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Phycotoxins: chemistry, mechanisms of action and shellfish poisoning

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Abstract. Phycotoxins are natural metabolites produced by micro-algae. Through accumulation in the food chain, these toxins may concentrate in different marine organisms, including filter-feeding bivalves, burrowing and grazing organisms, herbivorous and predatory fish. Human poisoning due to ingestion of seafood contaminated by phycotoxins has occurred in the past, and harmful algal blooms (HABs) are naturally occurring events. Still, we are witnessing a global increase in HABs and seafood contaminations, whose causative factors are only partially understood. Phycotoxins are small to medium-sized natural products and belong to many different groups of chemical compounds. The molecular mass ranges from ~300 to over 3000 Da, and the compound classes represented include amino acids, alkaloids and polyketides. Each compound group typically has several main compounds based on the same or similar structure. However, most groups also have several analogues, which are either produced by the algae or through metabolism in fish or shellfish or other marine organisms. The different phycotoxins have distinct molecular mechanisms of action. Saxitoxins, ciguatoxins, brevetoxins, gambierol, palytoxins, domoic acid, and, perhaps, cyclic imines, alter different ion channels and/or pumps at the level of the cell membrane. The normal functioning of neuronal and other excitable tissues is primarily perturbed by these mechanisms, leading to adverse effects in humans. Okadaic acid and related compounds inhibit serine/threonine phosphoprotein phosphatases, and disrupt major mechanisms controlling cellular functions. Pectenotoxins bind to actin filaments, and alter cellular cytoskeleton. The precise mechanisms of action of yessotoxins and azaspiracids, in turn, are still undetermined. The route of human exposure to phycotoxins is usually oral, although living systems may become exposed to phycotoxins through other routes. Based on recorded symptoms, the major poisonings recognized so far include paralytic, neurotoxic, amnesic, diarrheic shellfish poisonings, ciguatera, as well as palytoxin and azaspiracid poisonings.

Marine biotoxins in a changing environment

The term phycotoxin indicates natural metabolites produced by unicellular micro-algae (protists). Most phycotoxins are produced by dinoflagellates, although cyanobacteria have also been reported to produce saxitoxin; domoic acid is produced by diatoms. Some of the toxins were initially identified in associated organisms, e.g., okadaic acid in the sponge *Halichondria okadaii* [1], domoic acid in the red macroalga *Chondria armata* [2–5], or palytoxin in the soft coral *Palythoa toxica* [6].

Through accumulation in the food chain, these toxins may concentrate in a variety of marine organisms including filter-feeding bivalves, burrowing and

grazing organisms (tunicates and gastropods) as well as herbivorous and predatory fish. All marine biotoxins described in this chapter have been selected because they are found in seafood and have been identified as bioactive compounds potentially causing seafood poisoning.

Human poisoning due to ingestion of seafood contaminated by phycotoxins has occurred in the past, and historical records as well as the habits of some populations in coastal and tropical areas show that harmful algal blooms (HABs) are naturally occurring events [7]. In the last 30 years HABs have attracted increasing attention from the scientific community and the society. The occurrence of episodes of human poisoning due to ingestion of toxic seafood involving tens or hundreds of people in several areas of the world [7] has certainly called for more attention to HABs and their consequences on human health. The increased awareness has supported more research efforts in the area, which are contributing to a better understanding of HABs and contamination of seafood by algal toxins, as well as the chemistry, mechanisms of action and toxicity of phycotoxins. The accumulation of information in this field has led to the conclusion that we are witnessing a global increase in HABs and seafood contamination, and more effective and complex measures to prevent human intoxications are being developed and implemented worldwide.

The increased recording of occurrence of toxic algae and HABs in coastal waters in several areas in the world is certainly a result of a deeper attention paid to the phenomenon. Other factors, however, are being recognized as contributing to the increasing frequency of HAB outbreaks, their appearance in areas of the world where they had not been recorded in the past, as well as the intensity and duration of HABs, with their possible consequences on seafood contamination and human intoxications/poisoning [7].

The ongoing changes can be exemplified by the trend of recording of *Ostreopsis* species in the Mediterranean Sea, which has been essentially anecdotic in the past century, in keeping with the mainly tropical distribution of these algal species. Over the last 5 years, blooms of *Ostreopsis* in several parts of Mediterranean Sea have been recorded (Fig. 1), and in some cases these have been accompanied by human intoxications involving up to two hundred people, e.g., in Italy in 2005 [8, 9].

The factors proposed to be involved in the global increase in HABs include the eutrophication of coastal waters as a consequence of increased aquaculture and fertilizer runoff from agriculture, as well as other economic activities linked to urbanization, the changes in climatic conditions, the transportation of toxic algae and their cysts from one coastal area to another as a consequence of their presence in the ballast water of ships or through the movement of shellfish stocks [7]. Furthermore, a recent meta-analysis of published data and historical records provided indications that the regional loss of species diversity and ecosystem services in coastal oceans increases the occurrence of algal blooms [10]. HABs and the contaminations of seafood, undoubtedly represent relevant social issues, because of the problems they pose to human

Figure 1. Records of *Ostreopsis* blooms in coastal areas of the Mediterranean Sea in the years 2000–2008. The map has been compiled on the basis of data reported in literature and of information kindly communicated by the Italian National Reference Laboratory for Marine Biotoxins (Cesenatico, Italy). The detection of *Ostreopsis* species is indicated by open symbols, whereas the record of health problems and intoxications that are suspected to be caused by the *Ostreopsis* blooms are indicated by the black symbols.

health, economic activities, recreation and tourism. The many facets of the phenomenon and their complexity represent a powerful drive for a better understanding of the chemistry and biology of phycotoxins, as a basis for a more effective protection of human health and the support of several human activities.

In this chapter we summarize available information on the chemistry, mechanisms of action of phycotoxins and the human poisoning they may cause. The complexity of the subject approached here, and the vast literature devoted to it, constrain our account to major issues. We apologize to the many scientists whose contributions have not been directly quoted in this chapter, and point the interested reader to excellent reviews devoted to specific topics, whenever appropriate.

Chemistry of marine biotoxins

Firstly, we should note that marine biotoxins are naturally produced compounds and, therefore, many enzymatic systems in nature are capable of metabolizing them. This characteristic puts them in contrast to man-made compounds such as polychlorinated biphenyls (PCBs) and pesticides many of which are extremely stable compounds for which nature has no metabolic processes foreseen. Similar to PCBs, dioxins or polycyclic hydrocarbons, most groups of marine toxins have also many analogues. Thus, between naturally produced analogues and metabolites of these, marine biotoxins constitute a vast array of bioactive chemicals.

Historic perspective on the isolation of marine biotoxins

Although the effects of marine toxins have been known for hundreds of years, the toxic principles involved were not discovered until the 20th century. The identification and characterization has been a lengthy process for some toxins. For instance, in the late 19th century, reports describe paralytic shellfish poisoning (PSP) as a poisoning caused by the consumption of blue mussels [11], the toxic principles of which also occur in starfish [12], without the identity of toxic principles being revealed. Groundbreaking work was completed by Sommer and Meyer [13] to link this toxicity to the occurrence of micro-algae and to conceive an assay that has remained the reference tool to our days, the mouse bioassay for paralytic toxins. Onoue et al. [14] started work on the isolation of saxitoxin analogues as the toxic principles of PSP. The efforts were significantly advanced by Schantz et al. [15, 16]. However, it was not until 40 years after initial isolation efforts that the structure of saxitoxin was finally confirmed by Wong et al. [17]. The characterization process has been hampered for many toxins in a similar fashion due to the lack of compound mass for the studies. This lack can be understood from the fact that the organisms producing the toxin cannot always be cultured, and scientists thus rely on the natural occurrence of the compounds. In addition, the structure elucidation in early days was mostly based on chemical reaction of the compounds. The onset of more powerful non-destructive techniques such as nuclear magnetic resonance (NMR) has allowed the characterization of smaller quantities: while several hundreds of milligrams were required to characterize a toxin in the 1950/60s, nowadays 10–100 μg of compound may be sufficient to complete the structure elucidation of a novel compound. Thus, the discovery of domoic acid as a shellfish toxin was completed within weeks from the poisoning event [18]. More typically, it takes one to several years from the initial poisoning event to the identification of the chemical responsible for the toxic effect, e.g., for the identification of okadaic acid and azaspiracids [19, 20].

Chemical nature of marine biotoxins

This section describes the characteristics of a selected range of marine biotoxins to demonstrate the wide-ranging chemical diversity of these groups of compounds. From a natural products or biosynthesis point of view, the compounds described in this section belong to several classes including amino acids (domoic acid), alkaloids (saxitoxin) and polyketides (all others). Therefore, algal toxins are often referred to as small molecules. Thus, the selection of toxins excludes all compounds that are typically referred to as natural polymers (proteins, carbohydrates, nucleic acids). Indeed the molecular mass of phycotoxins typically ranges between 300 and 1500 Da; nevertheless, some compound groups such as palytoxins and maitotoxins are very sizeable molecules of 2677 and 3422 Da, respectively. Maitotoxin has been reported as the largest

non-proteinaceous natural toxin. The chemical nature and molecular size classification distinguish phycotoxins from the very large group of venoms from snakes, spiders or cone snails which are typically very potent mixtures of proteinaceous toxins. Table 1 gives an overview of some characteristics of the compound groups discussed in the specific sections.

In addition to the above-mentioned difficulties in isolation of a toxin for its initial identification, it should be noted that one of the problems with natural compounds is the possible co-occurrence of isomers (compounds with the same molecular weight but slightly different structural arrangements) and analogues (compounds that derive from the same structural skeleton but have some structural difference leading to a different molecular weight). The term "analogue" is often used synonymously with the terms "metabolite" or "derivative" (see also section below, "common routes of metabolism"). While the framework of this chapter is too limited to exhaustively describe all known analogues of the toxin groups dealt with, we give some examples of the complexity of analogues for several groups in the specific section (e.g., saxitoxin, okadaic acid and pectenotoxins). All toxin groups have 10 or more analogues, often up to 30 or more.

Saxitoxins

Saxitoxin (STX)-group toxins are closely related compounds based on a tetrahydro purine skeleton. The basic character of the hydro purine group renders the molecule highly water soluble. More than 30 saxitoxins, mainly from marine dinoflagellates and shellfish that feed on toxic algae, have been identified [21–23], at least 18 have toxicological relevance (Fig. 2). They are mainly produced by dinoflagellates belonging to the genus *Alexandrium*, e.g., *A. tamarensis*, *A. minutum* (syn. *A. excavata*), *A. catenella*, *A. fraterculus*, *A. fundyense* and *A. cohorticula*. Also other dinoflagellates such as *Pyrodinium bahamense* and *Gymnodinium catenatum* have been identified as sources of STX-group toxins [21]. In addition, some analogues have been identified in some cyanobacteria which may occur in fresh and brackish waters.

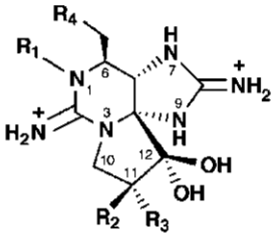
STX analogues do not exhibit a strong ultraviolet (UV) absorbance or fluorescence. They are typically stable to heat treatment up to 100 °C. Different acid and base treatments will lead to various transformations. In particular, all C11-epimeric pairs (e.g., GTX2 and 3 or GTX1 and 4) will interconvert and equilibrate to a constant ratio at high pH. Similarly, carbamoyl and sulfocarbamoyl derivatives will convert to decarbamoyl (dc) analogues through cleavage of the carbamoyl-ester group at high pH (e.g., GTX2 or C1 to dc-GTX2 and GTX3 or C2 to dc-GTX3). Under acidic conditions, the carbamoylester is relatively stable but the sulfate ester will be cleaved to convert sulfocarbamoyl groups into carbamoyl groups (e.g., C1 to GTX2 and C2 to GTX3). These transformations may only occur partially when shellfish tissues or human tissues or fluids contaminated with STXs are exposed to these conditions, as biological tissues typically buffer the pH. Since conversion reactions can result in a several fold increase in toxicity, a potential danger of these toxins was suggested [24]. To

Table 1. Characteristics of marine biotoxins: chemical formula, molecular weights, UV-absorption maxima, acidity constants and lipophilicity

Toxin	Chemical class	Formula	Molar weight	UV [nm]	$pKa_{1,2,3,4}$	Lipophilicity
Saxitoxin (STX)	Tetrahydro-purine alkaloid	$C_{10}H_{17}N_7O_4$	299	n/a	8.1, 11.5	Hydrophilic
Domoic acid (DA)	Cyclic amino acid, 3 carboxy groups	$C_{15}H_{21}NO_6$	311	242	2.1, 3.7, 5.0, 9.8	Hydrophilic
Okadaic acid (OA)	Polyether, spiro-keto assembly	$C_{44}H_{68}O_{13}$	804	n/a	4.9[§]	Lipophilic
Azaspiracid (AZA)	Polyether, second amine, 3-spiro-ring	$C_{47}H_{71}NO_{12}$	841	n/a	5.8[§]	Lipophilic
Palytoxin (PlTX)[*]	Polyether, 2 amide & a primary amine	$C_{129}H_{223}N_3O_{54}$	2677	263, 233	n/rep	Amphiphilic
Pectenotoxin-2 (PTX2)	Polyether, ester macrocycle	$C_{47}H_{70}O_{14}$	858	235	n/a[§]	Lipophilic
Gymnodimine	Cyclic imine, macrocycle	$C_{32}H_{45}NO_4$	507	n/a	n/rep	Lipophilic
Prorocentrolide	Cyclic imine, lactone macrocycle	$C_{56}H_{85}NO_{13}$	979	n/rep	n/rep	Lipophilic
13-Desmethyl Spirolide C	Cyclic imine, macrocycle	$C_{41}H_{61}NO_7$	691	n/a	n/rep	Lipophilic
Yessotoxin (YTX)	Ladder-shaped polyether	$C_{55}H_{82}O_{21}S_2$	1140	230	n/rep, 6.9[§]	Amphiphilic
Brevetoxin (BTX)-B	Ladder-shaped polyether	$C_{50}H_{70}O_{14}$	894	208	n/a	Lipophilic
Gambierol (Gb)	Ladder-shaped polyether	$C_{43}H_{64}O_{11}$	757	n/rep	n/a	Lipophilic
P-Ciguatoxin (CTX)-4B	Ladder-shaped polyether	$C_{60}H_{85}O_{16}$	1061	223	n/a	Lipophilic
Maitotoxin	Polyether, four fused ring systems	$C_{164}H_{256}O_{68}S_2Na_2$	3422	230	n/rep	Amphiphilic

[*] Palytoxin from *Palythoa toxica*

[§] Fux and Hess (unpublished observations) determined chromatographically (for YTX the pKa_1 was too low to be determined chromatographically, pKa_2 is given). n/a, not applicable; n/rep, not reported



R_1	R_2	R_3	R_4	Toxin
H	H	H	—O(C=O)NH_2	STX
H	H	OSO_3^-		GTX2
H	OSO_3^-	H		GTX3
OH	H	H		NEO
OH	H	OSO_3^-		GTX1
OH	OSO_3^-	H		GTX4
H	H	H	—O(C=O)$NHSO_3^-$	GTX5 (B1)
H	H	OSO_3^-		C1
H	OSO_3^-	H		C2
OH	H	H		GTX6 (B2)
OH	H	OSO_3^-		C3
OH	OSO_3^-	H		C4
H	H	H	—OH	dc-STX
H	H	OSO_3^-		dc-GTX2
H	OSO_3^-	H		dc-GTX3
OH	H	H		dc-NEO
OH	H	OSO_3^-		dc-GTX1
OH	OSO_3^-	H		dc-GTX4

Figure 2. Saxitoxins.

examine this phenomenon experimentally, B1 (GTX5) was incubated at conditions simulating the human stomach and analyzed by the mouse bioassay. After 5-h incubation at 37 °C, a twofold increase of toxicity corresponding to 9% conversion of toxin was observed in the artificial gastric juice at pH 1.1 and no apparent increase of toxicity in rat gastric juice at pH 2.2 [25].

