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Review

Bioactive peptides from marine sources: pharmacological properties and isolation procedures

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Abstract

Marine organisms represent a valuable source of new compounds. The biodiversity of the marine environment and the associated chemical diversity constitute a practically unlimited resource of new active substances in the field of the development of bioactive products.

In this paper, the molecular diversity of different marine peptides is described as well as information about their biological properties and mechanisms of action is provided. Moreover, a short review about isolation procedures of selected bioactive marine peptides is offered.

Novel peptides from sponges, ascidians, mollusks, sea anemones and seaweeds are presented in association with their pharmacological properties and obtainment methods.

Keywords: Reviews; Isolation; Marine sources; Peptides, bioactive

Contents

1. Introduction

It is a real fact that the importance of marine organisms as a source of new substances is growing. With marine species comprising approximately a half of the total global

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biodiversity, the sea offers an enormous resource for novel compounds [1], and it has been classified as the largest remaining reservoir of natural molecules to be evaluated for drug activity [2].

A very different kind of substances have been obtained from marine organisms among other reasons because they are living in a very exigent, competitive, and aggressive surrounding very different in many aspects from the terrestrial environment, a situation that demands the production of quite specific and potent active molecules.

The discovery of the bioregulatory role of different endogenous peptides in the organism as well as the understanding of the molecular mechanisms of action of some new bioactive peptides obtained from natural sources on specific cellular targets, contributed to consider peptides also as promising lead drug candidates. Recently marine peptides have opened a new perspective for pharmaceutical developments [3]. Cyclic and linear peptides discovered from marine animals have increased our knowledge about new potent cytotoxic, antimicrobial, ion channels specific blockers, and many other properties with novel chemical structures associated to original mechanisms of pharmacological activity. These facts introduce marine peptides as a new choice for the obtainment of lead compounds for biomedical research. This non exhaustive review is an intent to systematize some of the recent and novel information in this field.

2. Cyclic peptides and depsipeptides

2.1. Bioactive peptides from sponges

Sponges are a large and diverse group of colonial organisms that constitute the phylum Porifera with thousands of different species extensively distributed from superficial waters near the sea shores up to deep waters of the ocean. Sponges have been traditionally known as a source of novel bioactive metabolites like terpenoids, alkaloids, macrolides, polyethers, nucleoside derivatives and many other organic compounds. Synthetic analogues of the C-nucleosides Spongouridine and Spongothymidine isolated from a Caribbean sponge led later to the development of Cytosine Arabinoside, an anticancer compound [4]. More recently, the attention has been directed also to the search of bioactive peptides from sponges, being actually a well established sector in the research of marine natural products.

Active peptides from sponges most of them with unique unprecedent structures in comparison with these kind of compounds from other sources are often cyclic or linear peptides containing unusual amino acids which are either rare in terrestrial and microbial systems or even totally novel, and also frequently containing uncommon condensation between amino acids.

One of the first novel peptides isolated from sponges were the cell growth inhibitory tetradecapeptide Discodermins [5]. A review of cytotoxic peptides from sponges [6] describes some of the discoveries in this field at that time. Discodermins A–H and the structural related Polydiscamide A, obtained from sponges of the genus *Discodermia* are a group of cytotoxic peptides containing 13–14 known and rare amino acids as a chain, with a macrocyclic ring constituted by lactonization of a threonine unit with the carboxy terminal. They are all cytotoxic and were tested against P388 murine leukemia cells, A549 human lung cell line or by the inhibition of the development of starfish embryos with IC_{50} values from 0.02 to 20 μg/ml. Discodermin A, was further studied recently [7] showing a permeabilizing effect on the plasma membrane of vascular smooth muscle cells and tissues.

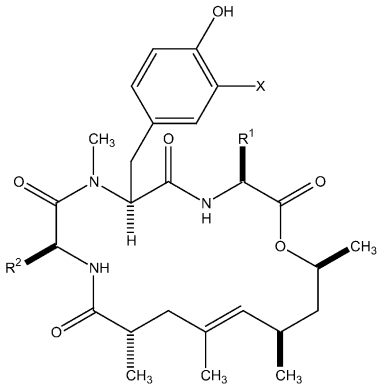

Fig. 1. Basic structure of Geodiamolides A–F peptides where X and R represent different atoms or radicals.

Geodiamolides A–G (Fig. 1) initially isolated from the Caribbean sponge *Geodia* sp. and the similar peptide Jaspamide are also a group of cytotoxic peptides in which three amino acids form a cyclic peptide with a common polyketide unit. Arenastatin A [8] a cyclic depsipeptide from *Dysidia arenaria* is a extreme potent cytotoxic compound with a IC_{50} of 5 pg/ml against KB cells.

Phakellistatins [9] are a group of proline rich cyclic heptapeptides. Phakellistatin 2 showed potent activity against P388 murine leukemia cells ($ED_{50} = 0.34$ μg/ml), but also against different melanoma cell lines.

Theonellamides A–F are also a group of cytotoxic compounds isolated by Matsunaga et al. [10] with novel amino acid residues and complex bicyclic macrocyclic rings. As it was demonstrated later, it is suggested that the origin of some of these peptides would be associated with a microbial source or with symbionts within the sponges, because of the similarity of some of these molecules to marine microbial metabolites.

New members have been added lately to the list of bioactive peptides from sponges and many of them were evaluated as cytotoxic exhibiting antitumor and antimicrobial activities.

A novel peptide, Polydiscamide A, and its derivatives were proposed as antibacterial and antitumor agents [11]. Discobahamin A and B are two bioactive antifungal peptides evaluated as inhibitors of the growth of *Candida*

albicans, isolated from the Bahamian deep water marine sponge *Discodermia* sp. [12].

The depsipeptides Halicylindramides A–C [13] and D and E [14] with antifungal and cytotoxic properties (against P388), were obtained from the Japanese marine sponge *Halichondria cylindrata*. Halicylindramides A–C are tetradecapeptides with an N-terminus blocked by a formyl group and the C-terminus lactonized with a threonine residue. Halicylindramide D is a tridecapeptide also with antifungal and cytotoxic properties, while Halicylindramide E is a truncated linear peptide with a C-terminal amide.

