Shellfish Toxins − Chemical Studies on Northern Adriatic Mussels

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Keywords: Diarrhetic shellfish poisoning / Marine biotoxins / Yessotoxins / Cytotoxic compounds / Natural products / Seafood poisoning / Toxicology

During our investigation on toxic mussels from the Northern Adriatic sea, initiated about ten years ago, a number of polyether toxins have been isolated and characterized, some of which represent new additions to the diarrhetic shellfish poisoning (DSP) class of biotoxins and seem to be specific to the Adriatic. In addition, we have also isolated new types of toxins, oxazinins and chlorosulfolipids, whose structures were elucidated by extensive use of 1D and 2D NMR spectroscopic techniques. Some of them could represent a further risk to public health due to their cytotoxic activity. A liquid-chromatography mass-spectrometry (LC-MS) method was proposed for the sensitive, specific and direct detection of yessotoxin and its analogs. In addition, the proposed method allows us highlight the possible presence of new analogs, and enables us to propose an effective structural hypothesis even when full structural elucidation of new toxins by NMR spectroscopy is hampered by the limited amount of material available.

1. Historical Background

Although shellfish are generally considered desirable components of a healthy diet, numerous cases of poisoning occur worldwide each year.[1] While most of these incidents are associated with the consumption of shellfish contaminated with viral, bacterial and parasitic microorganisms, a significant number of incidents are associated with natural toxins produced by microalgae.

When Virgil (70−19 BC) wrote the words *Nihil vilior alga* − there is nothing fouler than an alga − he presumably had the stench of decaying seaweed in mind. Macroscopic

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Patrizia Ciminiello was born in Naples, Italy, in 1958. She graduated in Biological Sciences in 1980 and Pharmaceutical Chemistry and Technology in 1985 at the University of Naples under the supervision of Prof. Ernesto Fattorusso. In 1987, she received a PhD degree in Chemical Sciences from the same university, where she is currently working as Full Professor. Her research has targeted the chemical investigation of metabolites from marine sources, especially the study of metabolites from algae and porifers, which has led to the isolation and structural determination, almost always with complete stereochemistry, of more than one hundred new compounds, many of which have unusual structural characteristics. Structural determination and stereochemical assignment of isolated compounds have been performed through extensive use of 1D and 2D NMR experiments as well as by mass and mass/mass techniques. Her current research interests focus on the stereostructural determination of marine biotoxins, related to the toxicity of some seafood.

Ernesto Fattorusso was born in Naples, Italy, in 1937. He graduated in Chemistry in 1960 at the University of Naples. In 1975 he became Full Professor of Organic Chemistry at the Faculty of Science of the University of Naples. In 1980 he moved to the Faculty of Pharmacy of the same university, where he was Dean from 1987 to 1990 and from 1997 to 2000. He has published 280 papers on the following topics: i) Structure and biogenesis of black, brown and reddish pigments from animals and plants; ii) Structure of bioactive metabolites from marine algae and invertebrates. The research on marine products deals with the isolation and structure determination of metabolites of invertebrates (sponges, ascidians, anthozoans, bryozoans, soft corals, hydroids) and algae. More than 500 new marine metabolites (steroids, terpenoids, carotenoids, alkaloids or glycolipids) were isolated. A number of the isolated metabolites showed to be biologically active (antibiotic, antifungal, cytotoxic, immunostimulating etc.). Recently he undertook a survey of some species of Mediterranean terrestrial flora that are used in popular medicine. Prof. Fattorusso is currently interested also in the chemistry of marine biotoxins. He is a member of the International Advisory Board of European Journal of Organic Chemistry.

MICROREVIEWS: This feature introduces the readers to the author's research through a concise overview of the selected topic. Reference to important work from others in the field is included.

algae can indeed be a nuisance, as when they get tangled around a boat's propeller or when an alien species invades and destroys native littoral communities, but many are of economic value, several are quite elegant, and none are particularly toxic. Indeed, it is now the microalgae that attract attention as being potentially more harmful. It has been known for a long time that some water blooms, as dense growths of these micro-organisms in freshwater are called, and red tides, which are analogous populations in the sea, may be harmful.[2−4] The account in the Bible of the first plague of Egypt is a convincing description of a dinoflagellate bloom killing fishes in the Nile.

The microscopic planktonic algae are primary producers that make up the base of both marine and freshwater food webs. They represent essential food for filter-feeding bivalve shellfish; in most cases, the proliferation of phytoplankton (so-called "algal blooms"; up to millions of cells per litre) therefore is beneficial for aquaculture and wild fisheries operation. However, in some situations algal blooms can have a negative effect, causing severe economic losses to aquaculture, fisheries and tourism operations and having major environmental and human health impacts. In fact, among the many algal secondary metabolites that have been identified, several are potent toxins; when blooms of toxin-producing microalgae occur, they result in a wide array of human illnesses, marine mammal and bird morbidity and mortality, and extensive fish losses.[5,6] Toxins associated with human intoxication originate primarily from three classes of unicellular algae: dinoflagellates, diatoms, and cyanobacteria. Dinoflagellates are responsible for the widest array of toxins. However, of the several thousand species of dinoflagellates known, only a few dozen species appear to be toxigenic.

Over the past century reports of blooms of toxic algae, both in fresh water and the sea, have become increasingly frequent. This may be in part attributable to greater awareness of the public, greater attention from the media, and the attraction of scientists to interesting chemical and biological problems. However, although it is difficult to produce unequivocal evidence, it seems that the increasing incidence of algal proliferations is real and that increasing eutrophication by human agencies is the main cause. Where formerly a few regions were affected in scattered locations, now virtually every coastal state is threatened, in many cases over large geographic areas and by more than one harmful or toxic algal species.

One major category of impact occurs when "blooms" of such toxigenic species appear unexpectedly in shellfish-producing regions. When toxic algal species are present, shellfish can be rendered unfit for human consumption. Filter-feeding bivalve molluscs, such as clams, mussels, oysters, or scallops, consume the phycoplankton, thereby accumulating phycotoxins in their edible tissues to levels which can be lethal to humans or other consumers. Typically, the contaminated shellfish are only marginally affected by the accumulated toxins, even though a single clam can sometimes contain sufficient amounts of toxin to kill a human.

Poisonous seafood neither looks nor tastes different from uncontaminated seafood, and cooking and other treatment of shellfish does not destroy the toxins. Shellfish farming areas infested by toxic algal species therefore need to run costly monitoring programmes to check for toxic algae in the water and, whenever these are present, regular tests for toxins in associated seafood products need to be carried out.

Since toxicity is the only common factor among the diverse phycotoxins, it is not surprising that a live-animal toxicity assay is the most widely used method for their detection in a regulatory setting. The most common test is a mouse bioassay in which a sample extract is injected into the intraperitoneal cavity, followed by an observation period to determine symptoms and time-to-death, which correlates with the amount of toxin present.

Filter-feeding shellfish can act as a vector for serious poisoning syndromes, that have been named paralytic, diarrhetic, neurotoxic, or amnesic shellfish poisoning (PSP, DSP, NSP, or ASP, respectively).[7] 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 are non-volatile and some are sensitive to pH, oxygen and light. 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. Clinical symptoms of toxicity are described in Table 1.

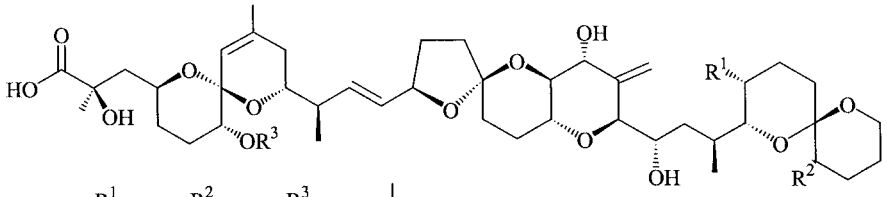
1.1. Paralytic Shellfish Poisoning (PSP)

Paralytic Shellfish Poisoning (PSP) is the most widespread algae-derived shellfish poisoning. The toxins responsible for PSP are a suite of heterocyclic guanidines collectively called saxitoxins, of which there are currently over 29 known congeners (Figure 1).[8] Their structures vary by different combination of hydroxyl and sulfate substitutions at four sites on the molecule. Based on substitutions at R^4, the saxitoxins (STXs) can be subdivided into four groups, the carbamate toxins, sulfocarbamoyl toxins, decarbamoyl, and deoxydecarbamoyl toxins Substitution at R^4 results in substantial changes in toxicity, with the carbamate toxins being the most potent.

A number of dinoflagellate species are known to produce the toxins: *Alexandrium spp.* (formerly *Gonyaulax* or *Protogonyaulax*), *Gymnodinium* and *Pyrodinium*.[9] Some strains of the freshwater blue-green algae *Anabaena* sp.[10] and *Aphanizomenon flos-aquae*,[11−13] also produce saxitoxin and neosaxitoxin.[10−12] Several studies suggest that saxitoxins can be produced autonomously by bacteria isolated from cultures of PSP-producing dinoflagellates.[13−15] In addition, endosymbiotic or cell-associated bacteria may

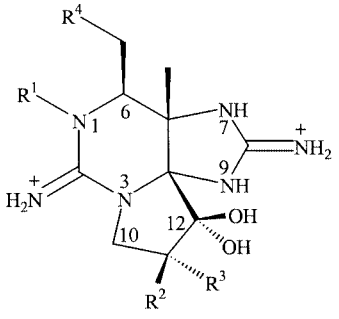
Table 1. Clinical symptoms of toxicity

Syndrome	Symptoms	
	Mild case	***Extreme case***
Paralytic Shellfish Poisoning (PSP)	Within 30 min: tingling and numbness of the perioral area and extremities; a sensation of prickling in fingertips and toes; headache, dizziness, nausea, vomiting, diarrhoea.	Loss of motor control; pronounced respiratory difficulty; choking sensation; death through respiratory paralysis may occur within 2–24 h after ingestion.
Diarrhetic Shellfish Poisoning (DSP)	After 30 min to a few h (seldom more than 12 h): diarrhoea, nausea, vomiting, abdominal pain, general discomfort.	Chronic exposure may promote tumour formation in the digestive system
Amnesic Shellfish Poisoning (ASP)	After 3–5 h: nausea, vomiting, diarrhoea, abdominal cramps.	Decreased reaction to deep pain; permanent loss of short-term memory; dizziness, hallucinations; seizures; disorientation.
Neurotoxic Shellfish Poisoning (NSP)	After 3–6 h: chills, headache, diarrhoea, muscle abdominal cramps; muscle weakness.	Paraesthesia; altered perception of hot and cold; difficulty in breathing, double vision, trouble in talking and swallowing.



