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Bioassay Guided Fractionation for the Isolation of Cytotoxic Compounds from a Caribbean Marine Sponge

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Abbreviations

BSLA	Brine shrimp lethality assay
CBD	Convention on Biological Diversity
CH_2Cl_2	Dichloromethane
CONAGEBIO	Comisión Nacional para la Gestión de la Biodiversidad (National Commission of Biodiversity Management)
DMSO	Dimethyl sulfoxide
ELSD	Evaporative light scattering detector
EMA	European Medicines Agency
EtOH	Ethanol
FDA	Food and Drug Administration
HPLC	High-performance liquid chromatography
INBio	Instituto Nacional de Biodiversidad (National Biodiversity Insitute)
LC_{50}	Median lethal concentration
MDR	Multiple drug resistance
MeOH	Methanol
NCI	National Cancer Institute
ND	No detection
NER	Nucleotide excision repair
P-gp	P-glycoprotein
R_f	Retention factor
SPE	Solid phase extraction
TLC	Thin layer chromatography
VEGF	Vascular endothelial growth factor
VLC	Vacuum liquid chromatography

Table of contents

1. INTRODUCTION

1.1. Porifera

Sponges (phylum Porifera) represent a significant component of benthic communities worldwide. They populate mainly marine habitats, but are also found in freshwater (Taylor et al. 2007). Sponges are ancient metazoans, whose origins date back to the Precambrian more than 600 million years ago (Hentschel et al. 2002). So far, approximately 15,000 species of sponges have been described, but their true diversity may be even higher. They populate the tropical oceans in great abundance and also inhabit temperate waters, from intertidal zones to the deep ocean (Thomas et al. 2010). Unpolluted littoral habitats harbor especially rich sponge faunas. Most littoral sponges are encrusting and form layers on hard surfaces, whereas benthic sponges, living on soft substrata, are upright and tall (Brusca & Brusca 1990). Only about 1% of the species live in freshwater (Belarbi et al. 2003).

Sponges are simple multicellular invertebrates that lack true tissues or organs. Main characteristics which define sponges are their unique aquiferous system and the highly totipotent nature of sponge cells (Brusca & Brusca 1990). The phylum Porifera consists of three classes, namely the Calcarea, Hexactinellida and Demospongiae. Sponge classification is mainly based on composition, size and shape of skeletal elements. The Calcarea have calciferous sclerites and occur predominantly in shallow waters. The skeleton of the Hexactinellida is characterized by siliceous spicules. Members of this class usually live in the deep sea. The Demospongiae, whose skeleton is composed of siliceous spicules and fibers of the protein spongin, constitute with approximately 85% the largest class in the phylum Porifera. Demospongiae are found from intertidal zones to the deepest seas (Hooper & Van Soest 2002).

Sponges are sessile filter feeders, which pump large volumes of water through a specialized canal system and ingest organic particles such as algae and bacteria from the seawater. The expelled water is left essentially sterile (Fieseler et al. 2004). Sponges are hosts for a wide variety of microorganisms such as bacteria, fungi and microalgae, which can make up more than half of the biomass of the sponge host. The role of these associated microbes varies from source of nutrition to mutualistic symbiosis. Since

sponges are sessile organisms which lack physical protection, they have developed potent chemical defense mechanisms during evolution to protect themselves from predators, competitors and infectious microorganisms (Thomas et al. 2010). Sponges produce bioactive secondary metabolites, which are structurally highly diverse and of great interest for the development of new drugs. They are well known for their cytotoxic properties in general, further, they show specific antitumor, antiviral, and antimicrobial activities. Among marine invertebrates, sponges are the richest source for novel pharmacologically active compounds (Hentschel & Bringmann 2010; Thomas et al. 2010; Wang 2006).

1.1.1. Anatomy and morphology

Sponges are among the oldest of the multicellular animals and show little differentiation and coordination of tissues. However, sponge morphology is very diverse. There are encrusting, branching, cup-shaped and massive types with a wide range of colors. Their size varies from a few millimeters to more than a meter in diameter (Taylor et al. 2007).

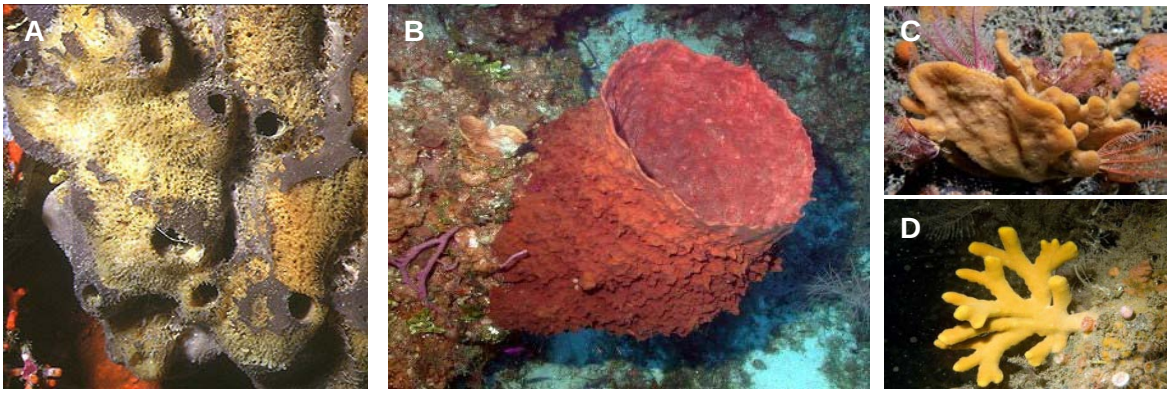


Figure 1: Diversity of marine sponges in size, shape and color. The common bath sponge *Spongia officinalis* (A), the giant barrel sponge *Xestospongia muta* (B), the branching sponge *Axinella dissimilis* (C and D). (Source: *Encyclopedia of Life*; www.eol.org)

Environmental factors such as water current and depth exert a great influence on size and shape of the sponges, and coloration can be due to symbiotic bacteria or unicellular algae (Brusca & Brusca 1990).

Cells of sponges are mostly totipotent, retaining a high degree of mobility and capable of changing form and function. Although sponges are large-bodied multicellular animals, they share many functional characteristics with unicellular organisms, for instance in terms of nutrition, gas exchange and reproduction. The main feature of sponges, beside the highly totipotent nature of their cells, is the aquiferous system, an arrangement of water channels traversing the body (Brusca & Brusca 1990). Specialized flagellated cells, called choanocytes, are arranged as single-layered epithelium lining the internal surface. Choanocytes pump a unidirectional water current through the sponge's body. The outer surface is constituted by epithelial cells called pinacocytes. Individual cells of this layer form pores (ostia), through which water passes from the exterior to the choanocyte-lined channels. Water is excreted through an apical opening called osculum. Between the internal flagellated epithelium (the choanoderm) and the external epithelium (the pinacoderm) lies the mesohyl, a greatly variable region which represents the connective tissue (Bergquist 1998).

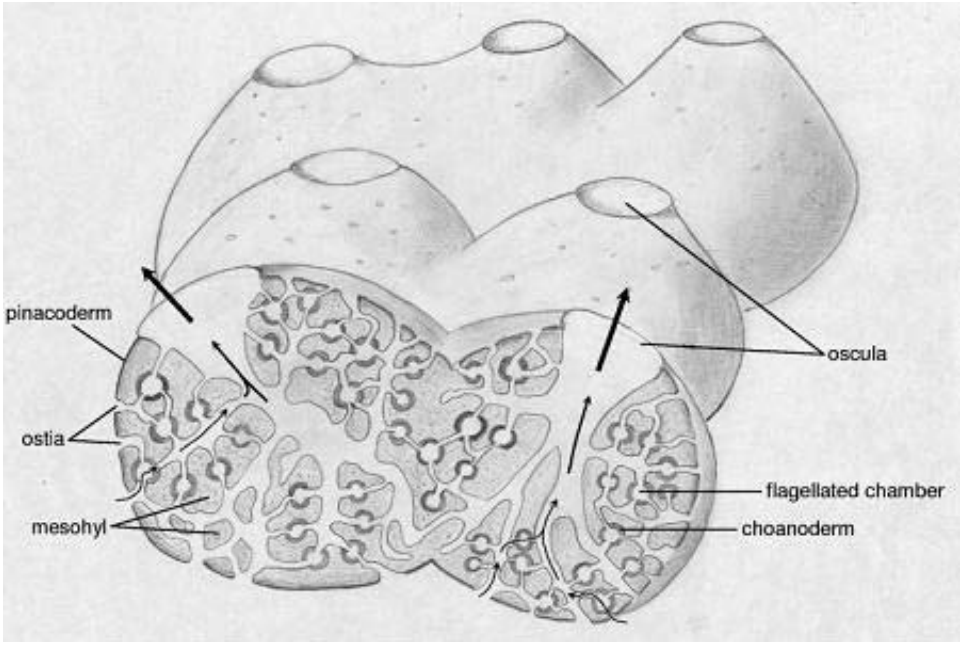


Figure 2: Schematic diagram of a sponge. Arrows indicate the direction of water flow through the sponge. Image used with kind permission from Taylor et al. (2007).

The mesohyl consists of a collagenous matrix and contains mobile phagocytic and secretory cells, as well as, in most cases, mineral skeletal elements (spicules). Spicules are secreted by sclerocytes and are composed of either hydrated silica or calcium carbonate. Special cells, called spongocytes, secrete fibrous collagen referred to as spongin, which supports the mineral skeleton. In the well-known bath sponges, spongin fibers exclusively form elaborate strong skeletons (Bergquist 1998).

The mesohyl ranges from thin to quite thick, and beside secretion of the skeleton it plays vital roles in digestion, gamete production, and transport of nutrients and waste products. With increasing mesohyl volume, the epithelial layers can increase their folding. A simple and continuous choanoderm, which is called asconoid condition, is found in some calcareous sponges. The syconoid condition is generated by simple folding of the pinacoderm and choanoderm. A greatly subdivided choanoderm into separate flagellated chambers produces the leuconoid condition. All members of the class Demospongiae, hence the majority of all sponges, show the complex leuconoid organization. In the mesohyl a great number of cell types may be found; most of them are able to change from one type to another as required. Large, highly motile cells called archaeocytes play a major role in digestion and food transport. These amoeboid cells - capable of phagocytosis - possess a variety of digestive enzymes (Brusca & Brusca 1990).

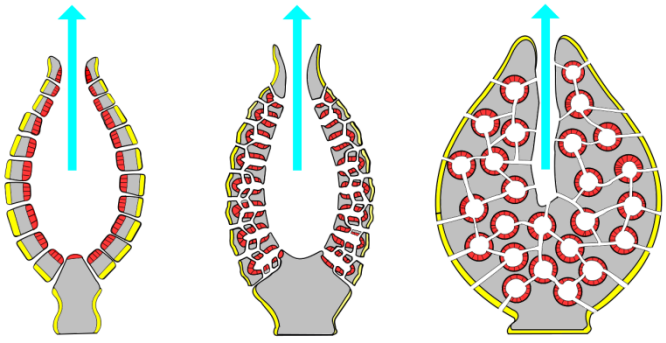


Figure 3: Porifera body structures. Yellow: pinacocytes; red: choanocytes; grey: mesohyl; blue arrow: water flow. Source: *Invertebrate Zoology* (Ruppert et al. 2004).

1.1.1.1. Feeding

Sponges are size-selective particle feeders and rely, unlike most metazoan, solely on intracellular digestion. They ingest particles such as organic molecules, bacteria and unicellular algae via phagocytosis and pinocytosis from the large volumes of water which is pumped through the body (Brusca & Brusca 1990). Some deep-sea sponges adapted to environments with a low particle concentration and still water conditions via developing a mechanism called carnivory. Carnivorous sponges show a fundamentally different body plan and are able to capture and digest swimming prey such as small crustaceans (Vacelet & Boury-Esnault 1995).

1.1.1.2. Reproduction

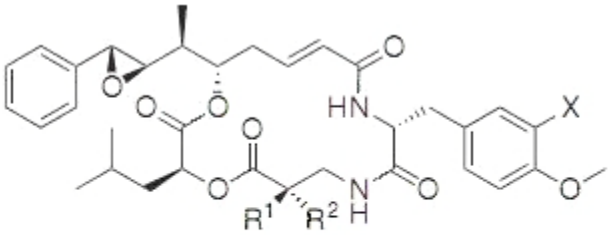
Sponges are capable of a variety of sexual and asexual reproduction strategies. With few exceptions, sponges are hermaphrodites. The majority is viviparous, releasing larvae; some species are oviparous and release zygotes into the water. Sponges do not feature gonads; sperms arise from choanocytes and occur in simple clusters, and eggs are formed from archaeocytes or choanocytes. Sperms are released from the osculum, and when they get into the aquiferous system of another sponge, they are phagocytosed by a choanocyte. The choanocyte then becomes amoeboid and transports the sperm to an egg. The carrier cell and the egg fuse with each other, and the sperm nucleus is transferred. Asexual processes of sponges include fragmentation, budding, and the formation of overwintering propagules called gemmules. Fragmentation primarily results from damage; a dislodged fragment may re-organize itself into a functional sponge. Budding is uncommon, but occurs in different manners in some species. The formation of gemmules happens in the mesohyl of a dying sponge from a cluster of nutrient-laden archaeocytes, which is encapsulated with a spongin shell. When environmental conditions permit, hatching takes place and a functional sponge is reconstituted from archaeocytes (Ruppert et al. 2004).

1.1.2. Associated microorganisms

Sponges are well known to harbor a large community of microorganisms, which can constitute up to 60% of the sponge tissue volume (Usher et al. 2004), exceeding the number of bacteria in seawater by two to three orders of magnitude (Friedrich et al. 2001). The bacterial load in well-irrigated sponges is generally lower than in sponges with a poor irrigation system (Wilkinson 1978). Sponges with numerous associated bacteria (referred to as 'high-microbial abundance sponges' or 'bacteriosponges') are massive with a high tissue density, and usually larger than their low-microbial abundance counterparts. The microbe population consists mainly of extracellular bacteria which are embedded in the mesohyl matrix, but in some Demospongiae bacteria are also found intracellularly (Vacelet & Donadey 1977; Hentschel et al. 2006). The microbial distribution within a typical sponge follows a general pattern. The outer, light-exposed layers are populated by photosynthetically active microorganisms such as cyanobacteria, which provide the sponge with nutrients and cause coloration of the tissue. Heterotrophic and probably also autotrophic bacteria are located in the internal regions (Wang 2006). Sponge-associated bacterial phyla are for example *Acidobacteria, Chloroflexi, Actinobacteria, Proteobacteria, Nitrospira, Bacteroidetes*, and the sponge-specific *Poribacteria* (Fieseler et al. 2004). Also eukaryotic microbes such as dinoflagellates, diatoms, microalgae, and fungi occur in sponges (Taylor et al. 2007).

The nature of sponge-microbe interactions varies from pathogenesis and parasitism (sometimes leading to sponge death) to microbes as the major food source (with the exception of phototrophic sponges), and to mutualistic or at least commensalistic associations. Sponges benefit greatly from the metabolic activities of their microbial partners. Cyanobacteria provide photosynthates and fixed nitrogen, and supply some phototrophic sponges commonly found on tropical reefs with more than 50% of their energy requirements. Sponges may also profit from nitrifying and sulfur-metabolizing microorganisms (Taylor et al. 2007). Further presumable benefits include stabilization of the sponge skeleton, processing of metabolic waste, and production of secondary metabolites (Hentschel et al. 2002).

Many sponge-derived bioactive compounds are suspected to be produced by symbiotic microorganisms (Thomas et al. 2010; Piel 2009; Wang 2006), since they show a high structural similarity to microbial natural products, e.g. complex polyketides or non-ribosomal peptides from sponges and microorganisms exhibiting only slight differences in the substitution patterns (Piel 2004). A striking example is the cryptophycin family, which comprises a group of cytotoxic compounds initially isolated from bacteria. Cryptophycin-1 was first isolated in 1990 from the cyanobacteria *Nostoc* spp. strain ATCC 53789, and re-isolated in 1994 along with several analogues from another *Nostoc* spp. strain. The cytotoxic compound arenastatin A, isolated from the marine sponge *Dysidea arenaria*, was reported contemporaneously, and turned out to be identical to cryptophycin-24 (Molinski et al. 2009).



1: R^1 = Me, R^2 = H, X = Cl Cryptophycin-1
2: R^1 = R^2 = X = H Arenastatin A/Crypt.-24
3: R^1 = R^2 = Me, X = Cl Cryptophycin-52

Figure 4: Chemical structure of cryptophycins.

