Bivalve Molluscs as Vectors of Marine Biotoxins Involved in Seafood Poisoning

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Abstract. Molluscs of many sorts, which are high in protein and trace minerals, have always been a substantial portion of the human diet. A great variety of mollusc species are therefore of commercial importance throughout the world. Episodes of poisoning occasionally happen to the consumers of molluscs, the main hazard being represented by bivalve molluscs. These organisms are filter-feeders, feeding mainly on a wide range of phytoplankton species. Among the thousands of species of microscopic algae at the base of the marine food chain, there are a few dozen which produce potent toxins. One major category of impact occurs when toxic phytoplankton are filtered from the water as food by shellfish, which then accumulate the algal toxins to levels which can be lethal to humans. Incidences of poisoning related to marine algal toxins come under the main categories of paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), diarrhetic shellfish poisoning (DSP), and amnesic shellfish poisoning (ASP), depending upon the toxins and the symptoms that they cause. Since the beginning of the 1990s, a research program has been initiated to examine the toxin profiles in mussels from the Adriatic Sea. Since then, a number of polyether toxins have been isolated and characterized, some of which represent new additions to the DSP class of biotoxins. During this investigation, new types of toxins have also been isolated. The recent application of LC–MS methods for the detection of Adriatic marine biotoxins made it possible to speed up the analysis of toxic samples.

3.1 Introduction

Molluscs, because of their ease of capture, edibility and beauty, have long been important to mankind. Molluscs of many sorts, which are high in protein and trace minerals, have always been a substantial portion of the human diet. Abalone, clams, cockles, muscles, octopus, oysters, periwinkles, scallops, snails, squid, whelks, winkles, and many more are all molluscs, and all make their contribution to the human diet.

Mankind has been deliberately culturing molluscs as food for a long time and the earliest known records of someone farming molluscs for food come from the Romans. It was in fact a Roman, a certain Sergius

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Orata, who established the first oyster farm in Lake Lucrinus near Naples, to the best of our knowledge the first mollusc farm in history, about 95 BC. Oysters were likely the first sea animal to be transported from one area to another and cultivated as food.

Although molluscs are generally considered desirable components of a healthy diet, numerous cases of poisoning, particularly frequent and recurring in bivalve molluscs, occur worldwide each year (Ahmed 1991). While most of these toxic events are associated with the consumption of bivalve molluscs contaminated with viral, bacterial, and parasitic microorganisms, a significant number of incidents are associated with natural toxins produced by microalgae.

All bivalves are filter-feeders, mainly feeding on a wide range of phytoplankton species. Mussels, like all filter-feeding bivalve molluscs, process large volumes of water. This is necessary because the amount of organic matter in seawater is low (average 1 mg l^{-1}). Mussels filter, on average, 7.5 l h^{-1} of seawater. As a consequence of this, they accumulate and concentrate many pollutants in seawater, particularly those which are particulate or associated with particles. Like all bivalves, mussels are notorious for their ability to accumulate very high concentrations of metals. They also accumulate other pollutants, such as fecal bacteria and radionuclides, as well as all the metabolites produced by phytoplanktonic species used as food.

There are several thousand different phytoplanktonic species and of these some 60–80 algae, mainly belonging to the classes dinoflagellates and diatoms, are known to produce toxins (Tibbetts 1998). They are normally present in small quantities and do not represent a problem for public health. However, sometimes, algal proliferation occurs. The term harmful algal blooms (HABs) was initially coined to describe high concentrations of algae that produce extremely potent poisons. However, the scientific community recognizes now that, because a wide range of organisms is involved and some species have toxic effects at low cell densities, not all HABs are "algal" and not all occur as "blooms". How and why these blooms occur is a complex issue, depending on oceanographic currents, winds and other factors.

During toxic blooms, fish and shellfish consume these algae, then accumulate and concentrate the toxins without apparent harm. One major category of impact occurs when toxic phytoplankton is filtered from the water as food by edible shellfish such as clams, mussels, oysters, or scallops, which then accumulate the algal toxins to levels, which can be lethal to humans or other consumers (Shumway 1990). Typically, the shellfish contaminated by toxic phytoplankton are only marginally affected, even though a single clam can sometimes contain sufficient toxin to kill a human. In general, the natural marine toxins are tasteless, odorless, and are heat- and acid-stable. Therefore, normal screening and food preparation procedures do not prevent intoxication if the shellfish is

contaminated. The myriad of toxic compounds that marine phytoplankton can produce are known as marine biotoxins (Botana 2000).

The number of documented toxic blooms has been found to be increasing globally over the past few decades (Hallegraeff 1993). This is undoubtedly the result of a number of factors, including increasing worldwide seafood consumption, increased public and scientific awareness of harmful events, improved detection and analytical capabilities, changing weather and global temperature, and coastal pollution. There is also a body of evidence to indicate human-induced transportation of the cysts or "seeds" of toxic marine and freshwater organisms such as dinoflagellates, or the dinoflagellates themselves located inside the "spat" (young bivalve shellfish sold commercially to global markets for aquaculture) and ship ballast water (Anderson 1989; Hallegraeff 1993). Dinoflagellate cysts are able to survive long journeys in the dark and cold ballast tanks, before being released into seas when the ships dump their ballast water prior to harbor entry. International regulations are now changing to require ship ballast water to be purged in the open ocean prior to docking.

It is also hypothesized that human-generated environmental changes, such as reef destruction and eutrophication, may be responsible for the apparent increase in reports of human cases of marine and freshwater toxin disease as well as the increased incidence of HABs reported worldwide. There is even evidence connecting the apparent global increase of algal blooms with global climate changes, as seen with the El Niño phenomenon (Maclean 1989).

3.2 Marine Biotoxins

Marine biotoxins, produced by phytoplankton usually during HAB events, are some of the most potent toxins in the world and extremely dangerous. For some toxins, doses at the microgram per kilogram level are more than sufficient to kill. When enough toxin is accumulated in fish or shellfish, small amounts of cooked or raw tissue, even the consumption of one or two small mussels, can kill a normal, healthy adult human. While some toxins are very potent, i.e., requiring only small amounts to produce illness or death, other less potent toxins may accumulate to such high levels that they can still cause harm.

The epidemiology of shellfish toxins around the world and their risk to human health have been well documented. In 1987 approximately 2,000 cases of human poisoning were thought to occur each year worldwide through the consumption of fish and shellfish contaminated with algal toxins, with a mortality rate of approximately 15% (Hallegraeff 1987), but more recently it was estimated to be approximately 60,000 people

affected each year (Tibbetts 1998). Approximately 90% of all known poisoning incidents from seafood are associated with molluscs, mainly bivalves (Soames-Mraci 1995).

The risk of poisoning from the consumption of fish or shellfish is serious and of concern to public health authorities in all coastal environments. The risk and threat to public health is so great that many countries have instituted some form of risk management plan to deal with marine biotoxins. These "sanitation" plans are difficult to design and implement because the properties of the toxins are only poorly understood and, in addition to this, their origins may also not be known. Moreover, there is still a very poor understanding of the target organs for toxicity and the nature of any dose–response relationship associated with this toxicity. For these reasons, it is still difficult to identify a safe level of exposure to the respective toxins and, therefore, to provide an estimate of the margin of safety at various levels of exposure. In addition, not all countries have thorough monitoring systems: with the international transport and sale of seafood there is always a possibility of falling victim to a biotoxin.

