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ARTICLE *in* CHEMICAL RESEARCH IN TOXICOLOGY · FEBRUARY 2007

Impact Factor: 3.53 · DOI: 10.1021/tx060192x · Source: PubMed

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Desulfoyessotoxins from Adriatic Mussels: A New Problem for Seafood Safety Control

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Received August 16, 2006

Two new desulfated yessotoxin (YTX) analogues were isolated from a toxic batch of Adriatic mussels collected in October 2004. Their stereostructures were elucidated through extensive NMR and MS-based analysis. The finding of these desulfocarboxyhomoYTXs in shellfish poses additional problems to those institutions entitled to control seafood safety, since desulfated YTXs fail the European Union method currently in force for checking toxicity in mollusks.

Introduction

Over the past decade, yessotoxins (YTXs) have been the most occurring Adriatic toxins, while diarrhetic shellfish poisoning (DSP) toxins [mainly okadaic acid (OA) and its derivatives], which were predominant in the early '90s, slowly subsided since 1995 and disappeared around the turn of the new millennium (*1*) (Figure 1). The difference in the danger of these two classes of toxins is substantial. OA is considered a most harmful compound because of its potent tumor promoting activity (*2*), whereas YTX, whose toxicology still presents many blind spots, is of significantly lower oral toxicity. (*3*) Nonetheless, YTX and OA show an almost equal lethal potency when tested through the mouse bioassay (intraperitoneal injection of shellfish extract into mice).

On account of the relative harmfulness to public health, the European Union (EU) has recently established different allowance levels in shellfish for DSP toxins and YTXs (16 μg of OA and 100 μg of YTX in 100 g of mollusk, respectively) (*4*).

Consequently, it was necessary to set up a new official procedure for analyzing seafood that would be capable of separating YTXs and DSP toxins in distinct layers (Scheme 1). In fact, the old official scheme did not allow any separation between YTXs and DSP toxins; therefore, the mouse bioassay (*5*)—the reference method in Europe to detect toxicity in seafood—could not lead to any confident assessment of the toxin(s) involved.

Materials and Methods

MS and NMR Spectral Measurements. NMR spectra were measured on a Varian Unity Inova700 spectrometer, and CD_3OD was used as an internal standard (δ_H 3.31). Negative ESI spectra were obtained on a API-2000 triple quadrupole mass spectrometer equipped with a turbo ion spray source (Applied biosystem; Thornhill, ON, Canada). Medium-pressure liquid chromatography (MPLC) was performed on a Büchi 861 apparatus (Develosil ODS and Toyopearl HW-40 SF columns). HPLC separations were performed on a Varian apparatus [Waters 490 MS UV detector (230 nm) and Luna 5 μm C18 column].

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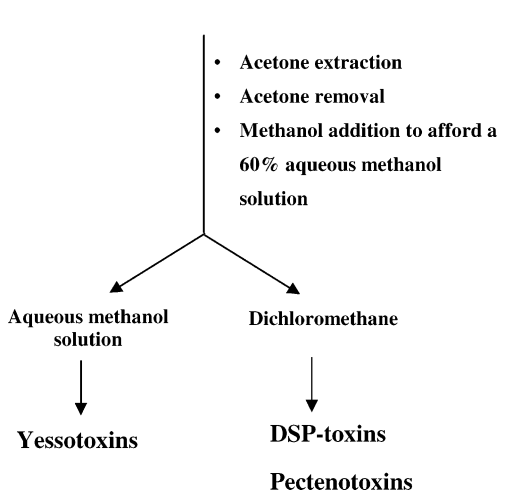
Scheme 1. Extraction Protocol for Toxic Shellfish Currently in Force in the European Community (*4*)[a]



[a] Such an experimental procedure allows us to now separate YTXs and DSP-toxins in different layers on the basis of their relative lipophilicity.

Isolation of 1 and 2. Compounds **1** and **2** were isolated from 150 kg of mussels and collected along northern Adriatic coasts (3 m depth) in October 2004. The mussel tissue was homogenized with a Waring blender and extracted twice with acetone and once with methanol. The organic phase was evaporated, dissolved in MeOH/H_2O 8:2, and then partitioned with *n*-hexane. The acqueous residue was evaporated and redissolved in MeOH/H_2O 6:4 and successively partitioned with CH_2Cl_2. Only the CH_2Cl_2 phase gave a positive result to the official mouse bioassay method for DSP toxins and was separated through a Develosil ODS column washing stepwise with MeOH:H_2O 1:1, 6:4, 8:2, and 9:1 and MeOH 100% in this order.

