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Structure Elucidation and Relative Toxicity of (24*R*)-24-Hydroxyyessotoxin from a Namibian Isolate of *Gonyaulax spinifera*

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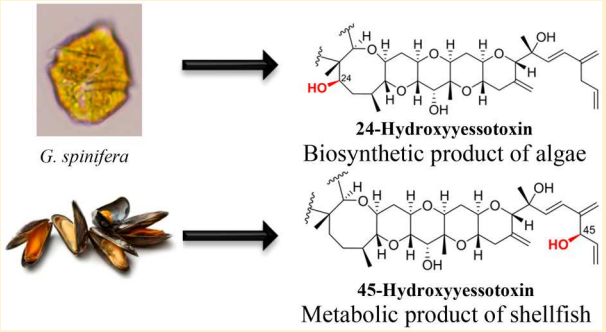
Supporting Information

ABSTRACT: Liquid chromatography−high-resolution mass spectrometry (LC-HRMS) analysis of a Namibian strain of *Gonyaulax spinifera* showed the presence of a number of yessotoxins (YTXs). Principal among these were YTX (**1**), homoYTX (**2**), and a tentative hydroxylated analogue that did not correspond to any previously confirmed YTX structures. Culturing the *G. spinifera* strain afforded sufficient biomass for purification of the new analogue through a series of solvent partitioning and chromatographic steps, yielding ∼0.9 mg as a solid. NMR spectroscopy, ion-trap mass spectrometry, and HRMS identified the new analogue as 24-hydroxyYTX (**7**). Purified 24-hydroxyYTX was quantitated by NMR, and its relative toxicity evaluated using two embryonic zebrafish toxicity assays. 24-HydroxyYTX demonstrated reduced toxicity compared to YTX.



Yessotoxins (YTXs) are a class of sulfated polyether toxins (Figure 1) produced by some marine dinoflagellates that can accumulate in filter-feeding shellfish.[1] YTX (**1**) was first isolated from shellfish,[2] and since then, various dinoflagellates have been reported to produce YTXs including *Protoceratium reticulatum* (Claparède & Lachmann) Bütschli, 1885,[3] *Lingulodinium polyedrum* (F. Stein) J. D. Dodge, 1989,[4] *Gonyaulax spinifera* (Claparède & Lachmann) Diesing, 1866,[5] and *Gonyaulax taylorii* M.C.Carbonell-Moore, 1996.[6] A number of YTX structures including 1a-homoYTX (**2**), 45-hydroxyYTX (**3**),[7] 45-hydroxyhomoYTX (**4**),[8] 44-carboxyYTX (**5**),[9] and 41a-homoYTX (**6**),[10] among others,[1] have been elucidated by NMR, while numerous other analogues have been observed but only tentatively identified by LC-MS.[11] Many YTX analogues are biosynthetic products of algae; however some variants such as **3** and **5** are metabolic products of shellfish. 45-hydroxyYTX (**3**) was first isolated and identified from Japanese scallops in 1996[12] and was subsequently detected in shellfish from a number of other locations.[7,8]

YTXs were originally classified as diarrhetic poisoning toxins due to their positive response when administered intraperitoneally in the traditional mouse bioassay for lipophilic toxins.[13] However, this toxicity is greatly diminished when YTXs are administered orally.[14] Recent studies have suggested cardiotoxicity[15] and immunotoxicity[16] in rodents via intraperitoneal administration. Due to evidence of reduced toxicity upon oral consumption, levels of YTXs regulated in seafood by the European Union were changed from a limit of 1 mg/kg to 3.75 mg/kg.[17]

Research on YTXs remains of interest despite further recommendations for less stringent regulatory limits for YTXs in seafood.[18] Several groups have studied possible synergistic effects with other lipophilic toxins. Aasen et al. reported no enhanced activity of YTX in a mouse bioassay when administered orally in combination with azaspiracid-1.[19] However, Ferron et al. reported a synergistic toxic effect on human intestinal cell models when YTX was combined with azaspiracid-1.[20] This indicates further work may be merited on the study of YTX toxicity. Continued development of improved analytical methods is necessary for detection of marine biotoxins in complex matrices, and profiling of toxin-producing algae for new analogues is also an important activity.[21,22] Methods using high-resolution mass spectrometry (HRMS) have been developed for profiling and quantitation of marine toxins including YTXs.[23,24] Furthermore, there is also interest in YTX as a synthetic target.[25,26]

In 2011 a bloom of dinoflagellates was reported in Walvis Bay, Namibia, from which a strain of *G. spinifera*, a known producer of YTXs,[27] was isolated.[28] Here we report on the purification, structure elucidation, and relative toxicity assessment of a new hydroxyYTX analogue from the Namibian *G. spinifera* strain.

