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Toxins from Adriatic blue mussels. A decade of studies*

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Abstract: A research program was initiated in 1990 to carefully examine the toxin profiles in mussels from the northern Adriatic Sea. Since then, 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 peculiar to the Adriatic Sea. During our investigation of toxic Adriatic mussels, we also isolated a new type of toxin, whose structure was elucidated by extensive use of 1D and 2D NMR techniques. Some of them could represent a further alarm for public health owing to their cytotoxic activity. The recent application of liquid chromatography/mass spectrometry (LC/MS) methods for detection of Adriatic marine biotoxins allowed us to hasten the analysis of toxic samples and to advance effective structural hypothesis even when full structure elucidation of new toxins by NMR spectroscopy is hampered by the limited amount of available material.

INTRODUCTION

Marine biotoxins and harmful algae represent a significant and expanding threat to human health and fisheries resources throughout the world [1–3]. This problem takes many forms, ranging from massive "red tides" or blooms of cells that discolor the water, to dilute, inconspicuous concentrations of cells that are noticed only because of the harm caused by the highly potent toxins these cells contain. The impacts of these phenomena include mass mortalities of wild and farmed fish and shellfish; human intoxications or even death from contaminated shellfish or fish; alterations of marine trophic structure through adverse effects on larvae and other life history stages of commercial fisheries species; and death of marine mammals, seabirds, and other animals.

The nature of the problem has changed considerably over the last two decades. 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.

The causes for this apparent expansion are unknown, but some believe that human alteration of the water quality of the coastal zone is an important factor [2].

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. These poisoning syndromes have been named paralytic, diarrhetic, neurotoxic, and amnesic shellfish poisoning (PSP, DSP, NSP, and ASP). Except for ASP, all are caused by biotoxins synthesized by dinoflagellates. The toxins involved in DSP are the most important for their wide distribution over the world [4].

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During the last decade, these types of poisonings have spread also to the northwestern Adriatic Sea, thus causing serious health and economic problems. This area has been subjected to recurring cases of red tides since 1975. The blooms have occurred most frequently in the coastal waters of Emilia Romagna, but sometimes they have interested much wider areas along the northwestern Adriatic coast. No toxic bloom had been described until 1989, when the first DSP episodes were reported in patients who ate mussels (*Mytilus galloprovincialis*) collected from the northern Adriatic Sea [5]. In fact, the presence of *Dinophysis fortii* cells in hepatopancreas of mussels and of lipid-soluble toxins of DSP type in mussel tissue collected in the coastal waters of Emilia Romagna region allowed us to prove that certain cases of diarrhea in consumers of mollusks were not due to bacteria or virus, but to biointoxication by DSP. 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. The phenomenon seriously threatens human health and causes severe economic losses for 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 structure elucidation of the causative toxins are prerequisites.

Because 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 an aqueous 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. This assay has been very successful for the water-soluble PSP toxins and has been in use for over 50 years. One of the problems with the mouse bioassay, however, is the inherent variability, which can exceed ±20 %, compared to most chemical techniques, which have uncertainties of less than 10 %. In addition, application to liphophilic toxins requires a different extraction method and has been less successful owing to severe matrix interferences giving both false positives and negatives. A number of rapid methods have been developed for seafood toxins. For all of these assays, however, it is generally recognized that confirmation of positives is still required.

Owing to the drawbacks of the mouse bioassay, instrumental methods are required. In fact, they have the potential for sensitive, precise, and fully automated quantitation of known toxins, as well as for confirmation of identity. Furthermore, methods based on chromatographic and spectroscopic techniques are required for chemical and biochemical studies, and for identification of new toxins.

Basing on these arguments, a research program based on instrumental analysis has been initiated in Italy since 1990 in order to carefully investigate DSP contamination in mussels of the Adriatic Sea. Thus, up to date, we have been analyzing toxic samples of shellfish collected along the Emilia Romagna coasts in correspondence to the highest level of toxicity.

