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Isolation of Adriatoxin, a New Analogue of Yessotoxin from Mussels of the Adriatic Sea

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Abstract

Diarrhetic shellfish toxin composition in the hepatopancreas of mussels from northern Adriatic sea was investigated. Along with yessotoxin (YTX), homoyessotoxin (homoYTX) and 45-hydroxyyessotoxin (45-OHYTX), identified by comparison of their chromatographic and spectral properties with those reported in the literature, we isolated a new analogue of YTX, adriatoxin (ATX), whose structure was determined on the basis of spectral evidence.

Keywords: Diarrhetic Shellfish Poisoning; DSP; Adriatoxin; ATX.

Blooms of toxic or harmful microalgae commonly called "red tides" represent a significant and expanding threat to human health and fisheries resources throughout the world. The impacts of these "harmful algal blooms" range from illness and death of human consumers of shellfish or fish that have accumulated algal toxins to ecosystem alteration and mortalities of marine mammals and other animals.

In the Adriatic Sea, DSP (Diarrethic Shellfish Poisoning) outbreaks have been known since 1989, when the first case of human gastroenteritis was reported [1]. To date, DSP phenomena in Italy have been related to the presence of okadaic acid (OA) [2-4], dinophysistoxin-1 (DTX-1) [5] and yessotoxins (YTXs) [6-8]. Since 1990, the Italian Health Authority has taken measures to prevent the risk of DSP contaminated-seafood consumption [9]. These involve the analysis of marine phytoplankton and shellfish to detect dinoflagellates and DSP toxin in the shellfish digestive glands.

However, as the toxins differ not only in chemical structure but also in toxicological effects, elucidation of toxin profiles by instrumental analyses is indispensable for evaluation of the health risks.

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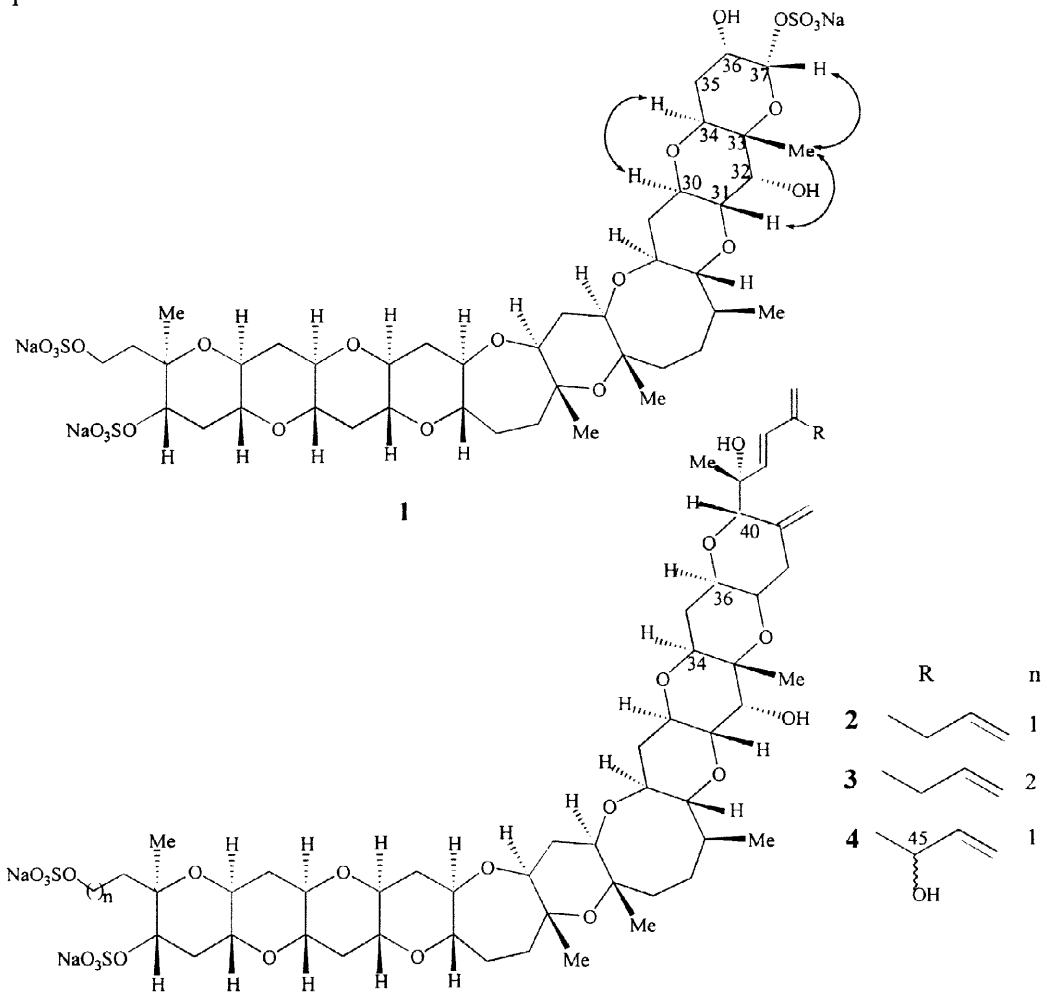
To this aim, our research group is now investigating the detailed toxin composition of mussels from farms of Northern Adriatic Sea.

