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Convenient Large-Scale Purification of Yessotoxin from *Protoceratium reticulatum* Culture and Isolation of a Novel Furanoyessotoxin

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Yessotoxins from a large-scale culture (226 L) of *Protoceratium reticulatum* strain CAWD129 were harvested by filtration followed by solid-phase extraction. The extract was purified by column chromatography over basic alumina and reverse-phase flash chromatography to afford pure yessotoxin (193 mg). Isolation of yessotoxin was greatly facilitated by selection of a strain which did not produce analogues that interfered with yessotoxin isolation. In addition to yessotoxin, numerous minor yessotoxins were detected by LC-MS in other fractions. From one of these, an early eluting minor analogue with the same molecular weight as yessotoxin and a similar mass spectrometric fragmentation pattern was isolated. This analogue was identified by NMR and mass spectrometry as a novel yessotoxin analogue containing a furan ring in the side chain. This finding reveals biosynthetic flexibility of the yessotoxin pathway in *P. reticulatum* and confirms earlier findings of production of many minor yessotoxin analogues by this alga. Production of these analogues appeared to be a constitutive trait of *P. reticulatum* CAWD129.

KEYWORDS: Yessotoxin; furanoyessotoxin; *Protoceratium reticulatum*

INTRODUCTION

Toxins originating from marine algae can accumulate in filter-feeding shellfish, sometimes causing health problems for shellfish consumers and economic hardship for shellfish producers. These toxins can also be transferred through the food chain, resulting in alternative routes of human exposure to the toxins and their metabolites and occasionally harming wildlife populations. A major goal in studying these toxins, their metabolism, disposition, and toxicology is to be able to set regulatory limits that protect public health while at the same time minimizing unnecessary closures of shellfish-harvesting areas. Progress in such studies is often severely constrained by the lack of availability of the amounts of high-purity toxin required for performing the necessary toxicological studies and for development of analytical methods.

Yessotoxins are a group of linear polycyclic polyethers that were first isolated from the Japanese scallop *Patinopecten yessoensis* (*1*), but have since been identified in a wide range of shellfish from around the world. The principal biogenic source of yessotoxins appears to be the dinoflagellate *Protoceratium reticulatum* (*2*), although yessotoxins have also been identified in the closely related *Lingulodinium polyedrum* (*3*) and detected in *Gonyaulax spinifera* (*4*). Yessotoxin (**1**) (**Figure 1**) itself is usually the most abundant analogue found in algal samples, but the situation is complicated by some strains producing large amounts of 1-homoyessotoxins (*5, 6*) and sometimes large numbers of minor yessotoxin analogues (*7*). These toxins are absorbed by mussels and other shellfish and converted to metabolites over time, resulting in a complex toxin profile (*8, 9*). Toxicological assessment suggests that although yessotoxins are very toxic by intraperitoneal injection, they possess very low oral toxicity (*1, 10–12*). However, this work is complicated by the apparent instability of pure yessotoxin under certain storage conditions (*13*), the presence of numerous metabolites in natural samples (*5, 7, 9, 14–28*) and, until recently, the poor availability of pure yessotoxin (*22*).

We have developed improved culturing and isolation procedures leading to convenient large-scale production of high-purity

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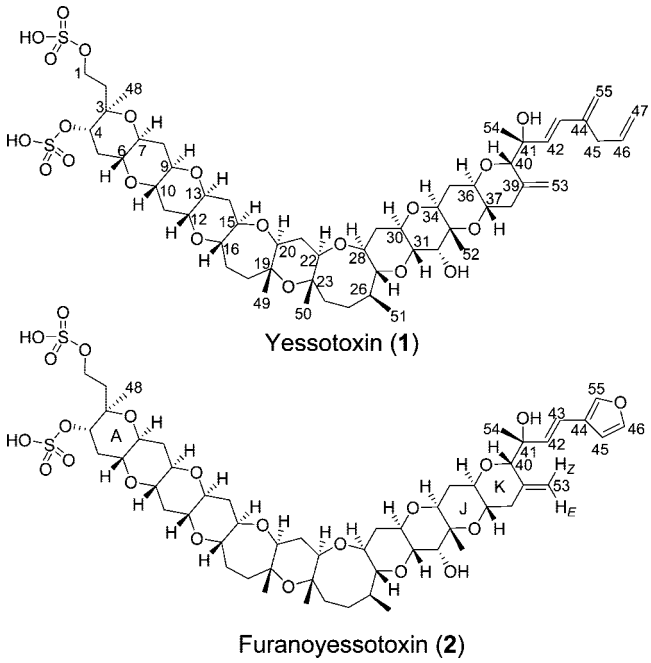

