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ISOLATION AND IDENTIFICATION OF SECONDARY METABOLITES FROM THE BRYOZOAN *Cryptosula zavjalovensis* FROM HOKKAIDO, JAPAN

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Erasmus Mundus MSc in Chemical Innovation and Regulation
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Work supervised by:
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DECLARATION OF AUTHORSHIP

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I declare that I am the author of this work, which is original. The work cites other authors and works, which are adequately referred in the text and are listed in the bibliography.

Loveille Jun A. Gonzaga

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Loveille

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ABSTRACT

The marine environment is an abundant and diverse source of biologically active compounds, which have great potential in pharmaceutics and medicine. Bryozoans are sessile colonial animals which are common and diverse in shallow waters. Despite their potential for novel biologically active secondary metabolites, there have been very few studies on bryozoans. This work will describe the isolation and identification of secondary metabolites from the marine bryozoan *Cryptosula zavjalovensis*, which belongs to one of the least studied genera of marine bryozoans. Samples were collected at the Akkeshi Marine Station, Akkeshi, Hokkaido, Japan and were extracted using ethanol. The ethanol extract was evaporated to dryness, dissolved in ethyl acetate and extracted with water to obtain an organic and an aqueous fraction. Preliminary toxicity profiling revealed that the ethyl acetate extract exhibited cytotoxicity toward human MCF7 breast cancer cells. The ethyl acetate extract was separated into three fractions by solid phase extraction using a gradient of methanol and water: E1 80:20 v/v, E2 100:0 v/v, and; E3 methanol and chloroform 50:50 v/v. Toxicity profiling of these extracts revealed E2 and E3 exhibiting toxicity toward human MCF7 breast cancer cells. The secondary metabolites were isolated using liquid chromatography-mass spectrometry and structures were determined by tandem mass spectrometry.

RESUMO

O meio marinho é uma fonte abundante e diversificada de compostos biologicamente ativos, que possuem potencial para produtos farmacêuticos e medicamentos. Apesar do seu potencial para novos metabolitos secundários biologicamente ativos, houve poucos estudos sobre os briozoários. Este trabalho descreve o isolamento e identificação de metabolitos secundários do bryozoário marinho Cryptosula zavjalovensis, que pertence a um dos gêneros menos estudados de bryozoários marinhos. As amostras foram recolhidas na Estação Marítima de Akkeshi, Akkeshi, Hokkaido, Japão e extraídas com etanol. O extracto de etanol foi subsequentemente evaporado à secura, dissolvido em acetato de etilo extraído com água, para obter uma fração orgânica e outra aquosa. O perfil de toxicidade preliminar revelou que o extrato de acetato de etilo exibia citotoxicidade em relação às células humanas de câncer de mama MCF7 humanas. O extracto de acetato de etilo foi então separado em três fracções por extracção em fase sólida utilizando um gradiente de metanol e água: E1 80:20 v/v, E2 100:0 v/v e; E3 metanol e clorofórmio 50:50 v/v. O perfil de toxicidade desses extratos revelou E2 e E3 exibindo toxicidade em relação a células de câncer de mama MCF7 humanas. Os metabolitos secundários foram isolados utilizando cromatografia líquida e espectrometria de massa e as estruturas foram determinadas por espectrometria de massa em tandem.

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LIST OF ABBREVIATIONS AND ACRONYMS

C18	Octadecyl
ESI	Electrospray Ionization
EtOAc	Ethyl acetate
H2O	Water
Hokudai	Hokkaido University
HPLC	High Performance Liquid Chromatography
LC-MS	Liquid Chromatography-Mass Spectrometry
m/z	Mass-to-charge ratio
MCF-7	Michigan Cancer Foundation-7
MeOH	Methanol
MS/MS	Tandem mass spectrometry
MS	First stage of mass spectrometry
MS2	Second stage of mass spectrometry
MS3	Third stage of mass spectrometry
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
ODS	Octadecylsilyl
SPE	Solid Phase Extraction
U.S. FDA	United States Food and Drug Administration
UAlg	University of Algrave

1. INTRODUCTION

The objective of this work was to identify natural products with potential medicinal applications from a type of bryozoan, a marine organism which can be found on the coast of Japan.

1.1. The importance of researching natural products

Secondary metabolites, also designated as natural products, are organic compounds that are produced by an organism with no apparent role for its growth, development, or reproduction. The production of secondary metabolites serves to adapt the organism to the surrounding environment and to improve the survival fitness of an organism as a defense mechanism specifically by exerting physiological effects on competing organisms (Williams, Stone, Hauck, & Rahman, 1989). Natural products have attracted research interest because of its various applications, particularly in drug discovery.

Natural products have long been extensively used in medicine considering that the earliest records from around 2600 B.C. show that oils from *Cupressus sempevirens* (cypress), *Glycyrrhiza glabra* (licorice), and *Commiphora* species (myrrh) were used to treat coughs, colds, and inflammation in Mesopotamia (Borchardt, 2002). Similarly, the use of natural products which were mostly plant-based was also well-documented in the Egyptian pharmaceutical record, Ebers papyrus, dating 1500 B.C. in the form of gargles, pills, infusions, and ointments (Dias, Urban, & Roessner, 2012); in the Chinese Materia Medica with the first record dating back to about 1100 B.C. (Huang, 1998), and; in the Indian Ayurvedic system dating back from before 1000 B.C. (Dev, 1999).

Traditionally, natural products have been sourced from the terrestrial environment due to their availability and accessibility. Experimentation by trial and error through palatability trials or untimely deaths searching for treatment to diseases was a dominant source of knowledge for the use of natural products from medicinal plants (Kinghorn, 2011). Most of the early medicines were thus based on these traditional medicines leading to subsequent chemical, toxicological, pharmacological, and clinical studies (Butler, 2004). These ethnopharmacological properties became a primary source for medicines for early drug discovery. In consequence, the current uses of most of these drugs are identical or related to the ethnopharmacological purpose of the plant-derived natural products (Fabricant & Farnsworth, 2001).

The success of plant-derived natural products led to the discovery of many important drugs such as the synthesis of the anti-inflammatory agent acetylsalicylic acid or aspirin, which was derived from the natural product salicin extracted from the bark of the willow tree (*Salix alba*); morphine and several other alkaloids from opium poppy (*Papaver somniferum*), and; anti-malarial drug quinine isolated from the bark of *Cinchona succirubra*, which was traditionally used as treatment for malaria, fever, malaria, throat diseases and cancer (DerMarderosian & Beutler, 2003).

Aside from plants, fungi have also been a source for natural products. Aside from being a food source and its use in alcoholic beverage preparation, fungi have already been used as medication in traditional medicine (Lorenzen & Anke, 1996). Early documented studies of natural products from fungi include the description of the quinoid pigments, L-tyrosine- and L-phenylalaine-derived terphenylquinones atromentin and polyporic acid, respectively (Stadler & Hoffmeister, 2015). The advancements in microbiology led to further use of fungi in enzymes, biological control, antibiotics and other pharmacologically-active products. Nevertheless, considered as one of the most important natural product discoveries from a fungus is penicillin from *Penicillium notatum*. Penicillin was a major turning point, being one of the first antibiotics, rendering a cure to the then incurable and infectious diseases.

The discovery of penicillin promoted intensive investigation and paved the way on drug discovery from microbial sources. In consequence, microorganisms have become a prolific source of natural products and led to the discovery of important products in the pharmaceutical industry. Some of these natural products include antibacterial agents like cephalosporins from *Cephalosporium acremonium*; immunosuppressive agents like cyclosporins from *Trichoderma* and *Tolypocladium* species and rapamycin from

Streptomyces species; cholesterol lowering agents like lovastatin from *Aspergillus* species, and; anthelmintic and antiparasitic drugs like ivermectins from *Streptomyces* species (Buss, Cox, & Waigh, 2003).

1.2. Natural Products from Marine Organisms

In comparison, traditional medicine sourced from the marine environment had been very few if there were any despite the world's oceans covering about 70% of earth's surface. Collecting marine organisms for obtaining natural products was limited to those which were easily accessible or those which can be harvested through skin diving. However, advancements in technology have opened the seas through explorations by modern snorkeling, scuba diving, manned submersibles and remotely operated vehicles (Dias, Urban, & Roessner, 2012). This resulted in the realization that the oceans possess unique biodiversity which can be a viable source of diverse natural products with potential for pharmaceutics (Haefner, 2003).

The heightened interest in investigating the marine environment led to the discovery of thousands of new natural products. Recently, there have been more than a thousand new compounds being discovered and reported annually (Blunt, Copp, Keyzers, Munro, & Prinsep, 2016). This demonstrates that the marine environment is an abundant and diverse source of biologically active compounds, some of which are novel chemical classes not found in terrestrial sources (Cragg & Newman, 2013). These compounds were found to have potential for pharmaceutics and medicine, particularly as anti-cancer agents. The first approved drug derived from a marine product called ziconotide (Mathur, 2000) was a non-narcotic analgesic, espite the focus on potential anti-cancer drugs. It was isolated as a constituent of the venom from tropical cone snails of the genus *Conus* (Bulaj, et al., 2003).

Despite being scarce in vertebrates (Jha & Zi-rong, 2004), natural products have been abundantly obtained from other marine organisms such as algae, sponges, cnidarians, bryozoans, mollusks, tunicates, echinoderms, marine microorganisms and phytoplankton, mangroves and other intertidal plants and microorganisms. The trend however, is that research efforts on the search of marine natural products are directed towards marine microorganisms, accounting for most of the new secondary metabolites from the marine environment in recent years (Blunt, Copp, Keyzers, Munro, & Prinsep, 2016). Antibiotic, antifungal, antiviral, and antiyeast activities of the marine microorganisms have been reported (Buck, Meyers, & Kamp, 1962).

Recent natural products isolated from marine microorganisms include cyanogramide, an unprecedented spirocyclic alkaloid with multidrug-resistance reversing activity, isolated from *Actinoalloteichus cyanogriseus* (Fu, Kong, Li, Wang, & Zhu, 2014); two new aplysiatoxin analogues, 3-methoxyaplysiatoxin and 3-methoxydebromoaplysiatoxin isolated from *Trichodesmium erythraeum* inhibited the Chikungunya virus (Gupta, et al., 2014), and; belizentrin, a novel and structurally unique macrolide obtained from *Prorocentrum belizeanum*, which had potent effects to neuronal network integrity eventually resulting to cell death (Domínguez, et al., 2014).

Algae have also been one of the prolific sources of natural products from the marine environment. Some of the recent natural products isolated from marine algae include a new diketosteroid, (E)-stigmasta-24(28)-en-3,6-dione, isolated from the green alga *Tydemania expeditionis* had moderate inhibitory activities against prostate cancer cells (Zhang, et al., 2012); spiralisones A-D isolated from the Australian marine brown alga *Zonaria spiralis* exhibited promising kinase inhibitory and antibacterial activity (Zhang, Xiao, Conte, Khalil, & Capon, 2012), and; a novel bromophenol, 5,5”-oxybis(methylene)bis(3-bromo-4-(2’,3’-dibromo-4’,5’-dihydroxylbenzyl)benzene-1,2-diol), isolated from the red alga *Vertebrata lanosa* had cellular antioxidant activities (Olsen, Hansen, Isaksson, & Andersen, 2013).

Sponges have also been a dominant source of new secondary metabolites although there is a growing realization that these bioactive metabolites might have been produced by microbial symbionts thereby requiring further analyses on sponge matrices (Blunt, Copp, Keyzers, Munro, & Prinsep, 2016). Moreover, numerous new bioactive metabolites have also been isolated from cnidarians, mollusks, tunicates, and echinoderms. However, despite its potential for novel natural products, there have been very few studies on secondary metabolites from bryozoans (Blunt, Copp, Munro, Northcote, & Prinsep, 2004).

1.3. Bryozoans

Bryozoans are invertebrates that can be both found in freshwater and marine environments, particularly in shallow areas. Most of the known bryozoans are marine bryozoans and are found in coastal waters attached to rocks, pilings, shells, algae, and other animals. On the other hand, the taxonomic diversity of freshwater bryozoans is rather low (Hayward & Ryland, 1985).

Phylum Bryozoa (Figure 1.1) is mostly diverse, probably exceeding 8000 living species with a good fossil record dating back to the Ordovician showing they are relatively unchanged in the past ca. 480 million years (Fortunato, 2015). Examples of bryozoans can be seen in Figure 1.2 and Figure 1.3.

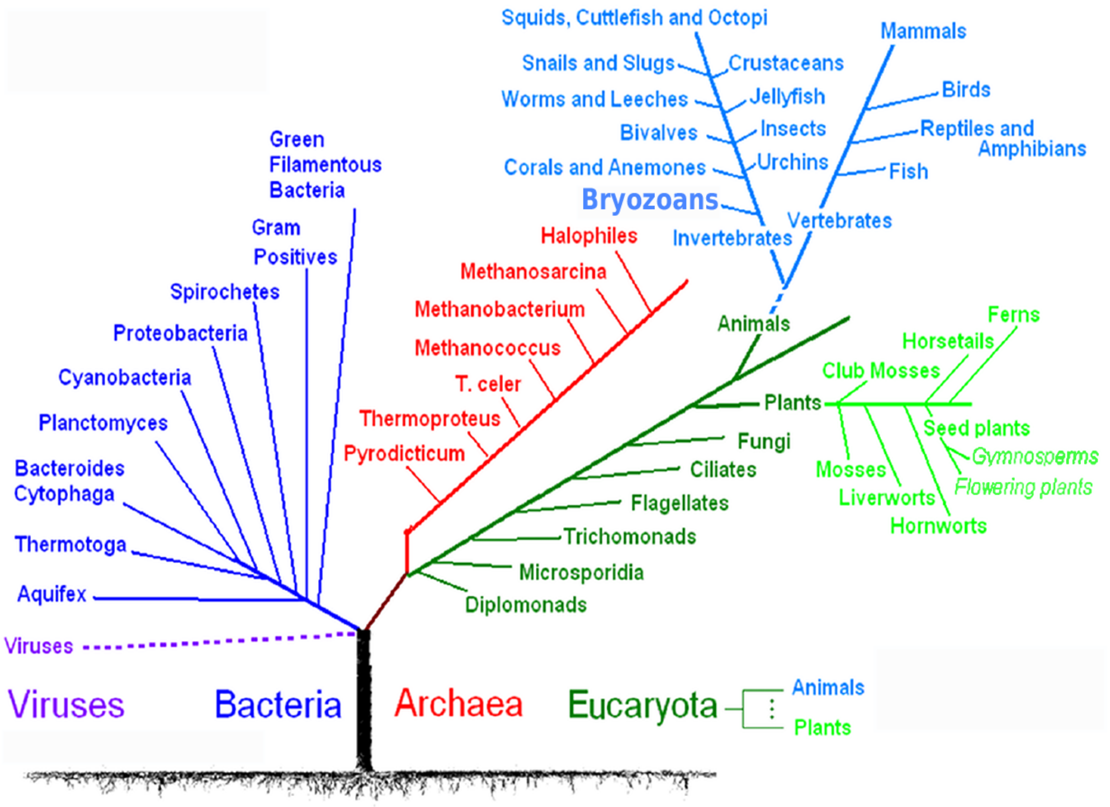


Figure 1.1 The Tree of Life, showing Phylum Bryozoa in Kingdom Animalia (adapted from www.greennature.ca).

Figure 1.2 A small Bugula neritina colony from San Francisco Bay, USA. The light tan organism on the left is another species of Bugula while red-orange organism below it is another bryozoan species, Watersipora subtorquata(Solórzano, 2011).

Figure 1.3 A colony of Watersipora subtorquata, mainly red with a blackened center, encrusting under an intertidal rock in San Francisco Bay, USA. On the lower left is another bryozoan, Schizoporella unicornis (Cohen, 2011).

Bryozoans are sometimes referred to as moss animals and are tiny, sessile, colonial animals. They have a distinguishing specialized feeding structure called a lopophore which is either a horseshoe- or circular-shaped tentacle structure extension of the body wall that surrounds the mouth. The basic body plan of a bryozoan consists of a polyp protected by a gelatinous or calcareous enclosure called a zooid.

These zooids may then integrate to form a colony. Bryozoan colonies can be encrusting (Figure 1.4), wherein flat and delicately lacy crusts are formed; arborescent (Figure 1.5), wherein colonies can be erect, branching, and tree-like, or; even free living. Due to these features, bryozoan colonies have often been mistaken as hydroids, corals, or seaweeds. While some bryozoan colonies encrust rocks, pilings, and algae, some are known to grow on the bottom of ships which results to the fouling of ships causing drag and reducing efficiency and maneuverability.

The morphological features of bryozoans had been historically the bases for taxonomy (Ruppert & Barnes, 1994). However, bryozoan taxonomy is continually revised and the taxonomic groupings does not necessarily represent the phylogenetic relationships between species (Sharp, Winson, & Porter, 2007).

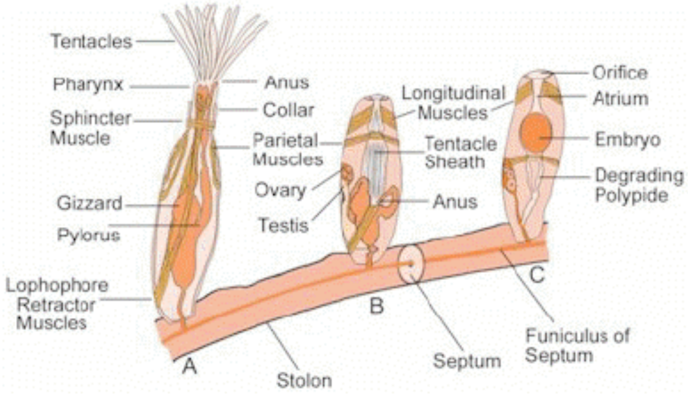


Figure 1.4 Sketch of an arborescent bryozoan (Smithsonian Marine Station at Fort Fierce, 2016).

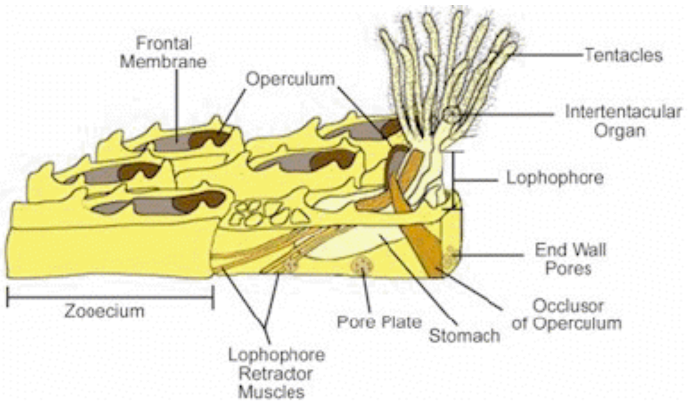


Figure 1.5 Sketch of an encrusting bryozoan (Smithsonian Marine Station at Fort Pierce, 2016).

Phylum Bryozoa is classified further into three major classes: class Phylactolaemata exclusively contains freshwater bryozoans; class Stenolaemata are exclusively marine bryozoans, with only one order with remaining living species, order Cyclostomata, while the rest are fossil species, and; class Gymnolaemata is a mostly marine class containing two orders, Cheilostomata and Ctenostomata. The three orders mentioned have been reported to have natural products and biological activity (Sharp, Winson, & Porter, 2007).

1.4. Natural Products from Bryozoans

Most of the metabolites isolated from bryozoans have been alkaloids (Blunt, Copp, Munro, Northcote, & Prinsep, 2004). Some of the metabolites obtained are macrolide lactones, indole alkaloids, isoquinoline quinones, sterols and other carbohydrates with a heteroatom such as nitrophenols and disulfides (Sinko, Rajchard, Balounova, & Fikotova, 2012).

In terms of chemical ecology, one of the most studied bryozoan species is *Bugula neritina*, from which bryostatins, a promising anticancer metabolite with significant biological activities, have been isolated (Pettit, Herald, Doubek, & Herald, 1982). Upon the isolation and the elucidation of the structure of bryostatin-1 (Figure 1.6), there had been numerous studies exploring its antitumor and anticancer potential.