OKADAIC ACID AND YESSOTOXIN

The toxin profile in Adriatic mussels has completely changed in the last years. Extraction of bulk samples of toxic mussels from Adriatic Sea showed that okadaic acid (OA, **1**, Fig. 1) was the main toxic component in the DSP outbreak of 1990. Its identity was confirmed by isolation and NMR spectroscopic identification [6]. This result represented the first sure evidence of presence of DSP toxins in mussels cultivated along the Italian coast.

However, recent research has demonstrated that other toxins are, at the moment, important contributors to DSP in Italy.

In 1995, in fact, discrepancies observed between mouse bioassays and high-performance liquid chromatography (HPLC) results in Italian mussels suggested the presence of other substances with either toxic or synergic effects of algal toxicity observed in mice. On that occasion, for the first time from Italian mussels, yessotoxin (**2**, YTX, Fig. 1) was isolated in relatively large amounts in addition to trace amounts of OA, by our research group [7]. Successively, homoyessotoxin (homoYTX, **3**) [8], 45-hy-

droxyyessotoxin (45-OHYTX, **4**) [9], and 45-hydroxyhomoyessotoxin (45-OHhomoYTX, **5**) [8] were also isolated and identified by comparison of their chromatographic and spectral properties with those reported in the literature.

A recent etiological study revealed that the biogenetic origin of YTX is different from those of OA and dinophysistoxin-1 (DTX-1). YTX is produced by the dinoflagellate *Protoceratium reticulatum* [10], while OA and DTX-1 are produced mainly by *Dinophysis* spp. [11]. In the past, YTX has been associated with DSP, because it often accompanied DSP toxins such as OA, DTX-1, and pectenotoxins (PTXs), and gives positive results when tested in the conventional mouse bioassay used for detecting DSP toxins. However, arguments existed as to whether or not YTX should be included in the DSP category. In fact, YTX differs from OA and DTX-1 in causing neither intestinal fluid accumulation in infant mice by intubation nor inhibition of protein phosphatase 2A [12]. The mouse lethality of YTX by intraperitoneal injection (0.1 mg kg^{-1}) is the strongest among all DSP toxins, but its oral toxicity is the weakest, as can be deduced by considering that the maximum oral dose of 1 mg/kg does not kill the mice [12]. In spite of the wealth of data on OA, the molecular mechanism underlying toxicity of YTXs is unknown. Indeed, very limited data are available regarding the effects of this group of components on cellular systems, being essentially confined to histopathological evaluations of few organs. Cardiac injury, for instance, appears the major end-point of IP injection of yessotoxin in mice [12]; an involvement of the nervous system in the YTX toxicity can also be hypothesized, on the basis of the chemical structure, since brevetoxins and ciguatoxins [13], both structurally strictly related to YTX, induce poisonings characterized by neurological and cardiological symptoms. As for the mechanism of action, it could be hypothesized that YTX, by analogy with brevetoxins and ciguatoxins, may act as depolarizing agent, opening membrane channels of excitable cells permeable to Na^+, and leading to a Na^+ influx [14]. It remains to be established, however, at which extent these toxins can be absorbed by the intestine, and then gain access to target organs.

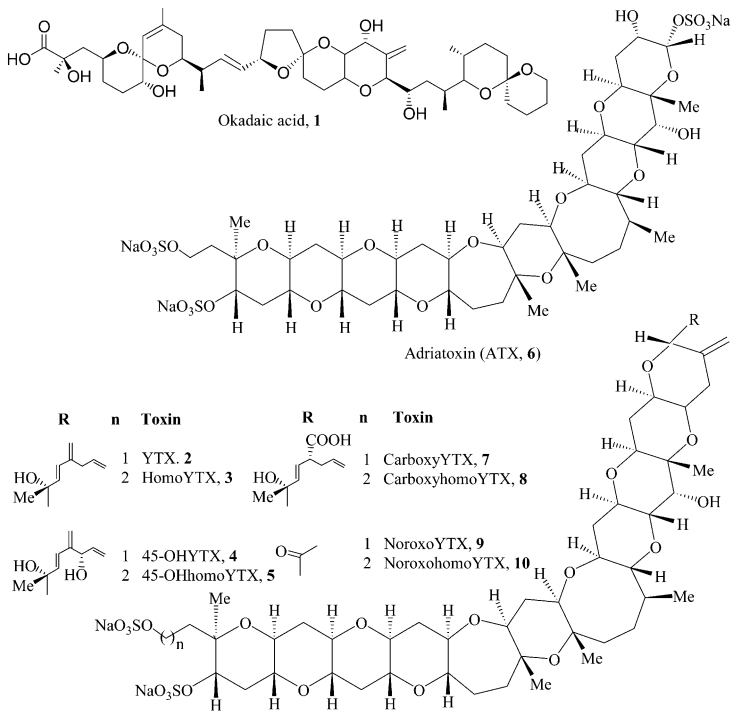


Fig. 1 Structures of OA and YTXs.