Three new antifungal cyclic peptides with unprecedented amino acids, Microsclerodermins C–E were isolated from two species of sponges, *Theonella* sp. and *Microscleroderma* sp. from the Philippines [15]. Further antifungal cyclic peptides from sponges are the Aciculitins A–C [16] and the Theonegramide [17]. Other examples are the Haligramides A and B, two new cytotoxic hexapeptides from the sponge *Haliclona nigra* [18], and Mozamides A and B from a theonellid sponge collected in Mozambique [19].

A bioassay guided fractionation of two species of marine sponges from the Pacific Ocean [20], led to the isolation of different new cytotoxic peptides like the cyclic depsipeptides Geodiamolides H–N and P. Geodiamolides J–N and P represented the first examples in the Geodiamolide family in which a serine residue has been incorporated.

Other cytotoxic and antiproliferative peptides from sponges are Hemiasterlin [21], Milnamide A [22] and Jasplakinolide [23].

HIV-inhibitory peptides from sponges constitute a recent discovery. The cyclic depsipeptides Papuamides A–D [24] isolated from sponges of the genus *Theonella*, containing a number of unusual amino acids are also the first marine-derived peptides reported to contain 3-hydroxyleucine and homoproline residues, and the previously underscribed 2, 3-dihydroxy-2,6,8-trimethyldeca-(4Z,6E)-dienoic acid moiety N-linked to a terminal glycine residue. Papuamides A and B inhibited the infection of human T-lymphoblastoid cells by HIV-1 sub(RF) in vitro with an EC_{50} of approximately 4 ng/ml. Another anti-HIV candidate from the sponge *Sidonops microspinosa* is the Microspinosamide [25] a new cyclic depsipeptide incorporating 13 amino acid residues, that is the first naturally occurring peptide containing a beta–hydroxy-*p*-bromophenylalanine residue. Microspinosamide inhibited the cytopathic effect of HIV-1 infection in an XTT-based in vitro assay.

Other novel peptides, Keramamides B–D were also isolated from sponges of the same previously mentioned genus *Theonella* [26] as well as Orbiculamide A [27] cytotoxic against P388 murine leukemia cells (IC_{50} = 4.7 ng/ml). Other active peptides also isolated from members of the same genus are the Swinholide A, Bistheonellides, Onnamide A, Theonellamide F, Theonellapeptolides, and Cyclotheonamides. Cyclotheonamides A and B [28] are potent antithrombin cyclic peptides which strongly inhibited various proteinases, particularly thrombin.

On the other hand, new polypeptides from sponges acting on membranes of excitable cells have been also useful to discover new targets for physiological mechanisms. Multiple-step purification procedure led to the new dimeric peptide Mapacalcine (Mr 19,064) from *Cliona vastifica* [29] which selectively blocked a non-L-type new calcium conductance characterized in mouse duodenal myocytes with an IC_{50} value of approximately 0.2 μM. Mapacalcine is suggested as a specific inhibitor of a new type of calcium current, first identified in duodenal myocytes.

The origin and role of bioactive peptides inside the sponges, in many cases are unclear [30]. Many of these compounds have potent activities not always clearly related to their in situ role. Examples are antitumorals, antivirals, immunosuppressive and antimicrobial agents, as well as neurotoxins, hepatotoxin, and cardiac stimulants. Additionally other studies indicate that different compounds isolated from marine macroorganisms like sponges and tunicates may be produced from dietary or by microorganisms like bacteria or symbiotic blue-green algae [31]. This fact illustrates the need for further research into the symbiotic association within these macroorganisms and also suggest to orientate more intensively the search of these compounds also directly to the marine microorganisms.

The isolation and structure elucidation of the first bioactive peptides isolated directly from a marine bacterium was reported in 1991 by McKee [32]. Some other peptides have been isolated from marine bacteria. However up to now there is no comparison with the amount of bioactive peptides isolated from marine macroorganisms, and the biologically active peptides from marine microorganisms. The potential of marine microorganisms for the obtainment of lead molecules is clearly immense due to other reasons, e.g. the feasibility to cultivate in laboratory conditions and to manipulate these initial sources of bioactive natural products.

Other examples of bioactive peptides isolated from microorganisms living in association with sponges are the bicyclic glycopeptide Theopalauamide [33] and the cytotoxic halogenated hexapeptide Cyclocinamide A with a potent in vitro selective activity against Colon-38 tumor cells [34].

2.2. Bioactive peptides from Ascidians

Cyclic and linear peptides and depsipeptides with novel structures and biological functions have been discovered also in ascidians usually called tunicates. Didemnins are a family of depsipeptides with antitumor, antiviral and immunosupressive activities primarily isolated from the Caribbean tunicate *Tridemnum solidum*, but later obtained from other species of the same genus [35]. Didemnin B (Fig. 2) was the most prominent member of the family with the most potent antitumor activity (in vitro L1210 IC_{50} = 2.5 ng/ml) [36] and it was the first marine natural product evaluated in human clinical trials.

In another study, Didemnin B together with some of the more promising marine antitumor candidates was evaluated

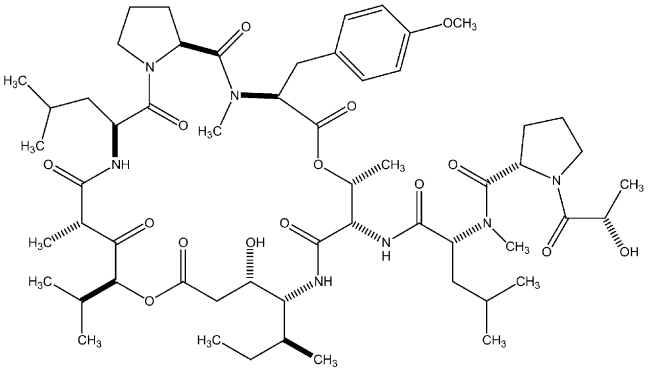

Fig. 2. Didemnim B from the tunicate *Trididemnum solidum*.

as an antiproliferative agent against human prostatic cancer cell lines [37]. At concentration levels of pmol/ml Didemnin B was more effective in the inhibition of prostate cancer cell proliferation than Taxol. Neurotoxic side effect for this compound was also detected and in this sense some caution in the use of it in the clinic was suggested.