The toxic residue was separated with a Toyopearl HW-40S column (eluent MeOH). The last purification was carried out through a HPLC-RP (Luna C-18, 10 μm) column eluted with a 1:2:2 CH_3CN:MeOH:H_2O mixture (UV detector, 230 nm). Compounds **1** and **2** eluted after 5.83 and 5.67 min, respectively. Throughout the purification, the presence of the toxin in the eluates was checked by the official mouse bioassay.

1-DesulfocarboxyhomoYTX. HR-ESIMS (negative ion mode) *m*/*z* 1107, 5156 (calculated for $C_{56}H_{83}O_{20}S$ $[M - Na^+]^-$; it requires 1007, 5198). ^1H NMR data are reported in Table 1. The degree of

10.1021/tx060192x CCC: $37.00
Published on Web 12/09/2006

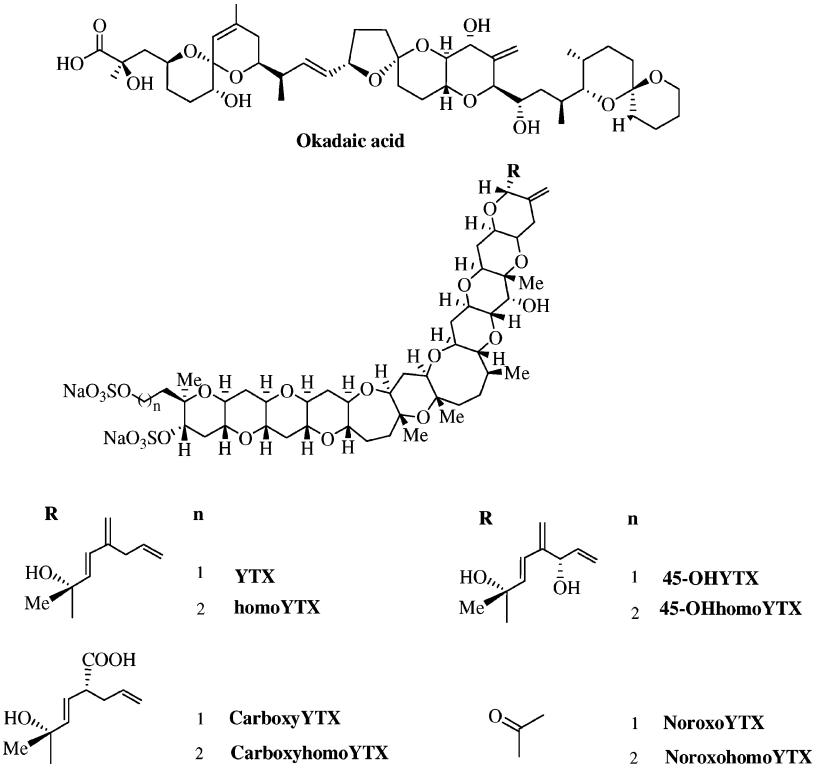


Figure 1. Main toxins responsible for seafood poisoning in the Northern Adriatic Sea over the last decades.

Table 1. ^{1}H NMR Spectroscopic Data of CarboxyhomoYTX and DesulfocarboxyhomoYTXs (CD_3OD)