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)

Figure 2. Chemical structure of DSP toxins (Okadaic acid group)



STX = saxitoxin
NEO = neosaxitoxin
GTX = gonyautoxin

				Carbamate toxins		N-Sulfocarbamoyl toxins		Decarbamoyl toxins		Deoxydecarbamoyl toxins	
R^1	R^2	R^3	R^4	—O(C=O)NH_2		—O(C=O)$NHSO_3^-$		—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		

Figure 1. Structures of paralytic shellfish toxins

play a role in the production of paralytic shellfish toxins by dinoflagellates.[16]

Saxitoxin binds with high affinity to site 1 on the voltage-dependent sodium channel, inhibiting channel opening.[17] The voltage-dependent sodium channel plays a critical role in neurotransmission at both the neuronal synapses and neuromuscular junctions. The polarity of the STX molecule largely excludes it from traversing the blood-brain barrier, therefore the primary site of STX action in humans is most likely at the neuromuscular junction.

The lethal dose in humans is 1−4 mg of STX equivalents.[18]

1.2. Diarrhetic Shellfish Poisoning (DSP)

The diarrhetic shellfish toxins are a class of acidic polyether toxins produced by dinoflagellates and are responsible

for a human illness, diarrhetic shellfish poisoning, associated with seafood consumption. This toxin class consists of at least eight congeners, including the parent compound, okadaic acid, which was first isolated from the black sponge, *Halichondria okadai*.[19]

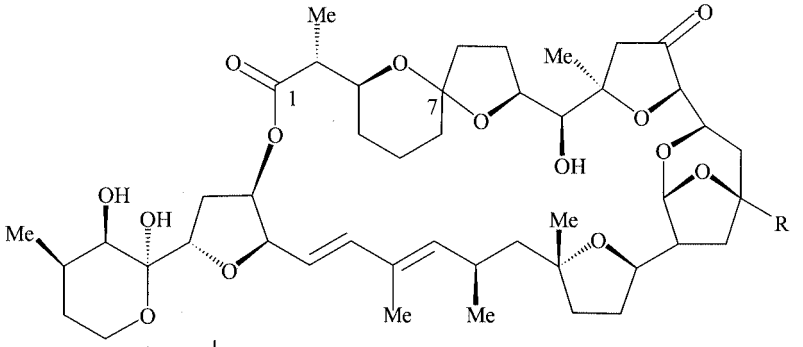
Okadaic acid (OA), dinophysistoxin 1 (DTX1) and dinophysistoxin 2 (DTX2) are the primary congeners involved in shellfish poisoning, with the other congeners believed to be either precursors or shellfish metabolites of the active toxins (Figure 2). DSP is widespread in its distribution, with essentially seasonal occurrence in Europe and Japan. The first incidence of human shellfish-related illness identified as DSP occurred in Japan in the late 1970s, where the dinoflagellate *Dinophysis fortii* was identified as the causative organism, and the toxin responsible was termed dinophysistoxin 1 (DTX1).[19,20]

The OA class toxins are diarrhetics[20,21] and tumor promoters.[22] The mechanism of action underlying these activities is explained mainly by their potent inhibitory action against ser/thr protein phosphatases.[23–25] Inhibitory activity is specific for classes PP2A and PP1, with PP2B being inhibited only at high concentrations and PP2C being insensitive.

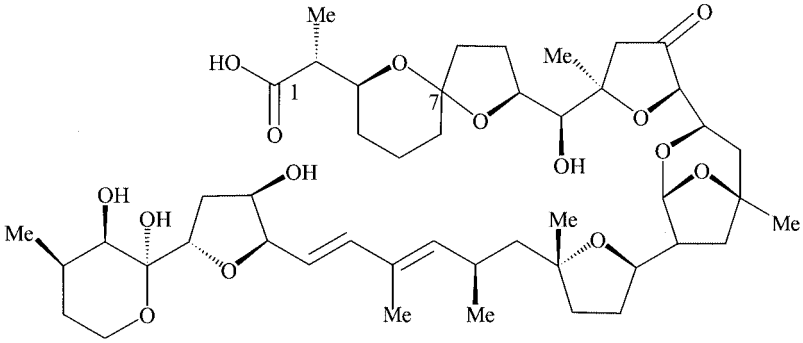
The second group of DSP toxins are named pectenotoxins (PTXs) (Figure 3).[26] Structurally, PTXs resemble OA in molecular weight and in having cyclic ethers and a carboxylic group in the molecule. Unlike in OA, however, the carboxyl moiety in PTX is in the form of a macrocyclic lactone (macrolide). Histopathological investigations in mice injected with PTX2 revealed severe mucosal injuries and fluid accumulation in the small intestine and revealed that it is hepatotoxic and induces rapid necrosis of hepatocytes.[27] In addition, PTXs show a potent cytotoxicity[20,28,29] and probably inhibit actin polymerization.[31]

1.3. Toxins Found in Association with DSP

The low specificity of the mouse bioassay for DSP is a drawback on the one hand, but, on the other hand, can be regarded as advantageous, because it detects all kinds of toxins that are soluble in organic solvents. Toxins that are frequently found in association with DSP are yessotoxins (YTXs), azaspiracids, and spyrolides.



R	C-7	
CH_2OH	*R*	Pectenotoxin 1 (PTX1)
CH_3	*R*	Pectenotoxin 2 (PTX2)
CHO	*R*	Pectenotoxin 3 (PTX3)
CH_2OH	*S*	Pectenotoxin 4 (PTX4)
COOH	*R*	Pectenotoxin 6 (PTX6)
COOH	*S*	Pectenotoxin 7 (PTX7)



C-7	
R	Pectenotoxin 2 seco acid (PTX2SA)
S	7-*epi*-Pectenotoxin 2 seco acid (7-*epi*-PTX2SA)

Figure 3. Structures of pectenotoxins (PTXs) and PTX2-seco acids (PTX2SA)

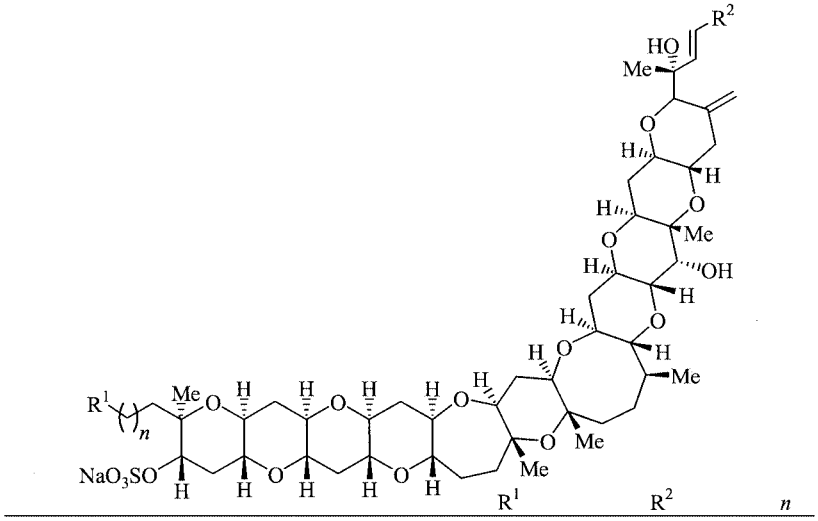
Yessotoxin (YTX) is a ladder-shaped polycyclic ether toxin isolated for the first time from the scallop *Patinopecten yessoensis* (Figure 4).[30] Subsequent studies revealed a number of analogs, as shown in Figure 4.

Further yessotoxin analogs have recently been isolated by our research group from mussels of the Adriatic sea. Their structures are discussed later.

A recent etiological study revealed that the origin of YTX is different from those of OA and dinophysistoxin 1 (DTX1). YTX is produced by the dinoflagellate *Protoceratium reticulatum*,[31] while OA and DTX1 are produced mainly by *Dinophysis* spp.[32] Because of its frequent coexistence with DTXs and PTXs, YTX was tentatively included in the DSP category. However, YTX does not cause diarrhoea and is less toxic by oral administration than OAs and PTXs.[33] It was found to be more than ten times less toxic to mice when administered orally than when injected intraperitoneally. Even at 10 mg/kg body weight, the highest dose ever tested orally, yessotoxin did not kill the mice. Therefore, it can be concluded that YTX is much less hazardous than OA or DTX1 to human health.

Nevertheless, the cell detachment and the lethal effect on infant mice observed in a toxicological study should be borne in mind.[33] In spite of the wealth of data on OA, the mechanism of action 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 intraperitoneal injection of the toxin.[34] An involvement of the nervous system in YTX toxicity can also be hypothesized on the basis of its chemical structure, since brevetoxins and ciguatoxins,[5] both of which are structurally strictly related to YTX, induce poisoning characterized by neurological and cardiovascular symptoms.[37] As for the mechanism of action, by analogy with brevetoxins and ciguatoxins, YTX may act as a depolarizing agent, opening the membrane channels of Na^+-permeable excitable cells and leading to a Na^+ influx.[38] It remains to be established, however, to what extent these toxins can be absorbed by the intestine, and then gain access to target organs.

