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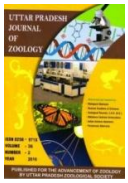

MARINE TOXINS (1940-2017)

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration between both authors. Author FJTM designed the study, wrote the protocol, and wrote the first draft of the manuscript. Author AGB managed the analyses of the study and the literature searches. Both authors read and approved the final manuscript.

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ABSTRACT

Marine toxins are secreted by marine organisms in order to act as a deterrent against attack from predators, parasites and fouling organisms. This paper gives a historical overview of the discovery of these chemical compounds - marine toxins –from 1940 till 2017 and describes the chemical structure of the main toxic substances as well as the analytical methods used for their identification. The use of this method as a means of studying the interaction between marine organisms and their environment, as well as evaluating the function and health of organisms at a molecular level has a number of benefits. As a consequence, there has been an increase in the application of chemical structural analysis in the marine sciences. This study draws attention to how complex chemical interactions in the marine environment are and why in depth research is required.

Keywords: Marine; natural; products; allomones; toxins.

1. INTRODUCTION

Marine organisms discharge chemical substances, called coactones or semiochemicals, into the marine environment and these allelochemicals can then affect the behaviour of organisms of the same (or different) species. This phenomenon is known as allelopathy [1]. When an organism from the same species is affected it is regarded as intra-specific allelopathy and the chemicals are called auto-toxins or pheromones while a reaction in another species is referred to as inter-specific allelopathy and the substances are known as allomones or kairomones. In addition, allomones are coactones which benefit the organism which produces them but not those which receive them. Many examples have been described in the marine environment, including toxins themselves, digestibility reducing factors, repellents, feeding-deterrents, anti-fouling compounds, and cytotoxins [1].

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Table 1. The main marine toxins discovered after1940

Toxin	Organism	Activity	Author and year of the discovery
Acetylcholine	*Buccinum undatum* (marine snail)	Inhibitory effect on heart of fish	Meyer, 1940 [2]
Murexine	*Murex trunculus* (marine snail)	Acetylcholine type activity. Neuromuscular blockade.	Erspamer et al., 1946 [3]
Tetrodotoxin	*Sphoeroides rubripes* (globefish)	Potent neurotoxin. It block sodium channels	Tsuda & Kawamura, 1953 [4]
Holothurin A	*Actinopyga agassizi* (sea cucumber)	Neurotoxin	Nigrelli et al., 1955 [5]
Senecioylcholine	*Thais floridana* (marine snail)	Murexine type activity	Whittaker, 1957 [6]
Nereistoxin	*Lumbriconereis heteropoda* (marine worn)	Neurotoxin	Okaichi & Hashimoto, 1962 [7]
Aplysine-20	*Aplysia kurodai* (marine slug)	Hypersalivation, ataxia, respiratory failure	Matsuda et al., 1967 [8]
Anabaseine	*Paranemertes peregrina* (marine worn)	Paralyzing factor	Kem, 1971 [9]
Saxitoxin	*Gonyaulax spp.* (red tide dinoflagellates)	Neurotoxin, insecticide	Wong et al., 1971 [10]
Surugatoxin	*Babylonia japonica* (marine snail)	Vision and language problems in people that eat shell-fish	Kosuge et al., 1972 [11]
Brevetoxin B	*Gymnodinium breve* (red tide dinoflagellates)	Neurotoxin	Lin et al., 1981 [12]
Pahutoxin	*Lactophrys triqueter* (trunkfish)	Murexine type activity	Goldberg et al., 1984 [13]
Tiglylcholine	*Thais clavigera* (marine snail)	Murexine type activity	Shiomi et al., 1998 [14]
Karmitoxin	*Karlodinium armiger* (marine dinoflagellate)	Cytotoxic and crustaceocide	Rasmussen et al., 2017 [15]

Toxic marine organisms have been known about for centuries. They were used by primitive cultures as weapons, as agents for ritual torture, and in medicine. As a result of this activity, the dangers of certain marine organisms are well documented. In recent years, much biological research has been carried out at molecular level, and marine toxins have been shown to be not only structurally unusual, but also to have interesting mechanisms of action. Thus, these toxins have become important research tools and interest in using such chemical compounds as biologically active substances has increased. Indeed, each of the three major seafood intoxications –red tides, pufferfish poisoning, and ciguatera- has presented a characteristic set of stimulations that have encouraged to further research. Consequently, the study of marine toxins has already contributed to a greater understanding of biochemical and disease mechanisms [16,17].