position	carboxy-homoYTX	1-desulfocarb-oxyhomoYTX (**1**)	4-desulfocarb-oxyhomoYTX (**2**)	position	carboxy-homoYTX	1-desulfocarb-oxyhomoYTX (**1**)	4-desulfocarb-oxyhomoYTX (**2**)
1	3.96, 4.00	3.54, 3.54	3.98, 3.98	25	1.46, 1.78	1.46, 1.78	1.46, 1.78
2	1.78, 1.83	1.59, 1.62	1.75, 1.79	26	1.70	1.70	1.70
2a	1.61, 1.78	1.63,1.74	1.57, 1.74	CH_3−26	1.02	1.02	1.02
CH_3−3	1.19	1.21	1.13	27	2.76	2.76	2.76
4	4.21	4.22	3.52	28	3.28	3.28	3.28
5	1.70, 2.56	1.72, 2.57	1.58, 2.04	29	1.50, 2.25	1.50, 2.25	1.50, 2.25
6	3.00	3.01	2.98	30	3.60	3.60	3.60
7	3.33	3.30	3.23	31	3.15	3.15	3.15
8	1.39, 2.16	1.34, 2.14	1.34, 2.14	32	3.82	3.82	3.82
9	3.12	3.12	3.11	CH_3−33	1.19	1.19	1.19
10	3.11	3.11	3.10	34	3.75	3.75	3.75
11	1.38, 2.22	1.38, 2.22	1.37, 2.21	35	1.46, 2.09	1.46, 2.09	1.46, 2.09
12	3.01	3.03	3.00	36	4.02	4.02	4.02
13	3.06	3.04	3.05	37	3.36	3.36	3.36
14	1.42, 2.28	1.42, 2.28	1.41, 2.28	38	2.41, 2.70	2.41, 2.70	2.41, 2.70
15	3.31	3.31	3.30	CH_2−39	4.89, 5.08	4.89, 5.08	4.89, 5.08
16	3.21	3.20	3.21	40	3.79	3.79	3.79
17	1.78, 1.93	1.78, 1.93	1.77, 1.91	CH_3- 41	1.34	1.34	1.34
18	1.79, 1.86	1.79, 1.86	1.79, 1.86	42	5.58	5.58	5.58
CH_3−19	1.24	1.25	1.25	43	5.70	5.70	5.70
20	3.41	3.41	3.41	44	2.89	2.89	2.89
21	1.72, 1.91	1.72, 1.91	1.72, 1.91	45	2.21, 2.43	2.21, 2.43	2.21, 2.43
22	3.49	3.49	3.49	46	5.78	5.78	5.78
CH_3−23	1.17	1.15	1.16	47	4.92, 5.03	4.92, 5.03	4.92, 5.03
24	1.47, 1.73	1.47, 1.73	1.47, 1.73				

purity of **1** (>90%) was evaluated on the basis of both HPLC and NMR analysis.

4-DesulfocarboxyhomoYTX. HR-ESIMS (negative ion mode) *m/z* 1107, 5237 (calculated for $C_{56}H_{83}O_{20}S$ $[M - Na^+]^-$; it requires 1007, 5198). ^{1}H NMR data are reported in Table 1. The degree of purity of **2** (>90%) was evaluated on the basis of both HPLC and NMR analysis.

Mouse Lethality Measurement. The lethality of both **1** and **2** was assayed in female mice (20 ± 0.5 g) by intraperitoneal injection of toxin solutions with 1% Tween 60 solution. The injection volume was 1.0 mL in all cases. Both compounds **1** and **2** were tested at a dose level of 0.5 mg kg^{-1}. Three mice were used for each compound and were observed for 24 h. All injected mice survived. For comparison, three mice were injected with YTX at a dose level of 0.1 mg kg^{-1}, two of whom died within 24 h after injection.

Results and Discussion

During the summer 2004, national institutions entitled to constantly monitor the toxicity in the Adriatic shellfish noted that the CH_2Cl_2 phase derived from shellfish extraction was becoming more toxic than the hydromethanolic one. When we analyzed a batch of toxic Adriatic mussels in October 2004, only the CH_2Cl_2 phase gave a positive result to the mouse bioassy. This pointed out a change in toxins profile marked by a decrease of YTXs and a comeback of DSP toxins.

