Glycoyessotoxin A, a New Yessotoxin Derivative from Cultures of *Protoceratium reticulatum*

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The dinoflagellate *Protoceratium reticulatum* produces toxins of the yessotoxin group currently included in the diarrhetic shellfish poisoning class. In this paper we report on the isolation and structural elucidation of a 32-arabinoside of yessotoxin, G-YTXA (**2**), obtained from laboratory cultures of *P. reticulatum* (strain GG1AM) that possesses a pentose unit, β-arabinofuranose, as a side chain.

Marine toxins present a variety and structural complexity unique in nature. In addition, these toxins possess a diversity of pharmacological actions that has made them an important tool for biomedical research. Consequently, the isolation and identification of new toxins, as well as understanding their mode of action, are of great interest to pharmacology, economics, and public health.[1−4]

Yessotoxins (YTXs) are one of the most recently identified groups of marine toxins. YTX (**1**) is a *trans*-fused polyether with an unsaturated side chain and two sulfate esters. It was initially isolated from the digestive glands of diarrhetic shellfish poisoning-infested scallops, *Patinopecten yessoensis*, but it actually originates from dinoflagellates of the genera *Protoceratium* and *Gonyaulax*.[5−7] Recently, YTX and related compounds have been detected in contaminated mollusks collected at the coasts of Japan, Italy, Norway, New Zealand, and Chile. Until now, the yessotoxin congeners reported within the YTX or homo-YTX series were metabolites derived by oxidation of the C-41−C-47 side chain or changes in the sulfate units of yessotoxin (**1**). Toxicological evaluation of YTX administered to mice showed that it is equally toxic when injected intraperitoneally as those algal toxins causing diarrhea. However, it is not diarrheagenic, because its activity is as much as 10-fold lower when administered orally compared with intraperitoneal injection. Ultrastructural studies revealed swelling of heart muscle cells leading to separation of the organelles.[8] Recent studies show that YTX is a powerful compound that opens the permeability transition pore of the inner mitochondrial membrane of rat mitochondria at nanomolar concentrations and causes the selective disruption of the E-cadherin-catenin system in epithelial cells. This finding raises some concern that an algal toxin found in seafood might disrupt the tumor-suppressive functions of E-cadherin.[9,10]

In this report, we describe the isolation and structural determination of a new type of analogue of YTX (**1**), G-YTXA (**2**), which possesses a carbohydrate side chain attached at carbon C-32.

The harvested cells of the dinoflagellate *Protoceratium reticulatum* were extracted with acetone. The combined extracts were subjected to gel filtration on Sephadex LH 20 ($CHCl_3$−MeOH−*n*-hexane 1:1:2) followed by C_{18} column chromatography (MeOH−H_2O, 17:3) and finally μ-Bondapak C_{18} HPLC purification with a gradient solvent system [(a) CH_3CN−H_2O−AcOH, 50:50:0.1; (b) CH_3CN−AcOH, 100:0.1] to yield YTX (**1**) (0.59 mg) and G-YTXA (**2**) (2.90 mg).

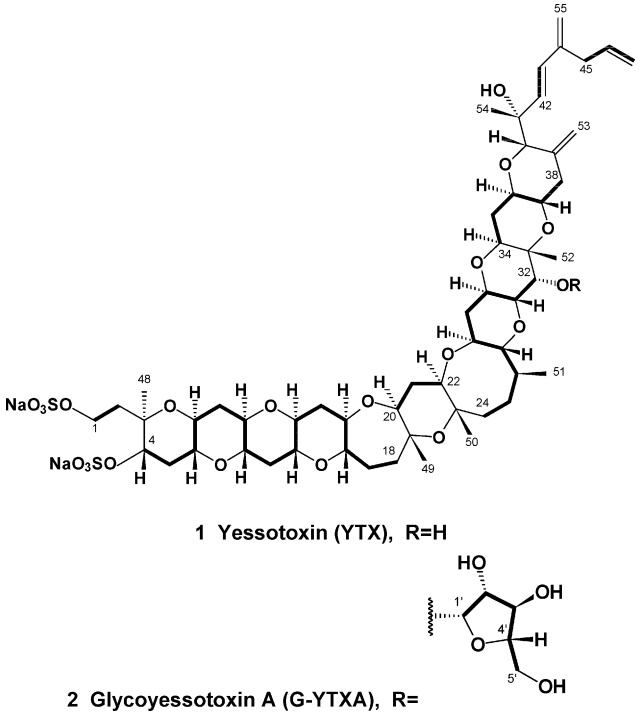

Figure 1. Bold lines show the partial structures obtained from COSY and TOCSY experiments, which were connected by quaternary carbon HMBC correlations. The relative stereochemistry between the sugar and aglycone is proposed but not shown by experimental data.