Nowadays, modern techniques of qualitative, quantitative and chemical structural analysis are used with ease to describe marine toxins, but the first chemical structure was analysed in the first half of the twentieth century when various methods for extracting and purifying organic chemical compounds first appeared. Thus, the investigation of the chemistry of marine toxins dates back to 1940. Table 1 shows the most significant dates, findings and participants in the development of this research since then.

2. METHODOLOGY

The data in this paper was compiled from a number of authentic online data bases, such as *Chemical Abstracts*, now part of *Scifinder* and *MarinLit*®, published by the University of Canterbury (New Zealand). A comprehensive bibliographic search was carried out using the keywords “marine and natural and products” and refining with the word “toxin”. Once the results were obtained, any duplicates were excluded. As a didactic method to improve the understanding of the manuscript, two molecular structures were added (Figs. 1 and 2).

3. THE HISTORY OF RESEARCH INTO MARINE TOXINS

In effect, everything began in 1940 when it was discovered that an extract obtained from the heart of a marine invertebrate , the slug *Buccinum undatum*, had an inhibitory effect on heart samples from two fish, *Raja clavata* and *Gadus morrhua*. Acetylcholine was the chemical substance responsible for this reaction [2].

In 1946 another choline derived compound, murexine was isolated in the hypobranchial glands of several marine snails, namely *Murex trunculus*, *Murex brandaris*, and *Tribonalia erinacea*. The toxin was found to have a concentration of 3-4 mg per gram of animal tissue, and was crystallized from acetone as a chloride. The product showed a high level of activity of acetylcholine [3].

In 1953 the deadly toxin found in the ovaries of Japanese pufferfish was isolated and given the name tetrodotoxin (TTX) [4]. A literature review concerning research into this toxin shows how problems related to its crystallization and understanding of its chemical structure were finally resolved by Tsuda, Hirata, Woodward, and Mosher [18] and the tetrodotoxin structure was revealed at the "International Symposium on the Chemistry of Natural Products", held in Kyoto (Japan) in 1964. It had become clear over time that the toxin is not produced by the pufferfish itself but by bacteria of the genus *Alteromonas*, *Vibrio* and *Shewanella*. The fish simply accumulates it in its tissues. Pharmacokinetic studies (absorption, distribution and accumulation) on the genus *Takifugu* were reported, and revealed that a higher concentration of TTX accumulates in the liver [19]. Other toxins with chemical structures similar to that of TTX were later found in prawns, fish, annelid worms and algae. In 1964 it was suggested that physiologically these toxins block sodium channels in human beings [20]. Since this discovery, tetrodotoxin has been widely used as a research tool in pharmacological and physiological research. This work has led to the identification of different biological functions for sodium channels.

In 1955 another toxin was isolated from the sea cucumber (*Actinopyga agassizi*). Using acid hydrolysis, the compound was broken down into 60 % sugars and 40% aglycones. The sugars were identified by paper chromatography as rhamnose, xylose and glucose in the proportion of 2:1:1 respectively. Three tetracyclic terpenoids similar to those shown in the structure of holothurin A (Fig. 1) were identified in the aglycones. This was the first steroidal saponin of animal origin ever be discovered [5].