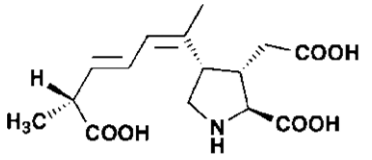
The marine organisms most often affected are mussels and oysters, but also puffer fish and marine snails (e.g., abalone) have been reported to accumulate dangerous concentrations. The hydrophilic character of the compounds may partially explain the relatively rapid depuration of these toxins from mussels.

This rapid depuration complicates the regulatory surveillance for these toxins, which is therefore usually complemented by observations of the algae responsible for *in situ* production.

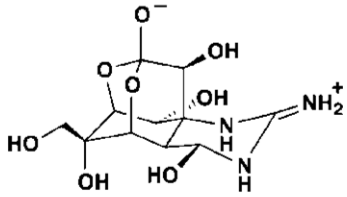
Complex toxin profiles, possible conversions and lack of reference materials have led most countries to maintain the mouse bioassay introduced by Sommer and Meyer [13] and validated as AOAC method (959.08) [26]. This assay can be used for the quantitation of levels above 350–400 μg/kg. Alternative methods have been proposed based on chromatography and fluorescence detection by Oshima [27], and Lawrence et al. [28], the latter also being officially validated as AOAC method (2005.06) [29]. These HPLC methods are technically challenging, time consuming and depend on a continuous supply of a large number of toxin standards as reference compounds. Due to the hydrophilic character of STXs, their complete chromatographic separation proved difficult until the introduction of hydrophilic interaction chromatography by Dell'Aversano et al. [30]. Also, the physicochemical determination of STXs has relatively high quantification limits, which are slightly lower than or similar to the detection limits of the mouse bioassay for complex toxin profiles.

Domoic acid group

Domoic acid (DA) is a small cyclic amino acid (311 Da), with three carboxylic acid groups (Fig. 3). These groups are responsible for its solubility in water and its relatively high polarity, resulting in early elution in reverse-phase chromatography [31]. The acid constants (pKa) of the three carboxylic acids and the cyclic amino group have been determined using NMR techniques by Walter et al. [32] (Tab. 1). Although numerous isomers and several analogues have been reported [33–38], so far only DA and its C5-diastereomer have been



Domoic acid



Tetrodotoxin

Figure 3. Small water-soluble toxins: domoic acid (DA) and tetrodotoxin (TTX).

shown to be of toxicological relevance [39]. DA transforms into its diastereomer through heat or long-term storage [40] and analysis has focused on determination of the sum of these two isomers as best estimate of the total toxicity. A conjugated double bond in the aliphatic side chain allows detection of DA by UV absorbance and both UV and MS detection are commonly used for the physicochemical determination of DA [41]. The conjugated double bond also leads to light sensitivity and is the cause of radical-mediated oxidative metabolism. As a contaminant in shellfish tissues, DA is heat stable and cooking does not typically destroy the toxin. However, protein coagulation leads to retraction of the tissues and DA as a water-soluble compound may be transferred significantly to cooking fluids [42]. Its stability under various conditions has been studied, and storage of raw or autoclaved tissues only resulted in ca. 50% degradation of the toxin after 5 months [43].

Domoic acid has been reported in a wide variety of seafoods, including mussels, scallops and anchovies. Due to the common occurrence of its source organism (the diatom *Pseudo-nitzschia* spp.), DA is spread worldwide. Thanks to the lightly diarrheic properties caused by the macro-alga *Chondria armata* (of which DA is the active ingredient), it has been used in Japan as anti-worming agent (reviewed in [39]). However, the severe poisoning in 1987 in Canada of over 100 people following consumption of mussels, including 3 fatalities, stopped this practice. The water-soluble character also results in relatively rapid depuration from shellfish (similar to STX), and regulatory surveillance is complemented by screening of shellfish production waters for *Pseudo-nitzschia* to allow early warning in an attempt to prevent human poisoning.

Azaspiracid group

Azaspiracid (AZA1) is an intermediately sized polyether toxin (841 Da, Fig. 4). The chemistry, ecology and toxicology of AZAs have been extensive-

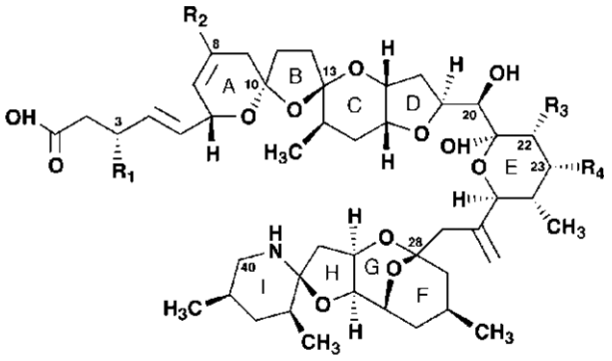


Figure 4. Azaspiracids: AZA1 ($R_{1,2,4}$ = H; R_3 = CH_3), AZA2 ($R_{1,4}$ = H; $R_{2,3}$ = CH_3). The initial structure proposed by Satake et al. [20] was corrected by Nicolaou et al. [51, 52]. The corrected structure is shown.

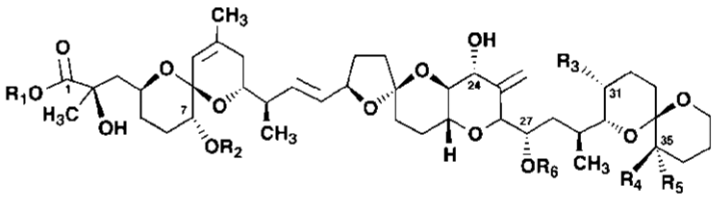
ly reviewed by Twiner et al. [44]. Although its geographical distribution was initially believed to be restricted to Europe, recent work has also demonstrated the compound in shellfish from North Africa, and in Canadian waters [44, 45]. AZA2 has recently also been identified in the sponge *Echinoclathria* sp., collected from Japanese waters, indicating that its producers occur worldwide [46]. However, so far poisoning directly attributed to AZA has only been reported from Europe, either due to environmental conditions not being appropriate for the producer to reach seafood in other locations, or due to the predominant screening for marine biotoxins with the mouse bioassay, an intrinsically unspecific method of detection of toxins. Also, the symptoms of AZA in human poisoning events are similar to diarrheic shellfish poisoning (DSP) from okadaic acid group compounds, and may therefore not always be followed up with further investigation. The mouse bioassay, initially introduced by Yasumoto et al. [19], for the detection of DSP toxins, also detects AZAs at similar levels [47].

Chemically, AZA is characterized by a cyclic amine group, a carboxylic acid and a unique tri-spiro ring assembly. Similar to okadaic acid, it is likely that the acid-labile character of the compound is related to the spiro-keto assembly (rings A, B and C in Fig. 4) [48]. Contrarily to okadaic acid, AZAs are also labile to strong bases, i.e., their destruction can be completed in methanolic solution through treatment with NaOH for 10 min at 76 °C. The mechanism for this reaction remains to be clarified. Due to the absence of conjugated double bonds or aromatic rings, the molecule has no chromophore or specific UV absorbance above 200 nm; therefore, physicochemical determination is mostly based on separation by liquid chromatography (LC) followed by detection using mass spectrometry (MS). An initial proposal of the chemical structure was made by Satake et al. [20], but a correction was made after chemical synthesis by Nicolaou et al. [49–52]. Approximately 20 analogues have been reported to occur naturally in shellfish [53]. However, only two of these, AZA1 and AZA2, have been reported to be produced by the previously unknown dinoflagellate *Azadinium spinosum* [54, 55]. Due to the minuscule nature of the causative organism (<20 μm), it cannot be easily identified using light microscopy and had only been discovered 12 years after the first poisoning event that was attributed to this toxin group [56]. The metabolism of AZA1 and AZA2 in mussels is presumed to follow an oxidative path at C3 and C23 and the methyl group at C22. Following the initial observation by Hess et al. [57] of increased AZA concentrations after heat treatment of AZA-contaminated mussel tissues, McCarron et al. [58] postulated that a carboxylic acid located at C22 is a product of such metabolism and that heat treatment leads to decarboxylation and further analogues of AZAs. In shellfish it is anticipated that the decarboxylation happens spontaneously over time. There are no reports on mammalian metabolism of AZAs. From the lipophilic nature of AZAs (Fux and Hess, unpublished observations), it is presumed that AZAs can pass the intestinal barrier, if they are sufficiently bioavailable. Stomach simulation experiments by Rehmann et al. [53] and Alfonso et al. [48] suggest that

there may be limited bioavailability due to the lipophilic character of AZA1; however, further *in vivo* studies will be required to clarify such behavior. Initial evaluation of the compounds using intraperitoneal (i.p.) injection in mouse bioassays suggests that the hydroxyl analogues are less toxic than the parent compounds [59]. Further structure-activity studies by Ito et al. [60] showed that a synthetic stereoisomer of AZA1 (C_{1-20} epi-AZA1) was three to four times less toxic than AZA1, and that a variety of smaller epitopes did not induce any effect similar to AZA1, thus suggesting that the entire skeleton is required to effectively interact with the biological target.

Okadaic acid group

Okadaic acid (OA) was originally found in the sponge *Halichondria okadaii* [1] but was only identified by Yasumoto et al. [19] as a shellfish contaminant following a series of poisoning events in 1976 (Fig. 5). In 1980, Yasumoto et al. [61] clearly demonstrated that DSP was associated with blooms of *Dinophysis fortii*, a dinoflagellate in which the authors also isolated an ana-



Toxin	R_1	R_2	R_3	R_4	R_5	R_6
OA	H	H	CH_3	H	H	H
DTX1	H	H	CH_3	CH_3	H	H
DTX2	H	H	H	H	CH_3	H
DTX3	H	acyl	as parent	as parent	as parent	H
Diol-ester	diol	H/acyl	as parent	as parent	as parent	H
DTX4	diol-ester	H	as parent	as parent	as parent	H
DTX5	diol-ester	H	as parent	as parent	as parent	H
27-*O*-acyl	H	H	CH_3	H	H	acyl

Figure 5. Okadaic acid (OA) and dinophysistoxin (DTX) derivatives; stereochemistry at C31 and C35 was clarified by Larsen et al. [313]. OA, DTX1 and DTX2 are the parent compounds independently produced by micro-algae. All other compounds listed are derivatives of these three, either identified in algae or in shellfish. DTX3-type compounds are ester derivatives (acyl group at C7–OH) of OA, DTX1 or DTX2 that have only been found in shellfish so far. Diol esters, DTX4 and DTX5 are derivatives of either OA, DTX1 or DTX2 detected in algae (but some recently in shellfish as well). The 27-*O*-acyl derivative has so far only been identified in a sponge.

logue of OA, dinophysistoxin-1 (DTX1). The same compound class was rapidly found as the causative agents of DSP in Europe [62]. Dinophysistoxin-2 (DTX2) has been discovered as a third main analogue by Hu et al. [63], explaining shellfish toxicity found in Irish mussels. OA and DTXs are produced by a variety of different dinoflagellates from the *Dinophysis* and *Prorocentrum* genera, including *D. acuta* and *D. acuminata*, as well as *P. lima* and *P. belizeanum*. Although the toxins of the OA group have been mainly reported from Japan and Europe, recent evidence in the gulf of Mexico demonstrates that *Dinophysis* in these regions may also produce the same compounds under appropriate environmental conditions [64]. Therefore, a global distribution of these toxins is now widely accepted and monitoring should occur during shellfish production.

Chemically, OA is one of the many polyether toxins among the phycotoxins (Fig. 5). Its structure is characterized by a carboxylic acid group and three spiro-keto ring assemblies, one which connects a five with a six-membered ring. OA, DTX1 and DTX2 withstand a wide pH range from mildly acidic to strongly basic, e.g., no degradation is found for up to 40 min at 76 °C in 0.3 M methanolic NaOH solution. Treatment with strong mineral acids, e.g., HCl, leads to rapid degradation: OA and DTX1 are completely destroyed within 20 min at 76 °C of 0.3 M methanolic HCl, even in the presence of shellfish matrix in the extract. However, without the addition of acid, the compounds are rather stable to heat. Also, recent work on stomach simulation experiments in the author's laboratory suggests that the food itself has a buffering capacity on the acid and the toxins may not be destroyed significantly in the gastric juice. In normal cooking procedures the toxins are not destroyed, although the coagulation of proteins in shellfish tissues may lead to redistribution within the organs of shellfish and some toxins may be released into the cooking fluids [65].

