A New Analogue of Yessotoxin, Carboxyyessotoxin, Isolated from Adriatic Sea Mussels

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A new analogue of yessotoxin (YTX), carboxyYTX, **3**, was isolated from the digestive glands of mussels cultured on the Italian Adriatic coast . Its structure was determined by MS and NMR spectroscopy.

Introduction

Worldwide infestation of bivalve shellfish by microalgal toxins poses a serious threat to both human health and shellfish industries. Yessotoxin, a disulfated polyether toxin reported from shellfish in Japan,[1] Norway,[2] Chile,[3] New Zealand,[3] and Italy[4] is one such toxin. Structurally YTX differs from others DSP-toxins but resembles the brevetoxins and ciguatoxins in having a ladder-shaped polycyclic ether skeleton. In the past YTX has been associated with diarrhetic shellfish poisoning (DSP), because it often accompanies DSP toxins such as okadaic acid (OA), dinophysistoxin-1 (DTX1), and pectenotoxins (PTX) and gives positive results when tested in the conventional mouse bioassay used for detecting DSP toxins. However, controversy has existed as to whether or not YTX should be included in the DSP category. Unlike DTX1 and OA, YTX shows neither diarrheagenicity nor inhibition of protein phosphatase 2A.[5] However, toxicological studies necessary to assess its risk to human health has been hampered until now, due to the extremely limited availability of the toxin.

Unlike OA, DTX1 and PTX2 produced by *Dinophysis spp.*,[6] the presence of YTX in shellfish very often did not correlate with the occurrence of *Dinophysis spp.*[7]

Analysis by a recently developed fluorometric determination method for HPLC[3] enabled Yasumoto and co-workers to identify YTX in the marine dinoflagellate *Protoceratium reticulatum* collected in New Zealand.[7] Since the dinoflagellate is commonly found in the coastal waters of many regions, more widespread occurrences of YTX in shellfish are predicted.

In the Adriatic Sea, occurrence of YTX (**1**) and its analogs was recently determined.[4] In addition, we found a new YTX-like structure, the adriatoxin (ATX, **2**), the chemical structure of which has been previously reported.[8]

In this paper, we report the structural determination of a new analogue of YTX, carboxyYTX, (COOHYTX, **3**), isolated from mussels from the Northern Adriatic Sea.

Results and Discussion

DSP-infested mussels *Mytilus galloprovincialis* were collected in July 1997 from one sampling site located along the Emilia Romagna coasts of Italy. The digestive glands of these mussels were found to be toxic by the mouse bioassay method for DSP.[9] The biotoxins present in the mussels (1150 g of hepatopancreas) were isolated following the previously reported procedures.[8] The toxic residue was fractionated by repeated bioassay-assisted column chromatography on ODS (aq. MeOH) and successively on TOYOPEARL (MeOH). The presence of YTXs was checked by TLC (Silica gel 60, $CHCl_3$/MeOH/H_2O 30:10:1) and by monitoring UV absorption at 230 nm. Further repeated chromatographic separation on reversed-phase columns allowed the isolation of YTXs free from contaminants. A good separation of the different YTXs was achieved on the final ODS column using the isocratic solvent mixture CH_3CN/MeOH/H_2O 1:2:2 as eluent, which allowed the isolation of YTX (**1**, 700 µg), homoYTX (**4**, 900 µg), 45-OHYTX (**5**, 600 µg), ATX (**2**, 300 µg) and carboxyYTX (**3**, 700 µg) in pure forms.