Currently, the identified toxins are classified according to the poisoning syndromes they cause (Yasumoto and Murata 1993); and the incidences of poisoning related to marine algal toxins, depending upon the toxins and the symptoms that they cause, come under the main categories of:

- Paralytic shellfish poisoning (PSP)
- Neurotoxic shellfish poisoning (NSP)
- Diarrhetic shellfish poisoning (DSP)
- Amnesic shellfish poisoning (ASP)

Except for ASP, all are caused by biotoxins synthesized by dinoflagellates.

The toxins responsible for these syndromes are not single chemical entities, but are families of compounds having similar chemical entities and effects. Chemically, they can range from polar, low molecular weight compounds to high molecular weight, lipophilic substances. Most algal toxins cause human illness by disrupting electrical conduction, uncoupling communication between nerve and muscle, and impeding critical physiological processes. To do so, they bind to specific membrane receptors, leading to changes in the intracellular concentration of ions such as sodium or calcium.

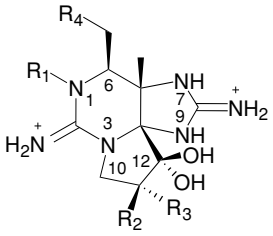
3.2.1
Paralytic Shellfish Poisoning

PSP is the most studied and understood of all the shellfish poisoning syndromes. Incidents of PSP have been recorded throughout the world for many centuries. Historically, PSP incidents are associated with dinoflagellates of the *Alexandrium* species (Schantz 1986). However, with developments in technology and research on marine algae, more species and classes of microorganisms are now being found to produce these toxins. Marine bacteria such as *Moraxella* (Kodama 1988) and *Alteromonas tetraodonis* (Gallacher and Birkbeck 1995) and freshwater cyanobacteria such as *Aphanizomenon flos-aquae*, *Anabaena circinalis*, *Lyngbya wollei*, *Cylindrospermopsis raciborskii* (Humpage et al. 1994; Falconer 1996; Lagos et al. 1997; Onodera et al. 1997), and *Protogonyaulax* (Ogata et al. 1989) have all been found to produce or influence the production of these toxins in algae. Infection of *Ostreopsis lenticularis* by *Pseudomonas* species was also found to affect the production of toxins (Gonzalez et al. 1995).

Paralytic Shellfish Toxins

The toxins responsible for PSP are a suite of heterocyclic guanidines collectively called saxitoxins, of which there are currently over 29 known congeners (Shimizu 2000). Their structures vary, having different combinations of hydroxyl and sulfate substitutions at four sites on the molecule (Fig. 3.1). Based on substitutions at R4, the saxitoxins can be subdivided into four groups: (1) neurotoxic and highly potent carbamate toxins which include the non-sulfated saxitoxin (STX) and neosaxitoxin (NEO) and gonyautoxins (GTX1–GTX4), which are singly sulfated and more lethal than the non-sulfated carbamate toxins, (2) weakly toxic *N*-sulfocarbamoyl-11-hydroxysulfate toxins (B1, B2, C1–C4), which are the least toxic to mammals of all the PSP toxins, (3) decarbamoyl (dc-) analogs, which are thought to arise from the metabolism of dinoflagellate toxins within the shellfish and (4) deoxydecarbamoyl (do-) toxins, that have been detected until now only in Australian populations of *G. catenatum* (Oshima et al. 1993).

STX blocks neurotransmission at the neuromuscular junction. It causes a blockage of neuronal and muscular Na^+ channels, preventing the propagation of action potentials and causing a relaxant action on vascular smooth muscle cells (Falconer 1993). STX binds specifically to site 1 of voltage-sensitive sodium channels (VSSCs) and requires the presence of both the α and the $\beta 1$ subunit of the channel.



STX = saxitoxin
NEO = neosaxitoxin
GTX = gonyautoxin

			R4							
			carbamate toxins		N-sulfocarbamoyl toxins		decarbamoyl toxins		deoxydecarbamoyl toxins	
R1	R2	R3					—OH		—H	
H	H	H	**1**	STX	**11**	GTX5, B1	**17**	dcSTX	**27**	doSTX
OH	H	H	**2**	NEO	**12**	GTX6, B2	**18**	dcNEO		
H	H	OSO_3^-	**3**	GTX2	**13**	C1	**19**	dcGTX2	**28**	doGTX2
H	OSO_3^-	H	**4**	GTX3	**14**	C2	**20**	dcGTX3	**29**	doGTX3
OH	H	OSO_3^-	**5**	GTX1	**15**	C3	**21**	dcGTX1		
OH	OSO_3^-	H	**6**	GTX4	**16**	C4	**22**	dcGTX4		
H	H	OH	**7**	11αOH-STX			**23**	11αOH-dcSTX		
H	OH	H	**8**	11βOH-STX			**24**	11βOH-dcSTX		
OH	H	OH	**9**	11αOH-NEO			**25**	11αOH-dcNEO		
OH	OH	H	**10**	11βOH-NEO			**26**	11βOH-dcNEO		

Fig. 3.1. Structure of paralytic shellfish toxins

Clinical Symptoms of PSP

PSP is a neurotoxic syndrome with a mortality rate of approximately 20% of those intoxicated. Symptoms of the disease develop fairly rapidly, within 0.5–2.0 h after ingestion of the shellfish, depending on the amount of toxin consumed. In humans, the peripheral nervous system is affected, with symptoms including tingling and numbness of extremities, muscular non-coordination, respiratory distress and muscular paralysis, leading to death by asphyxiation (Gessner et al. 1997). Transmission of nerve impulses to the muscles is inhibited and thus the diaphragm and other respiratory muscles in the lungs cease to assist breathing and the victim can die from respiratory arrest. When respiratory support is provided within 12 h of exposure, recovery is usually complete, with no lasting side-effects. In unusual cases, because of the weak hypotensive action of the toxin, death may occur from cardiovascular collapse despite respiratory support.

One of the most predominant toxins within this group is STX; and it is so potent that up to 50 humans can be poisoned from the level of poison contained in just one contaminated mussel (Soames-Mraci 1995 and references therein). There is currently no antidote for intoxication and

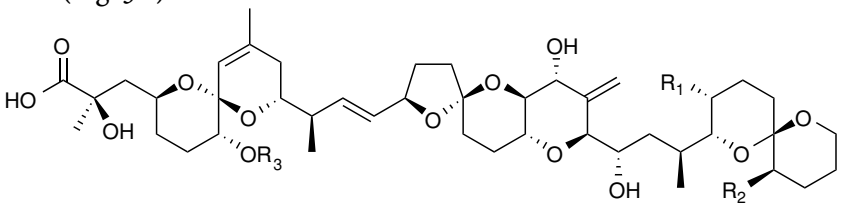
prognosis for the patient is entirely based upon the amount ingested by the victim.