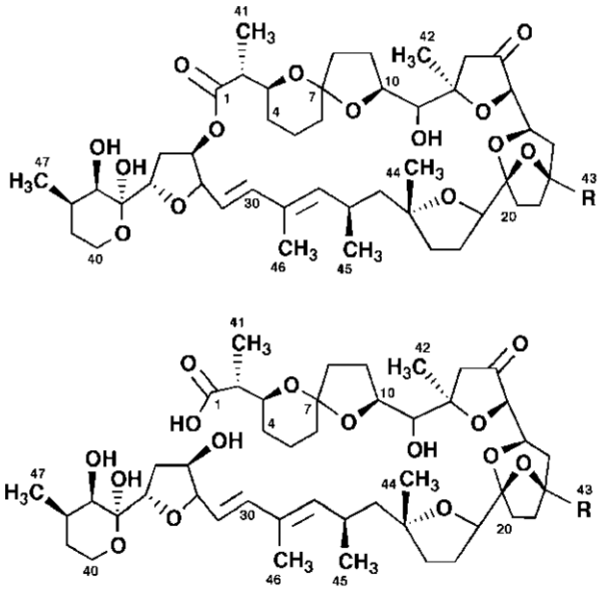
Different types of esters of OA and DTXs have been reported. In algae (so far mainly *P. lima* and *P. belizeanum*), esters of allylic diols with the carboxylic acid at C1 of OA and DTXs have been reported [63, 66, 67]; these esters were named DTX4, DTX5, etc. When the algae enter shellfish through natural filter-feeding, it is believed that these esters are rapidly degraded [68]. The shellfish then further metabolize OA and its analogues to form esters of OA and DTXs with fatty acids (at the C7–OH group). These esters were initially identified for DTX1 as shellfish derivatives [69] and their toxicity has been described to be similar to the parent compounds, although the onset appears later in the i.p. mouse model [70]. A further fatty acid ester of DTX1 at the C27–OH group has been reported in a sponge [71], and most recently, Torgersen et al. [72] also reported mixed esters of diols (at the C1 carboxyl end) and fatty acids (at the C7–OH position) in shellfish, suggesting that partial degradation and simultaneous metabolism may co-occur during digestion of algae by shellfish. The multitude of compounds potentially present in shellfish (free toxins, diol esters and their derivatives, fatty acids and mixtures of diol and fatty acid esters) leads to difficulty in determining the complete toxin

content in shellfish samples. This complexity has added to the difficulties in estimating the potency of these toxins and evaluation of their risk. The ester bond has not shown any degradation in long-term stability studies in the authors' laboratory; however, fatty acids have been reported to oxidize easily if they contain double bonds. Any of the esters discussed above (either at the C1–carboxyl or at the C7–OH) may be quantitatively cleaved through treatment with strong base, e.g., 0.3 M methanolic NaOH at 76 °C for 10–40 min; this characteristic, in combination with the stability of the parent compounds (OA, DTX1 and DTX2) to base treatment, has been extensively used to quantitatively determine the equivalent of parent compound present in any given shellfish sample [73].

A recent review of recorded poisoning events suggests that esters of OA and DTXs have very similar toxicity to the parent compounds in human poisoning [74]. OA and DTX1 are considered to be of approximately equal toxicity when injected i.p. into mice, while DTX2 has been reported to have only ~50–60% of the toxicity of OA [75], both by i.p. injection into mice and by assessment of their inhibitory character towards phosphoprotein phosphatases (see section "Mechanism of action of ocadaic acid and related compounds" below).

Pectenotoxin group

Pectenotoxins (PTXs, Fig. 6) are produced by *Dinophysis*, one of the main producers of OA and analogues. Pectenotoxin-2 (PTX2) is the main compound produced by *Dinophysis*. For this reason, PTXs were initially associated with diarrheic poisoning; however, subsequent studies clearly demonstrated that PTXs have a distinct mechanism of action that differs from that of OA and analogues (see section "Mechanism of action of pectenotoxins" below). PTXs are a group of polyethers with molecular weights similar to the OA and AZA groups, and PTXs also have two spiro-ketal ring assemblies. Contrarily to OA and AZA, active forms of PTXs represent a macrocyclic intramolecular ester and do not possess a free carboxylic acid group (Fig. 6). Thus, PTX2 behaves chromatographically like a neutral compound of high lipophilicity (Fux and Hess, unpublished observations). A comprehensive review of the occurrence, chemistry and shellfish metabolism of PTX analogues is given by Miles [76]. We describe here several analogues that exemplify the three main routes of metabolism in shellfish. In the Japanese scallop (*Patinopecten yessoensis*), PTX2 is successively metabolized to PTX1, PTX3 and finally PTX6 [77]. In all these analogues the macrocycle is maintained, which means their lipophilicity is only slightly altered. In contrast, in mussels (*Mytilus edulis*), PTX2 is metabolized to a seco-acid (PTX2sa), in which the macrocycle is opened [78]. This ring opening is clearly related to a loss in the bioactivity, as PTX2sa shows no activity when injected i.p. in mice. A further route of metabolism in mussels is the esterification of PTX2sa with fatty acids to yield PTX2sa fatty acid esters [79]. Although the toxicity of these compounds has not yet been evaluated, it is anticipated that it is relatively reduced as the parent PTX2sa already does not show any toxicity. Ito et al. [80], also showed evidence for a reduced oral tox-



Toxin	R (position 43)	C_7
PTX1	CH_2OH	7*R*
PTX2	CH_3	7*R*
PTX3	CHO	7*R*
PTX4	CH_2OH	7*S*
PTX6	COOH	7*R*
PTX7	COOH	7*S*
PTX2sa	CH_3	7*R*
7-epi-PTX2sa	CH_3	7*S*

Figure 6. Pectenotoxins. The complete macrocyclic ester of the PTX2 structure (and its derivatives) is shown in the top panel while the bottom-figure depicts the structure of the hydrolyzed seco-acid (sa) compound (PTX2sa), where the macrocycle is opened. C_7, configuration at position C7.

icity of PTX6, compared to PTX2, thereby suggesting that the main issue with pectenotoxins would be the presence of still non-metabolized PTX2. It is not clear whether such remaining PTX2 is bioavailable (due to its high lipophilicity it may not be effectively liberated during human digestion of shellfish tissues) or whether it withstands human digestive conditions. PTX2 has been shown to be rather labile, even under lightly acidic or lightly basic conditions and very rapid metabolism to non-toxic seco acids is likely if the compound is effectively liberated during digestion.

Yessotoxin group

Yessotoxin (YTX) and analogues are also polycyclic ether compounds; special characteristics consist of the 11 contiguously transfused ether rings, an unsaturated side chain and two sulfate ester groups (Fig. 7). The contiguously transfused rings make YTX and analogues chemical relatives of brevetoxins and ciguatoxins; this structural characteristic has also led to the classification of ladder-shaped polyethers. Although this rigid structure constitutes a rather unpolar part of the molecule, YTX is considered of intermediate lipophilicity, as it also features two sulfate ester groups. The biogenetic origin, its chemistry, synthesis and structure-activity relationships of analogues have been recently reviewed by Hess and Aasen [81].

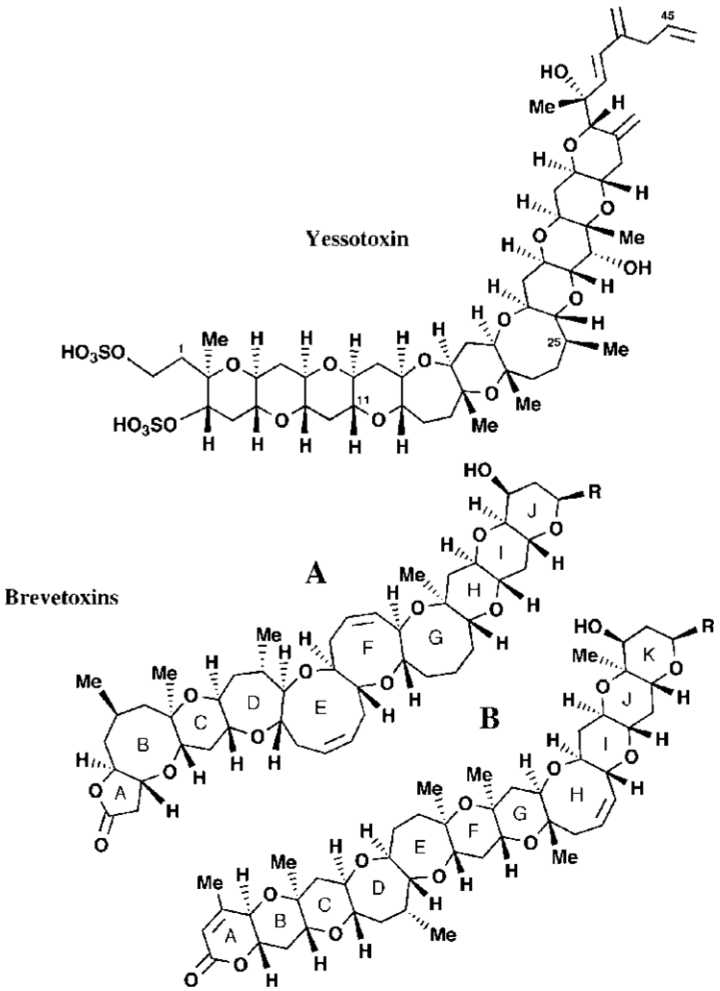


Figure 7. Ladder-shaped polyether toxins: yessotoxin (YTX) and brevetoxins (PbTXs) A and B.

YTX was first isolated from the digestive glands of scallops *Patinopecten yessoensis* in Japan [82]. Because of its discovery through the mouse bioassay originally developed for the detection of DSP [19], and due to its frequent co-occurrence with truly diarrheic toxins, YTX was initially mis-classified as one of the DSP toxins. Later, it was shown that YTX does not cause diarrheic effects when administered orally to mice [83–85]. Yessotoxin and its analogues are produced by the dinoflagellate algae *Protoceratium reticulatum* [86–89], *Lingulodinium polyedrum* [90] and, as recently reported, also by *Gonyaulax spinifera* [91]. Since the initial discovery of YTX, several more analogues of YTXs have been discovered in many parts of the world including Japan, Norway, Italy, Scotland and Chile [92]. Over the last few years, this toxin group has been shown to contain a large number of analogues, including 45-hydroxy-YTX, carboxy-YTX, 1-desulfo-YTX, homo-YTX, 45-hydroxy-homo-YTX, carboxyhomo-YTX, heptanor-41-oxo-YTX, heptanor-41-oxo-homo-YTX, trinor-YTX, adriatoxin, (44-*R,S*)-44,55-dihydroxy-YTX, and 9-methyl-YTXs [93]. Miles et al. [94] have described numerous analogues of YTX in *P. reticulatum*.

Although different toxicities have been reported for YTX itself when using different mouse strains (e.g., [82, 83]), crude estimates of relative toxicities can be obtained when using the same strain of mice for comparison of analogues, preferably in parallel. In this way, it is clear that YTX and homo-YTX have approximately the same toxicity [95], and that all other analogues have lesser toxicity than YTX, with hydroxyl and carboxy derivatives being approximately five times less toxic than the parent compounds. Some derivatives such as the trihydroxylated amides of 41-a-homo-YTX and the 1,3-enone isomer of heptanor-41-oxo-YTX have not shown any toxicity by i.p. injection into mice at levels >5000 μg/kg body weight [96, 97].

Another major pharmacological phenomenon directly related to the chemical structure is the large difference observed between toxicity of YTX in mice injected i.p. and those orally exposed to YTX [83]. This study showed that two out of three mice died when injected with a dose of 0.75 mg YTX/kg body weight, and three of three mice died when injected with a dose of 1 mg YTX/kg body weight, while all mice survived when exposed orally to a dose of 10 mg/kg. Similar observations were made by Munday and coworkers (quoted in [21]). The difference in i.p. and oral toxicity of YTX is probably related to low YTX absorption in the gastrointestinal tract. While the solubility of YTX in water facilitates bioavailability of YTX, it is probably also the reason for very short residence time in the gastrointestinal tract, thus diminishing overall absorption. The large differences in toxicity between YTX and its oxidized analogues 45-hydroxy-YTX and carboxy-YTX are likely to be related to the further increase in water solubility. A different approach to determining the relationship between structure and activity was taken by Ferrari et al. [98], where different YTX analogues were dosed onto cultured cells. The authors obtained different toxic equivalence factors with the 45-hydroxy and the 55-carboxy analogues being ~20–50 times less toxic. These differences

could be related to the more complex toxicology in live animals or to differences in the standards used.

Palytoxin group

With a continuous chain of 115 carbons, palytoxin (PlTX) is one of the largest polyether-type phycotoxins (Fig. 8). The many hydroxyl groups in the molecule characterize it as a polyol and together with the amine and amide groups are responsible for its hydrophilicity. The long carbon chain constitutes a lipophilic part. Thus, PlTX has a mixed hydrophilic and lipophilic character that also results in soap-like behavior at larger concentrations in aqueous solutions. The structure of PlTX was clarified in 1981 [99–101]. Recent reviews demonstrate that there is a lack of understanding on possible origins of PlTX and related compounds [102, 103]. While the compound was originally reported from the coelenterate zoanthids *Palythoa toxica* [6] and *Palythoa tuberculosa* [104], it is now clear that some micro-algae (*Ostreopsis siamensis* [105], *Ostreopsis ovata* [106] and *Ostreopsis mascarenensis* [107]) also produce PlTX and a number of related compounds. Symbiotic micro-organisms have been postulated as the true source of PlTX [108, 109] and bacterial involvement is still not excluded [110]. This toxin group was traditionally associated with fish poisoning and aerosol problems in the tropics. More recently, *Ostreopsis* spp. as well as PlTX and related compounds were also found in Southern Europe (Spain, Italy and Greece), mostly causing problems to people bathing at beaches on the Italian coast of Genoa [111], and by mouse assays of shellfish from Greece [112].

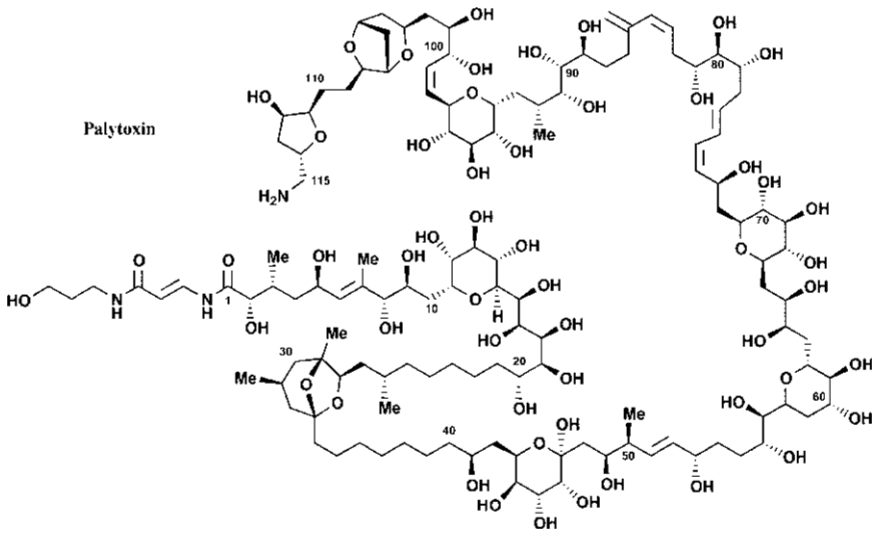


Figure 8. Palytoxin (PlTX).