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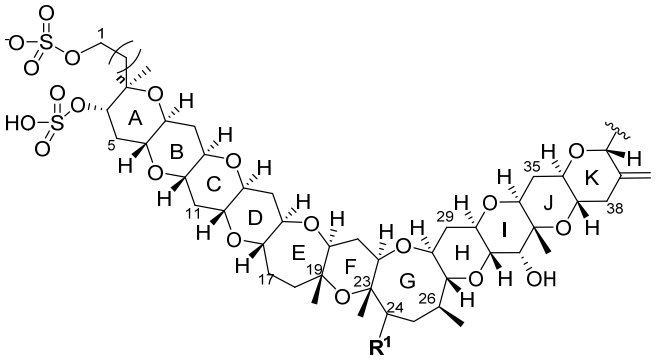


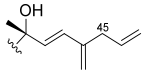
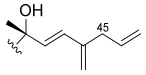
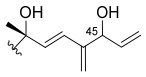
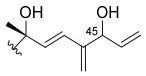
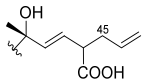
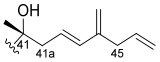
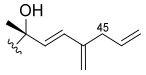
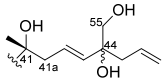
			R^1	n	$[M-H]^-$ *m/z*
1	YTX		H	1	1141.4717
2	1a-homoYTX		H	2	1155.4874
3	45-hydroxyYTX		H	1	1157.4666
4	45-hydroxy-1a-homoYTX		H	2	1171.4823
5	44-carboxyYTX		H	1	1173.4616
6	41a-homoYTX		H	1	1155.4874
7	24-hydroxyYTX		OH	1	1157.4666
8	41-ketoYTX		H	1	1047.3935
9	44,55-dihydroxy-41a-homo-YTX		H	1	1189.4929
10	TrinorYTX		H	1	1101.4404

Figure 1. Structure of YTX analogues mentioned in the text and calculated *m/z* values in negative ionization mode.

RESULTS AND DISCUSSION

LC-MS Profiling. LC-HRMS analysis of the Namibian *G. spinifera* strain revealed the presence of various YTX analogues. The most prominent were 1a-homoYTX and YTX, both confirmed by accurate mass and retention time matching with certified reference materials (CRMs), and an earlier eluting oxygenated YTX, tentatively identified as hydroxyYTX (**7**) due to its retention time (Figure 2). Tentative identification was supported by the measured accurate of *m/z* 1157.4662 $[M - H]^-$, corresponding to an oxygenated YTX ($C_{55}H_{81}O_{22}S_2^-$) (Δ −0.4 ppm). A series of minor YTX analogues were also detected with masses of *m/z* 1047.3939 $[M - H]^-$ (tentatively identified as 41-ketoYTX (**8**) by MS/MS), *m/z* 1061.4094 $[M - H]^-$ (unidentified), *m/z* 1089.4934 $[M - H]^-$ (tentatively identified as 44,55-dihydroxy-41a-homoYTX (**9**)), *m/z* 1101.4403 $[M - H]^-$ (tentatively identified as trinorYTX (**10**) by MS/MS), and *m/z* 1117.4350 $[M - H]^-$ (unidentified),[11,29] the most significant of which was 44,55-dihydroxy-41a-homoYTX.[11] Assuming equimolar response in the full-scan LC-HRMS measurements, the new hydroxyYTX analogue was ∼30% of the major component (1a-homoYTX), making it a viable target for isolation and structure elucidation. The potential presence of a hydroxylated YTX was of interest as 45-hydroxyYTX has been tentatively identified by LC-MS in an Italian strain of *P. reticulatum*[30] and in a strain of *G. spinifera* from South Africa.[31] However, 45-hydroxyYTX has been confirmed as a metabolite of YTX in shellfish.[32] The putative hydroxyYTX identified in the current work had an earlier LC retention time than the previously reported 45-hydroxyYTX, as determined through comparison with a matrix reference material containing YTXs (Figure S1).[33,34]

Structure Elucidation. Culturing of *G. spinifera*, methanolic extraction of the biomass, and multiple chromatographic steps yielded **7** as a colorless solid (∼0.9 mg). A series of LC-

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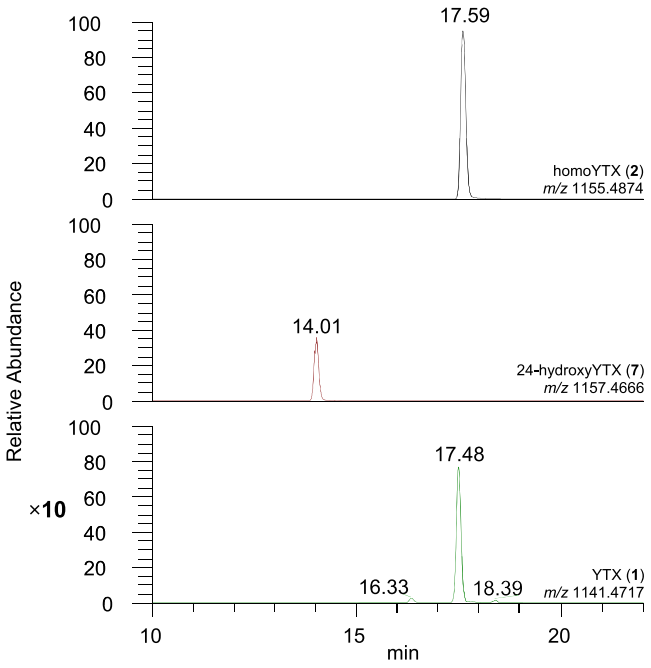

Figure 2. LC-HRMS chromatogram showing YTX analogues in a Namibian strain of *G. spinifera* (LC-MS method A).