Azaspiracid (AZA), so named because of its unusual azaspiro ring assembly, is the major toxin of azaspiracid poisoning (AZP).[35] In addition to AZA, four analogs − AZA2 to AZA5 − have been isolated and their structures determined, as shown in Figure 5.[36,37] The symptoms observed in the patients included nausea, vomiting, severe diarrhoea and stomach cramps and thus resembled those of diarrhetic shellfish poisoning (DSP). However, mouse symptoms induced by intraperitoneal injection of acetone extracts of mussel hepatopancreas were distinctly different from those normally associated with DSP toxins, showing prominent neurological symptoms, such as respiratory diffi-



	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

Figure 4. Structure of yessotoxins (YTXs)

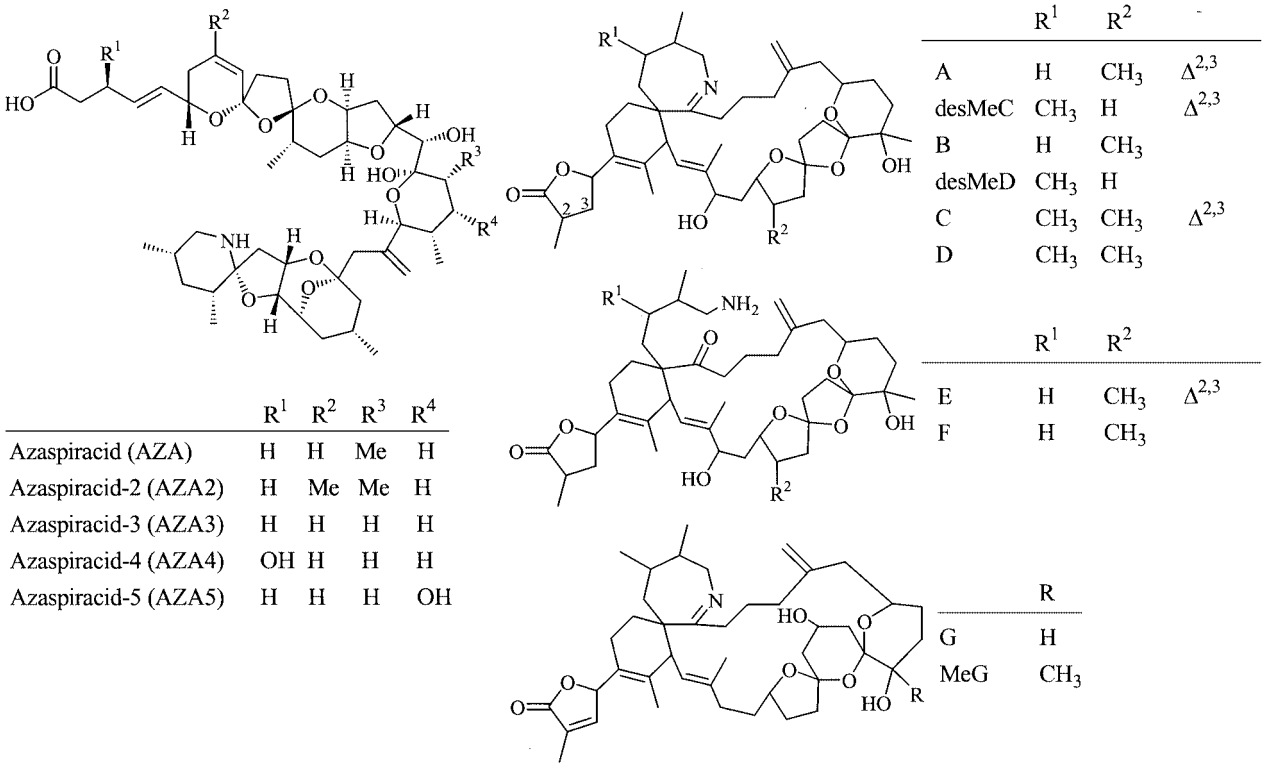


Figure 5. Other toxins isolated from shellfish

culties, spasms, paralysis of the limbs and death within 20 min at higher doses.[38]

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 (Figure 5).[39,40] These "fast acting toxins" cause rapid death upon intraperitoneal injection into mice, and also have a high oral potency with apparent neurotoxic symptomology, although the mode of action is currently unknown. The symptoms include piloerection, abdominal muscle spasms, hyperextensions 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, and occurrence in multiple shellfish species) strongly suggested a planktonic source.[41] 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*).[46] Various spirolides were detected in fractions of planktonic material from Nova Scotian aquaculture sites by liquid chromatography-mass spectrometry (LC-MS) analyses. In particular, the dinoflagellate *Alexandrium ostenfeldii* was shown to be the producer organism.[47]

1.4. Neurotoxic Shellfish Poisoning (NSP)

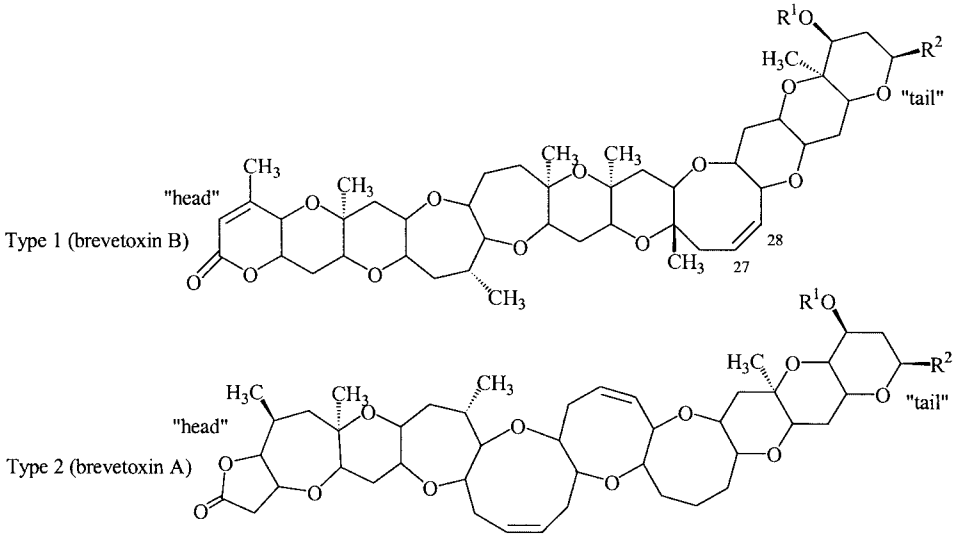
The occurrence of neurotoxic shellfish poisoning has historically been limited to the west coast of Florida, where blooms of the dinoflagellate *Gymnodinium breve* initiate offshore and are subsequently carried inshore by wind and current conditions.[42] In 1993, an unprecedented outbreak of shellfish toxicity in New Zealand resulted in the identification of additional *Gymnodinium* species which produce NSP-like toxins.[43] 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.[50−52]

The toxins responsible for NSP are a suite of ladder-like polycyclic ether toxins collectively called brevetoxins (Figure 6). It has been demonstrated that the toxins produced by the dinoflagellate *G. breve* are biochemically modified when accumulated in shellfish.[44−46]

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.[47] 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.

1.5. Amnesic Shellfish Poisoning (ASP)

Amnesic shellfish poisoning is the only shellfish poisoning produced by a diatom and is currently limited in its distribution to North America. The toxin responsible for



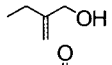
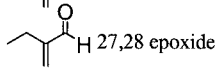
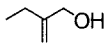
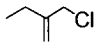
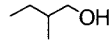
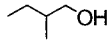
Toxin	Type	R^1	R^2
PbTx-1	2	H	
PbTx-2	1	H	
PbTx-3	1	H	
PbTx-5	1	$COCH_3$	
PbTx-6	1	H	 27,28 epoxide
PbTx-7	2	H	
PbTx-8	1	H	
PbTx-9	1	H	
PbTx-10	2	H	

Figure 6. Structures of brevetoxins

ASP is domoic acid (Figure 7), whose source was found to be the diatom *Pseudo-nitzschia multiseries* (formerly known as *Nitzschia pungens*).[48,49]

Domoic acid is a water-soluble tricarboxylic amino acid which acts as an analog of the neurotransmitter glutamate and is a potent glutamate receptor agonist. Several congeners of domoic acid have been identified so far, of which three geometrical isomers − isodomoic acids D, E, and F − and the C5′-diastereomer are found in small amounts in both the diatom and in shellfish tissue (Figure 7).[50,51]

Domoic acid binds with high affinity to both kainate and AMPA subtypes of the glutamate receptor.[52] Persistent activation of the kainate glutamate receptor results in greatly elevated intracellular Ca^{2+}.[53] 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.[54]

2. Occurrence of DSP Toxins in Phytoplankton and Mussels from the Northwestern Adriatic Sea

The northwestern coasts of the Adriatic sea, a commercially very important area in Italy responsible for about 90% of national mussel production, have repeatedly suffered from harmful algal blooms and shellfish poisonings due to consumption of toxic mussels.[55,56] The blooms have

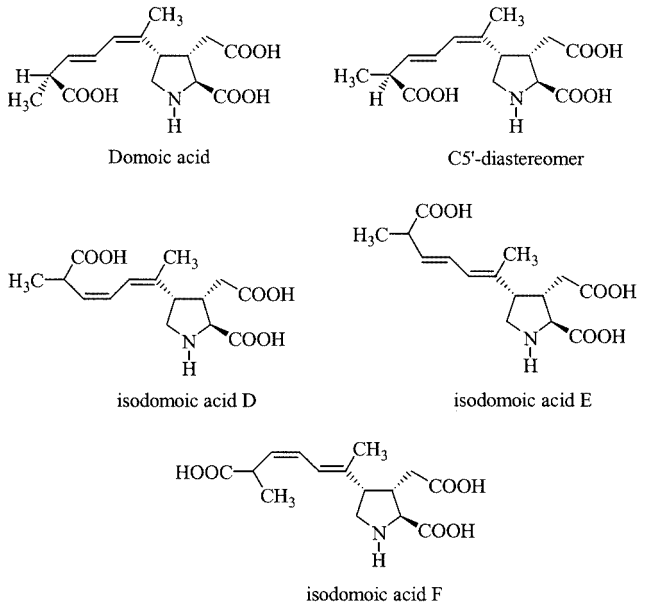


Figure 7. Chemical structures of domoic acid and some of its congeners

occurred most frequently in the coastal waters of Emilia Romagna, but sometimes they have covered much wider areas along the north-western Adriatic coast.

Therefore, a survey of the toxic and potentially toxic algae in the Adriatic sea and chemical research on the toxins responsible for toxicity of shellfish have been carried out in the last decade. These studies have highlighted that the main phycotoxins responsible for contamination of Adriatic molluscs fall into the class of DSP toxins. DSP outbreaks associated with harmful algal blooms were recognized as a problem only in 1989, when some cases of seafood poisoning were associated with the simultaneous presence of known producers of DSP toxins, such as *Dinophysis fortii*, *D. sacculus* and *D. tripos*, both in seawater and in the hepatopancreas of mussels.[57] Unfortunately, DSP outbreaks associated with blooms of harmful microalgae have occurred in the Adriatic sea with alarming frequency since then, extending over the coastal areas of Marche, Abruzzo, Veneto and Friuli-Venezia Giulia.