Cyclic imines

Several compound groups have been found in this category: gymnodimines, spirolides, pinnatoxins and pteriatoxins, pinnaic acids and halichlorines, prorocentrolides and symbio-imines [67, 113–116]. A selection of chemical structures for some of these groups are shown in Figure 9. A common structural feature of all these compound groups is the hexa- or heptacyclic imine ring, which is believed to contribute substantially to the bioactivity of these compounds. Using spirolides as an example, the opening of this ring has been related to loss of bioactivity [117]. However, this is not the only contributing factor as intricate stereochemical features may also play important roles, as demonstrated by McCauley et al. [118]. They were able to show that natural (+)-pinnatoxin A was very toxic, while synthetic (–)-pinnatoxin A was non-toxic. The unique neurotoxicity of cyclic imines is visible in mice following i.p. injection of the toxin, which leads to rapid death within minutes; this feature has led to grouping cyclic imines together as "fast-acting toxins". Due to the structural variety of the group, it is difficult to give physicochemical details in the frame

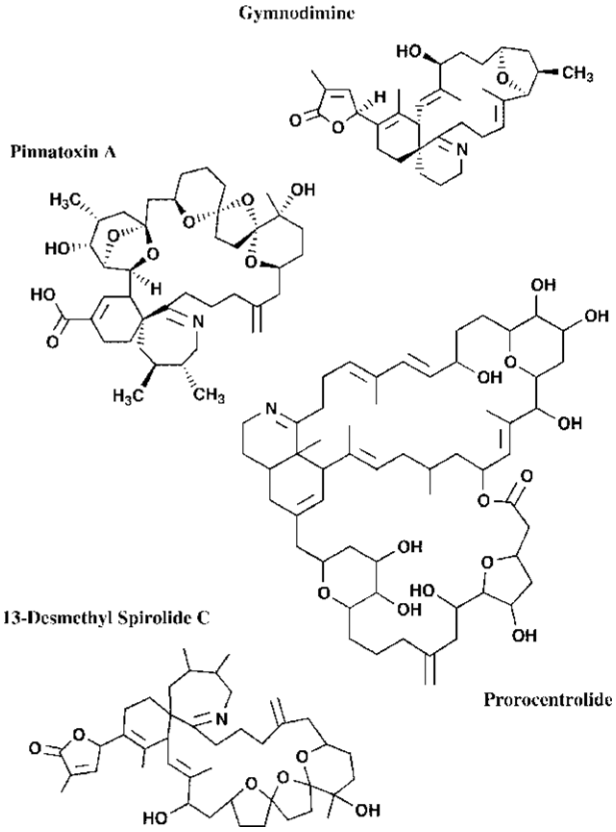


Figure 9. Cyclic imines.

of this chapter. Recent reviews give an overview of the chemistry and toxicology of these groups [119–121].

Brevetoxin group

Brevetoxins are a group of polyether toxins produced by the dinoflagellate *Karenia brevis*, and they also belong to the class of ladder-shaped polyethers, such as yessotoxin and ciguatoxin. Two types of abbreviations have been used (BTX and PbTX), sometimes leading to confusion. There are two basic skeletons (Fig. 7), a type-A skeleton, with 10 fused polyether rings, and a type-B skeleton with 11 fused polyether rings. BTX-A group compounds include the analogues PbTX-1, -7 and -10, while BTX-B compounds include PbTX-2, -3, -5, -6 and -9. The structure of PbTX-4 has never been confirmed, and PbTX-8 is an artifact from extraction procedures during the preparative isolation of brevetoxins [122]. *K. brevis* had undergone a number of name changes and was previously referred to as *Gymnodinium brevis*, *Gymnodinium breve* and *Ptychodiscus brevis*, the latter name leading to the abbreviation PbTX. Interestingly, another *Karenia* species, *K. mikimotoi*, produces a related ladder-shaped polyether toxin, gymnocin [123]. PbTX-2 (= BTX-B) is the main analogue produced by *K. brevis* and tends to be the main analogue found in seawater during *K. brevis* blooms; however, it is rapidly transformed into the ten times more toxic PbTX-3 (dihydro-PbTX-2), which is the main constituent in marine aerosols [124]. Brevetoxins had initially been only reported from U.S. and the Mexican gulf, but have subsequently also been found in New Zealand waters. Although the illness is known since the mid 19th century, full structure elucidation was only possible during the 1980s [125–127]. In Florida, *K. brevis* is known as a red tide organism, and the effects of the algae are threefold: aerosol exposure leading to skin damage and respiratory problems as well as accumulation in seafood leading to neurotoxic shellfish poisoning (NSP) (see sections below). While the brevetoxins produced by algae are very lipophilic compounds, some metabolites in shellfish have a slightly more hydrophilic character, due to the biotransformation to cysteine conjugates [128, 129]. Abraham et al. [130] have recently reported more polar metabolites from marine aerosols, in which the A-ring is opened, leading most likely to a reduced toxicity if the same structure activity relationship applies as found by Rein et al. [131]. Bourdelais et al. [132], have isolated an interesting compound from *K. brevis*, namely brevenal. This compound is potentially a biosynthetic precursor to brevetoxins but has been shown to completely inhibit PbTX action on Na^+ channels by competitive binding and is not toxic to fish. Dechraoui et al. [133] have studied the binding of analogues of this group to voltage-gated Na^+ channels, further contributing to the knowledge on the relative toxicity of analogues.

Ciguatera-related toxins

The toxins related to ciguatera fish poisoning comprise multiple groups. Although okadaic acid, palytoxin and other compounds have been implicated in some cases of ciguatera, we focus here on those toxins that are part of the

ciguatera complex that have not been described previously. Ciguatoxins (CTXs), gambierol and maitotoxin (MTX) are three groups of compounds among the toxic metabolites produced by *Gambierdiscus toxicus*, the main dinoflagellate responsible for contamination of fish by ciguatera toxins (Fig. 10). MTX is amphiphilic and is thus soluble in water, methanol and dimethyl sulfoxide. It is relatively stable in alkaline but not in acidic conditions [134]. MTX as polyhydroxy polyether with two sulfate ester groups is amongst the more hydrophilic polyethers, does not migrate up the food web, and is restricted to herbivorous fish (and potentially other grazing organisms). Gambierol and CTXs are more lipophilic polyethers and thus will persist and move up the food chain more easily to predatory (piscivorous) fish. The CTX analogue shown in Figure 10 is P-CTX-4B, one of the primary compounds produced by *G. toxicus* in the pacific. It appears to be a precursor to P-CTX-1 from moray eel [135, 136], which is the major constituent in most piscivorous fish in the Pacific, frequently contributing >90% to the overall toxic equivalents [137, 138]. A number of additional, often minor, analogues were isolated from the Indian, Pacific and Caribbean oceans [135, 136, 138–142]. The very low doses, which may already cause problems to consumers, result in challenges of ultra-trace detection in the range of 0.1 µg/kg to several µg/kg.

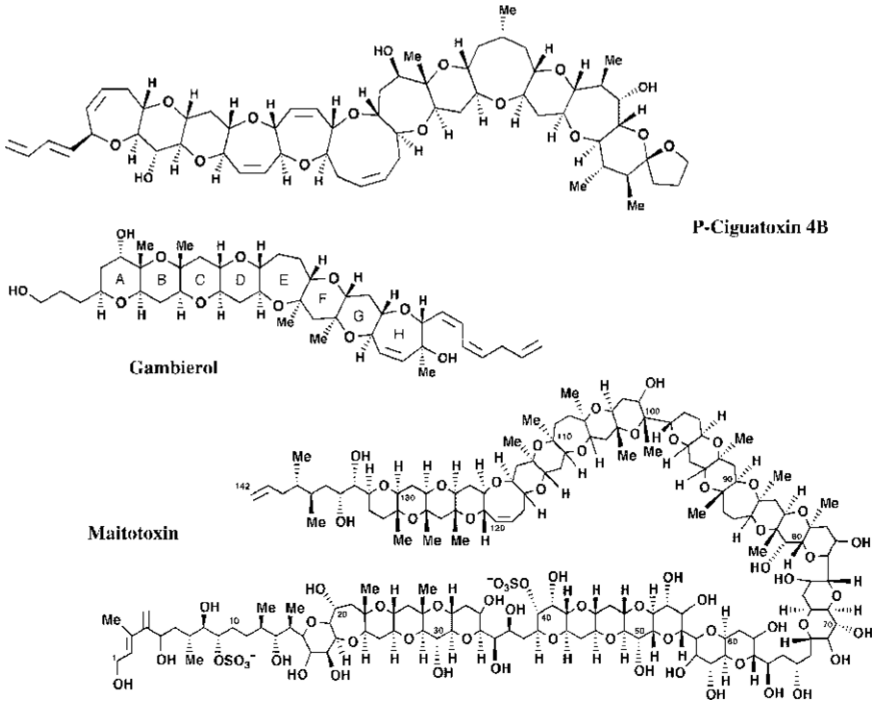


Figure 10. Ciguatera-related toxins: pacific (P-) ciguatoxin (CTX) 4B (P-CTX-4B), gambierol and maitotoxin (MTX).

For this reason, there are few methods available for the detection of these toxins, and worldwide, there are only few groups capable of analyzing CTXs, mainly located in Canada, US, Japan and Australia [143].

Preference of *G. toxicus* for warm water temperatures was demonstrated through correlation of sea surface temperatures with the occurrence of the organism [144]. Thus, CTXs are currently restricted to tropical and subtropical latitudes; however, distribution may well increase through rising sea surface temperatures in many areas. They are globally distributed across the Indian, Pacific and Caribbean oceans. A recent review by Dickey [143] describes difficulties in the analysis and diagnosis of this complex illness. The organism *G. toxicus* was discovered by Yasumoto et al. [145] and first described by Adachi and Fukuyo [146].

Common routes of metabolism of marine toxins

For a number of lipophilic shellfish toxins like PTXs, OA, DTXs, PbTXs, and spirolides, fatty acid ester derivatives have been reported to occur in shellfish tissue [69, 79, 129, 147]. However, until now no such esters have been reported for yessotoxins (YTXs) (although more than 80 different analogs of YTX have been reported so far [94]) or for AZAs [53]. In return, YTXs have shown hydroxy, dihydroxy and carboxy analogs, as discovered for AZAs [53, 93, 95, 148]. In respect to formation of analogs, AZAs show some similarities to YTXs and it is thus possible that more AZA analogues will be discovered in the future. Furanoside analogues have been reported for YTXs [149], and cysteine conjugates for brevetoxins [128]. Therefore, it is likely that further metabolites will still be discovered for several toxin groups; however, it should also be noted that many isolation efforts have been directed by bioactivity screening using either mouse bioassays or, more recently, cytotoxicity or functional assays. This approach facilitates the discovery of toxicologically relevant analogues, whereas a purely chemical structure-based approach, e.g., by LC-MS or ELISA methodology tends to discover all related chemical structures, irrespective of their toxicological relevance. It is arguable if one or the other approach should be favored; certainly the combination of approaches has allowed for the clarification of metabolism in fish or shellfish of many algal toxins and for such exciting discoveries as the brevenal antagonist of brevetoxins produced in the alga itself.

Mechanisms of action of phycotoxins

Our knowledge and understanding of the molecular mechanisms of action of the different marine biotoxins vary widely. In some cases, available data allow an appropriate comprehension of the events triggered by toxins and the description of the molecular bases of effects detected in living organisms. In

other instances, the picture is barely sketched, and many aspects remain undetermined. Our outline of the mechanisms of action of marine biotoxins is organized taking these differences into account. When we describe the toxins whose molecular mechanisms of action are not yet established our presentation is aimed at summarizing the most relevant findings about individual groups, with regards to their possible toxicological implications. Any association between effective concentrations/doses used *in vitro* and found *in vivo* is taken into account, if available. In mechanistic terms, particular attention is given to the molecular events triggered by lower toxin concentrations, implying high affinity binding to their primary molecular targets (receptors).

Molecular mechanisms of phycotoxins acting through binding to ion channels and other ion transfer systems

The better characterized mechanisms of action involve several groups of biotoxins whose recognized primary molecular targets are proteins involved in the movement of ions that are located at the level of the plasma membrane.

Biotoxins altering ion transport mechanisms in sensitive cells at the level of plasma membrane, perturb primarily, but not exclusively, the normal functioning of neuronal and other excitable tissues. The biotoxins considered in this section comprise components classified in different chemical groups, including saxitoxins (and tetrodotoxin), brevetoxins, ciguatera toxins (ciguatoxins, maitotoxin, gambierol), palytoxins, domoic acid and cyclic imines.

The primary molecular targets (receptors) of these toxins and/or their mechanism of action differ, depending on individual components, and a certain level of complexity is apparent, as a consequence of the primary event caused by individual toxins. Many basic biological processes, in fact, depend on the maintenance of specific ion gradients across the plasma membrane. Thus, an alteration of the intracellular concentration of one ion can trigger a secondary change in the intracellular concentrations of other ions in the course of the molecular events triggered by the interaction of a toxin with its receptor. A schematic representation of toxin receptors and their mechanisms of action are reported in Figures 11 and 12.

Mechanism of action of saxitoxins

The voltage-gated sodium channel (VGNC, Fig. 11A) is the recognized receptor of both saxitoxins (STXs, Fig. 2) and tetrodotoxin (TTX, Fig. 3). Before we consider the functional consequences of toxin interaction with the VGNC, we briefly outline the molecular organization and general functioning of these membrane proteins, which are also the target of other groups of algal biotoxins. Furthermore, the VGNC is a prototype for membrane proteins functioning as ion channels, and some description of this channel will be useful in analyzing the mechanism of action of groups of marine biotoxins that affect the functioning of other ion channels (see below).