Figure 1. Structures of yessotoxin (**1**) and furanoyessotoxin (**2**) depicted in their sulfonic acid forms.

yessotoxin. We anticipate that this will greatly facilitate future biological and chemical studies of yessotoxins. In addition, furanoyessotoxin (**2**) with the same molecular weight as **1** but possessing an unprecedented furanyl group in the side chain attached at C-40 was isolated.

MATERIALS AND METHODS

General. Flash chromatography was performed on a 19 × 4 cm, 40–63 μm LiChroprep RP-18 column (Merck, Darmstadt, Germany). Solvents were of analytical or HPLC grade. Milli-Q water was used for separation and purification steps. ELISA analysis for yessotoxins was performed as described by Briggs et al. (*29*). Samples of culture for analysis were thawed and sonicated for 30 s, and 50 mL was extracted by solid-phase extraction (SPE) on 500 mg, 6 mL Strata-X SPE cartridges (Phenomenex, Torrance, CA). The SPE columns were then rinsed with water and yessotoxins eluted with MeOH (10 mL) for analysis by LC-UV-MS^3 and ELISA. Quantitative analyses were performed against a secondary standard of yessotoxin from Cawthron Institute (Nelson, New Zealand) prepared in an earlier study (*22*). The standard, held in methanolic solution since the original isolation, showed no signs of decomposition and had been verified against a subsequently prepared certified reference material of yessotoxin (IMB NRC, Halifax, Canada) (P. McNabb, personal communication).

Algal Culturing and Harvesting. The strain used was *P. reticulatum* CAWD129 from the Cawthron Institute Culture Collection (http://www.cawthron.org.nz) isolated from the western coast of South Island, New Zealand. Culture size was increased to approximately 226 L in batch culture, grown in GP+Se medium in 14 L polycarbonate carboys with a 12/12 h photoperiod. The growth curve was followed with weekly cell counts on two representative culture vessels. Samples (100 mL) for toxin analysis were taken simultaneously with cell counts and preserved in glass bottles at −20 °C until analysis for yessotoxins. A composite sample from all culture vessels was also taken at harvest for analysis.

The stationary-phase culture was harvested at 43 days. Culture medium was filtered through a DFT Classic 10 μm wound glass filter (product G010A10S; Pall Corp., East Hills, NY), followed by a fine 125 mm GS-25 glass fiber flat filter (Advantec Toyo Kaisha, Tokyo, Japan), and the filtrate was pumped slowly through a column packed with 1 kg of Diaion HP20 resin. The resin was rinsed with water and drained dry. Toxins were extracted by immersing the resin in acetone (1.5 L) for 24 h at 4 °C with occasional swirling, followed by filtration on a Büchner funnel. This process was repeated two further times with the recovered resin. The three extracts were combined and evaporated in vacuo to give a pale yellow aqueous solution (ca. 900 mL), which was stored at −20 °C until required for isolation (ca. 2 weeks).

Analytical HPLC. HPLC-UV analysis of fractions was performed using a 250 × 4.6 mm i.d., 5 μm, Prodigy ODS(3) HPLC column, with a 4 × 3 mm guard column (Phenomenex). The mobile phase was MeCN/0.01 M ammonium acetate (28:72; 1 mL/min). Eluting yessotoxins were detected with a Hewlett-Packard 1040 M diode array UV detector scanning at 200–400 nm.