1.2. Drug development from marine natural products

For thousands of years, natural products have been playing an important role for treatment of human diseases all over the world. Plants have formed the basis of traditional medicine systems; the first records, written on clay tablets, are from Mesopotamia and date from about 2600 years before Christ. Well-known medicinal plants like *Papaver somniferum* (opium poppy) or *Glycyrrhiza glabra* (licorice) and oils of *Cedrus* species (cedar) and *Cupressus sempevirens* (cypress) were already used back then (Newman et al. 2000). Natural product-derived drugs were mainly obtained from terrestrial plants prior to the discovery of penicillin by Fleming in 1928 and its development in the early 1940's. The successful treatment of infections with β-lactams produced by fungi prompted intensive research in new antibiotics from microorganisms. Terrestrial bacteria and fungi are a plentiful source of structurally diverse bioactive substances. Today, therapeutic applications of metabolites from microorganisms and their derivates include treatment of infections (e.g. penicillins, cephalosporins, aminoglycosides, tetracyclins and many others), immunosuppression (e.g. cyclosporins and rapamycin) and anticancer therapy (e.g. pentostatin and epirubicin). Also cholesterol lowering agents like lovastatin and mevastatin, and the antidiabetic drug acarbose derive from microorganisms (Chin et al. 2006).

Marine organisms have a shorter history of utilization in the treatment of human disease. Approximately 60 years ago, the interest in marine natural products has increased. This was mainly due to refinements in technologies, e.g. scuba diving, which allowed the collection of source organisms. Investigations of marine natural products started with the pioneering work of Werner Bergmann (Molinski et al. 2009). In the early 1950s, Bergmann and co-workers reported on the bioactive nucleosides spongothymidine and spongouridine, which they had isolated from marine sponges (Bergmann & Feeney 1951; Bergmann & Burke 1955). These compounds led to the development of the anticancer agent cytarabine (Ara-C) and the antiviral compound vidarabine (Ara-A) and could be considered as the prototypes for all the modified nucleoside analogues that have been synthesized as antiviral and antitumor agents since that time (Newman et al. 2000). Cytarabine received approval by the US Food and Drug Administration (FDA) in 1969 and vidarabine in 1976. It has taken three decades for another marine-derived natural product to launch onto the market. The analgesic ziconotide (Prialt®), a synthetic equivalent of the

peptide ω-conotoxin MVIIA from the marine snail *Conus magnus* gained approval in 2004. Currently, it is labeled for the treatment of severe chronic pain in patients with cancer or AIDS. The anticancer agent trabectedin (Yondelis®), an alkaloid isolated from the tunicate *Ecteinascidia turbinata*, has been approved in the European Union in 2007 for patients with soft tissue sarcoma or relapsed platinum-sensitive ovarian cancer (Mayer et al. 2010). Finally, in November 2010, the anticancer agent eribulin mesylate has been approved by the FDA for the treatment of metastatic breast cancer. Eribulin mesylate is a synthetic analogue of halichondrin B, a polyether macrolide originally isolated from the marine sponge *Halichondria okadai* 25 years ago (Huyck et al. 2011). Approval from the European Medicines Agency (EMA) for eribulin was received in March 2011 (Eisai Co., Ltd. 2011).

One of the major reasons why the field of drug development from marine natural products evolved slowly, was – and still is – the problem of sustainable supply with compounds isolated from source organisms for *in vitro* and *in vivo* studies. The amounts of bioactive substances, which can be recovered, are in general exceedingly small. Levels of 1 mg of compound per 3 kg of biomass are not uncommon (Newman & Cragg 2004). The isolation and purification of the active principles from the complex matrix of natural products remains, despite great advances in separation technology such as preparative high-performance liquid chromatography (HPLC), often rate limiting. Due to the high structural complexity of the compounds with various chiral centers, synthesis is in many cases impractical. The problem of yielding sufficient quantities of pure material required for medicinal chemistry and development phases is particularly crucial for source organisms that have not been productively cultured, as is the case with marine sponges (Koehn & Carter 2005).

Due to the difficulties associated with the development of drugs from natural sources, many pharmaceutical companies appreciated the era of combinatorial chemistry, neglecting the use of natural products in drug discovery in favor of high-throughput synthesis of large compound libraries (Paterson & Anderson 2005). An additional factor for decreased emphasis of research into natural products during the past two decades was the emergence of possible uncertainties with regard to collection of biomaterials as a consequence of the Convention on Biological Diversity (CBD) (Koehn & Carter 2005). The CBD was adopted in 1992; its principal objectives are conservation of biological resources, their sustainable use, and fair sharing of benefits arising out of their utilization. Article 15 of the CBD sets out, that access to genetic resources is subject to the prior informed consent

of the country where such resources are located, and to mutually agreed terms regarding the sharing of benefits (Garrity et al. 2009).

Despite concerns about property rights and supply problems, almost half of the drugs approved since 1994 are based on natural products, and the interest in applying natural chemical diversity to drug discovery seems to be increasing again over the past years (Harvey 2008). This is partly based on the realization that modern technologies like combinatorial chemistry failed to provide new lead compounds in significant numbers, and partly due to new developments in analytical technology, spectroscopy and high-throughput-screening, which made research in natural products more compatible to the requirements of the pharmaceutical companies once again (Molinski et al. 2009).

The oceans, covering approximately 70% of the Earth's surface, harbor an exceedingly high biodiversity whose exploration has only begun relatively recently, but already yielded more than 18,000 active compounds. Marine sponges are the richest source of novel natural agents from the sea, providing lead compounds against a wide range of human diseases. Due to large-scale screening programs of the US National Cancer Institute (NCI), research in antitumor agents has made the greatest progress, but also many compounds with properties such as anti-inflammatory, antiviral, antibacterial, and antifungal activity have been detected from sponges (Hentschel & Bringmann 2010). Other resources of marine natural products are microorganisms and phytoplankton, algae, cnidarians, bryozoans, mollusks, tunicates, echinoderms and intertidal plants (Blunt et al. 2011).

The following description of approved marine-derived drugs and some promising agents in development demonstrates on the one hand the great potential of marine organisms to provide new leads for drug development, on the other hand it points out the difficulties and in some cases extremely long duration of the progress from discovery to introduction on the market.

1.2.1. Approved marine-derived drugs

1.2.1.1. Antimetabolites: Cytarabine and vidarabine

Cytarabine (cytosine arabinoside, Ara-C), an anticancer agent currently available as either conventional cytarabine or liposomal formulation, was approved by the FDA in 1969. The synthetic pyrimidine nucleoside was developed from spongothymidine, an arabinose-containing nucleoside originally isolated from the marine sponge *Tethya crypta* (Mayer et al. 2010). With the discovery of spongothymidine and spongouridine in the early 1950s by Bergmann and co-workers (Bergmann & Feeney 1951; Bergmann & Burke 1955), it was demonstrated for the first time that nucleosides using other sugars than ribose or deoxyribose naturally occur, and it was realized that biological activity of a nucleoside depends more on the base than on the sugar moiety. Hereupon, a vast number of derivates with modified sugar components were synthesized and tested as antiviral and antitumor agents. Compounds such as cytarabine, vidarabine, aciclovir, and azidothymidine can be traced back to these early discoveries (Newman et al. 2000).

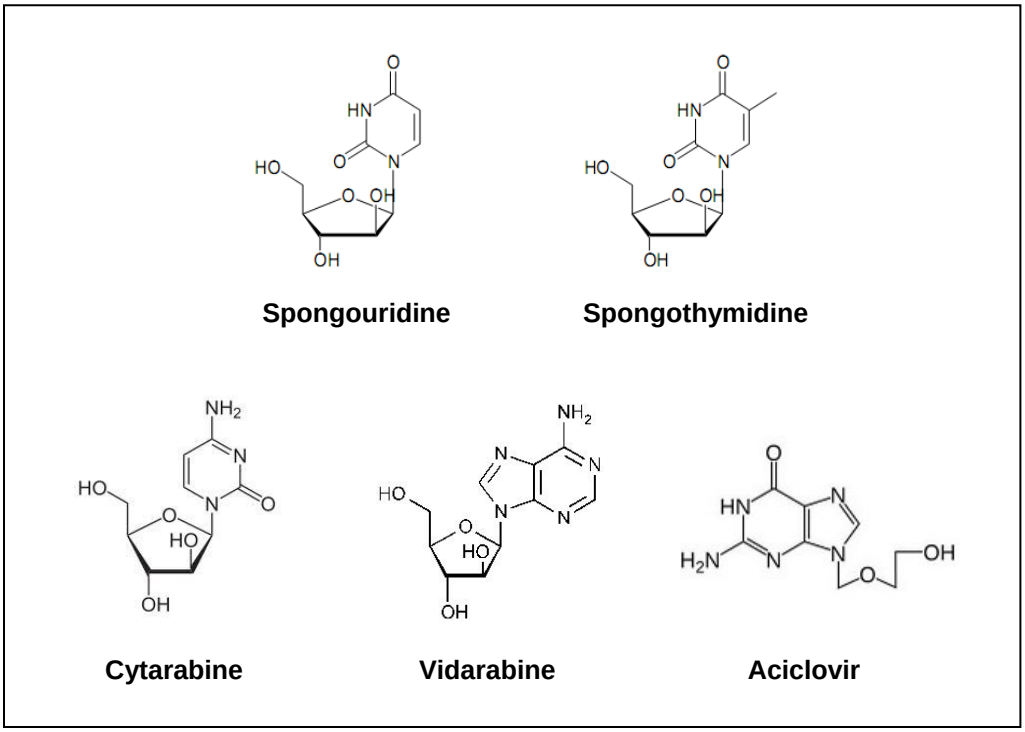


Figure 5: Naturally occurring nucleosides spongouridine and spongothymidine, and synthetic compounds cytarabine (cytostatic), vidarabine and aciclovir (virustatics).

Cytotoxicity of cytarabine is based on inhibition of DNA polymerase and synthesis. After phosphorylation, the compound competes with the physiologic substrate deoxycytidine triphosphate. The antimetabolite is indicated for the treatment of different types of leukemia (Mayer et al. 2010). In acute myeloid leukemia, combination therapy of cytarabine with another agent, usually an anthracycline or anthracenedione, has been the mainstay of therapy for almost 40 years (Fernandez 2010).

Vidarabine (adenine arabinoside, Ara-A) is a synthetic purine nucleoside with antiviral activity. The compound received the FDA approval in 1976 (Mayer et al. 2010). Adenine arabinoside is an inhibitor of viral DNA synthesis. Kinases encoded by viruses convert the agent into adenine arabinoside triphosphate, which inhibits viral DNA polymerase and hence DNA synthesis of herpes, vaccinia and varicella zoster viruses. Vidarabine has been used for the treatment of herpes virus infection. In the USA, its marketing has currently been stopped, as the newer drug aciclovir was found to be more efficient and less toxic. Though, vidarabine is probably capable of inhibiting aciclovir-resistant herpes and varicella zoster viruses (Sagar et al. 2010).

1.2.1.2. Ziconotide

Ziconotide (Prialt®), a marine-derived analgesic with a novel mechanism of action, which is applied for the treatment of severe chronic pain, received approval by the FDA in December 2004 and by the EMA in February 2005. Ziconotide is a synthetic form of ω-conotoxin MVIIA, one of the numerous toxic peptides found in the venom of the fish-hunting marine snail *Conus magnus* (Molinski et al. 2009). ω-Conotoxin MVIIA is a linear 25 amino acid peptide comprising three disulphide bridges, which stabilize its well-defined three-dimensional structure (Price-Carter et al. 1998). The conopeptide was found to be neuroactive in 1979 via a bioassay which elicited a characteristic tremor in mice after intracerebral injection (Olivera et al. 1985). Complete synthesis of the peptide was achieved in 1987 (Olivera et al. 1987).

Ziconotide acts as a reversible blocker of N-type voltage-sensitive calcium channels. These channels, occurring in the neural system, are expressed at high density on presynaptic terminals of primary afferent neurons that terminate in the dorsal horn of the spinal cord, an area which is important for processing pain. Activation of N-type channels

leads to calcium entry and subsequent release of pain-relevant neurotransmitters such as glutamate and neuropeptides. Binding of ziconotide to these channels inhibits calcium influx and thereby reduces neurotransmitter release at the first synapse in the nociceptive system, hence interrupting spinal transmission of pain information. As N-type calcium channels are widely distributed throughout the central nervous system, ziconotide-induced blockade can cause neurological side effects such as dizziness, confusion, ataxia, abnormal gait, memory impairment, nystagmus, or hallucinations (Schmidtko et al. 2010). Ziconotide has only a limited ability to cross the blood-brain barrier, and therefore requires intrathecal administration in order to achieve optimal analgesic efficacy with decreased potential for serious adverse effects. Ziconotide is non-addictive and does not lead to the development of tolerance (McGivern 2007). It is currently labeled for intrathecal management of severe chronic pain in patients with cancer or AIDS, who are intolerant of or refractory to other treatments such as systemic analgesics, adjunctive therapies, or intrathecal morphine (Mayer et al. 2010).

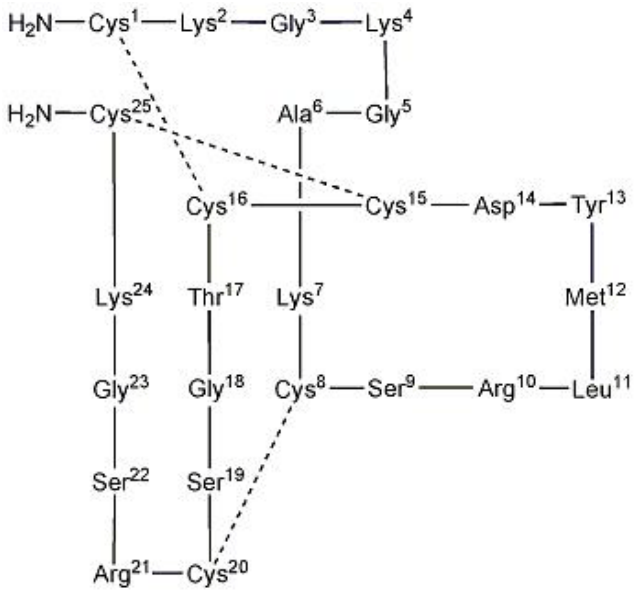


Figure 6: Ziconotide, a linear 25 amino acid peptide with three disulfide bridges.

1.2.1.3. Trabectedin (ET-743)

Trabectedin (Yondelis®), an alkaloid isolated from the tunicate *Ecteinascidia turbinata*, was approved for the treatment of refractory soft-tissue sarcomas by the EMA in July 2007 (Molinski et al. 2009). Although antitumor activity of extracts from the ascidian has been reported since 1969, the structure of the active compounds was not identified for almost two decades. The most abundant active component, ecteinascidin-743, is an alkaloid composed of three tetrahydroisoquinoline units and is related to the microbially derived safracins and saframycins (Rinehart et al. 1990). As the yield for ecteinascidin-743 from *Ecteinascidia turbinata* is very low, significant amounts of the tunicate had to be collected to obtain sufficient material for *in vitro* and *in vivo* animal studies. Additionally, total synthesis of the compound was performed, but this was not practicable to provide enough material for further development. A breakthrough was achieved by the Spanish company PharmaMar, whose chemists performed a realizable semi-synthesis from the marine *Pseudomonas fluorescens* metabolite cyanosafracin B (Newman & Cragg 2004; Cuevas et al. 2000).

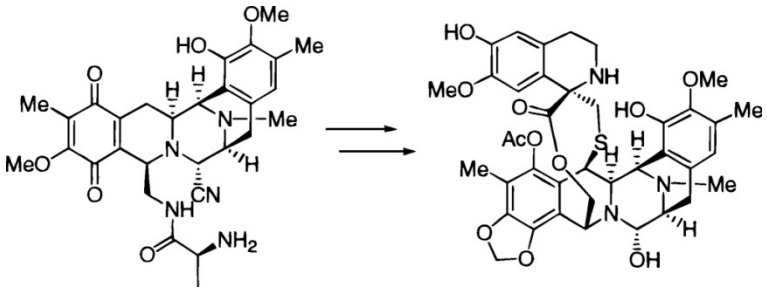


Figure 7: A practicable semi-synthesis of ecteinascidin-743 (right) from cyanosafracin B (left) was achieved by PharmaMar researchers.

Trabectedin interacts with DNA repair mechanisms, primarily through the nucleotide excision repair (NER) system. The compound binds to the DNA minor groove and alkylates guanine at the N2 position, preferentially at guanine-cytosine rich sequences, and induces bending of DNA towards the major groove. Trabectedin arrests cells in G2 phase. This is in contrast to other alkylating agents, which are generally active in S phase. The main adverse effects are myelosuppression, transient transaminase elevation, nausea, emesis, and fatigue (Chuk et al. 2009).