The scarcity of toxicological data on YTX and its analogs may be a quite dangerous lack, since they currently appear to be the major biotoxins of mollusks from the Adriatic Sea. There is, indeed, a great need to increase our knowledge of YTX toxicity so as to determine the acute and chronic risk associated with YTX-contaminated seafood. However, toxicological studies necessary to assess its human health risks have been hampered until now, owing to the extremely limited availability of the toxin.

NEW ANALOGS OF YESSOTOXIN

During the course of our studies, we succeeded in the isolation and structural determination of several new analogs of YTX from hepatopancreas of mussels of the Adriatic Sea, such as adriatoxin [15] (ATX, **6**), carboxyyessotoxin [16] (COOHYTX, **7**), carboxyhomoyessotoxin [17] (COOHhomoYTX, **8**), and 42,43,44,45,46,47,55-heptanor-41-oxohomoYTX [18] (noroxohomoYTX, **10**) (Fig. 1). All of the new analogs have been isolated in pure forms, and their chemical structures have been determined on the basis of spectral evidence, particularly mono- and two-dimensional ^{1}H NMR experiments, as well as MS/MS experiments. As for COOHYTX and COOHhomoYTX, which have an additional chiral carbon at position 44 in the eastern side chain, the absolute configuration of the centers has also been determined by applying the method proposed by Nagai and Kusumi [19] for the determination of the absolute configuration of α-chiral carboxylic acids.

These analogs represent new additions to the class of YTXs, and seem to be peculiar to our seas, since they have not been reported until now in any other country. However, their toxicology still remains to be investigated.

The results of our studies indicate that the composition and the relative abundance of YTXs in bivalves seem to vary regionally, seasonally, and annually, as observed for other DSP toxins. Therefore, we have to be vigilant on the occurrence of these toxins until their potential risks to human health are better understood on account of a variable toxicity spectrum based upon a different chemical structure.

LC/MS ANALYSIS OF YESSOTOXINS

Toxins in a shellfish extract are usually present at extremely low amounts. Thus, isolation of the pure compounds 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 handled. The work-up deals with the isolation of a few 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 provide for short analysis times, high sensitivities, and specific detection of individual components.

To partially overcome these problems and to hasten acquisition of the results, the combination of liquid chromatography and mass spectrometry (LC/MS) was considered. This is a valuable tool, which is able to afford either information on molecular weight and elemental composition as well as structural information through tandem MS experiments. At the same time, it is universal, selective, and highly sensitive. The advantage of this approach is that it is possible to detect 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 provide for derivatization of each toxin with an appropriate auxiliary reagent for fluorescence labeling followed by HPLC analysis. Unfortunately, there is no reagent that fits all DSP toxins. ADAM (9-anthryldiazomethane) is used for OA, DTXs, and PTXs [20], while YTXs are derivatized with a dienophile reagent, DMEQ-TAD {4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalinyl)ethyl]-1,2,4-triazoline-3,5-dione} [21]. 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, the method is not reliable for detection

of those derivatives that lack a conjugated diene functionality in the molecule, such as the Adriatic analogs noroxohomoYTX (**10**), carboxyYTX (**7**), carboxyhomoYTX (**8**), and adriatoxin (**6**). Therefore, we tested the suitability of the LC/MS method developed by Quilliam [22] for detection of most lipophilic toxins to separate and univocally detect all YTXs isolated so far, also at the presence of okadaic acid, which sometimes coexists in shellfish [23]. For this purpose, standard solutions at known concentration of YTX and OA, as well as solutions of a number of YTX analogs from mussels of the northern Adriatic Sea were employed. The expected toxins were easily detected, although most of them eluted as overlapping peaks. However, this drawback could be overcome because toxins with different molecular masses were monitored by extracted ion chromatograms (XICs) of the *pseudo*-molecular ions, thus allowing their unambiguous identification even if they were chromatographically unresolved.