3.2.2 Diarrhetic Shellfish Poisoning

DSP is a human illness, associated with seafood consumption and characterized by acute gastrointestinal disturbance. It is caused by a class of acidic polyether toxins produced by dinoflagellates. DSP is widespread in its distribution, with essentially seasonal occurrence in Europe and Japan. The first incidence of human shellfish-related illness identified as DSP occurred in Japan in the late 1970s, when the dinoflagellate *Dinophysis fortii* was identified as the causative organism and the toxin responsible was termed dinophysistoxin 1 (DTX1; Yasumoto et al. 1980). DSP toxins are produced by several other *Dinophysis* species including *D. acuta*, *D. fortii*, *D. acuminata*, *D. norvegica*, *D. mitra* (Yasumoto and Murata 1990), and *D. caudata* (Eaglesham et al. 2000), in addition to being produced by benthic species such as *Prorocentrum lima* (Bravo et al. 2001).

Diarrhetic Shellfish Toxins

This toxin class consists of at least eight congeners, including the parent compound, okadaic acid (OA), which was first isolated from the black sponge, *Halichondria fortii* (Tachibana et al. 1981). OA, DTX1 (Murata et al. 1982) and dinophysistoxin 2 (DTX2; Hu et al. 1992) are the primary congeners involved in shellfish poisoning, with the other congeners believed to be either precursors or shellfish metabolites of the active toxins (Fig. 3.2).



R_1	R_2	R_3	
CH_3	H	H	okadaic acid (OA)
CH_3	CH_3	H	dinophysistoxin 1 (DTX1)
H	CH_3	H	dinophysistoxin 2 (DTX2)
CH_3	CH_3	Acyl	dinophysistoxin 3 (DTX3)

Fig. 3.2. Chemical structure of main DSP toxins (okadaic acid group)

The OA class toxins are diarrhetic (Terao et al. 1986) and tumorigenic (Fujiki and Suganuma 1993). The mechanism of action underlying these activities is explained mainly by their potent inhibitory action against ser/thr protein phosphatases (Sasaki et al. 1994). Inhibitory activity is specific for classes PP2A and PP1, with PP2B being inhibited only at high concentrations and PP2C being insensitive.

Clinical Symptoms of DSP

Oral ingestion of the DSP toxins can lead to the gastrointestinal disturbances of acute diarrhea, nausea, vomiting, and abdominal pain, with symptoms often beginning within 30 min of consuming contaminated shellfish. No human mortalities to date have been reported from any cases of DSP poisoning, although there has been considerable morbidity resulting in hospitalization. The clinical symptoms of DSP may often have been mistaken for those of bacterial gastric infections and the problem may be much more widespread than currently thought.

OA is a potent tumor promoter (Fujiki and Suganuma 1993) and chronic exposure may promote tumor formation in the digestive system.

3.2.3
Toxins Found in Association with DSP Toxins

Other toxins have long been included in the DSP group for a number of reasons. First of all because they coexist in DSP-contaminated shellfish with OA and its congeners and, on account of their lipophilic nature, they are coextracted from the shellfish digestive gland together with okadaic acid group toxins. They are also included in the DSP group because they exert toxic effects following intraperitoneal (i.p.) injection in mice, thus producing positive results in the mouse bioassay, largely used as a screening method in monitoring programs. In addition, they are produced, as DSP toxins, by dinoflagellates; and indeed some of them are produced by the same toxinogenic algal species which also transmit OA and DTX1. However, they do not fit in the definition of DSP toxins, because they lack diarrhogenicity in mammals.

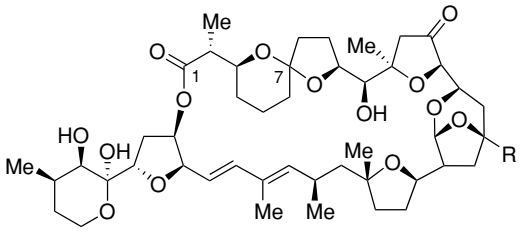
These toxins are represented by pectenotoxins (PTXs), yessotoxins (YTXs), and azaspiracids (AZAs).

Pectenotoxins

PTXs are a group of toxins isolated from algae commonly known to produce other DSPs such as OA and DTX1 (Yasumoto and Murata 1993). Examples of such algae include the dinoflagellates *D. acuta*, *D. fortii*,

D. acuminata, and *D. caudata*. The PTXs are often found in combination with other DSPs in shellfish and a debate exists over whether these toxins should be classified as DSP toxins. Some research groups have found mild diarrhetic effects caused by the administration of PTXs, while others have found no such evidence. Additionally, many DSPs have been found to be potent phosphatase inhibitors, but some PTX toxins were found to be inactive against PP1 and PP2A (Lun et al. 1993).

Structurally, PTXs resemble OA in molecular weight and in having cyclic ethers and a carboxylic group in the molecule (Fig. 3.3). Unlike in OA, however, the carboxyl moiety in PTX is in the form of a macrocyclic lactone (macrolide). It is believed that several of the pectenotoxins are derived from a parent pectenotoxin, where the parent molecule is metabolized within the scallops to form other pectenotoxin analogs.



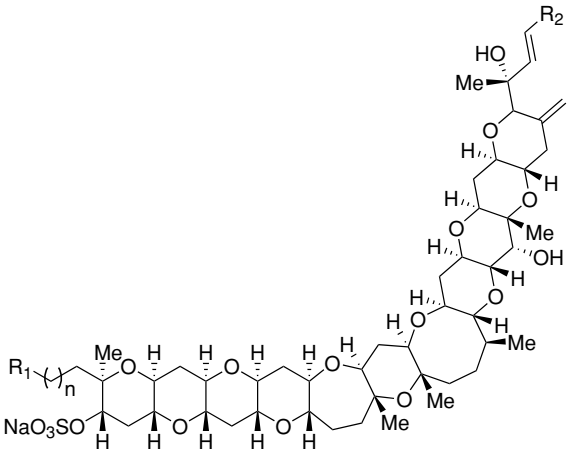
R	C-7		C-7	
CH_2OH	*R*	pectenotoxin 1 (PTX1)	*R*	pectenotoxin 2 seco acid (PTX2SA)
CH_3	*R*	pectenotoxin 2 (PTX2)	*S*	7-epi-pectenotoxin 2 seco acid (PTX2SA)
CHO	*R*	pectenotoxin 3 (PTX3)		
CH_2OH	*S*	pectenotoxin 4 (PTX4)		
COOH	*R*	pectenotoxin 6 (PTX6)		
COOH	*S*	pectenotoxin 7 (PTX7)		

Fig. 3.3. Structure of pectenotoxins (PTXs) and PTX2-seco acids (PTX2SAs)

Histopathological investigations of PTX2 to mice caused severe mucosal injuries and fluid accumulation in the small intestine and revealed that it is hepatotoxic and induces rapid necrosis of hepatocytes (Ishige et al. 1988). PTXs have a potent cytotoxicity (Jung et al. 1995) and probably inhibit actin polymerization (Spector et al. 1988).

Yessotoxins

YTXs are lipophilic polyether compounds. The group consists of yessotoxin, a ladder-shaped polycyclic ether toxin isolated for the first time from the scallop *Patinopecten yessoensis* (Murata et al. 1987) and a number of analogs including the homoyessotoxins, as shown in Fig. 3.4.