In 1957 it was reported that an extract from the hypobranchial gland of the marine snail *Thais floridana*, subjected to column chromatography, produced a substance whose acetylcholine activity was different to the characteristic acetylcholine activity or the murexine activity, which, as mentioned earlier, were previously identified in other related organisms. The biologically active compound was treated with alkali, which produced choline, and a volatile unsaturated acid, which showed a maximum absorption in ultraviolet spectrophotometry (UV) of 220 *nm*. The acid was transformed by catalytic hydrogenation, producing isovaleric acid, from which it was concluded that the toxin has the structure of senecioylcholine [6].

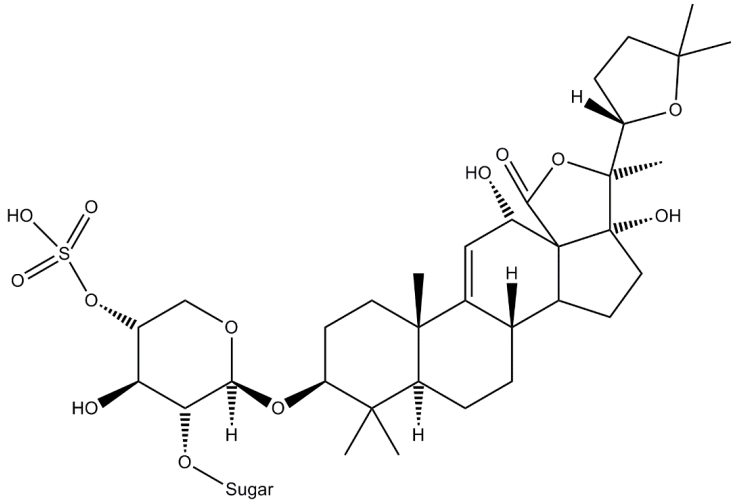

Fig. 1. Holothurin A

In 1962 the discovery of Nereistoxin, NTX [4-(N,N-dimethylamino)-1,2-dithiolane] was published. This compound, with an alkaloidal structure, was isolated from an extract obtained from the salivary glands of the marine annelid worm *Lumbriconereis heteropoda.* lt accumulated in the live worm to concentrations of the magnitude of 600-1050 ppm. The minimum lethal dose (MLD) for the Japanese rice fish (*Oryzias latipes*) is approximately 0.3 ppm. Added to oxalic acid, it forms a salt –an oxalate- which, when administered subcutaneously to rats, gives rise to a median lethal dose (LD_{50}) of 33.6 ppm. In addition, it has a noticeable anaesthetic effect on certain insects [7].

In 1967 aplysine-20, which is a mono-bromide diterpenic compound, was isolated from the sea hare, *Aplysia kurodai*. Its structure was determined by nuclear magnetic resonance (NMR) spectroscopy and X-ray diffraction, and confirmed by chemical methods [8]. However, fifty one years after its discovery, it is now believed that it is not biosynthetized by *de novo* biogenesis, but the mollusc ingests it in its natural diet and bio accumulates it [21].

In 1971 came the discovery of anabaseine (3,4,5,6-tetrahydro-2,3'-bipyridine), a new alkaloid, which can cause paralysis. It was found in extracts from several worms of the genus *Paranemertes,* as well as in other nemertines. The toxin accumulates in the outer coat (integument) of its body in quantities 15 times greater than in the internal organs (viscera), and the proboscis of the animal contains 70 times the amount necessary to paralyse an annelid worm of its own size [9]. It is structurally similar to nicotine and anabasine, meaning it attacks the nervous system [22].

In 1971 the discovery of saxitoxin, STX, was made. This is a heterocyclic compound (2,6-diaminotetrahydropurine with an additional fused ring) which is biosynthetized, for example, by the dinoflagellate *Gonyaulax catenella* [10]. The molecular structure of saxitoxin was confirmed by X-ray diffraction methods in 1974 [23]. It is a powerful neurotoxin which can also block the sodium channel. Harmful algal blooms (HABs) occur when colonies of algae grow out of control while producing toxic or harmful effects on different marine organisms, including shellfish. The human illnesses caused by HABs are known as paralytic shellfish poisoning (PSP) and though its effects are rarely fatal the occurrence of HABs is considered to have a negative impact on any area affected, especially if it is a tourist destination.