MS experiments comparing YTX (**1**) and the new analogue (**7**) helped localize the position of the extra oxygen (Figure 3). In negative ion mode the first major fragment observed for YTXs typically corresponds to a loss of SO_3 $[M - SO_3]^-$, which corresponds to *m/z* 1061 and 1077 for **1** and **7**, respectively. The fragmentation of the $[M - SO_3]^-$ ion follows with a retro-ene reaction,[35] forming the fragment *m/z* 924 for **1**[11] and *m/z* 940 for **7**. Compared to **1**, fragments 16 Da higher were observed for **7**, *m/z* 871, 815, 759, 729, 673, and 647 corresponding to cleavages in rings K down to H (Figure 3a). Fragment *m/z* 647, corresponding to the first cleavage of ring G, marks the last +16 Da fragment. Below this point, the fragmentation diverges between **1** and **7**, indicating modification on a carbon below C-27. On ring G, the fragments observed for **7** were not consistent with those found in **1**. However, fragments *m/z* 559 and lower were present in both **1** and **7**, indicating the presence of an additional oxygen between C-24 and 26 in the form of a hydroxylated analogue (Figure 3).

The structure of **7** was established by comparing it with **1** in a series of NMR experiments (^{1}H, ^{13}C, COSY, TOCSY, ROESY, HSQC, and HMBC) on the purified compounds. In accord with the chemical formula indicated by HRMS, the ^{13}C and HSQC NMR spectra revealed the presence of 55 carbon signals for **7** (6 methyl, 17 methylene, 25 methine, and 7 nonprotonated carbons). Comparison to the signals for **1** (6 methyl, 18 methylene, 24 methine, and 7 nonprotonated carbons) indicated that a methylene had been converted to a methine, suggesting hydroxylation at C-24 or C-25.

Comparison of the ^{1}H spectra revealed a distinct shift of 26-Me (deshielded by 0.05 ppm), but no change in multiplicity, and minor shifts for 23-Me (deshielded by 0.01 ppm) and 19-Me (shielded 0.01 ppm). All the other methyls were equivalent for **1** and **7** (Figure 4a). Hydroxylation at C-26 was excluded due to the doublet splitting of 26-Me that remained unchanged (Figure 4a). The ^{1}H NMR spectra also showed major differences in the signals at 3.3−3.6 ppm (Figure 4b). Compound **1** showed a doublet of doublets at 3.49 ppm

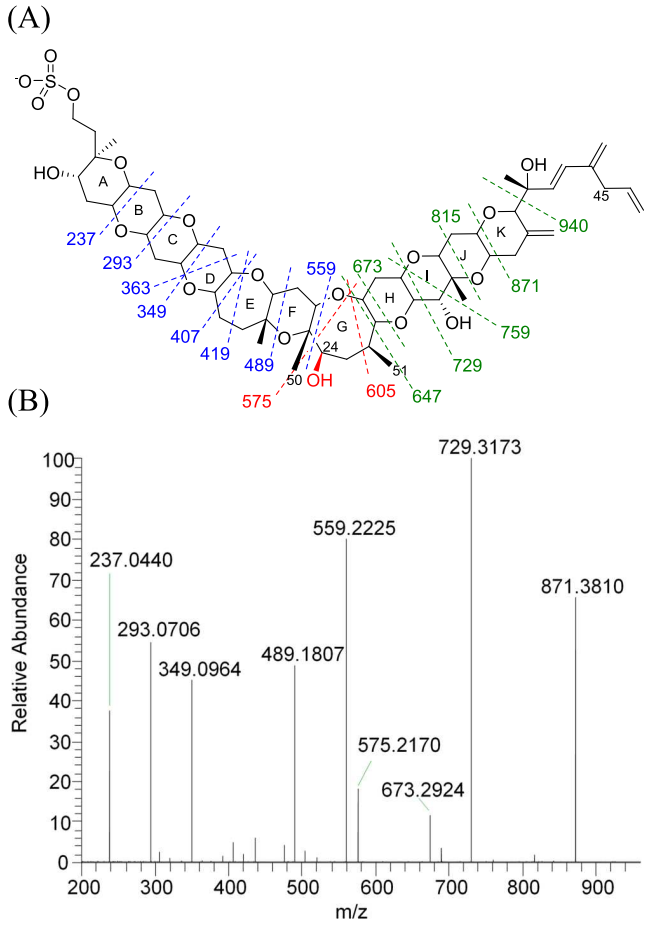

Figure 3. (a) LC-MS/MS (LTQ) negative ion fragmentation of YTX and 24-hydroxyYTX (method B). Blue fragments are consistent between YTX and 24-hydroxyYTX, green corresponds to YTX-specific fragments M+16 (= M+O), and red fragments are unique to 24-hydroxyYTX. (b) HRMS/MS fragmentation of 24-hydroxyYTX (method A, collision energy 150 eV).