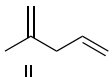
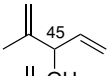
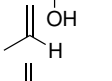
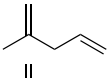
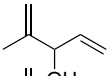
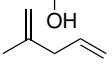
	R_1	R_2	n
yessotoxin (YTX)	OSO_3Na		1
45-hydroxyyessotoxin (45-OHYTX)	OSO_3Na		1
45, 46, 47-trinoryessotoxin (norYTX)	OSO_3Na		1
2-homoyessotoxin (homoYTX)	OSO_3Na		2
2-homo-45-hydroxyyessotoxin (45-OHhomoYTX)	OSO_3Na		2
1-desulfoyessotoxin (desulfoYTX)	OH		1

Fig. 3.4. Structure of some yessotoxins (YTXs)

Further yessotoxin analogs have been more recently isolated by our research group from Adriatic Sea mussels. Their structures are reported in Fig. 3.9.

Recent etiological study revealed that the origin of YTX is different from that of OA and DTX1. YTX is produced by the dinoflagellate *Protoceratium reticulatum* (Satake et al. 1997a), while OA and DTX1 are produced mainly by *Dinophysis* spp (Lee et al. 1989).

In several countries, YTXs are included within the class of DSP toxins for regulatory purposes because they coextract with other DSP toxins and are often found in association with DSPs in shellfish. However, studies on yessotoxin have shown it not to cause diarrhea or inhibit PP2A (Ogino et al. 1997). In the EU, the YTXs have been reclassified and are no longer included in recommended guidelines for DSPs, but are regulated within their own subgroup.

Yessotoxin was found to be more than ten times less toxic to mice via the oral route, compared with i.p. injections. Even at 10 mg kg^{-1} body weight, the highest dose ever tested orally, YTX did not kill the mice.

However, in spite of the wealth of data on OA, the molecular mechanism underlying the toxicity of YTXs is unknown. Indeed, very limited data are available regarding the effects of this group of components on cellular systems. Histopathological analysis revealed that a target organ of YTX is the heart: marked intracytoplasmatic edema in cardiac muscle cells was observed in mice after i.p. injection of the toxin (Terao 1990). An involvement of the nervous system in YTX toxicity can be also hypothesized on the basis of the chemical structure, since brevetoxins and ciguatoxins (Yasumoto and Murata 1993), both structurally strictly related to YTX, induce poisoning characterized by neurological and cardiovascular symptoms (Dechraoui et al. 1999).

As for the mechanism of action, by analogy with brevetoxins and ciguatoxins, YTX may act as a depolarizing agent, opening membrane channels of Na^+-permeable excitable cells and leading to a Na^+ influx (Gawley et al. 1992). It remains to be established, however, to what extent these toxins can be absorbed by the intestine and then gain access to the target organs.

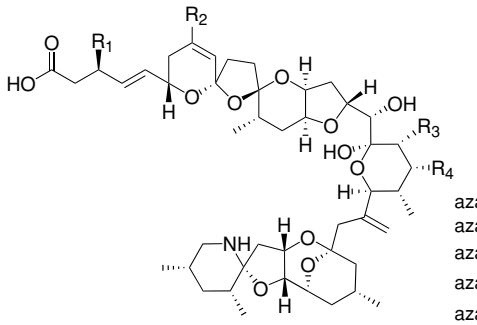
Azaspiracids

Azaspiracid poisoning (AZP) is a newly identified syndrome. The causative toxin, azaspiracid, so named because of its unusual azaspiro ring assembly, was first identified from Irish mussel extracts in association with a shellfish poisoning incident that took place in the Netherlands during 1995 (Satake et al. 1998). In addition to AZA, four analogs, AZA2–AZA5, were isolated and their structures determined, as shown in Fig. 3.5 (Ofuji et al. 1999, 2001). The symptoms observed in the patients included nausea, vomiting, severe diarrhea and stomach cramps and thus resembled those of DSP. However, mouse symptoms induced by i.p. injection of acetone extracts of mussel hepatopancreas were distinctly different from those normally associated with DSP toxins, showing prominent neurological symptoms, such as respiratory difficulties, spasms, paralysis of the limbs and death within 20 min at higher doses (Ito et al. 2000).

3.2.4
Neurotoxic Shellfish Poisoning

A long history of toxic microalgal blooms exists in the Gulf of Mexico, blooms that have caused massive fish kills and respiratory irritation in humans. It was later realized that the toxin in these blooms could also be

passed to humans via shellfish, to cause a syndrome named neurotoxic shellfish poisoning. Reports of NSP were limited for a long time to the west coast of Florida, where blooms of the dinoflagellate *Gymnodinium breve* initiate offshore and are subsequently carried inshore by wind and current conditions (Steidinger et al. 1998). In the early 1990s, outbreaks of shellfish toxicity were reported in New Zealand and Australia and resulted in the identification of additional *Gymnodinium* species which produce NSP-like toxins (Haywood et al. 1996). Recently, other fish-killing flagellate species, *Chattonella marina*, *C. antiqua*, *Fibrocapsa japonica*, and *Heterosigma akashiwo*, have also been reported as producers of this class of polyether toxins (Sagir Ahmed et al. 1995; Khan et al. 1997; Hallegraeff et al. 1998).



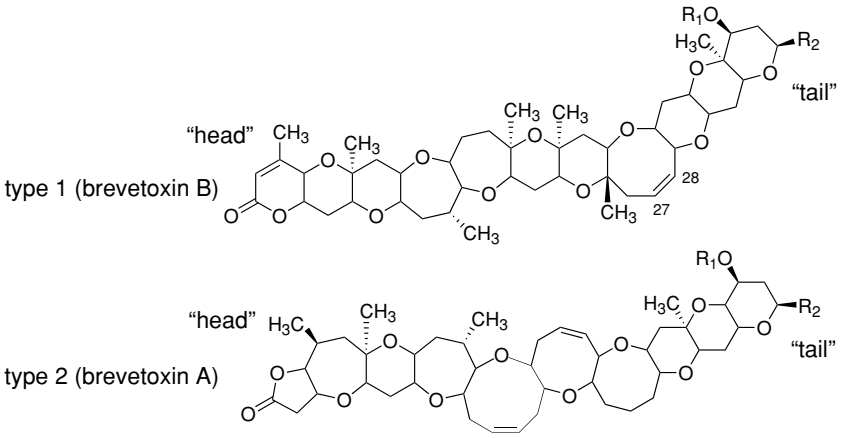
	R_1	R_2	R_3	R_4
azaspiracid (AZA)	H	H	Me	H
azaspiracid-2 (AZA2)	H	Me	Me	H
azaspiracid-3 (AZA3)	H	H	H	H
azaspiracid-4 (AZA4)	OH	H	H	H
azaspiracid-5 (AZA5)	H	H	H	OH

Fig. 3.5. Chemical structure of azaspiracids

Neurotoxic Shellfish Toxins

The toxins responsible for NSP are a suite of ladder-like polycyclic ether toxins collectively called brevetoxins (Fig. 3.6). Brevetoxin congeners fall into two types, based on backbone structure: the brevetoxin B backbone (type 1) and brevetoxin A backbone (type 2). Although the ring systems in the middle of the molecules differ somewhat, type 1 and type 2 toxins share a lactone in the A ring ("head" of the molecule) and a conserved structure on the "tail" ring, both of which are required for their toxicity (Baden 1989).

These toxins are depolarizing substances that open voltage-gated sodium (Na^+) ion channels in cell walls, leading to uncontrolled Na^+ influx into the cell (Baden 1983). This alters the membrane properties of excitable cell types in ways that enhance the inward flow of Na^+ ions into the cell; and this current can be blocked by external application of tetrodotoxin (Poli et al. 1986; Trainer et al. 1991).