In 1972, surugatoxin (SGTX) was discovered. An aqueous extract was obtained from the mid-gut digestive gland of the Japanese ivory mollusk *Babylonia japonica* and purified by gel filtration, also called size-exclusion chromatography. So, the extract was passed through a column of *Sephadex* C-25. It was found to be a complex heterocyclic compound, possessing atropine-like biological activity [11].

Ciguatera is a fish poisoning associated with tropical coral reefs. In 1977, the organism causing the illness was identified as a dinoflagellate, *Gambierdiscus toxicus* [24] and the toxic compounds were given the name of ciguatoxins (CTX). Another dinoflagellate produces the so-called Florida or Gulf of Mexico red-tide toxins, and the compounds involved were called neurotoxic shellfish poisoning (NSP) toxins, or brevetoxins. Both ciguatoxins and brevetoxins were a new class of polycyclic polyethers. In 1981, the structural elucidation of brevetoxin B, by X-ray crystallography, was published [12]. This substance comes from the dinoflagellate *Gymnodinium breve*, which manifests itself by red-coloured blooms. The complexity of the toxin mixtures and the difficulties involved in the purification of the different compounds have contributed to the problems associated with research into brevetoxins. However, it is believed that the deaths of bottlenose dolphins in the Florida Panhandle region may have been the result of exposure to blooms of the dinoflagellate *Karenia brevis* [25].

Pahutoxin was identified in 1984. This is another substance which, structurally speaking, belongs to the family of choline esters. It was found in venom taken from *Lactophrys triqueter*, the smooth trunkfish, a species of box fish found on and near reefs in the Caribbean Sea, Gulf of Mexico and subtropical parts of the western Atlantic Ocean. Choline ester chloride salts, containing a number of fatty acids (C_{16}, C_{17} and C_{18}), were detected using gas chromatography mass spectrometry (GC-MS) and different analytic methods [13].

In 1998 the structure of tiglylcholine, an isomer of senecioylcholine, was revealed. It is another choline ester that was identified in venom extracted from the hypobranchial glands of the muricides (from the Latin murex = hard shell), gastropod molluscs, two common species of which are *Thais clavigera* and *Thais bronni*. The structural elucidation of this molecule was obtained by a spectroscopic study (^{1}H-NMR, ^{13}C-NMR and FAB-MS) and it was shown to be lethal to rats at low concentrations (LD_{50} = 0.92 ppm) [14].