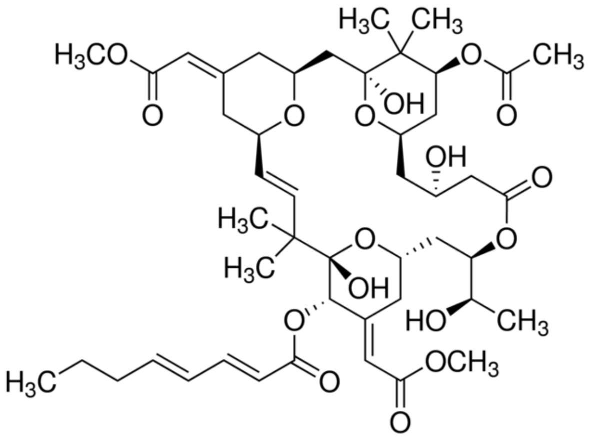


Figure 1.6 The structure of bryostatin-1.

Bryostatin 1, a macrocyclic lactone and a protein kinase C activator, have undergone Phase I clinical study in patients with advanced cancer to obtain a safe dosage although side effects included myalgia (Prendiville, et al., 1993). When bryostatin 1 was administered to patients with advanced malignancies in prolonged infusion schedules, there had been limited clinical activity observed and it was suggested for future studies to combine it with other cytotoxics or targeted therapies (Marshall, et al., 2002). A Phase II clinical study combined bryostatin 1 with cisplatin but the toxicity profile, primarily severe myalgias, precludes tolerability thereby preventing any further investigation using this combination (Morgan, et al., 2012).

Despite *B. neritina* being a single species, the natural products isolated vary among populations. That is, different populations of *B. neritina* produce different bryostatins (Davidson & Haygood, 1999). However, it was subsequently discovered that bryostatins are produced by a bacterial symbiont, *Endobugula sertula*. *E. sertula* is a unique bacterial symbiont of *B. neritina* and produces bryostatins which protect *B. neritina* larvae against predation from fish (Lopanik, Lindquist, & Targett, 2004). These results show a different perspective on natural products from bryozoans, which should consider the variable presence of symbionts and the environmental factors with which these metabolites are produced.

The bryozoan, *Flustra foliacea* has yielded several brominated alkaloids (Figure 1.7) including flustramine A and B (Carle & Christophersen, 1980), flustramine C, flustraminol A and B (Carle & Christophersen, 1981) flustramide A (Wulff, Carle, & Christophersen, 1982), and dihydroflustramine C, which had strong activity against Bacillus subtilis (Wright, 1984).

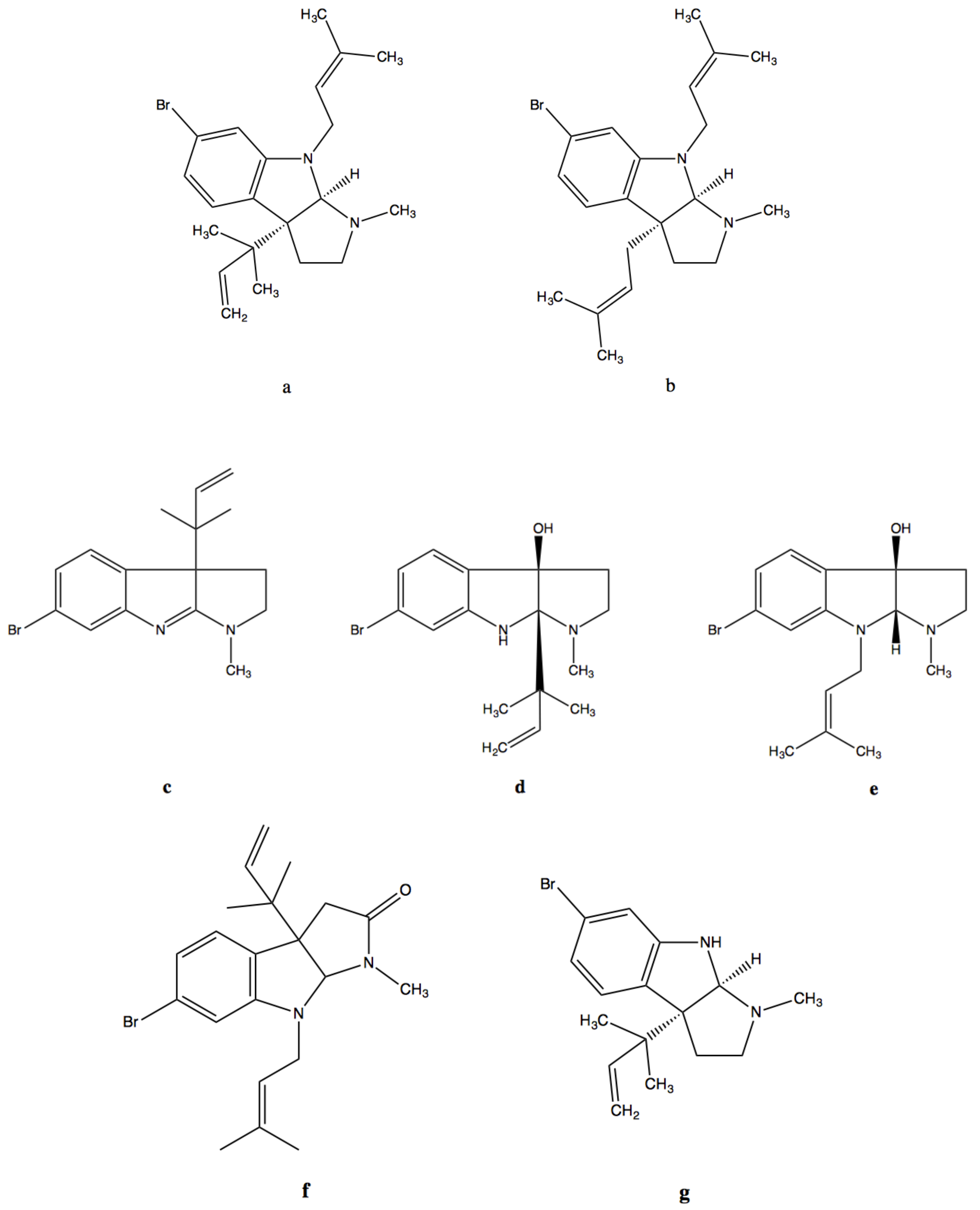


Figure 1.7 Structures of a. flustramine A, b. flustramine B, c. flustramine C, d. flustraminol A, e. flustraminol B, f. flustramide A, and g. dihydroflustramine C.

Alkaloids (Figure 1.8) from the bryozoan *Pterocella vesiculosa* have also been isolated. These include pterocellin A and B (Yao, Prinsep, Nicholson, & Gordon, 2003) with pterocellin A exhibiting potent antitumor activity towards murine leukemia cell line (Wang, Prinsep, & Martinus, 2016); pterocellins C-F (Prinsep, 2008), and; 5-bromo-8-methoxy-1-methyl-β-carboline (Till & Prinsep, 2009).

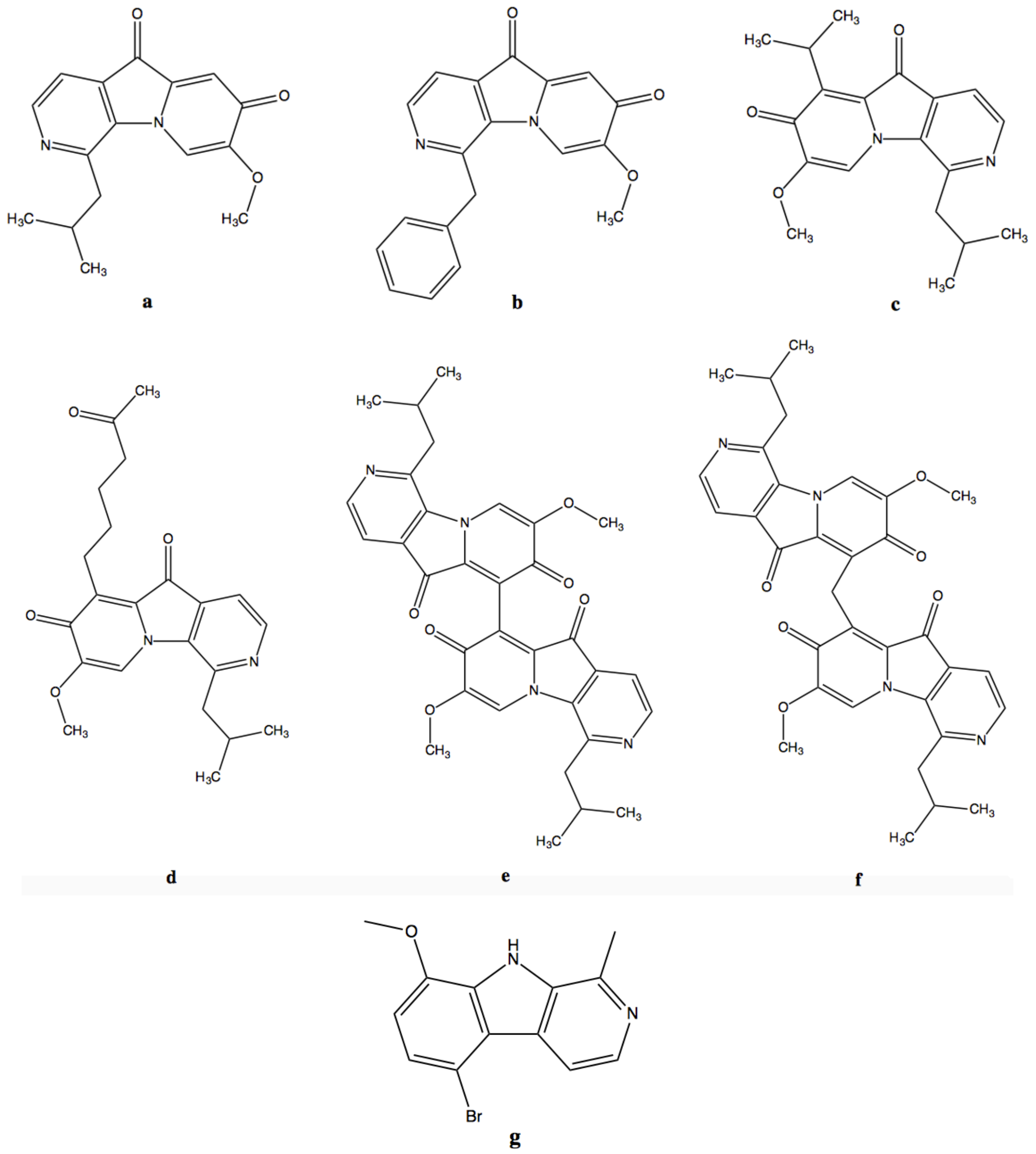


Figure 1.8 Structures of a. pterocellin A, b. pterocellin B, c. pterocellin D, d. pterocellin C, e. pterocellin E, f. pterocellin F, and g. 5-bromo-8-methoxy-1-methyl-β-carboline.

Although not as common, two nitrophenols, desmethylphidolopin and 3-nitro-4-hydroxybenzyl alcohol (Figure 1.9), have been isolated from the northeast Pacific bryozoan *Phidolopora pacifica* (Tischler, Ayer, & Andersen, 1986).

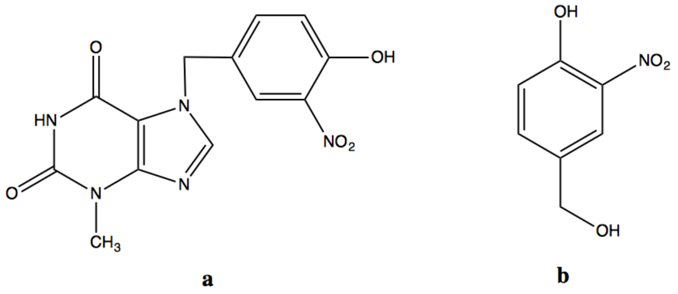


Figure 1.9 Structures of a. desmethylphidolopin and b. 3-nitro-4-hydroxybenzyl alcohol.

Bugula neritina, *Flustra foliacea*, *Pterocella vesiculosa*, and *Phidolopora pacifica* are marine bryozoans that belong to the class Gymnolaemata and order Cheilostomata, one of the three orders of bryozoans where secondary metabolites have been reported (Sharp, Winson, & Porter, 2007).

1.5. Cryptosula zavjalovensis

One of the least studied genera of bryozoans is genus *Cryptosula*, which is classified under the same order Cheilostomata as shown in **Error! Reference source not found.**. Currently, there are only five recorded species under this genus. The first recorded species is *Cryptosula pallasiana*, originally classified as *Eschara pallasiana*, and is cosmopolitan with worldwide distribution. Worldwide distribution of bryozoans, as with other fouling species, is suggested to have come from ships and may develop to be invasive species, particularly in ports and harbors (Almeida, Souza, Gordon, & Vieira, 2015).

Bryozoans under the genus *C. pallasiana* are encrusting. The overall color of *C. pallasiana* colonies is white, orange, or pink. The aperture of *C. pallasiana* is often described as bell-shaped. *C. pallasiana* has no spines, no avicularia which are jaw-like structures that opens and closes, and no ovicells for brooding embryos, all of which are conspicuous features in many bryozoans.

A recent study determined the chemical constituents of *C. pallasiana* from which seven alkaloids were isolated (Figure 1.8 a-g), of which two are new natural products, *p*-methylsulfonylmethyl-phenol (Figure 1.8 f) and 7-bromo-2,4-(1H,3H)-quinazolineione (Figure 1.8 g), with *p*-methylsulfonylmethyl-phenol exhibiting strong cytotoxicity against leukemia cell line (Tian, et al., 2014).

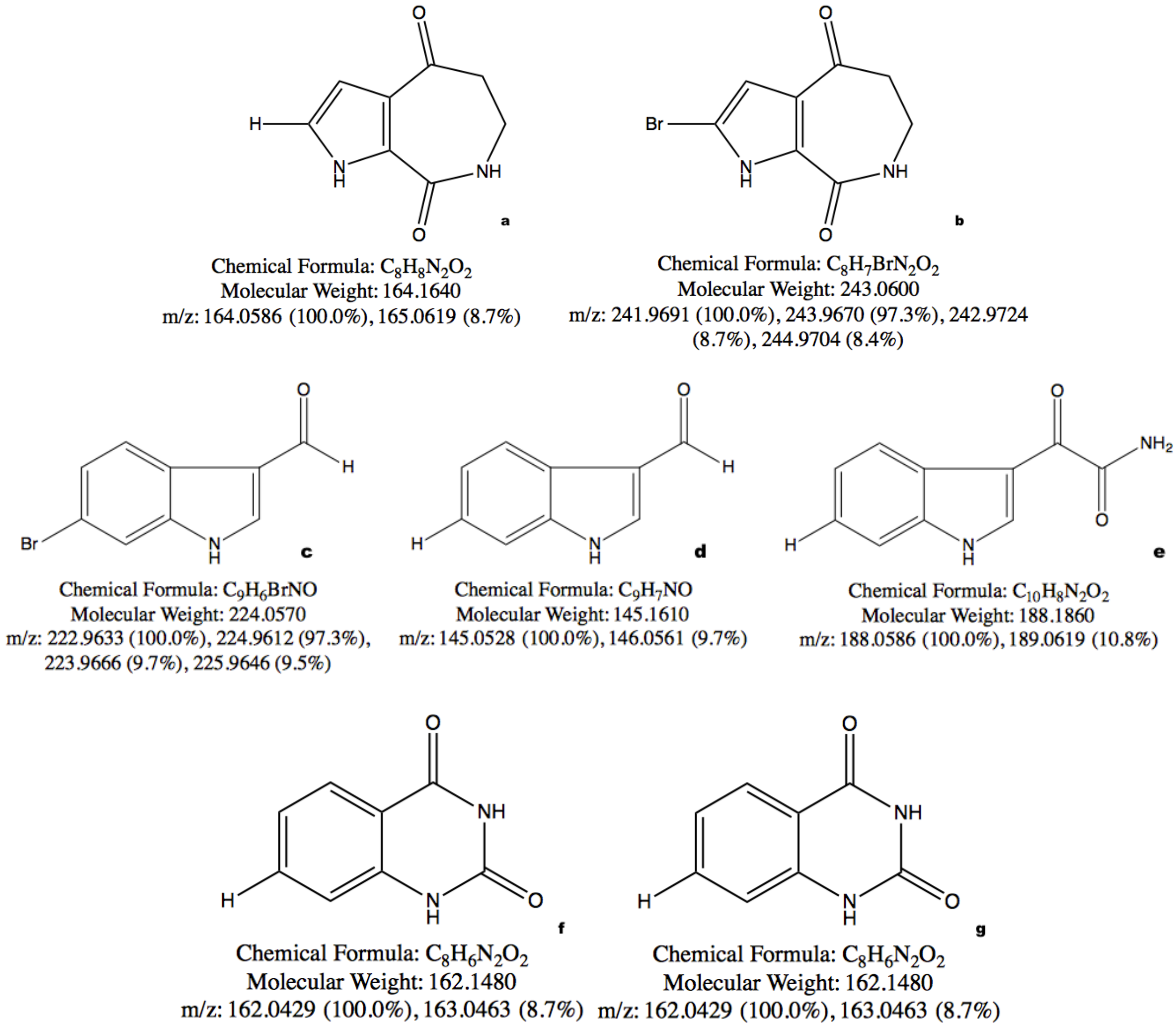


Figure 1.10 Structures of a. aldisin, b. 2-bromoaldisin, c. 6-bromoindole-3-carbaldehyde, d. indole-3-carboxyaldehyde, e. indole-3-oxoacetamide, f. 2,4(1H,3H)-quinazolinedione, and g. 7-bromo-2,4(1H,3H)-quinazolinedione, with some pertinent properties.

Cryptosula zavjalovensis may be difficult to differentiate from *C. pallasiana*. *C. zavjalovensis* colonies are also encrusting and have a similar orange color. However, inspection under a microscope shows that *C. zavjalovensis* (Figure 1.11) has a distinct zooid structure. This difference is even clearer upon inspection under a scanning electron microscope.

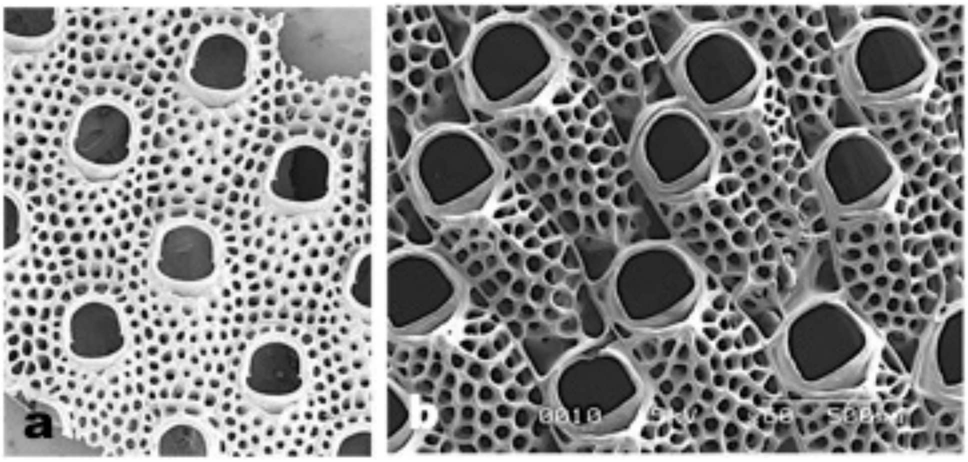


Figure 1.11 A scanning electron microscope image of a. Cryptosula pallasiana and b. Cryptosula zavjalovensis (Bock, 2009).

Moreover, *C. zavjalovensis* emits a lemon-like odor upon close inspection. This may be hypothesized to be emitted as part of a defense mechanism from predators. It is therefore of interest to investigate the natural products produced by *C. zavjalovensis*, which may, directly or indirectly, justify such a defense mechanism.

Aside from the study mentioned, research on *Cryptosula* is considerably scarce, more so on *C. zavjalovensis* which is not as globally distributed as *C. pallasiana*. *C. zavjalovensis* have been reported in North Pacific locations such as Vladivostok, Russia; Alaska, U.S.A. (Dick, Grischenko, & Mawatari, 2005) and; Hokkaido, Japan (Grischenko, Dick, & Mawatari, 2007).