HomoYTX,[10] 45-OHYTX[11][12] and YTX[1][4] were identified by comparison of their chromatographic behavior with authentic samples previously isolated in our laboratory and by comparison of their reported spectral properties. The identity of ATX was established as previously reported.[8]

Negative FABMS gave an ion peak at *m/z* 1195, corresponding to $(M - Na)^-$ of carboxyYTX (**3**), 32 mass units higher than that of the corresponding $(M - Na)^-$ of YTX (*m/z* 1163). Its molecular formula was deduced as $C_{55}H_{80}Na_2O_{23}S_2$ from the negative HRFABMS data on the pseudomolecular ion at $m/z = 1195$ [$(M - Na)^-$, $m/z = 1195.4446$, calculated 1195.4429]. The ^1H NMR spectrum of **3** in a CD_3OD was very similar to that of YTX. Detailed analysis of ^1H-^1H COSY and HOHAHA spectra of **3** led to the identification of spin systems corresponding to the entire polycyclic part of YTX.

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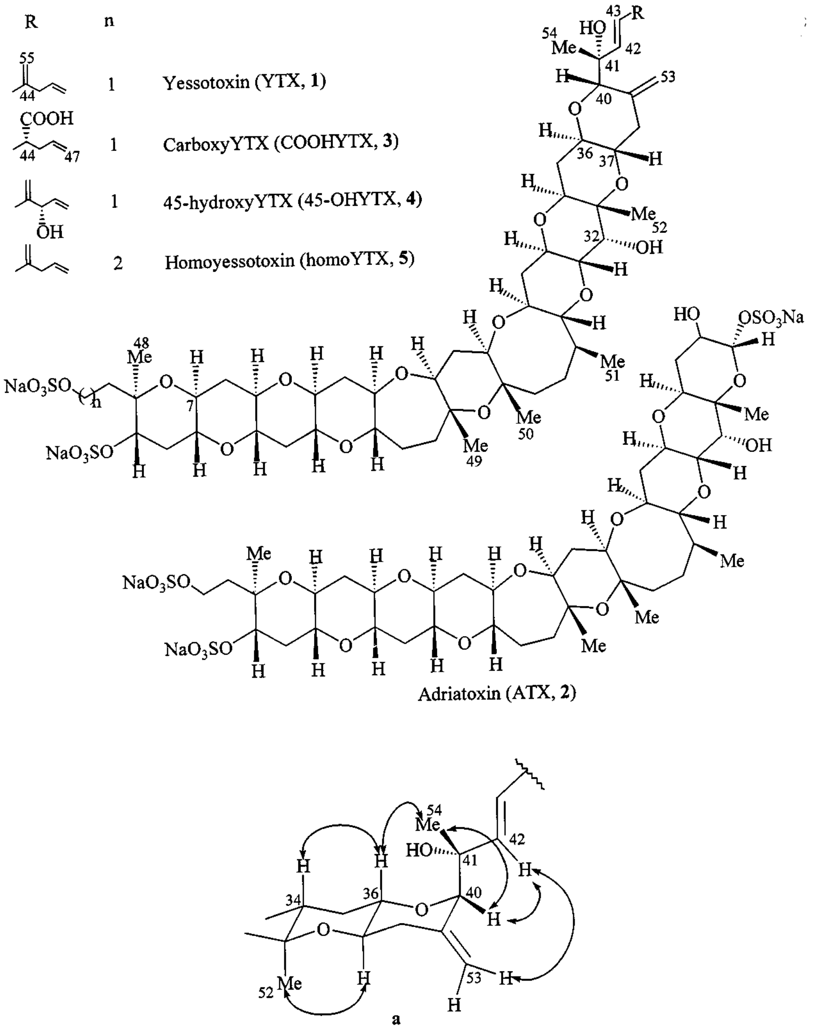

Figure 1. Structures of Yessotoxins; (a) conformer and stereostructure around the last rings of the eastern part of the molecule