G-YTXA (**2**) has the molecular formula $C_{60}H_{88}O_{25}Na_2S_2$, as revealed by negative pseudomolecular ions by ESIMS at *m/z* 1295 $[M - Na]^-$ and 636 $[M - 2Na]^{2-}$ in the negative ion mode. NMR spectral data for compound **2**, G-YTXA, showed a considerable analogy with those for YTX, as indicated by the characteristic proton chemical shift of H-1 at δ_H 4.18. A detailed analysis of $^1H-^1H$ COSY, TOCSY, and HSQC experiments for compound **2** established the following partial structures: C-1→C-2, C-4→C-18, C-20→C-22, C-24→C-32, C-34→C-38, C-42→C-43, and C-45→C-47 (Figure 1). HMBC correlations showed the connections between the different fragments that started in the quaternary carbons. Therefore, C-3 (δ_C 76.5) showed connectivities with protons H_2-2 (δ_H 2.18 and 1.91), H-4 (δ_H 4.19),

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Table 1. ^{13}C and ^{1}H NMR Data for G-YTXA (**2**) in CD_3OD

carbon	δ_C	δ_H	carbon	δ_C	δ_H
1	65.0	4.18	31	79.2	3.25
2	40.3	2.18/1.91	32	74.5	4.18
3	76.5		33	76.8	
4	78.7	4.19	34	74.0	3.90
5	32.8	2.55/1.74	35	31.7	2.11/1.50
6	78.4	3.06	36	73.3	4.06
7	70.8	3.33	37	73.0	3.38
8	36.5	2.15/1.37	38	39.2	2.64/2.40
9	78.3	3.11	39	143.4	
10	78.3	3.10	40	85.2	3.85
11	36.2	2.23/1.38	41	78.6	
12	77.7	3.03	42	136.8	5.80
13	78.4	3.09	43	130.8	6.27
14	38.0	2.26/1.40	44	146.2	
15	81.2	3.34	45	37.9	2.95 (2H)
16	82.2	3.21	46	137.6	5.87
17	30.3	1.91/1.79	47	116.7	5.08/5.03
18	41.2	1.78 (2H)	48	16.5	1.26
19	78.6		49	24.0	1.24
20	82.7	3.44	50	20.5	1.14
21	33.0	1.89/1.76	51	22.3	1.03
22	87.5	3.44	52	16.4	1.22
23	77.0		53	115.7	5.01/4.75
24	47.0	1.74/1.48	54	26.2	1.36
25	32.7	1.72/1.46	55	116.7	5.02/4.96
26	40.7	1.71	1′	102.8	5.43
27	89.5	2.80	2′	74.3	3.93
28	84.0	3.28	3′	73.1	3.85
29	40.0	2.25/1.50	4′	87.5	4.13
30	73.7	3.73	5′	63.3	3.57/3.55

and the angular methyl H_3-48 (δ_H 1.26). The C-19 resonance (δ_C 78.6) showed correlations with H_2-18 (δ_H 1.78), H-20 (δ_H 3.44), and methyl group H_3-49 (δ_H 1.24), while C-23 (δ_C 77.0) was correlated with H-22 (δ_H 3.44), H_2-24 (δ_H 1.74 and 1.48), and methyl group H_3-50 (δ_H 1.14). In turn, C-33 (δ_C 76.8) showed correlations with H-32 (δ_H 4.18), H-34 (δ_H 3.90), and methyl group H_3-52 (δ_H 1.22), and C-39 (δ_C 143.4) was correlated with H_2-38 (δ_H 2.64 and 2.40), the olefinic methylene H_2-53 (δ_H 5.01 and 4.75), and the methine H-40 (δ_H 3.85). Finally, C-41 (δ_C 78.6) was correlated with the above-mentioned H-40, the vinylic proton H-42 (δ_H 5.80), and the methyl group H_3-54 (δ_H 1.36), and C-44 (δ_C 146.2) was correlated with the proton H-43 (δ_H 6.27), the allylic methylene H_2-45 (δ_H 2.95), and the olefinic methylene H_2-55 (δ_H 5.02 and 4.96). These correlations, together with most of the chemical shift values, confirmed a backbone for compound **2** identical to that of YTX with the differences being a downfield displacement in the proton signals for H-32 (δ_H 4.18) and H-34 (δ_H 3.90) versus δ_H 3.89 and 3.80, respectively, in YTX (Table 1). Moreover, the presence of new signals corresponding to five carbons each connected to an oxygen atom in the form of an anomeric methine, three oxymethines, and one oxymethylene suggested the presence of a sugar moiety in the molecule. This was also supported by the negative ESIMS fragmentation ion peaks at *m/z* 1163 $[M - C_5H_9O_4 + H - Na]^-$ and 1141 $[M - C_5H_9O_4 + 2H\text{-}2Na]^-$ in accordance with the loss of a pentose unit. In addition, the linkage of the sugar moiety was established at C-32 in accord with the mutual HMBC correlations between the anomeric carbon C-1′ (δ_C 102.8) with the H-32 methine (δ_H 4.18) and the methine carbon C-32 (δ_C 74.5) with the anomeric methine H-1′ (δ_H 5.43). These data suggested that **2** is a glycoside of **1** by addition of a pentose unit.