MS/MS experiments were carried out for further confirmation. The fragmentations in this experiment are known to occur by losing neutral species and fragment ions derived from the "western part" of the molecule in which a negative charge is localized by the presence of the sulfate esters [24]. At a low-collision energy, all investigated disulfated YTXs gave an intensive product ion corresponding to loss of SO_3. Further MS/MS experiments were carried out at higher collision energy by using the desulfated fragment ion as precursor ion. The fragmentation patterns for YTX and homoYTX appeared very similar, all the peaks differing for only 14 mass units in the 2 spectra. It has to be noted that the region of the spectra under *m/z* 950 contained ions relative to the fragmentation of the backbone skeleton. Because fragmentation occurs at specific sites of the ether rings, the same product ions were observed for all YTX- or homoYTX-like compounds that we analyzed. 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.

The so-developed technique allowed determination of OA and all YTX and homoYTX derivatives so far isolated in a single chromatographic run of 25 min and showed it to be both selective and sensitive with a detection limit of 70 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 quantitation 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.

IDENTIFICATION OF NEW YTX ANALOGS, A DESULFOYTX AND NOROXOYTX, BY LC/MS ANALYSIS

Together with rapid detection of known compounds at part-per-billion levels, the method allows us to highlight the possible presence of new analogs, thus representing a key potentiality for natural product chemists 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 from one sampling site located along the Emilia Romagna coast of Italy gave quite interesting results, since, besides the already known composition [18], consisting of homoYTX, 45-OHhomoYTX, carboxyhomoYTX, and noroxohomoYTX, it revealed that a novel YTX analog was present in the mixture [23] on the basis of the following evidence. XIC of the ion at *m/z* 1061.5 showed the presence of two peaks eluting at slightly different retention times (Fig. 4). Assignment of one of the peak to 42,43,44,45,46,47,55-heptanor-41-oxohomoYTX was an easy task, comparing the retention time and mass spectrum with those of an authentic sample, whereas the other peak could not be associated to any of the samples at our disposal. So, LC/MS/MS experiments (CE = 45 %) were carried out using ion at *m/z* 1061.5 as precursor ion. An inspection of the MS/MS spectrum of the unknown peak revealed the typical fragmentation pattern of the backbone skeleton of YTX, but no sulfonate loss was observed for this peak, thus suggesting the presence of only one sulfate ester group in the molecule. The whole of this data suggested that the peak under investigation was due to a desulfoYTX, the only uncertainty being in the desulfated position. NMR experiments are required to unambiguously assign 1-desulfo [25]

or the alternative 4-desulfoYTX 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 gave the same interesting results [26]. A slight modification of the solid-phase extraction (SPE) clean-up of the extract reported by Goto et al. [27] was used for eliminating substances that interfere with ionization of analytes or cause deterioration of the columns upon repeated injections. In particular, in the conditioning and loading steps of the SPE procedure, the phosphate buffer, suggested by Goto et al. to control the charge state of the sulfate moiety, was substituted with a formate buffer, which appeared to be more suitable for the subsequent LC/MS analyses. The eluates of the SPE clean-up were all investigated by LC/MS, in full scan mode (negative ions).

Besides some already known YTXs whose retention times and mass spectra were perfectly coincident when compared with those of individual reference samples, injected in 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 associated with any of the already known YTXs.

In order to perform structural investigation on the newly detected compound, LC/MS/MS experiments were carried out. The characteristic fragmentation of the polycyclic backbone skeleton of YTX was 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 originates ion peak at *m/z* 925.3, was indicative of a CH_2=C=O neutral loss; this 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, **9**, the homolog in the YTX series of the noroxohomoYTX (**10**), which we have previously isolated and fully characterized (Fig. 1). This hypothesis was supported by a comparison of the chromatographic and mass spectral properties of the involved compounds: **9** and **10** eluted in the same experimental conditions at almost the same retention time, and their MS/MS spectra appeared to be almost superimposable, as long as they were shifted of 14 mass units (Fig. 2).