corresponding to H-22;[36] however, for **7** the signal at 3.48 ppm is a doublet. 2D NMR spectra (COSY and TOCSY) revealed that the signal at 3.48 ppm in **7** did not correspond to H-22 (Figure S2). The COSY NMR spectrum of YTX revealed that the signal at 3.49 ppm (H-22; dd; J = 4.5, 11.9 Hz) correlated with signals at 1.94 and 1.73 ppm (H-21 α and H-21 β, respectively), confirming its correlation to H-22. However, the COSY NMR spectrum of **7** showed that the signal at 3.48 ppm (H-24; d; J = 10.2 Hz) correlated with signals 1.98 (H-25 β), 1.39 (H-25 α), and 1.14 ppm (23-Me). Moreover, in the TOCSY NMR spectrum of **1** the signal at 3.49 ppm (H-22; dd; J = 4.5, 11.9 Hz) correlated with signals at 3.43 (H-20), 1.94 (H-21 α), and 1.73 (H-21 β). However, the TOCSY NMR spectrum of **7** revealed that the signal at 3.48 ppm (H-24; d; J = 10.2 Hz) correlated with signals at 3.23 (H-28), 2.76 (H-27), 1.98 (H-25 β), 1.82 (H-26), 1.39 (H-25 α), and 1.08 (26-Me) ppm. Therefore, even though the signals are superimposed, they belong to two different spin systems. The peak at 3.48 ppm belongs to H-24 in **7**. C-24 in **1** is a methylene, and the protons appear as two distinct signals (1.48 ppm, 1.75 ppm, m), while C-24 in **7** is an oxygenated methine revealing a change in multiplicity and a major shift (deshielded >1.73 ppm).

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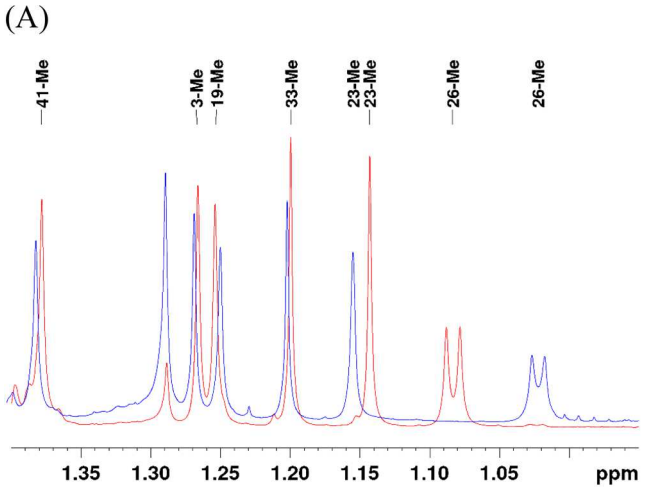

Figure 4. ^{1}H spectra (700 MHz, CD_3OD) for YTX (**1**) (blue) and 24-hydroxyYTX (**7**) (red) overlaid showing ranges for (a) 1.40 to 0.95 ppm and (b) 3.6 to 3.4 ppm.

The HSQC NMR spectrum of **1** showed correlations between the ^{13}C signal at 47.0 ppm (CH_2; C-24) and ^{1}H signals at 1.48 (m; H-24 β) and 1.75 (m; H-24 α). In **7** the ^{13}C signal for C-24 was present as a methine at 84.2 ppm and correlated only to the proton at 3.48 ppm (d; H-24), confirming that the hydroxy group is positioned on C-24.

The configuration at C-24 was determined by examination of the coupling constants and correlations observed in the ROESY NMR experiment (Figure S3). The ROESY spectrum revealed that H-24 correlated with signals at 3.39 (H-22 α), 1.39 (H-25 α), and 1.82 (H-26 α) ppm (Figure 5). There were no correlations to either 23-Me(β) or 26-Me(β). Furthermore, on the alpha face, the signal at 3.23 ppm (H-28 α) correlated with signals at 3.39 (H-22 α) and 1.82 (H-26 α) ppm. On the beta face, the signal at 1.98 ppm (H-25 β) correlated with signals 1.14 (23-Me β) and 2.76 (H-27 β). As a result, the hydroxy group must have a β orientation, thus assigning the configuration of this new analogue as (24*R*)-24-hydroxyYTX based on the known absolute configuration as YTX (**1**). The complete assignment of 24-hydroxyYTX is reported in Table 1. Comparison of YTX and 24-hydroxyYTX signals in both ^{1}H and ^{13}C experiments showed major ppm shifts in C-22 to C-26 as well as 23-Me.

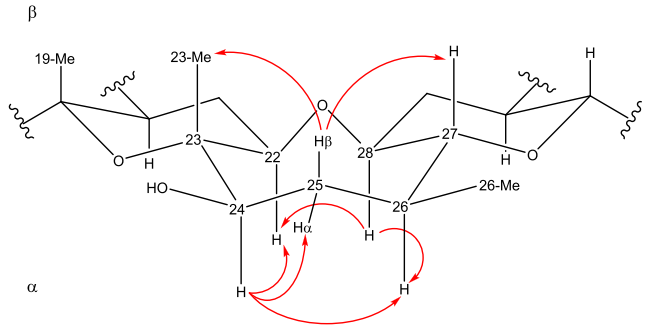

Figure 5. 3D representation of 24-hydroxyYTX and observed ROESY correlations (red arrows).