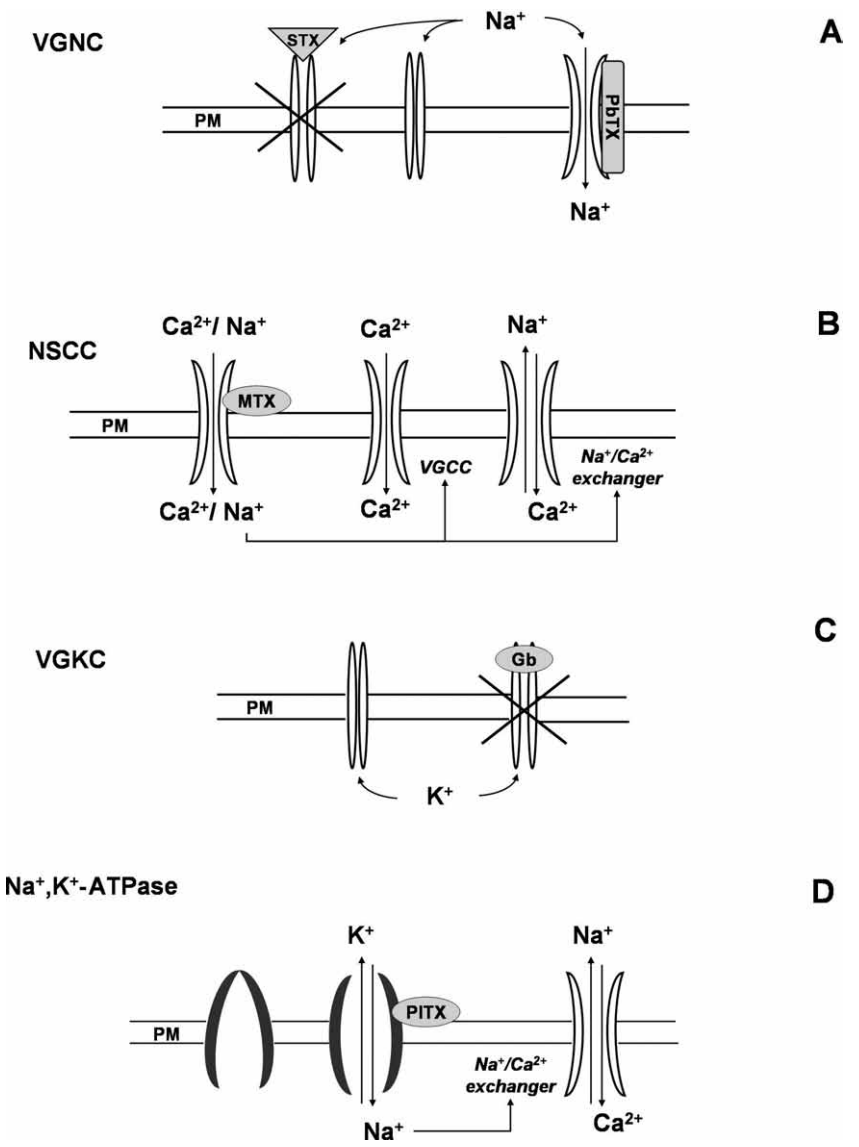


Figure 11. Schematic representation of the molecular mechanisms of action of saxitoxins, brevetoxins, ciguatoxins, maitotoxin, gambierol, palytoxins. (A) Mechanism of action of saxitoxin (STX) and brevetoxins (PbTXs), exerted by binding to voltage-gated sodium channels (VGNC). (B) Mechanism of action of maitotoxin (MTX) by binding to non-selective cation channels (NSCC). In the case of membrane depolarization caused by sodium entry, a secondary increase of intracellular Ca^{2+} concentrations would be induced by opening of voltage-gated calcium channels (VGCC), and some Na^+/Ca^{2+} exchangers. (C) Mechanism of action of gambierol (Gb) by binding to voltage-gated potassium channels (VGKC). (D) Mechanism of action of palytoxin (PlTX) by binding to Na^+,K^+-ATPase: sodium entry determines a secondary increase of intracellular Ca^{2+} concentrations, due to the activity of Na^+/Ca^{2+} exchangers. PM, plasma membrane. See text for details.

The VGNC is a large family of plasma membrane proteins that are expressed primarily in excitable cells (reviewed in [150]). The functional channel consists of an oligomer comprising one large α subunit and one or two smaller β subunits. The α subunit is endowed with both the voltage-sensing function and the ion-transporting structures, whereas the β subunits have roles in the modulation of functional properties and subcellular location of the α subunits.

The structure of α subunits comprises four homologous domains that are formed by six transmembrane segments (S1–S6). Segment S4 is primarily involved in voltage sensing, whereas segments S5 and S6 are involved in the formation of the ion channel itself. The transmembrane segments of the four domains are connected by loops of the polypeptide chain that are mostly exposed to the aqueous environment at the two sides of the plasma membrane. The long loop connecting segments S5 and S6 includes a portion that is inserted within the membrane itself and the arrangement of the loops from the four domains of the VGNC forms the pore that controls the passage of the ion through the channel. The changes in the ion concentration inside the cell are the basis of the voltage sensing by segment S4, which undergoes a conformational change. This change results in the opening of the pore and the entry of sodium ions into the cell, leading to membrane depolarization and the response of excitable cells.

The VGNC is the target of many neurotoxins, interacting with different sites on the channel protein. STXs and TTX have different chemical structures and belong to separate toxin groups, but share their mechanism of action because they both interact with site 1 of the VGNC, which undergoes essentially the same structural change. Site 1 is shaped by a short portion of the amino acid stretches connecting the S5 and S6 transmembrane segments, giving rise to a cavity that accomodates the toxin. The interaction of one STX molecule (and of TTX) with the site 1 of the α subunit in the sodium channel [151] essentially plugs the channel at the level of the pore, as originally proposed by Hille [152], thereby blocking its ion conductance [153]. The loss of sodium conductance in excitable cells then prevents membrane depolarization and the transmission of the action potential, representing the molecular basis of the toxic effects of STXs. As a consequence of VGNC blockade, a progressive loss of neuromuscular function ensues.

Although the binding of STXs and the consequent blocking of the ionic flux is recognized as the mechanistic basis of the symptoms recorded in humans intoxicated by these toxins, the possibility that other ion fluxes might be affected by STXs, either directly or indirectly, should be borne in mind. The significant homologies among channels for different cations [150], in fact, could be the basis for other biological effects of this class of toxins. For instance, the action of STX on potassium and calcium channels has been reported [154, 155], but the effective doses in those molecular systems are three to four orders of magnitude higher (10^{-6}–10^{-5} M) than those affecting VGNC (10^{-10}–10^{-8} M), and the observed effect could be explained by a lower

affinity interaction of STXs with these channels. Thus, the toxicological relevance of the effects exerted by high STX concentrations through binding to channels other than the VGNC *in vivo* is questionable.

Finally, it has long been recognized that STXs can bind to soluble proteins, such as saxiphilin (reviewed in [156]), and this could add an additional set of molecular responses induced in sensitive systems.

Mechanism of action of brevetoxins and ciguatoxins

The VGNC is the primary molecular target of other phycotoxins, such as brevetoxins (PbTXs) and some ciguatoxins (CTXs), that cause the opening of the ion pore and sodium entry into the cells. These algal toxins interact with site 5 on the VGNC, leading to changes in the gating properties of the channel (Fig. 11A). Site 5 comprises sequences of the transmembrane segments S5 and S6, and the binding region of PbTXs would essentially span the entire transmembrane segments [150].

The components classified among ciguatera-related toxins include an heterogeneous array of natural compounds produced by *Gambierdiscus toxicus*, possessing distinct chemical properties (see section "Ciguatera-related toxins" above). Among the toxins produced by this algal species, only CTXs appear to selectively target the VGNC [157], whereas maitotoxin (MTX) and gambierol have separate molecular targets (see below).

The binding of toxins to the site 5 of the VGNC determines an increase in the activation threshold to more negative membrane potentials and blocks the inactivation of voltage of the VGNC, leading to enhancement of the sodium entry into the cell. The increased membrane permeability to sodium initially determines excitatory cellular responses (including release of neurotransmitters at some synapses), but loss of cell excitability eventually ensues, leading to paralysis [158, 159].

Mechanism of action of maitotoxin

Although the ingestion of food contaminated with MTX and gambierol has resulted in some symptoms of ciguatera in humans, providing the basis to include these compounds among ciguatera toxins [158, 159], the primary molecular mechanisms of action of MTX and gambierol do not appear to involve their interaction with VGNC (Fig. 11B and C).

The mechanism of action of MTX has long been recognized to involve increased calcium entry into cells, lending support to the conclusion that MTX acts through binding to the plasma membrane calcium channels, resulting in channel opening and enhanced calcium influx (reviewed in [160, 161]). In some systems, however, MTX has been shown to induce both sodium and calcium entry into cells [162], supporting the conclusion that the toxin would actually target a non-selective cation channel [163–165], as depicted in Figure 11B. The increased intracellular sodium concentrations, resulting from the opening of the non-selective cation channel, might then determine membrane depolarization and secondary increases in intracellular Ca^{2+} concentrations,

due to opening of voltage-dependent calcium channels, or some Na^+/Ca^{2+} exchangers [161].

In any case, the increase in intracellular calcium concentrations is considered the major event in the mechanism of action of MTX, and the proximal cause of effects observed in biological systems exposed to this toxin. The increase in intracellular Ca^{2+} concentrations detected in cells exposed to MTX is several-fold [164, 165], and the ion reaches levels that are known to trigger specific calcium-dependent responses (reviewed in [160]), such as neurotransmitter secretion and contraction in excitable tissues [166, 167], stimulation of hormone secretion [168, 169], increased metabolism of phosphoinositides [170–172] and activation of protein kinases [172, 173]. The toxicological relevance of some of these responses *in vivo*, however, remains to be established.

Although MTX has been a useful pharmacological tool in investigations probing Ca^{2+}-dependent cellular processes, a better characterization of molecular responses to MTX is needed to fully understand its mode of action and the molecular bases of the effects exerted in living animals. In particular, MTX is a potent cytotoxic agent [173–175], and exposure of biological systems to this compound is expected to result in disruption of cellular functioning and tissue damage.

Mechanism of action of gambierol

Only a few studies have been carried out on the molecular mechanism of action of gambierol, focusing the attention to the effects that this compound might exert on ion movement across the plasma membrane. The most recent data obtained by the patch clamp technique in different cellular systems have shown that voltage-gated potassium channels (VGKC) are blocked by gambierol with an IC_{50} in the 10^{-9}–10^{-8} M range [176, 177]. In those studies, VGNC were found to be insensitive to gambierol concentrations three to four orders of magnitude higher (10^{-6}–10^{-5} M) than the IC_{50} measured for gambierol inhibition of VGKC [176, 177]. VGNC could be targeted by gambierol at concentration ranges higher than 10–100 μM [178]. This effect on VGNC has been explained as a secondary event induced by the membrane depolarization and lowering of the action potential threshold following the proximal blockade of VGKC exerted by the toxin [177]. The possibility of low affinity binding of high gambierol concentrations to ion channels other than VGKC *in vivo* can not be excluded at the moment, although any toxicological relevance of the effects exerted by high gambierol concentrations through binding to VGNC *in vivo* is uncertain.

As opposed to other toxins produced by *G. toxicus*, gambierol does not appear to be cytotoxic up to a 50 nM concentration, although it induces a stress response in affected cells [179].

Based on available information, the VGKC appears the relevant target of gambierol in living animals (Fig. 11C), and the blockade of potassium efflux in sensitive cells would be the proximal event leading to some of the symptoms

recorded in humans intoxicated by food contaminated by this toxin, such as disturbances of taste and pain [158, 159, 176].

Mechanism of action of palytoxins

The Na^+,K^+-ATPase is the recognized receptor of palytoxin (PlTX) (Fig. 11D). The binding of PlTX to the N-terminal segment of the α subunit of the Na^+,K^+-ATPase, located on the extracellular portion of the molecule, converts the ion pump into a non-selective cation channel [180–182]. The details of the interaction between the toxin and its receptor have not been fully characterized, but its occurrence has been exploited for an extensive description of molecular features of the Na^+,K^+-ATPase and its functioning [183–187].

The identification of the Na^+,K^+-ATPase as the receptor of PlTXs stemmed from the pioneering studies by Habermann, showing that K^+ efflux was induced as an early response of erythrocytes exposed to picomolar concentrations of PlTX [188], and was fully established by demonstrating that PlTX-dependent ionic fluxes are induced upon expression of the Na^+,K^+-ATPase in yeast [189], and transmembrane cation fluxes are induced by PlTX in a cell-free system when *in vitro* synthesized Na^+,K^+-ATPase is incorporated in artificial membranes [190].

Plasma membrane proteins involved in ion transport, other than the Na^+,K^+-ATPase, have been proposed to contribute to molecular responses induced by PlTX in some sensitive systems. These include some non-selective cation channels [191], whose characterization has remained elusive so far. Moreover, VGNC and calcium channels have also been indicated as primary targets of PlTX [191, 192]. Although the contribution of these molecules to the effects exerted by PlTX in excitable cells may not be excluded at the moment, a large body of evidence would indicate that the Na^+ influx and K^+ efflux through the Na^+,K^+-ATPase should be the most relevant and proximal events in the mechanism of action of PlTXs.

Taking into consideration that the Na^+,K^+-ATPase is ubiquitous in animals, the sensitivity of both excitable and non-excitable cells to palytoxins is expected. In mechanistic terms, several events can be induced by the potassium efflux and sodium entry into the cell, resulting in membrane depolarization (Fig. 11D). The available data indicate a high level of complexity in the array of molecular and functional alterations caused by PlTX *in vitro* and *in vivo*, as outlined below.

In the first instance, sodium-dependent transport of calcium ions across the plasma membrane would be triggered, mostly by the involvement of Na^+/Ca^{2+} exchangers [191, 193, 194]. The increased intracellular Ca^{2+} concentrations would then trigger other Ca^{2+}-induced responses, such as muscle contraction and, in the heart, arrhythmias [181, 182, 191, 193, 195]. The lowering of intracellular pH, due to Na^+ efflux through Na^+/H^+ exchangers, could be another secondary event triggered by PlTXs [196–198].

The metabolism of eicosanoids is also affected by PlTX, by enhancing the production and release of prostaglandins in many biological systems

[199–201]. The increased release of prostaglandins from the endothelium and smooth muscle cells determines norepinephrine release and contraction of the rabbit aortas [202].

Other molecular processes have been shown to contribute to cellular responses to PlTX. A possible role of PlTX in carcinogenesis was originally proposed based on indications of some effect as a tumor promoter [199, 203]. This early observation led to investigations aimed at understanding the molecular mechanism responsible for that effect, and studies were primarily devoted to ascertaining whether the toxin could alter protein kinases involved in the control of cell proliferation, such as extracellular signal-regulated kinases (ERK) and the c-Jun-NH_2-terminal protein kinases (JNK) [204]. The major conclusions of those studies are that altered ion fluxes through the Na^+,K^+-ATPase induced by PlTX determine the activation of some ERK and JNK isoforms by different mechanisms, including increased prostaglandin production, the activation of pathways responsible for phosphorylation of ERK and JNK, respectively, and the inhibition of ERK dephosphorylation (reviewed in [205]).