Figure 1.12 Taxonomy of the bryozoan species: Bugula neritina, Flustra foliacea, Pterocella vesiculosa, Phidolopora pacifica, and Cryptosula zavjalovensis.

1.6. Objectives and Methodology

The aim of this work has been to isolate and identify natural products from *Cryptosula zavjalovensis*. While pursuing this objective, samples were collected from the coast of Hokkaido, Japan, separated into fractions by solvent extraction, solid phase extraction and chromatography, and finally analysis by LC-MS.

1.6.1. Separation by Solvent Extraction

Extraction of the natural products from *Cryptosula zavjalovensis* was done using solvent extraction. Solvent extraction or liquid-liquid extraction is a method to separate a compound which relies on the solubility of the components. This technique is widely used in the laboratory for refining, isolating, and purifying compounds.

In this study, the organic phase of the extract was separated using ethyl acetate and the aqueous phase using water. This is a rough separation of the components of the extract where the hydrophilic natural products will be in the aqueous layer while the hydrophobic natural products will be in the organic layer. To enhance this separation, this process was repeated for the separated organic and aqueous layers.

1.6.2. Solid Phase Extraction and Reversed-Phase Chromatography

The extract obtained from the organic layer was further separated by means of solid phase extraction (SPE). SPE was used to separate the different classes of compounds by their polarity, thereby separating the more polar to non-polar compounds into different fractions.

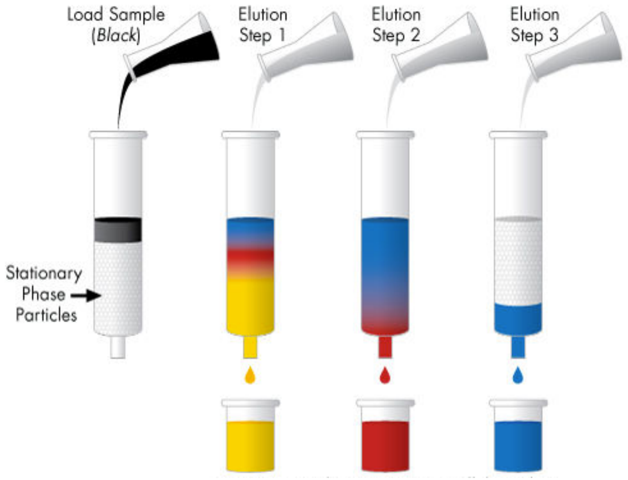


Figure 1.13 An example of a solid phase extraction (Waters, 2017)

SPE is a form of step-wise chromatography designed to partition the components in a liquid phase sample onto a stationary phase of either a sorbent or resin. Figure 10 shows how SPE can separate the sample into its different components.

As the components in the organic layer are hydrophobic, reversed-phase SPE was done to fractionate the components because it retains most components having hydrophobic properties. Reversed-phase SPE utilizes a non-polar stationary phase and a polar mobile phase. The study used silica-based sorbent in a cartridge as the stationary phase and a step-gradient of methanol as the mobile phase to fractionate the organic extract based on hydrophobicity. That is, the most polar components of the sample were eluted first.

In a similar process, the extract obtained from the aqueous layer was further separated using reversed-phase chromatography. Reversed-phase chromatography utilizes a hydrophobic stationary phase and a polar mobile phase. Octadecyl (C18) silica gel contained in a column was used as the stationary phase and a step-gradient of methanol as the mobile phase to fractionate the aqueous extract. This also resulted to the more hydrophilic components of the sample being eluted first.

Fractionation of the organic and aqueous extracts using solid phase extraction and reversed-phase chromatography, respectively, served as sample preparation prior to liquid chromatography and mass spectrometry.

1.6.3. Analysis by Liquid Chromatography-Mass Spectrometry (Electrospray Ionization)

The fractions obtained were further separated in a liquid chromatography-mass spectrometry (LC-MS) system. Columnar liquid chromatography, specifically high-performance liquid chromatography (HPLC), was utilized. HPLC employs liquid mobile and stationary phases. The mobile phase solvents together with the samples were forced through a tightly packed column containing a thin film, made of small particles with molecules bonded to their surface, having liquid-like properties. In the study, an octadecylsilyl (ODS) column with 3 µm silica particles was used as the stationary phase and a gradient of acetonitrile and water as the mobile phase.

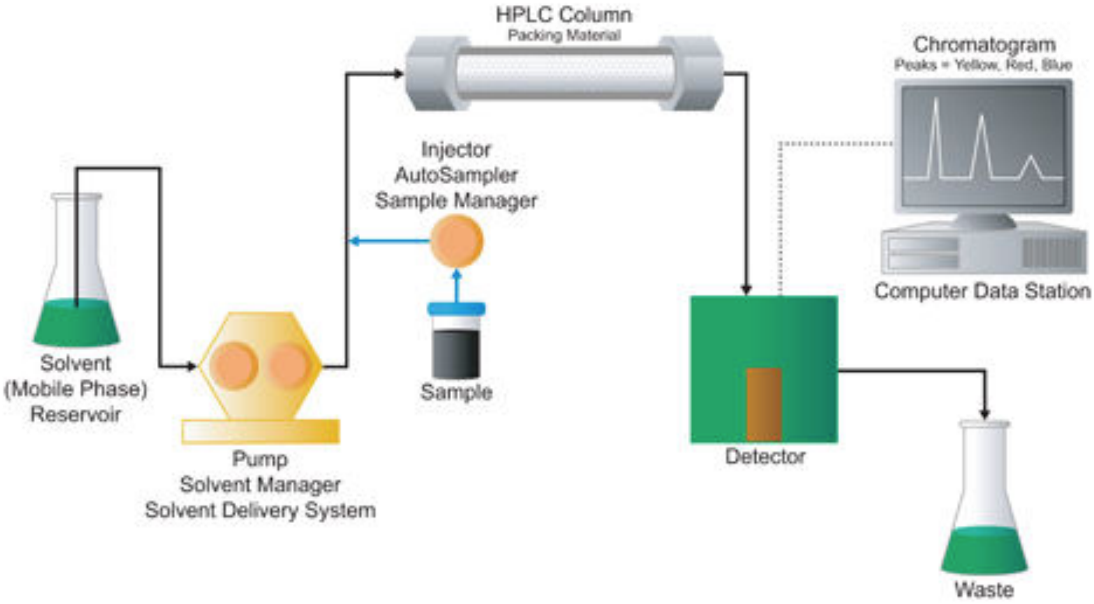


Figure 1.14 Components of a basic High-Performance Liquid Chromatography (HPLC) System (Waters, 2017).

Typically, a detector at the end of the column monitors the separated components of the sample. The electrical signals from the detector are translated into chromatograms, which enable to identify and quantify the concentration of the components. However, it might be difficult to ascertain if a certain peak is pure and contains only a single chemical.

Coupling the HPLC with mass spectrometry helps identify these peaks as it tells the masses of the chemicals present in every peak. Thus, after separation of the components from HPLC, these components enter the mass spectrometer to be ionized. The ions formed are accelerated into the mass analyzer, where the ions are subjected to electric and magnetic field to alter their paths thereby separating the ions depending on their mass-to-charge (m/z) ratios. A detector at the end of the mass analyzer measures the intensity of the ionic beam. This is then translated into a chromatogram of the ionic beam intensity as a function of the mass-to-charge ratio of each ionic component. Moreover, quantitative analysis can be done to determine the concentration of the component by relating it to the height of the mass spectrometric peak.

In the study, electrospray ionization served the ion source of the LC-MS system. Components separated from the HPLC were bombarded with ions or were collided with other ions to produce ions for mass spectrometric analysis. Electrospray ionization uses electrical energy to assist the transfer of ions from solution into the gaseous phase prior to mass spectrometric analysis. Thus, ionic species in the sample can be analyzed with increased sensitivity. Moreover, neutral compounds can be converted to ionic form in solution or in gaseous phase by protonation or cationization (Ho, et al., 2003).

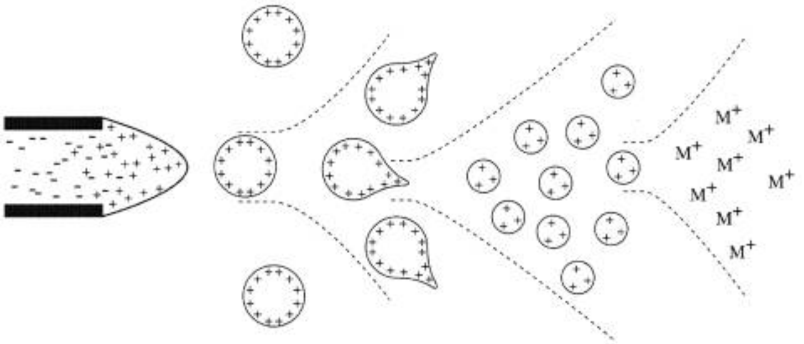


Figure 1.15 Electrospray ionization mechanism (Ho, et al., 2003).

ESI can ionize very fragile biomolecules and detect non-covalent complexes with no dissociation. In addition, it can analyze large biomolecules in a small mass-to-charge ratio range. This feature is advantageous particularly in identifying novel or unknown compounds or natural products, as in the case of this research.

2. EXPERIMENTAL SECTION

2.1. General Experimental Procedures

The ethanol, methanol, chloroform, ethyl acetate, acetonitrile, and formic acid used were procured from SIgma-Aldrich (Japan) and Carlo Erba Reagents (Portugal). All the reagents used were analytical or HPLC grade and used without further purification. In Japan, the liquid chromatography-mass spectrometry (LC-MS) analyses at were done using an Agilent 1100 Series HPLC System coupled with a Bruker Daltonics micrOTOF-HS mass spectrometer (ESI). In Portugal, the liquid chromatography-mass spectrometry (LC-MS) and tandem mass spectrometry (MS/MS) analyses were done using a Bruker® QqTOF Impact II Mass Spectrometer with ESI and CaptiveSpray nanoBooster™ with a ThermoScientific™ Dionex™ UltiMate™ WPS-3000TPL RSLCnano autosampler.

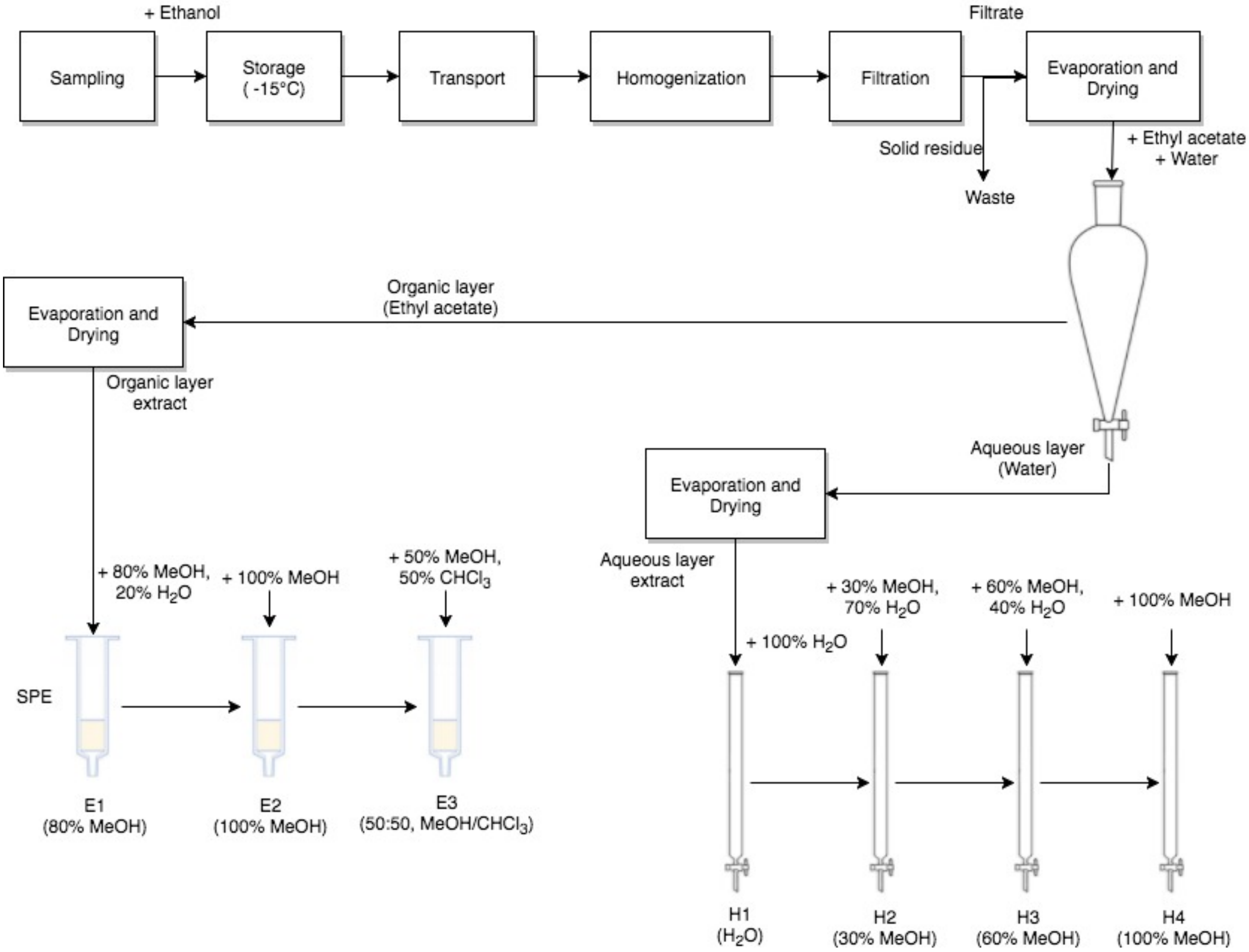


Figure 2.1 A process flow diagram of the methodology.

2.2. Sample Collection

Samples of *Cyrptosula zavjalovensis* (60 g) were collected at the Akkeshi Marine Station, Hokkaido, Japan (Figure 2.2) during low tide in August 2016, with tide heights at 0.2 to 0.4 m. Collection was done in two locations by the pier of the marine station (Figure 2.3): **1** at the first piling of the pier and **2** by the rocks at the end of the pier. The samples were collected from algae and rocks where the bryozoan colonies have encrusted. Algae with encrusting bryozoans were cut and bryozoans in rocks were scraped and were placed in containers with seawater and were brought to the marine laboratory for separation. The bryozoan samples were delicately separated and removed from the algae and rocks using forceps. Foreign particles in the samples were also removed. The bryozoan samples collected were inspected under a compound light microscope (Olympus) for zooid structure and *C. zavjalovensis* samples were separated. These samples also emitted a lemon-like odor, which is distinct to the species.

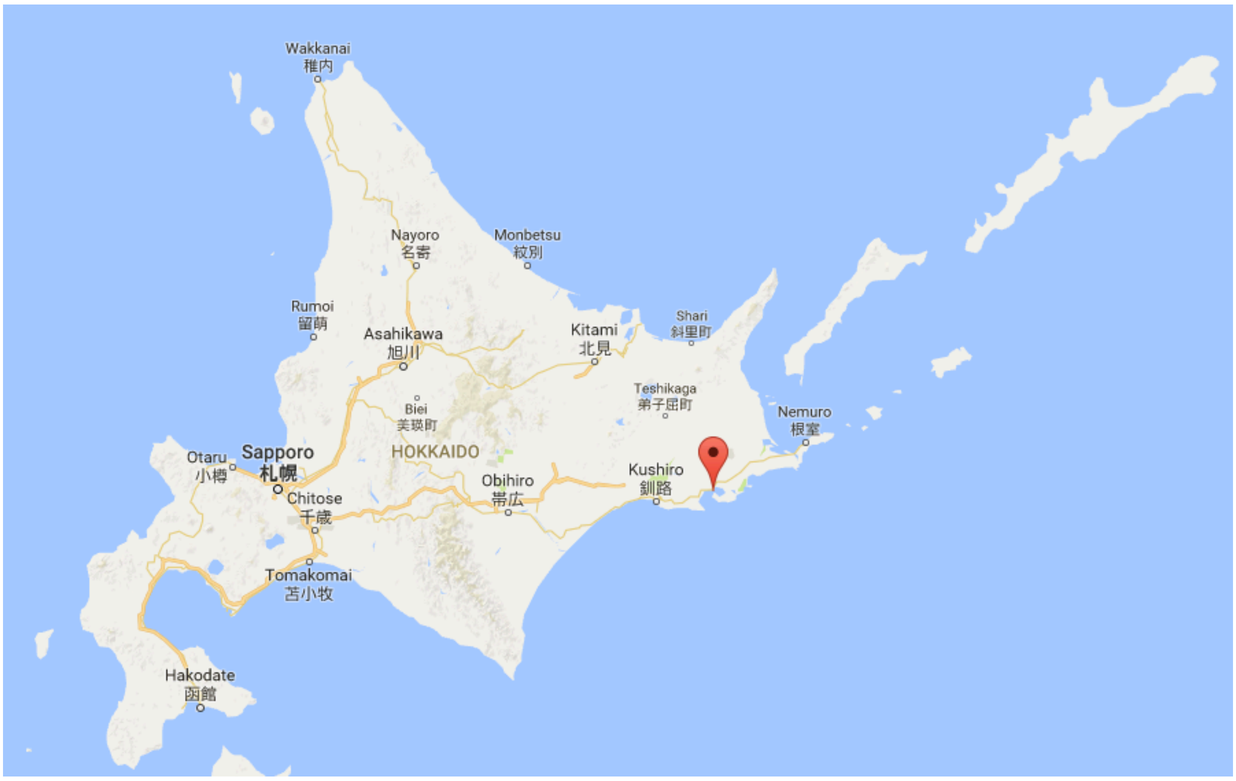


Figure 2.2 Location of Akkeshi in the island of Hokkaido, Japan (Google, 2017).

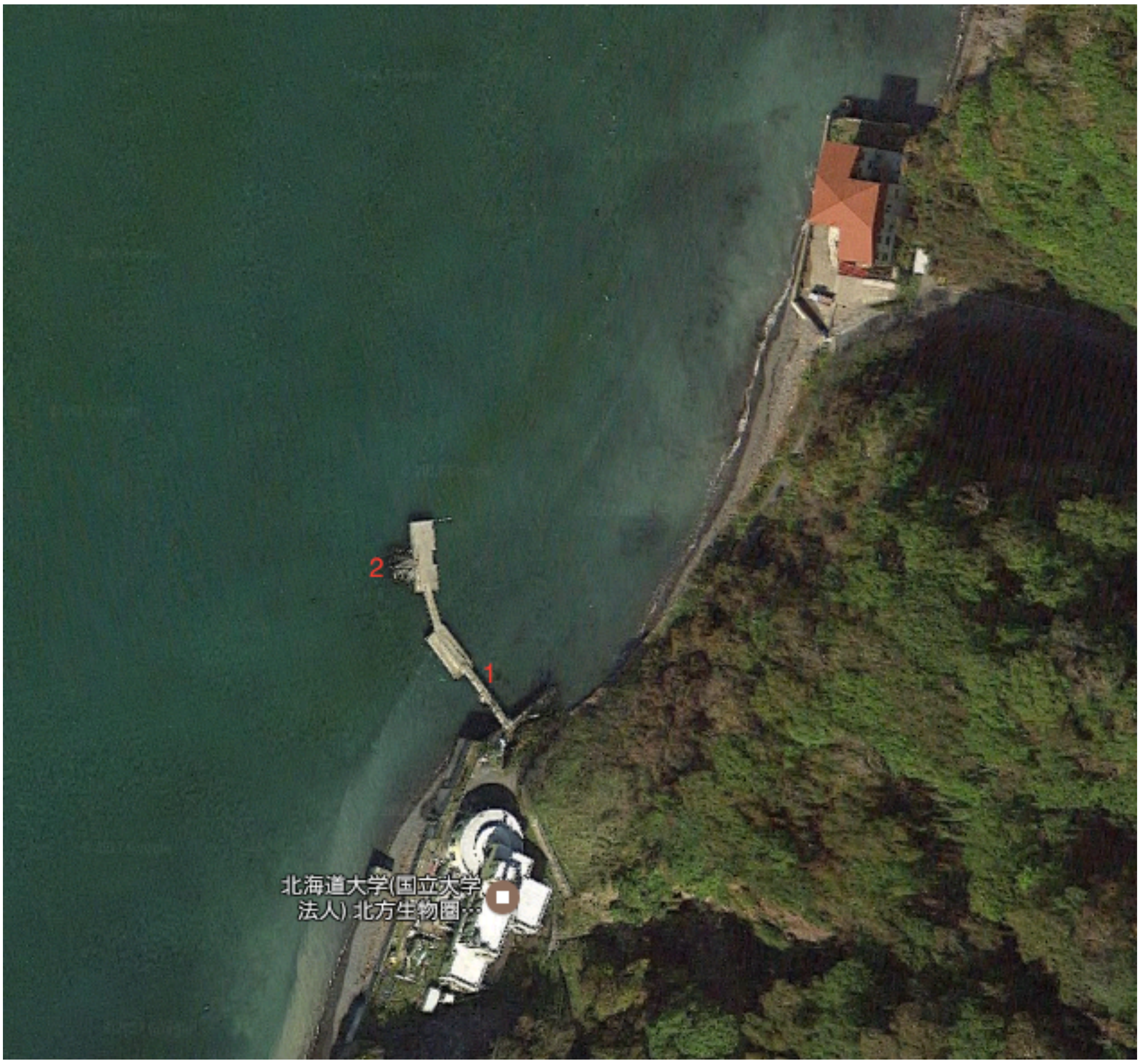


Figure 2.3 The sampling locations at the 1 first piling and 2 by the rocks of the pier of the Akkeshi Marine Station, Hokkaido, Japan (Google, 2017).