All ether rings in **3** were found to be *trans*-fused, as in the case of YTX, on the basis of the typical coupling constants (9−10 Hz) of angular protons for antiperiplanar substitution on oxycarbon. NOE correlations observed between angular protons, between angular protons and angular methyl groups (H-7/Me-48, H-37/Me-52), and between angular methyl groups (Me-49/Me-50), allowed us to determine, not only the positions of the ether linkages, but also the stereostructure of the fused rings. Strong NOE correlations between H-40 and Me-54, as well as between H-36 and Me-54, observed in the ROESY spectrum of **3**, allowed the extentsion the above part structure as far as the quaternary C-41. Analogously to YTX, in addition to the Me-54, C-41 possesses a hydroxyl group as evidenced by the downshifted resonance of the methyl group (δ 1.38). As was the case for YTX, the observed NOEs between H-36 and Me-54 also allowed deduction of the diaxial relationship between C-41 and H-36. The additional NOEs between H-42 and H-40, H-42 and H-53 as well as the above reported correlation between H-40 and Me-54, and Me-54 and H-36 indicated that, as in YTX,[12] the conformation of Me-54 and C-42 were *gauche* to H-40 and that of 41-OH was *anti* to H-40 (see Figure 1). All these data allowed assignment of the relative configuration of that part of the molecule which **3** has in common with YTX.[12]

Therefore, the structural difference between **1** and **3,** must arise in the remaining part of the side-chain. Characteristic signals arising from protons of the exomethylene group at C-44, easily recognizable in the ^{1}H NMR spectrum of **1** were absent in that of **3**. Instead, a signal at δ = 2.92 was observed. The 2D COSY data showed that this signal (H-44) was coupled to the protons of a methylene group (C-45) resonating at δ = 2.25 and 2.46 respectively, as well as to the vinyl proton (C-43) at δ = 5.77. Further consideration of the 2D COSY and HOHAHA data allowed extention at both extremities of the above partial structure from C-42 to C-47, which (on the basis of the above evidence) suggests the further partial structure: $-CH=CH-CH-CH_2-CH=CH_2$, linked through the vinylic carbon atom to C-41.

The remaining 45 mass units still needed to be accounted for and, as inferred by the molecular formula, this suggested the presence of a carboxylic group in the molecule. This was proved by its transformation into a chiral amide

Table 1. Comparison of ^{1}H NMR chemical shifts (δ) of YTX with those of CarboxyYTX in CD_3OD

Position	YTX	CarboxyYTX	Position	YTX	CarboxyYTX
1	4.21, 4.21	4.21, 4.21	29	1.58, 2.32	1.58, 2.32
2	1.99, 2.21	1.99, 2.21	30	3.64	3.64
4	4.24	4.24	31	3.22	3.22
5	1.75, 2.59	1.75, 2.59	32	3.89	3.89
6	3.10	3.10	34	3.80	3.80
7	3.35	3.35	35	1.53, 2.14	1.53, 2.14
8	1.44, 2.22	1.44, 2.22	36	4.09	4.09
9	3.17	3.17	37	3.43	3.43
10	3.15	3.15	38	2.47, 2.75	2.47, 2.75
11	1.45, 2.30	1.45, 2.30	40	3.92	3.89
12	3.03	3.03	42	5.86	5.61
13	3.12	3.12	43	6.35	5.77
14	1.47, 2.34	1.47, 2.34	44	–	2.92
15	3.35	3.35	45	3.00, 3.00	2.25, 2.46
16	3.26	3.26	46	5.91	5.82
17	1.84, 1.99	1.84, 1.99	47	5.10, 5.12	4.95, 5.09
18	1.83, 1.89	1.83, 1.89	48	1.31	1.31
20	3.46	3.46	49	1.29	1.29
21	1.80, 1.97	1.80, 1.97	50	1.20	1.20
22	3.53	3.53	51	1.07	1.07
24	1.54, 1.77	1.54, 1.77	52	1.25	1.25
25	1.50, 1.75	1.50, 1.75	53	4.84, 5.05	4.84, 5.05
26	1.73	1.73	54	1.43	1.38
27	2.81	2.81	55	5.01, 5.09	–
28	3.34	3.34			

by the method of Nagai and Kusumi,[13] reported below. This functionality was obviously positioned at C-44, although the configuration at this center still had to be established. Thus, the structure reported in formula **3**, apart from the stereochemistry at C-44, was assigned to carboxyYTX.