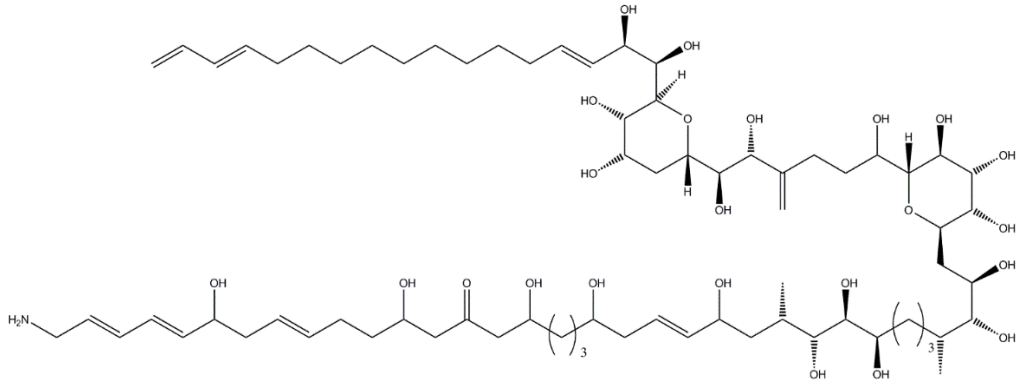

Fig. 2. Karmitoxin

When colonies of the marine algae, genus *Karlodinium*, meet certain favourable conditions harmful algal blooms (HABs) occur. These HABs have been related to large-scale fish kills all over the world. In 2017, an extract was obtained from the species *Karlodinium armiger* and karmitoxin (Fig. 2), an amine-containing polyhydroxy-polyene compound, was identified. Its complex molecular structure was found owing to a study of ^{13}C enriching and higher resolution nuclear magnetic resonance spectroscopy (in one and two dimensions) [15]. Although karmitoxin is structurally related to amfidinols and karlotoxins, it differs from them in that its carbon skeleton is the largest ever seen in this type of substances and it is a primary amine. At concentrations of the order 10^{-9} mol L^{-1} it produces paralysis and eventual death in the marine planktonic crustacean, *Acartia tonsa* (Copepoda), which is its predator. It is also cytotoxic at the same concentrations for the fish cell line RTgill-W1.

4. EXTRACTION AND CHEMICAL ANALYSIS OF MARINE TOXINS

The isolation and identification of marine toxins required the development of extraction and purification techniques. These compounds could only be studied once enough biological matter could be gathered (kilograms), either from organisms collected directly from their natural environment, or using fermentation, photobioreaction, or marine aquaculture. The extraction could then be carried out with the use of water (for hydrosoluble toxins) or organic solvents (for liposoluble toxins). The preliminary separation techniques used in laboratories were performed with adsorption chromatography, using flow columns at low or medium pressure (CC), preparatory plates (TLC or HPTLC) or liquid-liquid partition methods [26]. These techniques can be variously applied to provide either low, medium or high polarity compounds. This was the case of the fractions obtained from the growth medium used for *P. roqueforti* fungus fermentation [27]. Another important technique to separate toxins from an extract has been developed under the name of "Gel Filtration". This technique can be used to separate families of chemical substances of similar molecular weight. A typical method is *Sephadex*-type size-exclusion chromatography (SEC). There are several resins available on the market, such as LH-20, which can be used to perform this technique, [28].

The structure elucidation of organic compounds using spectroscopic methods has advanced throughout the twentieth century. If crystals of the toxin can be obtained, an X-ray diffraction study can be carried out to deduce its three-dimensional structure. If not, if the purified toxin has a non-crystalline appearance, then the literature recommends performing ^{1}H- and ^{13}C-nuclear magnetic resonance (NMR) spectra and mass spectra (MS). From these spectra, the researcher can determine the structure of the toxin [29].

Obtaining the three-dimensional structure of toxins by spectroscopy only became possible after the development of high-resolution nuclear magnetic resonance techniques and complex two-dimensional experiments called COSY, TOCSY, NOESY, HSQC, HMBC, DEPT-90 and DEPT-135 [30].

On the other hand, the invention of sophisticated analytical methods provided tools to make possible the qualitative and quantitative chemical analysis of toxins and other substances contained in a given fraction [27]. Instrumental techniques include high-resolution liquid chromatography (HPLC or UHPLC) and gas chromatography (GC). Once connected to modern detectors, such as mass spectrometry apparatus, these tools were able to resolve countless analytical problems (UHPLC-MS/MS or GC-MS). Thus, for example, methane chemical ionisation gas

chromatography-mass spectrometry (GC-MS) was used to study pahutoxin and choline chloride esters of 16C, 17C and 18C fatty acids from Caribbean trunkfish (*Lactophrys triqueter*) toxin [13], and a large number of biomolecules produced in liquid-state fermentation by the marine-derived fungus *Penicillium roqueforti* were recently identified and quantified by GC-MS [31].

5. DISCUSSION

Marine organisms use chemistry for many different purposes. Obvious objectives are the formation of cellular structures, genetic expression (DNA) and primary metabolism. In addition, secondary metabolism is used by organisms to produce, accumulate and disseminate biologically active chemicals into the environment, which are essential for the survival of both the organism itself and others of the same or a different species [32]. There are an estimated 22,000 known natural marine products [1] and their value as potential drugs for the pharmaceutical industry is well documented; in fact, in recent years, companies have been set up to try to exploit this potential in a sustainable manner. However, only a few marine metabolites have as yet been developed commercially.