Didemnim A and B also show antiviral activity against different kinds of virus like herpes simplex, parainfluenza, and dengue virus, among others [38].

Different new members of the family, also cyclic depsipeptides related to Didemnins have been later isolated from extracts of this tunicate. Some of them are Didemnins X, Y, M, Nordidemnin N, and Epididemnin A, as well as twenty-two new Didemnin analogues were prepared semisynthetically and their biological activities evaluated, including cytotoxicity and antiviral and immunosuppressive properties [39]. Lissoclinamides constitute another large family of cyclic peptides [40] isolated from ascidians (*Lissoclinum patella*) showing relevant antineoplastic as well as other pharmacological properties against human fibroblast and bladder carcinoma cell lines. Some members of the family contain an oxazoline ring, one proline, one valine, two phenylalanine residues, and thiazole and/or thiazoline rings [41].

Another recently discovered cytotoxic peptide from an ascidian is Styelin D [42], a 32-residue C-terminally amidated antimicrobial peptide, isolated from blood cells (hemocytes) of the ascidian *Styela clava*. The molecule contains extensive post-translational modifications, and novel and unusual amino acids. Styelin D exhibited activity against Gram-negative and Gram-positive bacteria, that is retained in high salinity (200 mM NaCl). This author indicates that these kind of peptide antibiotics from marine organisms could be useful for the development of topical microbicides in conditions of physiological or elevated NaCl concentrations.

Tamandarins A and B are also two cytotoxic depsipeptides recently discovered from a marine ascidian of the family *Didemnidae* [43]. The cytotoxicity of Tamandarin A was evaluated against various human cancer cell lines and shown to be slightly more potent than Didemnin.

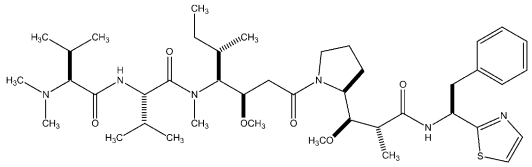

Fig. 3. Dolastatin 10 from the sea hare (mollusk) *Dolabella auricularia*.

2.3. Bioactive cyclic peptides from Mollusks

Cytotoxic cyclic peptides have been also found in mollusks. Dolastatins are a group of cyclic and linear peptides isolated from the marine mollusk *Dolabella auricularia*, with prominent cell growth suppressing activity. From mollusks it has been also isolated another prominent family of peptides, in this case highly compact and stable linear peptides, known as conotoxins but with specific actions on ion channels and membrane receptors of excitable cells, as it will be shown later in Section 3.1.

Dolabella auricularia is a mollusk devoid of shell and for this reason initially appears to lack defenses against predators, but this is only a preliminary supposition. Accumulated evidences support the fact that *Opisthobranchia* mollusks have developed very powerful chemical defenses [44]. The earliest information about the isolation of some of these compounds was reported by Pettit in 1981 [45]. The pentapeptide Dolastatin 10 (Fig. 3) when isolated in 1987 [46] was reported as the most active anticancer natural substance at that time with a ED_{50} of 4.6×10^{-5} μg/ml against the P388 cell line. Another study showed that Dolastatin 10 inhibits tubulin polymerization and tubulin dependent GTP hydrolysis [47].

Dolastatin 10 has been evaluated with promising perspectives in a phase I clinical study in patients with solid tumors. Subsequently its noticeable antitumor activity was well documented in various in vitro and in vivo tumor models [48]. More than a dozen of Dolastatins have been isolated up to now. The study of the cytotoxic mechanism of action of members of the family of Dolastatins exhibit particularities. Recent studies have shown for example that the depsipeptide Dolastatin 11 arrests cells at cytokinesis by causing a rapid and massive rearrangement of the cellular actin filament network and induces hyperpolymerization of purified actin [49]. The effects of Dolastatin 11 were most similar to those of the sponge-derived depsipeptide Jasplakinolide, but Dolastatin 11 was about three-fold more cytotoxic than Jasplakinolide in the cells studied.

Another new cytotoxic cyclic hexapeptide from a marine mollusk is the Keenamide A, isolated from *Pleurobranchus forskalii* [50]. Keenamide A exhibited significant activity against the P-388, A-549, MEL-20, and HT-29 tumor cell lines.

New classes of anticancer drugs candidates, isolated from marine organisms, have been shown to possess powerful cytotoxic activity against multiple tumor types. According to structural characteristics, cytotoxic compounds from marine

organisms include very different kind of metabolites like macrolides, terpenes, nitrogen including compounds like complex alkaloids, and many others. However among the most active of them are the cyclic peptides and depsipeptides or linear peptides bearing uncommon amino acids isolated from sponges, tunicates, and mollusks. In conformity with Schmitz et al. [51] the most active cytotoxins ($ED_{50} < 10^{-3}$ μg/ml) are the depsipeptides. They include Didemnims B, D, E, X, Dehydrodidemnimn B, Dolastatins 3, 10, 11, 15 and Geodiamolides A, B, C, and D.

2.4. Isolation methods of cyclic peptides

Purification procedures of these kind of cyclic peptides and depsipeptides isolated from sea animals like ascidians, sponges and mollusk, usually include initial extraction with methanol (MeOH), partitions of this extract with organic solvents of increasing polarities to render diverse organic fractions, and chromatographic steps on silica and Sephadex LH-20 columns, and the use of reversed phase C_{18} HPLC for the final purification.

As an example, a typical procedure of a bioassay guided isolation applied to the obtainment of Onchidin B, a cyclodepsipeptide from the mollusk *Onchidum* sp. [52] include an initial extraction from the freeze-dried animal with methanol. The methanol extract is concentrated in vacuum and the resulting viscous concentrate is partitioned between 10% aqueous methanol and hexane. The methanolic portion is then extracted with carbon tetrachloride (CCl_4), and later with dichloromethane (CH_2Cl_2). Each organic layer is concentrated in vacuum to yield the hexane, CCl_4, and CH_2Cl_2 extracts.