On the basis of this evidence, the proposed LC/MS method represents a key potentiality for natural product chemists to discover new YTX analogs and provides a more comprehensive approach than HPLC-fluorescence detection (FLD) methods currently in use [21]. Secondly, this technique can be usefully employed for structure elucidation of new toxins whenever great structural analogies occur between 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 advanced even when full structure elucidation of new toxins by NMR spectroscopy is hampered by the limited amount of available material.

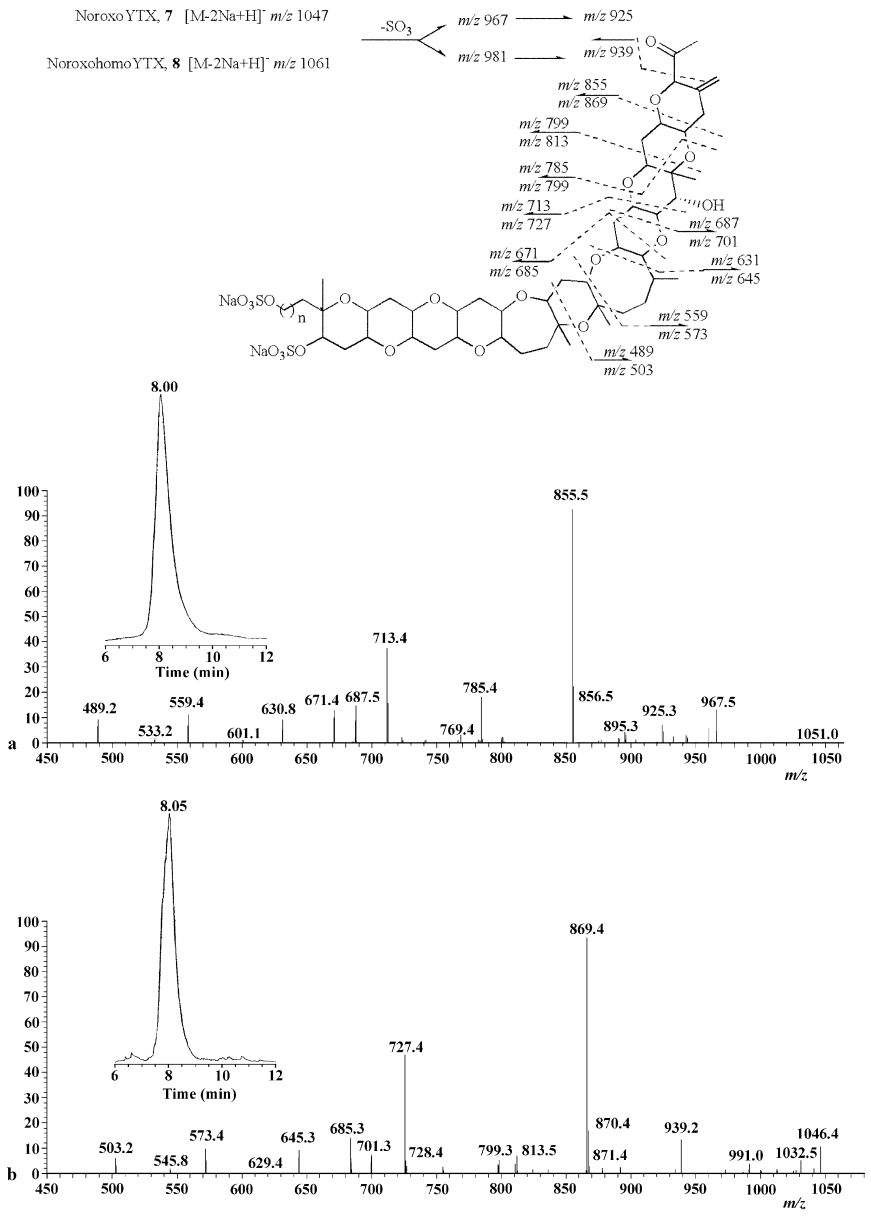


Fig. 2 Product ion chromatograms and MS/MS spectra of noroxoYTX, **9** (a) and noroxohomoYTX, **10** (b) obtained using a collision energy of 45 % and the $[M\text{-}H\text{-}80]^-$ ions at *m/z* 967.5 and 981.5, as precursor ions, respectively. Characteristic product ions observed in the two tandem mass spectra are shifted of 14 mass units and indicate that fragmentation occurs in the molecules at specific sites of the polycyclic backbone skeleton.