In contrast to the experiments showing the tumor-promoting effect of PlTX, cytolysis has been found in several systems exposed to this toxin [180, 188, 206]. An altered Na^+,K^+-ATPase is recognized as the proximal cause of cytolysis, indicating that altered ion homeostasis and osmotic stress are involved in the process [180, 188, 206]. However, cytolysis can not represent the simple outcome of cell swelling accompanying altered Na^+ and K^+ fluxes in cells exposed to PlTX, because the cytolytic response is delayed compared to potassium efflux from the cells [188].

In conclusion, only some of the many molecular and functional effects of PlTX found in experimental systems can be integrated in a coherent picture providing a mechanistic explanation of the toxic responses observed in intact animals, and some of the effects detected in cellular systems most likely reflect cell-specific responses, under controlled experimental conditions. Still, the large body of evidence shows a complex array of events that are secondary to PlTX binding to Na^+,K^+-ATPase and conversion of the pump to a nonspecific ion channel.

Mechanism of action of domoic acid

The mechanism of action of domoic acid (DA) involves toxin binding to non-*N*-methyl-D-aspartate (non-NMDA) glutamate receptors (Fig. 12). Glutamate is one of the major neurotransmitters in the brain [207], and its importance in normal functioning of the central nervous system has been a primary drive for the extensive investigations on the mechanism of action of DA.

DA binds to non-NMDA receptors in several regions of the central nervous system, and the effects ensue from a coordinated and synergistic action of receptors functioning at the two sides of the synapsis, resulting in altered neurotransmission (see [39, 208, 209] for excellent recent reviews). Non-NMDA receptors include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA)

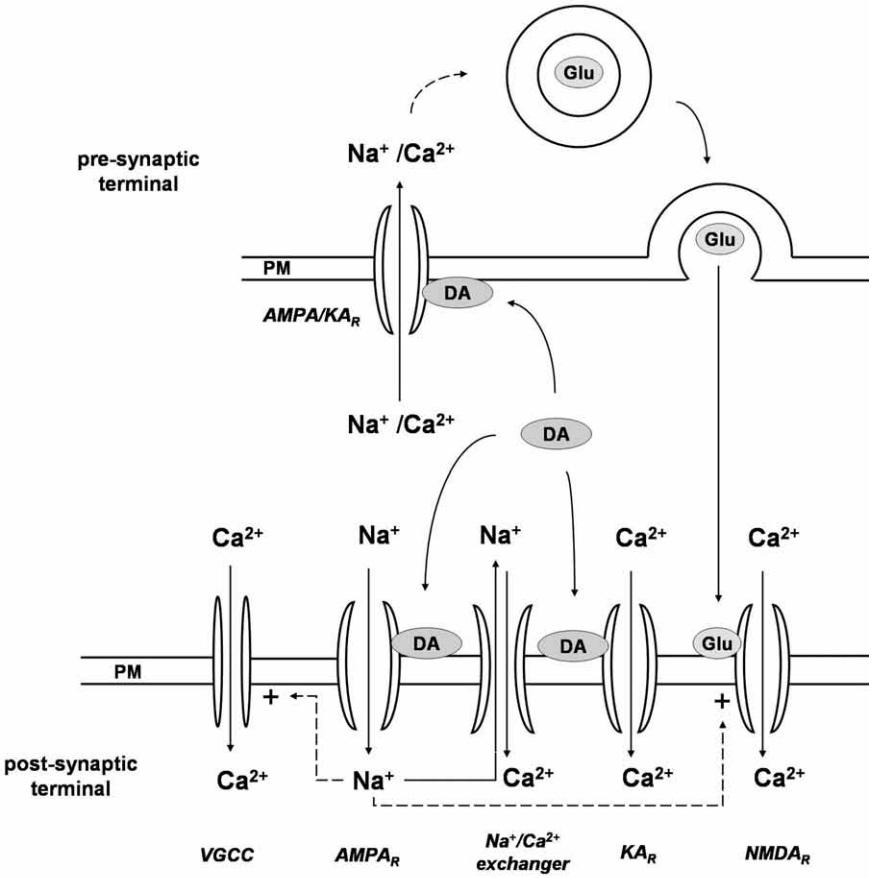


Figure 12. Schematic representation of the molecular mechanisms of action of domoic acid (DA). Mechanism of action of DA by binding to AMPA ($AMPA_R$) and kainate (KA_R) receptors. Glu, glutamate; $NMDA_R$, NMDA receptor; PM, plasma membrane; VGCC, voltage-gated calcium channels. See text for details.

and kainate receptors, which represent ligand-dependent ion channels, and the binding of DA determines the opening of the channels [210, 211]. When DA binds to AMPA channels, influx of Na^+ into the cell occurs, whereas influx of extracellular Ca^{2+} ions into the cell is induced by DA binding to kainate receptors [210, 211]. The molecular mechanism of action of DA, however, may not be reduced to these two receptor systems, because other ion channels and receptors are recognized to participate in the response induced by the toxin [39, 208]. In particular, DA also induces the influx of extracellular Ca^{2+} through voltage-gated calcium channels [212, 213], and by reverse action of Na^+/Ca^{2+} exchangers [213]. In both cases, the effect would follow Na^+ influx through AMPA receptor and membrane depolarization [213]. Furthermore,

NMDA receptors could contribute to the processes leading to increased intracellular Ca^{2+} concentrations in affected cells, as NMDA receptors would be activated by glutamate released from the synapsis, following DA binding to pre-synaptic AMPA/kainate receptors [214–218].

The mechanism of action of DA would then involve a primary stimulation of influx of Na^{+} and Ca^{2+} ions in neurons, as a consequence of DA binding to post-synaptic AMPA and kainate receptors (Fig. 12). The membrane depolarization would then enhance the influx of extracellular Ca^{2+} ions through voltage-gated calcium channels [212, 213] and NMDA receptors [214–218]. The calcium conductance of NMDA receptors would be caused by the binding of glutamate released from nerve endings, following a reduction of the voltage-dependent Mg^{2+} block of NMDA receptors [217, 218]. The glutamate secretion, in turn, would be induced by the pre-synaptic action of DA, but the molecular details of this response have not been fully clarified, and different proposals have been put forth [39, 208]. The increased intracellular Na^{+} concentrations would also stimulate an influx of extracellular Ca^{2+} ions through Na^{+}/Ca^{2+} exchangers (Fig. 12). The combination of the molecular transducers of DA response would lead to an overall increase in intracellular Ca^{2+} concentrations, which would trigger Ca^{2+}-dependent responses [39, 208, 209].

The action of glutamate receptors under normal conditions would contribute to coordinated neurotransmission among neurons in the central nervous system. The desensitization of receptors that contributes to normal glutamate-based neurotransmission is impaired in receptors bound to DA [39, 211, 219], and this condition then results in unrestrained signaling of DA-bound receptors, leading to Ca^{2+} overload in cells exposed to the toxin [39, 211]. The prolonged calcium load then determines a loss in the regulatory mechanisms of cell functions involving controlled intracellular calcium homeostasis, leading to cell damage and overt neurotoxicity, which represent the major effects of DA *in vitro* and *in vivo* [39, 208, 209, 211, 217, 220–224]. Thus, DA in the brain would determine a sustained signaling through glutamate receptors in some neurons, leading to altered neurological and behavioral activities, which are apparent in the symptoms recorded in humans and animals that have been poisoned by this toxin (see section “Amnesic shellfish poisoning” below).

Mechanism of action of cyclic imines

The components of this group of algal toxins share several structural features that have been the basis of the hypothesis that they might have a common mechanism of action [120]. The investigation on the molecular effects of cyclic imines, however, has been very limited so far, and therefore it seems premature to make any firm proposal about their mechanism of action. Within this constraint, the few available data on spirolides would suggest that these toxins exert their effects through alteration of ion conductance at the level of the plasma membrane (primarily Ca^{2+}), acting on some acetylcholine receptors [225]. The increased frequency in the detection of this class of compounds in

shellfish indicates the need for greater research efforts to characterize their mechanism(s) of action.

Mechanism of action of okadaic acid and related compounds

It has long been recognized that okadaic acid (OA) and related compounds bind and inhibit serine/threonine phosphoprotein phosphatases (PPases), and, among them, the 2A isoform (PP2A) shows a particularly high affinity for the toxin [226, 227]. The mechanism of action of OA and related compounds, therefore, involves the inhibition of PPases, leading to stabilization of the phosphorylated states of proteins that are substrate of OA-sensitive enzymes (Fig. 13).

The phosphorylation of amino acids in proteins is a widespread process through which the functioning of individual proteins is controlled in eukaryotic cells, so that different activities are usually associated with the phosphorylation pattern of individual proteins, and the functional states existing under defined conditions in biological systems may be more than the simple active/inactive opposition [228–230]. Although hundreds of PPases are believed to be expressed in eukaryotes [231], the inhibition of only some isoforms of PPases (primarily PP1 and PP2A) by OA has dramatic consequences, because these isoforms are responsible for the dephosphorylation of many enzymes and regulatory proteins in living systems [232]. The original study of Haystead et al. [233] provided the clear demonstration that, in OA-treated cells, OA

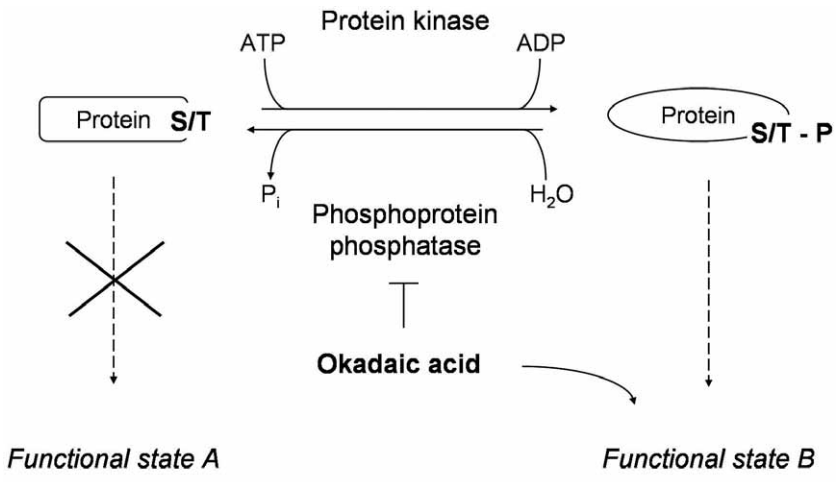


Figure 13. Schematic representation of the mechanism of action of okadaic acid (OA). The phosphorylation reaction has been referred to a serine/threonine residue (S/T) of the protein, in a reaction involving ATP, as the phosphate donor, and ADP as the product. The dephosphorylation reaction is a hydrolysis, leading to release of orthophosphate (P_i).

leads to increased levels of phosphorylated proteins and altered cell functioning and, indeed, a simple literature search reveals that the phosphorylation states of cellular proteins are frequently affected by exposure of biological systems to OA. This toxin, therefore, can disrupt the molecular mechanisms controlling functions in biological systems. Many examples regarding single regulatory pathways could be described, but only two general phenomena are discussed, as they refer to the two major responses found in animals exposed to OA.

The tumor-promoting effect of OA and related compounds has been described in two models of two-stage carcinogenesis [234, 235]. Taking into consideration that cell proliferation is stimulated by protein phosphorylation cascades [204], the stabilization of phosphorylated forms of key regulatory proteins, as a consequence of inhibition of PPases due to OA, could represent a molecular explanation for the tumor-promoting effect of this toxin in some biological systems.

OA, however, does not represent a carcinogen on its own [234, 235], and its tumor-promoting effects have been observed in systems that were subjected to tumor initiation by exposure to a chemical carcinogen, before being challenged with OA [234, 235], in keeping with the general process of two-stage carcinogenesis [236]. In turn, the evidence obtained in many systems *in vitro* and *in vivo*, indicates that cells exposed to OA do not respond by a stimulation of cell proliferation, but the toxin actually induces cell death (reviewed in [237]). The death response induced by OA has been observed with both normal and transformed cells, and the disruption of several regulatory pathways due to inhibition of PPases in cells exposed to OA can explain the death responses reported in literature. The disruption of proper cell functioning and cell death is actually considered the molecular basis of the diarrhea that represents the major symptom of animals and humans poisoned by OA and related toxins (see below).

In vitro studies have shown that OA does not significantly affect ion currents in intestinal cell monolayers and in stripped rabbit colonic mucosa, but it attenuates the cellular response to secretagogues, such as forskolin and carbachol, supporting the conclusion that OA does not act as a secretagogue in the intestine [238]. The analysis of OA effect on transepithelial electrical resistance showed that the toxin significantly decreased the resistance of cell monolayers, lending support to the notion that it disrupts the barrier function of intestinal cells and increases paracellular permeability [238]. E-cadherin is the protein responsible for cell-cell adhesion of intestinal epithelia [239], and the destruction of E-cadherin has been described in epithelial cells exposed to OA [240], indicating that cell disposal of the E-cadherin system would contribute to increased paracellular permeability of intestinal epithelial cells. The observation that OA decreases transepithelial electrical resistance without any measurable effect on ion currents of intestinal tissue has been confirmed in an animal study [241], providing convincing evidence that the mechanism by which OA induces diarrhea in animals includes sub-mucosal fluid collec-

tion in the intestine, followed by its flowing into the intestine through the paracellular pathway of the epithelium and its secretion into the intestinal lumen [241].

Mechanism of action of yessotoxins and azaspiracids

The chemistry (see above) and toxicity of these two groups of compounds are quite different. Animal studies have shown that azaspiracids (AZAs) are toxic by both i.p. injection and the oral route [44, 242], and the oral toxicity of AZAs has been confirmed in humans [242]. A different picture is available for yessotoxins (YTXs), which are toxic when injected i.p. in the mouse, while only limited alterations have been recorded after oral administration [83–85, 243]. A recent study has shown that only little gastrointestinal absorption of YTX occurs, as low (10^{-9}–10^{-8} M) toxin concentrations have been detected in the blood of mice that received comparatively large doses of the compound orally [243]. In line with the low toxicity of YTX found in animal studies, no episodes of human intoxications have been linked to ingestion of shellfish contaminated with this group of compounds so far, and the acute toxicity of YTXs remains a matter of debate.

The precise mechanisms by which AZAs and YTXs trigger their effects in biological systems are undetermined at the moment, although many studies have been carried out by different groups, using primarily cultured cells. The difficulties in extrapolating the results obtained *in vitro* with regard to their toxicological relevance *in vivo* should then be borne in mind. In the account that follows, therefore, the attention is focused on the molecular events that have been recorded in experimental systems by the use of toxin concentrations up to 10^{-8} M, which are compatible with exposure upon ingestion of food contaminated with AZAs and YTXs, based on the toxin levels found in animal tissues after oral administration of the compounds [242, 243]. For reviews reporting the molecular effects detected *in vitro* by exposing cells to high (10^{-7}–10^{-6} M) AZA and YTX concentrations, the reader is referred to some recent reviews [244–246].