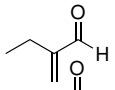
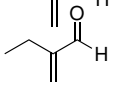
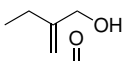
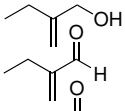
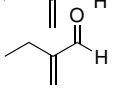
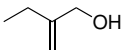
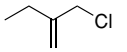
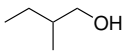
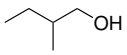
toxin	type	R_1	R_2
PbTx-1	2	H	
PbTx-2	1	H	
PbTx-3	1	H	
PbTx-5	1	$COCH_3$	
PbTx-6	1	H	 27,28 epoxide
PbTx-7	2	H	
PbTx-8	1	H	
PbTx-9	1	H	
PbTx-10	2	H	

Fig. 3.6. Structure of brevetoxins

Clinical Symptoms of NSP

In humans, the symptoms of NSP intoxication include respiratory distress, as well as eye and nasal membrane irritation, caused principally by exposure to sea-spray aerosols and by direct contact with toxic blooms while swimming. There have been no reported fatalities from NSP, although the toxin kills test mammals when administered by various routes, including oral.

3.2.5 Amnesic Shellfish Poisoning

ASP is the only shellfish poisoning produced by a diatom. The syndrome of ASP was first recognized in 1987 on Prince Edward Island, Canada, where there were three deaths and 105 acute human poisonings from blue mussels (Perl et al. 1990; Teitelbaum et al. 1990). The chain-forming diatom *Pseudo-nitzschia multiseries* (formerly known as *Nitzschia pungens*) was recognized as the causative agent of that toxic event (Subba-Rao et al. 1988; Bates et al. 1989). It is now known that different diatom species induce ASP. These diatom species are distributed worldwide.

Amnesic Shellfish Toxins

The toxin responsible for ASP is domoic acid (DA; Fig. 3.7), a water-soluble excitatory tricarboxylic amino acid belonging to the kainoid class of compounds, which acts as a glutamate antagonist on the kainate receptors of the central nervous system. Several congeners of DA have been identified so far, of which three geometrical isomers, isodomoic acids D, E, and F and the C5′-diastereomer were found in small amounts in both the diatom and in shellfish tissue (Fig. 3.7; Wright et al. 1990; Walter et al. 1994).

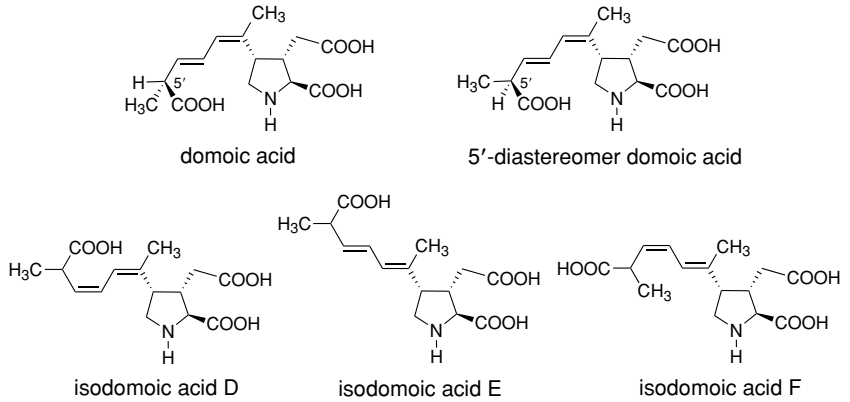


Fig. 3.7. Chemical structures of domoic acid and some of its congeners

Domoic acid binds with high affinity to both kainate and AMPA subtypes of glutamate receptor (Hampson et al. 1992). Persistent activation of the kainate glutamate receptor results in greatly elevated intracellular Ca^{2+} (Xi and Ramsdell 1996). This induces lesions in areas of the brain where glutaminergic pathways are heavily concentrated, particularly in the CA1 and CA3 regions of the hippocampus, areas responsible for learning and memory processing (Peng and Ramsdell 1996).

Clinical Symptoms of ASP

The clinical symptoms of ASP include abdominal cramps, vomiting, diarrhea, incapacitating headaches, disorientation and short-term memory loss. In the most severe case of poisoning, patients are victim to seizure, coma, profuse respiratory secretion, unstable blood pressure, and death. The loss of memory in patients intoxicated with mussel toxin appears to be similar to patients with Alzheimer's disease. However, the loss of memory in mussel-intoxicated patients is not affected by the age of patients, whereas symptoms of Alzheimer's disease intensify with advancing age and are generally noted in older people. Further, the findings that intellect and higher cortical functions are not influenced by DA intoxication distinguish the mussel-induced intoxication from Alzheimer's disease.

3.2.6
Spirolides and Shellfish Syndrome Related to Dinoflagellates

Spirolides are pharmacologically active macrocyclic imines that were first isolated and characterized from lipophilic extracts of scallop and mussel viscera harvested from aquaculture sites in Nova Scotia, Canada (Fig. 3.8; Hu et al. 1995, 1996). These "fast-acting toxins" cause rapid death upon i.p. injection into mice and also have a high oral potency with apparent neurotoxic symptomatology, but the mode of action is currently unknown. The symptoms include piloerection, abdominal muscle spasms, hyper-extensions of the back and arching of the tail to the point of touching the nose.

The biological origin of spirolides was unknown until recently, although the evidence (geographical extent, seasonality, occurrence in multiple shellfish species) strongly suggested a planktonic source (Cembella et al. 1998). This hypothesis was also supported by the high degree of structural homology between spirolides and other macrolides of marine dinoflagellate origin, including gymnodimine (from *Gymnodinium mikimoto*) and prorocentrolides (found in *Prorocentrum lima*; Wright and Cembella 1998). Using liquid chromatography–mass spectrometry (LC–MS) analyses, various spirolides were detected in fractions of planktonic material from Nova Scotian aquaculture sites. Particularly, the dinoflagellate *Alexandrium ostenfeldii* was shown to be the producing organism (Cembella et al. 2000).