LC-UV-MS^3 Analysis. LC-UV-MS^3 analysis was performed on an LCQ Deca ion trap mass spectrometer fitted with an ESI interface (ThermoQuest, Finnigan, San Jose, CA) coupled to a Surveyor HPLC and PDA detector. The column was a 150 × 2 mm i.d., 5 μm, Prodigy ODS(3) (Phenomenex). A 0.2 μm in-line filter (Alltech, Deerfield, IL) was installed before the analytical column, and the temperature of the column oven was maintained at 35 °C. Gradient elution was performed using MeOH/0.1% aqueous ammonium formate (3:17) containing 0.1% formic acid (solvent A) and 100% MeOH (solvent B). Linear gradients were run from 70% B to 100% B over 10 min, held for 3 min, and then reset to the initial conditions. The flow rate was 200 μL/min, the injection volume was 10 μL, and the PDA detector scanned from 200 to 600 nm. MS data were acquired in negative mode using a data-dependent LC-MS^n method. The ESI voltage, capillary temperature, sheath gas pressure, and auxiliary gas were set at 4 kV, 275 °C, 35 psi, and 0 psi, respectively.

HR-MS. High-resolution mass spectrometry (HR-MS) was performed in negative ion mode on a Bruker Daltonics MicrOTOF spectrometer. The samples were dissolved in 4:1 MeOH/water containing 1% formic acid and infused via a syringe pump at 4 μL/min. Cluster ions from sodium formate (2 mM) were used for mass calibration. Mass spectra were acquired with a time-of-flight analyzer from *m*/*z* 500 to 1500. A standard solution of yessotoxin was checked prior to analysis of furanoyessotoxin. Capillary voltage and skimmer cone voltage were set at −120 and −40 V, respectively.

Purification of Yessotoxin (1). The aqueous solution (ca. 900 mL) containing yessotoxin was dried in vacuo to give a brown oily residue. The residue was applied to a 13 × 2.5 cm column of ICN Alumina B, Super I, activity III, pre-equilibrated with CH_2Cl_2/MeOH (1:1). The column was successively eluted with CH_2Cl_2/MeOH (1:1, 300 mL), MeOH (100 mL), and finally NH_4OH (1%)/MeOH (1:1, 240 mL). The methanolic ammonia fraction was immediately dried by rotary evaporation to yield a pale yellow residue.

The residue was taken up in 350 mL of water and washed with EtOAc (200 mL), and the water was extracted with BuOH (3 × 200 mL). The BuOH fraction was dried with anhydrous Na_2SO_4, filtered, and evaporated in vacuo. The residue was applied to a flash column pre-equilibrated with 40% MeOH and eluted with a stepwise gradient of 40, 45, 50, and 55% MeOH (500 mL each), 60% MeOH (250 mL), and 100% MeOH (100 mL); 50 mL fractions were collected except for the final fraction (100 mL). All fractions were analyzed by HPLC and LC-MS. Fractions 31−41 contained the majority (ca. 90%) of the yessotoxin.

Fractions 31−41 were combined and applied to a flash column pre-equilibrated with 35% MeCN and eluted successively with 35 and 40% MeCN (500 mL), 45% MeCN (300 mL), and 100% MeCN (100 mL); 50 mL fractions were collected except for the final fraction (400 mL). HPLC analysis showed that the yessotoxin eluted in fractions 3−13 with the majority (ca. 93%) of the yessotoxin in fractions 3–7.

Fractions 3–7 were combined and applied to a flash column pre-equilibrated with 25% MeCN and eluted successively with 25, 27.5, 30, and 35% MeCN (500 mL) and 100% MeCN (150 mL); 50 mL fractions were collected except for the final fraction (400 mL). HPLC analysis showed yessotoxin to have fully eluted by the final 30% MeCN fraction (fraction 27), with the majority detected in fractions 18−25 (ca. 96%). These fractions were evaporated in vacuo to afford a white residue, which was passed through a small column containing Chelex 100 cation exchange resin, sodium form, in MeOH (2 × 5 mL), blown down under a stream of warm nitrogen, and lyophilized to yield pure

yessotoxin disodium salt as a white powder (192.53 mg) when weighed in a sealed vial after vacuum-drying at room temperature. HR-MS [M − H]$^-$ *m/z* 1141.4720, calcd *m/z* 1141.4706 for $C_{55}H_{81}O_{21}S_2^-$ (1 ppm).