Negative FAB MS/MS provided additional evidence to the proposed structure **3**. The spectrum was obtained by use of the molecular-related ion at m/z = 1195 as a precursor ion and showed many intense fragment peaks. The fragmentations in this experiment are known to occur by loss of neutral species. Thus, all the fragments ions were derived from the "western" part of the molecule in which a negative charge was localized by the presence of the sulfate esters. Characteristic fragmentation, summarized in Figure 2, at specific sites of the rings provided invaluable information regarding ring size, once more confirming the similarity with YTX, and supporting the structure proposed by NMR spectroscopic data.

The final task was to establish the absolute stereochemistry at C-44, and all other chiral centres. Our strategy was based on application of the method for chiral carboxylic acids recently proposed by Nagai and Kusumi.[13] CarboxyYTX was therefore treated with *S*- and *R*-phenylglycine methyl ester (PGME) hydrochloride in DMF solution to afford the corresponding (*S*) and (*R*) PGME amides (**7** and **8**, respectively).

The stereochemical determination was based on the chemical shift differences of the protons at C-42, C-43, C-45 and C-46 [*R*-PGME amide: H-42 δ = 5.48; H-43 δ = 5.58; H_2-45 δ = 2.03 and 2.11; H-46 δ = 5.68; *S*-PGME amide: H-42 δ = 5.44; H-43 δ = 5.49; H_2-45 δ = 2.29 and 2.35; H-46 δ = 5.76; Δδ: H-42 −0.04; H-43 −0.09; H_2-45 +0.26 and +0.24; H-46 +0.08]. The Δδ (*S* − *R*) values obtained by analysis of the ^{1}H NMR spectra of the PGME amides **7** and **8** are reported in Figure 3. As pointed out by

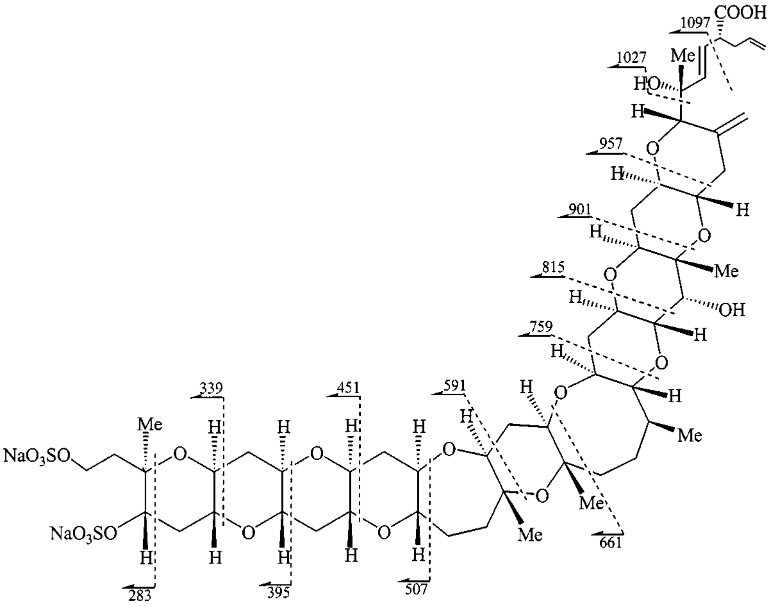

Figure 2. Characteristic fragment ions observed in the tandem mass spectrum of carboxyYTX, **3**; in the CID spectrum for the molecular ion, all the fragmentations were accompanied by loss of sulfonate (SO_3) which denotes that fragment ions are linked by one of the sulfate esters

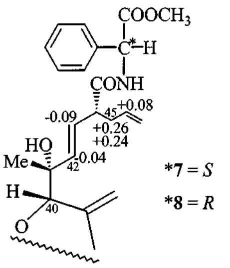

Figure 3. Δδ ($S - R$) values (in ppm) for PGME amide derivatives of compound **3**

Nagai and Kusumi in their original paper, the differences are not very marked; however, they are completely consistent and indicate the *R* configuration at the C-44 of compound **3**.