In order to prevent or minimize such damage, continuous monitoring of the toxicity of shellfish and structural elucidation of the causative toxins are essential. The mouse assay mentioned above is the most widely used method for their detection. It is rapid but poorly reproducible, and sometimes gives false positives mostly due to the presence of high levels of fatty acids. Its main drawback is, however, the lack of information of toxin profiles.

Based on these arguments, a research program biased towards instrumental analysis was initiated in Italy in 1990 in order to carefully investigate DSP contamination of mussels of the Adriatic sea. A number of toxic samples of shellfish collected along the Emilia Romagna coasts in correspondence to the highest level of toxicity have been analysed.

2.1. Isolation of Known Marine Biotoxins from Adriatic Mussels

The toxin profiles in Adriatic mussels have changed completely in the last decade. Initially, DSP phenomena were related to the presence of okadaic acid (OA)[58] in shellfish and to high population densities of *Dinophysis* spp. in seawater. The identity of OA was confirmed by our group through isolation and NMR spectroscopic identification.[58] This result was the first strong evidence for the presence of DSP toxins in mussels cultivated along the Italian coast. Later, the presence of DTX-1 was also shown by LC-MS analysis.[59] In 1995, yessotoxin (YTX) appeared for the first time in extracts of toxic Adriatic mussels together with comparable amounts of OA.[60] Since then, its role in mussel-related poisonings has become dominant. Besides yessotoxin, a number of YTX analogs, such as homoyessotoxin (homoYTX),[61] 45-hydroxyyessotoxin (45-OHYTX),[62] and 45-hydroxyhomoyessotoxin (45-OHhomoYTX),[61] were also isolated from the mussel *Mytilus galloprovincialis* (see Figure 4) and characterized by comparison of their chromatographic and spectral properties with those reported in the literature.

2.2. Isolation and Structural Determination of New Yessotoxin Analogues

During the course of our studies, we succeeded in the isolation and structural determination of several new analogs of YTX from the hepatopancreas of mussels of the Adriatic sea, such as adriatoxin (ATX),[63] carboxyyessotoxin (COOHYTX),[64] carboxyhomoyessotoxin (COOHhomoYTX),[65] and 42,43,44,45,46,47,55-heptanor-41-oxohomoYTX (noroxohomoYTX)[66] (Figure 8). All the new analogs were isolated in pure forms. On account of the very limited amount of the isolated compounds (a few hundreds of micrograms, as a rule) structural determination studies had to be carried out with just a few spectroscopic data, such as those provided by one- and two-dimensional ^{1}H NMR spectroscopy and MS/MS experiments.

ATX was isolated from the digestive glands of DSP-infested mussels *M. galloprovincialis*, collected at the beginning of July 1997.[63] Its structural elucidation was mainly carried out by comparing the ^{1}H NMR spectroscopic data of YTX and ATX. A preliminary analysis of the ^{1}H NMR spectrum of ATX showed a close resemblance to that of YTX, but the lack of the characteristic signals of the side chain at C-40 was immediately evident. A careful analysis of the complex ^{1}H-^{1}H COSY spectrum allowed us to ascertain that the two analogs share the same partial structure from C-1 to C-32, and that ATX lacks the entire side chain and the ring M. The location of the third sulfate group, whose presence was indicated by the fragmentation pattern in the mass spectrum, as well as that of an additional hydroxyl group, whose presence was implied by the molecular formula, were deduced by the analysis of the chemical shift values of the oxymethine protons in the ring L.

All ether rings in ATX were found to be *trans*-fused, as in the case of YTX, on the basis of the typical coupling

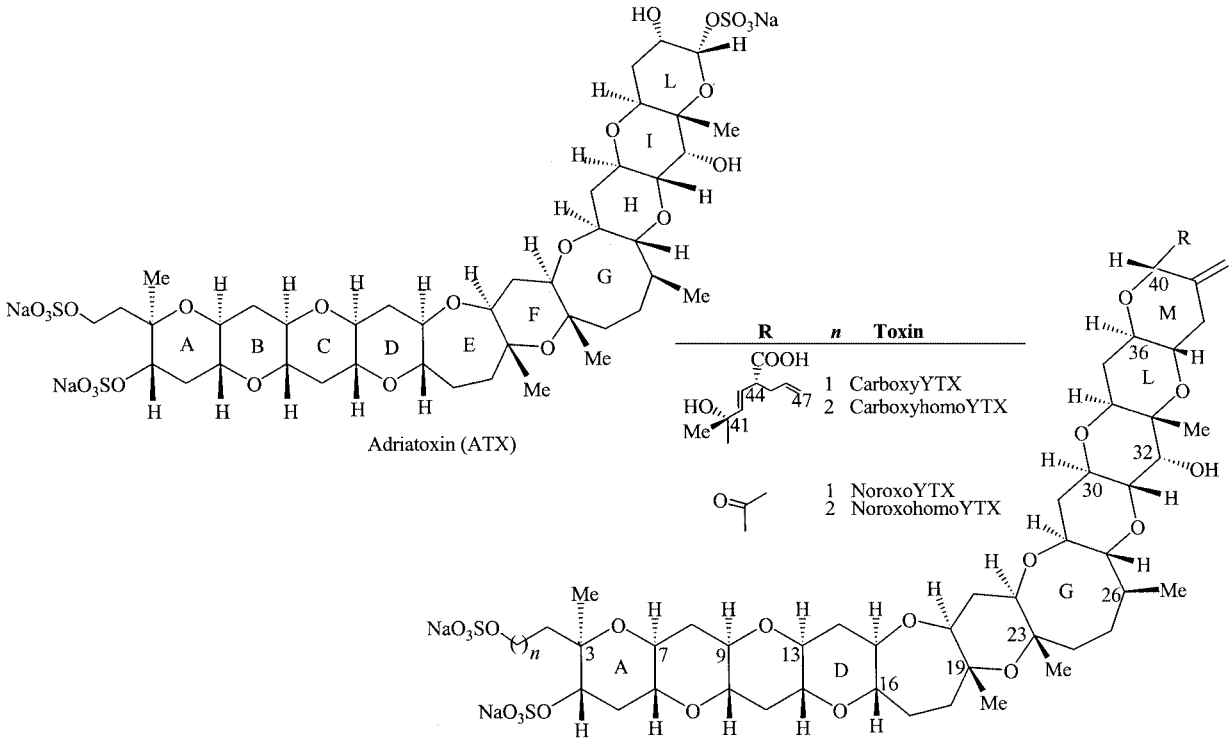


Figure 8. New YTX analogs from Adriatic mussels

constants (9−10 Hz) of angular protons for antiperiplanar substitution on oxy-bound carbons. The observed NOEs between methyls or hydrogens located on angular carbon atoms on both sides of the ether oxygens also supported the *trans*-fusion of the rings, and allowed us to determine the relative stereostructure of the fused rings.

As for the structure elucidation of COOHYTX, a detailed analysis of the COSY and HOHAHA spectra allowed us to identify the spin systems corresponding to the entire polycyclic skeleton of YTX.[64] Therefore, the structural difference between the two analogs had to concern the eastern side-chain. Analysis of the mass spectrum suggested the presence of a carboxylic function in the molecule, which was positioned at C-44 by identification of the side-chain spin system. The presence of a carboxylic group in the molecule was proved by its transformation into an amide by the method of Nagai and Kusumi that we applied for the determination of the absolute configuration at C-44.[67]

COOHHomoYTX was found to be 14 mass units larger than COOHYTX.[65] The ^{1}H-^{1}H COSY spectrum allowed us to identify an extra methylene located at position 2a. The ^{1}H connectivities from H_2−1 to H_2−2a were easily determined from the ^{1}H-^{1}H COSY and HOHAHA spectra and were identical with those of homoYTX.[61] ROE correlations observed by the ROESY spectra of COOHhomoYTX were also identical to those observed for COOHYTX, thus indicating that they have the same stereostructure.

The structure of COOHhomoYTX was further tested for collision-induced dissociation (CID) by negative ion MALDI MS/MS experiments carried out on the [M − Na]$^-$ ion (*m*/*z* = 1209) of COOHhomoYTX. Fragment ions were generated by bond cleavage at the sites characteristic of ether rings, as established in previous experiments on yessotoxin[68] and maitotoxin,[69] in which it was demonstrated that the fused polycyclic ethers of yessotoxin and maitotoxin give rise to characteristic fragmentations from which the sizes of the ether rings and/or substituents on the rings could be deduced directly. All prominent product ions in the MS/MS spectrum of COOHhomoYTX were 14 mass units larger than the corresponding fragment ions observed in the MS/MS spectrum of COOHYTX, thus confirming, once again, that an extra methylene should be present on the sulfated side chain.

NoroxohomoYTX was isolated from the hepatopancreas of toxic mussels *M. galloprovincialis* collected in October 1998 from one sampling site located along the Emilia Romagna coasts.[66]

A preliminary analysis of the ^{1}H NMR spectrum of noroxohomoYTX showed a close resemblance to those of other YTXs, strongly suggesting that it possessed the same basic polycyclic ether skeleton as YTXs. There were two basic differences, however, in the ^{1}H NMR spectrum of NoroxohomoYTX: first of all, the lack of the characteristic signals of the side chain at C-40 in the olefinic region of spectrum, and secondly the presence of an additional methyl signal at relatively low field (δ = 2.22 ppm).

The polycyclic skeletal structure was mainly confirmed on the basis of homonuclear 2D NMR spectroscopic data obtained from COSY and HOHAHA experiments. In addition, the analysis of these spectroscopic data suggested that the toxin was of the homoyessotoxin type, namely with

an extra methylene added to the structure of YTX in the western side-chain of the molecule.

On the basis of the mass spectrum and ^{1}H NMR spectroscopic data a terminal acetyl functionality was positioned at C-40. Negative-ion FAB MS/MS provided essential information to confirm the structure. The ROESY spectrum revealed the relative stereochemistry of the molecule to be identical to that of YTX,[60] as well as to that of all other YTX analogs so far isolated, as the same key ROE correlations were observed.[61−66] In addition, the ROE correlations between H-36 and Me-42 indicated the diaxial relationship between C-41 and H-36, thus defining the stereochemistry at C-40.

Two other new analogs from mussels of the Northern Adriatic sea were identified through LC-MS analysis, by applying the method developed by us for the determination of YTXs.[70] Together with rapid detection of known compounds at parts-per-billion levels, as reported below, the method allows us to highlight the possible presence of new analogs, thus representing a key potentiality to discover new YTX analogs useful for structure-bioactivity relationship studies.