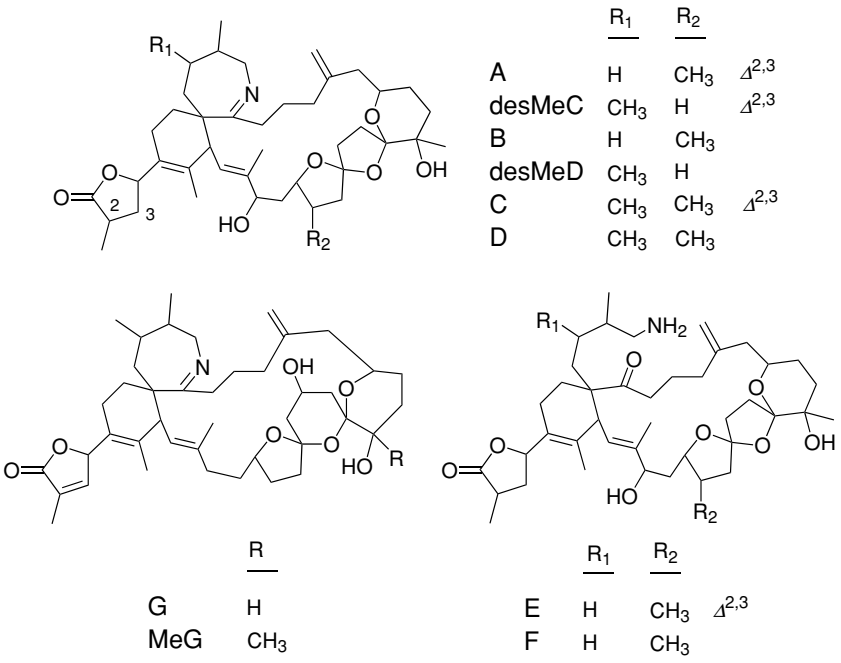


Fig. 3.8. Chemical structure of spirolides

3.3
DSP Toxins in Phytoplankton and Mussels from the Northwestern Adriatic Sea

The northwestern coast of the Adriatic Sea has been subject to recurring cases of red tides since 1975. DSP outbreaks associated with harmful algae blooms were, however, recognized as a problem only in 1989, when the first case of human gastroenteritis was related to the simultaneous presence of known producers of DSP toxins both in seawater and hepatopancreas of mussels (Boni et al. 1992). The evidence that certain cases of diarrhea in consumers of molluscs were not due to bacteria or virus but to biointoxication by DSP came from the isolation of lipid-soluble DSP-type toxins in mussel tissue collected in the coastal water of the Emilia Romagna region. This phenomenon has occurred in the Adriatic Sea with alarming frequency since then, subsequently extending over the coastal areas of Marche, Abruzzo, Veneto, and Friuli-Venezia Giulia.

The continuance of mussel toxicity causes a serious threat to human health and severe economic losses for the Adriatic shellfish industries, whose production areas, which cover 90% of the national total production of mussels, have been forced to remain closed for some months.

In order to prevent or minimize such damage, continuous monitoring of toxicity in shellfish and structural elucidation of the causative toxins are prerequisites. The most commonly used assay method is the mouse bioassay developed by the Japanese Ministry of Health and Welfare (Japanese Ministry of Health and Welfare 1981). One mouse unit (MU) is defined as the minimum quantity of toxin needed to kill a mouse within 24 h.

Major disadvantages of this assay are the lack of specificity (no differentiation between the various components of DSP toxins), the subjectivity of death time of the animals and the maintenance and killing of laboratory animals.

A research program based on instrumental analysis was, therefore, initiated in 1990 by our research group, to examine the toxic profiles in mussels from the northern Adriatic Sea. Until now, a number of toxic samples of shellfish collected along the Emilia Romagna coasts and corresponding to the highest level of toxicity have been analyzed.

In the first part of this study, OA was recovered as the causative toxin in the toxic episode of 1990, identified through ^{1}H NMR spectroscopy (Fattorusso et al. 1992). This result represented the first certain evidence of the presence of DSP toxins in mussels cultivated along the Italian coast.

Subsequently, DTX1 was also detected, using ionspray LC–MS (Draisci et al. 1995). However, recent research has demonstrated that other toxins are, at the moment, important contributors to DSP in Italy. In 1995, in fact, for the first time from Italian mussels, YTX was isolated in relatively large amounts, in addition to trace amounts of OA, by our research group (Ciminiello et al. 1997).

Later, besides a relatively large amount of 45-hydroxyyessotoxin (Ciminiello et al. 1999), two new analogs, homoYTX (which presents an extra methylene group to the structure of YTX in the western part of the molecule) and 45-hydroxyhomoYTX (Satake et al. 1997a) were also isolated from Italian mussels.

3.3.1 New YTX Analogs Isolated from Adriatic Mussels

Very recently, in our laboratory, we isolated from the hepatopancreas of Adriatic mussels and chemically characterized several new YTX-like structures (Fig. 3.9), such as adriatoxin (ATX; Ciminiello et al. 1998), carboxyyessotoxin (COOHYTX; Ciminiello et al. 2000a), carboxyhomoyessotoxin (COOHhomoYTX; Ciminiello et al. 2000b) and 42,43,44,45,46,47,55-heptanor-41-oxohomoyessotoxin (noroxohomoYTX; Ciminiello et al. 2001a).

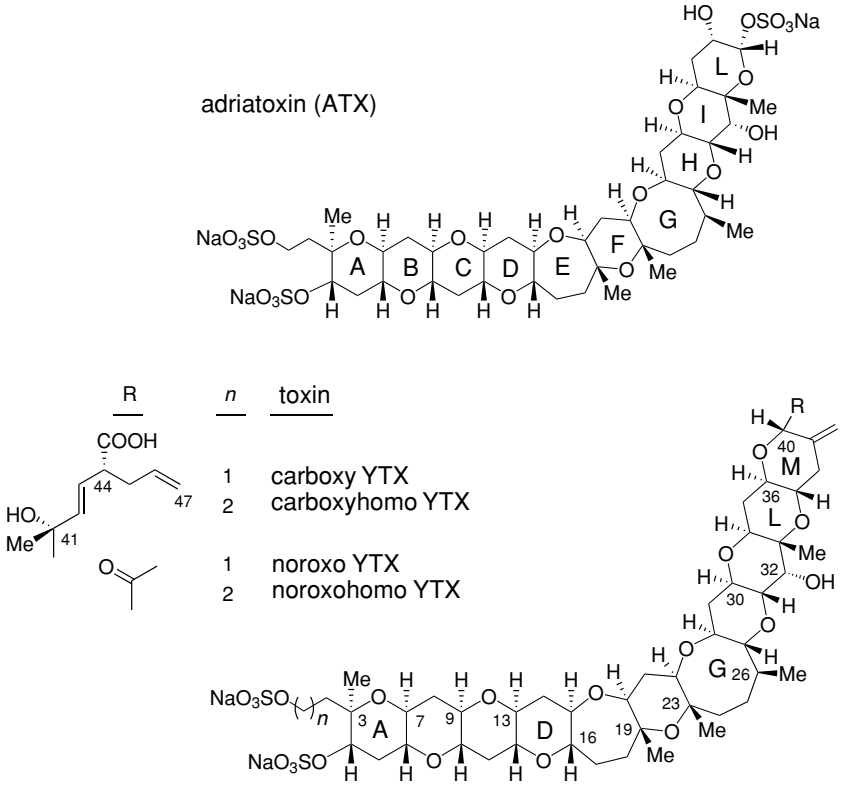


Fig. 3.9. New YTX analogs from Adriatic mussels

All the new analogs have been isolated in a pure form and show a close resemblance to YTX, the main differences being located in the eastern part of the molecule. Their chemical structures have been determined on the basis of spectral evidence, particularly mono-dimensional and two-dimensional ^{1}H NMR experiments, as well as MS/MS experiments.

The toxicology of these new biotoxins is unknown. The results of our studies indicate, however, that the composition and the relative abundance of YTXs in bivalves seem to vary regionally, seasonally and annually, as observed for other DSP toxins.

3.3.2 LC–MS Method for Analysis of YTXs

The chief obstacles in facing the acute and chronic risk associated with YTX-contaminated seafood are the limited availability of toxins for

toxicological studies and the lack of a rapid and efficient analytical method for following the variation of the toxin profile in molluscs.