The *C. zavjalovensis* samples were weighed and placed in vials containing 10 g of sample each vial. Each vial was filled with ethanol (30mL) to store the samples for transport to the laboratory in Hokkaido University (Hokudai), Sapporo, Japan and University of Algarve (UAlg), Faro, Portugal. Vials were kept in a freezer (-15 °C) until analysis.

Figure 2.4 Optical microscopy image from a sample of Cryptosula zavjalovensis, showing its characteristic zooid structure.

2.3. Preliminary Tests at Hokkaido University

Preliminary tests were conducted at the Okino Laboratory, Faculty of Environmental Earth Science, Hokkaido University, Sapporo, Japan. These tests were done to determine the presence and bioactivity of the secondary metabolites from *Cryptosula zavjalovensis*.

2.3.1. Solvent Extraction

The sample was homogenized by mashing using a mortar and pestle. The sample was then filtered using vacuum filtration and was washed with ethanol (100mL) three times. The ethanol was removed from the filtrate by means of a rotary evaporator to obtain a crude extract (340 mg).

The crude extract was then partitioned into an organic and aqueous fraction using ethyl acetate (100 mL) and Milli-Q® water (100 mL), respectively. This was done three times to obtain better separation between the two fractions. The organic and aqueous fractions were dried using a rotary evaporator to obtain an ethyl acetate (EtOAc) extract (21.3 mg) and Milli-Q® water (H_2O) extract (300 mg), respectively.

2.3.2. Solid Phase Extraction and Reversed-Phase Chromatography

The EtOAc extract was fractionated by solid phase extraction using a Strata® SPE to obtain three fractions: a gradient of methanol (MeOH) and H_2O, **E1** 80:20, **E2** 100:0 v/v, and; **E3** methanol and chloroform 50:50 v/v. The wash and elution volume used was 4 mL.

The aqueous extract was fractionated by reversed-phase chromatography using octadecyl (C18) as a stationary phase with a gradient composed of MeOH and H_2O to obtain four fractions: **H1** 0:100, **H2** 30:70, **H3** 60:40, **H4** 100:0 v/v. The wash and elution volume used was 6 mL, which was thrice the depth volume for the stationary phase to be totally submerged.

The extracts were dried using a rotary evaporator and were weighed and placed in pre-weighed vials prior to liquid chromatography-mass spectrometry (LCMS) and cytotoxicity sample preparation and for storage.

2.3.3. Cytotoxicity Screening

Cytotoxicity was evaluated using MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay. In the mitochondria of living cells, MTT is reduced to formazan as shown in Figure 2.4. Formazan crystals, which are purple in color, are dissolved in dimethyl sulfoxide (DMSO) and the optical density is measured at 570 nm.

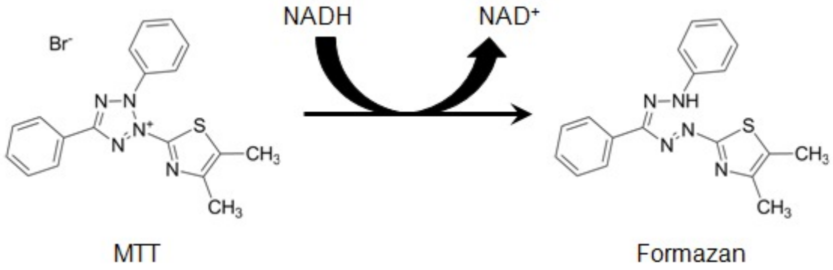


Figure 2.5 The reduction of MTT into formazan.

MTT was dissolved in Dulbecco’s phosphate buffered saline (DPBS) at a concentration of 5 mg/mL and was frozen at –20 °C until use. The wells of the plate were seeded with 90 µL of the cell suspension to make a concentration of 1 x 10^4 cells per well. MCF-7 breast cancer cell line (Culture Collections, Public Health England) was used in the analysis. After a 24-hour incubation, 10 µL of the samples were added at a concentration of 1 µg/mL and 10 µg/mL. Cisplatin was used as a positive control.

Incubation was done for 72 hours. After incubation, the medium was aspirated from the plate. The healthy cells should have adhered at the bottom of the well. MTT solution (100 µL) was added to each well was incubated for 3 hours. Healthy cells manifested purple formazan crystals. The MTT solution was then aspirated and DMSO (100 µL) was added to dissolve the formazan crystals. This was then incubated for 10 minutes at 37 °C and shaken or 10s before it was read under Thermo Labsystems Multiskan JX plate reader at an absorbance of 570 nm.

The cytotoxicity effect is calculated as:

$$\%Cell\ Cytotoxic\ Effect = \left[1 - \frac{Optical\ density\ of\ treated\ cells}{Optical\ density\ of\ control\ cells}\right] \times 100$$

2.3.4. Liquid Chromatography-Mass Spectrometry

Each extract obtained was diluted in filtered ethanol to obtain a concentration of 100 µg/mL filtered ethanol for LCMS analysis. 500 µL of each sample solution was placed in an LCMS vial for analysis.

The LCMS system was calibrated using an ESI tuning mix solution: 570 µL acetonitrile (MeCN), 30 µL Milli-Q® water, and 20 µL ESI tuning mix solution. For the analyses of the EtOAc extracts, the HPLC system was equipped with a Cadenza® CD-C18 column (2 x 150mm, 3µm, 25°C, 0.2 mL/min) under the following conditions: 0-15 min, gradient elution of 50-80% MeCN with 0.1% (v/v) formic acid in H_2O; 15-30 min, isocratic elution of 80% MeCN with 0.1% (v/v) formic acid in H_2O. Samples were analyzed in both positive and negative ionization methods.

For the analyses of H_2O extracts, the HPLC system was equipped with a Cosmosil HILIC® column (4.6 x 250mm, 5µm, type Waters, 25°C, 0.2 mL/min) under the following conditions: 0-30 min, gradient elution of 10-80% MeCN with 0.1% (v/v) formic acid in H_2O; 30-35 min, isocratic elution of 80% MeCN with 0.1% (v/v) formic acid in H_2O. Samples were analyzed in both positive and negative ionization methods.

Post-processing of the chromatograms was done using HyStar®. Potential peaks were noted and matched in the MarinLit® database, an extensive database of marine natural products, for possible identification.

2.4. Analyses at the University of Algarve

Samples were brought to the University of Algarve for further analyses. Preliminary tests results obtained from Hokkaido University helped develop the tests done at UAlg. Since the bioactive compounds were present in the organic extracts, extraction and analyses done were focused on the organic extracts.

2.4.1. Solvent Extraction

The sample was homogenized by mashing using a mortar and pestle. The sample was then filtered using vacuum filtration and was washed with ethanol (100mL) three times. The ethanol was removed from the filtrate by means of a rotary evaporator to obtain a crude extract (186 mg).

The crude extract was then partitioned into an organic and aqueous fraction using ethyl acetate (100 mL) and Milli-Q® water (100 mL), respectively. This was done three times to obtain better separation between the two fractions. The organic fraction was dried using a rotary evaporator to obtain an ethyl acetate (EtOAc) extract (60 mg).

2.4.2. Solid Phase Extraction

The EtOAc extract was fractionated by solid phase extraction using a Strata® SPE to obtain three fractions: a gradient of methanol (MeOH) and H_2O, **E1** 80:20, **E2** 100:0 v/v, and; **E3** methanol and chloroform 50:50 v/v. The elution volume used was 4 mL.

The fractions were dried using a rotary evaporator and were weighed and placed in pre-weighed vials prior to liquid chromatography-mass spectrometry (LCMS) and cytotoxicity sample preparation and for storage.

2.4.3. Liquid Chromatography-Mass Spectrometry

Each extract obtained was diluted in filtered ethanol to obtain a concentration of 100 µg/mL filtered ethanol and 150 µg/mL filtered ethanol for LCMS analysis. 500 µL of each sample solution was placed in an LCMS vial for analysis.

LC-MS and tandem mass spectrometry (MS/MS) analyses were done at the Mass Spectrometry Facility, Centro de Química Estrutural, Instituto Superior Técnico, Universidade de Lisboa. For the analyses of the EtOAc extracts, the HPLC system was equipped with a Hamilton® PRP-1 column (2.1 x 150mm, 5µm, 25°C, 0.2 mL/min) under the following conditions: 0-15 min, gradient elution of 50-80% MeCN with 0.1% (v/v) formic acid in H_2O; 15-30 min, isocratic elution of 80% MeCN with 0.1% (v/v) formic acid in H_2O. Samples were analyzed in both positive and negative ionization methods.

2.4.4. Tandem Mass Spectrometry

Peaks identified from the LC-MS chromatograms and spectra were analyzed further using MS/MS. The ions of these selected peaks were collided with a neutral atom or molecule during collision-induced dissociation using a collision energy of 27 eV to obtain fragment ions.

Larger fragment ions were further dissociated and collided with a neutral atom or molecule to obtain smaller fragment ions. Both the m/z ratios of the fragment ions and the leaving groups during MS/MS analysis were detected until a third stage of mass spectrometry. Post-processing of the chromatograms and spectra was done using Thermo Xcalibur® software.

3. RESULTS AND DISCUSSION

3.1. Preliminary Tests

3.1.1. Cytotoxicity Screening

Preliminary testing for cytotoxicity of the extracts were tested using MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay against MCF7 breast cancer cells. This MTT assay was used to screen the extract for the presence of bioactive compounds. Active metabolism of viable cells convert MTT into the purple-colored formazan and upon cell death, this ability to metabolize to MTT is lost. Consequently, color formation is a marker of cell viability.

Cisplatin, a known chemotherapeutic drug, has been used as a positive control. It has shown anticancer activity against various types of tumors such as bladder, head and neck, lung, ovarian and testicular cancers (Dasari & Tchounwou, 2014). Its United States Food and Drug Administration (US FDA) approval and clinical introduction led the interest on the success of platinum complexes as anticancer drugs (Kelland, 2007).

Cisplatin has already been used against breast cancer cells and sometimes used in combination with other drugs such as taxanes, vinca alkaloids, and 5-fluorouracil for its synergistic and effective effects as well as to minimize cisplatin resistance (Prabhakaran, Hassiotou, Blancafort, & Filgueira, 2013). Moreover, the cisplatin-related toxicities are dose-dependent (Florea & Büsselberg, 2011), making it an ideal positive control.

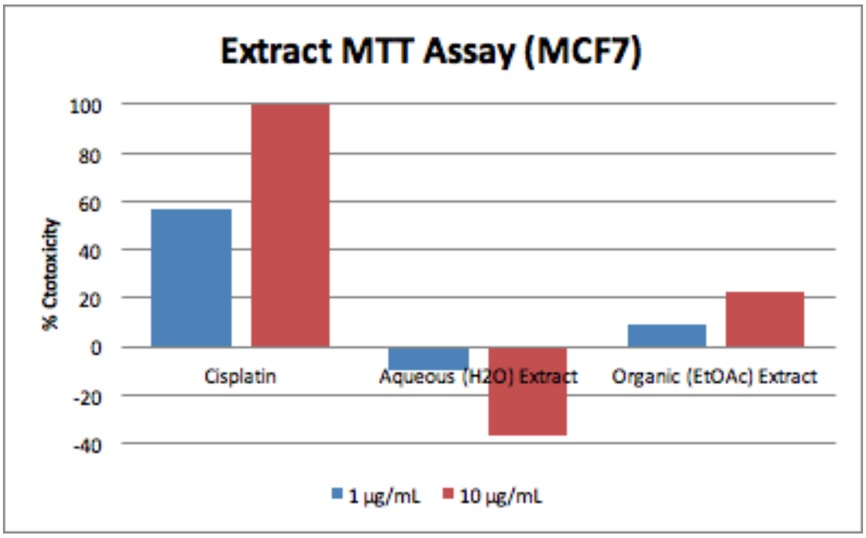


Figure 3.1 MTT assay results of the aqueous (water) and organic (ethyl acetate) extracts.

Figure 3.1 shows the cytotoxicity results of the aqueous and organic extracts. The organic extract exhibited cytotoxicity against breast cancer cells. An increase in the extract concentration from 1 to 10 µg/mL resulted to an increase of cytotoxicity from 9% to 23%, respectively.

On the other hand, the aqueous extract strangely exhibited negative cytotoxicity, that is, there were more live breast cancer cells in the treated cells than that of in the control cells. Increasing the extract concentration from 1 to 10 µg/mL resulted to a decrease in cytotoxicity from –10% to –36%, respectively. Although this result would point to a proliferation of the cancer cells, that is unlikely. As it was not possible to repeat the experiment due to time constraints at Hokudai, a similar assay is being performed in UAlg.

It should be noted that MTT reduction is a marker for cell viability and not specifically for cell proliferation, although MTT reduction is often used to measure cell proliferation. With the use of proper controls, however, it can describe cytotoxicity and cell proliferation (Huyck, Ampe, & Van Troys, 2012). Thus, the use of cisplatin as positive control for the study. Nevertheless, bioactivity was observed with the organic extract.

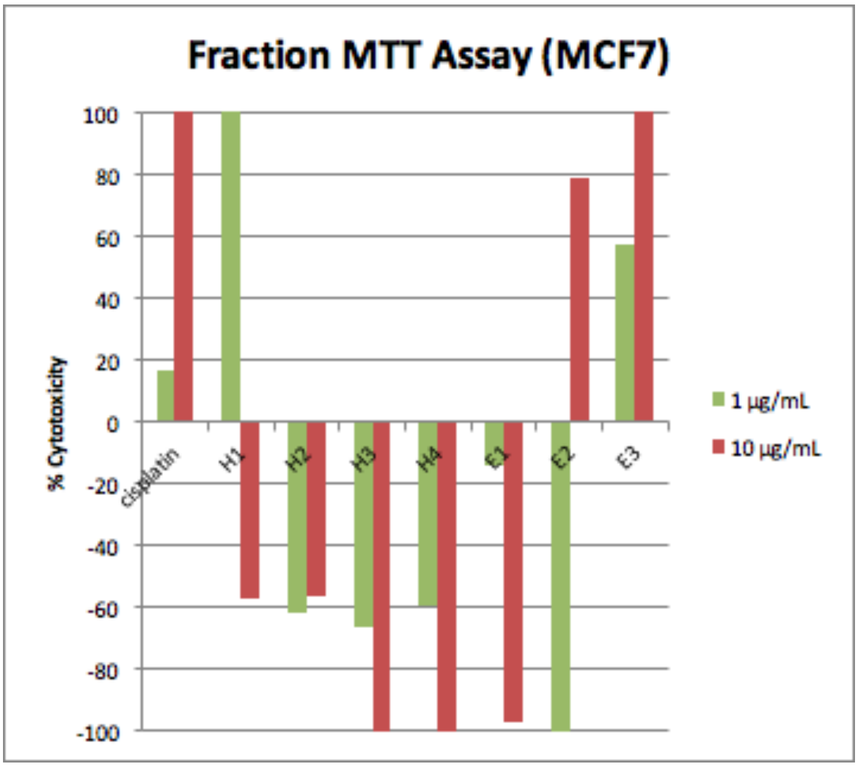


Figure 3.2 MTT assay results of the aqueous fractions: H1, H2, H3, H4, and; organic fractions: E1, E2, E3.

To narrow down the search for bioactive compounds, fractions from both the organic and aqueous extracts were tested for cytotoxicity. Figure 3.2 shows the cytotoxicity results of the fractions. Aqueous extract fractions consistently exhibited negative cytotoxicity. However, the fractions from the organic extract exhibited varying cytotoxicity results. Fraction E1, like the aqueous fractions, exhibited negative cytotoxicity. At a concentration of 1 µg/mL, E2 exhibited negative cytotoxicity. However, at a concentration of 10 µg/mL, it exhibited 79% cytotoxicity indicative of the presence of bioactive compounds. At both concentrations of 1 and 10 µg/mL, fraction E3 exhibited cytotoxicity at 58% and 107%, respectively. As before, a negative cytotoxicity result is unclear thus necessitating the need repeat these results and conduct further cytotoxicity tests.

Nevertheless, these preliminary cytotoxicity results narrow down the search for the bioactive compounds in the organic extract, specifically in fractions E2 and E3. Consequently, this will guide the search for bioactive compounds during further LC-MS analysis.

3.1.2. Liquid Chromatography-Mass Spectrometry

Aqueous fractions H1-H4 and organic fractions E1-E3 were separated into their components by means of LC-MS, in both positive and negative ionization methods.

LC-MS chromatograms for aqueous fractions H1-H4 revealed no promising peaks in both positive and negative ionization modes. This, together with the cytotoxicity screening results, supported the move to focus only on the organic fractions for the search of bioactive compounds.

There were also no significant peaks observed from the organic fractions using the negative ionization method. Using the positive ionization method for the organic fraction, however, yielded some notable peaks, as shown in Figure 3.3 and Figure 3.5, which are reproducible as shown in Figure 3.4 and Figure 3.6, respectively.

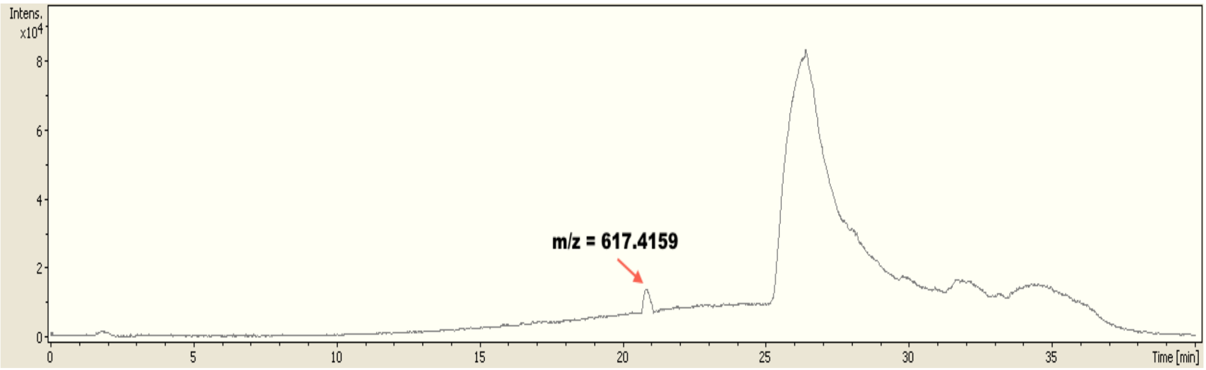


Figure 3.3 Positive ionization LC-MS chromatogram of fraction E2.