A cytotoxic effect of 10^{-9} and 10^{-8} M AZA1 (Fig. 4) has been described in a variety of cellular systems [247–250]. In some of those model systems, alterations of F-actin-based cytoskeletal structures have been found upon cell treatment with nanomolar concentrations of AZA1 [247, 248], and the effects of the toxin on cytoskeletal structures showed some degree of cell specificity [248, 249]. Furthermore, AZA1 was found to alter cell adhesion [248, 249], and to induce the accumulation of a fragmented form of E-cadherin, termed $ECRA_{100}$ [249]. AZA1 treatment of primary cultured neurons has been shown to cause increased nuclear levels of phosphorylated (active) JNK, and an inhibitor of JNK could prevent the cytotoxic effect of AZA1 in that experimental system [251]. Thus, the cytotoxic effect of AZA1 could be brought about by an array of molecular events, whose mechanistic links remain to be defined.

In a different line of investigation, the analysis of the effects of AZA1 on the transcription profiles of T lymphocytes has shown that a 24-h exposure of these cells to 10 nM AZA1 affects the expression of several genes, including a coordinated up-regulation of those coding for enzymes involved in cholesterol and fatty acid synthetic pathways [252]. At the moment, it is unclear how the various effects found *in vitro* can be linked to the observed toxic effects *in vivo*.

Turning to the molecular effects elicited by YTX (Fig. 7) in cultured cells, three major responses triggered by toxin concentrations up to 10^{-8} M have been reported. Some of those effects have been observed only in some cell lines, indicating a certain degree of cell specificity in the responses elicited by YTX in sensitive systems. The induction of cell death as the most prominent effect of YTX has been observed with cell lines of different histological types and is detected 1–2 days after addition of 10^{-10}–10^{-8} M YTX to cell cultures [253–257], suggesting that a general alteration would be induced by YTX in sensitive lines, leading to cell death. The increase in intracellular Ca^{2+} concentrations is another effect triggered within minutes of YTX addition to some cells, due to the opening of voltage-sensitive calcium channels [246]. The increase is limited, leading to less than doubling of the intracellular Ca^{2+} concentrations, and in primary cultures of neuronal cells it is induced by YTX concentrations that are about 10^{-8} M [255]. In other systems, YTX concentrations higher than 10^{-7} M were needed to elicit the same kind of response [246]. The functional consequences of the very limited increase in intracellular Ca^{2+} concentrations elicited by YTX are presently undetermined, and experiments carried out in two different systems have shown that it should not be involved in the induction of cell death by YTX [255, 257].

Another molecular effect elicited by YTX is the disruption of the E-cadherin system in epithelial cells [258–260]. The effect is induced by very low YTX concentrations (10^{-10}–10^{-9} M) and is detected by the accumulation of a 100-kDa fragment of E-cadherin ($ECRA_{100}$), that has lost the intracellular C-terminal domain of the protein [259]. YTX does not enhance E-cadherin degradation per se, but interferes with the normal turnover of the plasma membrane protein, preventing endocytosis and complete disposal of the protein fragment produced after the initial proteolytic attack, and leading to accumulation of proteolytic fragments in vescicular structures in the proximity of the plasma membrane [260]. Taking into consideration that the half-life of E-cadherin was about 8 h in both control and YTX-treated cells, the detection of $ECRA_{100}$ at levels comparable to those of the intact protein 16–20 h after YTX addition to cultured cells indicates that the blockade of endocytosis by the toxin should be a rapid and early response of sensitive cells [260]. Interestingly, decreased levels of a proteolytic fragment of E-cadherin lacking the intracellular domain of the protein have also been found in the colon of mice after oral administration of YTX [261], indicating that YTX can decrease the disposal of E-cadherin both *in vitro* and *in vivo*.

Notwithstanding the recognized differences between AZAs and YTXs, the experimental findings described above reveal that similar effects can be trig-

gered by low concentrations (10^{-10}–10^{-9} M) of AZA1 and YTXs in the same cell line. Among the molecular responses induced by AZAs and YTXs described so far, cell death appears ubiquitous, whereas disruption of the F-actin cytoskeleton and the inhibition of endocytosis of membrane proteins appear cell specific. In the case of the effects exerted on E-cadherin, the knowledge on the molecular details pinpoints the fact that the effect of AZA1 was undistinguishable from that induced by YTX in epithelial cells [249]. Thus, it has been proposed that AZAs and YTXs might share their molecular mechanism(s) of action in some target cells and/or biological settings [249].

The extensive structural features distinguishing AZAs from YTXs make the possibility that the two classes of compounds share the same receptor unlikely, although it can not be excluded at the moment. In turn, two different molecular mechanisms of action of AZAs and YTXs could converge toward some shared effectors that could mediate common responses with identical endpoints, and cross-talks would then occur at multiple levels, as has been found in the case of other algal toxins [179]. This working hypothesis is presented in Figure 14, where AZAs and YTXs are considered to induce their effects by interacting with different receptors, thereby triggering responses through the involvement of distinct sets of effectors. The two pathways would include, however, mechanistic links between some effectors, either directly or indirectly. In this hypothetical model, for instance, the effects exerted by both AZAs and YTXs on the E-cadherin system would converge on the inhibition of a key endocytic step, and could occur through several pathways. The most direct pathways for AZAs and YTXs in our model would include steps 1, 3, 12, and 6, 7, 12, respectively (Fig. 14). Alternatives would be possible, however, through disruption of the F-actin cytoskeleton, as a consequence of converging mechanisms to effector $E_{AZA/YTX}$, and the subsequent steps 10 and 13 (Fig. 14). This latter pathway, however, would represent a cell-specific alternative, as cell treatment with pectenotoxin-6, which disrupts the F-actin cytoskeleton (see below), does not lead to accumulation of the $ECRA_{100}$ fragment of E-cadherin [262], indicating that a general alteration of actin cytoskeleton may not be sufficient to cause the effect on E-cadherin. A third possible mechanism would incorporate the recent evidence that AZA1 induces the accumulation of early endosomes in the proximity of the plasma membrane [263]. These should be the vescicles accumulating $ECRA_{100}$ in YTX-treated cells [260], and we recently observed that AZA1 inhibits endocytosis of plasma membrane proteins (Bellocci et al., in preparation). In this third mechanism, AZA1 and YTX would bring about their response through the common effector $E_{AZA/YTX}$, and steps 11 and 14 (Fig. 14). Interestingly, the alteration of proteins involved in snRNP functioning and mRNA maturation could be another response shared by AZA1 and YTX [263, 264], a possibility that has been embedded in the model of Figure 14.

Similar considerations could be put forward about the death responses, which would result from multiple cellular events, such as the disruption of cel-

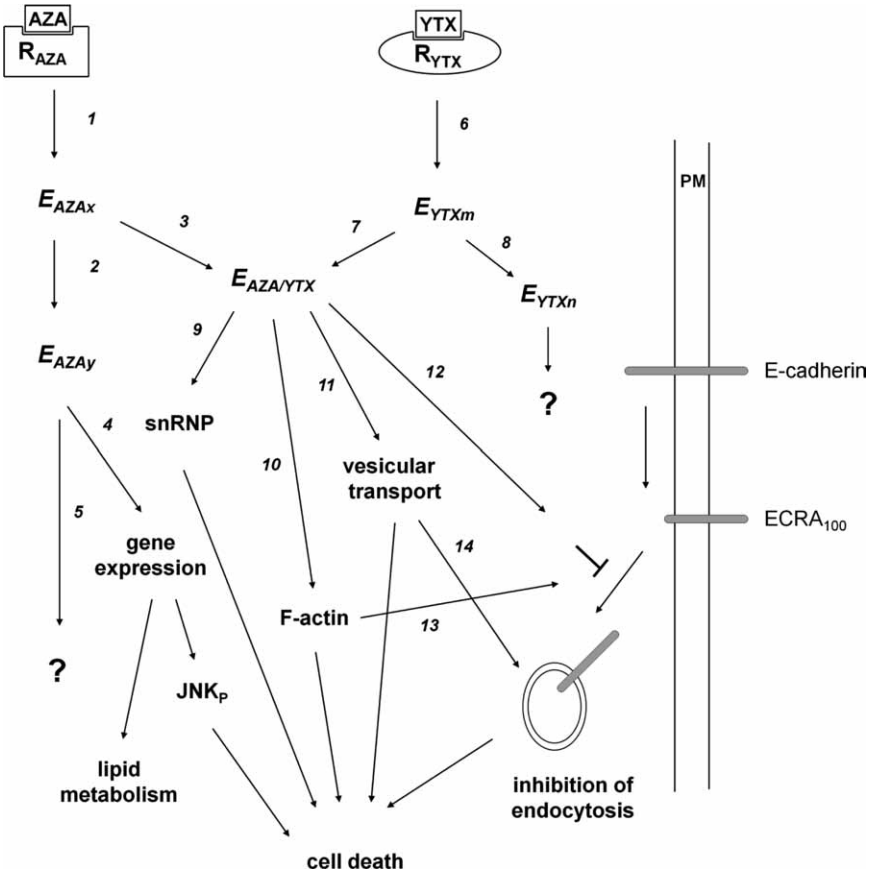


Figure 14. Hypothetical model of the cross-talks between the mechanisms of action of azaspiracids (AZAs) and yessotoxins (YTXs). The subcellular location of the AZA and YTX receptors (R_{AZA} and R_{YTX}), as well as the number and mechanistic links between effectors of the pathways ($E_{AZAx,y}$ and $E_{YTXm,n}$) are entirely speculative. Some steps of the different pathways have been numbered. PM, plasma membrane. See text for explanation.

lular ultrastructures (cytoskeleton) and processes (endocytosis and mRNA maturation), including cell-specific events. The redundancy in signaling pathways, as hypothesized in the schematic model of Figure 14, is common in transduction systems in eukaryotes, and could contribute to cell-specific responses to AZAs and YTXs.

We wish to stress the hypothetical nature of the pathways reported in Figure 14, and remark that the similarity among some molecular responses elicited by AZAs and YTXs could be exploited for studies on cross-talks between distinct mechanisms of action of algal toxins.

Mechanism of action of pectenotoxins

Few data are available on the toxicity of pectenotoxins (PTXs, Fig. 6), indicating that this group of compounds is less toxic when administered by the oral route, as opposed to i.p. injection [265]. It has been hypothesized that this difference would stem from low absorbance of these toxins in the gastrointestinal tract. Chromatographic studies by Fux and Hess (unpublished observations) confirm the nonpolar character of PTX2, the primary algal metabolite, which may result in low bioavailability in the intestinal tract, and studies by Miles et al. [78] clearly demonstrate rapid transformation of PTX2 into inactive PTX2-seco acid in aqueous systems. However, *in vivo* data on absorption and toxicokinetics of PTXs are lacking.

Taking into consideration these limitations, it is recognized that PTXs interact with F-actin, leading to alterations in the ultrastructure and functioning of the cellular cytoskeleton. Initial evidence suggesting that F-actin might be a target of PTXs stemmed from the histopathological damages found in the liver of mice injected with PTX1, which resembled those induced by phalloidin [266]. The observation that PTX1 disrupts stress fibers then provided the first direct indication that PTXs alter F-actin [267]. This observation was later confirmed with PTX2 [268], leading to the conclusion that PTXs cause actin depolymerization [269–272].

More recently, detailed crystallographic analysis of the interaction between PTX2 and actin has shown that the toxin and actin form a 1:1 complex [273]. F-actin is composed of two helices containing polymers of non-covalently bound actin monomers, and PTX2 could interfere with polymerization by associating with actin monomers at a site that is close to the "inner" filament axis, inhibiting the lateral subunit interactions critical for filament assembly [273]. PTXs have been shown to induce cell death in many cell lines, in a wide concentration range (10^{-9}–10^{-6} M), through multiple mechanisms [274–282], and F-actin depolymerization induced by PTX2 appears the causative event leading to cell death [276].

Shellfish poisoning

The route of human exposure to phycotoxins is most often oral, through ingestion of food contaminated with toxins. In some instances, however, living systems may become exposed to phycotoxins through other routes, as in the case of breathing aerosols containing toxins and/or direct skin contact with toxins. In this section, we summarize the major symptoms of and, whenever available, possible remedies for the most relevant poisonings that may occur in humans exposed to phycotoxins, independently of the routes of toxin entry into the body. The poisonings have been classified mostly on the basis of recorded symptoms, and our description follows the existing classification.

Paralytic shellfish poisoning (PSP)

Saxitoxins (STXs) are the causative agents of this poisoning, which results from ingestion of contaminated shellfish [283, 284]. The same kind of poisoning is due to ingestion of tetrodotoxin (TTX), as a consequence of eating raw meat from puffer fish. The gonads and liver of the puffer fish can accumulate bacterial TTX as a consequence of its feeding behavior: if the puffer fish viscera are not carefully dissected from the rest of the meat, this meat can become contaminated and cause poisoning when ingested [285].

PSP has been recorded worldwide, and its symptoms are experienced within 30 minutes from ingestion of contaminated seafood, related to the severity of the poisoning [284]. Mild symptoms include altered perception (burning or tingling sensation and numbness of the lips, that can spread to the face and neck), headache, dizziness and nausea. More severe symptoms include incoherent speech, a progression of altered perception to arms and legs, a progressive loss in the coordination of limbs, and general weakness. Respiratory difficulty is a late symptom, as a consequence of muscular paralysis progressing in the whole body, and death may be the outcome of PSP by respiratory paralysis [284]. The blockade of ion conductance due to binding of STX to the voltage-gated sodium channel (VGNC) is the mechanistic basis of the symptoms of PSP (Fig. 11A).

Artificial respiration has been the most appropriate remedy for PSP so far, as patients subjected to mechanical intervention have a high probability of a full and rapid recovery [283, 286].

Neurotoxic shellfish poisoning (NSP)

Brevetoxins (PbTXs) are the causative agents of this poisoning, which may occur after both inhaling aerosol containing the toxins or as a consequence of eating contaminated seafood. When poisoning is through the respiratory tract, the exposure usually occurs on or near the waters where a bloom of PbTX producers has developed. NSP has been recorded primarily in the southeastern coast of the United States, the Gulf of Mexico, and New Zealand [283, 284, 287].