Nevertheless, irrespective of whether or not marine metabolites have an industrial application, an understanding of their three-dimensional chemical structure and the bio-genetic pathways that living creatures use to produce them is already of great value in the field of marine chemical ecology. For some time, these metabolites were classed under the definition of marine natural products (MNPs), but this definition is defective as it ignores the ecological function or role that they have. That is why more precise words such as allomone, kairomone or pheromone have increasingly been applied to them [1].

In recent years, marine toxins have been the subject of much research as their presence can cause socio-economic problems, especially in tourist areas. Table 1 shows some of the main discoveries made during the period 1944-2017. Their significance is even greater in that studies in cancer epidemiology, carried out in the late twentieth century, have provided strong evidence for the importance of environmental toxins in the aetiology of human cancer [33].

Animals that are mobile or have hard shells or spines do not usually use toxic chemicals to defend themselves. This is the case of spine-covered sea urchins as well as many marine snails, whose shells protect them. On the other hand, nudibranchs, or sea slugs as they are also known, are an example of organisms which, having no physical protection, use powerful chemical defences [21]. Likewise, the spotted trunkfish (*Lactophrys bicaudalis*) secretes a colourless toxin from glands on its skin when touched. Such organisms usually obtain their toxins from the sponges, bryozoans and sea squirts that they eat [32], but cases have been reported in which their venom is produced by *de novo* biogenesis [34]. Nudibranchs also contain defensive compounds in their soft egg ribbons. An example is the case of jaspisamides, chemical compounds which as well as being produced by the Okiwanna sponge *Jaspis* sp., have been isolated from nudibranch egg masses [35,36]. They possess a characteristic macrolide portion, comprising three contiguous oxazole units, which exhibit potent toxicity towards eukaryotic cells [37]. On the other hand, it is now known that a large number of marine toxins are produced by toxic microscopic microorganisms [38]. Generally, these are chemicals which accumulate throughout the food chain. Since microorganisms constitute over half the ocean's biomass, they are a threat to seafood safety. For this reason, it is necessary to develop programs to study marine microbes and their toxins.

6. CONCLUSION

A great number of marine organisms are known to contain toxic substances, many of which have chemical compounds with unique structures and biological activities. These toxins are usually regarded as self-defence mechanisms since marine organisms are often dangerously exposed to attack by predators or parasites. They either biosynthetize the toxin by *de novo* biogenesis, or ingest it in their diet and then it is bioaccumulated as it is or its molecular structure transformed.

Marine environmental toxicology is the study of the interactions of marine organisms with their environment, using allomones, in general, and toxins, in particular, to characterize these interactions. There are many advantages to using this method to assess the function and health of organisms at the molecular level and toxicology is, indeed, finding an increasing number of applications in the marine sciences. These range from understanding the biochemical response of organisms to abiotic pressure to researching the response of organisms to other biota that compete with them for space and nutrients.

Advances in separation methods and analytical instrumentation, including one- and two-dimensional ^{1}H- and ^{13}C-NMR spectroscopy, high resolution mass spectrometry and analytical/ preparative chromatography, have clearly played a significant role

in the rate of progress of marine toxin research over the past 77 years. Nevertheless, there are still many unanswered questions regarding the intoxication mechanism and the metabolic transformation of toxic compounds. As a consequence, the isolation of toxic chemicals from poisonous marine organisms has become of increasing interest to scientists, and this field of toxicology has continued to expand. The future is promising, as there is an evergrowing awareness of the need to study the marine environment in depth. The proof of this is the creation of several Faculties of Marine Sciences that focus on the oceans as their field of study. At the same time, students are presenting their master´s degree projects and doctoral theses on marine natural products. All these developments suggest a promising future for this field of science.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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