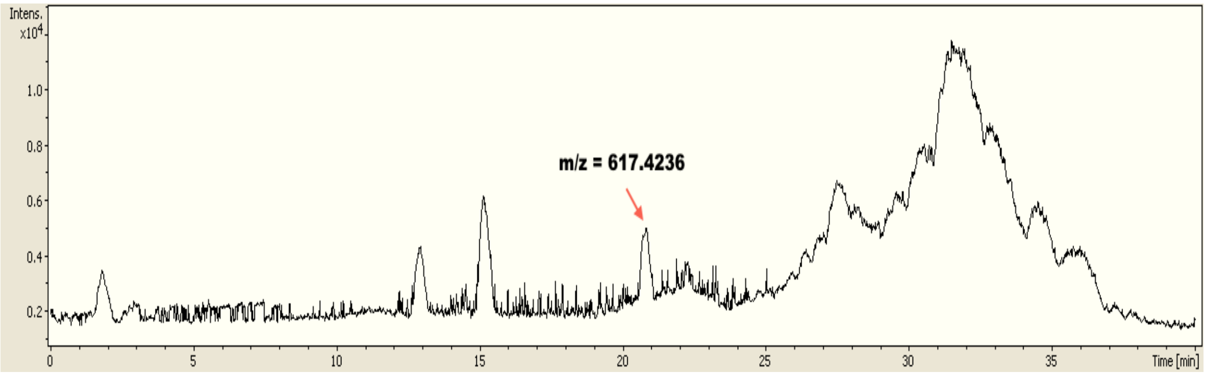


Figure 3.4 Reproducibility of peak mass-to-charge ratio (m/z) = 617.

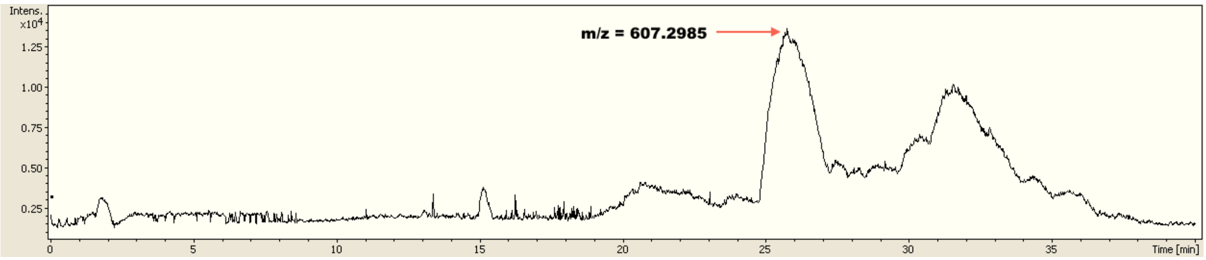


Figure 3.5 Positive ionization LC-MS chromatogram of fraction E3.

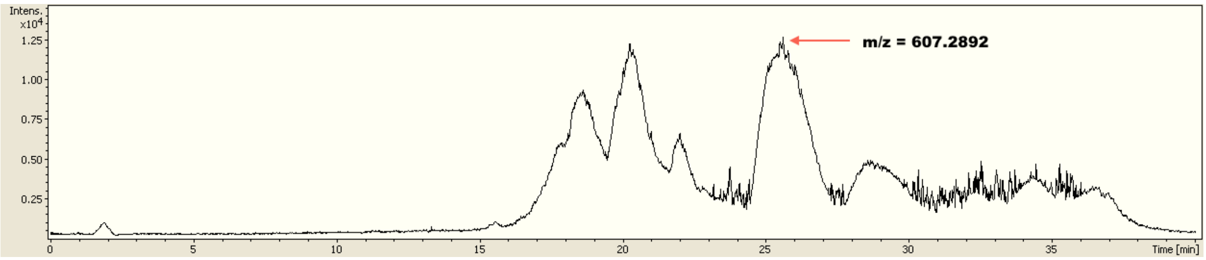


Figure 3.6 Reproducibility of peak m/z = 607.

These peaks were then matched in the MarinLit® database for identification. A compound search in the database yielded no results. This implies that these peaks are compounds which have not yet been isolated nor recorded in the database and consequently, may be novel compounds.

3.2. Results from the University of Algarve

3.2.1. Liquid Chromatography-Mass Spectrometry

A similar procedure for LC-MS analysis was done the at the Mass Spectrometry Facility, Centro de Química Estrutural, Instituto Superior Técnico, Universidade de Lisboa, this time using a Hamilton® PRP-1 column. The column used during the preliminary tests, Cadenza® CD-C18, is ideal for isomer separation and impurities detection. In addition, the selectivity of this column is ideal for small molecule pharmaceutical testing or drugs of abuse. However, the number of peaks and compounds identified using this column suggests that there could be another column which can better separate the extracts.

The problem with C18 columns is that labile compounds can be irreversibly bound to the silanol groups present. Having no silanol groups present, using a Hamilton PRP-1 column eliminates this problem since Hamilton polymers are made entirely of polystyrene-divinylbenzene. Recovery and quantification of labile and reactive compounds is enhanced. Moreover, unlike silica-based C18 columns, PRP-1 has no stationary coating thereby maintaining its performance characteristics longer (ChromTech, 2017).

This change of column resulted to the identification of significant peaks in both positive and negative ionization modes. Figure 3.7 shows the LC-MS total ion chromatogram of fraction E2 in negative ionization mode while **Error! Reference source not found.** shows the LC-MS total ion chromatogram in positive ionization mode.

Several peaks have been identified. Some of the peaks identified in the negative ionization mode were: retention time (RT) = 4.23 minutes, m/z = 586.04 (Figure 3.8); RT = 5.38 min, m/z = 612.12 (Figure 3.9); RT = 6.56 min, m/z = 565.99 (Figure 3.10), m/z = 613.97 (Figure 3.11). Additionally, in the positive ionization mode: RT = 5.37 min, m/z = 568.26 (Figure 3.13), and; RT = 6.18 min, m/z = 225.20 (**Error! Reference source not found.**).

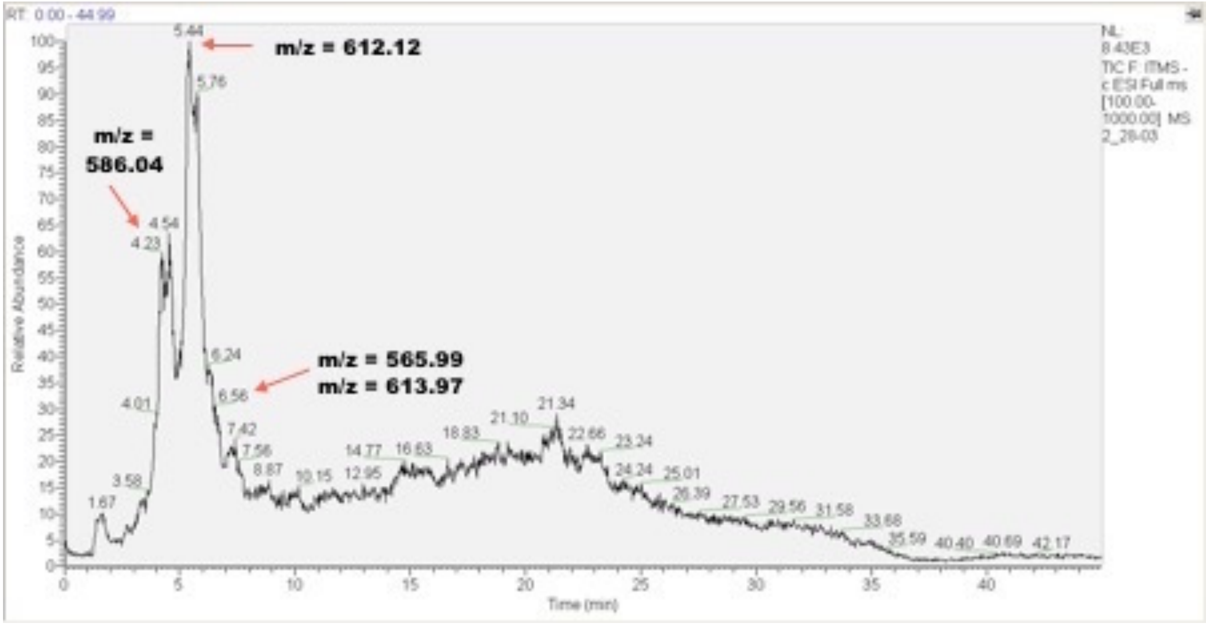


Figure 3.7 Identified peaks from the LC-MS total ion chromatogram of fraction E2 in negative ionization mode.

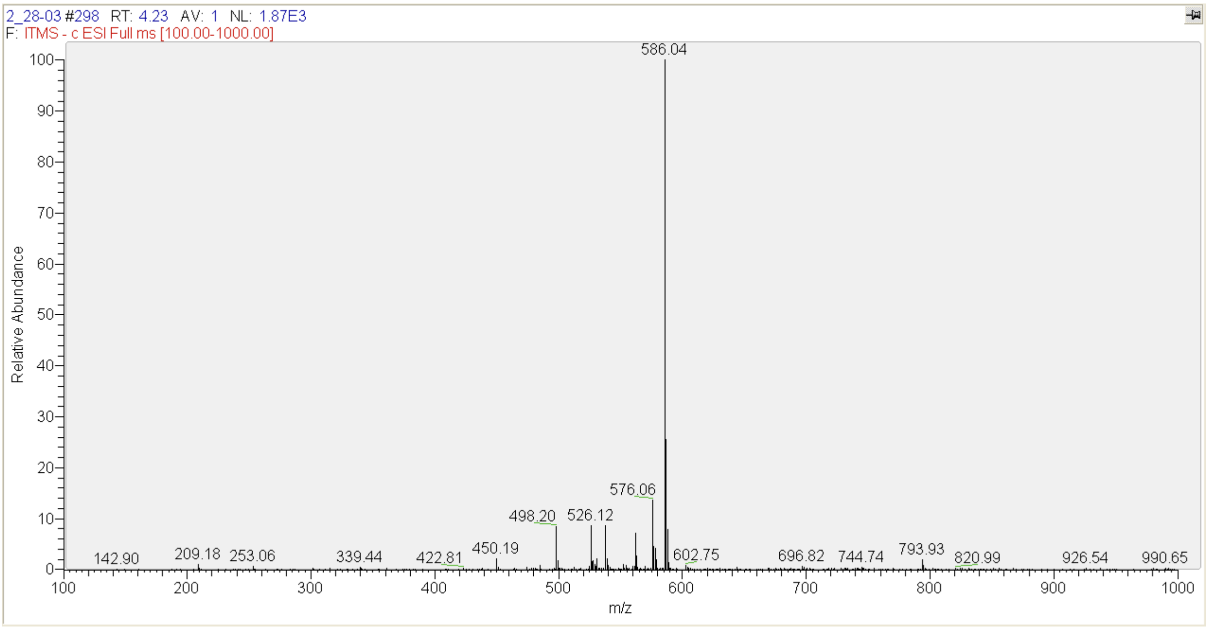


Figure 3.8 Mass spectrum with peak m/z = 586, RT = 4.23 min.

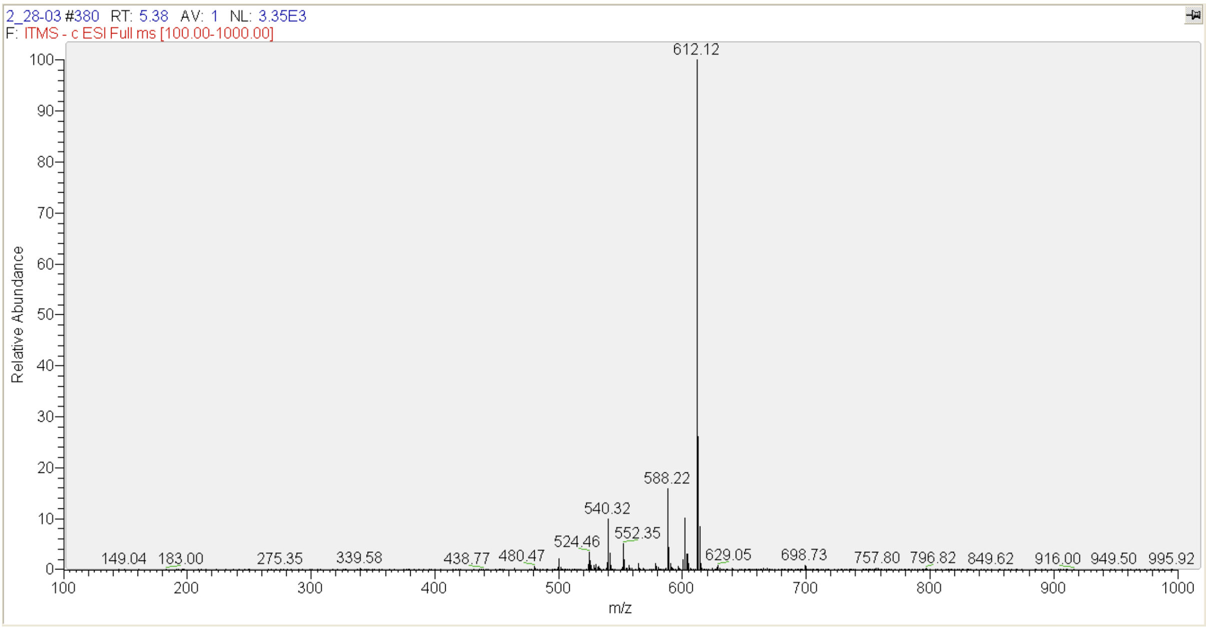


Figure 3.9 Mass spectrum with peak m/z = 612, RT = 5.38 min.

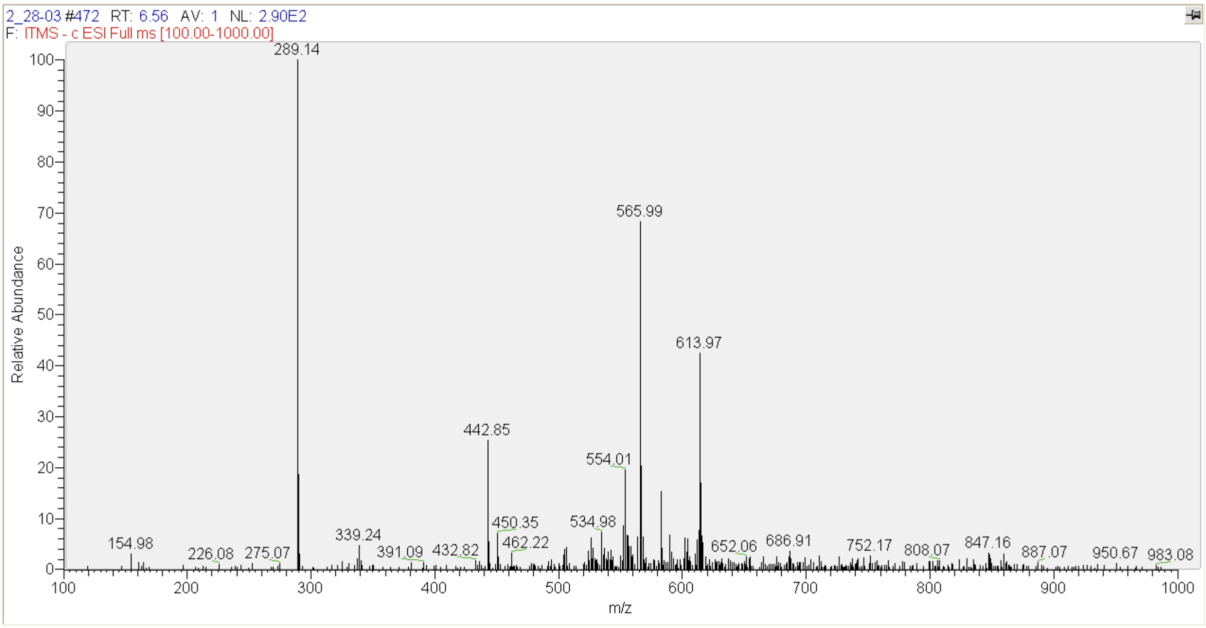


Figure 3.10 Mass spectrum with peak m/z = 566, RT = 6.56 min.

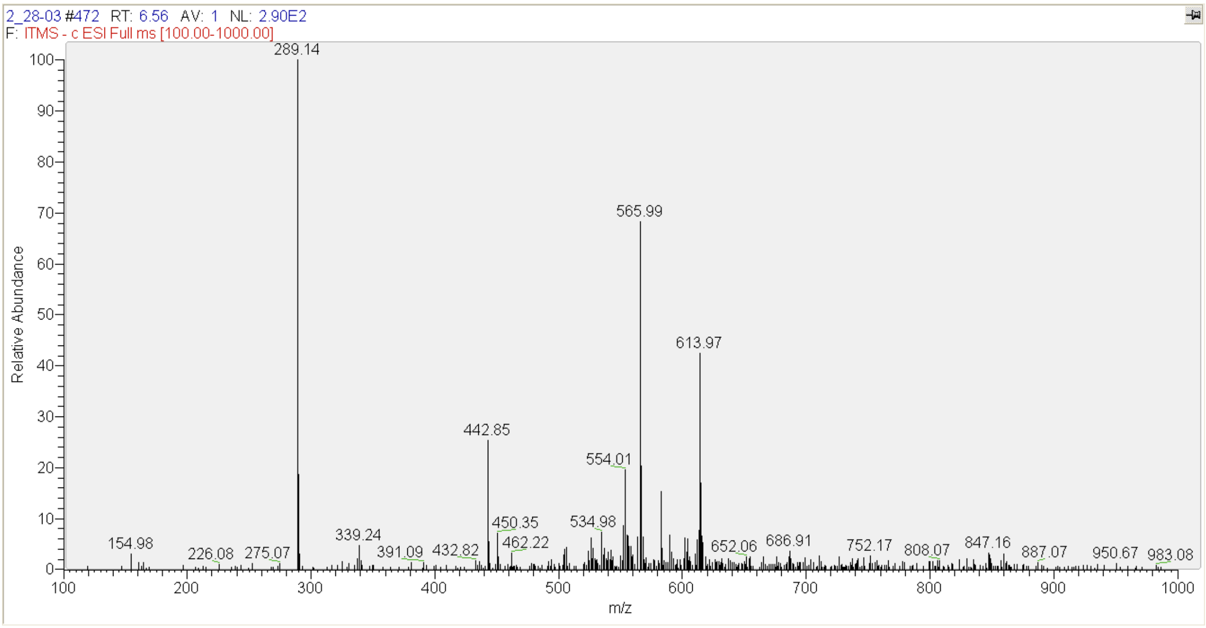


Figure 3.11 Mass spectrum with peak m/z = 614, RT = 6.56 min.

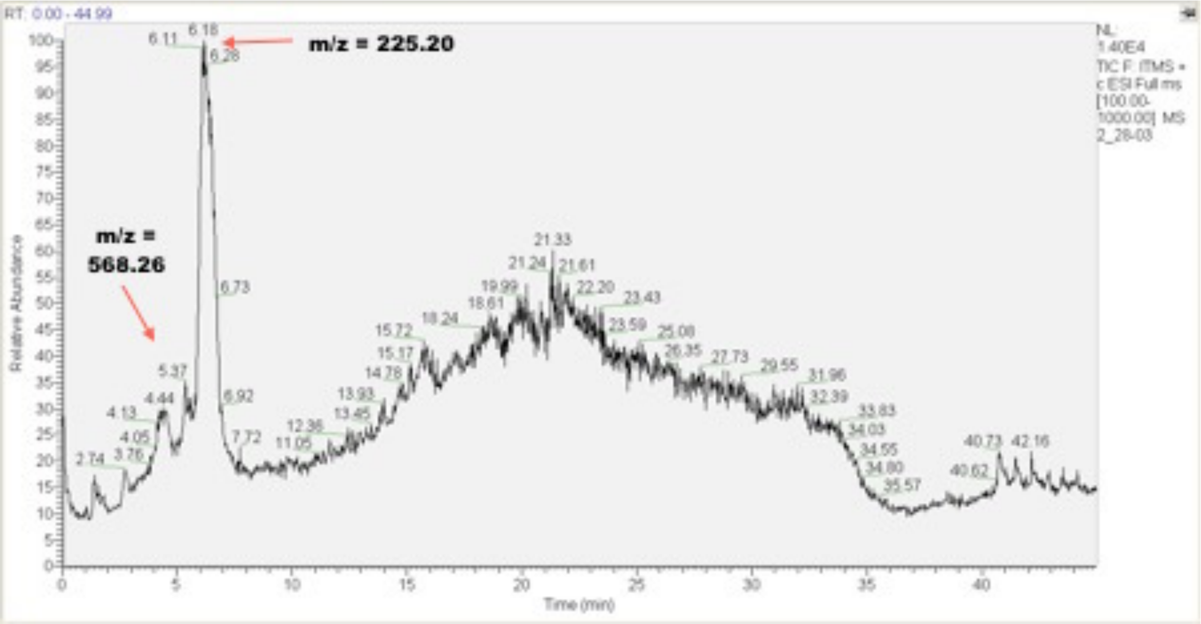


Figure 3.12 Identified peaks from the LC-MS total ion chromatogram of fraction E2 in positive ionization mode.