Although there have been previous observations of hydroxylated YTXs in marine microalgae[11,30] by LC-MS, this is the first specific report defining the identity of a hydroxyYTX from algae. 45-hydroxyYTX (**3**) is a known metabolite of shellfish;[32] therefore it would be unexpected for it to also be a biosynthetic product of algae. A sample of the *P. reticulatum* extract analyzed in an earlier study[30] was provided for LC-HRMS comparison against the 24-hydroxyYTX (**7**) identified in this work. No clear peak for either **3** or **7** was observed in the sample provided; however **1** and **2** were confirmed as previously reported (Figure S4). Trace levels of tentative hydroxylated YTXs were observed at retention times different from **1** and **7**; however, it was not possible to identify these due to the lower concentrations present in the sample. Recently, a strain of *G. spinifera* from Walker Bay, South Africa, was reported[31] to produce **3** as part of a YTX toxin profile similar to the Namibian strain of *G. spinifera* (**2** most significant, with lower amounts of a hydroxyYTX and **1**). The identity of the hydroxyYTX reported was determined using the EU harmonized standard operating procedure for LC-MS determination of lipophilic marine biotoxins,[37] which includes a transition for **3**. Due to relative proximity of the locations where the respective strains of *G. spinifera* were isolated, similarities in the profiles reported, and the ease of misidentification of hydroxyYTX analogues using conventional LC-MS methods in the absence of individual toxin standards, it should be considered whether the hydroxylated compound reported[31] is actually **7**.

The toxin profile of the *G. spinifera* strain studied in this work is of interest in terms of the biosynthesis of these compounds. Considering the reported biosynthesis of YTX[38] and that the most abundant analogue in this culture is homoYTX, the presence of an equivalent homo analogue of 24-hydroxy-YTX might reasonably be expected. However, no additional homoYTX analogues were detected by LC-HRMS. The presence of a mixed algal culture is unlikely, as the strain analyzed was cultured from a single-cell isolate,[39] and an equivalent toxin profile was reported by Pitcher et al.[31] in the *G. spinifera* from South Africa. Future work should consider the biosynthesis of YTXs by these particular strains of *G. spinifera.*

Relative Toxicity. To assess the toxicity of 24-hydroxyYTX (**7**), a stock solution was accurately quantitated by qNMR[40] and a reference material was prepared by making accurate dilutions in MeOH. Toxicity was compared to accurately quantitated **1** *in vivo* by means of embryonic zebrafish (*Danio rerio*) toxicity testing. Embryonic zebrafish toxicity assays have been used as an alternative model for testing of algal toxins.[41,42] As YTX toxicity has already been described using

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Table 1. NMR Spectroscopic Data (^{1}H 700 MHz, ^{13}C 175 MHz, CD_3OD) for 24-HydroxyYTX (7)

position	δ_C type	δ_H (J in Hz)	position	δ_C type	δ_H (J in Hz)
1	65.0, CH_2	4.20, m	26	36.8, CH	1.82, m
2	40.3, CH_2	1.91, 2.20, m	26-Me	22.4, CH_3	1.08, d (6.8)
3	76.6, C		27	89.0, CH	2.76, t (9.4)
3-Me	16.2, CH_3	1.27, s	28	83.5, CH	3.23, m
4	78.7, CH	4.20, m	29	39.7, CH_2	1.50, 2.25, m
5	32.8, CH_2	1.72, q (11.8), 2.56, dt (11.8, 4.5)	30	73.3, CH	3.59, ddd (4.4, 9.8, 11.7)
6	78.5, CH	3.05, m	31	79.7, CH	3.19, dd (9.8, 2.9)
7	70.6, CH	3.32, m	32	73.8, CH	3.85, d (2.7)
8	36.5, CH_2	1.39, 2.17, m	33	76.8, C	
9	78.3, CH	3.13, m	33-Me	15.3, CH_3	1.20, s
10	78.3, CH	3.13, m	34	73.3, CH	3.76, dd (12.6, 4.0)
11	36.2, CH_2	1.39, 2.25, m	35	31.7, CH_2	1.48, m, 2.10, dt (10.9, 4.2)
12	77.6, CH	3.03, m	36	73.2, CH	4.05, ddd (11.5, 9.6, 4.6)
13	78.1, CH	3.06, m	37	73.0, CH	3.38, m
14	38.0, CH_2	1.43, 2.29, m	38	39.0, CH_2	2.43, dd (12.5, 4.5), 2.71, td (12.2, 6.2)
15	81.1, CH	3.33, m	39	143.2, C	
16	82.4, CH	3.21, m	b	115.7, CH_2	4.77, s, 5.00, s
17	30.2, CH_2	1.79, 1.95, m	40	85.1, CH	3.86, s
18	40.8, CH_2	1.83, 1.90, m	41	78.4, C	
19	78.5, C		41-Me	26.2, CH_3	1.38, s
19-Me	23.4, CH_3	1.25, s	42	136.7, CH	5.81, d (16.0)
20	82.2, CH	3.40, m	43	130.6, CH	6.28, d (16.0)
21	32.5, CH_2	1.78, 1.96, m	44	145.5, C	
22	84.0, CH	3.39, m	44-methylene	116.6, CH_2	4.96, s, 5.04, s
23	79.6, C		45	37.8, CH_2	2.96, dd (6.6, 1.6)
23-Me	15.1, CH_3	1.14, s	46	137.6, CH	5.87, ddt (16.8, 10.1, 6.5)
24	84.2, CH	3.48, d (10.2)	47	116.6, CH_2	5.06, m, 5.08, dd (1.7, 1.6)
25	41.5, CH_2	1.39, 1.98, m			