LC-MS data on the toxic mixture obtained from *M. galloprovincialis* collected in 1998, besides the already known composition,[66] consisting of homoYTX, 45-OHhomoYTX, carboxyhomoYTX, and noroxohomoYTX, revealed the presence of an unknown peak. An inspection of the MS/MS spectrum of this peak revealed the typical fragmentation pattern of the backbone skeleton of yessotoxin, but no sulfonate loss was observed for this peak, thus suggesting the presence of only one sulfate ester group in the molecule. Taken together these data suggested that the peak under investigation was due to a desulfoYTX, the only uncertainty being in the desulfated position.[70] NMR experiments are required to unambiguously assign 1-desulfo-[71] or the alternative 4-desulfo-YTX to the above peak. However, to the best of our knowledge, this is the first report of a desulfoYTX derivative from Italian mussels.

The LC-MS analysis of DSP-infested mussels (*M. galloprovincialis*) collected in June 2001 produced a similar result. Besides some already known yessotoxins, whose retention times and mass spectra were perfectly coincident when compared with those of individual reference samples injected under the same experimental conditions, the total ion current (TIC) chromatogram showed a significant chromatographic peak of a potentially new analog. The associated full-scan mass spectrum displayed a signal at m/z = 1047.1 which could not be found for any of the already known YTXs.

In order to determine the structure of this new compound, LC-MS/MS experiments were carried out. The characteristic fragmentations of the polycyclic backbone skeleton of yessotoxin were observed, thus suggesting that the unknown compound belonged to the YTX series. Furthermore, the loss of 42 mass units from the $[M - H - SO_3]^-$ ion, which gives rise to the ion peak at m/z = 925.3, was indicative of the loss of $CH_2{=}C{=}O$, which suggested that the eastern side-chain was constituted by a $-COCH_3$ moiety.

The emerging structural features were suggestive of the new compound being 42,43,44,45,46,47,55-heptanor-41-oxoyessotoxin (noroxoYTX),[72] the homologue of the YTX series of the noroxohomoYTX[66] that we had previously isolated and fully characterized. This hypothesis was supported by a comparison of the chromatographic and mass-spectral properties of the involved compounds. MS/MS spectra of the two compounds appeared to be almost superimposable provided they were shifted by 14 mass units.

These analogs represent new additions to the class of yessotoxins, and seem to be peculiar to our seas, since they have not been reported until now in any other country.

In spite of the wealth of data on OA, the molecular mechanism underlying the toxicity of YTXs is far from being thoroughly understood. Indeed, very limited data (essentially confined to histopathological evaluations of a few organs) are available regarding the effects of yessotoxin on cellular systems while practically nothing is known about the toxicology of its analogs. The scarcity of toxicological data of these compounds and of YTX itself may be quite dangerous considering the key role that these toxins play in the poisoning of Adriatic mussels. The chief obstacle facing acute and chronic risk associated with YTX-contaminated seafood is the limited availability of the toxins for toxicological studies, as well as the lack of a rapid and efficient analytical method to follow the variation of the toxin profiles in molluscs.

With the aim of overcoming these issues, we recently developed an analytical procedure based on LC-MS techniques for the rapid detection of all YTXs so far isolated.[70] This method could be employed not only for the analysis of toxic mussels, but also for the screening of the algal cultures in order to select the producer organisms of the compounds to be submitted to toxicological studies. The following two sections report the results obtained in this regard.

2.3. LC-MS Method for YTXs Determination

Toxins in a shellfish extract are usually present in extremely low amounts. Thus, isolation of the pure compound responsible for toxicity is the first critical stage of the study. In order to obtain an amount of pure toxin sufficient for NMR analysis, a huge batch of shellfish (usually 200−300 kg) must be processed. The work up deals with the isolation, in some cases, of a few hundred micrograms of toxins from a complex mixture of the main metabolites.

Although the NMR method for toxin analysis has considerable utility, there is still a need for the further development of analytical methods which are characterized by short analysis times, high sensitivities, and specific detection of individual components.

To partially overcome these problems and to speed up the acquisition of results, a combination of liquid chromatography and mass spectrometry (LC-MS) was considered. This is a valuable tool that is able to afford information on the molecular formula as well as structural information through tandem MS experiments. At the same time it is

Table 2. Retention times and main ions observed in full scan MS and MS/MS experiments for YTX and its derivatives

Toxin	t_R	Rel t_R [a]	$[M - 2Na + H]^-$	$[M - 2Na - SO_3 + H]^-$ [b]
YTX	8.98	0.86	1141.5	1061.5
HomoYTX	9.01	0.86	1155.5	1075.5
45-OH YTX	8.73	0.84	1157.5	1077.5
45-OH HomoYTX	8.76	0.84	1171.5	1091.5
CarboxyYTX	8.18	0.79	1173.5	1093.5
CarboxyhomoYTX	8.21	0.79	1187.5	1107.5
42,43,44,45,46,47,55-Heptanor-41-oxohomoYTX	8.04	0.77	1061.5	981.5

[a] Retention times are relative to okadaic acid eluting at 10.38 min under the same LC-MS conditions. Rel $t_R = t_R(X)/t_R(OA)$. [b] Fragment ions obtained in LC-MS/MS experiments carried out at a collision energy of 35% using $[M - 2Na + H]^-$ as the precursor ion.

universal, selective and highly sensitive. The advantage of this approach is the possibility of detecting intact, underivatized toxins and related compounds in relatively crude extracts of both shellfish and plankton samples. In the case of Adriatic toxins, this technique was extremely appropriate, especially considering that the most common analytical methods for specific detection of DSPs require derivatization of each toxin with an appropriate auxiliary reagent for fluorescence labeling followed by HPLC analysis. Unfortunately, there is no reagent which fits all DSP toxins. 9-Anthryldiazomethane (ADAM) is used for OA, DTXs, and PTXs,[73] while YTXs are derivatized with the dienophile reagent 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD).[74] It has to be noted, however, that for the application of the latter method, the presence of a conjugated diene functionality in the side chain of YTX-like compounds is a prerequisite. Thus, it is not reliable for the detection of those derivatives that lack a conjugated diene functionality in the molecule, such as the Adriatic analogs noroxohomoYTX, carboxyYTX, carboxyhomoYTX, and adriatoxin. Therefore, we tested the suitability of the LC-MS method developed by Quilliam[75] for detection of most lipophilic toxins to separate and univocally detect all yessotoxins isolated so far, especially in the presence of okadaic acid which sometimes coexists in shellfish.[70] For this purpose standard solutions of YTX and OA at known concentration as well as solutions of a number of YTX analogs from mussels of the North Adriatic sea were employed. The expected toxins were easily detected and eluted at the retention times summarized in Table 2.

Figure 9 shows the reversed phase gradient elution LC-MS analysis of a wide range of yessotoxins in a blend of contaminated mussel tissue extracts with added OA standard. The YTXs were eluted 1.3−2.3 min before OA, which can be used as the reference peak.

As shown in Figure 9, the peaks of most toxins overlapped and attempts to improve the chromatographic separation by varying the mobile-phase composition were unsuccessful. However, toxins with different molecular masses were monitored by extracted ion chromatograms (XIC) of the $[M - 2Na + H]^-$ ions, thus allowing their unambiguous identification even if they were chromatographically unresolved.

MS/MS experiments were carried out for further confirmation. As shown in Figure 10, at a collision energy (CE) of 35% all investigated disulfated YTXs give an intensive product ion at $[M - 2Na - 80 + H]^-$ corresponding to loss of SO_3. As expected from the high selectivity of the MS/MS technique, no interference with the analytes was observed in the product-ion chromatograms.

Further MS/MS experiments were carried out by increasing the collision energy to 45%. The resulting MS/MS spectra of YTX and homoYTX are shown in Figure 11. The fragmentation patterns of the two molecules appear very similar, with all the peaks differing by only 14 mass units in the two spectra (Figure 11). It should be noted that the region of the spectra below $m/z = 950$ contains ions relative to the fragmentation of the backbone skeleton. Since fragmentation occurs at specific sites of the ether rings, the same daughter ions were observed for all YTX- or homoYTX-like compounds that we analysed. Consequently, this part of the MS/MS spectrum can be considered as a fingerprint, thus allowing YTX and homoYTX analogs to be distinguished from each other.

This technique allowed determination of OA and all YTX and HomoYTX derivatives so far isolated in a single chromatographic run of 25 min and was shown to be both selective and sensitive, with a detection limit of 68.4 pg for YTX. The above approach seems to be appropriate for unambiguous identification of all YTXs and represents the first step toward their quantitative determination. For quantification of YTX analogs, preparation of their appropriate standards is required; unfortunately the low purity and the poor amount of available material prevented us from performing quantitative studies.

On the basis of the above evidence, the proposed LC-MS method represents a key tool for natural products chemists to discover YTX analogs and provides a more comprehensive approach than the currently used HPLC analysis on fluorescence-labeled derivatives.[74] Secondly, as described in the previous section for identification of desulfoYTX and noroxoYTX, this technique can be usefully employed for structure elucidation of new toxins whenever great structural analogies occur between the toxins under investigation and known compounds. MS/MS spectra, interpreted by reference to model compounds, provide substantial structure information. Thus, effective structural hypothesis can be