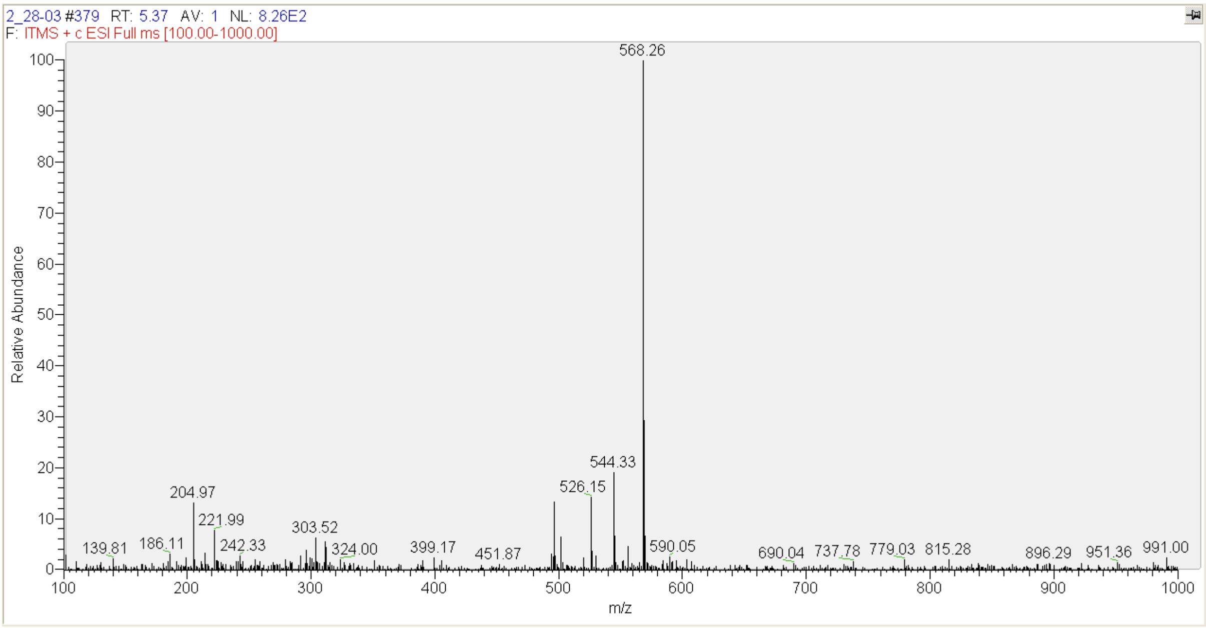


Figure 3.13 Mass spectrum with peak m/z = 568, RT = 5.37 min.

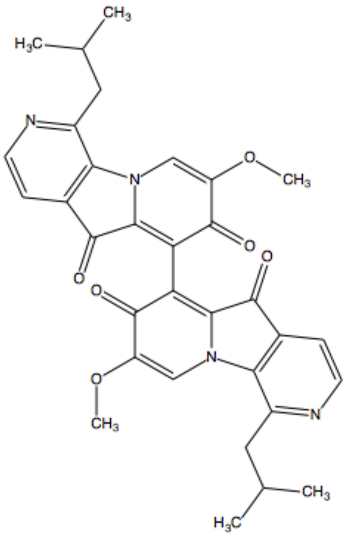
3.2.2. Tandem Mass Spectrometry

Peaks of interest were identified and served as precursor ions during tandem mass spectroscopy to begin the structure elucidation process, where the fragment ions help give an idea of the structure of the compound. Three stages of tandem mass spectrometry were performed to better separate the fragment ions.

During the first stage of mass spectrometry (MS), the ion source formed ions and were separated according to their mass-to-charge ratio. The ions formed for a specific mass-to-charge ratio, served as the precursor ion during the second stage of mass spectrometry (MS2). The second stage of mass spectrometry involved the creation of fragment ions from the precursor ion. The fragment ions produced from MS2 were then selected, and if possible, were further separated into smaller fragment ions during the third stage of mass spectrometry (MS3).

The leaving group from MS2 is the difference between the precursor ion and the fragment ion from MS2. Similarly, the leaving group from MS3 is the difference between the fragment ion from MS2 and fragment ion from MS3. Leaving groups from the precursor ions provide information on the functional groups or the fragment of the precursor ion which can easily be detached.

The m/z values of the peaks identified were compared to previously isolated and identified natural products from other marine bryozoans. Being of the same genus, natural products from *Cryptosula pallasiana* were compared. The alkaloids isolated from *Cryptosula pallasiana*, shown in Figure 1.10, do not match with any of the m/z data from this study. Further search of similar natural products showed that the peak with m/z = 566 might be similar with that of an alkaloid isolated from the New Zealand bryozoan *Pterocella vesiculosa* (Prinsep, 2008), specifically pterocellin E, shown in Figure 3.14.



Chemical Formula: $C_{32}H_{30}N_4O_6$
Molecular Weight: 566.6140
m/z: 566.2165 (100.0%), 567.2199 (34.6%), 568.2232 (5.8%), 567.2136 (1.5%), 568.2208 (1.2%)

Figure 3.14 Structure and pertinent properties of pterocellin E.

Using pterocellin E as a basis for structure, structures for the identified peaks are proposed, as well as the fragment ions and leaving groups.

3.2.2.1 Peak m/z = 566

The selected fragment ion from MS2 for m/z = 566 (Figure 3.15) is shown in Figure 3.16, and was further fragmented through MS3, shown in Figure 3.17. The proposed structures of the precursor ion, fragment ions, and the leaving groups are shown in Figure 3.30.

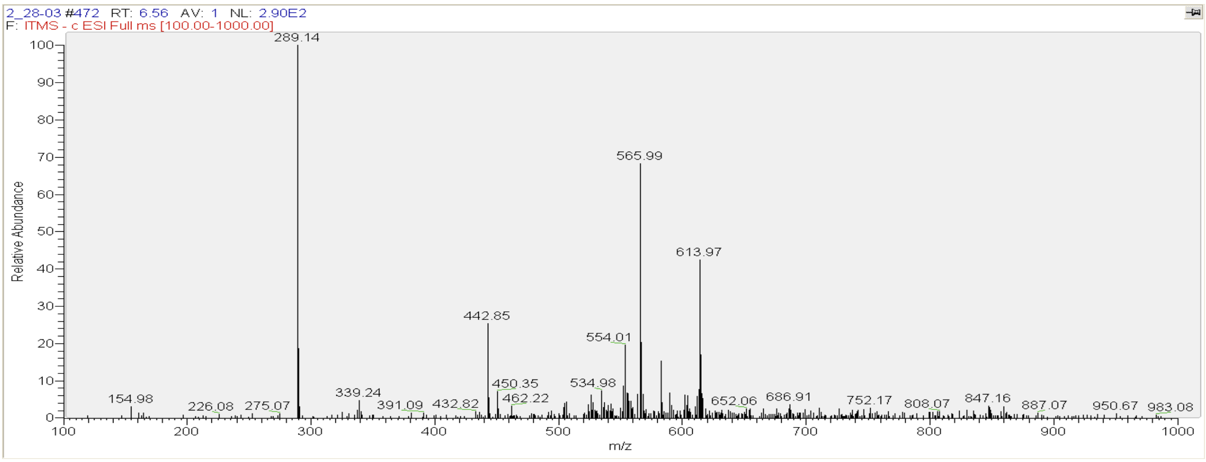


Figure 3.15 Precursor ion. Peak m/z = 566, RT = 6.56 min.

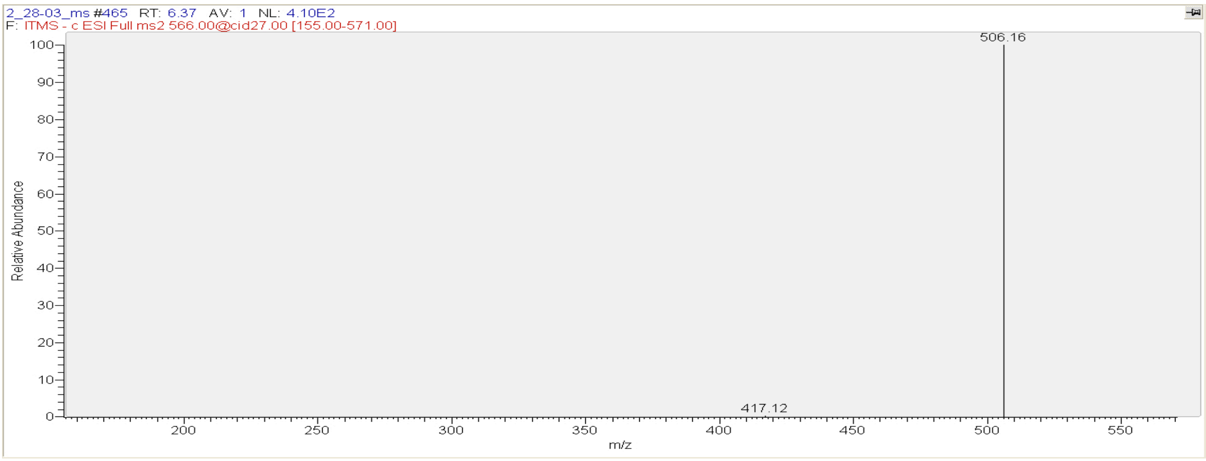


Figure 3.16 Fragment ion after MS2. Peak m/z = 506.16, RT = 6.37 min.

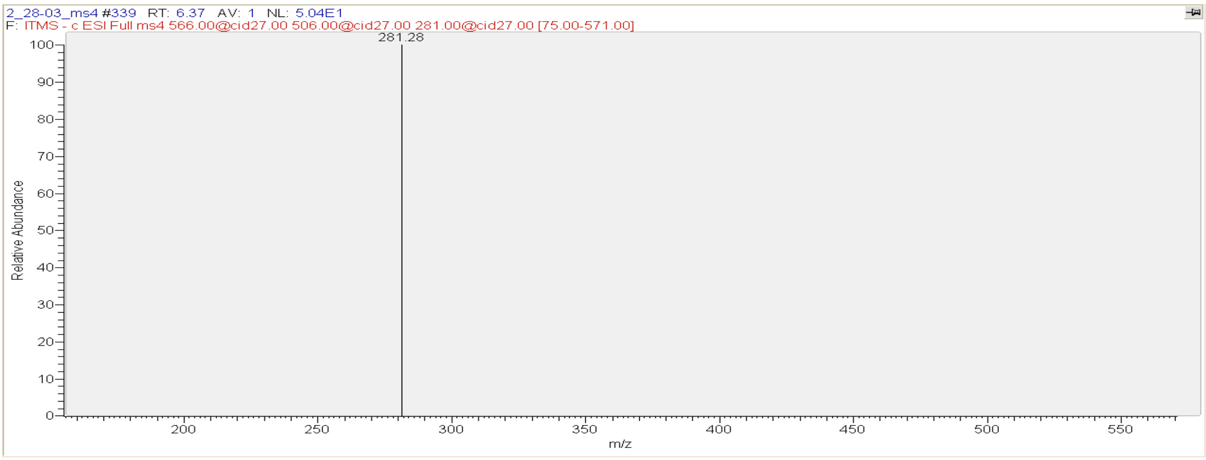


Figure 3.17 Fragment ion after MS3. Peak m/z = 281.28, RT = 6.37.

3.2.2.2 Peak m/z = 586

The selected fragment ion from MS2 for m/z = 586 (Figure 3.18) is shown in Figure 3.19, and was further fragmented through MS3, shown in Figure 3.20. The proposed structures of the precursor ion, fragment ions, and the leaving groups are shown in Figure 3.31.

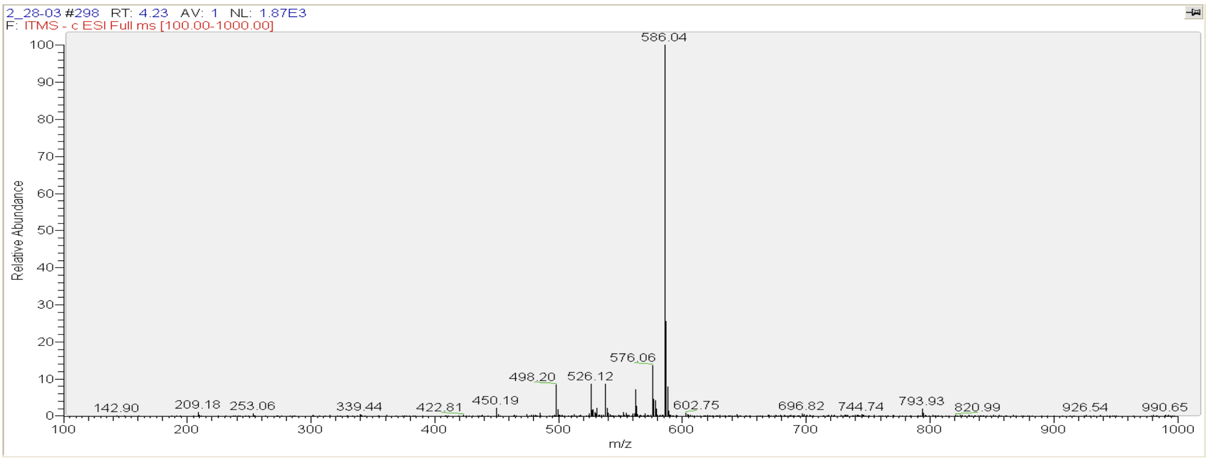


Figure 3.18 Precursor ion. Peak m/z = 586, RT = 4.23 min.

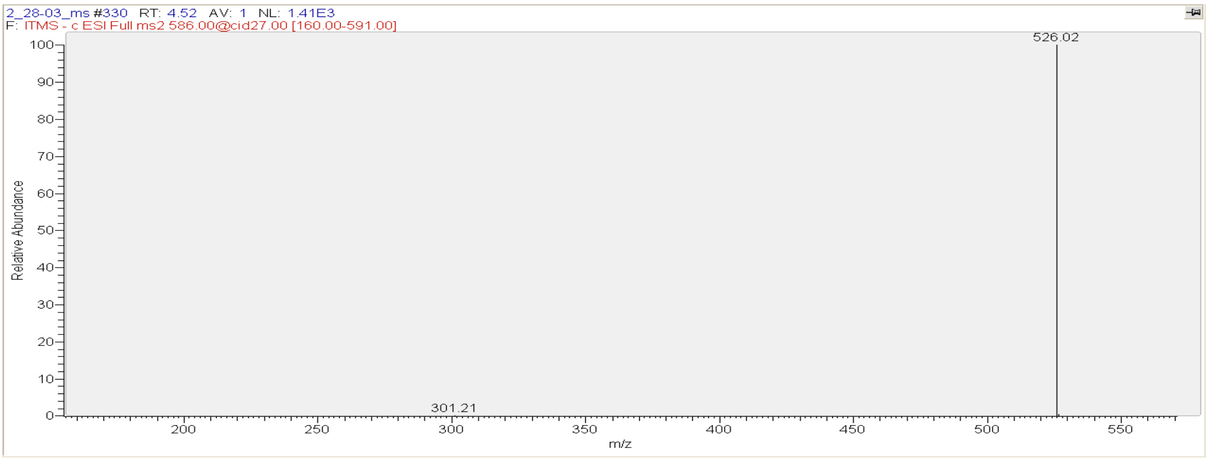


Figure 3.19 Fragment ion after the MS2. Peak m/z = 526.02, RT = 4.52 min.

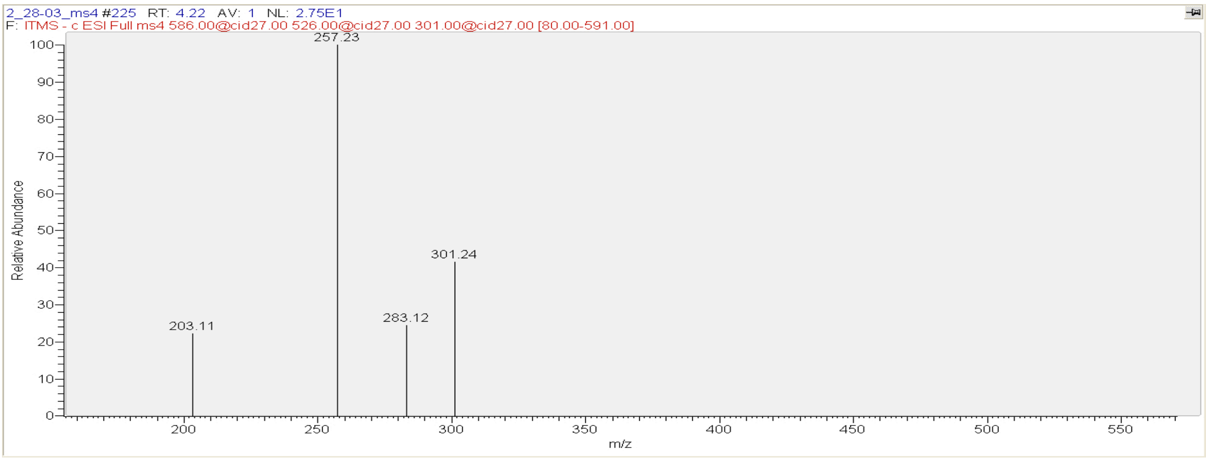


Figure 3.20 Fragment ion after the MS3. Peak m/z = 301.24, RT = 4.22.

3.2.2.3 Peak m/z = 612

The selected fragment ion from MS2 for m/z = 612 (Figure 3.21) is shown in Figure 3.22, and was further fragmented through MS3, shown in Figure 3.23. The proposed structures of the precursor ion, fragment ions, and the leaving groups are shown in Figure 3.32.

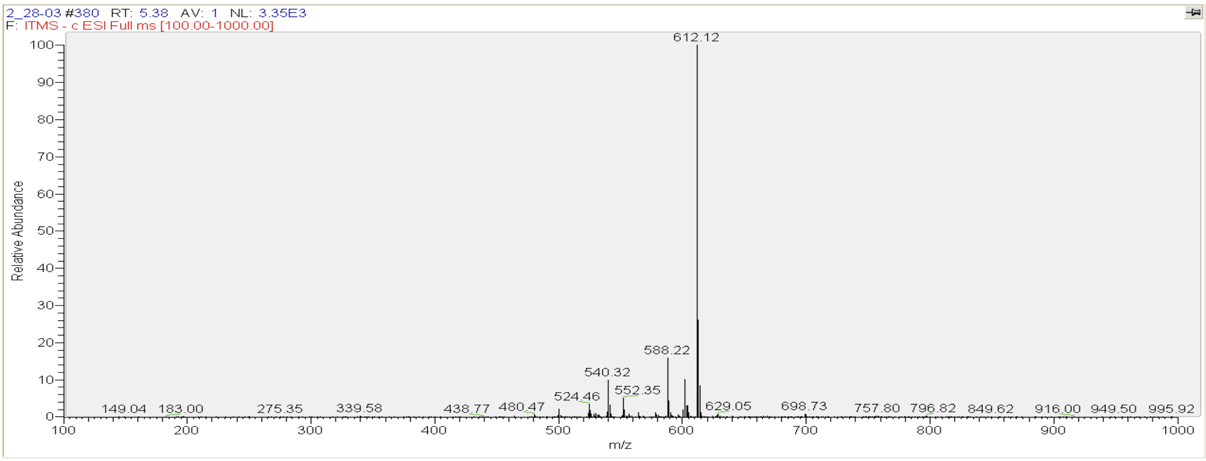


Figure 3.21 Precursor ion. Peak m/z = 612, RT = 5.38 min.