In the European Union and several other countries, trabectedin is approved for the treatment of relapsed soft tissue sarcoma, and, in combination with pegylated liposomal doxorubicin, for the treatment of platinum-sensitive recurrent ovarian cancer. In the USA and in Switzerland, it holds orphan drug status for the treatment of advanced recurrent soft tissue sarcoma and ovarian cancer. Trabectedin is under development for prostate cancer, breast cancer and pediatric soft tissue sarcoma (Cassier et al. 2010).

1.2.1.4. Eribulin mesylate (E7389)

Eribulin mesylate (Halaven™; Eisai), approved by the FDA in November 2010 and by the EMA in March 2011 (Eisai Co., Ltd. 2011) for the treatment of metastatic breast cancer, is the product of nearly 25 years of struggle in the laboratory and stands for a hard-won victory for the total synthesis of natural products. Eribulin is a truncated synthetic analogue of halichondrin B, a cytotoxic compound discovered in 1986 from extracts of the marine sponge *Halichondria okadai*. Halichondrin B occurs in very low concentrations. The structure of the molecule is highly complex, comprising 32 stereocenters (Ledford 2010).

Halichondrin B was shown to inhibit cell growth at nanomolar concentrations by a tubulin-based mechanism of action (Bai et al. 1991). In 1992, total synthesis of halichondrin B via complex reactions comprising approximately 90 steps was reported (Aicher et al. 1992). The discovery of the deep-water sponge *Lissodendoryx* n. sp. 1, which also sequesters halichondrins, led to the harvest of approximately 1 tonne of this sponge by trawling from natural habitats. Isolation of the cytotoxic compounds resulted in a yield of 310 mg halichondrin B and a comparable amount of isohomohalichondrin B, which was sufficient material for initial preclinical trials only. Aquaculture feasibility trials were carried out to establish an option for the supply with halichondrins. The survival and growth of the sponge culture was highly dependent on season, water temperature and depth, further, the production of halichondrin B was lower than in wild-type samples of *Lissodendoryx* (Munro et al. 1999). A breakthrough was achieved by the synthesis of simplified analogues, which showed equipotent anticancer activity compared to the natural product (Molinski et al. 2009). Besides retaining the biological activity of the parent compound, the development candidate eribulin mesylate (E7389) also showed favorable pharmaceutical characteristics including water solubility and chemical stability (Mayer et al. 2010).

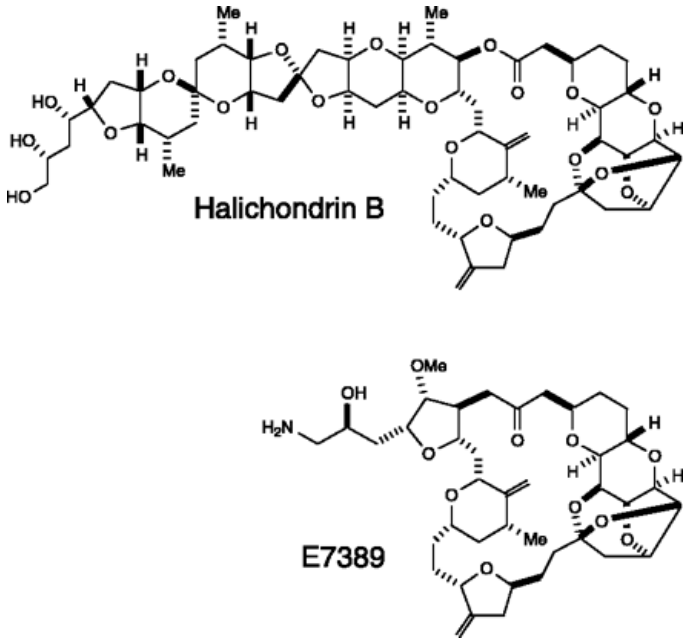


Figure 8: Halichondrin B, a cytotoxic compound isolated from marine sponges, and the truncated synthetic analogue eribulin mesylate (E7389).

Eribulin is, like the widely used taxans and the vinca alkaloids, a tubulin-targeted agent, but inhibits the microtubule dynamics through a distinct mechanism (Jordan et al. 2005). It inhibits the growth of microtubules and sequesters tubulin into non-productive aggregates, which leads to G2/M cell cycle arrest, disruption of mitotic spindles and apoptotic cell death after prolonged mitotic blockade (Huyck et al. 2011). Dose-limiting toxicities of eribulin mesylate include leukopenia, fatigue and neuropathy. In Phase II studies, the response rate against breast cancer in heavily pre-treated patients was approximately 10% (Cortes et al. 2010; Vahdat et al. 2009). Eribulin possesses the potential to overcome acquired drug resistance in metastatic breast cancer; a Phase III trial showed an average improvement of 2.5 months in overall survival. Several Phase II trials are ongoing for further evaluation of the efficacy of eribulin in other cancer types including soft tissue sarcoma, colon cancer, prostate cancer and non-small-cell lung cancer (Huyck et al. 2011).

1.2.2. Agents in development

1.2.2.1. Plitidepsin

Plitidepsin (Aplidin®) is a cytotoxic depsipeptide originally isolated from the tunicate *Aplidium albicans*. The compound is currently in Phase II clinical trials for the treatment of solid and hematologic malignancies. It has been granted the orphan drug designation for the treatment of acute lymphoblastic leukemia and multiple myeloma by the EMA and the FDA (Rockwell & Y. Liu 2010). Plitidepsin induces apoptosis through a mechanism which is not fully elucidated yet; oxidative stress and activation of the Rac1-JNK pathway seem to play a major role. Additionally, the compound exhibits antiangiogenic activity through inhibiting the expression of vascular endothelial growth factor (VEGF) and its receptor (Dumez et al. 2009). Plitidepsin is currently obtained by multistep total synthesis from constituent amino acids, due to the fact that supply from nature is limited by the rare occurrence of the source organism and the lack of feasible aquaculture conditions (Molinski et al. 2009).

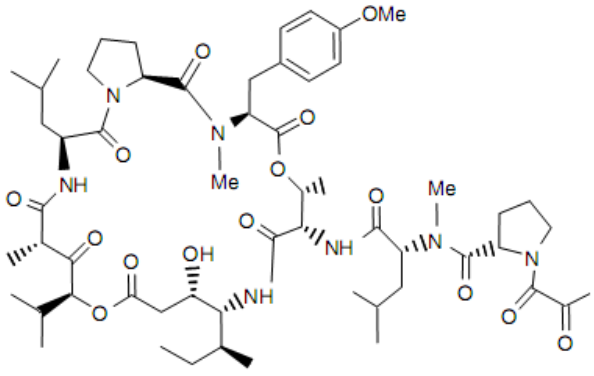


Figure 9: Plitidepsin (Aplidin®), a depsipeptide isolated from the tunicate *Aplidium albicans*.

1.2.2.2. (+)-Discodermolide

(+)-Discodermolide is another example for a highly potent and promising antitumor compound, which can be isolated from the source organism only in minute amounts. It features a highly complex structure, thus representing a daunting target for large-scale chemical synthesis. The polyketide, possessing cytotoxic and immunosuppressive properties, was isolated from the Caribbean sponge *Discodermia dissoluta* and first reported in 1990. Its mechanism of action – microtubule stabilization – is similar to paclitaxel, but (+)-discodermolide is ten times more potent. Additionally, it is strongly effective against P-glycoprotein (P-gp)-expressing multiple drug resistant (MDR) tumor cells (Koehn & Carter 2005). The compound was shown to inhibit *in vitro* cancer cell lines, which are resistant to paclitaxel (Valderrama et al. 2010). Several research groups developed methods for the synthesis of (+)-discodermolide, e.g. Smith et al. (2000), Paterson et al. (2000) and Harried et al. (2003). After the development of an efficient large-scale synthetic route by a working group of the pharmaceutical company Novartis (Mickel et al. 2004), (+)-discodermolide entered into clinical trials, but further studies were halted due to toxicity problems. As the work on the synthesis of various analogues is continuing, it is very likely that a derivate of (+)-discodermolide may become a clinical candidate in the future (Kingston 2009).

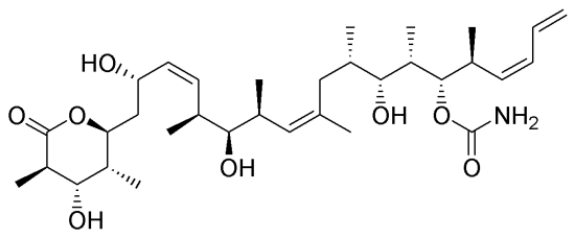


Figure 10: Discodermolide, isolated from the marine sponge *Discodermia dissoluta*. (Photo courtesy of Florida Atlantic University, Harbor Branch Oceanographic Institute.)

1.2.2.3. Hemiasterlin

Hemiasterlin, first reported in 1994, is an antimitotic tripeptide which was isolated from the sponge *Hemiasterella minor* (Talpir et al. 1994). The related isomers hemiasterlin A and B, which were found in other sponges, were described one year later (Coleman et al. 1995) and a fourth analogue was reported in 1999 (Gamble et al. 1999). All hemiasterlins showed high cytotoxicity against a variety of cultured cell lines, acting through induction of mitotic arrest in the metaphase. A simpler and more potent synthetic analogue of hemiasterlin, HTI-286, completed an open-label Phase I clinical trial in patients with advanced solid tumors. As no objective responses were observed, Phase II trials have been halted (Molinski et al. 2009). Currently, the novel synthetic analogue E7974 is undergoing phase I clinical trials for cancer. This compound was synthesized in the course of a lead optimization program of the Eisai Research Institute (Andover, Massachusetts, USA). E7974 acts via a tubulin-based antimitotic mechanism, predominantly targeting α-tubulin. This is in contrast to other tubulin-targeted agents, such as the taxanes and vinca alkaloids, which bind to β-tubulin. E7974 shows high *in vivo* antitumor efficacy even in tumor models which are resistant to paclitaxel; it retains strong potency in cells harboring mutations of β-tubulin, as it is the case with cells resistant to the taxanes. Further, it shows low susceptibility to P-gp-mediated efflux. Based on these promising findings, E7974 was selected for clinical evaluation for the treatment of solid tumors (Kuznetsov et al. 2009).

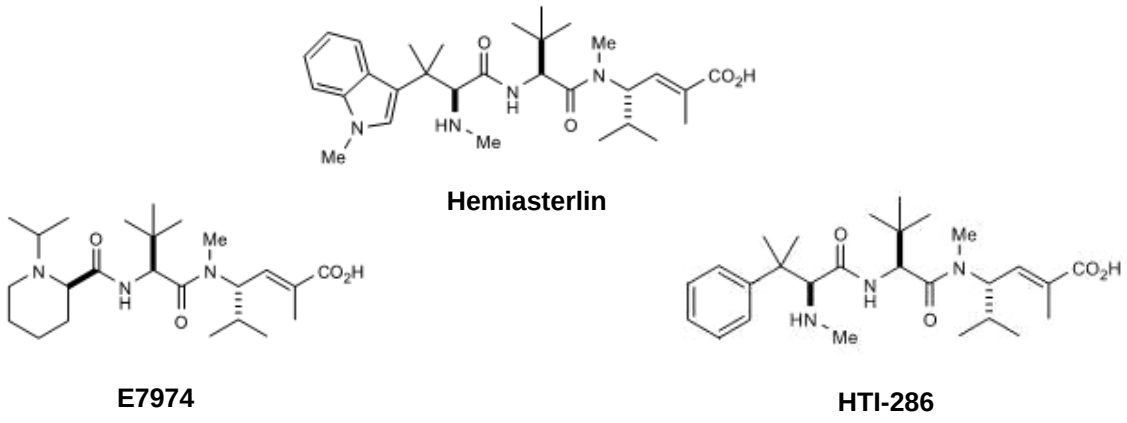


Figure 11: Chemical structures of the naturally occurring compound hemiasterlin (isolated from the sponge *Hemiasterella minor*), and the synthetic analogues E7974 and HTI-286.

1.2.2.4. Dolastatin analogues

The dolastatins, whose discovery had its beginnings in 1972, are a class of related cytotoxic peptides isolated from the sea hare *Dolabella auricularia*. Among these compounds, dolastatin 10 exhibited exceptionally potent *in vitro* cytotoxic activity against various cancer cell lines (Pettit et al. 1987). The antimitotic agent acts via inhibition of tubulin polymerization in a similar way to the vinca alkaloids (Bai et al. 1990). Dolastatin 10 entered Phase I clinical trials in the 1990's and progressed to Phase II trials under the auspices of the NCI for the treatment of several solid tumors. As no significant antitumor activity could be detected, the compound was withdrawn from clinical trials (Kingston 2009; Molinski et al. 2009). Until recently, soblidotin, a simplified analogue of dolastatin 10, and tasidotin, a synthetic dolastatin 15 derivate, were in clinical trials, but have been discontinued as well (Mayer et al. 2010; Midwestern University 2011).

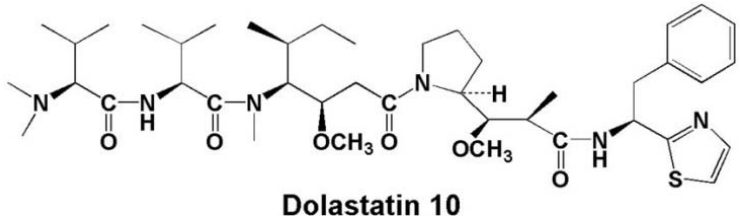


Figure 12: The antimitotic peptide dolastatin 10, isolated in the 1970's from the sea hare *Dolabella auricularia*, was the first member of the dolastatin family which entered clinical trials.

1.2.2.5. Cyclodepsipeptides with antiviral and antitumor activity

A number of cyclic depsipeptides, isolated from various marine sponges, e.g. papuamides and mirabamides, have been shown to exhibit antiviral activity *in vitro* via inhibition of HIV entry into cells. Other cyclodepsipeptides, e.g. jasplakinolide, arenastatin A and spongidepsin, possess potent *in vitro* cytotoxic activity and may be of interest in the development of new anticancer agents. The compounds kahalalide F, a depsipeptide isolated from mollusks and from the green alga *Bryopsis* sp., and plitidepsin (Aplidin®, see chapter 1.2.2.1) have already shown promising results in phase I and II clinical trials

(Andavan & Lemmens-Gruber 2010). The cryptophycins, isolated from the cyanobacteria *Nostoc* spp. and from the sponge *Dysidea arenaria* (see also chapter 1.1.2), are highly potent cytotoxic agents (Beck et al. 2005). The synthetic analogue cryptophycin-52 (see Figure 4) entered clinical trials, but failed to produce an objective response in a phase II study for advanced non-small-cell lung cancer (Edelman et al. 2003). Several new cryptophycin analogues have been synthesized and undergone preclinical efficacy studies over the past years, some of them being considered as second-generation clinical candidates (Liang et al. 2005; Liu et al. 2009).

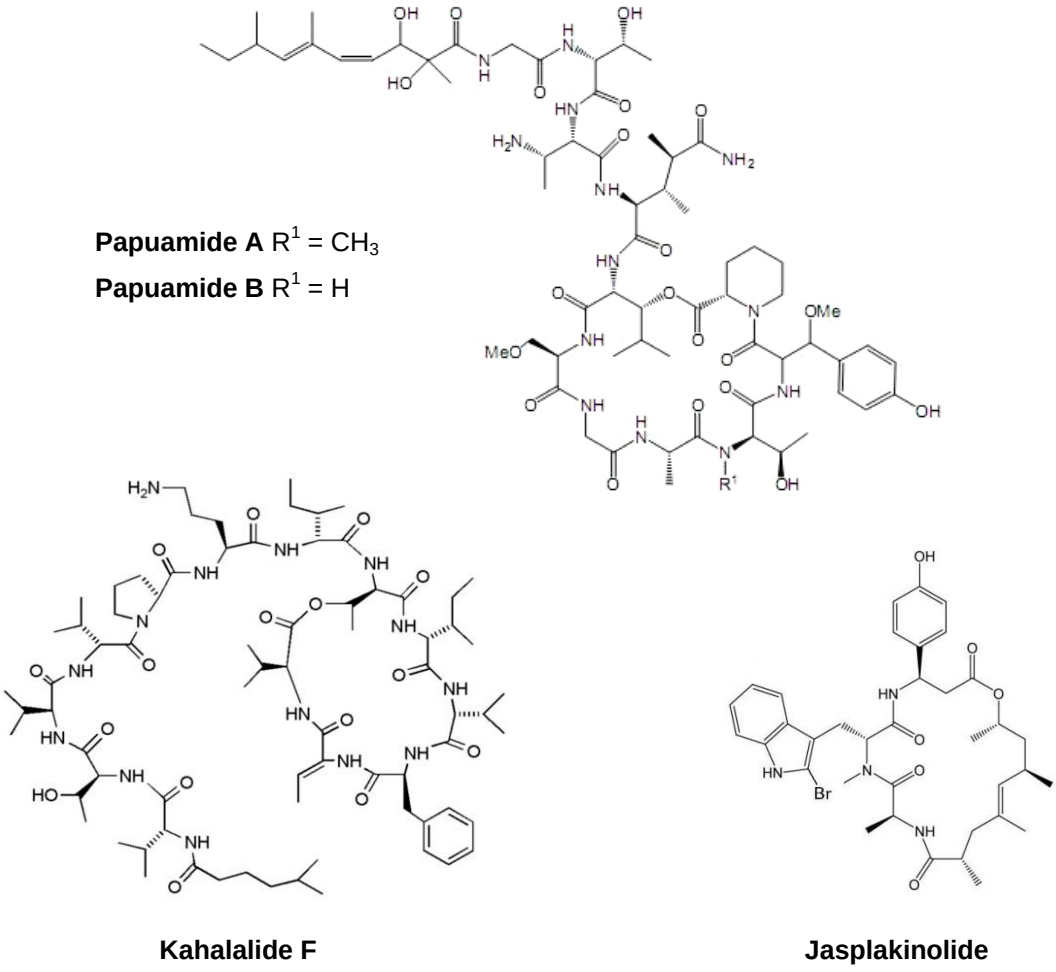


Figure 13: Chemical structures of cyclodepsipeptides produced by marine organisms.