On the basis of the very reasonable assumption that compound **3** and YTX are biogenetically related and therefore have the same absolute configuration for the common part of the molecule,[14] the determination of the absolute configuration at C-44 allowed us to define the absolute stereochemistry of carboxyYTX as shown in **3**.

Mouse bioassay performed on carboxyYTX suggested that its toxicity was comparable with that of 45-OHYTX (0.5 mg/kg).

The present results confirm that YTX and its analogues are the principal toxins in mussels collected from the Adriatic Sea in recent years. An interesting aspect of this study is that the toxin profile in mussels from Adriatic Sea differs from that of other countries, where the DSP phenomenon has been studied in some detail and where this new analogue, as well as homoYTX[10] and adriatoxin,[8] recently isolated from Adriatic mussels, have (as yet) not been reported. Apparently, the phytoplankton species responsible for the production of yessotoxins in the Adriatic Sea, differ from those present in other countries. 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 standards. Therefore, much effort must be directed to the isolation of an adequate amount of toxins belonging to the yessotoxin group to allow studies relating to their mechanism of action and toxicological effects

Experimental Section

General: NMR spectra were measured on a Brüker AMX-500 spectrometer and the solvent was used as an internal standard (CD_3OD $\delta = 3.34$). FABMS were obtained at 70 eV on a Kratos MS50 mass spectrometer (CsI ions, glycerol matrix). Negative FAB MS/MS was carried out with an AutoSpecTOF mass spectrometer (glycerol matrix). Medium pressure liquid chromatographies (MPLC) were performed on a Büchi 861. High performance liquid chromatographies (HPLC) were performed on a Varian 2510 apparatus, equipped with Waters 490 MS UV and Waters 470 fluorescence detectors.

Collection, Extraction and Isolation: Specimens of the toxic mussel *Mytilus galloprovincialis* were collected along the coastal area of the northern Adriatic Sea (Cesenatico) in June 1997 at 3 m depth, which corresponded to the upper levels of mussel farms in this area. After collection, the mussels were placed in refrigerated boxes and sent to our laboratories where the hepatopancreases were removed, homogenized with a Waring blender, and stored at −20° until used. The digestive glands (1150 g) were extracted with acetone (2 × 20 liters) at room temperature. The extracts obtained after removal of the solvent were dissolved in $MeOH/H_2O$ (8:2, 3 liters), and the solution extracted with *n*-hexane (3 × 3 liters). The aqueous methanolic layer was finally partitioned between $MeOH/H_2O$ (4:6, 1 liter) and CH_2Cl_2 (3 × 1 liter). The dichloromethane-soluble material (15.2 g) was then passed through a Develosil ODS column (Nomura Chemicals, 36 × 460 mm) eluting successively with 80%, 90% and 100% of methanol/water. The toxic residue in the last eluate was chromatographed again on a Toyopearl HW-40 SF column (Tosohaas, 26 × 460 mm), with 100% methanol as eluent, which resolved the toxins into several fractions. YTXs were eluted between 300 and 400 mL. The toxic fraction containing YTX was rechromatographed on a RP-8 column (Merck, 15 × 230 mm) with 60% and 80% aqueous methanol. Final purification of the 60% methanol fraction on a RP 18 column (Phenomenex, mod. Luna, 4.6 × 250 mm, 5μ) with $CH_3CN/MeOH/H_2O$ 1:1:2 as eluent at a flow rate of 0.8 mL/min, afforded YTX (**1**, 700 μg), homoYTX (**4**, 900 μg), 45-OHYTX (**5**, 600 μg), ATX (**2**, 300 μg) and carboxyYTX (**3**, 700 μg) in pure form. YTX (**1**),[1][4] homoYTX (**4**),[10] 45-OHYTX (**5**),[11][12] and ATX (**2**)[8] were identified by comparison of MS and NMR spectroscopic data reported in the literature.