ROESY data confirmed the relative stereochemistry of all chiral centers of the aglycon. ROE correlations revealed a stereostructure of this part of the molecule identical in all respects to YTX, including the β configuration of the

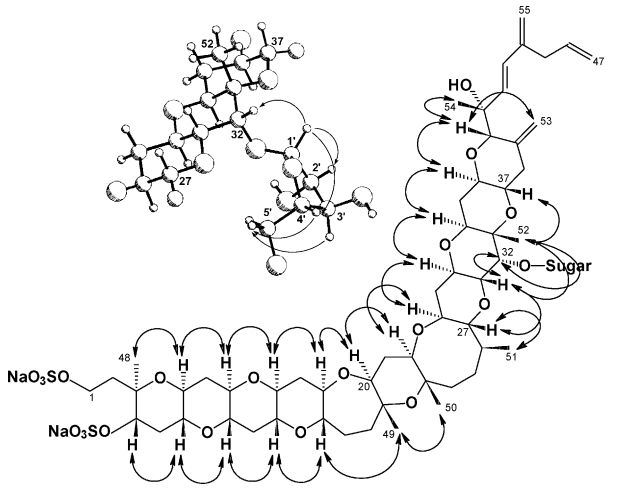

Figure 2. Significant ROE correlations for G-YTXA as well as partial structure corresponding to C-27→C-37(C-52) and carbohydrate side chain fragment.

H-32 proton as manifested by the correlations between this methine and the H-31 and H_3-52 protons (Figure 2).

The pentose sugar, the C-1′→C-5′ fragment, was identified by a 1D TOCSY experiment, and its furanose nature was indicated by the HMBC correlation between the oxymethylene carbon at C-4′ (δ_C 87.5) and the anomeric methine H-1′ (δ_H 5.43). This was confirmed by the results obtained in a ^{13}C-coupled HSQC experiment, in which each sugar peak correlation was split into two correlation peaks along the ^{1}H dimension by the large $^{1}J_{C-H}$ coupling constant.[11] Thus we obtained the following series of $^{1}J_{C-H}$ values: $J_{C-1'-H-1'}$ = 182.2 Hz, $J_{C-2'-H-2'}$ = 151.8 Hz, $J_{C-3'-H-3'}$ = 154.3 Hz, $J_{C-4'-H-4'}$ = 149.9, $J_{C-5'-H-5'}$ = 139.9 Hz, and $J_{C-5'-H-5'}$ = 140.0 Hz. These magnitudes of coupling constants indicate the presence of a furanosyl system versus a pyranosyl ring.[12−14] Furthermore, these $^{1}J_{CH}$ data provided additional information to facilitate the sugar structure. The large value of $^{1}J_{C-1'-H-1'}$ suggested a quasi-equatorial C-1′−H-1′ bond as an N-type sugar,[15−18] and the chemical shift of the anomeric carbon C-1′ at δ_C 102.8 was in accordance with a β orientation.[19,20] HMBC, ROESY, and GROESY data confirmed the final arrangement of the sugar moiety. Thus, the linkage between C-1′ and C-32, indicated by the HMBC experiment, was confirmed by the presence of a 1D ROESY cross-peak of H-1′ (δ_H 5.43) with H-32 (δ_H 4.18). In the furanose ring, the coupling constant values analysis between sugar protons ($J_{1'-2'}$ = 3.8 Hz, $J_{2'-3'}$ = 5.8 Hz, $J_{3'-4'}$ = 4.4 Hz) showed that protons H-1′, H-2′, and H-4′ were on the same face, while H-3′ must be located on the opposite face. Additional ROESY correlations of H-1′ with the protons H-2′ (δ_H 3.93, strong) and H-4′ (δ_H 4.13, weak), together with the weak correlation between protons H-3′ (δ_H 3.85) and H_2-5′ (δ_H 3.57 and 3.55), substantiate these conclusions (Figure 2). This result can be explained only if the sugar possesses a β-arabinofuranose structure.