2. OBJECTIVE

In drug discovery from natural sources, bioassay guided fractionation is an appealing strategy for the isolation of active compounds (Houssen & Jaspars 2006; Koehn & Carter 2005). In the present study, this method was applied for the isolation of the cytotoxic component of a marine sponge extract. The assay used to determine toxicity was the brine shrimp lethality assay (BSLA). This test is based on the ability of extracts, fractions, or pure compounds to kill *Artemia salina* (brine shrimp) nauplii.

The first task of this work was the selection of a sponge sample with considerable toxicity for bioassay guided fractionation. 40 extracts of Caribbean marine sponges, yielded via ultrasonic-assisted solvent extraction with CH_2Cl_2/MeOH (1:1), were provided from INBio (access permit granted by CONAGEBIO[♣]), and were tested for their cytotoxicity against *Artemia salina*. The sample with the lowest median lethal concentration (LC_{50}) value in the BSLA, also showing high toxicity against human and murine cancer cells, was selected for fractionation.

The aim of this study was the isolation of the toxic component from the selected sponge sample. The extract was initially fractionated via vacuum liquid chromatography (VLC). As a first approach, to get an overview about the composition of the fraction with the highest toxicity, both analytical high performance liquid chromatography (HPLC) and semi-preparative HPLC were applied. For further fractionation, the preparative HPLC instrument BioXplore© (Varian) was used to separate the purified ethanol extract.

The individual fractions obtained by the VLC and HPLC separations were tested in the BSLA and LC_{50} values were calculated. Additionally, thin layer chromatography (TLC) was used to monitor the process and identify the toxic compound.

[♣] Costa Rica is a country with an exceptionally great diversity of species and ecosystems. For the conservation and sustainable use of its biodiversity, a very comprehensive legal framework has been established, which includes the Biodiversity Law, approved in 1998. This law states, that any research program or bioprospecting requires an access permit, which has to be granted by the National Commission of Biodiversity Management (CONAGEBIO) (INBio 2011; Solís & Madrigal 1999).

3. EXPERIMENTAL

3.1. Sponge samples

Starting materials were extracts of 40 Caribbean marine sponges (the extracts collection was used thanks to CONAGEBIO's *ex-situ* access permit **R-CM-INBio-121-2010-OT**), which were adsorbed on a styrene-divinylbenzene resin (Diaion™ HP-20). The marine sponges were collected in 2002 and 2003 in the Caribbean Sea of Costa Rica by scuba diving at a depth of 8 – 30 meters.

The further investigated sponge sample (INBio code 290-13443, laboratory code 999; election based on cytotoxicity assay, see below) was collected at 8 m depth (flat bottom) at Cahuita Point, Costa Rica, on September 10, 2002. The sponge was hard and easy to tear, had an encrusting, tree like shape and a lumpy surface, the color was off-white inside and purple on the upper surface. A voucher specimen was deposited at INBio, Santo Domingo de Heredia, Costa Rica. The sponge belongs to the order Haplosclerida (class Demospongiae), probably to the genus Haliclona (classified by Dr. Sven Zea, National University of Colombia, Department of Biology and Center of Marine Sciences CECIMAR).

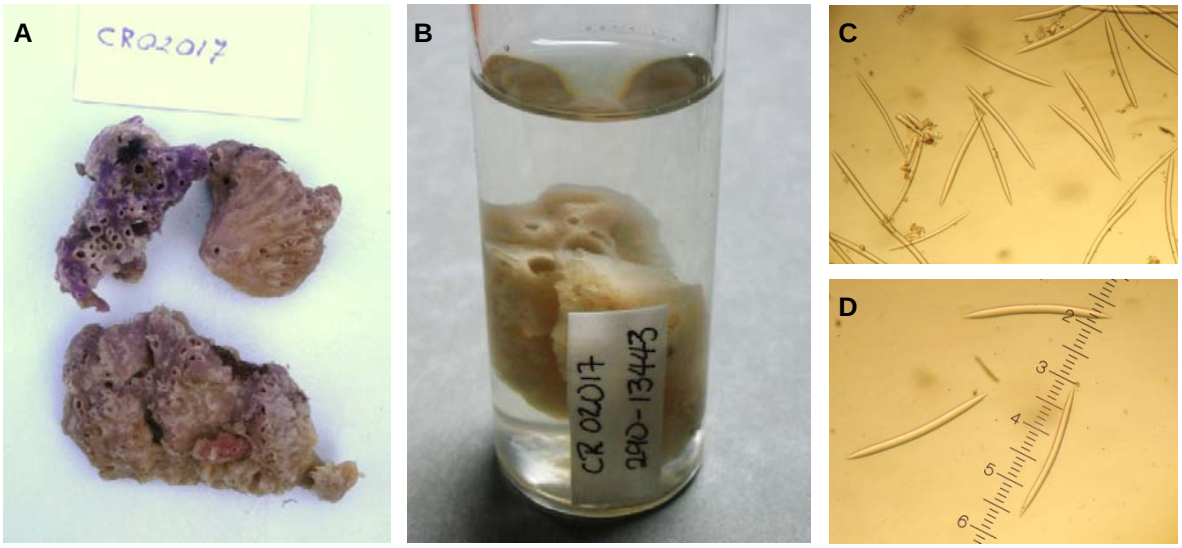


Figure 14: Voucher specimen (A and B) and characteristic spicules (C and D) of the further investigated marine sponge.

3.2. Materials

Solvents for purification and fractionation:

Dichloromethane	Reactivos quimicos Gamma®
CAS 75-09-2	Laboratorios quimicos ARVI S.A.
	(CH_2Cl_2, 99.5%)
Ethanol 95 %	Fábrica Nacional de Licores
CAS 64-17-5	Grecia, Alajuela
Isopropanol	Reactivos quimicos Gamma®
CAS 67-63-0	Laboratorios quimicos ARVI S.A.
	(C_3H_8O, 99.5%)
Methanol	Methanol for liquid chromatography LiChrosolv®
CAS 67-56-1	Merck Chemicals
	(CH_3OH, 99.8%)
Distilled water	

Adsorbent resin:

Supelco Diaion™ HP-20 (Particle size 250-850 µm) **Supelco Diaion™ HP-20SS** (Particle size 75-150 µm)	Sigma-Aldrich®, Bellefonte, PA Matrix: styrene-divinylbenzene Surface area: ~500 m^2/g

Thin layer chromatography (TLC):

Stationary phase:

Silica on TLC Alu foils	Silica gel matrix, 20 cm x 20 cm, with fluorescent indicator 254 nm Fluka® Analytical, Sigma-Aldrich® Chemie GmbH Steinheim, Germany

Solvents for mobile phases:

Dichloromethane	Reactivos quimicos Gamma®
CAS 75-09-2	Laboratorios quimicos ARVI S.A.
	(CH_2Cl_2, 99.5%)
Methanol	Methanol for liquid chromatography LiChrosolv®
CAS 67-56-1	Merck Chemicals
	(CH_3OH, 99.8%)
Toluene	Fisher Scientific Company
CAS 108-88-3	Fair Lawn, NJ
	($C_6H_5CH_3$, 99%)
p-Dioxane	Spectrum Chemical Mfg. Corp.
CAS 123-91-1	Gardena, CA; New Brunswick, NJ
	($C_4H_8O_2$, 99.0%)
1-Butanol	Reactivos quimicos Gamma®
CAS 71-36-3	Laboratorios quimicos ARVI Ltda.
	($C_4H_{10}O$, 99.4%)
Glacial acetic acid	Reactivos quimicos Gamma®
CAS 64-19-7	Laboratorios quimicos ARVI Ltda.
	Grado reactivo A.C.S.

Distilled water

Spray reagents:

Vanillin-sulfuric acid (Jork et al. 1994)

Potassium permanganate (Krebs et al. 1969)

Cytotoxicity assay:

***Artemia salina* cysts**	Acuario Flora y Fauna Guadalupe, Costa Rica
Sea salt	Instant Ocean® – Synthetic Sea Salt Aquarium Systems Sarrebourg, France; Mentor, Ohio
Dry yeast	Levadura en grano Yerona DIPROA S.A. Moravia, San José, CR
Antichlor (sodium thiosulfate)	Nutrafin® Aqua plus Hagen/Canada
Dimethyl sulfoxide (DMSO) **Reagent A.C.S.** CAS 67-68-5	Spectrum Chemical Mfg. Corp. Gardena, CA; New Brunswick, NJ ($(CH_3)_2SO$, 99.9%)
Thermostat	PENN-PLAX® Therma-Flow “PC” Plus Heater Automatic moisture-proof aquarium heater
Aquarium air pumps	PENN-PLAX® AirTech 2K1™ AirTech 2K2 ™
pH meter	pH/mV/Temp. Meter SUNTEX Model SP-701
Light source	Sylvania Cool White 20 W Model F20T12/CW
Stereo microscope	Carl Zeiss Stemi 2000-C
Eppendorf adjustable pipettes	10-100 µl 100-1000 µl Multichannel (12-channel), 30-300 µl

3.3. Methods

3.3.1. Sponge extraction

The sponges were extracted by ultrasonic-assisted solvent extraction with CH_2Cl_2/MeOH (1:1).

Immediately after collection of the marine sponges, they were preserved in a solution of 50% seawater and 50% EtOH for transport. Further sample preparation included discarding the stabilizing solution, homogenization of the sponges, and extraction of the sponge material with CH_2Cl_2/MeOH (1:1) in the ultrasonic bath for 30 minutes. Afterwards the mixture was transferred into a percolator; CH_2Cl_2/MeOH (1:1) was added and after 8 h the solvent was filtered off. The sponge material was extracted two more times in the percolator (12 h and 4 h, respectively), and the filtrates were combined. Solvent was removed using a rotary evaporator. Finally the extract was allowed to absorb onto the resin HP-20 which was then evaporated to dryness in a fume hood, and the samples were stored at -20°C.

3.3.2. Chromatography

3.3.2.1. Vacuum liquid chromatography (VLC)

VLC is a form of column chromatography which uses reduced pressure to increase the flow rate of the mobile phase. Operating VLC, the column is eluted with the appropriate solvents and allowed to run dry after each fraction is collected.

In the present work, the basic mechanism of VLC was adopted for small-scale purification of crude sponge extracts to obtain ethanol fractions for testing the cytotoxicity by means of the brine shrimp lethality assay (BSLA). Furthermore, the technique was used for the fractionation of two ethanol extracts that were active in the bioassay. The mode of separation was reversed-phase chromatography, a styrene-divenylbenzene polymer serving as stationary phase.

Small-scale purification of 40 sponge samples:

With a portion of each sponge extract, a pre-purification step was carried out in order to obtain purified material, in which secondary metabolites were concentrated and separated from sugars, salts, fats and other concomitant substances. For each sample approximately 2 ml of the resin Diaion™ HP-20, charged with crude sponge extract, were used.

Purification was carried out with a Supelco Visiprep™ Solid Phase Extraction (SPE) Vacuum Manifold, 12-Port Model and 6 ml glass reaction tubes with Teflon® frits and a bed of approximately 2 cm HP-20 resin. The extract was eluted with 15 ml distilled water (fraction 1), EtOH (fraction 2) and CH_2Cl_2/EtOH (1:1) (fraction 3). The obtained fractions were collected in 20 ml glass vials and evaporated using a Speed Vac Concentrator Savant SC 210A, stored in a freezer at -20°C over night and then lyophilized via a FTS Systems™ Lyophilisator. The ethanol fractions were later subjected to cytotoxicity testing via the BSLA (see chapter 3.3.3).

Figure 15: Supelco Visiprep™ SPE Vacuum Manifold, used for small-scale purification of the crude sponge extracts.

Fractionation of two active samples:

The large-scale purified samples 998 and 999 (see following chapter) were fractionated via VLC, and the fractions were tested for their toxicity. First, the purified ethanol fractions were absorbed on the resin HP-20 by re-dissolving the extracts in a small amount of EtOH, adding resin HP-20 (five times the quantity of extract), and gently removing the solvent by a rotary evaporator.

Further, ethanol slurry of HP-20SS (resin which consisted of a smaller grain size) was filled into a chromatography column (30 cm l x 1 cm id). The resin was vacuum dried, and the column was washed with water and equilibrated with EtOH/water (2:8). Following, approximately 5 ml of the dried, extract-charged resin was poured onto the 12 cm bed. The column was eluted with 15 ml EtOH/water (2:8) (fraction 1), EtOH/water (1:1) (fraction 2), EtOH/water (8:2) (fraction 3), EtOH (fraction 4), and isopropanol/CH_2Cl_2 (8:2) (fraction 5) by applying vacuum. The fractions were collected in 20 ml glass vials, dried using a Speed Vac Concentrator, and subjected to the BSLA.

3.3.2.2. Purification via pressurized column chromatography

Based on the results of the cytotoxicity assay (see chapter 4.1.3), the samples 998 and 999 were chosen for further separation; therefore, a larger quantity of purified extract was required. The purification was carried out by means of the “charging station”, which is a particular equipment capable of cleaning up large amounts of crude extracts within a short time. The instrument holds two columns with a capacity of 250 ml each, and works with a maximal pressure of 2000 psi (~13.8 MPa). Solvents are conducted from the tank to the previously filled column and pressed through it.

Approximately 80 ml of the HP-20 resin charged with crude extract (4-5 g) were filled into the high-pressure column, further fresh HP-20 resin was poured in and it was locked. Then the device was placed in-line and the extract was eluted with 500 ml of distilled water (fraction 1), 1000 ml EtOH (fraction 2) and 1000 ml CH_2Cl_2/EtOH (1:1) (fraction 3). The collected fractions were evaporated to dryness using a rotary evaporator and weighed.

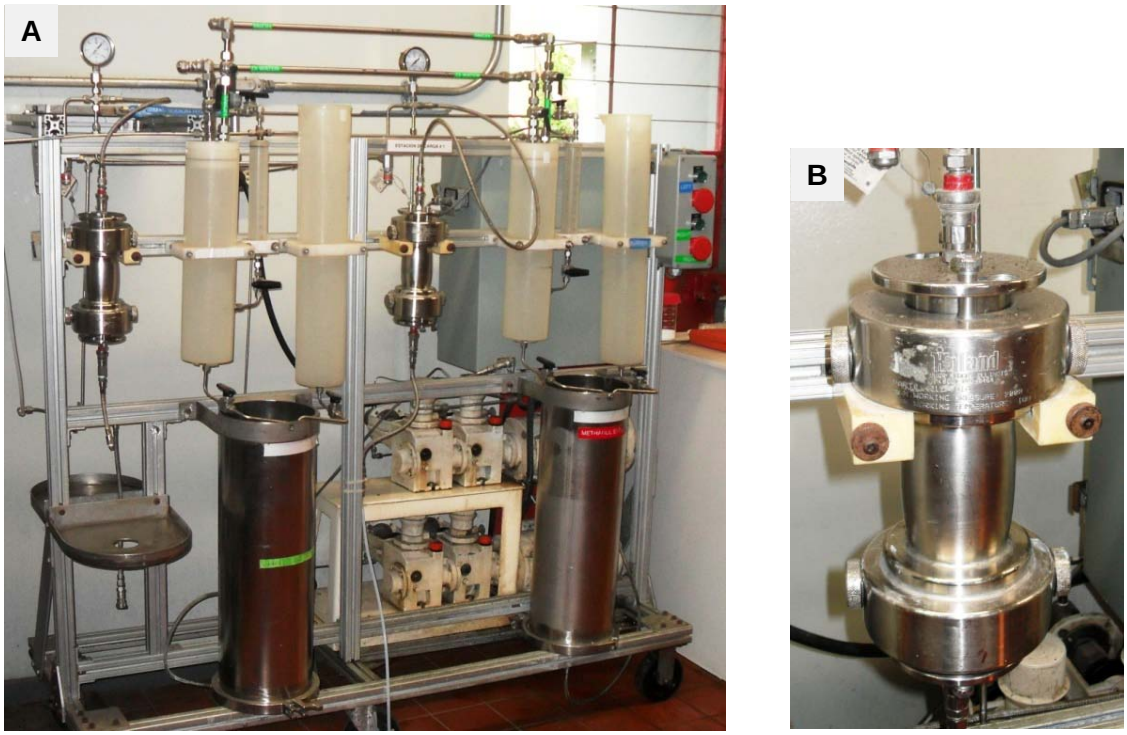


Figure 16: The charging station (A), equipped with a high-pressure column (B), was used for purification of crude extracts.