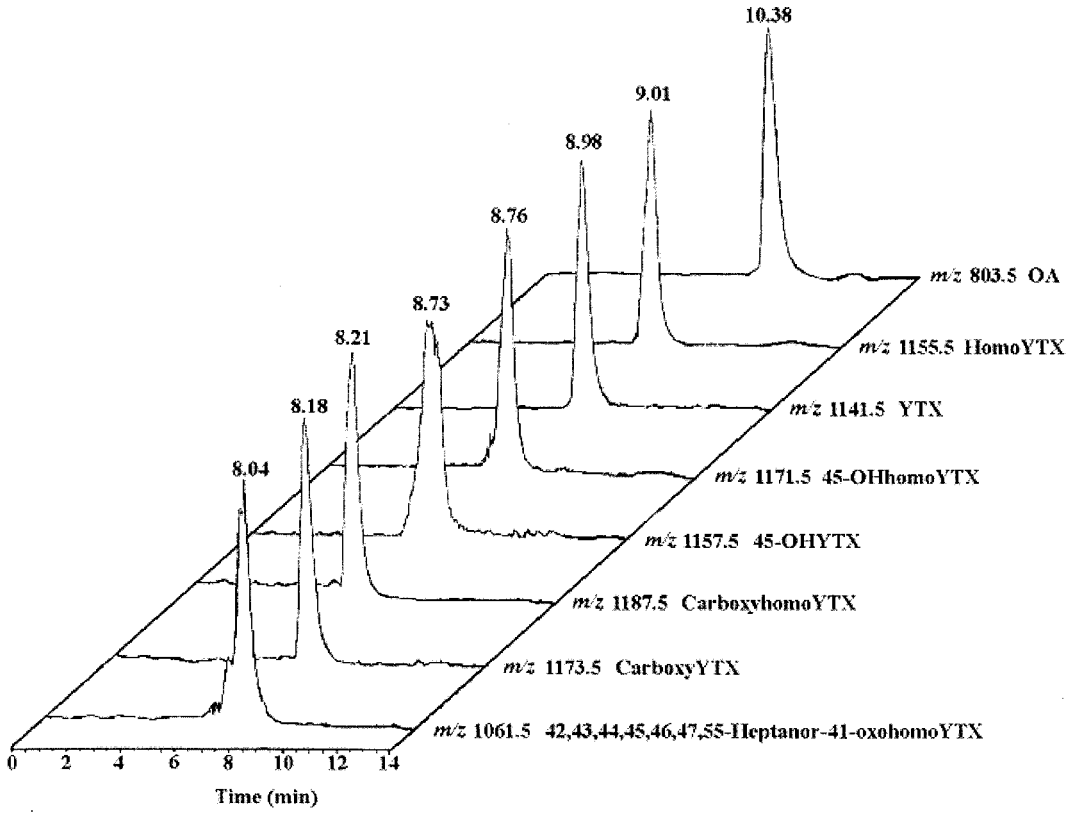


Figure 9. LC-ESI (negative ion) ion trap MS analysis of a wide range of yessotoxins in a blend of mussel tissue extract with added OA standard; selected monitoring of yessotoxins with different molecular masses was carried out by extracted ion chromatograms (XIC) of the $[M - 2Na + H]^-$ ions; for OA the XIC is relative to the $[M - H]^-$ ion

advanced even when full structural elucidation of the new toxins by NMR spectroscopy is hampered by the limited amounts of material available.

2.4. An Adriatic Strain of *P. reticulatum* as Producer of Some New YTX Analogs

The 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, Yasumoto et al. identified the marine dinoflagellate *Protoceratium reticulatum*, collected in New Zealand, as the producer organism of yessotoxin.[31] YTX was later detected in Adriatic *P. reticulatum*[76] and, together with 45,46,47-trinoryessotoxin, in strains of the same species collected in Japan.[77] In 1999, Draisci et al. reported the presence of YTX and homoYTX in *Gonyaulax polyedra* collected in the Northwestern Adriatic sea.[78] However, the origin of all the other YTX analogs was still unknown, thus raising the question as to 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* (*Gonyaulax grindley*) collected along the Cesenatico coast (Emilia Romagna, Italy) in June 2001 was investigated.[79] A detailed toxin profile of this strain was obtained by high performance liquid chromatography coupled with electrospray ionization ion trap mass spectrometry (HPLC-ESIMS).

In order to investigate the presence of YTX analogs in the crude extract, extracted ion chromatograms were obtained for all known YTX derivatives isolated so far. However, the high background signals in some ion traces for some other YTX analogs prevented us from detecting unambiguously even some of the known derivatives. Thus a clean-up step by solid phase extraction (SPE) was carried out in order to reduce interference and matrix effect.

All SPE eluates were analyzed by LC-MS and the propanol/water eluate was shown to contain noroxoYTX, carboxyYTX, 45-OHYTX, YTX, and homoYTX. A quantitative analysis was also carried out. Each toxin was quantitatively determined in shellfish tissue by direct comparison with individual standard solutions of yessotoxin at similar concentrations injected under the same experimental conditions. Thus, in this study, yessotoxin was the only toxin which was accurately quantitated. Instead, quantitation of YTX analogs was carried out by assuming that they had the same molar response as YTX.

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

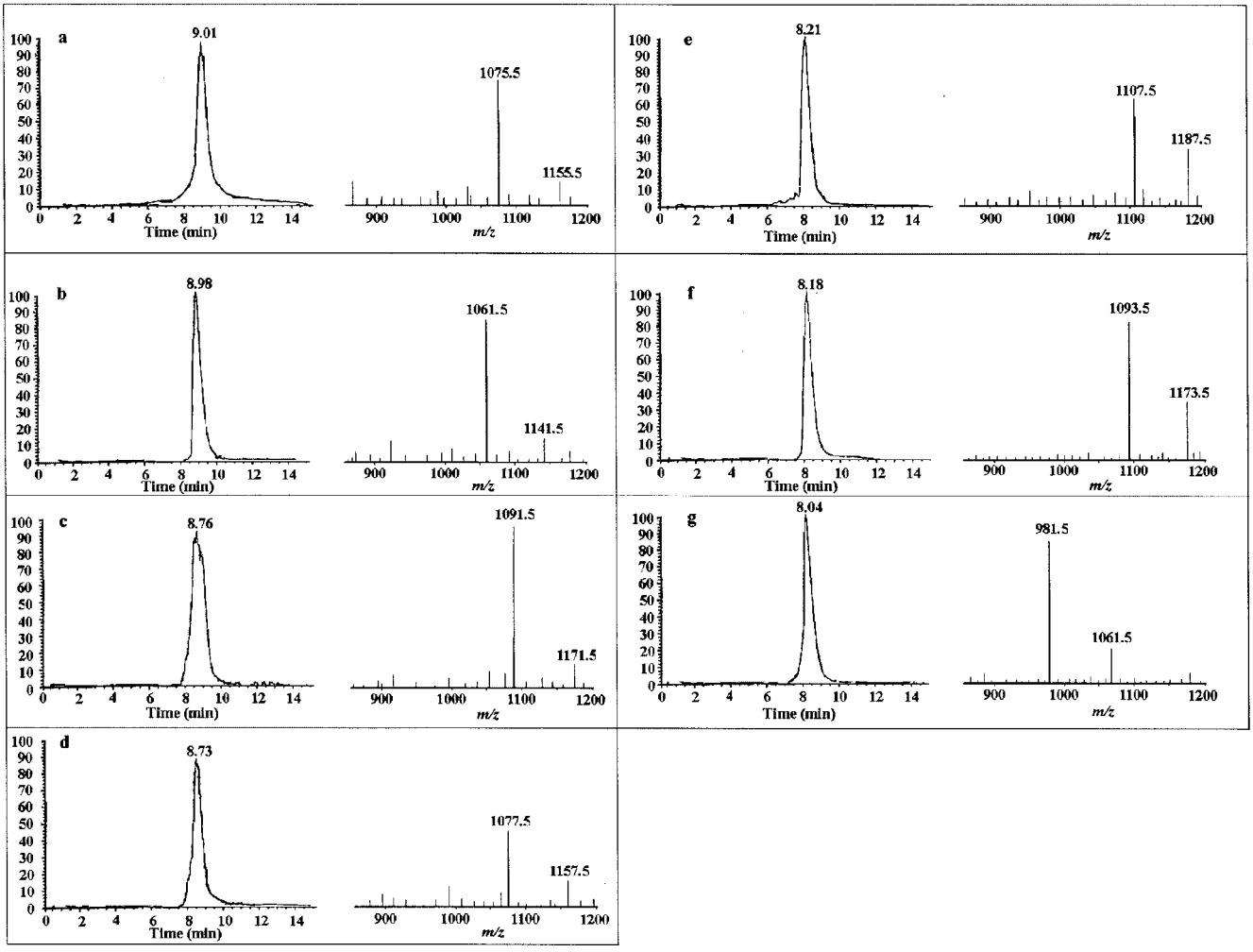


Figure 10. Product-ion chromatograms and relevant MS/MS spectra of homoYTX (a), YTX (b), 45-OHhomoYTX (c), 45-OHYTX (d), carboxyhomoYTX (e), carboxyYTX (f), and 42,43,44,45,46,47,55-heptanor-41-oxohomoYTX (g), using $[M - 2Na + H]^-$ as the precursor ion and a collision energy of 35%; a fragment ion $[M - 2Na - 80 + H]^-$ corresponding to loss of an SO_3 group is observed for each derivative

of the two homologue series. Furthermore, these findings indicate that most of the Adriatic YTX derivatives are true products of the dinoflagellate and are not derived from metabolic conversion of YTX in shellfish. Finally, it has to be noted that the toxin profile of *P. reticulatum* revealed by LC-MS analysis appeared to be more complex than that previously determined by other workers.[31,76–78] This is probably due to the different analytical methods employed, although a different toxin productivity in different strains cannot be ruled out.

3. Cytotoxins from Contaminated Adriatic Blue Mussels

During our investigation on toxic Adriatic mussels we also isolated two new types of toxins – oxazinins and chlorosulfolipis – that are completely different in structure from the polyether DSP toxins isolated so far, but can still represent a further threat to public health due to their cytotoxic activity.

3.1. Oxazinins

A chemical investigation of the digestive glands of *M. galloprovincialis* from the North Adriatic sea led to the isolation of three novel compounds, oxazinin-1, oxazinin-2 and oxazinin-3 (Figure 12).[80] They are characterized by unique structural features, which, to the best of our knowledge, have not been found in any other naturally occurring compound.