ADRIATIC STRAIN OF *P. RETICULATUM* AS PRODUCER OF SOME NEW YTX ANALOGS

Identification of organism(s) responsible for 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 biogenetic origin of YTX

[10]. Successively, YTX was determined also from Adriatic *P. reticulatum* [28] and together with 45,46,47-trinoryessotoxin in strains of the same species collected in Japan [29]. In 1999, Draisci et al. reported the presence of YTX and homoYTX from *Gonyaulax polyedra* collected in the northwestern Adriatic Sea [30]. However, the biogenetic origin of all the other YTX analogs was still unknown, thus raising an issue whether they were derived from metabolic conversion of YTX 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 coasts (Emilia Romagna, Italy) in June 2001 was investigated. Detailed toxin profile of this strain was obtained by HPLC coupled with electrospray ionization ion trap mass spectrometry (HPLC-ESIMS).

In order to investigate the presence in the crude extract of YTX analogs, XICs were obtained for all known YTX derivatives isolated so far. However, the high background signal 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 SPE was carried out in order to reduce interference and matrix effects.

All SPE eluates were analyzed by LC/MS and the propanol/water eluate showed that they contain noroxoYTX, carboxyYTX, 45-OHYTX, YTX, and homoYTX, respectively. The retention times and mass spectra of each of the above peaks were compared with those of individual reference samples, injected in the same experimental conditions, and resulted in being perfectly coincident. In order to fully confirm the assignment, multiple-stage tandem mass (MS^n) experiments were carried out. These were achieved by trapping daughter ions at each successive stage of multiple tandem MS experiments and fragmenting them to produce new daughter ions. The MS conditions were optimized in order to discriminate in favor of trapping the ions of interest at each successive stage and the optimized method was used to confirm the presence of each YTX analog in plankton samples. As expected from the high selectivity of the MS/MS technique, no interference with the analytes was observed in the product ion chromatograms.

This was the first identification of *P. reticulatum* as the biogenetic origin of some of the YTX derivatives so far isolated from Italian mussels: 45-OHYTX, homoYTX, carboxyYTX, and noroxoYTX. Interestingly, Adriatic *P. reticulatum* is able to produce compounds belonging to both YTX and homoYTX series, whereas previous studies were suggestive of two different organisms being responsible for production of each homolog series.

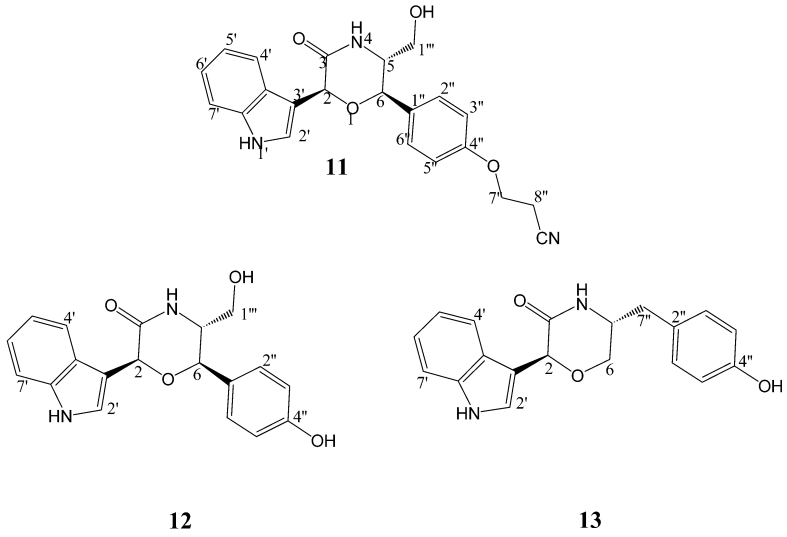
Furthermore, these findings indicate that most of the Adriatic YTX derivatives are true products of the dinoflagellate and do not derive from metabolic conversion of YTX in shellfish. Finally, it has to be noted that the toxin profile of *P. reticulatum* revealed by LC/MS analysis appeared to be more complex than that previously determined by other authors [10,28–30]. Likely, this is due to the different analytical method employed, although a different toxin productivity in different strains cannot be ruled out.