Most of the previously reported examples of YTX derivatives were isolated from red tide episodes, although their presence in cultures of *Protoceratium* had been established. Recently, while we were finishing this work, the Shimizu group reported on the isolation from the culture medium of *Protoceratium* of the first examples of glycoside derivatives of homoyessotoxin, protoceratines II to IV;[21] our compound is the first glycoside in the yessotoxin series. The discovery of G-YTXA together with those isolated by the Shimizu group expands the possibility that new and related

metabolites presenting different carbohydrate side chains will be discovered, which substantially increases the complexity of the monitoring programs for the YTX toxin group.

Experimental Section

General Experimental Procedures. Optical rotation was determined on a Perkin-Elmer 241 polarimeter. The NMR spectra were obtained with Bruker AMX 500 and AVANCE 400 MHz instruments. Chemical shifts were reported relative to TMS, and coupling constants were given in Hz. Mass spectrometric measurements were performed using a Thermo Finnigan LCQ-Advantage ion trap mass spectrometer, equipped with electrospray ionization (ESI). ESI was performed with a 4.5 kV spray voltage and 120 °C capillary temperature, flow 60 mL/min for sheath gas and 20 mL/min for auxiliary gas. HPLC was carried out with a Waters system equipped with a differential diffractometer detector. Si gel CC and TLC were performed on Si gel Merck 60 G. TLC plates were visualized by spraying with phosphomolybdic acid 20 wt % in ethanol solution and heating.

Cultures. The GG1AM strain of *P. reticulatum* used in this study was obtained from the collection of phytoplankton cultures at the Centro Oceanográfico de Vigo. Cultures initiated with 500 cells/mL were grown in 120 L cylindrical metacrilate tanks with 100 L of L1 medium without silicates sterilized with 0.25 mL/L of sodium hypochlorite 40% for 2 h (excess of chloride was neutralized with 0.25 mL/L of sodium thiosulfate), at a salinity of 34 psu and 17 ± 1 °C under a 12:12 h light:darkness cycle with light of 100−150 μmol photons/m^2·s (PAR). For growth measurements, 5 mL samples were collected every 4 days and fixed with Lugol's solution. Cell counts were made for each sample with Sedgewick-Rafter counting slides. After a period of culture, the *P. reticulatum* cells were harvested by filtration and continuous centrifugation at 7000 rpm. The fresh cells (70 g) were sonicated and extracted with acetone. The solvent was evaporated and the resultant extract (0.86 g) chromatographed.

Chromatographic Separation. The organic extract was chromatographed by gel filtration on a Sephadex LH-20 column eluted with a mixture of $CHCl_3$−MeOH−*n*-hexane (1:1:2). The toxic fraction was rechromatographed over a medium-pressure reversed-phase Lobar LiChroprep RP-18 column using MeOH−H_2O (17:3) as eluent. The final purification was achieved by HPLC reversed-phase chromatography on a μ-Bondapak C_{18} column and gradient solvent system: (a) CH_3CN−H_2O−AcOH (50:50:0.1), (b) CH_3CN−AcOH (100:0.1), at 2.5 mL/min to yield YTX (**1**) (0.59 mg) and G-YTX (**2**) (2.90 mg).

Compound 2 (G-YTXA): $[\alpha]^{25}_D$ −6° (*c* 0.29, $CHCl_3$); ^{13}C and ^1H NMR data, Table 1; EISMS negative mode *m/z* 1295 $[M - Na]^-$, 1273 $[M - 2Na + H]^-$, 1163 $[M - C_5H_9O_4 - Na + H]^-$, 1141 $[M - C_5H_9O_4 - 2Na + 2H]^-$, 636 $[M - 2Na]^{2-}$.

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