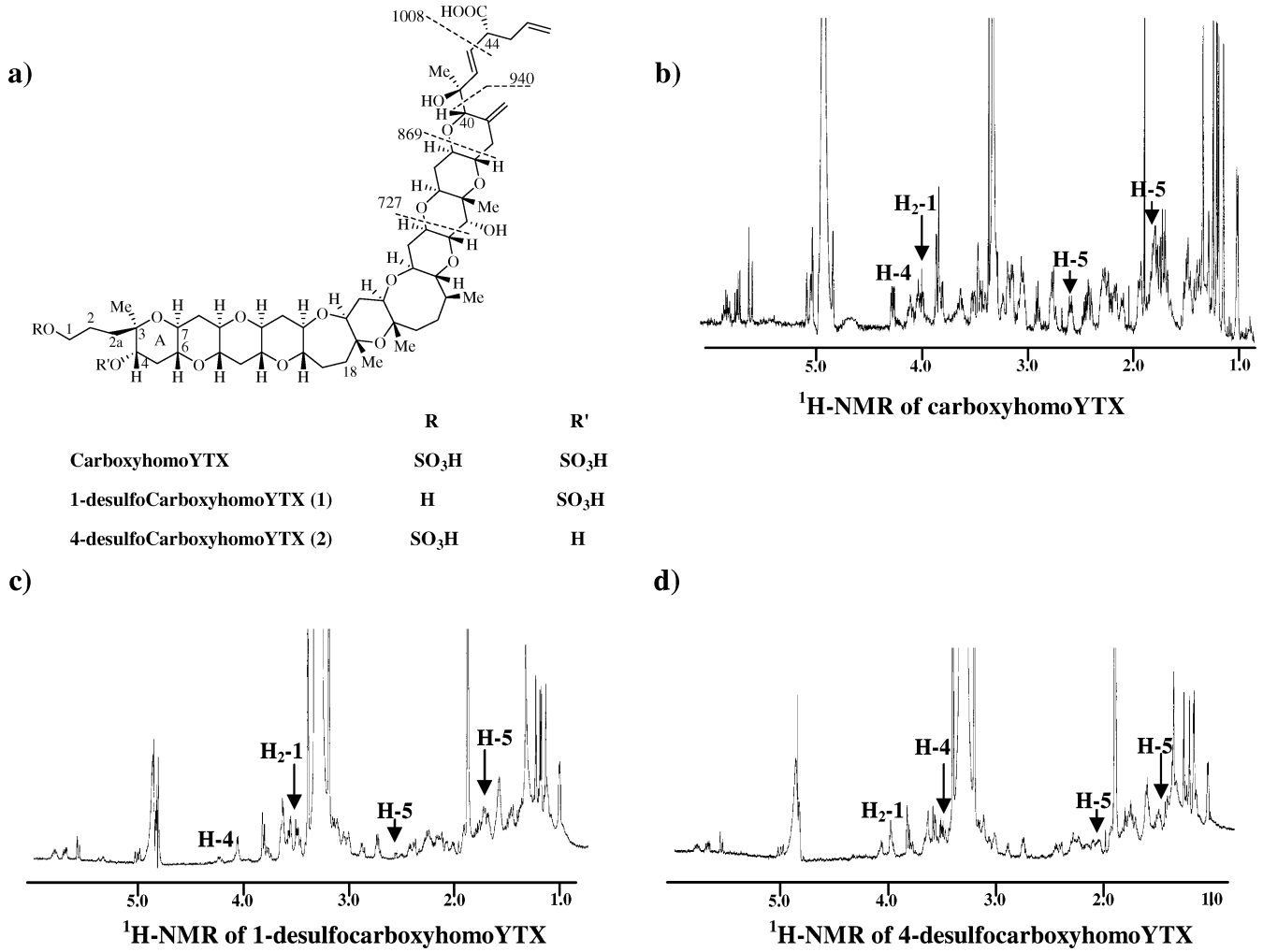


Figure 2. (a) Stereostructure of carboxyhomoYTX, 1-desulfocarboxyhomoYTX (**1**), and 4-desulfocarboxyhomoYTX (**2**) showing the fragmentation pattern provided by MS/MS experiments performed in negative-ion mode using the $[M - H]^-$ ion at *m*/*z* 1107 of each toxin as the precursor ion. The fragmentation patterns paralleled that of a pure carboxyhomoYTX sample when the $[M - H - SO_3]^-$ ion (*m*/*z* 1107) was selected as the precursor ion. (b) ^{1}H NMR spectrum of carboxyhomoYTX (in CD_3OD), where H_2-1, H-4, and H-5 have been highlighted. (c) ^{1}H NMR spectrum of 1-desulfocarboxyhomoYTX (in CD_3OD): The arrow shows the absence of the typical H_2-1 resonance when C-1 is sulfated. (d) ^{1}H NMR spectrum of 4-desulfocarboxyhomoYTX (in CD_3OD): The arrows show the absence of the typical H-4 and H-5 resonances when C-4 is sulfated.

Initially, the toxic CH_2Cl_2 fraction obtained from only 100 g of mussels was analyzed through LC/MS (*6*): Surprisingly, no peak related to DSP toxins was recorded nor were any of the other main lipophilic toxins usually coextracted in the CH_2Cl_2 phase, such as pectenotoxins and azaspiracids.

A new type of toxin had to be taken into account. Therefore, we performed a massive mussel extraction, which turned out to be a most laborious and time-consuming procedure, because of the large quantity of starting material (about 150 kg) that had to be treated, in order to isolate the contaminating toxins in sufficient amount for their NMR characterization. Eventually, we afforded only two toxic fractions containing 52 and 73 μg of pure **1** and **2**, respectively (see the Materials and Methods).

It is worth mentioning that the small quantity of isolated biotoxins does not correspond to its real occurrence in the natural sample. In fact, a large part of toxin is unavoidably lost during several steps of extraction and purification as well as consumed by the mouse bioassay in order to follow up toxicity throughout the purification. On this basis, we can definitively assume that the overall yield for the two biotoxins after the purification is around 10%. Despite the small quantity of the isolated toxins, their structural elucidation was successfully carried out by MS and NMR techniques.