3.3.2.3. Thin layer chromatography (TLC)

TLC was employed for monitoring the composition of the fractions obtained by VLC and HPLC. TLC was carried out on silica gel TLC plates with fluorescent indicator 254 nm. The chromatograms were prepared by applying sample solutions with a concentration of 10 mg/ml and developed under saturated conditions. Running distance was 8 cm.

Mobile phase:

- dichloromethane – methanol (95:5)
- toluene – p-dioxan – glacial acetic acid (45:12:2)
- 1-butanol – glacial acetic acid – water (4:1:1)

The spots were first detected under UV-light (254 and 365 nm). For better visualization, the TLC plates were sprayed with the detection reagents vanillin-sulfuric acid and

potassium permanganate respectively, and heated until coloration appeared. Characterization was performed by calculation of the retention factor (R_f) according to the following equation:

$$R_f = \frac{Distance\ traveled\ by\ compound}{Distance\ traveled\ by\ solvent}$$

3.3.2.4. High Performance Liquid Chromatography (HPLC)

HPLC was used for further separation of the fractions obtained by VLC, which were active in the BSLA. Several water/MeOH gradients were tested using an XTerra® RP-18 column (3.9 x 150 mm). The best conditions were obtained by the gradient shown in Table 1. Further, this gradient was applied for semi-preparative HPLC separation employing a 10 x 250 mm column. Degassing of solvents was achieved by placing the container into an ultrasonic bath before use. Samples were diluted in MeOH at a concentration of 25 mg/ml.

Instrument:	Waters	600E Multisolvent delivery system (pump and controller)
		2487 Dual λ Absorbance Detector
	Rheodyne	7725i Manual Injector
		7161 injection valve
	Software:	Millennium[32]

Columns: XTerra® (Waters, Ireland)

RP-18 5 µm	RP-18 10 µm
3.9 x 150 mm	10 x 250 mm
Part.No. 186000480	Part.No. 186001028
SN 01913729713638	SN 11813634012601

Fractionation conditions:

Mobile phase A:	Distilled water
Mobile phase B:	MeOH
Flow:	1 ml/min (column 3.9 x 150 mm) 3 ml/min (column 10 x 250 mm)
Injection volume:	20 µl (column 3.9 x 150 mm) 100 µl (column 10 x 250 mm)
UV Detection:	254 and 280 nm

Table 1: Gradient system developed for semi-preparative HPLC separation.

Time (min)	Mobile phase A (v/v)	Mobile phase B (v/v)
00 → 30	65 → 00	35 → 100
30 → 35	00 → 00	100 → 100
35 → 40	00 → 65	100 → 35
40 → 45	65 → 65	35 → 35

3.3.2.5. BioXplore© fractionation technology

The BioXplore© equipment is an industrial HPLC system which can be applied for the fractionation of different types of samples, e.g. plant material, marine organisms and culture broths. The unit is equipped with two detectors, an evaporative light scattering detector (ELSD) and a variable wavelength detector. In the present work, the BioXplore© technology was employed for fractionating the purified ethanol extract of sample 999. Approximately 50 mg of extract were dissolved in 20 ml ethanol. The compounds were eluted applying a step gradient of ethanol and distilled water (see Table 2 and Figure 18), and ELSD peak-based collection was carried out manually. The obtained fractions were evaporated to dryness and tested in the BSLA.

Instrument:	BioXplore 300, Model ST 400, custom made HPLC by Varian
	Sedex 55 Evaporative Light Scattering Detector
	Knauer Variable Wavelength Detector
	Varian ProStar HPLC Fraction Collector
	Rheodyne 3725-038 Injector
	Demicap filter capsules, Peplyn Plus Membrane
	LC ReSponder™ Control Software
Column:	Grace Vydac Flanged MODcol®
	50.8 x 250 mm
	Packing material: Kromasil C18, 10 µm
	Volume of stationary phase: 500 ml

Figure 17: Industrial HPLC unit BioXplore 300, custom made by Varian.

Fractionation conditions:

Mobile phase A:	Distilled water
Mobile phase B:	Ethanol 95%
Flow:	80 ml/min
Injection volume:	20 ml
Detection:	ELSD

Table 2: Gradient system applied for BioXplore© separation.

Time (min)	Mobile phase A (v/v)	Mobile phase B (v/v)
0 → 5	90	10
5 → 12	65	35
12 → 19	40	60
19 → 26	15	85
26 → 51	0	100

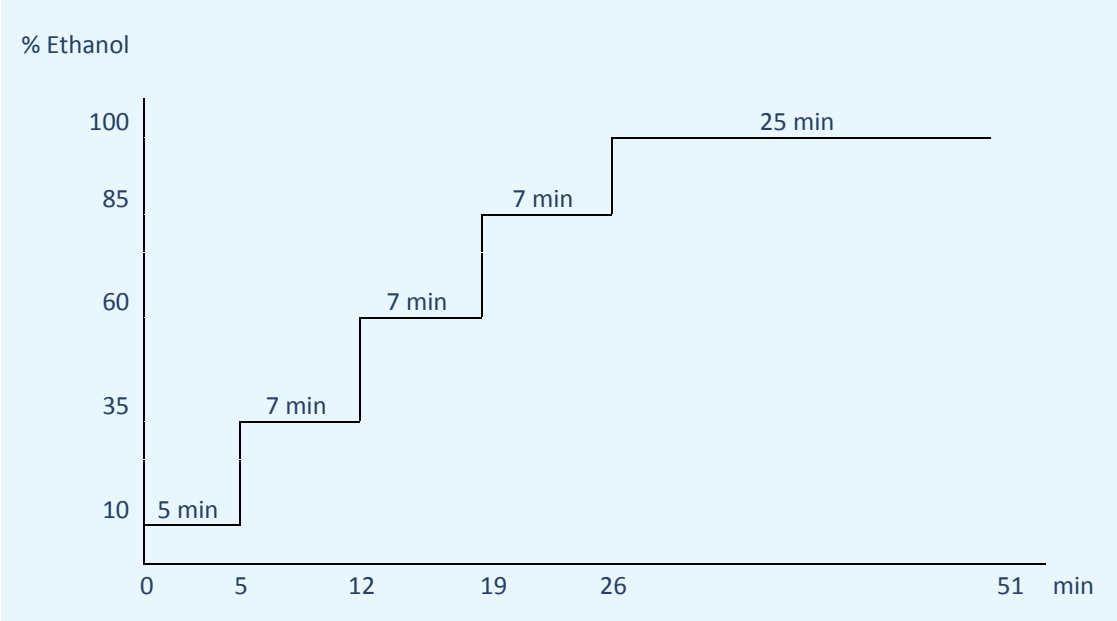


Figure 18: Step gradient applied for fractionation via BioXplore© technology.

3.3.3. Cytotoxicity test: Brine shrimp lethality assay (BSLA)

The BSLA was used for the toxicity assessment of purified sponge extracts and fractions thereof. The test is based on the ability of extracts and compounds to kill laboratory-cultured *Artemia salina* larvae.

Artemia salina is a small crustacean of the Artemiidae family, reaching a size of approximately 15 mm (Schminke 2007). Dried cysts of the brine shrimp are commercially available, remain viable for years and can be hatched easily (Vanhaecke et al. 1981).

Artemia salina cysts were purchased from the aquaristic shop Acuario Flora y Fauna, Guadalupe, Costa Rica.

Figure 19: Magnified view of *Artemia salina*.
(Source: www.flickriver.com/photos/xavipat/4451152486)

3.3.3.1. Test conditions

In the present work, the test was carried out as a microplate assay in 96-well microtiter plates, developed by Solis et al. (1993). A preliminary screening was performed to detect samples with significant activity. For this purpose, four different concentrations (500, 250, 125 and 62.5 µg/ml) of purified sponge extracts were tested in duplicate. For the definitive test, eight different concentrations (1000, 500, 250, 125, 62.5, 31.3, 15.6 and 7.8 µg/ml) were used and three replicates were accomplished. Control wells with 5% dimethyl sulfoxide (DMSO) in artificial seawater, used as solubilizer, were included in each

experiment. Extracts of *Persea americana* (avocado) seeds, whose activity in the BSLA has been confirmed (Leite et al. 2009), served as positive control.

3.3.3.2. Hatching of nauplii

Artificial seawater was prepared in a 2 liter glass beaker by dissolving 28 g of synthetic sea salt (Instant Ocean®) in 1 liter of commercially available purified drinking water. 3 mg of dry yeast and 1 drop of antichlor (sodium thiosulfate) solution were added. The pH value was adjusted to 8.5 with 1N NaOH. Approximately 100 ml of the artificial seawater were taken and stored in the refrigerator (+6°C) for further preparation of the samples and microplates (see chapter 3.3.3.3 and 3.3.3.4). The beaker with the remaining solution was put into a water tank at 28-30°C, and the artificial seawater was strongly aerated via two aquarium air pumps. Approximately 1 g of dried *Artemia salina* cysts were added and incubated for 24 hours under continuous aeration and illumination.

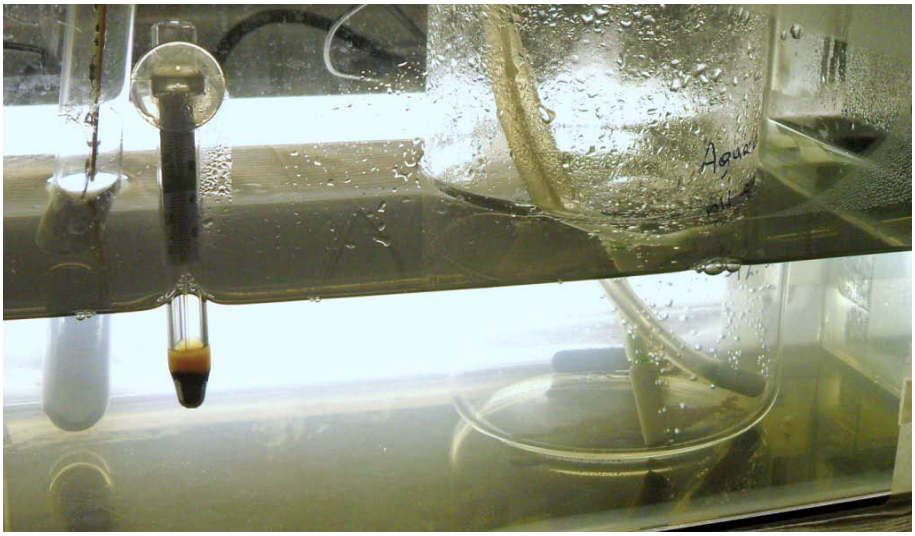


Figure 20: Hatching of *Artemia salina* nauplii at 28-30°C under aeration and illumination for 24 h.

The hatched nauplii are phototropic and accumulate at the lightened side. By stopping aeration, they could easily be collected with a graduated pipette and were transferred into a petri dish.

3.3.3.3. Sample preparation

Stock solutions of 2000 µg/ml (and 1000 µg/ml for the pre-selection step, respectively) of the sponge extracts were prepared in artificial seawater. For this purpose, 1-3 mg of the ethanol fractions obtained in the purification step were precisely weighed into 1.5 ml Eppendorf tubes. The extracts were dissolved in the corresponding volume of DMSO (5% of the total volume), and an appropriate volume of seawater was added (examples given in Table 3). Dissolution of the extracts in DMSO was facilitated by ultrasonication. After adding the seawater, the solution was well agitated by means of a vortex mixer.

Table 3: Preparation of 2000 µg/ml stock solutions for the BSLA.

Sample	weight (mg)	total volume (µl)	volume DMSO (µl)	volume water (µl)
985	1.4	700	35	665
988	1.2	600	30	570
994	1.0	500	25	475
998	1.5	750	38	713
999	1.4	700	35	665
1016	1.8	900	45	855
1018	1.4	700	35	665
1024	2.2	1100	55	1045
1347	1.9	950	48	903
1475	1.5	750	38	713
Persea	2.1	1050	53	998

3.3.3.4. Assay arrangement

Serial dilutions were made directly in the wells of 96-well microtiter plates in 100 µl seawater. Control wells with 5% DMSO in artificial seawater (blank) and *Persea americana* seed extract (positive control) were included in each experiment. Nauplii were added to the dilutions by catching them from the petri dish with an Eppendorf pipette. Into every well 10 nauplii (each in 10 µl artificial seawater) were transferred.

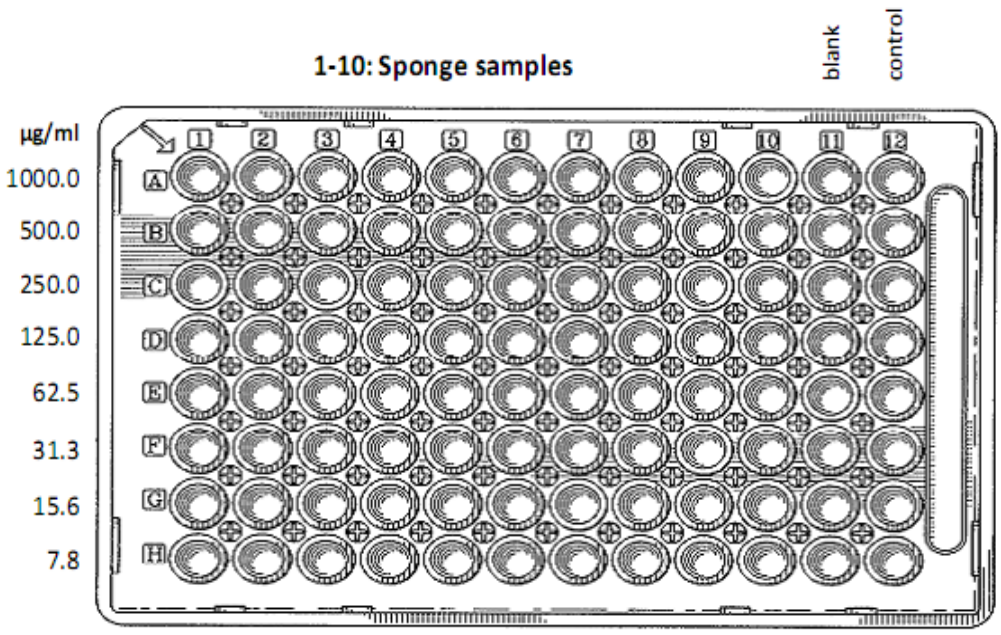


Figure 21: Arrangement of samples and resulting concentrations obtained by serial dilutions and subsequent transfer of 10 nauplii to each well. Final volume: 200 µl.
Blank: 5% DMSO in artificial seawater
Control: *Persea americana* seed extract

3.3.3.5. Incubation and evaluation

The microplates were covered and incubated at approximately 25°C in darkness. After 24 hours, the plates were examined under a stereo microscope and the numbers of dead nauplii in each well were counted and noted. Nauplii were considered as dead if they did not show any movement during several seconds of observation.

LC_{50} values and 95% confidence intervals were determined via probit analysis using the LC_{50} Program Version 2.5, a modified version of the program originally supplied by Stephan (1977). If the obtained data were insufficient for probit analysis, the binomial test was applied.

3.3.4. Cellular studies

The cytotoxicity of the investigated sponges (MeOH extracts and CH_2CL_2/MeOH extracts) against several cell lines was studied using a disk diffusion assay. Differential cell killing among the following cell types was examined: Two leukemias (murine L1210 and human CCRF-CEM), three solid tumors (murine Colon 38, human Colon H116 and human lung H125), as well as a murine and a human normal cell line (hematopoietic progenitor cell, CFU-GM). Both antiproliferative response and differential activity have been the end-points in this *in vitro* assay. Solid tumor selectivity relative to normal cells and/or to their respective leukemia cells were positive outcomes, and human selectivity was prioritized over murine selectivity. The assay was designed to determine differences in the relative sensitivity of leukemias, solid tumors and normal cells for a given sample.