this model[41] and shown to affect cardiovascular function in other animal models,[15] both a zebrafish embryonic developmental toxicity assay and a zebrafish embryonic cardiotoxicity model were used to assess both **1** and the new **7**. Exposure of developing zebrafish embryos to both **1** and **7** caused an edema phenotype (Figure S5), as reported in the previous study.[41] However, **7** was slightly but significantly less toxic in this assay, with a resultant EC_{50} of 51 nM (95% confidence interval, 42−62) as compared to an EC_{50} of 7.0 nM (95% confidence interval, 3.5−14) for **1**. Toxicity curves are shown (Figure 6a). Both compounds exhibited bradycardia in the zebrafish model, consistent with what has been observed with YTX in the rat model.[15] Compounds **1** and **7** both reduced heart rate relative to carrier controls (Figure 6b). However, as observed in the zebrafish embryo toxicity model, **7** was not as toxic.

CONCLUSIONS

(24*R*)-24-HydroxyYTX was isolated from a Namibian strain of *G. spinifera* and structurally elucidated through a combination of NMR and LC-MS experiments. Embryonic zebrafish toxicity assays demonstrated that this new YTX analogue was less toxic than YTX. The production of hydroxylated analogues of YTXs, both as biosynthetic products of microalgae and as metabolic products of shellfish feeding on YTX-producing algae, requires consideration when assessing YTX uptake and transformation in seafood, and when performing regulatory monitoring for hydroxylated YTXs in seafood. This also highlights the challenge when assigning tentative identities to new toxin analogues on the basis of LC and MS experiments alone, a situation that is equally applicable to other toxin classes.

This work expands knowledge on the occurrence of toxin-producing harmful algal species in Western Africa, which is of importance considering the increased international exploitation of seafood as a commodity for human consumption.

EXPERIMENTAL SECTION

General Experimental Procedures. All NMR spectra were obtained with a Bruker Avance III 700 MHz instrument. Experiments for structure elucidation were performed in CD_3OD at 20 °C with a 1.7 mm TXI cryoprobe. Chemical shifts were reported relative to the methyl of CHD_2OD (^{1}H 3.31, ^{13}C 49.00). Standard Bruker pulse sequences were used for ^{1}H, ^{13}C, COSY, TOCSY (120 ms), HSQC, HMBC, and ROESY. 24-HydroxyYTX (**7**) was dissolved in ∼30 μL of CD_3OD. A sample of YTX (**1**) (∼0.2 mg)[36] was dissolved in ∼30 μL of CD_3OD. Quantitative NMR experiments were performed by dissolving the purified **7** in 700 μL of CD_3OD and running at 20 °C with a 5 mm TXI cryoprobe using benzoic acid as an external standard.[40] LC-MS (method A): The YTX profile of the Namibian *G. spinefera* culture was assessed by LC-HRMS using a Q Exactive HF Orbitrap mass spectrometer (ThermoFisher Scientific) equipped with a heated electrospray ionization interface (HESI) connected to an Agilent 1200 G1312B binary pump, G1367C autosampler, and G1316B column oven (Agilent). Separations were performed on a Phenomenex Luna C_{18} column (50 × 2 mm, 1.7 μm) eluting with 25−75% B over 25 min, hold (5 min), and re-equilibration (back to 25% over 10 min) (A: 5 mM NH_4Ac in deionized H_2O; B: 5 mM NH_4Ac in 95% CH_3CN) at 150 μL min^{-1}. The MS was operated in negative ion mode and calibrated in the range m/z 150−2000. The spray voltage was 2.70 kV, the capillary temperature was 350 °C, and the sheath and auxiliary gas flow rates were 40 and 15 (arbitrary units), respectively. The MS was operated in negative full scan (FS) mode: scan range m/z 500−1500, resolution setting at 60 000, AGC target 3 × 10^6, max IT 200 ms; all ion fragmentation: scan range m/z 100−1500, resolution setting at 60 000, AGC target 3 × 10^6,

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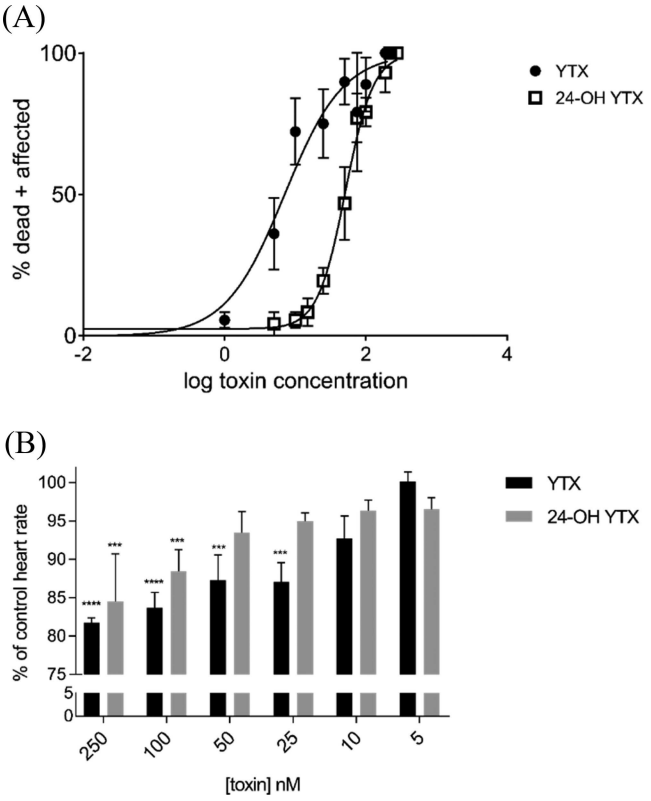