The CCl_4 extract was submitted to flash silica gel column chromatography eluted with a gradient from dichloromethane to methanol resulting in five fractions. Fraction two was further purified on a reversed phase C_{18} HPLC and resulted in purification of Onchidin B.

Variants of this procedure additionally include for example steps of desalting of organic (alcoholic) fractions with the resin Amberlite XAD-2, and a separation step in a Sephadex LH-20 column eluted with methanol–water, just before the HPLC chromatography on the reversed phase C_{18} column [53,54].

3. Linear peptide toxins from *Conus* and sea anemones

The animals' use of venoms for feeding, defence and other interspecies interactions results from an evolutionary fine process that has taken place in different taxa along millions of years.

As the use of venoms is the strategy of different species to survive in a specific environment it is not surprising that the components of these venoms might exert very specific and potent effects. For this same reason animal venoms are for scientists a source of interesting bioactive molecules commonly known as toxins. The use of toxins is an expanding field and up to the moment they have been used as lead structures for diagnostic, therapeutic, and other purposes. Moreover, they are being used as useful chemical tools in the field of scientific research to study the molecular mechanisms underlying different functions.

The ion channels are a common target for many peptide toxins. As many neurological disorders are the result of mutations at this level (termed "channelopathies") such mutant ion channels represent an important therapeutic target for the discovery of new drugs.

Several studies have shown that some of the most active components of venoms are of proteinic nature probably because of different reasons. Their large surfaces allow more bonding contact with their target receptors, they seem to be very potent and additionally for the biosynthesis of proteins the required machinery is already present in animals [55]. Moreover, because of structural foldings, peptidic toxins can adopt relatively stable compact conformations, making them resistent to enzymatic actions and exerting very specific effect at the level of their target receptors because of their complex structure [3].

3.1. Conus toxins

There are approximately 500 species of predatory cone snails within the genus *Conus* (Phyllum *Mollusca*) and it has been estimated that the venom of each *Conus* species has among 50 and 200 components [56].

Conus represent a valuable source of neuropharmacologically active peptides named conotoxins. According to Olivera [57] these peptides take part in the defence, prey capture and some other biotic interactions. They consist of a mixture of peptides, of relatively short strings of amino acids and are rich in disulfide bonds. The majority of these peptides consist of about 8–35 amino acids in length [58]. The presence of disulfide bonds seems to lock the peptide into a relatively rigid shape, which probably increase the binding ability of conotoxins to very specific targets [59]. The relatively small size of these peptides represents a great advantage for their chemical synthesis [60]. In addition *Conus* venoms also contain a heterogeneous group of peptides that are not disulfide rich (e.g. the conantokins), large polypeptides (>10 kDa) or small molecules such as biologically active amines.

According to Cruz et al. [60] the list of macromolecular targets of these peptides includes six types of ion channels and receptors: acetylcholine receptors (α-, αA- and ψ-conotoxins), NMDA receptors (conantokins), sodium (μ-, μO- and δ-conotoxins) calcium (ω-conotoxins) and potassium (k-conotoxins) channels, vasopressin (conopressins) and neurotensin (contulateins) receptors (Table 1). However, the discovery of new conopeptides having specific structural and biological features is enhancing.

The α-conopeptides represent the best structurally studied class and they act as antagonists of the nicotinic acetylcholine receptors (AchRs) [61,62]. According to Arias and

Table 1
Types of *Conus* peptides and molecular targets

Molecular target	Cysteine arrangement	Class	Mode of action	Prototypical example
Acetylcholine receptors	CC–C–C	α-Conotoxins	Competitive inhibitor	α-GI
	CC–C–C–C–C	αA-Conotoxins	Competitive inhibitor	αA-EIVA
	CC–C–C–CC	ψ-Conotoxins	Noncompetitive inhibitor	ψ-PIIIE
Sodium channel	CC–C–C–CC	μ-Conotoxins	Block current	μ-PIIIA
	C–C–CC–C–C	μO-Conotoxins	Block current	μO-MrVIB
	C–C–CC–C–C	δ-Conotoxins	Delay inactivation of Na^+ channel (site VI)	δ-TxVIA
Calcium channel	C–C–CC–C–C	ω-Conotoxins	block current of specific subtypes	ω-MVIIA
Potassium channels	C–C–CC–C–C	κ-Conotoxins	Blocker	κA-SVIA
NMDA receptor	Linear	Conantokins	Inhibitor	Conantokin-G
Vasopressin receptors	C–C	Conopressin	Agonist	Conopresin-S
Neurotensin receptors	Linear	Contulateins	Agonist	Contulakin-G

Blanton [63] these neurotoxins are capable to discriminate between muscle and neuronal type AchRs and even among distinct AchR subtypes and the binding toxin-receptor shows specific characteristics It has been postulated that these peptides could be of interest in the treatment of anxiety, Parkinson's disease, pain, hypertension, cancer and also as muscle relaxants [61].

Conantokins are a specific class of linear peptides which inhibit *N*-methyl-D-aspartate (NMDA) receptors This kind of receptors plays an important role in the pathophysiology of central nervous system (CNS) disorders. All conantokins are linear peptides except the conantokin isolated from *C. radiathus* which has a three amino acid disulfide bridged loop near the C-terminus [61]. As it was indicated by Olivera [57] these peptides were originally purified following their characteristic to induce a sleep-like state in mice under 2 weeks of age, whereas in mice older than 3 weeks a hyperactivity is observed. They represent a novel class of NMDA receptor antagonists that can modulate CNS excitability. They offer an interesting alternative in the treatment of epilepsy, pain, stroke and Parkinson's disease [61].

Different types of conotoxins act on the voltage-sensitive sodium channels. The best studied of these compounds are μ-conotoxins which act as selectively blockers on vertebrate skeletal muscle subtypes of voltage-dependent sodium channel [58]. μ-Conotoxins which act on neuromuscular sodium channels might be useful to treat neuromuscular disorders [61]. δ-Conotoxin-GmVIA, purified from *Conus gloriamaris*, induces convulsive-like contractions when injected into land snails but has no detectable effects in mammals. It acts specifically on molluskan sodium channels [64]. It slows down the inactivation process of this current and induces action potential broadening. μO-Conotoxins include a family of peptides which block sodium currents of voltage-sensitive sodium channels. μO-Conotoxins MrVIA and MrVIB isolated from *C. marmoreus* potently block the sodium conductance in *Aplysia* neurons. Although these peptides exhibit a pharmacological action which is similar of the μ-conotoxins, they are structurally unrelated [65].