Disc diffusion assay

The test material (extract dissolved in DMSO) was placed on a filter disk which was then put on top of the agar plates that have been seeded with either tumor cells or normal cells. The zone of inhibition was examined after 7-10 days of incubation (depending upon the cell type) and quantified in terms of zone units. The diameter of the filter disk (6.5 mm) was arbitrarily taken as 200 units; samples were of further interest if the zone of inhibition exceeded 300 units. A difference of ≥ 250 units between any solid tumor and either leukemia or normal cells defined solid tumor selective samples.

The cellular studies were carried out by Dr. Frederick Valeriote, Ford Cancer Center, Detroit, USA.

4. RESULTS

The aim of the study was the isolation of the toxic compound via bioassay guided fractionation from an extract of a marine sponge with high cytotoxicity. The following flow chart shows the individual steps applied for this investigation.

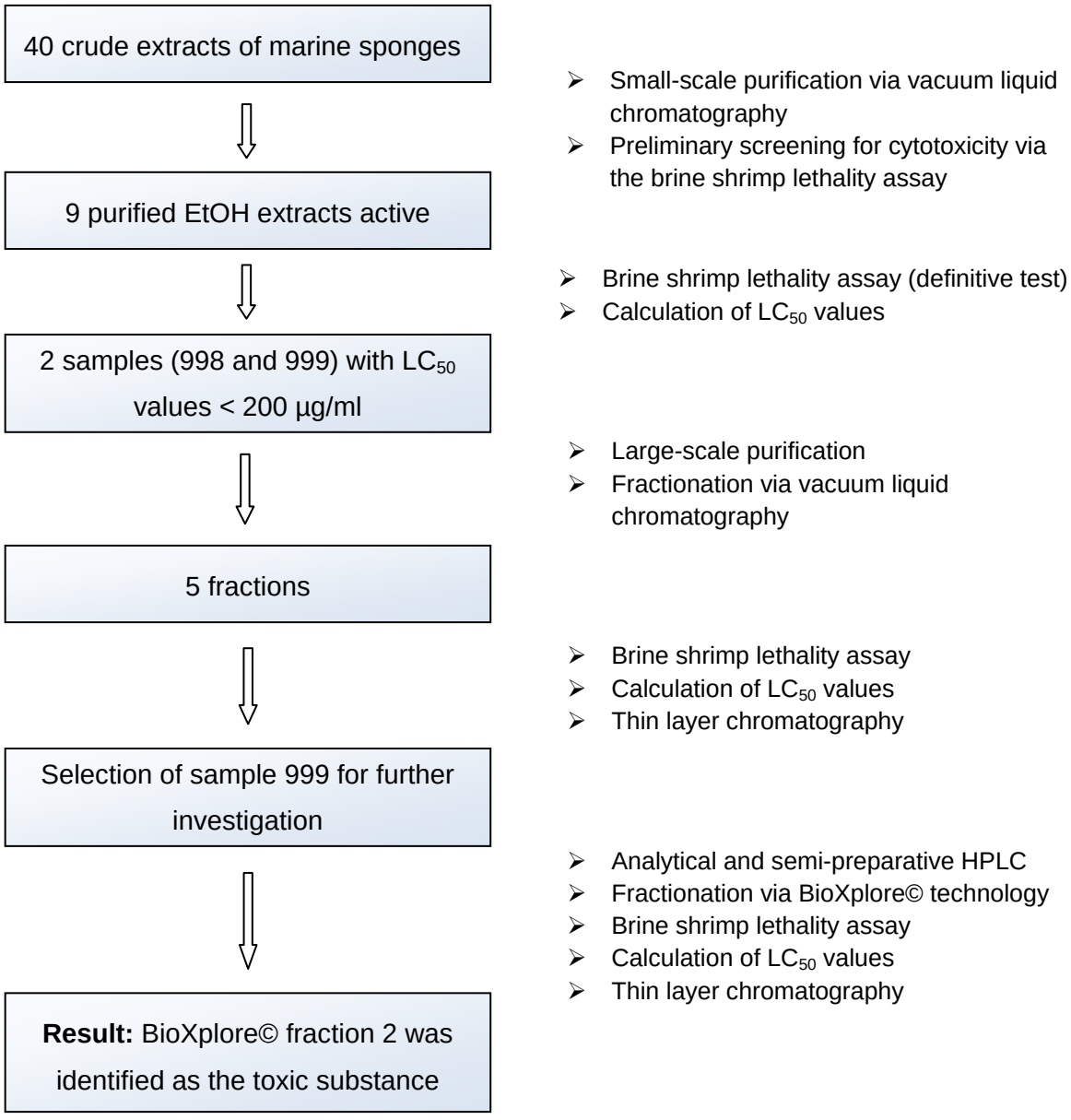


Figure 22: Sequence of individual working steps applied in the present study.

4.1. Selection of sponge sample for further investigation

From a total of 40 sponge extracts (see chapter 3.1) the most suitable sample for bioassay guided fractionation had to be chosen. As significant cytotoxicity was the main criterion for further investigation, all the extracts were screened via the brine shrimp lethality assay (BSLA) to assess the grade of toxicity (see chapter 3.3.3).

First, the crude extracts were subjected to a clean-up process to obtain purified ethanol extracts, in which the secondary metabolites of interest were allowed to concentrate. With these extracts, a preliminary BSLA was carried out to exclude sponge samples without notable toxicity against *Artemia salina*. The initial screening was performed under simplified conditions (see chapter 3.3.3.1). Only those extracts, which showed significant activity against *Artemia salina* at concentrations of ≤ 500 µg/ml, were further investigated. They were tested in the BSLA under the extended conditions and LC_{50} values were determined.

For these two samples, which demonstrated the highest activity, a larger amount of crude extract was purified in order to obtain material for further investigation. The purified ethanol extracts were fractionated via vacuum liquid chromatography (VLC) (see chapter 3.3.2.1). The fractions were subjected to the BSLA and analyzed by means of thin layer chromatography (TLC) (see chapter 3.3.2.3).

Based on the results obtained by the BSLA, the extract of the marine sponge with the code 999 was finally selected for further investigation.

4.1.1. Small-scale purification of sponge samples

Small amounts of the crude CH_2Cl_2/MeOH (1:1) sponge extracts, absorbed on Diaion® HP-20 resin, were purified using a Supelco Visiprep™ SPE Vacuum Manifold as described in chapter 3.3.2.1. The respective yields are given in Table 4.

Table 4: Purification yields of 40 sponge extracts absorbed on approx. 2 ml resin. Fraction 1: water; fraction 2: EtOH; fraction 3: CH_2Cl_2/EtOH (1:1).

Sample number	**Fraction 1 (mg)**	**Fraction 2 (mg)**	**Fraction 3 (mg)**
983	269.3	**69.1**	81.9
984	161.5	**66.7**	69.9
985	41.0	**6.8**	12.5
986	100.9	**54.4**	89.3
987	10.3	**6.2**	14.6
988	120.1	**22.4**	43.4
991	51.2	**11.9**	22.8
992	74.2	**12.0**	21.4
994	40.5	**7.4**	12.8
995	61.5	**19.8**	32.6
997	4.4	**12.8**	16.2
998	60.6	**30.6**	21.1
999	69.2	**25.4**	43.1
1000	77.4	**11.7**	16.0
1006	61.7	**27.8**	31.6
1007	25.6	**11.7**	36.7
1008	27.3	**19.4**	23.9
1009	22.7	**7.4**	8.2
1016	133.5	**107.6**	80.7
1017	35.6	**71.9**	28.6

Sample number	**Fraction 1 (mg)**	**Fraction 2 (mg)**	**Fraction 3 (mg)**
1018	143.3	**58.7**	54.9
1019	150.9	**108.3**	124.0
1020	90.8	**77.4**	120.3
1021	201.4	**49.9**	76.9
1022	76.3	**70.3**	86.1
1023	36.8	**20.7**	34.8
1024	62.6	**24.7**	54.9
1344	108.3	**20.7**	46.2
1346	85.7	**30.6**	100.2
1347	211.3	**42.1**	94.2
1468	389.5	**105.3**	133.0
1469	51.1	**35.2**	49.3
1470	41.9	**27.8**	45.8
1471	96.9	**50.4**	87.0
1472	125.7	**89.1**	147.0
1473	165.9	**66.1**	108.7
1474	50.1	**33.7**	72.3
1475	32.6	**24.8**	40.5
1476	49.4	**47.0**	66.2
1477	53.9	**26.5**	34.7

4.1.2. Preliminary screening for cytotoxicity

The purified ethanol extracts (fraction 2) were subjected to the BSLA as described in chapter 3.3.3. Each sample was tested at concentrations of 500, 250, 125 and 62.5 µg/ml in duplicate. Table 5 shows the results of this initial selection step. The numbers indicate dead nauplii counted at each concentration of the respective sample.

Individually occurring deaths at any concentration were disregarded, as they could be considered as coincidence. At this stage, the obtained data were not sufficient for calculation of reliable LC_{50} values. Thus, samples were just classified as active (at least three deaths in both replicates, or more than five in one test at 500 µg/ml), or not active (ND, no detection). Eight samples showed significant activity (sample number 987, 988, 998, 999, 1016, 1018, 1347, and 1475), and were therefore chosen for further investigation. The significant higher death rate of sample number 1024 at lower concentrations can be explained by poor dissolution of the extract. Therefore it was also selected for further investigation.

In the blank sample (5% DMSO in artificial seawater), a survival rate of 100% was observed. An investigated mortality of 100% in the positive control (*Persea americana* seed extract) indicate an LC_{50} of < 62.5 µg/ml, which is in accordance with the results obtained by Leite et al. (2009).

Table 5: Activity of sponge samples (purified ethanol extracts) against *Artemia salina*. Numbers indicate dead nauplii counted. A: active; ND: no detection; R: retest.

Sample number	**500 µg/ml**	**250 µg/ml**	**125 µg/ml**	**62.5 µg/ml**	**Toxicity**
983	0 0	0 0	0 0	0 0	ND
984	0 0	0 0	0 1	0 1	ND
985	0 0	0 0	0 0	0 0	ND
986	0 0	1 0	0 0	0 0	ND
987	**8** **2**	**0** **0**	**0** **0**	**0** **0**	**A**
988	**7** **7**	**7** **6**	**5** **6**	**5** **6**	**A**
991	2 0	1 0	0 0	0 0	ND
992	0 0	0 0	0 0	0 0	ND
994	0 0	0 0	1 1	0 0	ND
995	0 0	1 0	0 1	1 1	ND
997	0 0	0 0	0 0	0 0	ND
998	**6** **9**	**5** **7**	**3** **4**	**2** **2**	**A**
999	**9** **9**	**7** **6**	**3** **4**	**2** **4**	**A**
1000	0 0	0 0	0 0	0 0	ND
1006	0 0	0 0	0 0	0 0	ND
1007	0 0	0 0	0 0	0 0	ND
1008	0 0	0 0	0 0	0 0	ND
1009	0 1	0 0	0 0	0 0	ND
1016	**8** **7**	**2** **3**	**1** **2**	**1** **2**	**A**
1017	2 0	0 0	0 0	0 0	ND
Blank	0 0	0 0	0 0	0 0	ND

Sample number	**500 µg/ml**	**250 µg/ml**	**125 µg/ml**	**62.5 µg/ml**	**Toxicity**
1018	**9** **7**	**5** **2**	**2** **2**	**2** **1**	**A**
1019	0 0	0 1	0 0	0 1	ND
1020	0 0	0 0	0 1	0 1	ND
1021	0 0	0 0	0 1	0 0	ND
1022	0 0	0 0	0 0	0 0	ND
1023	0 0	1 0	0 0	0 0	ND
1024	**1** **0**	**4** **1**	**5** **2**	**7** **3**	**R**
1344	0 0	0 0	0 0	0 0	ND
1346	0 0	0 1	0 0	0 0	ND
1347	**4** **3**	**2** **1**	**1** **1**	**1** **2**	**A**
1468	0 0	0 0	0 0	0 0	ND
1469	0 0	0 0	0 0	0 1	ND
1470	3 0	1 0	0 0	0 0	ND
1471	0 0	0 0	0 0	0 0	ND
1472	0 0	0 0	0 0	0 0	ND
1473	0 1	0 0	0 0	0 0	ND
1474	3 2	0 0	0 0	1 0	ND
1475	**8** **1**	**3** **0**	**0** **0**	**0** **0**	**A**
1476	1 0	1 0	0 0	0 0	ND
1477	0 0	0 0	0 0	0 0	ND
Persea	10 10	10 10	10 10	10 10	A

4.1.3. Determination of LC_{50} values for active samples

Those samples, which were classified as active in the preliminary screening, were subjected to the BLSA over the full range of concentrations (1000, 500, 250, 125, 62.5, 31.3, 15.6 and 7.8 µg/ml). LC_{50} values and 95% confidence were calculated via probit analysis for samples with steady results (see chapter 3.3.3.5). The results for the sponge samples examined in more detail can be seen from Table 6. Remarkably low LC_{50} values were calculated for sample 998 and 999 (191 µg/ml and 115 µg/ml respectively), which were therefore chosen for further investigation. Sample 1347 also showed significant activity with an LC_{50} value of 290 µg/ml. Sample 987 was moderately toxic (LC_{50} value of 725 µg/ml). For sample 988, 1016 and 1018 no reliable LC_{50} values could be determined, because they showed inconsistent activity within the dilution series and the repetitions, respectively. Two samples (1024 and 1475) showed low activity within the observed concentration range. Hence, no LC_{50} values could be calculated.

Table 6: LC_{50} values and 95% confidence intervals calculated from results obtained by the BSLA for further investigated samples.

Sample number	**LC_{50} (µg/ml)**	**95% confidence interval**
987	725	528-1178
988	*not evaluable*	
998	191	131-280
999	115	79-167
1016	*not evaluable*	
1018	*not evaluable*	
1024	> 1000	*not evaluable*
1347	290	194-459
1475	> 1000	*not evaluable*
Blank	*no detection*	
Persea	9 (binom.)	*not evaluable*

4.1.4. Purification and fractionation of two active samples

The crude extracts of the marine sponges with the code 998 and 999 were purified as described in chapter 3.3.2.2. The amounts of extracts recovered in this purification process are shown in the following table.

Table 7: Purification yields of two sponge extracts absorbed on approx. 80 ml resin. Fraction 1: water; fraction 2: EtOH; fraction 3: CH_2Cl_2/EtOH (1:1).

Sample number	**Fraction 1 (mg)**	**Fraction 2 (mg)**	**Fraction 3 (mg)**
998	1831	**1716**	893
999	1698	**1579**	682

The purified ethanol extracts (fraction 2) were further fractionated by VLC (see chapter 3.3.2.1). The respective yield of each fraction is given in Table 8.

Table 8: Fractionation yields of approx. 5 ml HP-20 resin charged with purified extract. Fraction 1: EtOH/water 2:8; fraction 2: EtOH/water 1:1; fraction 3: EtOH/water 8:2; fraction 4: EtOH; fraction 5: isopropanol/CH_2Cl_2 8:2.

Sample number	**Fraction 1 (mg)**	**Fraction 2 (mg)**	**Fraction 3 (mg)**	**Fraction 4 (mg)**	**Fraction 5 (mg)**
998_2	17.9	28.6	30.4	71.0	155
999_2	14.9	16.7	50.4	49.1	108

4.1.5. Cytotoxicity assay of VLC fractions

The fractions of the samples 998 and 999 obtained by VLC were tested for their toxicity at eight different concentrations by the BSLA in triplicate. Testing conditions are described in chapter 3.3.3.1. LC_{50} values and 95% confidence intervals of the fractions which demonstrated toxicity can be seen from Table 9.

Table 9: LC_{50} values and 95% confidence intervals calculated from results obtained by the BSLA for the samples 998 and 999.
998_2 and 999_2: Purified ethanol extracts.
2-1 to 2-5: VLC fractions of the corresponding extract.

Sample number	**LC_{50} (µg/ml)**	**95% confidence interval**
998_2	203	141-297
998_2-1	*no detection*	
998_2-2	*no detection*	
998_2-3	*no detection*	
998_2-4	680	492-1097
998_2-5	71	51-99
999_2	94	63-140
999_2-1	*no detection*	
999_2-2	*no detection*	
999_2-3	50	31-125
999_2-4	37	27-49
999_2-5	203	127-348

For sample 998 no toxicity was observed in the fractions 1, 2, and 3 (eluted with EtOH/water mixtures of different percentage). Fraction 4 (EtOH) caused dead nauplii at 1000 and 500 µg/ml, but the major toxicity was detected in the least polar fraction (fraction 5, isopropanol/CH_2Cl_2 (8:2)) with an LC_{50} of 71 µg/ml. Sample 999 was significantly more toxic, showing LC_{50} values of 50 µg/ml, 37 µg/ml and 203 µg/ml in fraction 3 (EtOH/water (8:2)), 4 (EtOH) and 5 (isopropanol/CH_2Cl_2 (8:2)) respectively. Based on these results, sample 999 was selected for further fractionation.