The combination of liquid chromatography and mass spectrometry (LC–MS) has proven to be the most powerful tool for the detection and quantitation of toxins in plankton and shellfish at trace levels, the identification of new toxins, the investigation of toxin production by plankton and the study of toxin metabolism in shellfish (Quilliam 1996).

This technique appears to be extremely useful for the detection and quantitation of DSP toxins. In fact, the most common analytical methods so far employed for the specific detection of DSPs provide for the derivatization of each toxin with an appropriate auxiliary reagent for fluorescence labeling followed by HPLC analysis. However, there is no reagent which fits all DSP toxins. 9-Anthryldiazomethane (ADAM) is used for OA, DTXs, and PTXs (Lee et al. 1987), while YTXs are derivatized with a dienophile reagent, 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD; Yasumoto and Takizawa 1997). It has to be noted that, for the application of latter method, the presence of a conjugated diene functionality in the side-chain of YTX-like compounds is a prerequisite. Thus, the method is not reliable for the detection of those derivatives which lack a conjugated diene functionality in the molecule, such as the Adriatic analogs noroxohomo-YTX, carboxyYTX, carboxyhomoYTX, and adriatoxin.

With the aim of setting up a suitable method for the rapid and unambiguous detection of all YTXs isolated so far and also the presence of OA which sometimes coexists in shellfish, we tested the suitability of the LC–MS method developed by Quilliam et al. (2001) for the detection of most lipophilic toxins (Ciminiello et al. 2002a). For this purpose, standard solutions at a known concentration of YTX and OA as well as solutions of a number of YTX analogs from North Adriatic mussels were employed.

Thus developed, this LC–MS technique allowed the determination of OA and all YTXs and homoYTXs derivatives so far isolated in a single chromatographic run of 25 min and showed itself to be both selective and sensitive with a detection limit of 68.4 pg for YTX (Fig. 3.10).

Together with the rapid detection of known compounds at parts per billion levels, the method makes it possible to highlight the potential presence of new analogs and can be usefully employed for the structural elucidation of new toxins whenever great structural analogies occur between the toxins under investigation and known compounds.

The potential of LC–MS analysis for the detection of new toxins can be illustrated by two new YTX analogs from Northern Adriatic mussels we recently identified in toxic mixtures obtained from *Mytilus galloprovincialis* collected in 1998 and 2001, respectively (Ciminiello et al. 2002a,b).

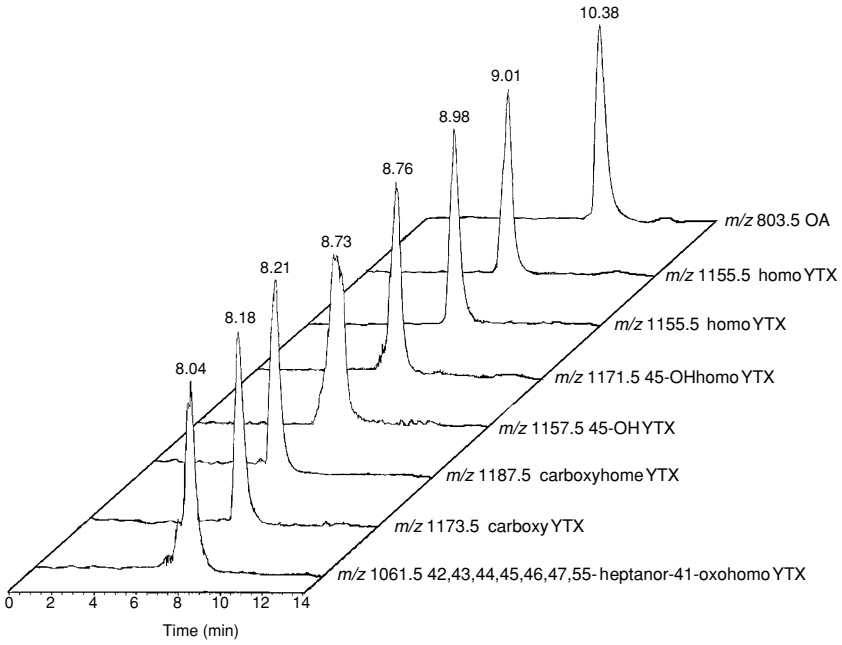


Fig. 3.10. LC-ESI (negative ion)-ion trap–MS analysis of a wide range of yessotoxins in a blend of mussel tissue extract added of OA standard solution. Selected monitoring of yessotoxins with different molecular masses was carried out by extracted ion chromatograms (XICs) of the $[M–2Na^{+}H]^{-}$ ions. For OA the XIC is relevant to the $[M–H]^{-}$ ion

Along with known YTXs identified by comparison of their retention times and mass spectra with those of appropriate standards, new marine toxins, a desulfoYTX and 42,43,44,45,46,47,55-heptanor-41-oxoyessotoxin, were detected. MS/MS experiments were used to gain structural information.

3.3.3 LC–MS Analysis of an Adriatic Strain of *P. reticulatum*

The LC–MS method developed for the determination of YTXs could be employed not only for the analysis of toxic mussels, but also for the screening of algal cultures in order to select the producer organisms of the compounds to be submitted to toxicological studies.

Identification of the organism(s) responsible for the production of YTX derivatives is of critical importance for the future regulation and management of toxic shellfish. In 1997, the marine dinoflagellate *Protoceratium reticulatum* collected in New Zealand was indicated as the

biogenetic origin of YTX (Satake et al. 1997b). Subsequently, YTX was detected both in Adriatic *P. reticulatum* (Boni et al. 2001) and together with 45,46,47-trinoryessotoxin in strains of the same species collected in Japan (Satake et al. 1999). In 1999, the presence of YTX and homoYTX in *Gonyaulax polyedra* collected in the northwestern Adriatic Sea was reported (Draisci et al. 1999). However, the origin of all the other YTX analogs was still unknown, thus raising an issue whether they were metabolites of YTX formed in mussels or true products of different dinoflagellate species.

To ascertain their origin, a cultured strain of *P. reticulatum* (*G. grindley*) collected along the Cesenatico coasts (Emilia Romagna, Italy) in June 2001 was investigated (Ciminiello et al. 2003). Careful analysis of this strain obtained by high performance liquid chromatography coupled with electrospray ionization ion trap mass spectrometry (HPLC–ESI MS), suggests that *P. reticulatum* from the Northwestern Adriatic Sea is responsible for the production, together with YTX, of homoYTX, 45-OHYTX, carboxyYTX, and noroxoYTX.

This is the first identification of *P. reticulatum* as the producer of some of the YTX derivatives so far isolated from Italian mussels. Interestingly, Adriatic *P. reticulatum* is able to produce compounds belonging to both the YTX and homoYTX series, whereas previous studies were suggestive of two different organisms being responsible for production of the two homologous series.

Furthermore, these findings indicate that most of the Adriatic YTX derivatives are true products of the dinoflagellate and do not derive from the metabolic conversion of YTX in shellfish.

3.4 Detection of Domoic Acid in Adriatic Shellfish by Hydrophilic Interaction Liquid Chromatography–Mass Spectrometry

A hydrophilic interaction liquid chromatography–mass spectrometry (HILIC–MS) method has been very recently developed by us to allow for the rapid, unambiguous identification and quantitation of DA in shellfish sample (Ciminiello et al. 2005).