Specifically ω-conotoxins, selective for calcium channels, were purified using their ability to produce a shaking behavior in mice as it was described by Olivera [66]. They include ω-conotoxins GVIA and MVIIA from *C. geographus* and *C. magus*, respectively, among others. Four new ω-toxins were isolated from the venom of *C. catus* and their potencies in different bioassays were compared [67]. Because of the therapeutical potential of this type of neurotoxins in the management of severe pain they are of great interest. The synthetic peptide SNX-111 corresponds to the sequence of an ω-conopeptide MVIIA obtained from the venom of *Conus magus* and it acts as a highly potent and selective antagonist of N-type calcium channels [68]. The team of Nadasdi et al., synthesized and developed a structure-activity study of analogs to SNX-111. Neurex Corporation presented a patent (No. 5364842) [69] for ω-conopeptides or derivatives (SNX-111 or SNX-185) to be used as potent analgesics.

From the venom of *Conus purpurascens* the first cone snail toxin that blocks potassium channels (κ-conotoxin PVIIA) was isolated. The overall fold of κ-conotoxin is similar to that of calcium channel-blocking ω-conotoxins but differs in this aspect from potassium channel-blocking toxins from sea anemones, scorpions and snakes [70].

In addition, there have been isolated few new conotoxins which target at different sites. For instance, Serotonin receptors are another molecular target for conotoxins and specifically σ-conotoxin GVIIIA selectively inhibits the 5-HT3 receptor [71].

3.2. Sea anemone linear polypeptides

3.2.1. Channel toxins

Sessile animals like sea anemones have developed along the evolution specific chemical mechanisms for the complex interactions in which they participate in the marine environment. The toxicity of these animals is in part associated to the presence of nematocysts, a specialized stinging organell, characteristic of the *Coelenterate* Phyllum.

From nematocyst rich tissues, secretions as well as different parts or the whole body from sea anemones have been obtained different compounds of proteinic nature including pore-forming toxins or cytolisins [72–74], phospholipases

[75], Na^+ channel toxins [76–78], K^+ channel inhibitors [79–83], other neurotoxins [84] and even proteinase inhibitors [85,86]. Nevertheless, the exact origin of both neurotoxins and cytolysins is not quite clear and others sources besides nematocysts seem to contribute to the toxicity of sea anemones [74].

Different authors have suggested that it is possible that the cytolisins and the toxins that target ionic channels together act synergistically [87].

According to the molecular weight (MW) and some pharmacological properties, sea anemone sodium polypeptide toxins were earlier classified in four groups [88]:

1. Basic polypeptides with MW $\leq$ 3000.
2. Polypeptides in the MW range between 4000 and 6000. These two groups include toxins that act on the voltage-activated Na^+ channel.
3. Polypeptides in the MW range between 6000 and 7000. This group posses proteinase inhibiting activity and they show great homology with other proteinase inhibitors isolated from mammalian species.
4. Polypeptides in the MW range $\geq$ 10 000. These compounds show mainly cytolytic activity.

It has not been corroborated subsequently if all the four groups of polypeptides are present in each sea anemone specie.

More recently, a different type of polypeptide neurotoxins acting on the voltage-dependent K^+ channels was reported [79–83].

The studies on sea anemones toxins were started by Shapiro [89] and Béress and Béress [76] who isolated toxins from *Condylactis gigantea* and *Anemonia sulcata*, respectively. These studies led to the discovery of new compounds capable to act at the level of voltage activated sodium channels. After that time many others polypeptide toxins have been isolated and chemically characterized from different sea anemone species which similarly to toxins from *A. sulcata* and *C. gigantea* inhibit the Na^+ channel inactivation [90,91].

There are two more recent classification of the sodium channel toxins, one of them take into account their sequence homologies and the second one is according to the specific binding site and to their specific physiological effect in the sodium channel.

According to sequence homologies sodium channel toxins can be classified as types I and II [78]:

- Type I toxins are represented by ATXI, ATXII and ATXV from *A. sulcata*, AftI and AftII from *Anthopleura fuscoviridis*, ApA and ApB from *A. xantogrammica*, ApC from *A. elegantissima* [90,92] and BgII and BgIII from *Bunodosoma granulifera* among others [93,94].
- Type II toxins include ShI from *S. helianthus* and toxins from *Heteractis* sp.

These two types of toxins are cross linked by three disulfide bridges, have molecular weights about 5 kDa and show the same electrophysiological activity.

Other toxins have been isolated which do not fall nearly into one of these classes.

In Fig. 4, different type I sea anemone toxins are depicted. As it can be seen a considerable similarity exist in the amino acid sequence among them.

Voltage-dependent sodium channels constitute an important target for different groups of neurotoxins which have contributed to the structural and functional characterization of this channel [95]. At least six different neurotoxin receptor sites have been described for the mammalian Na^+ channels and sea anemone peptide neurotoxins and α-scorpion toxins share receptor site 3 on sodium channels [96,97], which involves the extracellular loop IVS3–S4 and additional unidentified contacts in the IS5–S6, and IVS5–S6 loops of the ionic channel [98]. Their main effect is to delay sodium channel inactivation which leads to neurotoxic (repetitive firing) and cardiotoxic (arrhytmia) effect.

In general, sea anemone toxins are basic proteins that bind across to the IVS3–S4 loop through electrostatic, hydrogen-bonding, or Van der Waals interactions [98]. Many of these toxins induce a positive inotropic effect in different mammalian heart preparations and an increase in the action potential (AP) duration, that can be explained by modulation via Na^+/Ca^{2+} exchange that results from the increased cellular Na^+ load [99].