4.1.6. Thin layer chromatography (TLC)

The fractions of sample 999 obtained in the purification process (999_1, 999_2, 999_3) and the fractions obtained via VLC (1-5, sub-fractions of 999_2) were further analyzed by TLC as described in chapter 3.3.2.3. The plates were developed with three different mobile phases and detected under UV light and subsequently with two spray reagents.

Butanol – glacial acetic acid – water (4:1:1) turned out to be the most suitable solution for the development of the plates, and vanillin-sulfuric acid was the more expedient visualization reagent. The purple spots with an R_f value of 0.56 were most probably the substance of interest, as toxicity for the fractions showing this spot was detected in the BSLA. Further, the intensity of the spots correlated with the activity of the respective fractions (compare marked spots and LC_{50} values of the corresponding fractions in Figure 23).

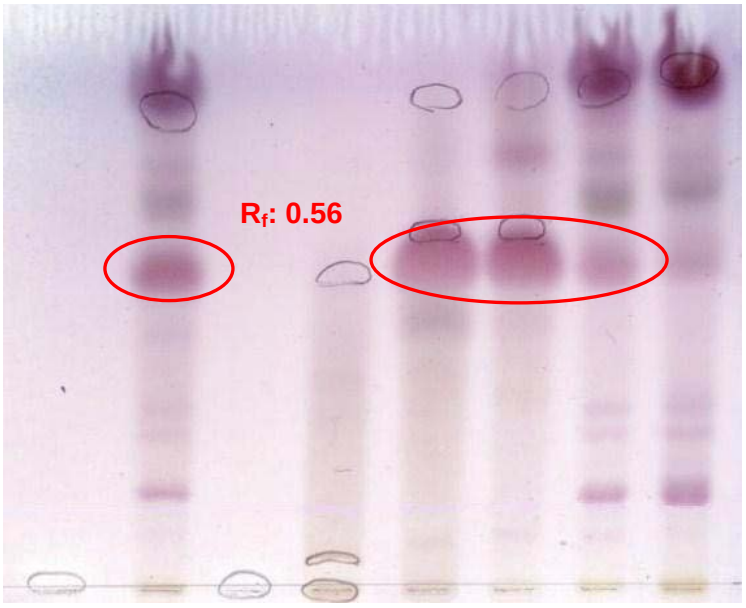


Figure 23: TLC of fractions obtained by VLC.
Stationary phase: Silica gel F_{254}
Mobile phase: Butanol – glacial acetic acid – water (4:1:1)
Detection: UV_{254}, vanillin-sulfuric acid reagent

4.2. Isolation of the active compound of sample 999

As a first approach, high performance liquid chromatography (HPLC) and semi-preparative HPLC were applied for further fractionation of sample 999. Finally, the industrial HPLC system BioXplore© was used to separate the purified ethanol extract. The individual fractions obtained by the HPLC separations were tested in the BSLA, and TLC was used to monitor the process.

4.2.1. Semi-preparative HPLC

After fractionation of the purified sponge extracts via VLC and cytotoxicity testing of the resulting fractions against *Artemia salina*, the most toxic fraction 999_2-4 was further characterized by HPLC as described in chapter 3.3.2.4. In a first step, an analytical separation method was developed for further application of the semi-preparative fractionation. The chromatogram obtained by this method is shown in Figure 24. Six fractions (shown in different colors in the figure below) were collected manually in several runs from a total amount of approximately 20 mg.

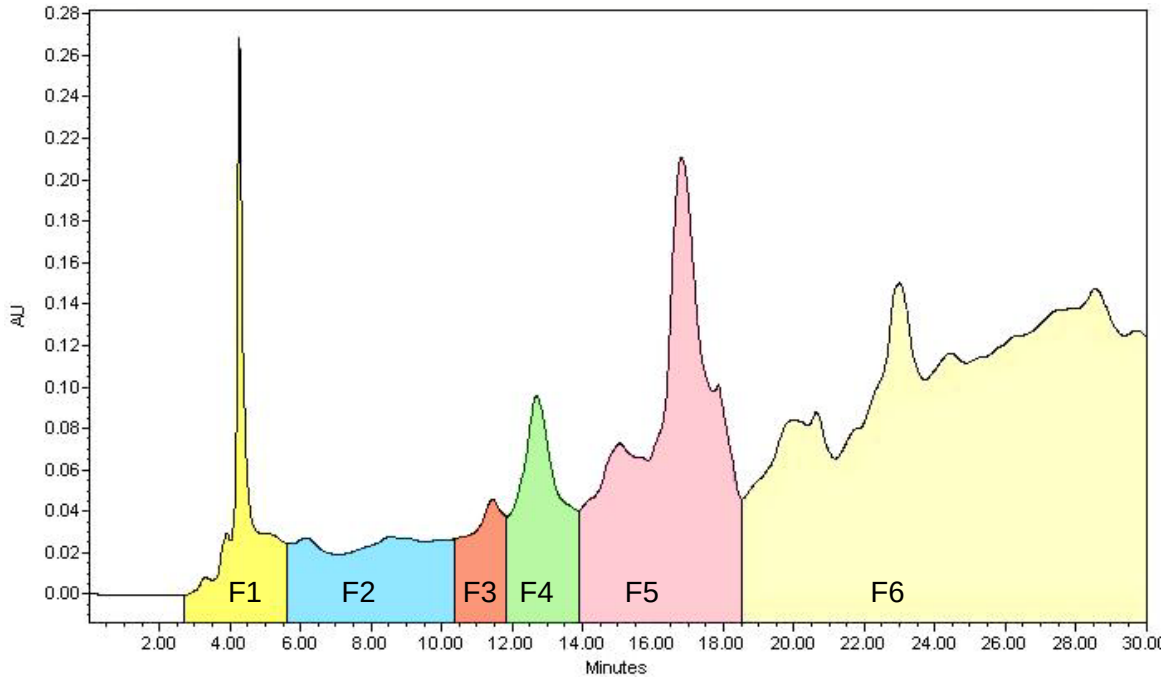


Figure 24: HPLC chromatogram of fraction 999_2-4; conditions as stated in chapter 3.3.2.4.

The fractions obtained by semi-preparative HPLC were evaporated to dryness and weighed. After purification steps via re-dissolving in methanol and filtrating with Supelco Supelclean™ LC-18 SPE tubes (bed wt. 500 mg, volume 6 ml) and Acrodisc® LC PVDF syringe filters (0.45 µm, 25 mm), the recovery was approximately 200%. As it was not significantly affecting the final results, no further separation of the contaminant was necessary.

4.2.2. Cytotoxicity assay of HPLC fractions

The activity of the HPLC fractions against *Artemia salina* was tested at eight different concentrations in triplicate, and LC_{50} values were calculated for the toxic fractions. The obtained results are shown in Table 10. Fraction 5 and 6 showed activity in the BSLA, with similar LC_{50} values compared to the unfractionated extract. This can be explained by dilution with the non-filterable contaminant (see above).

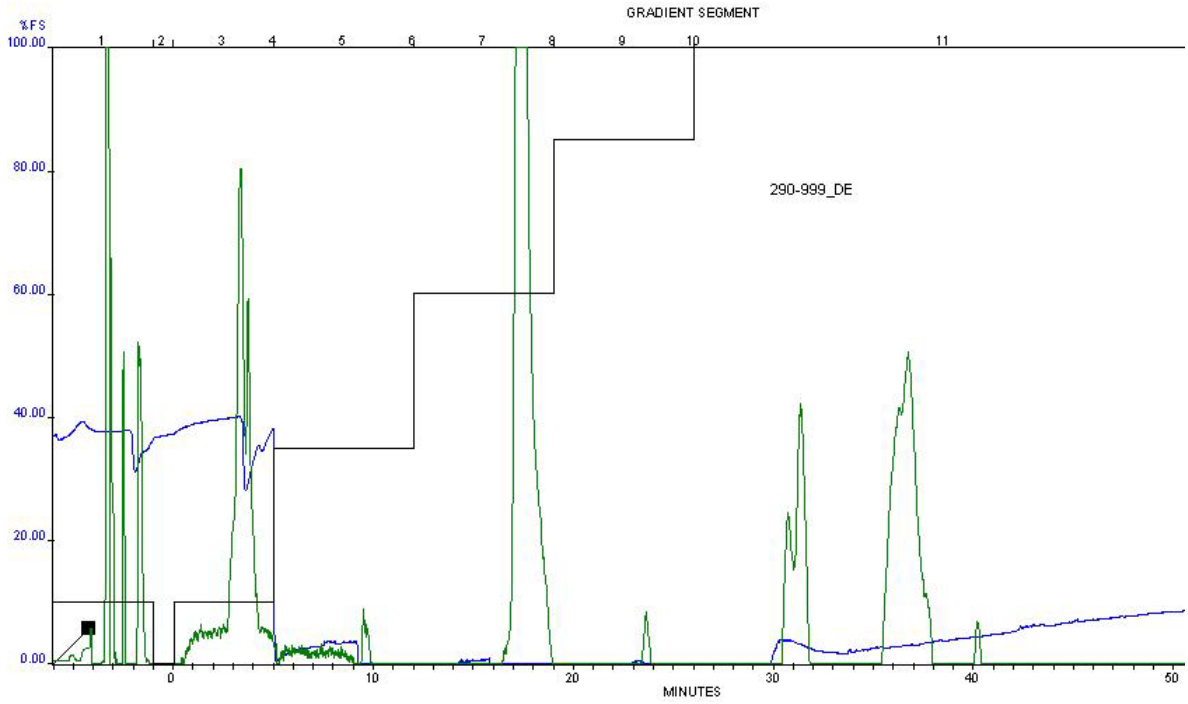
Table 10: LC_{50} values and 95% confidence intervals calculated from results obtained by the BSLA for HPLC fractions.

Sample number	**LC_{50} (µg/ml)**	**95% confidence interval**
999_2-4	114	63-250
999-HPLC_1	*no detection*	
999-HPLC_2	*no detection*	
998-HPLC_3	*no detection*	
999-HPLC_4	*no detection*	
999-HPLC_5	136	102-182
999-HPLC_6	111	81-151

The chromatogram obtained by UV detection (see Figure 24) did not show a specific peak which could be assigned to the toxic component. Therefore, in a next step the preparative HPLC system BioXplore© was applied, which was equipped with an evaporative light scattering detector (ELSD) in addition to a variable wavelength detector.

4.2.3. Fractionation via BioXplore© technology

The advantage of the BioXplore© technology was used in order to separate more fractions from more volume within less time. Approximately 50 mg of purified sponge extract 999_2 were fractionated by means of the BioXplore© HPLC unit (see chapter 3.3.2.5 for system information). The extract was dissolved in 20 ml EtOH, and the whole solution was injected. Elution was achieved with a step gradient of EtOH and water as described in chapter 3.3.2.5. Six fractions were collected based on the signal provided by the ELSD (green peaks in the figure below), evaporated to dryness and weighed.



	F 1	F 2	F 3	F 4	F 5	F 6
min	1-10	16-19	23-24	30-32	35-38	40-41
weight (mg)	19.7	17.2	1.8	6.1	7.0	1.8

Figure 25: HPLC chromatogram of the purified sponge extract 999_2. BioXplore© fractionation technology, water/EtOH step gradient; see chapter 3.3.2.5 for further conditions. Green peaks: ELSD signal; blue: UV-detection. ELSD peak-based collection of six fractions at the times indicated.

ELS detection indicated clearly the elution of the compounds, and signal strength correlated well with the yielded amounts (compare size of the green ELSD peaks and corresponding weights given below).

4.2.4. Cytotoxicity assay of BioXplore© fractions

The fractions obtained through separation by BioXplore© technology were tested in the BSLA at eight different concentrations in triplicate, and LC_{50} values were calculated. Table 11 shows the results of this assay.

Table 11: LC_{50} values and 95% confidence intervals calculated from results obtained by the BSLA for BioXplore© fractions of the purified sponge extract 999_2.

Sample number	**LC_{50} (µg/ml)**	**95% confidence interval**
999_2	162	110-241
999-BX_1	*no detection*	
999-BX_2	73	31-125
998-BX_3	*no detection*	
999-BX_4	*no detection*	
999-BX_5	*no detection*	
999-BX_6	*no detection*	

Fraction 2, which was collected from minute 16-19, yielded an LC_{50} of 73 µg/ml and was the only toxic fraction. For further analysis of this fraction and for comparison of the VLC-, HPLC- and BioXplore©-fractions among each other, TLC was applied.

4.2.5. Comparison of fractions via TLC

The fractions obtained by VLC, semi-preparative HPLC and BioXplore© fractionation were further identified by TLC. As mobile phase, butanol – glacial acetic acid – water (4:1:1), which already proved to be appropriate for the VLC fractions (see chapter 4.1.6, Figure 23), was used. Visualization was carried out via vanillin-sulfuric acid reagent. In the obtained chromatogram (Figure 26), a spot with an R_f value of 0.53 could be seen at each fraction for which toxicity in the BSLA has been detected.

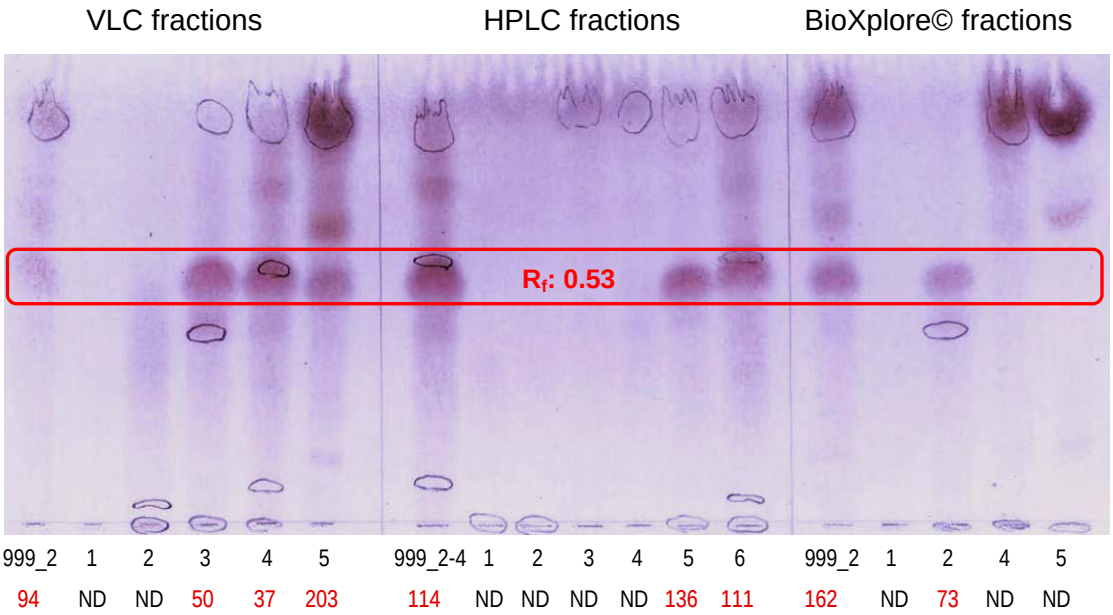


Figure 26: TLC of the fractions obtained by VLC, semi-preparative HPLC and BioXplore© fractionation.

Stationary phase: Silica gel F_{254}
Mobile phase: Butanol – glacial acetic acid – water (4:1:1)
Detection: UV_{254}, vanillin-sulfuric acid reagent

LC_{50} values (µg/ml) as calculated in chapter 4.1.5 (VLC fractions), chapter 4.2.2 (HPLC fractions) and chapter 4.2.4 (BioXplore© fractions).

4.2.6. Cellular studies

The results of the cellular studies (carried out by Dr. Frederick Valeriote, Ford Cancer Center, Detroit, USA) for the extracts of the marine sponge with the code 999 can be seen from Table 12. Murine L1210 and human CCRF-CEM are leukemia cells, murine colon 38, human colon H116 and human lung H125 are solid tumor cells, and murine CFU-GM is a normal cell. An extract is of sufficient activity to be of further interest, if the zone of inhibition exceeded 300 units (see chapter 3.3.4). The methanol extract of sample 999 was of significant toxicity to all cell types which were tested.

Table 12: Results obtained by the cellular studies for sample 999.