The determination of the structure of the new molecules was established by extensive NMR spectroscopy, including 2D NMR experiments, such as ^1H-^1H COSY, HOHAHA, HMQC, and HMBC, which allowed us to identify and then combine all the isolated spin systems. NMR spectroscopic data were also conclusive to assign the relative stereochemistry of the new compounds. Additional evidence to con-

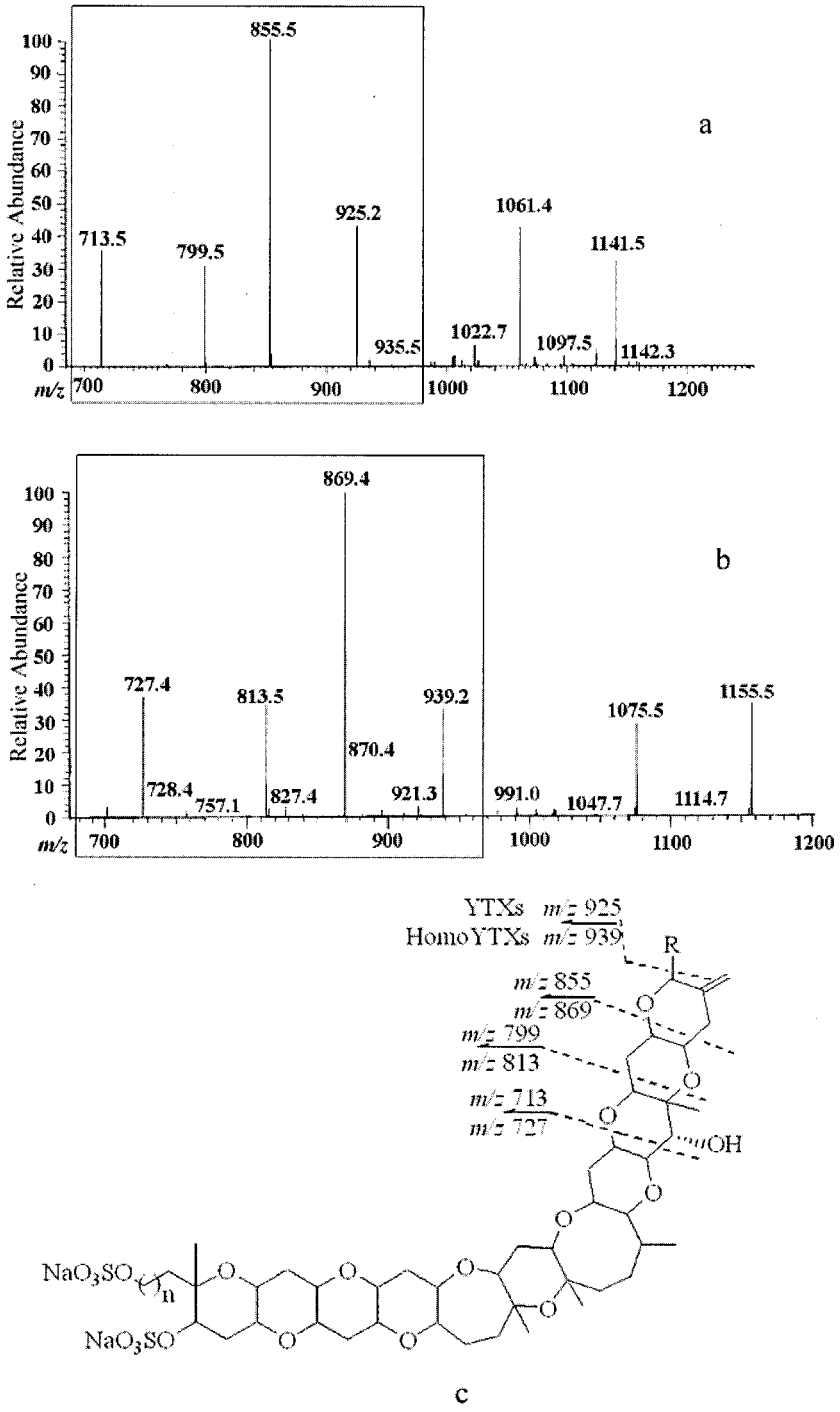


Figure 11. (a) MS/MS spectrum at a collision energy of 45% for YTX; the $[M - 2Na + H]^-$ ion at m/z = 1141.1 was used as the precursor ion; the fragment ion at m/z = 1061.4 is due to loss of SO3; characteristic ions relative to fragmentation of the polycyclic backbone skeleton for all YTX-like compounds are contained in the frame; (b) MS/MS spectrum at a collision energy of 45% for homoYTX; the $[M - 2Na + H]^-$ ion at m/z = 1155.3 was used as the precursor ion; the fragment ion at m/z = 1075.5 is due to loss of SO_3; the characteristic ions relative to fragmentation of the polycyclic backbone skeleton for all homoYTX-like compounds are contained in the frame; (c) characteristic fragment ions observed for YTX- and homoYTX-like compounds in the mass range 700−1200 a.m.u. of tandem mass spectra; all the fragmentations were accompanied by loss of SO_3, meaning that the fragment ions linked one of the sulfate esters

firm the relative stereochemistry suggested by the NMR spectroscopic data was obtained by molecular mechanics and dynamics calculations performed in the CHARM force field. The absolute stereochemistry of oxazinin-1 has also been assigned by application of the 9-AMA (9-anthrylmethoxyacetic acid) shift-correlation method for β-chiral primary alcohols and preparatory molecular mechanics calculations.[81]

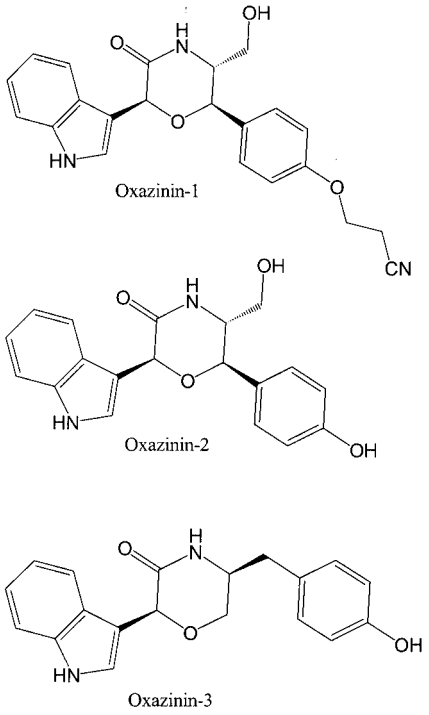


Figure 12. Structure of oxazinins

The new compounds were tested for cytotoxic activity and oxazinin-1 was shown to inhibit the growth of WEHI 164 and J774 cell lines in vitro.

3.2. Chlorosulfolipids

Very recently we have reported the isolation of a new class of cytotoxins containing polychlorinated sulfolipids from *M. galloprovincialis*.

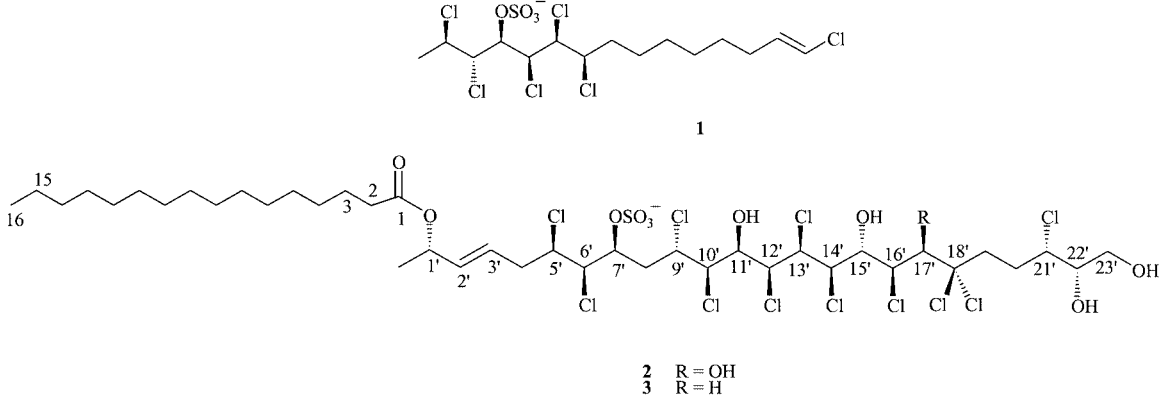
Chlorosulfolipids are unusual naturally occurring compounds which were first isolated at the end of the 1960s from some species of freshwater microalgae such as *Ochromonas danica* and *Ochromonas malhamensis*, where they constitute 15% of the total lipids.[82] They were later also detected in three species of Xanthophyceae, *Tribonema aequale*,[83] *Botrydium granulatum* and *Monodus subterraneus* and in two species of Chlorophyta, *Elakatothrix viridis* and *Zygnema*.[84] In 1979 Mercer and Davies examined about 30 species chosen from a wide range of algal classes and orders for their chlorosulfolipid content. They found that, in marked contrast to the freshwater algae, the chlorosulfolipid level in marine species was usually not detectable or at least an order of magnitude lower than that of the lowest freshwater species examined.[85]

Despite the quite large number of algae examined, just a few chlorosulfolipids were structurally characterized, and the stereochemical details were never studied. On the basis of the available structural data, algal chlorosulfolipids were divided into two different series: polychlorodocosane 1,14-disulfates and polychlorotetracosane 1,15-disulfates, with the number of chlorine atoms ranging from one 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.[86]

During our investigation on toxic Adriatic mussels we succeeded in the isolation of three unique cytotoxic compounds (Figure 13), which can be included in the class of chlorosulfolipids even though, structurally, they are quite different from the previously reported ones. Interestingly, the presence of chlorosulfolipids in Adriatic mussels should not be considered accidental, as we have been reporting them since 1998. It is likely that these cytotoxic compounds originate, just like typical biotoxins, from harmful microalgae that are accumulated by the continuous filter-feeding process of mussels. The first isolated compound is a hexachloromonosulfate (**1**),[87] while **2**[88] and **3**,[89] characterized very recently, contain eleven chlorines and a fatty acid acyl moiety.

The structural determination of the new cytotoxins, including their absolute stereochemistry, was performed by extensive NMR analysis and molecular mechanics and dynamics calculations. Particularly interesting was the stereostructural determination of the two latter highly functionalized compounds. A more detailed description of the structural identification of **3** is given below.

The chromatographic behavior and preliminary evaluation of the NMR spectroscopic data of **3** allowed us to recognize its close relationship with compound **2** previously isolated from the same source. This was further supported by the negative ion HRESIMS spectrum, which provided



1

2 R = OH
3 R = H

Figure 13. Structure of chlorosulfolipids from Adriatic mussels

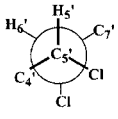
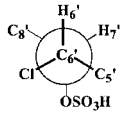
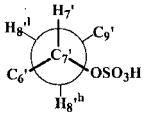
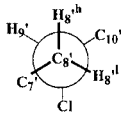
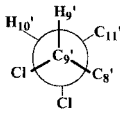
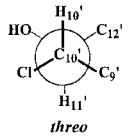
an intense pseudomolecular ion cluster characteristic of highly polychlorinated compounds; analysis of the molecular formulas of the two compounds suggested **3** to be smaller than **2** by one oxygen atom. Consequently, the most obvious and immediate hypothesis was that **3** had the same structure of **2** but lacked one of the five hydroxyl groups present in **2**. The determination of the entire planar structure of the molecule was established from the corresponding acetylated compound, as its ^{1}H NMR spectra showed a better dispersion of the signals in the low-field region.