Chemical modification studies of sea anemone toxins were carried out by several laboratories to determine the role of different amino acids in the channel binding but do not exist a general conclusion about the role of each residue, however different residues that seem to play a role in the interaction with toxin receptor such as Arg-12, Arg-14, Lys-49 and Lys-37 were identified [92,100,101]. In general, basic amino acids of toxins and acidic residues of the domains I and IV of the sodium channel alpha subunit have been implicated in toxin binding.

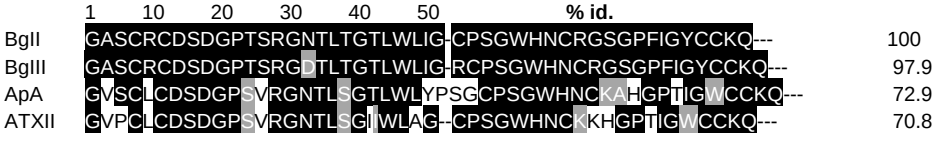

Fig. 4. Comparison of the sequences of some anemone toxins: BgII and BgIII from *Bunodosoma granulifera* ApA from *Anthopleura xanthogrammica* and ATXII from *Anemonia sulcata*. Identical amino acids are indicated with a black background, homologous amino acids are indicated with a gray background, dashes represent gaps. At the right column the percentage of identity in comparison to BgII (modified from Goudet et al. [93]).

Concerning to the K^+ channels more recently a new type of blocking toxins from different sea anemones was obtained such as BgK from *B. granulifera* [79], ShK from *Stichodactyla helianthus* [80], AsKS and AsKC from *A. sulcata* [81], HmK from *Heteractis magnifica* [82] and AeK from *Actinia equina* [83] which are structurally different in comparison with other K^+ channel toxins isolated from snake, scorpion and bee venoms. These sea anemone toxins are formed by about 35–37 amino acids and they present three disulfide bridge [87]. They inhibit K^+ channels by binding to a receptor site in the external vestibule of the channel occluding physically the pore and thus blocking the ionic current [102]. Because of their action mechanism and their specificities sea anemone toxins and scorpion toxins have been used to identify the pore region and to measure the external mouth of the channel pore [102]. A common characteristic of both sea anemone and scorpion potassium channel blockers is the presence of a functional diad formed by lysine and a hydrophobic residue [103,104]. This diad of toxins residues determinants for the potassium channel interactions consist in a Lys-25 and Tyr-26 for BgK, whereas for ShK the diad is composed by a Lys-22 and Tyr-23 [104,105].

Both BgK and ShK compete with dendrotoxin-I (from snake venom) for binding to rat brain synaptosomal membranes, and suppress K^+ currents in rat dorsal root ganglion neurons in culture [79,80]. BgK blocks K^+ currents in snail neurons [106] and Kv1 channels in *Xenopus* oocytes almost equipotently at nanomolar concentrations [87]. In contrast, ShK blocks different Kv1 channels at nano and subnanomolar concentrations [87,103]. Even at 100 nM, BgK, and ShK did not affect the Kv3.1 channel [87]. Additionally, they potently blocked at nanomolar concentrations the intermediate conductance Ca^{2+}-activated potassium channel IKCa1, a well-recognized therapeutic target present in erythrocytes, human T lymphocytes and colon [107]. In addition ShK is a potent blocker of Kv1.3 potassium channel in Jurkat T-lymphocytes [108]. As the Kv1.3 channel has been implicated in the T-lymphocyte proliferation and lymphokine production, blockers of this channel are of interest too as potential immunosuppressants [109].

From *A. sulcata* the blood depressing substances BDS-1 and BDS-II are the first specific blockers for the Kv3.4 potassium channel [110].

HmK, the potassium channel toxin from the sea anemone *H. magnifica* [82] was reported to inhibit the binding of [^{125}I]-α-dendrotoxin (a ligand for voltage-gated K^+ channel) to rat brain synaptosomal membranes with a k*I* of about 1 nM blocks K^+ currents through Kv1.2 channels expressed in a mammalian cell line and facilitates acetylcholine release at the avian neuromuscular junctions.

3.2.2. Cytolisins

According to their molecular weight sea anemones cytolytic polypeptides can be classified in four groups [74]:

- About 5–8 kDa peptides with antihistamine activity.
- About 20 kDa pore-forming proteins inhibited by sphingomyelin.
- About 30–40 kDa cytolysins with or without phospholipase A_2 activity.
- A putative group of proteins represented at present solely by an 80 kDa *Metridium senile* cytolysin.

Group 1: Cytolysins belonging to this group have been found in *Tealia felina* [111], *Radianthus macrodactylus* [112] and *Condylactis aurantiaca* [111,112]. The cytolysins of this group in general are not inhibited by sphingomyelin and are less hemolytically active than the groups II and III cytolysins. Cytolysins isolated from *T. felina* and *R. macrodactylus* show antihistamine activity.

The structural characteristics of this group of cytolysins is not well known. In comparison with group II cytolysins they possess cysteine residues and lack tryptophan.

Group 2: This is the best studied group of sea anemone cytolysins. They are known as actinoporins [77] taking into account that their source are sea anemones (*Actinaria*) and their ability to form pores in natural and model lipid membrane. This pore formation includes at least two steps: the first one involves the binding of a soluble toxin monomer to the lipid bilayer, this process is fast, it is concluded in few seconds and is irreversible [113,114]. The second step involves slower oligomerization in the plane of the membrane, which eventually leads to the formation of the functional pore [115,116].

They are all monomeric, cysteinless proteins with isoelectric point values mostly above nine. Three or four molecules oligomerise in the presence of a lipid membrane to form cation-selective pores of about 2 nm hydrodynamic diameter [117,118].

Hemolysis has been mainly used to characterize the activity of actinoporins [119].

Actinoporins are very potent toxins that affect almost all kind of eukaryotic cells. They use sphyngomyelin, a lipid that is ubiquitous in animal cells, as a low affinity receptor [120].

The most studied actinoporins are Equinatoxins II, IV and V from *A. equina* [121], Sticholysin I and II [73] from *S. helianthus* and HMgIII from *H. magnifica* [72]. The primary structure of these actinoporins is characterized by a conserved putative N-terminal amphiphilic α-helix, a triptophan-rich stretch, and an RGD-motif [74]. The binding of these polypeptides to the membrane is associated with a partial conformational change of the toxin without a significant change in its secondary structure.