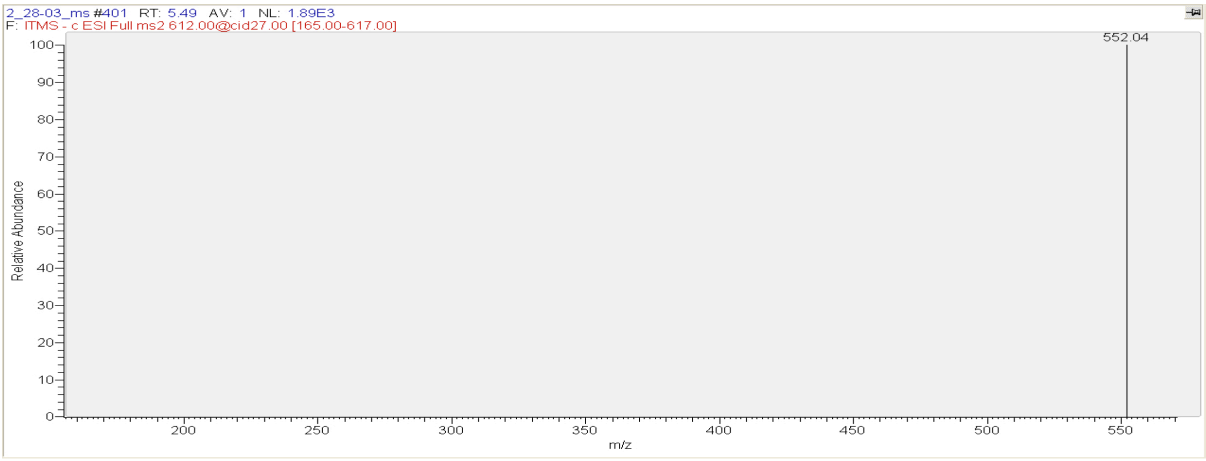


Figure 3.22 Fragment ion after the MS2. Peak m/z = 526.02, RT = 4.52 min.

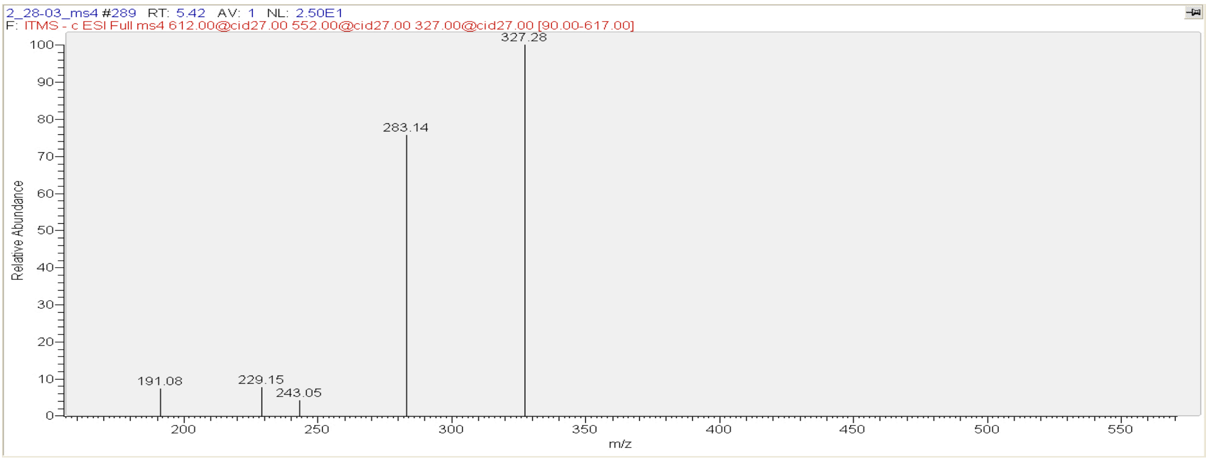


Figure 3.23 Fragment ion after the MS3. Peak m/z = 301.24, RT = 4.22 min.

3.2.2.4 Peak m/z = 614

The selected fragment ion from MS2 for m/z = 614 (Figure 3.24) is shown in Figure 3.25, and was further fragmented through MS3, shown in Figure 3.26. The proposed structures of the precursor ion, fragment ions, and the leaving groups are shown in Figure 3.33.

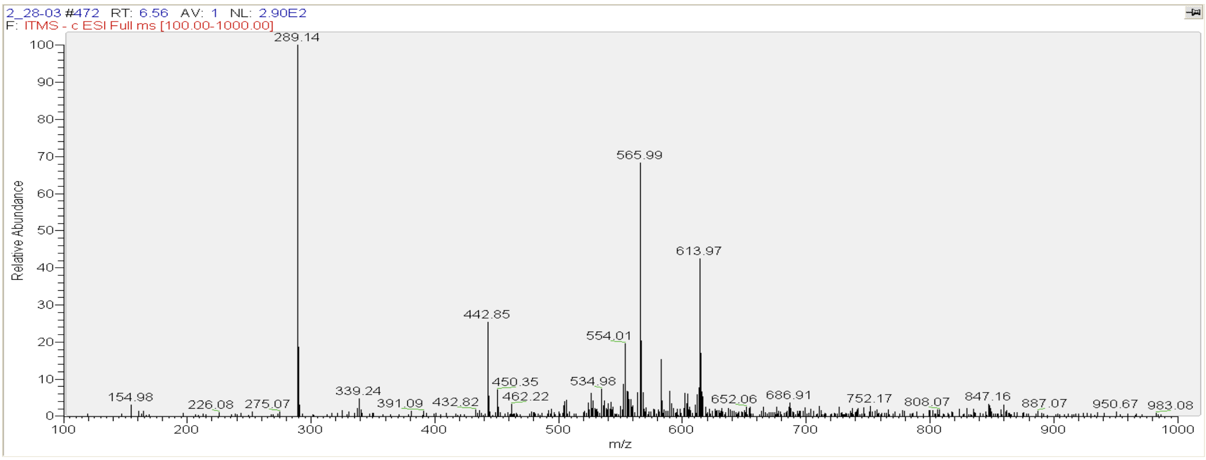


Figure 3.24 Precursor ion. Peak m/z = 614, RT = 6.56 min.

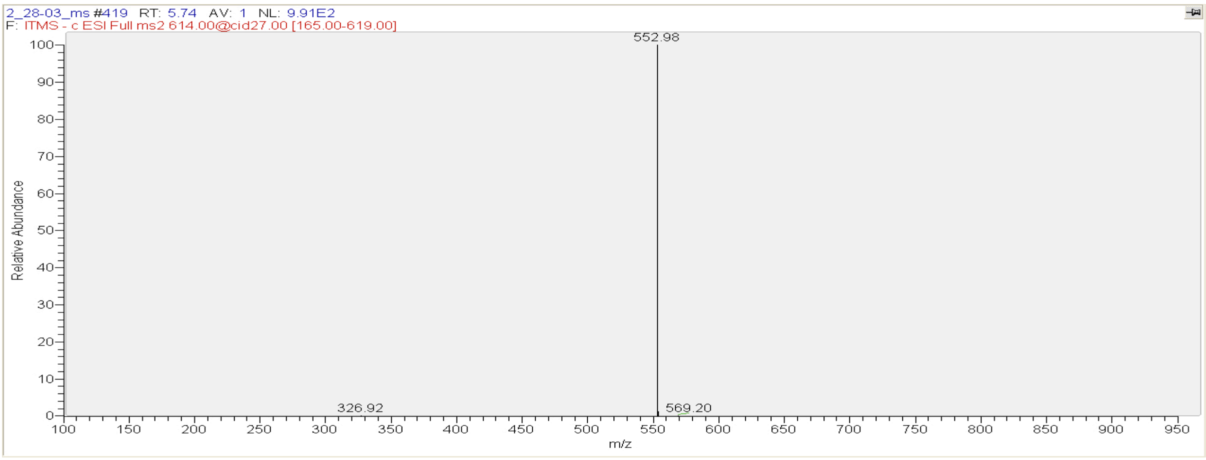


Figure 3.25 Fragment ion after the MS2. Peak m/z = 552.98, RT = 5.74 min.

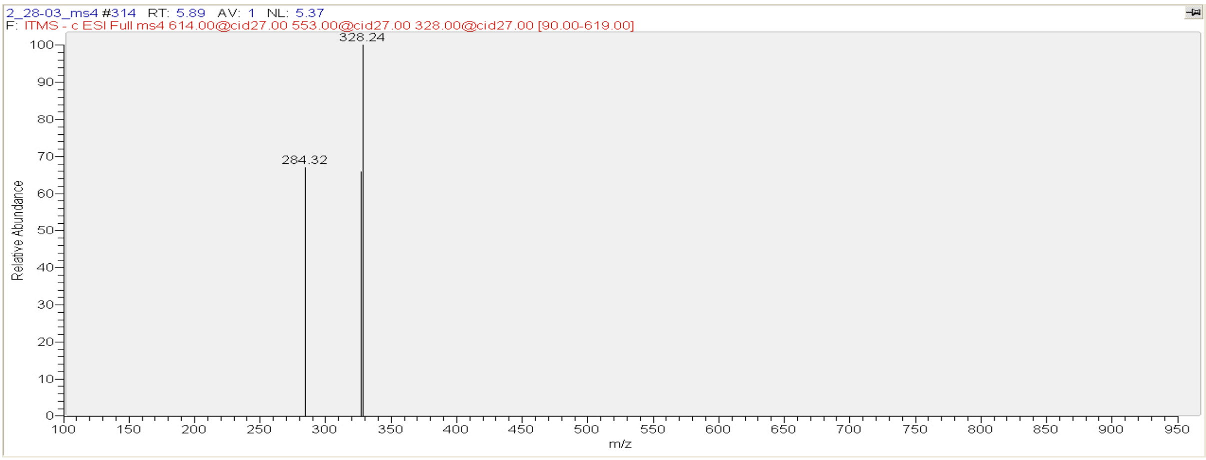


Figure 3.26 Fragment ion after the MS3. Peak m/z = 328.24, RT = 5.89 min.

3.2.2.5 Peak m/z = 568

The selected fragment ion from MS2 for m/z = 614 (Figure 3.27) is shown in Figure 3.28, and was further fragmented through MS3, shown in Figure 3.29. The proposed structures of the precursor ion, fragment ions, and the leaving groups are shown in Figure 3.34.

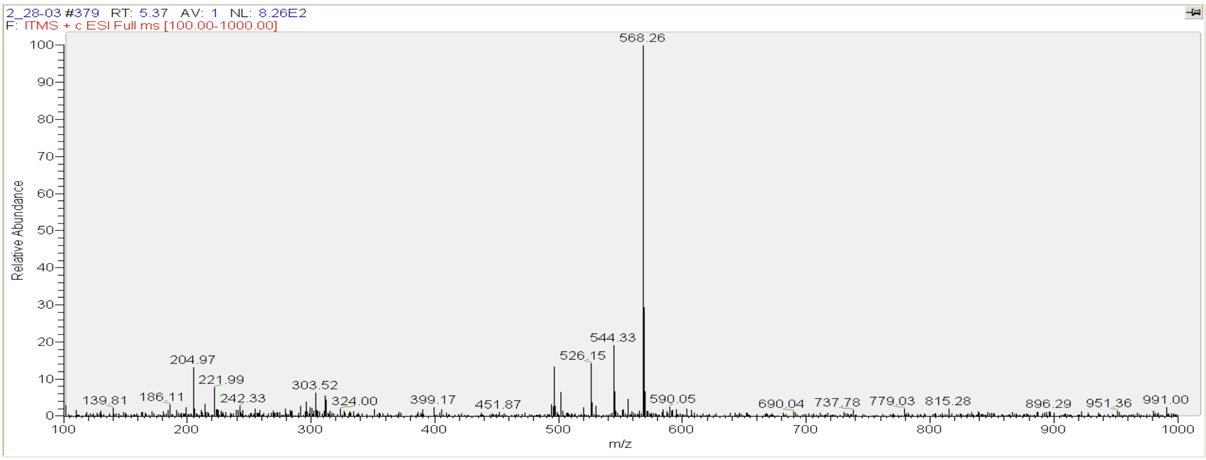


Figure 3.27 Precursor ion. Peak m/z = 568, RT = 5.37 min.

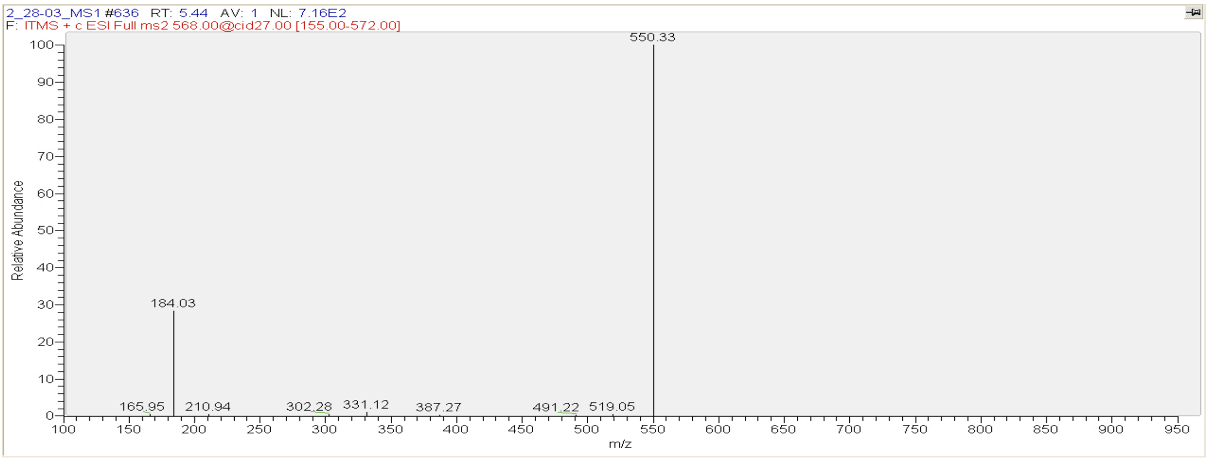


Figure 3.28 Fragment ion after the MS2. Peak m/z = 550.33, RT = 5.44 min.

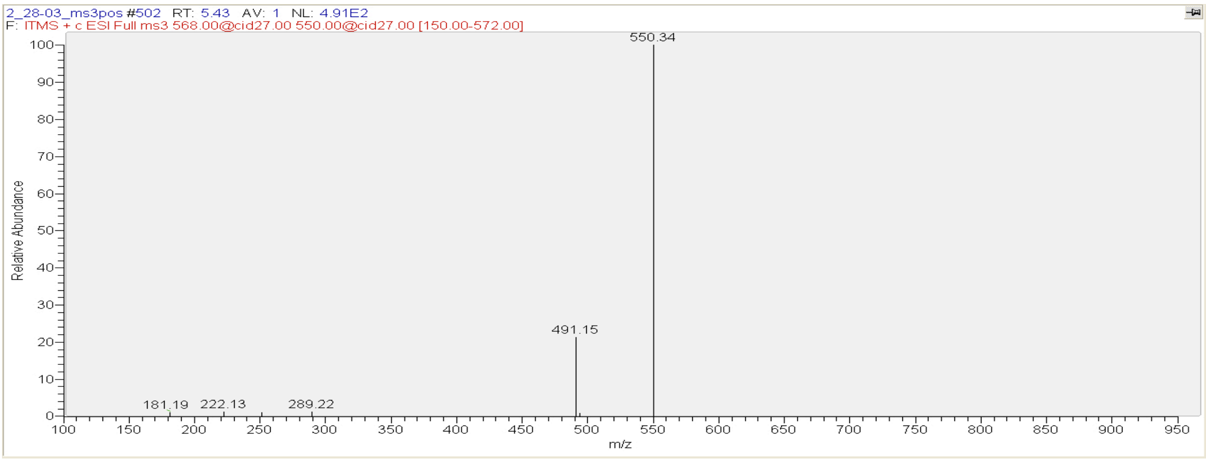
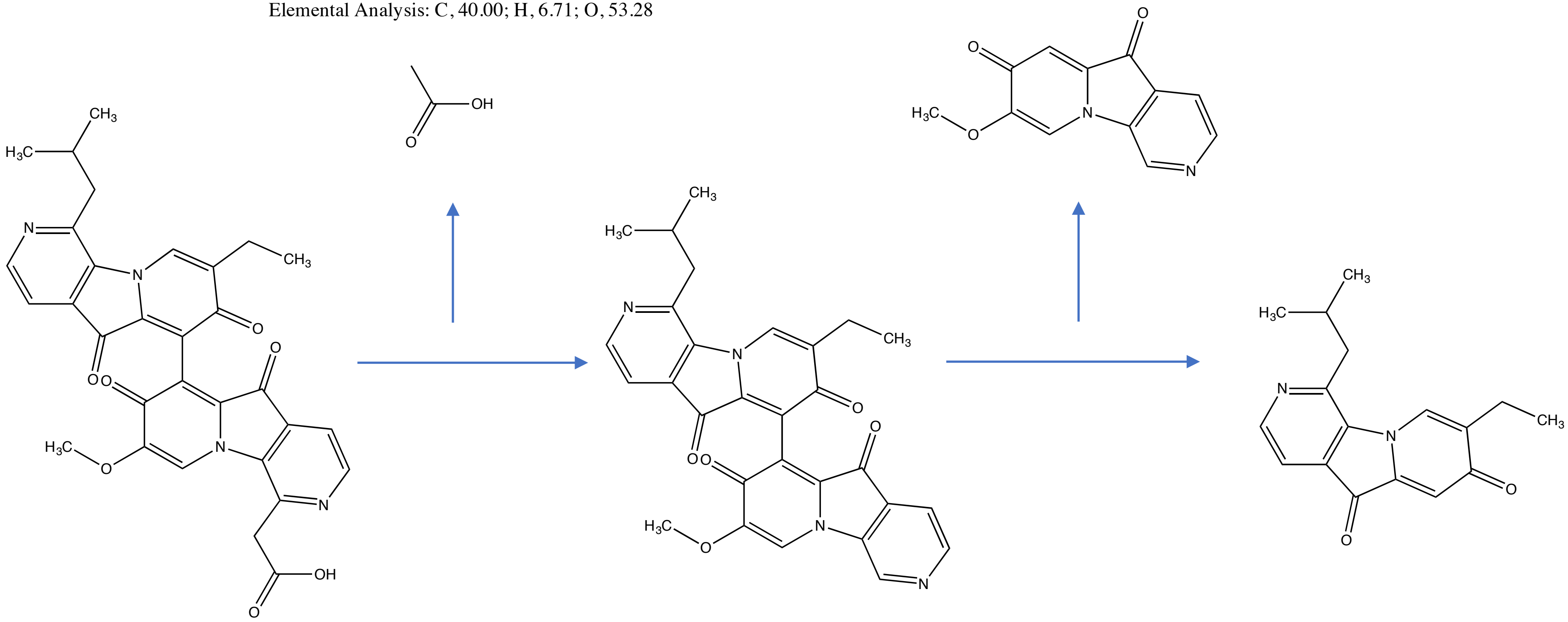


Figure 3.29 Fragment ion after the MS3. Peak m/z = 491.15, RT = 5.43 min.