In this paper we report the structural determination of a new analog of YTX, **1**, that we named adriatoxin (ATX).

ATX was isolated from the digestive glands (224 g) of DSP-infested mussels *Mytilus galloprovincialis,* collected at the beginning of July 1997 from one sampling site located along the Emilia Romagna coasts of Italy, when the highest level of toxicity were recorded in this area. Causative agents of the contamination could be associated with the simultaneous presence of the following toxic or potentially toxic species, detected according to the Utermöhl method [10]: *Alexandrium* spp., *Dinophysis* spp., *Gonyaulax fragilis, Gonyaulax polyedra* and *Protoceratium reticulatum.*

The DSP toxins were extracted following the previously reported procedures [6]. The toxic residue was loaded on a Develosil ODS column and multiple chromatographic steps were required to purify the lipophylic toxins. The presence of YTXs in the eluates was previously checked by mouse bioassays, TLC analysis, and by monitoring ultraviolet absorption at 230 nm. The YTXs fraction was then further refined by a repetition of reversed-phase columns. A good separation was achieved on an ODS column with CH_3CN-MeOH-H_2O 1:2:2 as eluent, which allowed the isolation of YTX (**2**, 100 μg), homoYTX (**3**, 600 μg), and 45-OHYTX (**4**, 400 μg) in pure form.



Furthermore, from this chromatographic purification, we obtained an additional toxic fraction. TLC of this partially purified fraction showed two more polar compounds, apparently related to YTX, which were further purified on the same ODS column with CH_3CN:MeOH:H_2O (1:1:3) as eluent, thus obtaining 400 μg of pure ATX, **1**. The structural analysis of the other new toxin is currently under investigation.

Negative FABMS of **1** provided ion peaks at m/z 1093 $(M-Na)^-$, 1071 [major peak $(M-2Na+H)^-$], 1049 $(M-3Na+2H)^-$, 969 $(M-SO_3Na-2Na+H)^-$, suggesting the presence of three sulfate groups.

The structural elucidation of **1** was mainly carried out by comparing the ^1H NMR data between YTX and ATX (Table 1). Preliminary analysis of ^1H NMR spectrum of **1** showed a close resemblance to that of YTX, but the lack of the characteristic signals of the side chain at C-40 appeared immediately evident in the proton spectrum of **1**.

^1H NMR assignments were done by ^1H-^1H COSY measurements in CD_3OD (Table 1). Proton connectivities, chemical shifts, signal shapes, and NOEs of protons from H-1 to H-2, H-4 to H18, H-20 to H-22, H-24 to H-32 in **1** were identical to those of the corresponding signals in YTX. Thus, they shared the same structure at that part, and the structural alteration resided somewhere on the terminal part of the molecule.