The obtained results showed that the HILIC–MS technique is suitable for combined analysis of DA and PSP toxins in a single 30-min chromatographic run, using gradient elution. Isocratic elution allows detection of DA in 10 min.

Application of the developed method to the analysis of a number of samples of *Mytilus galloprovincialis*, collected over the period 2000–2005 in the Adriatic Sea, indicated the presence of DA in some of the analyzed

samples as a new toxin which has entered the Adriatic mussels' toxin profile.

This is the first time that DA has been detected in Adriatic shellfish, although in all analyzed samples the toxin appeared to be present at levels well below the regulatory limit (20 μg g^{-1} in edible tissue). The obtained results represent a warning for DA as one of the toxins which need to be carefully monitored in Adriatic shellfish.

3.5 Cytotoxins from Contaminated Adriatic Blue Mussels

In the course of our investigation into toxic Adriatic mussels, we have also isolated and structurally characterized, besides YTXs, new types of toxins, oxazinins and chlorosulfolipids, which are completely different in structure from the polyether DSP toxins isolated so far, but may represent a further alarm for public health, due to their cytotoxic activity.

3.5.1 Oxazinins

Three new compounds, oxazinin-1, oxazinin-2, and oxazinin-3, have been isolated from the toxic digestive glands of the Adriatic mussel *Mytilus galloprovincialis* (Ciminiello et al. 2001b; Fig. 3.11). They are characterized by unique structural features, which to the best of our knowledge have not been found in any other naturally occurring compound.

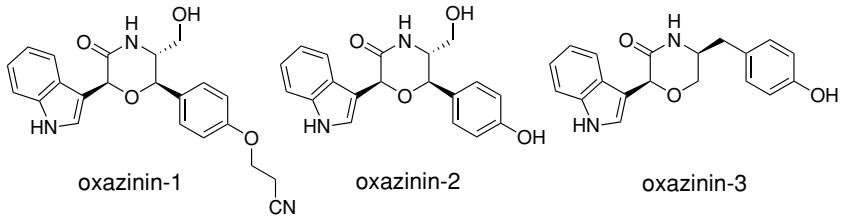


Fig. 3.11. Structure of oxazinins

The determination of the structure and the relative stereochemistry of the new molecules was based on spectroscopic evidence including extensive 2D-NMR experiments and molecular mechanical calculations. The absolute stereochemistry of oxazinin-1 has been assigned (Ciminiello et al. 2001c) by application of a method proposed by Latypov et al. (1998)

for the assignment of the absolute configuration of most β-chiral primary alcohols.

3.5.2 Chlorosulfolipids

Very recently, we reported the isolation from *M. galloprovincialis* of a new class of cytotoxins constituted by polychlorinated sulfolipids.

Some chlorosulfolipids were previously isolated from species of microalgae (Mercer and Davies 1979; Chen et al. 1994). These compounds, whose structure has so far been assigned devoid of stereochemical details, have been divided into two series: the polychlorodocosane 1,14-disulfates and the polychlorotetracosane 1,15-disulfates with a number of chlorine atoms, which range from zero to six in various combinations of positions on the aliphatic chain. In most cases, the exact location of the chlorine atoms could not be determined. They represent a unique class of products in that they are essentially polar at both ends of the molecule.

During our investigation into toxic Adriatic mussels, we succeeded in isolating three unique cytotoxic compounds (Fig. 3.12) which can be included in the class of chlorosulfolipids, even if they are structurally quite different from the previously reported ones.

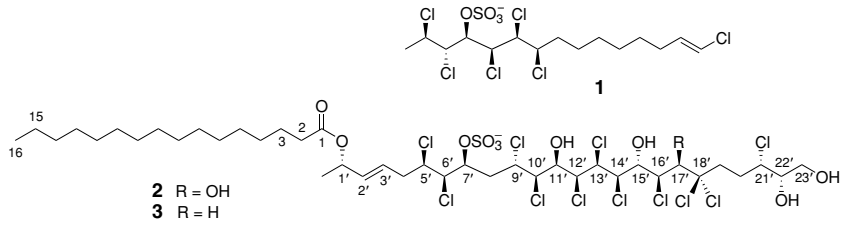


Fig. 3.12. Structure of chlorosulfolipids from Adriatic mussels

The first isolated compound is a hexachloromonosulfate, **1** (Ciminiello et al. 2001d), while **2** (Ciminiello et al. 2002c) and **3** (Ciminiello et al. 2004), very recently characterized, contain 11 chlorines, in addition to a fatty acid acyl moiety.

Structure determination of the new polychlorinated sulfolipids has been carried out by extensive use of 1D- and 2D-NMR techniques, supported by ESI MS and MS/MS experiments, as well as molecular mechanics and dynamics calculations.

Elucidation of the absolute stereochemistry of the three molecules appeared to change, particularly due to the presence of many stereogenic carbons, from 6 in compound **1** up to 15 in compound **2**.

The relative stereochemistry of the polychlorinated sulfolipids was determined by using the *J*-based configuration analysis method (Murata et al. 1999; Matsumori et al. 1999).

This method was first applied to assign the relative stereochemistry of **1**; and in order to verify its confident applicability to our complex chlorosulfolipids, molecular mechanics calculations were carried out using the CHARMm force field. The obtained results fully substantiated the stereochemical assignments based on the Murata method.

The absolute stereochemistry of the chlorosulfolipids was defined by a modified Mosher's method (Ohtani et al. 1991).

3.6 Conclusions

The results of our studies have revealed a very interesting, complex and changeable scenario of shellfish toxicity in Italy. It is evident from our data that there is a variety of YTX analogs in some shellfish-producing areas. An important aspect to be considered is that the presence in Adriatic shellfish of several toxins of the YTX class creates complications due to the lack of toxicity data for this type of toxin and also makes quantification difficult in the absence of analytical reference compounds. It is indispensable to address toxicological investigations into all the YTX-like compounds. Much effort should therefore be directed at the accumulation of these toxins to be utilized in toxicological studies.

The great variety of closely related toxin structures and the varying toxicities present significant challenges to the analytical chemist interested in developing a method for their detection and quantitation.

Our results confirmed the LC–MS technique as being a very promising alternative tool to animal testing. This chemical analysis method has played an essential role in all phases of toxic investigations, including the identification of new toxins by bioassay-directed fractionation, the detection and quantitation of toxins in plankton and shellfish and the investigation of toxin production by plankton. However, improvement and inter-laboratory studies will be necessary before this technique can become a generally accepted tool in regulatory analysis. A serious problem hampering the further development and validation of analytical methodology for biotoxins is that pure analytical standards and reference materials are hardly or not readily available. This is particularly true for YTXs, which are really very rare materials: they are not commercially available and few laboratories possess even very small amounts of YTX and much less of its analogs.

Moreover, the presence of further toxic compounds in edible shellfish, such as oxazinin-1, chlorosulfolipids, and DA, in addition to contamination

of DSP toxins, increases the potential risk to human health. To prevent the damage caused by pollution from harmful marine algae (both to public health and to the shellfish industries), it is necessary to implement careful monitoring, both at markets and at shellfish farms. Monitoring, in turn, cannot be run without good knowledge of the causative organisms and the nature of implicated toxins. Therefore, an accurate analysis of toxic mussels is indispensable in order to identify new toxins, even other than DSP polyether toxins, and to isolate a larger amount to clarify in depth their toxicological effects.

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