Figure 6. (A) Dose−response curve for YTX (●) and 24-hydroxyYTX (□) in the zebrafish embryo toxicity test. SEM values for each concentrated tested are plotted ($n \geq 4$). Linear regression analysis was used to fit dose−response curves to the data sets and determine EC_{50} values. (B) Heart rate toxicity analysis. SEM values normalized to the heart beats within 15 s observed in carrier control are shown ($n \geq 3$). Replicate means were analyzed by two-way ANOVA followed by a Dunnett's multiple comparison test (values significantly different than carrier controls indicated by $****p < 0.0001$, $***p < 0.0002$).

maximum IT 200 ms, normalized collision energy (35 eV); and parallel reaction monitoring mode for MS/MS spectra: m/z 1141.5, 1155.5, 1157.5, with resolution setting at 30 000, AGC target 2×10^5, max IT 100 ms, isolation window 4.0 m/z, and collision energy of 150 eV. LC-MS (method B): Fragmentation patterns were assessed using linear ion trap mass spectrometry (Thermo Scientific LTQ-XL) with an HESI source in negative mode. Instrument calibration was performed according to the manufacturer's recommendations, and instrument default settings were used unless otherwise stated. Test solutions dissolved in methanol were introduced by infusion at 5 μL min^{-1}. The instrument was tuned using CRM-YTX. The HESI source voltage was 3 kV, temperature at 40 °C, sheath gas at 15 arbitrary units, aux and sweep gases off. Data for **1** and **7** were acquired using normal scan rate in full scan from m/z 150 to 2000, from which target ions were isolated. Once isolated, CID normalized collision energy was increased until sufficient fragmentation was achieved, and additional ions were isolated for further fragmentation as required. Method C: Analysis of fractions during the purification process was performed by selected reaction monitoring (SRM) on an Agilent 1200 HPLC connected to a Sciex API 4000 Qtrap. Chromatography conditions were as described for method A. Mass spectrometry conditions: source temperature 350 °C, DP −60 V, and CE −65 eV for a range of SRM transitions corresponding to known YTX analogues: m/z 1155 > 1075; 1047 > 967; 991 > 911; 1203 > 1123; 1189 > 1109; 1175 > 1095; 1169 > 1089; 1191 > 111.; 1187 > 1107; 1173 > 1093; 1290 > 1210; 1304 > 1224; 1085 > 1005; 1171 > 1091; 1157 > 1077; 1101 > 1021; 1141 > 1061.

Toxins and Other Materials. Distilled H_2O was further purified using a UV purification system (Thermo Scientific) or a Milli-Q water purification system (Millipore Ltd.). MeOH and CH_3CN (Optima LC-MS grade) were from Fisher Scientific. Hexanes, chloroform, and butanol were from Caledon. CD_3OD (99.8%) was from Cambridge Isotope Laboratories. Ammonium hydroxide, calcium chloride dihydrate, HEPES buffer, magnesium sulfate heptahydrate, potassium chloride, and sodium chloride were from Sigma−Aldrich. Sephadex LH-20 was from Amersham Biosciences, and C18 silica (40 μm) was from Bakerbond. YTX (CRM-YTX-c (lot # 20151125)) and in-house purified YTX), homoYTX (CRM-hYTX (lot # 20111102)), and a freeze-dried mussel tissue (CRM-FDMT1)[34] were from the National Research Council Canada (Biotoxin Metrology). YTX used for NMR and LTQ experiments was purified in-house from material provided for developing the original CRM-YTX.

Culturing of *Gonyaulax spinifera*. A clonal isolate of *G. spinifera* obtained from Namibia, Africa,[28] was grown in L1 medium in 250 mL flasks at 18 °C under a 14:10 h light:dark photoperiod. An approximate photon flux density of 80−100 μmol quanta m^{-2} s^{-1} cool white light was maintained. Light was measured outside the flask using a Li-Cor model LI-185B quantum/photometer. Cultures were scaled-up from 100 mL stock cultures to 300 L in a photobioreactor under the same environmental conditions using full-strength L1 medium and harvested in late exponential growth phase by gravity filtration through a 10 μm Nitex mesh sieve. Cells were concentrated by centrifugation at 2100*g* for 15 min at 4 °C in 200 mL polypropylene centrifuge tubes to yield 85 g of biomass.