OXAZININS

During our investigation of toxic Adriatic mussels, we have also isolated new types of toxins, which are completely different in structure from the polyether DSP toxins isolated so far, but can represent a further alarm for public health, owing to their cytotoxic activity.

A chemical investigation of the digestive glands of *M. galloprovincialis* from the northern Adriatic Sea led, in fact, to the isolation of three novel compounds, oxazinin-1 (**11**), oxazinin-2 (**12**), and oxazinin-3 (**13**) (Fig. 3). Their structures, including the relative stereochemistry, were established by spectroscopic means including extensive 2D NMR and molecular mechanics calculations [31]. The absolute stereochemistry of oxazinin-1 has also been assigned by application of the 9-AMA shift–correlation method for β-chiral primary alcohols [32].

The new compounds were analyzed for cytotoxic activity and oxazinin-1 (**11**) was shown to inhibit the growth of WEHI 164 and J774 cell lines in vitro.



11 **12** **13**

Fig. 3 Structures of oxazinins.

CHLOROSULFOLIPIDS

Besides the long-known biotoxins, very recently we have reported isolation and stereostructure elucidation of a new class of cytotoxins constituted by polychlorinated sulfolipids. Chlorine-substituted docosane and tetracosane disulfates, whose structures were assigned devoid of stereochemical details, have been previously reported in the microalgae *Ochromonas danica* and *Poterioochromonas mahlamensis* (Chrysophyta, Chrysophyceae) [33], where they represent almost half of the total membrane lipids. The chlorosulfolipids of *P. malhamensis* were found to be predominantly of the tetracosane-1,14-diol disulfate variety [34] and displayed broad-spectrum antimicrobial activity [35].

During our investigation of toxic Adriatic mussels collected in 1998, we isolated the novel cytotoxic polychlorinated sulfolipid **14** (Fig. 4), a new type of toxin [36], which is completely different in structure from the polyether DSP toxins isolated so far. The structural determination of the new toxin, including its absolute stereochemistry, has been performed by extensive NMR analysis and molecular mechanics and dynamics calculations. The relative stereochemistry was elucidated by successful application of the *J*-based configuration analysis. It is a recently powerful method developed 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 ($^{3}J_{H,H}$), often in combination with nuclear Overhauser effect (NOE)/rotational nuclear Overhauser effect (ROE) data [37]. This approach 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 consecutive or alternating stereogenic centers can be ideally divided. The absolute stereochemistry of **14** was established by application of the Mosher method [39] on the desulfated derivative.

To date, the presence of this type of compound appears to be not incidental in Adriatic shellfish, as we have recently isolated another new cytotoxic polychlorinated sulfolipid (hexadecanoic acid 5′,6′,9′,10′,12′,13′,14′,16′,18′,18′,21′-undecachloro-11′,15′,17′,22′,23′-pentahydroxy-1′-methyl-7′-sulfooxy-tricos-2′-enyl ester, **15**, Fig. 4) [38].

Its gross structure has been elucidated by spectral analysis, including various 2D NMR experiments. Determination of the absolute stereochemistry at the 15 centers and 1 stereogenic axis, namely, 15 chiral carbons and the carbon–carbon double bond, in the chlorosulfolipid **15** appeared particularly challenging. As for the chiral carbons, we took advantage of the fact that most of them were adjacent or alternating. So, *J*-based configuration analysis could be again successfully applied.

