Theobald N., Shoolery J. N., Djerassi C., Erdman T. R. and Scheuer P. J., Minor and trace sterols in marine invertebrates. 24-Ethyl-.DELTA.5,24(28),28-cholestatrien-3.beta.-ol - a naturally occurring allenic marine sterol, Journal of the American Chemical Society, 1978, 100, 5574-5575.

Thomas T. R. A., Kavlekar D. P., and LokaBharathi P. A., Marine drugs from sponge-microbe association- a review, Marine Drugs, 2010, 8, 1417-1468.

Thompson M. N., Gallimore W. A., Townsend M. M., Chambers N. A. and Williams L. A. D., Bioactivity of amphitoxin, the major constituent of the jamaican sponge *Amphimedon compressa*, Chemistry & Biodiversity, 2010, 7, 1904-1910.

Toth S. I. and Schmitz F. J., Two new cytotoxic peroxide-containing acids from a new guinea sponge, *Callyspongia* sp., Journal of Natural Products, 1994, 57, 123-127.

Tsukamoto S., Kato H., Hirota H. and Fusetani N., Seven new polyacetylene derivatives, showing both potent metamorphosis-inducing activity in ascidian larvae and antifouling activity against barnacle larvae, from the ,marine sponge *Callyspongia truncate*, Journal of Natural Products, 1997, 60, 126-130.

Umeyama A., NaganoC., and Arihara S., Three novel C21 polyacetylenes from the marine sponge *Callyspongia sp*., Journal of Natural Products, 1997, 60,131–133.

Umeyama A., Matsuoka N., Mine R., Nakata A., Arimoto E., Matsui E., Shoji N., Arihara S., Takei M. and Hashimoto T., Polyacetylene diols with antiproliferative and driving Th1 polarization effects from the marine sponge *Callyspongia* sp., Journal of Natural Medicines, 2010, 64, 93-97.

Uno M., Ohta S., Ohta E. and Ikegami S., Callyspongins A and B: novel polyacetylene sulfates from the marine sponge *Callyspongia truncata* that inhibit fertilization of starfish gametes, Journal of Natural Products, 1996, 59, 1146-1148.

Urban S. and Capon R. J. , A new lipid from an australian marine sponge, *Callyspongia* sp., Lipids, 1997, 32, 675-677.

Vacelet J. and Gallissian M. F., Virusßlike particles in cells of the sponge verongia cavernicola (demospongiae, dictyoceratida) and accompanying tissues changes, Journal of Invertebrate Pathology, 1978, 31, 246-254.

Voogd de N. J., Haftka J. J. H. and Hoeksema B. W., Evaluation of the ecological function of amphitoxin in the reef-dwelling sponge Callyspongia (Euplacella) biru (Haplosclerida: Callyspongiidae) at southwest Sulawesi, Indonesia, Contributions to Zoology, 2005, 74, 51-59.

Voogd de N. J., The mariculture potential of the Indonesian reef-dwelling sponge *Callyspongia (Euplacella) biru*: growth, survival and bioactive compounds, Aquaculture, 2007, 262, 54-64.

Voogd de N. J., Haftka J. J. H. and Hoeksema B. W., Contributions to Zoology, 2005, 74, 51-59.

Wang G.Y.S., Kuramoto M. and Uemura D., Three novel anti-microfouling nitroalkyl pyridine alkaloids from the okinawan marine sponge *Callyspongia* sp., Tetrahedron Letters, 1996, 11, 1813–1816.

Wächter G.A., Franzblau S.G., Montenegro G., Hoffmann J.J., Maiese W.M. and Timmermann B.N., Inhibition of *Mycobacterium tuberculosis* growth by saringosterol from *Lessonia nigrescens*, Journal of Natural Products, 2001, 64, 1463-1464.

Wei R. B., Xi T., Li J., Wang P., Li F.C., Lin Y.C., Qin S., Lobophorin C and D, new kijanimicin derivatives from a marine sponge-associated actinomycetal strain AZS17, Marine Drugs, 2011, 9, 359-368.

Wilkinson C. R., Microbial associations in sponges: ecology, physiology and microbial populations of coral reef sponges, Marine Biology, 1978, 49, 161-167.

Woldemichael G.M., Gutierrez-Lugo M.T., Franzblau S.G., Wang Y., Suarez E. and Timmermann B.N., *Mycobacterium tuberculosis* growth inhibition by constituents of *Sapium haematospermum*, Journal of Natural Products, 2004, 67, 598-603.

Yang B., Dong J., Zhou X., Yang X., Lee K. J., Wang L., Zhang S. and Liu Y., Proline-containing dipeptides from a marine sponge of a *Callyspongia* Species, Helvetica Chimica Acta, 2009, 92, 1112-1117.

Youssef D. T. A., Yoshida W. Y., Kelly M. and Sheuer P. J., Polyacetylenes from a red sea sponge *Callyspongia* Species, Journal of Natural Products, 2000, 63, 1406-1410.

Youssef D. T. A., R. W. M. van Soest and Fusetani N., Callyspongamide A, a new cytotoxic polyacetylenic amide from the red sea sponge *Callyspongia fistularis*, Journal of Natural Products, 2003, 66, 861-862.

Yuliana, N. D., Khatib, A., Choi, Y. H. and Verpoorte, R. Metabolomics for bioactivity assessment of natural products, Phytotherapy Research: PTR, 2011, 25,157-169.

Zheng L., Yan X., Han X., Chen H., Lin W., FS Lee and X. Wang. Identification of norharman as the cytotoxic compound produced by the sponge (*Hymeniacidon perleve*)-associated marine bacterium *Pseudoalteromonas piscicida* and its apoptotic effect on cancer cells, Biotechnology and Applied Biochemistry, 2006, 44,135-142.