They are highly lethal in mammals and this activity is attributed to cardiorespiratory arrest and coronary vasospasm. They are also highly cytotoxic and lytic to a variety of cells and their vesicular organelles. They also produce significant degranulation of granulocytes and platelets.

Group 3: This group include proteins with phospholipase A_2 like activity.

The venom of the sea anemone *Aiptasia pallida* possesses hemolytic activity which is produced by at least three synergistic venom proteins. One of these proteins is a phospholipase A_2 which exists in two forms, α and β [75].

Phospholipase activity was also reported in Up1, from the crude extract of the sea anemone *Urcinia piscivora* [122,123]. Up1 is a potent hemolysin on erythrocytes of rat, guinea pig, dog, and humans causing numerous holes and breaks on membranes as indicated by scanning electron microscopy. Its hemolytic activity is not inhibited by cholesterol [122]. According to Cline [123] it produces a potent positive inotropic effect of the left atria of the heart of rat and guinea pig with little or no increase in heart rate in vivo. It also produces ileal contractions similar to acetylcholine and such action was inhibited by atropine. Intravenous administration of Up1 produced a fall in blood pressure in normotensive rats partially reduced by atropine.

Group 4: This group is represented by metridiolysin, an 80 kDa haemolytic and lethal protein from *M. senile*. It is a cholesterol inhibitable cytolysin [124]. The toxin exhibits preference for the lysis of horse and dog erythrocytes, which was correlated with cholesterol contents in erythrocyte membranes [125]. According to a study [126] the toxin does not show high specificity for interactions with lipids, and it was concluded that this toxin is capable of forming channels at low toxin concentrations and larger pores at high concentrations which results in lipid bilayer membrane destruction.

3.3. Isolation of toxins from Conus and sea anemones

Peptide toxins with specific action on ion channels from marine animals like coelenterates (sea anemones) and mollusks (*Conus*) are mainly linear basic peptides. For that reason the isolation procedure has to include steps for basic peptide purification. Sometimes when a toxic acidic peptide is detected, a step of anion exchange chromatography is included in the protocol to separate basic from acidic toxins.

The general procedure used to fractionate the complex mixture of different peptide toxins contained in crude *Conus* venoms, comprise gel filtration and reverse-phase HPLC as it was used to purify the potent sleeper peptide Conotoxin GV which contains five residues of γ-carboxyglutamate in a chain of 17 amino acids, isolated from the fish hunting marine snail *Conus geographus* [127,128]. The same general procedure was recently applied by the same research group to isolate the new peptide mr10a from *Conus marmoreus*, with potent antinociceptive properties [129]. In brief, the crude venom was size fractionated using Sephadex G25 with acetic acid as elution buffer, and after lyophilization the active fraction was fractionated on a C18 HPLC column with a linear gradient of acetonitrile in trifluoroacetic acid, with a further rechromatography in the same column to eliminate contaminants and a final purification step on a Vydac protein-SCX (strong cation exchange) column.

On the other hand, isolation methods of sea anemone sodium channel toxins used since the beginning, included combinations of traditional chromatographic methods for protein group separation as size exclusion and ion exchange chromatography. An early now classical procedure described by Béress et al. [130] for ion channel toxins isolation from *A. sulcata*, comprise alcoholic extraction of the crude toxic material from freshly collected sea anemones, batch-wise adsorption of the toxins on a cation exchanger followed by gel filtration on Sephadex G-50, then ion-exchange chromatography on QAE Sephadex A25 and SP Sephadex C25 and desalting on Sephadex G25.

A very similar procedure was described for the isolation of toxins from the sea anemone *Radianthus paumotensis* [131].

Sequence studies of ATX I, ATX II and ATX V toxins previously isolated from *A. sulcata* [130] indicated that these toxins were not pure proteins, each having microheterogeneities in the sequence. This facts led to the discovery of sea anemone isotoxins, sometimes with only one different amino acid in a specific position of the chain but with a dramatic influence in the pharmacological activity in contrast. A description in details of these isolation procedures is given by Béress et al. [132] which indicated that the separation of the isotoxins could not be achieved even with the use of very precise gel filtration and ion exchange chromatographic techniques. The use of a RP C-18 HPLC column enabled the resolution of the sea anemone toxins from *A. sulcata* into different isotoxins.

The presence of isotoxins in sea anemone venoms became later a well known fact.

Even though that the presence of some toxins in typical structures denominated nematocysts from coelenterates is well documented in the literature [133,134], the precise localization of sea anemone toxins inside the animal is not always clear or another origin not associated with nematocysts has been suggested. Thus, other different crude starting extracts have been used like sea anemone secretions [79] and mainly the whole animal body extract [99], which is the most frequently used initial source. Whole body extracts are very complex mixtures of a large number of molecules containing not only toxins but also many other components from tissues and sea water salts. This has to be taken into account for the isolation procedure, which has its own peculiarities in comparison with the same process from crude snake, scorpion, and bee venoms as a source of toxins.

The method described above with slight variants has been later used for the obtainment of other sodium and subsequently for potassium channel sea anemone toxins. Some of the modifications or additions later included are the use of “tentacle” ion exchangers like Fractogel®, Bio-Gel, or TSK HPLC cation exchangers, the employment of the research-grade Serdolit® AD-2 as an initial additional alternative of normal pressure hydrophobic adsorption step and the repeated use of the same chromatographic step or rechromatography to improve the separation, and the extensive use of all the possibilities of RP-HPLC chromatography [79,81,135].

3.4. Isolation of cytolysins

Purification of cytolytic toxins or cytolysins from sea anemones aqueous extracts commonly include a first gel filtration step (usually Sephadex G50 or similar) followed by cation exchange chromatography (CM-32 cellulose or other equivalent exchanger) as essential combination to achieve the resolution of the complex crude materials. As an example, this protocol was used by Kem and Dunn [136], and Lanio et al. [137] to isolate protein cytolysins from the sea anemone *S. helianthus*. Sticholysins I and II (StI and StII) purified by Lanio et al. [137] by this procedure, are two cysteinless polypeptides containing a high proportion of nonpolar amino acids, with a highly basic isoelectric point and molecular weight of 19 392 and 19 283 (±2) Da, respectively [120]. Similar procedures have been also used for different authors to purify other members of the family of sea anemone cytolisins referred before.