Peak 566 (neg)

Leaving group: 60
Chemical Formula: $C_2H_4O_2$
Exact Mass: 60.02
Molecular Weight: 60.05
m/z: 60.02 (100.0%), 61.02 (2.2%)
Elemental Analysis: C, 40.00; H, 6.71; O, 53.28

Leaving group: 225
Chemical Formula: $C_{12}H_8N_2O_3$
Exact Mass: 228.0535
Molecular Weight: 228.2070
m/z: 228.0535 (100.0%), 229.0568 (13.0%)
Elemental Analysis: C, 63.16; H, 3.53; N, 12.28; O, 21.03



Chemical Formula: $C_{31}H_{26}N_4O_7$
Exact Mass: 566.1801
Molecular Weight: 566.5700
m/z: 566.1801 (100.0%), 567.1835 (33.5%), 568.1869 (2.7%), 568.1869 (2.7%), 567.1772 (1.5%), 568.1844 (1.4%)
Elemental Analysis: C, 65.72; H, 4.63; N, 9.89; O, 19.77

Chemical Formula: $C_{29}H_{24}N_4O_5$
Exact Mass: 508.1747
Molecular Weight: 508.5340
m/z: 508.1747 (100.0%), 509.1780 (31.4%), 510.1814 (2.7%), 510.1814 (2.0%), 509.1717 (1.5%), 510.1789 (1.0%)
Elemental Analysis: C, 68.49; H, 4.76; N, 11.02; O, 15.73

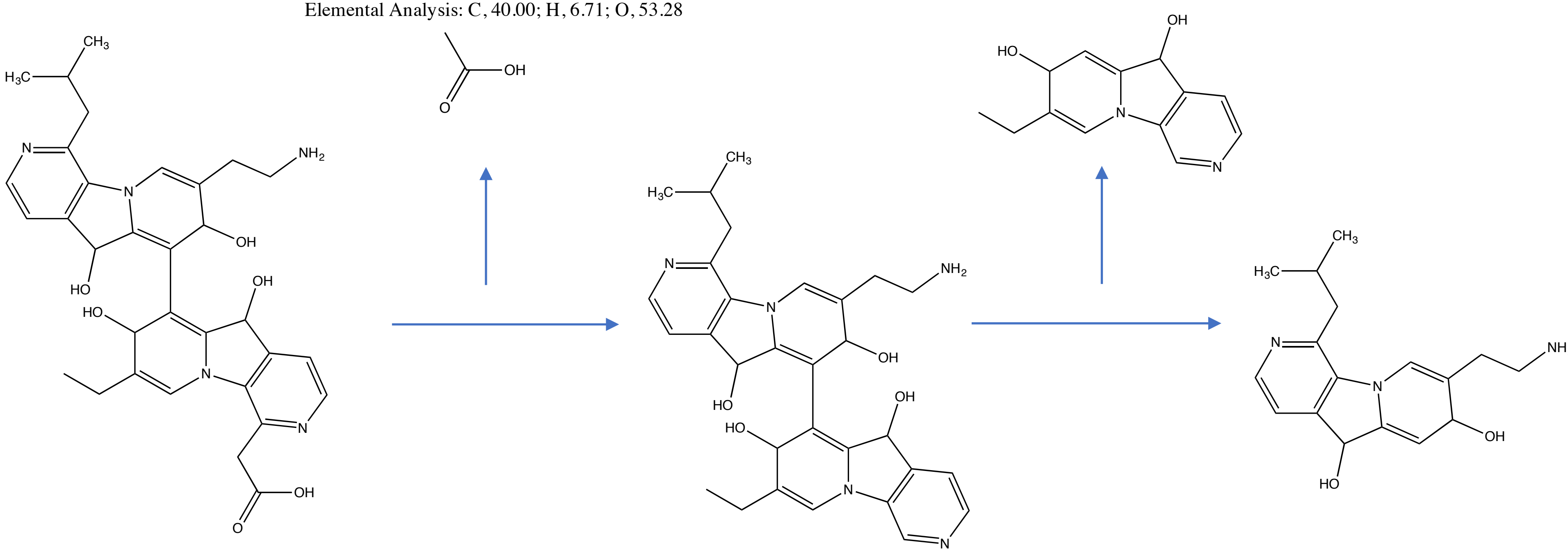
Chemical Formula: $C_{17}H_{18}N_2O_2$
Exact Mass: 282.1368
Molecular Weight: 282.3430
m/z: 282.1368 (100.0%), 283.1402 (18.4%), 284.1435 (1.6%)
Elemental Analysis: C, 72.32; H, 6.43; N, 9.92; O, 11.33

Figure 3.30 Proposed structure for peak m/z = 566 and its fragment ions and leaving groups.

Peak 586 (neg)

Leaving group: 60
Chemical Formula: $C_2H_4O_2$
Exact Mass: 60.02
Molecular Weight: 60.05
m/z: 60.02 (100.0%), 61.02 (2.2%)
Elemental Analysis: C, 40.00; H, 6.71; O, 53.28

Leaving group: 225
Chemical Formula: $C_{13}H_{14}N_2O_2$
Exact Mass: 230.1055
Molecular Weight: 230.2670
m/z: 230.1055 (100.0%), 231.1089 (14.1%)
Elemental Analysis: C, 67.81; H, 6.13; N, 12.17; O, 13.90



Chemical Formula: $C_{32}H_{37}N_5O_6$
Exact Mass: 587.2744
Molecular Weight: 587.6770
m/z: 587.2744 (100.0%), 588.2777 (34.6%), 589.2811 (5.8%), 588.2714 (1.8%), 589.2786 (1.2%)
Elemental Analysis: C, 65.40; H, 6.35; N, 11.92; O, 16.33

Chemical Formula: $C_{30}H_{35}N_5O_4$
Exact Mass: 529.2689
Molecular Weight: 529.6410
m/z: 529.2689 (100.0%), 530.2723 (32.4%), 531.2756 (2.7%), 531.2756 (2.4%), 530.2659 (1.8%)
Elemental Analysis: C, 68.03; H, 6.66; N, 13.22; O, 12.08

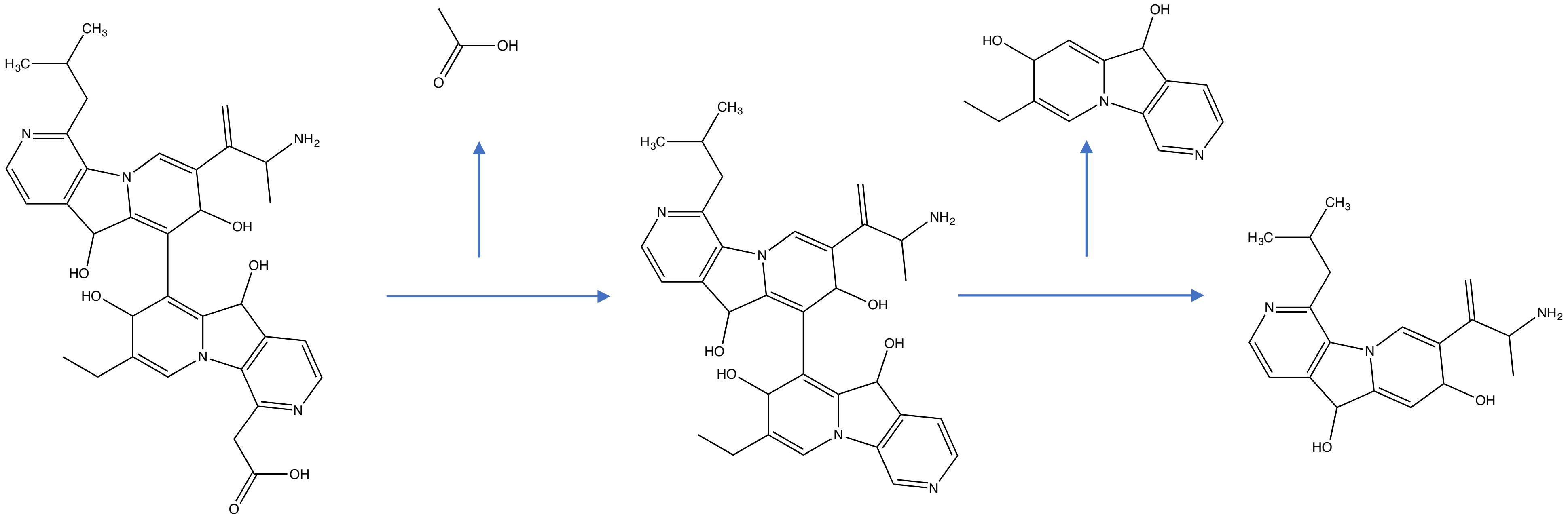
Chemical Formula: $C_{17}H_{23}N_3O_2$
Exact Mass: 301.1790
Molecular Weight: 301.3900
m/z: 301.1790 (100.0%), 302.1824 (18.4%), 303.1857 (1.6%), 302.1761 (1.1%)
Elemental Analysis: C, 67.75; H, 7.69; N, 13.94; O, 10.62

Figure 3.31 Proposed structure for peak m/z = 586 and its fragment ions and leaving groups.

Peak 612 (neg)

Leaving group: 60
Chemical Formula: $C_2H_4O_2$
Exact Mass: 60.02
Molecular Weight: 60.05
m/z: 60.02 (100.0%), 61.02 (2.2%)
Elemental Analysis: C, 40.00; H, 6.71; O, 53.28

Leaving group: 225
Chemical Formula: $C_{13}H_{14}N_2O_2$
Exact Mass: 230.1055
Molecular Weight: 230.2670
m/z: 230.1055 (100.0%), 231.1089 (14.1%)
Elemental Analysis: C, 67.81; H, 6.13; N, 12.17; O, 13.90



Chemical Formula: $C_{34}H_{39}N_5O_6$
Exact Mass: 613.2900
Molecular Weight: 613.7150
m/z: 613.2900 (100.0%), 614.2934 (36.8%), 615.2967 (6.6%), 614.2871 (1.8%), 615.2943 (1.2%)
Elemental Analysis: C, 66.54; H, 6.41; N, 11.41; O, 15.64

Chemical Formula: $C_{32}H_{37}N_5O_4$
Exact Mass: 555.2846
Molecular Weight: 555.6790
m/z: 555.2846 (100.0%), 556.2879 (34.6%), 557.2913 (5.8%), 556.2816 (1.8%)
Elemental Analysis: C, 69.17; H, 6.71; N, 12.60; O, 11.52

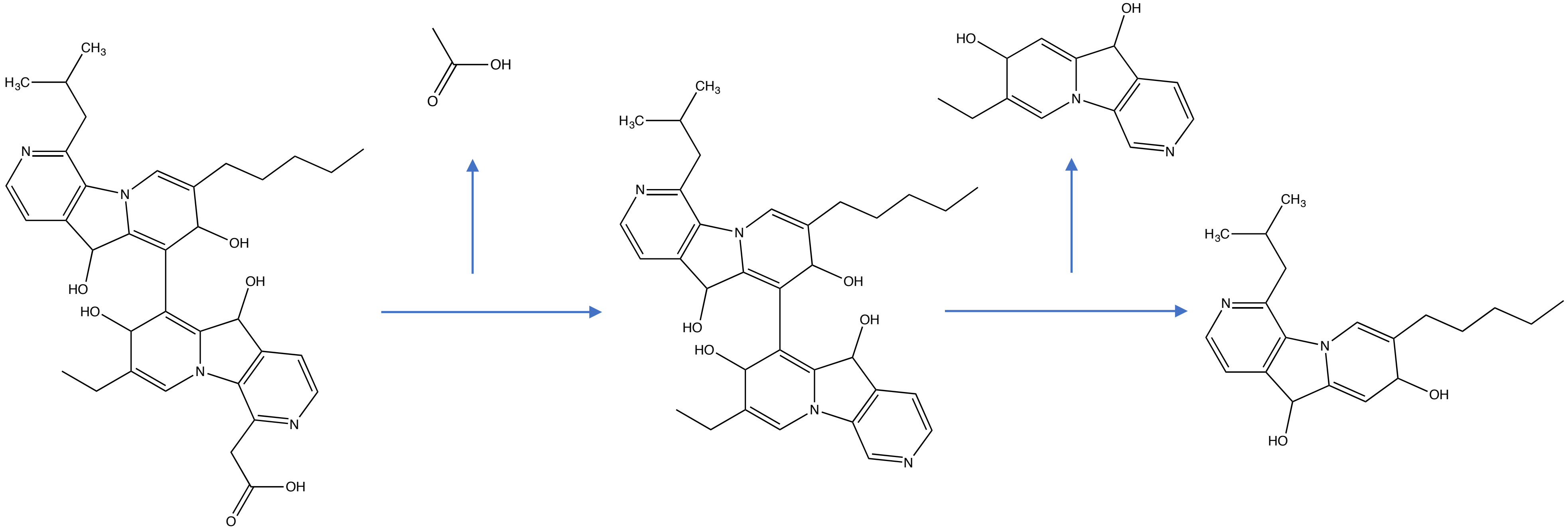
Chemical Formula: $C_{19}H_{25}N_3O_2$
Exact Mass: 327.1947
Molecular Weight: 327.4280
m/z: 327.1947 (100.0%), 328.1980 (20.5%), 329.2014 (2.0%), 328.1917 (1.1%)
Elemental Analysis: C, 69.70; H, 7.70; N, 12.83; O, 9.77

Figure 3.32 Proposed structure for peak m/z = 612 and its fragment ions and leaving groups.

Peak 614 (neg)

Leaving group: 60
Chemical Formula: $C_2H_4O_2$
Exact Mass: 60.02
Molecular Weight: 60.05
m/z: 60.02 (100.0%), 61.02 (2.2%)
Elemental Analysis: C, 40.00; H, 6.71; O, 53.28

Leaving group: 225
Chemical Formula: $C_{13}H_{14}N_2O_2$
Exact Mass: 230.1055
Molecular Weight: 230.2670
m/z: 230.1055 (100.0%), 231.1089 (14.1%)
Elemental Analysis: C, 67.81; H, 6.13; N, 12.17; O, 13.90



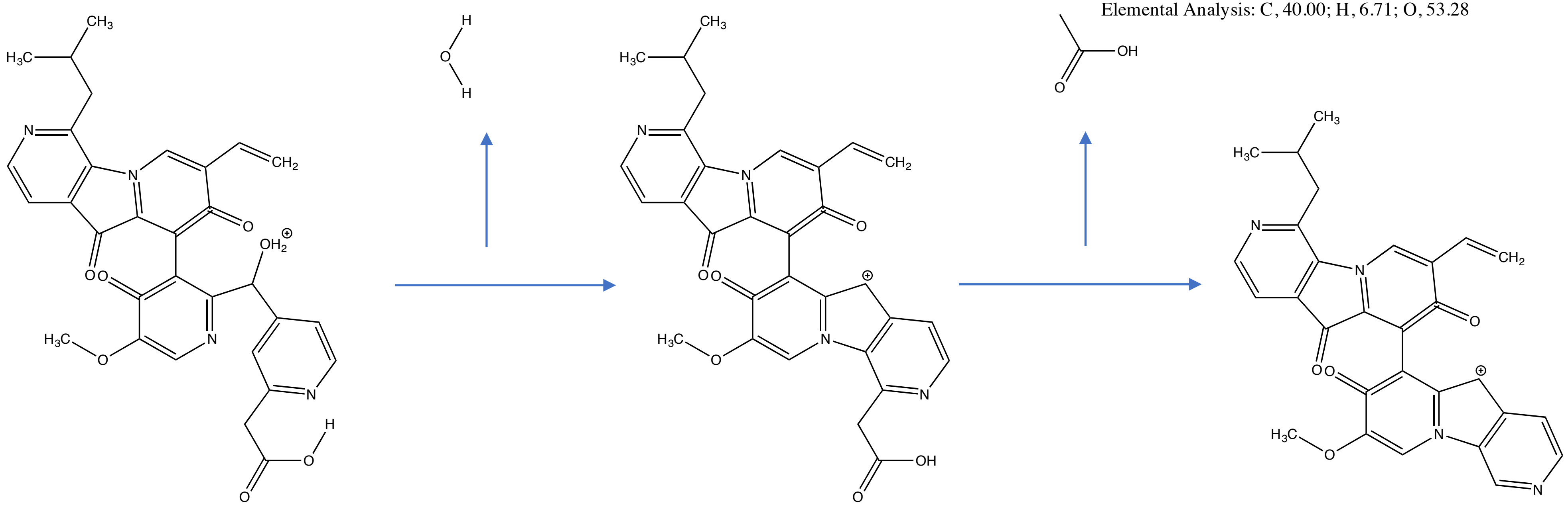
Chemical Formula: $C_{35}H_{42}N_4O_6$
Exact Mass: 614.3104
Molecular Weight: 614.7430
m/z: 614.3104 (100.0%), 615.3138 (37.9%), 616.3171 (7.0%), 615.3075 (1.5%), 616.3147 (1.2%)
Elemental Analysis: C, 68.38; H, 6.89; N, 9.11; O, 15.62

Chemical Formula: $C_{33}H_{40}N_4O_4$
Exact Mass: 556.3050
Molecular Weight: 556.7070
m/z: 556.3050 (100.0%), 557.3083 (35.7%), 558.3117 (3.5%), 558.3117 (2.7%), 557.3020 (1.5%)
Elemental Analysis: C, 71.20; H, 7.24; N, 10.06; O, 11.50

Chemical Formula: $C_{20}H_{28}N_2O_2$
Exact Mass: 328.2151
Molecular Weight: 328.4560
m/z: 328.2151 (100.0%), 329.2184 (21.6%), 330.2218 (2.2%)
Elemental Analysis: C, 73.14; H, 8.59; N, 8.53; O, 9.74

Figure 3.33 Proposed structure for peak m/z = 614 and its fragment ions and leaving groups.

Peak 568 (pos)



Leaving group: 18

Leaving group: 59
Chemical Formula: $C_2H_4O_2$
Exact Mass: 60.02
Molecular Weight: 60.05
m/z: 60.02 (100.0%), 61.02 (2.2%)
Elemental Analysis: C, 40.00; H, 6.71; O, 53.28

Chemical Formula: $C_{31}H_{28}N_4O_7^+$
Exact Mass: 568.20
Molecular Weight: 568.59
m/z: 568.20 (100.0%), 569.20 (33.5%), 570.20 (2.7%), 570.20 (2.7%), 569.19 (1.5%), 570.20 (1.4%)
Elemental Analysis: C, 65.49; H, 4.96; N, 9.85; O, 19.70

Chemical Formula: $C_{31}H_{25}N_4O_6^+$
Exact Mass: 549.18
Molecular Weight: 549.56
m/z: 549.18 (100.0%), 550.18 (33.5%), 551.18 (5.4%), 550.17 (1.5%), 551.18 (1.2%)
Elemental Analysis: C, 67.75; H, 4.59; N, 10.20; O, 17.47

Chemical Formula: $C_{29}H_{23}N_4O_4^+$
Exact Mass: 491.17
Molecular Weight: 491.53
m/z: 491.17 (100.0%), 492.17 (31.4%), 493.18 (2.7%), 493.18 (2.0%), 492.17 (1.5%)
Elemental Analysis: C, 70.86; H, 4.72; N, 11.40; O, 13.02

Figure 3.34 Proposed structure for peak m/z = 566 and its fragment ions and leaving groups.

4. CONCLUSIONS

This study aimed to identify and isolate secondary metabolites from the bryozoan Cryptosula zavjalovensis from Hokkaido, Japan. There have been no previous studies on the natural products from this bryozoan species and these compounds are of interest for their bioactivity. Specifically, it aimed to determine if these compounds are bioactive and if this bioactivity might translate into medicinal and pharmaceutical applications through cytotoxicity testing.

From these objectives, it can be concluded from this study that:

Secondary metabolites similar to that of pterocellin E have been identified. The elucidation of the structure of these compounds, however, are still at the early stages using tandem mass spectrometry and are thus not definitive. The structures proposed are based merely on the similarities of the properties of the identified compounds and that of pterocellin E. Further tests that can be used to elucidate the structure include Infrared (IR) spectroscopy and carbon-13 nuclear magnetic resonance (^{13}C NMR) to identify functional groups and proton nuclear magnetic resonance (H NMR) to identify molecular fragments.

Compounds extracted from *Cryptosula zavjalovensis* are bioactive. The cytotoxicity screening showed that the bioactive compounds are contained in the organic extract, specifically in fractions E2 and E3. At a concentration of 1 µg/mL fraction E3 exhibited 58% cytotoxicity while at a concentration of 10 µg/mL fraction E2 exhibited 79% cytotoxicity whereas E3 exhibited 107% cytotoxicity against MCF-7 breast cancer cells. In order to better understand the bioactivity and cytotoxicity of these extracts, it is suggested that further cytotoxicity tests should be done on other cell lines.

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