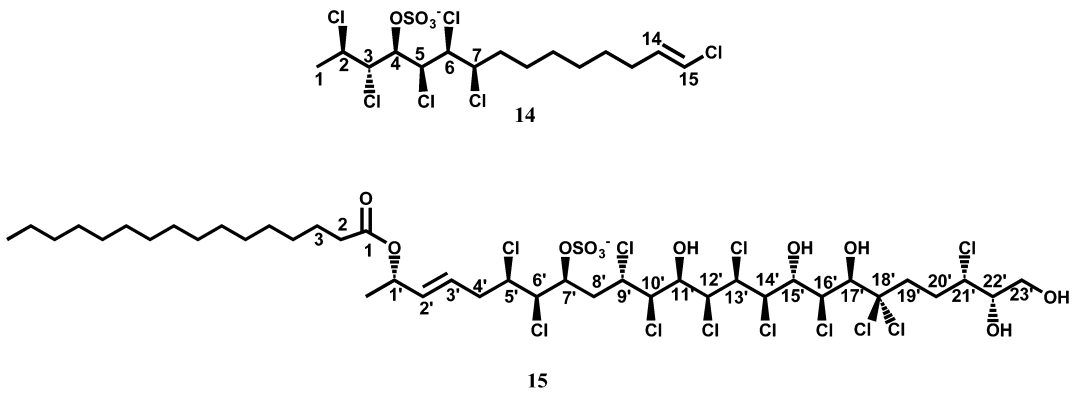


Fig. 4 Structures of chlorosulfolipids.

Using ^{1}H NMR, heteronuclear long-range coupling (HETLOC), and PS-HMBC experiments, homonuclear and heteronuclear *J* values of the functionalized portions C5′–C16′ and C21′–C22′ of the molecule were successfully determined and evaluated, thus allowing us to solve the relative configurations of all the stereogenic centers, apart from the C16′–C17′ bond, where the observed *J*-values were inconsistent, probably because of the presence of the bulky CCl_2 group at C17′.

To overcome the failed assignment of the C16′–C17′ relative configuration by Murata method, we took advantage of the presence of a hydroxyl group at C17′; so, we determined the absolute configuration at this position by application of the modified Mosher method suggested by Kakisawa [39]. After derivatization with the Mosher's reagents, the mixture of esters MTPA-derivatives was purified in order to obtain three-MTPA esters where the Mosher derivatization occurred at three key positions, namely C11′, C17′, and C22′.

NMR analysis of these esters allowed not only the assignment of the absolute stereochemistry at C17′, but also to upgrade our knowledge on the two separate fragments C5′–C16′ and C22′–C23′, changing their stereochemistries from relative to absolute ones.

To determine full stereochemistry of **15**, the assignment of the absolute configuration of the isolate asymmetric center at C1′ was still required. For this purpose, we appealed to the Mosher's method again, after reduction of the ester functionality at C1′ with $LiAlH_4$. However, because of the small amount of starting material, we decided to apply the Mosher's method modified by Reguera, which requires the use of only one enantiomer [either the (*R*)- or (*S*)-MTPA] [40]. The obtained results suggested the *S* configuration at C1′.

Bearing in mind the relative stereochemistries established by *J*-based configuration analysis and the absolute configurations at C11′, C17′, C22′, and C1′, established by Mosher's method, the whole absolute configuration of the molecule was consequently assigned as 1′*S*, 5′*R*, 6′*R*, 7′*S*, 9′*S*, 10′*R*, 11′*R*, 12′*S*, 13′*S*, 14′*R*, 15′*R*, 16′*S*, 17′*R*, 21′*S*, 22′*S*.

Because of their potential risk this new class of cytotoxic compounds could represent for human health, pharmacological studies are needed. To this aim, the accumulation of a sufficient amount of these compounds from toxic Adriatic mussels seems to be a prerequisite.

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 YTXs, which occur in the Adriatic

Sea, differ from those present in other countries. Thus, investigation of the phytoplankton species responsible for the production of YTXs 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 owing 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 to be addressed to further toxicological studies.

Moreover, the presence of the above-reported cytotoxic compounds in edible shellfish, in addition to contamination of DSP toxins, increases potential risk to human health, considering that this finding extends the Adriatic Sea toxin profile. To prevent the damages owing to pollution from harmful marine algae, both to the public health and to the shellfish industries, 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, even other than DSP polyether toxins, and isolate a larger amount for deeply clarifying their toxicological effects.

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