4. Peptides and proteins from seaweeds

Biologically active peptides and proteins have been isolated not only from marine animals, but also from seaweeds. In a screening of medicinal potentialities of seaweed were discovered different proteins and peptides exhibiting bioactives properties [138].

Although in a minor scale in comparison with marine animals, some useful proteins like lectins and phycobiliproteins (PBP) have been reported from seaweeds.

Lectins are glycoproteins with aggregation and recognition functions in the organism that posses them, and they have proved to be useful in the field of clinical diagnosis and other health applications. Lectins have been isolated before from primitive marine organisms like sponges [139].

From the red marine alga *Bryothamnion triquetrum* a member of a new structural family of lectins was isolated. It contains 91 amino acids and two disulfide bonds. The primary structure of the *B. triquetrum* lectin does not show amino acid sequence similarity with known plant and animal lectin structures [140].

Mitogenic and antineoplastic isoagglutinin glycoproteins have been equally described from the red alga *Solieria robusta* [141]. A mitogenic hexapeptide with the sequence Glu-Asp-Arg-Leu-Lys-Pro denominated SECMA 1 was purified from the alga *Ulva* sp. which is active on human foreskin fibroblasts [142].

In another field of application, a novel class of nontoxic bioactive oligopeptides, found in marine red algae and cyanobacteria [143], were proposed to be useful for both the diagnosis and therapy of certain brain and central nervous system disorders resulting from malfunctions of systems controlled by the neurotransmitter γ-aminobutyric acid (GABA). Several of these oligopeptides were more active in competing for specific super(3)H-muscimol binding to the mammalian GABA sub(A) receptors than GABA itself, demonstrating their potential as a new class of potent GABA analogs.

As it was shown before, an origen associated with symbionts from some new metabolites isolated from marine macroorganisms is suspected. This is also the case in cyclic peptides isolated from some algae. Ceratospongamides are two isomers of a new bioactive thiazole-containing cyclic heptapeptide, *cis*,*cis*-Ceratospongamide and *trans*,*trans*-Ceratospongamide. They were isolated from the marine red alga (Rhodophyta) *Ceratodictyon spongiosum* containing the symbiotic sponge *Sigmadocia symbiotica*. *trans*,*trans*-Ceratospongamide exhibits potent inhibition of secretory phospholipase (sPLA) expression in a cell-based model for antiinflammation ($ED_{50} = 32$ nM), whereas the *cis*,*cis* isomer is inactive [144].

On the other hand, phycobiliproteins play an important role in the photosynthetic process of blue–green, red and cryptophyte algae. Light is efficiently collected by phycobiliproteins assembled into macromolecular structures, the phycobilisomes (PBS). Phycobiliproteins are oligomeric proteins, built up from two chromophore-bearing polypeptides. They include three main groups of compounds: phycoerythrins (PEs) with a red pigment joined to the protein molecule, phycocianins with a blue pigment and alophycocianins, absorbing them all at different wavelength of the spectrum [145,146]. Phycobiliproteins are spontaneously fluorescent “in vivo” and “in vitro” molecules. This property is specially used in the field of biotechnological applications [147] where they are used in a wide variety of biomedical diagnostic systems mainly in immunochemical methods. Phycoerythrin is the most used phycobiliprotein [148], specifically in fluorescent immunoassays [149], fluorescent immunohistochemistry [150] and other methodologies.

Phycobiliproteins have also antioxidant properties [151] with applications in cosmetology, and in the food industry.

4.1. Isolation of phycobiliproteins

Isolation methods and molecular characterization of phycobiliproteins from seaweeds are described in the literature [152,153]. Because of its applications, phycoerythrin has been the most studied phycobiliprotein. One typical procedure [154] for the isolation of these proteins and in particular of phycoerythrins includes a combination of different steps of density gradient centrifugation, column chromatography and electrophoresis. In brief, the first step is the isolation of an intact phycobilisome fraction from the crude seaweed extract containing phycobiliprotein pigments by a method [155] including disruption of cells and a discontinuous sucrose gradient centrifugation. PBS dissociation is later achieved, and another step of sucrose density gradient centrifugation is used for the obtainment of two main colored fractions, a blue-colored one (fraction A), and the other pink-violet (fraction B). Fractions A and B are submitted separately to chromatography on columns of the anionic DEAE-cellulose DE52 developed with a linear gradient of

an appropriate buffer containing mercaptoethanol. The pigment complexes from each major column are pooled and then loaded onto linear sucrose density gradients and centrifuged. Pure fractions of phycoerythrin, as judged by their electrophoretic composition are further purified by centrifugation on a linear sucrose density gradient.

Another purification procedure in a large preparative level using also an anionic chromatographic column of DEAE cellulose, and sodium dodecyl sulfate poly-acrylamide gel electrophoresis (SDS-PAGE) is used to obtain B-phycoerythrin and R-phycocyanin from a microalga [156]. An alternative procedure to purify R-phycoerythrin from a Mediterranean red algae [157] propose the use of hydroxyapatite produced in the laboratory that can be used batch-wise with large amounts of extracts and therefore can be scaled up.

5. Concluding remarks

Peptidic compounds analyzed here are obtained from very different marine organisms exhibiting different chemical structures and displaying a large variety of pharmacological effects on specific targets. They have been produced along of millions of years of evolution in response of the necessity to evolve in a very competitive environment. Because of the peculiarities of the life in the sea, many of these molecules can be found only in a single source.

From another side, these compounds seem to be very useful and promising for biomedical research to clarify many normal and pathological mechanism of action in the human body as well as in the design of very specific and potent new pharmaceuticals for a wide variety of diseases.

A multidisciplinary and cooperative effort with the use of more sensitive and fast methods in the analysis of the structure of peptides, e.g. exact description of the molecular weight and the sequence, as well as in the pharmacological evaluation, will speed up drastically the discovery of novel active peptides from marine sources.

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