The HR-ESIMS spectrum of **1** suggested $C_{56}H_{83}O_{20}S$ as its molecular formula (negative ion mode *m*/*z* 1107.5156; calculated for $C_{56}H_{83}O_{20}S$ $[M - Na^+]^-$). At a first glance, the ^{1}H NMR spectrum highlighted a YTX-like structure and more precisely a close similarity of **1** to carboxyhomoYTX (*7*), which we previously isolated (Figure 2b,c). Interpreting COSY, TOCSY, and ROESY spectra allowed us to individuate a polycyclic portion superimposable with YTXs as well as to establish the coincidence of the eastern moiety of **1** with carboxyhomoYTXs. This was further supported by fragmentation patterns that emerged from MS/MS experiments (Figure 2a).

In the western part of the toxin, instead, we detected some significant differences with carboxyhomoYTX. A new spin system stretching from C-1 to C-2a was singled out from complex COSY and TOCSY experiments. ^{1}H resonances of this system and particularly those of H_2-1 were consistent with a desulfatation of the typical sulfate at C-1 in YTXs (Table 1). The new toxin was therefore classified as 1-desulfocarboxyhomoYTX (Figure 2a).

About its stereochemistry, we could confidently assume that **1** had the same stereochemistry as carboxyhomoYTX on the basis of the coincidence of ^{1}H NMR signals of the whole polycyclic portion of **1** and of the ester side chain with

carboxyhomoYTXs both in resonating values and multiplicity. In addition, all of the ROE correlations in **1** were consistent with the assumed stereochemistry.

Once we characterized **1**, the structure determination of **2** was a relatively easy task. In fact, the HR-ESIMS spectrum of **2** suggested the same molecular formula as **1** (negative ion mode *m*/*z* 1107,5237; calculated for $C_{56}H_{83}O_{20}S$ $[M - Na^+]^-$) and the ^{1}H NMR spectrum of **2** unfolded its close analogy with **1** and consequently with carboxyhomoYTX (Figure 2b−d). The main difference between **2** and carboxyhomoYTX was detected at C-4, where a high-field shift of H-4 resonance in **2** was observed (Table 1). Starting from H-4, the whole spin system through C-18 was individuated by interpreting COSY and TOCSY experiments. On the basis of the above data, **2** was identified as 4-desulfocarboxyhomoYTX (Figure 2a). The stereochemistry of **2** was assigned through the same evidence as **1**. Particularly, as for ring A—where ^{1}H NMR resonances for **2** and carboxyhomoYTX were not coincident—the stereochemistry was assigned on the basis of a ROESY spectrum. The observed ROEs between CH_3-3 and H-7 as well as between H-4 and H-6 suggested the ring A stereochemistry of all YTXs so far known.

We tried to assess the mouse lethality of both **1** and **2**, but we were significantly hampered by the scarce amount of pure compound available. In fact, in order to set up a pharmacological experiment as reliable as possible, we could test each toxin only at a dose level of 0.5 mg kg^{-1}. For all of the injected mice that survived within 24 h after the injection, we can conclude that the mouse lethality of both **1** and **2** has to be considered $>$0.5 mg kg^{-1}.

Conclusions

The presence of **1** and **2** in Adriatic mussels gives rise at least to the following objects that would be worth studying over in the future: Despite their small structural innovation, the new desulfoYTXs are not recovered in the YTX layer but in the DSP one, when extracted according to the official EU protocol. Hence, an overhaul of the EU control test is urgently needed. Toxicity of desulfocarboxyhomoYTXs must be investigated. An aspect to be considered is the lack of a sulfate group. This indeed decreases the hydrophilicity of desulfoYTXs in comparison to YTXs. So, the biomembrane's permeability and, consequently, the toxicity level could be affected. Finally, it would also be worth assessing if the lack of a sulfate ester in carboxyhomodesulfoYTXs represents an outcome of the mollusk metabolism or is connected to the metabolic pathways of the producing alga.

Acknowledgment. This work is a result of research supported by PRIN 2004 and the EU Project BIOTOXmarin 2005. NMR and MS experiments were performed at "Centro di Servizi Interdipartimentale di Analisi Strumentale", Università di Napoli Federico II. The assistance of the staff is gratefully appreciated.

Supporting Information Available: ^{1}H−^{1}H COSY spectra and ^{1}H−^{1}H TOCSY spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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TX060192X