Cell types	***MeOH extract (units)***	***CH_2CL_2/MeOH extract (units)***
Murine L1210	650	250
Human CCRF-CEM	550	—
Murine colon 38	650	350
Human colon H116	500	100
Human lung H125	500	200
Murine CFU-GM	550	200

5. DISCUSSION

Marine sponges are well known to produce structurally diverse cytotoxic compounds as a defense strategy. These bioactive secondary metabolites are an important source of novel pharmacologically active agents. They provide lead compounds for the treatment of a variety of human diseases, most of them showing antitumor potential (Hentschel & Bringmann 2010). In the present study, 40 extracts of marine sponges were tested for their cytotoxicity, and the sponge extract with the highest toxicity was selected for further investigation. The active component of this extract was isolated via bioassay guided fractionation. The bioassay applied to investigate the activity of marine sponges was the brine shrimp lethality assay (BSLA).

The sponges studied in this work were collected in 2002 and 2003 in the Caribbean Sea of Costa Rica and extracted via ultrasonic-assisted solvent extraction. Ultrasonic-assisted solvent extraction is a modified maceration method, where the extraction of metabolites is facilitated and yields are improved. Ultrasonication induces a mechanical stress on the cells through formation of cavitations in the sample, resulting in cellular breakdown and thus increased solubilization of intracellular metabolites (Seidel 2006). The solvents mainly used for sponge extraction are MeOH (Bao et al. 2007; Ridley & Faulkner 2003) and a 1:1 mixture of MeOH and CH_2Cl_2 (Coello et al. 2009; Pettit et al. 2004), respectively. Further, EtOH (Oku et al. 2003) and mixtures of EtOH/$CHCl_3$ have been used (Ponomarenko et al. 2007). In this study, the solvent mixture CH_2Cl_2/MeOH (1:1) was chosen to obtain a broad spectrum of secondary metabolites in terms of polarity.

For purification and fractionation of the sponge extracts, vacuum liquid chromatography (VLC) was applied. The development of VLC proceeded in the 1970s, arousing from the need to have a simple, inexpensive chromatographic system capable of producing good resolution in a short time. Coll et al. first investigated the potential of this technique in 1977 by applying it to the isolation of a cembrenoid diterpene from an Australian soft coral. Detailed descriptions of the method were published some years later (Targett et al. 1979; Coll & Bowden 1986). Manipulations, e.g. solvent changes are easy, because the head of the column is at atmospheric pressure (Hostettmann et al. 1998). The application of VLC increased in the last decade, mainly for the fractionation of natural products prior to other chromatographic systems such as medium-pressure liquid chromatography (MPLC) and

high performance liquid chromatography (HPLC). In some cases it is used as the only separation step (Sticher 2008).

Purification of the crude sponge extracts was carried out via elution with water, EtOH and CH_2Cl_2/EtOH (1:1) in order to obtain purified ethanol extracts, in which the secondary metabolites of interest were allowed to concentrate. Through this extraction steps, highly polar substances, e.g. salts and sugars, and non-polar substances, e.g. fats were separated. Small-scale purification of 40 sponge extracts was performed with a Supelco Visiprep™ SPE Vacuum Manifold, 12-Port Model, which allowed the purification of up to 12 sponge extracts simultaneously. It therefore helped to obtain purified material for the initial cytotoxicity screening via the BSLA within a short time.

The BSLA is a useful tool for the preliminary toxicity assessment (Carballo et al. 2002; Lopes et al. 2010). It was proposed by Michael et al. (1956), later developed by Vanhaecke et al. (1981) and Sleet and Brendel (1983). The BSLA is based on the ability of test materials to kill laboratory-cultured *Artemia salina* (brine shrimp) nauplii. It allows to determine LC_{50} values of active compounds and extracts and to detect a broad spectrum of pharmacologic activities. The method is rapid, reliable and inexpensive, and is therefore a very suitable bioassay for screening and fractionation monitoring of active materials (Meyer et al. 1982).

Brine shrimp are typically found in hypersaline lakes and ponds, habitats of low species diversity and simple trophic structures (Lenz & Browne 1991). They possess uncommon adaptability to extreme environmental conditions such as large ranges of salinity (5-250 g/L) and temperature (6 to 36°C), as well as varied nutrient sources. Reproduction can be performed either sexually or parthenogenetically, with nauplii or cysts production. Nauplii are free-swimming larvae spawned under optimal conditions. Resistant embryos (cysts) are produced when environmental conditions get unfavorable, such as increase in salinity with eventual desiccation and decreasing temperatures (Nunes et al. 2006). Dried cysts of the brine shrimp can be stored for years without losing their viability, and, upon immersion in seawater, the nauplii will hatch within 24 hours (Vanhaecke et al. 1981) and can be used for the BLSA.

Toxicity tests with the crustacean *Artemia salina* have been applied in many different fields, e.g. for the detection of fungal toxins (González et al. 2007), cyanobacteria toxins (Lopes et al. 2010), plant extract toxicity (Krishnaraju et al. 2006; Kusuma et al. 2011),

detection of pesticides (Bustos-Obregon & Vargas 2010) and warfare agents (Lumor et al. 2011), cytotoxicity testing of dental materials (Milhem et al. 2008) and marine natural products (Carballo et al. 2002; Mansoor et al. 2007), and in ecotoxicity testing (Nunes et al. 2006). In contrast to the mainly used BSLA, an alternative assay with *Artemia salina* is based on the inhibition of hatching of the cyst (hatchability assay) (Carballo et al. 2002). In the lethality assay - which was performed in the present work - *Artemia salina* larvae were exposed to test samples in saline solution and lethality was evaluated after 24 hours. The inexpensiveness and commercial availability of *Artemia salina* cysts, as well as the low cost and ease of performing the test, make the brine shrimp lethality assay a very useful bench-top method (Krishnaraju et al. 2006).

In the present work, the BSLA was performed in 96-well microtiter plates as developed by Solis et al. (1993). Other tests, carried out in petri dishes (Vanhaecke et al. 1981), vials (Meyer et al. 1982) or multiwell culture plates (Carballo et al. 2002) use sample solutions of 5 to 10 ml. Disadvantages thereof are, that relatively large amounts of test material are required and preparation of the dilutions is time-consuming. In contrast to these methods, the microplate assay is performed with small sample volumes (e.g. 200 µl). The varying concentrations of test solutions are directly prepared in the microplate wells via serial dilutions. The employment of micro-technique therefore allows an economic screening of large numbers of samples and dilutions (Solis et al. 1993).

In the BSLA, the cytotoxicity was evaluated on an extract of *Persea americana* (avocado) seeds, which was used as positive control. Activity of *Persea americana* seed extracts in the BSLA was investigated by Leite et al. (2009). In the preliminary screening, the detected activity for 2 samples (2 replicates each) differed widely (8 and 2 dead nauplii in sample 987, 8 and 1 in sample 1475 at a concentration of 500 µg/ml). The solubilizer used was 5% DSMO. The high variability of the results may be due to poor dissolution and inhomogeneity of the test solutions respectively. Since DSMO was not the appropriate solvent for all sponge extracts, other solvents, e.g. MeOH (as used by Meyer et al. 1982) could be tested in further studies.

From a total of 40 sponge extracts, two of the samples (998 and 999) demonstrated high toxicity (LC_{50} values < 200 µg/ml) with high reproducibility. These two samples were further fractionated via VLC, the obtained fractions were subjected to the BSLA and LC_{50} values were calculated. The samples 998 and 999 demonstrated activities against *Artemia salina*

in two and in three fractions, respectively. Sample 998 showed low toxicity in fraction 4 (EtOH) with an LC_{50} value of 680 µg/ml and high toxicity in fraction 5 (isopropanol/CH_2Cl_2 (8:2)) with an LC_{50} value of 71 µg/ml. Fraction 3 (EtOH/water (8:2)), 4 (EtOH) and 5 (isopropanol/CH_2Cl_2 (8:2)) of sample 999 showed high toxicity with LC_{50} values of 50 µg/ml, 37 µg/ml and 203 µg/ml, respectively. Based on these results, sample 999 was chosen for further investigation.

The fractions of sample 999 obtained by VLC were further analyzed via thin layer chromatography (TLC). TLC is a simple, rapid and inexpensive separation method which can be used both as an analytical and a preparative technique. With regard to natural product isolation, analytical TLC is widely applied for the detection of compounds through several separation steps. It further helps to select an appropriate system for column chromatography, and assists in assessing the degree of purity of isolated compounds (Houssen & Jaspars 2006). In the chromatogram obtained from the VLC fractions of sample 999 (see chapter 4.1.6, Figure 23), a spot with an R_f value of 0.56 could be seen in the fractions 3 and 4, which showed high toxicity in the BSLA. The spot was also visible - to a lesser extent - in fraction 5, which showed lower toxicity. As the intensity of the spots directly correlates with the activity in the BSLA, this spot is most likely the toxic component.

For further characterization and separation of sample 999, the most toxic fraction (fraction 4) obtained by VLC was first subjected to analytical HPLC separation, further semi-preparative HPLC was applied. HPLC is a powerful separation technique which has become a mainstay for natural product isolation and purification, regardless to their source. Due to various modes available, e.g. normal-phase, reversed-phase, size exclusion, and ion exchange HPLC, it is applicable to purify most classes of natural products. Reversed-phase HPLC (RP-HPLC) is the most common technique used for analysis and purification of compounds from a complex matrix, especially when the identity of the compounds of interest is unknown (Latif 2006). The fractions obtained by semi-preparative HPLC were subjected to the BSLA and LC_{50} values were calculated. Compared to the results of the VLC fractions obtained by the cytotoxicity assay, the LC_{50} values were consistently higher. The decrease of toxicity in the VLC fraction (999_2-4, LC_{50} 37 µg/ml vs. 114 µg/ml) may be due to instability of the active component. The toxic HPLC fractions showed similar activity compared to the unfractionated extract, which can be explained by dilution with the non-filterable contaminant (see chapter 4.2.2).

For further separation of sample 999, the advantage of the BioXplore© Varian equipment to separate more fractions from more volume within less time was used. The BioXplore© is an industrial HPLC system which can be applied for removing concomitant compounds such as salts, carbohydrates and major fatty acids of crude extracts, as well as for fractionating different types of samples. Plant material, marine organisms, culture broths and other extracts can be separated. Up to 48 fractions are automatically collected within one run, optionally by time or by volume. Columns with a capacity of 500 ml or 1000 ml can be applied, providing a loading capacity of several grams. The unit is equipped with two detectors in parallel, a variable wavelength detector and an evaporative light scattering detector (ELSD). In contrast to UV detection, which depends on the capacity of compounds to absorb light, the extinction coefficients of the analytes have no effect on the response of ELSD. The signal resulting from ELSD directly correlates with the concentration of compounds present in the sample (Houssen & Jaspars 2006). From the chromatogram obtained by separation with the BioXplore© instrument can be seen, that the ELSD signal clearly indicates the elution of the compounds, whereas almost no UV signal could be observed. As the compounds present in the extract are not UV absorptive, the chromatogram obtained by the Waters HPLC instrument with solely UV detection did not show the elution of the toxic substance.

The fractions of the BioXplore© separation were collected based on the ELSD signal and tested via the BSLA. For fraction 2, the peak collected from minute 16-19, an LC_{50} value of 73 µg/ml was calculated. Decrease of toxicity was clearly observable for the unfractionated sponge extract 999_2, which showed an LC_{50} value of 162 µg/ml vs. 94 µg/ml in the BSLA performed with the VLC fractions (see chapter 4.1.5, Table 9). A similar decrease of toxicity could already be seen in the BSLA for the HPLC fractions and was probably due to instability of the active component.

Finally, the fractions obtained by VLC, HPLC and BioXplore© technology were subjected to TLC. The obtained chromatogram showed spots with an R_f value of 0.53. This characteristic spot could be seen in each fraction, for which toxicity in the BSLA has been observed. Hence, this substance (fraction 2 of the BioXplore© fractionation) could be identified as the toxic component.

In former cellular studies carried out by Dr. Frederick Valeriote (Ford Cancer Center, Detroit, USA), high toxicity for the methanol extract of sponge sample 999 against several human and murine cell lines (see chapter 4.2.6, Table 12) has been investigated.

The results obtained in this work proved high toxicity of the investigated marine sponge against *Artemia salina,* and demonstrated the great potential of bioassay guided fraction for the isolation of pharmacologically active compounds from the complex matrixes of natural products. The present study further confirms the suitability and efficiency of the BSLA carried out as a microplate assay for the assessment of cytotoxicity. Additionally, the great capacity of the BioXplore© technology for the fractionation of extracts has been shown.

In further studies, the validation of the purity and the structural elucidation of the cytotoxic compound via nuclear magnetic resonance (NMR) spectroscopy are being accomplished by the research team of the Bioprospecting Strategic Action Unit of INBio.

6. SUMMARY

Marine sponges (Porifera) comprise a rich source of novel pharmacologically active compounds. A major part of them shows cytotoxic properties, hence, these substances are potential antitumor agents.

The aim of this study was the isolation of the toxic component from a selected sponge extract after preliminary screening a potential toxic activity of a set of marine sponges. Therefore, purified ethanol extracts of 40 Caribbean marine sponges, which were obtained by ultrasonic-assisted solvent extraction with CH_2Cl_2/MeOH (1:1) and purified via vacuum liquid chromatography (VLC), were tested applying the brine shrimp lethality assay (BSLA). The BSLA, which was carried out as a microplate assay, allowed to determine the toxicity of the marine sponge extracts against *Artemia salina* (brine shrimp) larvae and also, to calculate LC_{50} values via probit analysis. The extract with the highest toxic activity, which was obtained from a marine sponge of the order Haplosclerida, showed an LC_{50} value of 94 µg/ml and was further investigated via bioassay guided fractionation.

The isolation of the toxic component from the selected sponge extract was performed by various chromatographic methods. VLC was applied for initial fractionation, and further separation was carried out via high-performance liquid chromatography (HPLC). The fractionation of the individual components was finally achieved by means of the preparative HPLC system BioXplore© (Varian). The individual fractions obtained by VLC and HPLC were tested for their toxicity via the BSLA. Further, thin layer chromatography (TLC) was used to monitor the fractionation processes. Based on the results obtained by the BSLA, one specific BioXplore©-fraction was identified as the toxic component of the investigated sponge extract.

This study was considered as a highly important basic work for further structural elucidation of the compound of interest, which is being accomplished at the Bioprospecting Strategic Action Unit of INBio.

7. ZUSAMMENFASSUNG

Meeresschwämme (Porifera) stellen eine reiche Quelle neuartiger pharmakologisch aktiver Verbindungen dar. Ein großer Teil davon zeigt zytotoxische Eigenschaften, weshalb diese Substanzen als potenzielle Antitumor-Wirkstoffe gelten.

Ziel dieser Arbeit war, die toxische Komponente eines Meeresschwamm-Extrakts zu isolieren, welcher zuvor aus einem Set von Extrakten mittels Toxizitäts-Screening ausgewählt wurde. Dazu wurden aufgereinigte Ethanol-Extrakte von 40 karibischen Meeresschwämmen, die durch Extraktion mit dem Lösungsmittelgemisch CH_2Cl_2/MeOH (1:1) gewonnen und mittels Vakuum-Flüssigchromatographie (VLC) aufgereinigt wurden, anhand eines Bioassays (brine shrimp lethality assay, BSLA) auf Zytotoxizität getestet. Mit dem BSLA, welcher in Form eines Mikroplatten-Assays durchgeführt wurde, konnte die Toxizität der Extrakte gegenüber *Artemia salina* (Salinenkrebs)-Larven bestimmt und LC_{50}-Werte anhand der Probit-Analyse berechnet werden. Der Extrakt mit der stärksten toxischen Aktivität, gewonnen von einem Meeresschwamm der Ordnung Haplosclerida, zeigte einen LC_{50}-Wert von 94 µg/ml und wurde mittels Bioassay-geleiteter Fraktionierung näher untersucht.

Die Isolierung der toxischen Komponente des ausgewählten Meeresschwamm-Extrakts erfolgte mit Hilfe verschiedener chromatographischer Methoden. Begonnen wurde mit einer Fraktionierung mittels VLC, und die weitere Auftrennung erfolgte anhand von Hochleistungs-Flüssigchromatographie (HPLC). Die Separation der einzelnen Komponenten wurde schließlich mit Hilfe des präparativen HPLC-Systems BioXplore© (Varian) erreicht. Die mit Hilfe von VLC und HPLC erhaltenen Fraktionen wurden im BSLA auf ihre Toxizität getestet und der Fraktionierungs-Prozess mittels Dünnschicht-chromatographie (TLC) überprüft. Basierend auf den Ergebnissen des BSLA konnte eine spezifische BioXplore©-Fraktion als die toxische Komponente des untersuchten Meeresschwamm-Extrakts identifiziert werden.

Diese Arbeit stellte die Grundlage für die Strukturaufklärung der zytotoxischen Verbindung dar, welche an der Bioprospecting Strategic Action Unit von INBio in einer weiteren Studie durchgeführt wird.

8. REFERENCES

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FIGURES

Page

TABLES

Page

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Ausbildung

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Fremdsprachen:

Englisch: sehr gut
Spanisch: gut