Mouse Assay: Assessment of toxicities of the samples was undertaken by the official mouse bioassay method.[9] The official method expresses toxicity by a mouse unit (MU), which is defined as the amount of a toxin, or toxins required to kill a male ddY mouse of 20 g body weight in 24 h. According to this definition, one MU corresponds to 4.0 μg of OA. Midgut glands lethal to mice above 0.5 MU/g were regarded as toxic.

Detection of Toxins: During the chromatographic purification, elution of the toxins was monitored with a UV spectrometer, by thin layer chromatography (TLC) and by mouse bioassay. Wavelengths of the UV spectrometer were set at 235 nm for OA and 210 nm for YTX. TLC was carried out on silica gel 60 plates (Merck, precoated) with $BuOH/MeOH/H_2O$ (8:1:1) or with $CHCl_3/MeOH/H_2O$ (50:10:1). Detection of the toxins was made by heating the plates after spraying with 50% sulfuric acid. Mouse bioassays were carried out following the official method for the determination of diarrhetic shellfish poisoning.[9]

CarboxyYTX (3): ^1H NMR (CD_3OD): See Table 1. FABMS (negative ions, glycerol matrix): *m/z*: 1195 $(M - Na)^-$, 1093 $(M - 2Na - SO_3 + H)^-$. HRFABMS (negative mode) *m/z*: 1195.4446 [calcd. for $C_{55}H_{80}O_{23}S_2Na$ $(M - Na)^-$ 1195.4429]. FAB MS/MS experiment (negative ions) was carried out on a AutoSpecTOF instrument with a glycerol matrix. The spectrum is shown in Figure 2.

Preparation of PGME Amides of Compound 3: A solution of compound **3** (0.3 mg, 2.5×10^{-4} mmol) in DMF (200 μL) at 0°C, was treated with (*S*)-PGME hydrochloride (0.07 mg, 1.5 equiv), benzotriazolyloxytris(pyrrolidinyl)phosponium hexafluorophosphate (PyBoP) (0.22 mg, 1.5 equiv.), 1-hydroxybenzotriazole (HOBT) (0.06 mg, 1.5 equiv.) and *N*-methylmorpholine (1 μL, 4 equiv.), and the mixture was stirred at room temperature for 3 h. After the addition of brine, the reaction mixture was extracted three times with EtOAc/benzene (2:1) and the organic phase was washed in sequence with 5 mL of 1.2 N HCl, water, saturated aqueous $NaHCO_3$, and water. The organic phase was dried over Na_2SO_4, filtered and the solvent removed under reduced pressure to give the (*S*)-PGME amide **7** (0.33 mg, 95% yield). Using (*R*)-PGME hydrochloride, the same procedure afforded the (*R*)-PGME amide **8** in the same yield. No racemization occurred during the reaction.

Compound 7: [(*S*)-PGME amide]: ^{1}H NMR: δ = 5.44 (H-42), 5.49 (H-43), 2.29 and 2.35 (H_2-45), 5.76 (H-46). $\Delta\delta$ ($S - R$) values obtained by analysis of ^{1}H NMR spectra of the PGME amides **7** and **8** are reported in Figure 3.

Compound 8: [(*R*)-PGME amide]: ^{1}H NMR: δ = 5.48 (H-42), 5.58 (H-43), 2.03 and 2.11 (H_2-45), 5.68 (H-46). $\Delta\delta$ ($S - R$) values obtained by analysis of ^{1}H NMR spectra of the PGME amides **7** and **8** are reported in Figure 3.

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