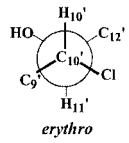
The ^{1}H-^{1}H COSY and HOHAHA spectra allowed the identification of three spin systems, one related to the acylic portion of the molecule and two for the functionalized part of the molecule, the first of which ranges from CH_3-1′ to C17′ and the second from C19′ to C23′. Their assembly was allowed by some key correlations present in the HMBC spectra. In particular, the long-range couplings between C-19 and both H-18 and H-20, as well as between C-20 and H-18 emerging from the HMBC spectrum, were useful to connect the partial structural units from C-1 to C-24 for both compounds.

To fully establish the gross structure of **3** it remained necessary to assign the exact location of the many functional groups on the carbon chain. This was made by an accurate analysis of both 1D and 2D NMR experiments. In particular, the location of the hydroxyl groups was determined by a comparison between the ^{1}H NMR spectra of the chlorosulfolipid before and after acetylation, and confirmed by inspection of the HMBC spectra of the acetylated compounds, which show long-range couplings between each carbonyl and the vicinal oxymethine or oxymethylene protons.

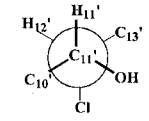
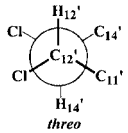
Among the ten still-unassigned sp^3 methine carbons in the ^{13}C NMR spectrum, only one resonates at very low field

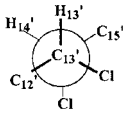
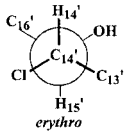
Table 3. $^3J_{H,H}$, $^2J_{C,H}$ and $^3J_{C,H}$ of the C5′−C17′ and C21′−C22′ portions of compound **3** (all coupling constants were measured by PS-HMBC; H^h is the proton signal resonating at higher field and H^l is that resonating at lower field)

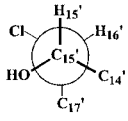
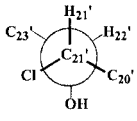
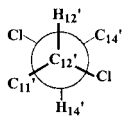







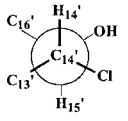


C5'-C6' axis		C6'-C7' axis		C7'-C8' axis		C8'-C9' axis		C9'-C10' axis		C10'-C11' axis	
$^3J_{H5'-H6'}$	small	$^3J_{H6'-H7'}$	small	$^3J_{H7'-H8'^l}$	small	$^3J_{H8'^h-H9'}$	small	$^3J_{H9'-H10'}$	small	$^3J_{H10'-H11'}$	large
$^3J_{H5'-C7'}$	small	$^3J_{H6'-C8'}$	small	$^3J_{H7'-H8'^h}$	large	$^3J_{H8'^l-H9'}$	large	$^3J_{H9'-C11'}$	small	$^3J_{H10'-C12'}$	small
$^3J_{C4'-H6'}$	small	$^3J_{C5'-H7'}$	small	$^3J_{H7'-C9'}$	small	$^3J_{H8'^l-C10'}$	small	$^3J_{C8'-H10'}$	large	$^3J_{C9'-H11'}$	small
$^2J_{C5'-H6'}$	small	$^2J_{C6'-H7'}$	small	$^3J_{C6'-H8'^h}$	small	$^3J_{H8'^h-C10'}$	small	$^2J_{C9'-H10'}$	large	$^2J_{C10'-H11'}$	large
$^2J_{C6'-H5'}$	small	$^2J_{C7'-H6'}$	small	$^3J_{C6'-H8'^l}$	small	$^3J_{C7'-H9'}$	small	$^2J_{C10'-H9'}$	small	$^2J_{C11'-H10'}$	large
				$^2J_{C7'-H8'^l}$	small	$^2J_{C9'-H8'^l}$	large				
				$^2J_{C7'-H8'^h}$	large	$^2J_{C9'-H8'^h}$	small				



C11'-C12'axis		C12'-C13' axis		C13'-C14'axis		C14'-C15' axis		C15'-C16' axis		C21'-C22' axis	
$^3J_{H11'-H12'}$	small	$^3J_{H12'-H13'}$	large	$^3J_{H13'-H14'}$	small	$^3J_{H14'-H15'}$	large	$^3J_{H15'-H16'}$	small	$^3J_{H21'-H22'}$	small
$^3J_{H11'-C13'}$	small	$^3J_{H12'-C14'}$	small	$^3J_{H13'-C15'}$	small	$^3J_{H14'-C16'}$	small	$^3J_{H15'-C17'}$	large	$^3J_{H21'-C23'}$	small
$^3J_{C10'-H12'}$	small	$^3J_{C11'-H13'}$	small	$^3J_{C12'-H14'}$	small	$^3J_{C13'-H15'}$	small	$^3J_{C14'-H16'}$	small	$^3J_{C20'-H22'}$	small
$^2J_{C11'-H12'}$	small	$^2J_{C12'-H13'}$	large	$^2J_{C13'-H14'}$	small	$^2J_{C14'-H15'}$	large	$^2J_{C15'-H16'}$	small	$^2J_{C21'-H22'}$	small
$^2J_{C12'-H11'}$	small	$^2J_{C13'-H12'}$	large	$^2J_{C14'-H13'}$	small	$^2J_{C15'-H14'}$	large	$^2J_{C16'-H15'}$	large	$^2J_{C22'-H21'}$	small

(C-7′, δ = 74.3 ppm); this pointed to the location of the sulfate group at this position on account of its higher deshielding effect when compared with that of a chlorine atom. Consequently, the eleven chlorines were unequivocally placed: nine on the remaining nine methine groups and two on the only unprotonated sp^3 carbon (C18′; δ = 93.4 ppm).

The relative stereochemistry was elucidated by successful application of the *J*-based configuration analysis. This is a powerful method developed recently by Murata for the elucidation of relative stereochemistry in acyclic structures using carbon-proton spin-coupling constants ($^{2,3}J_{C,H}$) and proton-proton spin-coupling constants ($^3J_{H,H}$), often in combination with NOE/ROE data.[90,91] It allows the determination of the predominant staggered rotamer(s), with the correct relative configuration, among the six possible staggered conformers of each two-carbon fragment in which a chiral molecule with 1,2- or 1,3-asymmetric methine systems can be ideally divided.

The small amount of available material hampered a quantitative evaluation of $^2J_{C,H}$ and $^3J_{C,H}$ required for relative stereochemistry assignment. In this case, the heteronuclear coupling constants were only qualitatively (small or large) determined simply by comparing the intensities of the cross peaks in the PS-HMBC spectrum. According to Murata, this qualitative elaboration is allowed taking into account the significant differences in $^2J_{C,H}$ and $^3J_{C,H}$ values between *anti* and *gauche* rotamers. The combination of $^3J_{H,H}$, $^2J_{C,H}$ and $^3J_{C,H}$ allowed us to unambiguously individuate the correct stereochemical relationship for each pair of vicinal or alternate asymmetric carbons (Table 3). Where a H/H *anti* arrangement occurs the $^3J_{H,H}$, $^2J_{C,H}$ and $^3J_{C,H}$ values did not support an unequivocal assignment of the spatial disposition of the substituents. In these cases, as suggested by Murata, the relative configurations could be assigned by evaluation of the ROE correlations. In this way, we were able to solve the relative stereochemistry of the fragments C-5′/C-16′ and C-21′/C-22′of the molecule, which were 5′*R*,6′*R*,7′*S*,9′*S*,10′*R*,11′*R*,12′*S*,13′*S*,14′*R*, 15′*R*,16′*S* or its enantiomer, and 21′*S*,22′*S* or its enantiomer (Table 2).

To fully clarify the stereochemistry of our compound we still had to determine the configuration of the isolated stereogenic carbon in position 1′ and to upgrade the relative stereochemistry of the fragments C-5′/C-16′ and C-21′/C-22′ to the absolute one. Unfortunately, the paucity of **3** prevented us from applying current methods of absolute stereochemistry elucidation, which involve reaction with chiral auxiliaries. However, considering that the assigned relative configurations of the fragments C-5′/C-16′ and C-21′/C-22′ in **3** parallel those of the corresponding chiral carbon sequences in **2**, whose absolute configuration is known, and on account of the very reasonable assumption that **2** and **3** are biogenetically related, we can assume that they share the same absolute stereochemistry, 5′*R*,6′*R*,7′*S*, 9′*S*,10′*R*,11′*R*,12′*S*,13′*S*,14′*R*,15′*R*,16′*S*,21′*S*,22′*S*. Compound **3** was tested for its antiproliferative activity on WEHI 164 and J774, and it was found to inhibit the growth of all cell lines evaluated at 72 h with an IC_{50} of 10.4 and >20 μg/mL, respectively.

Due to the potential risk this new class of cytotoxic compounds could represent for human health, pharmacological studies are needed. However, the accumulation of sufficient amounts of these compounds from toxic Adriatic mussels is a prerequisite for this.

4. Conclusions

Our studies have revealed a very interesting, uncommon and changeable scenario of shellfish toxicity in Italy. The toxin profile in mussels from the Adriatic sea differs from that of other countries where the DSP phenomenon has been deeply studied and where the new analogs of YTX have not been reported until now. Apparently, the phytoplankton species producing yessotoxins that occur in the Adriatic sea differ from those present in other countries. Thus, investigation of the phytoplankton species responsible for the production of yessotoxins in the Adriatic sea represents an unavoidable future direction.

Another aspect to be considered is that the presence in 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 now evident that there is a variety of YTX analogs in some shellfish-producing areas and toxicological investigations should be addressed to all the YTX-like compounds. Therefore many efforts must be directed to the accumulation of these toxins in order to perform further toxicological studies.

Moreover, the presence of the above-reported cytotoxic compounds in edible shellfish, in addition to contamination of DSP toxins, increases the potential risk to human health as this finding extends the Adriatic sea toxin profile. To prevent damage, both to public health and to the shellfish industries, due to pollution from harmful marine algae it is necessary to implement careful monitoring, both at markets and shellfish farms. Therefore an accurate analysis of toxic mussels is indispensable in order to identify new toxins, especially non-DSP polyether toxins, and isolate these toxins in amounts sufficient to clarify their toxicological effects.

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