Isolation of YTXs. Biomass (85 g wet) was extracted by sonication with MeOH (3 × 250 mL) on ice and centrifuged. The methanolic supernatants were combined and evaporated *in vacuo*. The residue (5.4 g) was dissolved in 70% MeOH/H_2O (100 mL) and partitioned with hexanes (3 × 100 mL). The MeOH/H_2O fraction was evaporated *in vacuo* and dissolved in H_2O (150 mL) for partitioning with $CHCl_3$ (3 × 100 mL) and BuOH (3 × 100 mL). The $CHCl_3$ and BuOH fractions were combined, and the solvent was removed *in vacuo*. The residue (2.2 g) was dissolved in a minimum volume of MeOH and subjected to two successive Sephadex LH-20 columns (3 × 57 cm) eluting with MeOH (no pressure, ~2 mL min^{-1}). Fractions of 3.5 min were collected and YTXs eluted in fractions 15−49. Following analysis by LC-MS (method C), selected fractions were combined and evaporated to dryness (337 mg). The fraction containing YTXs was subjected to a C_{18} flash chromatography column (1.2 × 13 cm) eluting with 100 mL volumes in a step gradient (10, 20, 30, 40, 50, and 60% MeOH in H_2O; N_2 pressure: 15 psi). Fractions of 1.3 min were collected. Hydroxylated YTXs eluted in fractions 71−84, and hYTX (**2**) and YTX (**1**) coeluted in fractions 85−168. Selected fractions were combined and evaporated to dryness. The 24-hydroxyYTX fraction was subjected to HPLC purification (Luna C_{18}; 3 μm; 250 × 4.6 mm, Phenomenex) eluting with A [H_2O (5 mM NH_4Ac)]/B (CH_3CN) in a step gradient (37% B for 15 min; 47% B for 10 min; 70% B for 10 min; and equilibration) at 0.85 mL min^{-1}. UV was monitored at 210, 230, and 254 nm. 24-HydroxyYTX eluted at 20.5 min to afford 0.87 mg (determined by quantitative ^1H NMR) of pure **7**.

*24-HydroxyYTX (**7**):* ^1H (700 MHz) and ^{13}C (175 MHz) NMR data (CD_3OD), Table 1; HRMS m/z 1157.4662 $[M - H]^-$ (calcd for $C_{55}H_{81}O_{22}S_2$, 1157.4666).

Larval Zebrafish Toxicity Assays. All larvae were derived from breeding AB/Tub hybrid wild-type zebrafish (*Danio rerio*) housed following Canadian Council for Animal Care (CCAC) guidelines in a ZebTec recirculating water aquaria system (Tecniplast USA, Exton, PA, USA). The aquaria were kept at a constant 28.5 °C under a 14 h light:10 h dark lighting cycle. In the zebrafish embryo toxicity assay zebrafish embryos at 6 h postfertilization (hpf) were placed in 150 μL of HE3 media (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2 \cdot 2H_2O$, 0.33 mM $MgSO_4 \cdot 7H_2O$, 10 mM HEPES, pH 7.2) in a well of a flat-bottom 96-well polystyrene plate with one embryo per well. The exposure was initiated by adding 150 μL of a 2× solution of the desired concentration of either **1** or the purified **7** in HE3 media with 1% MeOH (v/v). Experimental replicates consisted of 12 embryos

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tested at each concentration (250, 100, 50, 25, 10, and 5 nM) of YTX or 24-hydroxyYTX. For all dilutions the MeOH concentration were held constant at 0.5% (v/v). Plates were sealed with a clear-transparent film (Thermoseal RTS, Excel Scientific) to prevent evaporation and incubated at 28.5 °C on a 14 h:10 h light:dark photoperiod. Lethality (as defined by absence of heart beat) and phenotypic observations were made every 24 h under a stereomicroscope until larvae reached 120 hpf. Values of % dead + % phenotypically affected at 120 hpf ($n \geq 4$) were plotted against the log of the toxin concentration to determine the EC_{50} using a nonlinear regression analysis with corresponding 95% confidence intervals.

For the larval heart rate toxicity assay zebrafish embryos were manually dechorionated using Dumont #3 forceps (Fisher Scientific) at 24−26 hpf and allowed to recover in HE3 media at 28.5 °C for at least 2 h. At 29 hpf the dechorionated larvae were incubated in toxin in a 96-well flat-bottomed polystyrene plate as above and incubated at 28.5 °C on a 14:10 h light:dark photoperiod. At 52 hpf the plates containing larvae and toxin were removed from the incubator, the film removed, and the plate allowed to sit upon the lit stage of a dissecting stereomicroscope for 1 h to come to room temperature. Heart beats within a 15 s interval were manually counted using the stereomicroscope for at least three larvae per concentration tested. A percentage of control heart rate for each experimental replicate was calculated by dividing the mean of the heart rates observed for each concentration of toxin by the mean of the heart rate observed for the carrier control [HE3 media w/0.5% methanol (v/v)] for each plate. At least three replicates were performed for every concentration of YTX and 24-hydroxyYTX tested. A two-way ANOVA with Dunnett's multiple comparison test was used to test significance between the values obtained for every toxin concentration and the carrier control.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.9b00318.

LC-HRMS/MS chromatogram showing YTX profile in Namibian *G. spinifera*; comparison of COSY and TOCSY spectra for **1** and **7**; ROESY spectrum between 3.6 and 3.4 ppm; LC-MS/MS chromatograms showing extracted traces of hydroxyYTX and YTX from reference materials and an Italian *P. reticulatum* extract; representative phenotypes of zebrafish larvae exposed to dilution of either **1** or **7**; NMR spectra for **7** (^{1}H, ^{13}C, COSY, TOCSY, HSQC, HMBC, and ROESY); NMR spectra for **1** (^{1}H and ^{13}C); LTQ MS fragmentation pathways of **1** and **7**; NMR spectroscopic data for **1** and **7** (PDF)

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Notes

The authors declare no competing financial interest.

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