The symptoms due to contaminated shellfish appear minutes/hours after its ingestion, and are more severe than those found when contaminated aerosol is involved. In the former case, symptoms are both gastrointestinal (nausea, diarrhea, and abdominal pain) and neurological (circumoral paresthesia and hot/cold temperature reversal). In more severe cases, the muscular system may be affected (altered heart contractions, convulsions, and respiratory difficulties). Death from NSP has never been reported in humans, and symptoms resolve within a few days after exposure to the toxins [283, 284].

When exposure to PbTXs is through contaminated aerosols, symptoms involve primarily conjunctival irritation, copious catarrhal exudates, rhino-

rrhea, nonproductive cough, and bronchoconstriction [288–291]. In the normal population, these symptoms are usually reversed by leaving the beach area or entering an air-conditioned area [291]. The molecular mechanism at the basis of brevetoxin poisoning is related to the increased ion conductance that these compounds induce on interacting with, and opening, the VGNC (see Fig. 11A). Thus, an initial phase of increased neurotransmission (firing of action potentials and release of acetylcholine at muscular endplates) and muscular contraction is followed by a progressive inhibitory action [283].

No specific therapy is available for NSP, but some of the effects of PbTXs can be reversed by infusion with hyperosmotic solutions [292]. The finding that brevenal is an antagonist of PbTX action [132] opens interesting perspectives for the therapeutic use of drugs counteracting the effects of these natural toxins.

Amnesic shellfish poisoning (ASP)

The symptoms due to eating domoic acid (DA)-contaminated shellfish appear within the first few hours after ingestion, and in more severe cases may persist for months [39, 208, 209, 220, 293]. Initial symptoms affect the gastrointestinal tract, with nausea, vomiting, abdominal cramps and diarrhea. These are followed by headache and other neurological symptoms that often result in disturbances of memory, an effect that has led to the naming of this type of shellfish poisoning. The most severe cases may result in death [39, 208, 209, 220, 293].

The neurological symptoms of ASP have been shown to evolve in the weeks (months) following poisoning, and anterograde memory disturbances can be accompanied by confusion, disorientation, peripheral nerve damage and changes in memory threshold [220, 293, 294]. Postmortem histopathological analysis of the brain in an individual who had suffered ASP, and whose death was unrelated to the poisoning, showed atrophy of the hippocampus [220, 293].

Overall, the symptoms of ASP are related to damage of structures of the nervous system, which can be fully explained by unrestrained post-synaptic activity and neuronal toxicity induced by DA stimulation of non-NMDA glutamate receptors (Fig. 12). Because of the agonistic properties of DA in this receptor system, the toxic effects of this algal toxin can be antagonized by glutamate receptor antagonists *in vitro* and *in vivo* [216, 222, 295–297].

Diarrheic shellfish poisoning (DSP)

The contamination of seafood by okadaic acid (OA) and related compounds is very common in European and Asia-Pacific Countries [283]. The symptoms of DSP appear within 1 h after ingestion of contaminated seafood, and affect the gastrointestinal tract, producing nausea, vomiting, abdominal cramps and diarrhea [283]. The symptoms do not last long and usually disappear within a few

days. No death has been recorded due to DSP. No long-term effects have been described in humans, although experiments in animal systems have recorded a tumor-promoting effect of OA in two-stage carcinogenesis models [234, 235]. In those cases, the effect depended on protocols of toxin administration involving repeated dosing at least twice per week over periods of several months [234, 235]. The conditions of those studies do not appear to be easily comparable to human intake of OA and related compounds, and a study aimed at probing whether residual levels of OA in shellfish might increase the risk of cancer among regular shellfish consumers has yielded inconclusive results [298].

The mechanism of action of OA and the molecular events detected in biological systems exposed to this toxin provide a clear explanation of the DSP symptoms (Fig. 13). Taking into consideration, however, the vast array of molecular responses elicited by this group of compounds, through alteration of regulatory pathways in the cells, further investigations are needed to better clarify possible long-term effects of exposure to OA. When gastrointestinal symptoms of DSP are severe, their treatment is symptomatic, e.g., rehydration.

Ciguatera

Globally, ciguatera is probably one of the most frequent poisonings with estimates reaching from 50 000 to 500 000 events per annum [299]. This poisoning is caused by eating fishes in tropical marine areas, and the toxins responsible for this poisoning are components produced by *G. toxicus* (see sections above) that are accumulated in fish through the food chain. A vast literature exists on ciguatera, and the reader is referred to some excellent reviews for detailed descriptions [158, 159, 283].

As toxins possessing different chemical properties and mechanisms of action have been involved in ciguatera, a discussion of this poisoning in humans demands some attention to specific issues related to toxin groups and the molecular bases of their effects. Furthermore, despite the increasing understanding of the multiple processes set in motion by ciguatoxins (CTXs), maitotoxin (MTX) and gambierol, there are still significant uncertainties about the specific contribution given by each group of toxins. Based on this consideration, we first describe the most relevant symptoms of ciguatera, and then discuss the possible cause-effect relationships that have been proposed for individual toxin groups.

Ample spectra of symptoms have been recorded for ciguatera, with a manifestation that starts within hours after the ingestion of contaminated food, often resolves within 1 week, but may last up to some years. Furthermore a higher susceptibility to ciguatera toxins has been observed in both humans [300] and animals [175, 301] that had experienced previous exposure to these toxins. The symptoms are both gastrointestinal and neurological, with nausea, diarrhea and vomiting representing early signs of poisoning, accompanied by neurological symptoms, such as numbness and circumoral paresthesia, inver-

sion of thermal sensations ("dry ice sensation"), and metallic taste. In severe cases, paresthesia may progress to other parts of the body, accompanied by difficulties in breathing, and death may ensue due to respiratory paralysis, heart dysrythmias or cerebral edema [158, 159, 283].

Although any attempt to link the different symptoms of ciguatera to any specific group of toxins should be considered an oversimplification at the moment, some preliminary indication can be made on the basis of available knowledge on molecular mechanisms of action of CTXs, MTX and gambierol. It is recognized, for instance, that many symptoms of ciguatera are similar to those found in NSP, and they are most likely due to the effect of CTXs, by opening the VGNC, whereby the increased sodium conductance would lead to increased neurotransmission, followed by a progressive inhibitory action. MTX, however, could contribute to increased neurotransmission with regard to muscle contraction, and the symptoms of heart dysrythmias. MTX also causes extensive cell damage in animal studies, and has a potent cytotoxic effect in cultured cells, and could therefore contribute to neurotoxic symptoms [173–175]. The role of gambierol in ciguatera is less clear, but its impairment of VGKC functioning in taste buds [176] suggests that it might have a role in the taste disturbances that have been recorded. MTX does not move up the marine food chain (probably related to its solubility in water) and its contribution to ciguatera-poisoning is more likely when the poisoning arises from the consumption of herbivorous fish.

No specific therapy has been proposed for ciguatera so far, and its treatment remains symptomatic. In particular, intravenous infusion of mannitol has been used, the protective effect of which has been attributed to the inhibition of edema formation in several tissues [302]. In keeping with the shared mechanism of action of brevetoxins and ciguatoxins, brevenal has been reported to inhibit ciguatoxin-induced neurotoxic effects and might represent an appropriate therapeutic tool [303].

Palytoxin poisoning

The potential of palytoxins (PlTXs) to cause human poisonings is a matter of discussion [304]. On the one hand, human illness and even fatal cases have been linked to PlTXs that could have entered the human body as a consequence of eating contaminated food [304], or through small skin injuries [305]. On the other hand, hundreds of human cases have been reported in coastal areas of some Mediterranean countries (Italy, France, Greece and Spain) in recent years, and have been linked to blooms of algae producing PlTXs (*Ostreopsis* species [8, 9, 111, 306]). Although a formal proof that PlTXs were responsible for those human cases is still lacking, circumstantial evidence would suggest that this could be the case, because intoxications involved people residing at or close to the coastal areas where the harmful algal blooms (HABs) were taking place, and PlTXs were found in the *Ostreopsis* algae present in those coastal

areas, supporting the proposal that the PlTXs present in the aerosols were the causative agents of those intoxications [8, 9].

The symptoms of PlTX poisoning are to be considered within these constraints, and can be differentiated based on the proposed route of entry into the body. When intoxication was apparent following ingestion of contaminated food [304], gastrointestinal symptoms (nausea, vomiting, diarrhea) have been recorded, together with neurological disturbances (circumoral paresthesia and paresthesia of the extremities, muscle spasms and pain, respiratory problems), and death has been observed in a few instances. Tissue damage has been documented in several cases, suggesting a cytotoxic effect of PlTXs in these tissues [180, 188, 206].

In many cases, the presence of other toxins contaminating the ingested food, and/or a lack of knowledge regarding the toxins present in the materials have led to uncertainties about the actual involvement of PlTXs in the recorded cases [304], although PlTXs have been found in seafood in coastal areas where blooms of *Ostreopsis* algae were ongoing.

When PlTX exposure was attributed to toxic aerosols, in turn, the mucosae were primarily affected, and symptoms included conjunctivitis, respiratory distress, rhinorrhea, cough and fever [8, 9]. The symptoms of PlTX poisoning through contact, in turn, were both local (swelling and paresthesia around the site of injury) and systemic (dizziness, weakness, myalgia, irregularities of the ECG and indications of rhabdomyolysis), indicating that PlTX or its analogues were absorbed into the blood stream through the skin injuries [305].

Some of the recorded symptoms of PlTX poisoning, particularly the neurological ones, can be explained by alterations of ion conductance in excitable cells. The clarification of the molecular bases of the recorded symptoms, however, is far from being complete.

As in the case of ciguatera poisoning, no specific therapy exists for palytoxin poisoning, and the treatment of suspected poisoning has been symptomatic, including the intravenous infusion of mannitol [307].

Azaspiracid poisoning

The symptoms of azaspiracid (AZA) poisoning in humans are very similar to those described for DSP, including nausea, vomiting, abdominal cramps and diarrhea, which disappear within a few days after the ingestion of contaminated shellfish [242]. As in the case of OA responses (see above), the gastrointestinal effects of AZAs, including the destruction of intestinal epithelia [308], might be explained by the alterations they induce in cytoskeletal structures and the E-cadherin system, with disruption of cell-cell and cell-matrix interactions, as well as perturbation of the intestinal barrier function [247–249].

An animal study aimed at probing the carcinogenic potential of AZAs [309] has provided limited information on this issue, and more data are needed to consider a possible carcinogenic risk posed to humans by exposure to AZAs.

Uncertainties existing on the risks posed by some phycotoxins

We are not aware of cases of human intoxication that can be attributed with certainty to ingestion of food contaminated with either YTXs, or PTXs, or cyclic imines. Animal studies aimed at characterizing the toxicity of these three groups of compounds have shown that symptoms of acute toxicity can be detected only after oral administration of high doses (in the mg/kg range) [121, 265, 310]. Thus, the inclusion of YTXs, PTXs, and cyclic imines among toxins that can pose risks to the consumer through ingestion of contaminated seafood is debated.

The many facets of the ongoing discussions are not described here, although it seems appropriate to call attention to a few items that indicate the need for further studies on the mechanisms of action and toxicity of those compounds.

YTXs, for instance, are cytotoxic at the very low concentrations (nanomolar) that can be found in animals after oral administration. Furthermore, YTX has been shown to inhibit the endocytosis of E-cadherin *in vitro* and this could occur also *in vivo* (see above). Taking into consideration that proteins located at the level of the plasma membrane comprise hormone and neurotransmitter receptors, ion channels and pumps, transporters, adhesion proteins, etc., that are often involved in the control of relevant cellular and biological functions (see for instance [311]), the possibility that low YTX concentrations might alter endocytosis (and phagocytosis) *in vivo* should be evaluated [312].

The molecular mechanism of action of PTXs has been well characterized in cultured cells, but it is not clear whether effective doses of this group of compounds can exist *in vivo* after ingestion of contaminated food.

Final remarks

The information on chemistry, mechanism of action and toxicity of phycotoxins has accumulated rapidly over the last few years, but many relevant aspects remain to be characterized.

It should be noted that most of the toxins discussed above are rather chemically complex molecules, and chemical synthesis is typically not a viable route to obtaining large amounts of these compounds for extensive studies. In most cases, chemical synthesis is used to clarify the structure, and to establish basic structure-activity relationships; however, preparative isolation still plays a major role in the supply of large amounts of compounds for analytical method development, routine analysis and toxicological evaluation. Preparative isolation is also difficult due to the difficulty in separating chemically closely related analogues, and poor yields in the preparative separation and purification of trace analytes (ng/kg to mg/kg range). In addition, the algae responsible for the biosynthesis of a toxin are not always known from the beginning, are sometimes not (easily) culturable and may only grow slowly or produce little toxin in culture. Thus, isolation often relies on natural occur-

rence, which is most often poorly predictable and involves monitoring with unspecific techniques, such as animal assays. Therefore, the knowledge on these toxins remains limited and requires further studies.

There are many gaps in the knowledge of marine toxins, beginning with their poorly understood biological and ecological roles (chemical defense, intercellular signaling or storage?). Also the biosynthetic routes to the compounds have virtually not been explored. The many remaining gaps in the chemical behavior of phycotoxins can be categorized into the following areas: physical constants (log P_{ow}, pKa, thermal stability, etc.), chemical behavior in different pH environments and reactivity in biological systems (toxicokinetics).

Our understanding of the molecular events induced by several phycotoxin groups in different biological systems, mostly in cultured cells, is steadily increasing, and the molecular details of the mechanisms by which algal toxins trigger their effects have been remarkably characterized in a few cases. Still, many relevant features of the series of molecular events triggered by phycotoxins are not known yet, and investigations in this area are needed for a deeper understanding of their modes of action. Within this frame, the recognition that phycotoxins are increasingly used as research tools for the characterization of basic biological processes will attract the interest of researchers. The characterization of the molecular targets of those toxins for which they are not discovered may lead to the discovery of novel targets, which will most likely lead to new therapies and remedies for diseases unrelated to phycotoxin poisoning.

Moreover, the toxicity of phycotoxins, particularly in the case of their acute effects, has been characterized for many groups of compounds, but significant gaps remain, with particular regard to long-term toxicity, the possible toxicity of repeated ingestion of low doses of toxins, and the combined toxicity of mixtures of toxins belonging to different chemical groups (that often co-occur in the same naturally contaminated seafood).

The lack of the pure toxins and the poor understanding of the reactivity results in many delays in method development and validation, and in poor understanding of their toxicity. However, the authors are confident that rising awareness of the potential of marine toxins in biotechnology and medicine will contribute to the rapidly increasing knowledge on these interesting compounds.

Many exciting challenges await the investigators in the field of phycotoxins.

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