Table 1.
Comparison of ^1H Nmr Chemical Shift (δ) of YTX with those of ATX in CD_3OD

position	YTX	ATX	position	YTX	ATX
1	4.21; 4.21	4.21; 4.21	25	1.50; 1.75	1.50; 1.75
2	1.99; 2.21	1.99; 2.21	CH_3-26	1.07	1.07
CH_3-3	1.31	1.31	27	2.81	2.81
4	4.24	4.24	28	3.34	3.34
5	1.75; 2.59	1.75; 2.59	29	1.58; 2.32	1.58; 2.32
6	3.10	3.10	30	3.64	3.64
7	3.35	3.35	31	3.22	3.21
8	1.44; 2.22	1.44; 2.22	32	3.89	3.86
9	3.17	3.17	CH_3-33	1.25	1.34
10	3.15	3.15	34	3.80	3.78
11	1.45; 2.30	1.45; 2.30	35	1.53; 2.14	1.49; 2.12
12	3.03	3.03	36	4.09	3.42
13	3.12	3.12	37	3.43	4.97
14	1.47; 2.34	1.47; 2.34	38	2.47; 2.75	=
15	3.35	3.35	CH_2=39	4.84; 5.05	=
16	3.26	3.26	40	3.92	=
17	1.84; 1.99	1.84; 1.99	CH_3-41	1.43	=
18	1.83; 1.89	1.83; 1.89	42	5.86	=
CH_3-19	1.29	1.29	43	6.35	=
20	3.46	3.46	CH_2=44	5.01; 5.09	=
21	1.80; 1.97	1.80; 1.97	45	3.00; 3.00	=
22	3.53	3.53	46	5.91	=
CH_3-23	1.20	1.20	47	5.10; 5.12	=
24	1.54; 1.77	1.54; 1.77			

^1H connectivities from H-34 to H-37 protons, some of them heavily overlapping, were determined by ^1H-^1H COSY, and confirmed by an HOHAHA experiment.

The whole of NMR and mass spectral data allowed us to define the molecular formula of ATX as $C_{42}H_{63}O_{24}S_3Na_3$.

At this point, to fully define the structure of ATX it remained to locate the third sulfate ester group, whose presence was indicated by the fragmentation pattern in the mass spectrum, and

an hydroxyl group, whose presence was implied by the molecular formula. Of course, these two functions could be linked only at C-36 and C-37. Analysis of the chemical shift value of H-36, which resonated at relatively high field region (δ 3.42) suggested the hydroxyl group to be located at this position and, consequently the sulfate ester at C-37.

All ether rings in **1** were found to be *trans*-fused, as in the case of yessotoxin, on the basis of the typical coupling costants (9-10 Hz) of angular protons for antiperiplanar substitution on oxycarbons. Observed NOE's between angular protons, angular proton and a methyl, and angular methyls on both sides of ether oxygens also supported *trans*-fusion of rings, and allowed us to determine the relative stereostructure of the fused rings.

The α-orientation of 32-OH was deduced from the NOE between H-32 and the methyl at C-33, and from the coupling constant of H-32 (J=2.7 Hz) with the axial H-31. In the same way, a NOE between the methyl at C-33 and H-37 indicated the equatorial orientation of the sulfate ester at this last position. On the contrary, the absence of a NOE between H-36 and H-34 suggested the axial orientation of the OH-group at C-36. However, the overlapping of many signals in the midfield region, and the very small amounts of **1** (400 μg) prevented us from confirming the configuration at C-36 through the analysis of the coupling costants.

Mouse assay results obtained on **1** suggested that its lethality was slightly reduced in comparison with YTX, perhaps due to the reduced affinity to the lipid membrane as the result of the increased polarity of the molecule. The limited availability of ATX, however, prevented us to exactly determine the mouse lethality.

The discovery of this new analog of YTX from mussels of Adriatic sea extends the problem associated with the toxicology of YTXs, because of the poor knowledge about implication of the YTXs in human illness. The extremely limited amount of available material has prevented until now an accurate toxicological evaluation of YTX. Many efforts must be directed to the isolation of an adequate amount of YTX and its analogs to allow studies on their mechanism of action and toxicological effects.

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