Purification of Furanoyessotoxin (2). LC-MS analyses of fractions 16–18 from the initial flash column revealed the presence of an early eluting yessotoxin-like compound (**2**) exhibiting the same molecular ion and essentially the same fragmentation as **1** (*m/z* 1141.4 [M − H]$^-$). These fractions were combined, evaporated in vacuo, and reconstituted in MeOH for HPLC purification.

Purification of **2** was achieved by semipreparative HPLC on a 250 × 10 i.d. mm, 5 μm, Prodigy ODS(3) HPLC column fitted with a 4 × 3 mm guard column (Phenomenex). The mobile phase was a 3:1 mixture of MeOH/MeCN (2:1) and 0.01 M ammonium acetate, at 4 mL/min. Eluting yessotoxins were detected with a Hewlett-Packard 1040 M diode array UV detector scanning at 200–400 nm, and injection volumes were typically 100 μL. HPLC fractions containing the compound of interest were combined, evaporated in vacuo, and found to be sufficiently pure for full NMR structural characterization. HR-MS [M − H]$^-$ *m/z* 1141.4377, calcd *m/z* 1141.4342 for $C_{54}H_{77}O_{22}S_2^-$ (3 ppm).

NMR Spectroscopy. NMR spectra were obtained from solutions of purified yessotoxin (**1**) (9 mg) and furanoyessotoxin (**2**) (ca. 600 μg) in CD_3OD (99.8+ atom % D; Aldrich) with a Bruker 400 MHz spectrometer fitted with a 5 mm dual inverse probe. NMR data were processed using Topspin V1.3 (Bruker Biospin 2005). Chemical shifts were determined at 30 °C and spectra calibrated relative to internal CHD_2OD (3.31 ppm) and CD_3OD (49.0 ppm). Resonances observed for **1** were entirely consistent with those reported previously for yessotoxin (*22*).

RESULTS AND DISCUSSION

During recent preparation of a reference material, ca. 75% of lyophilized yessotoxin disodium salt from a previous study (*22*) had been observed to have decomposed (M. A. Quilliam, and J. Walter, personal communication) after 2 years of dry storage at −20 °C, to give a complex mixture of yessotoxin-like degradation products (unpublished observations). Material from the same batch, but stored over the same period in MeOH for use as a working standard for LC-MS analysis, had not undergone detectable decomposition (P. McNabb, personal communication). An earlier sample of yessotoxin isolated from mussels for use as a reference standard, and stored dry under a range of conditions, decomposed completely within 2 years (D. J. Stirling and L. R. Briggs, personal communications; unpublished observations), although no decomposition had been detectable at a 6 month check with LC-MS (D. J. Stirling, personal communication). Therefore, in the present study the length of time that yessotoxin and its analogues were stored in the dry state was kept to a minimum throughout the procedures, and final storage was as a solution in MeOH at −20 °C. We suggest this as a general precaution to be taken with purified yessotoxin analogues until the factors involved in their decomposition have been fully identified. We also urge researchers to exercise caution when interpreting toxicological or analytical results obtained using specimens of yessotoxin analogues that may have been stored for more than 6 months in the dry state.

To continue chemical and biological investigations, further supplies of yessotoxin were required to replace the decomposed material. The previous large-scale isolation of yessotoxin from *P. reticulatum* (*22*) was difficult due to the presence of relatively small amounts of closely coeluting cocontaminants, which were subsequently identified as 41a-homoyessotoxin, 9-methyl-41a-homoyessotoxin, and nor-ring-A-yessotoxin (*23*). Numerous other minor yessotoxin analogues were also present in the culture extract (*7*), although these eluted earlier and did not interfere with isolation of yessotoxin, and similar analogues were observed in a culture of *P. reticulatum* from the Oslofjord, Norway (*25*). The aim of this study was to develop a more convenient procedure suitable for large-scale isolation of yessotoxin and to use the method on bulk cultures to produce yessotoxin for preparation of reference standards and toxicological studies.

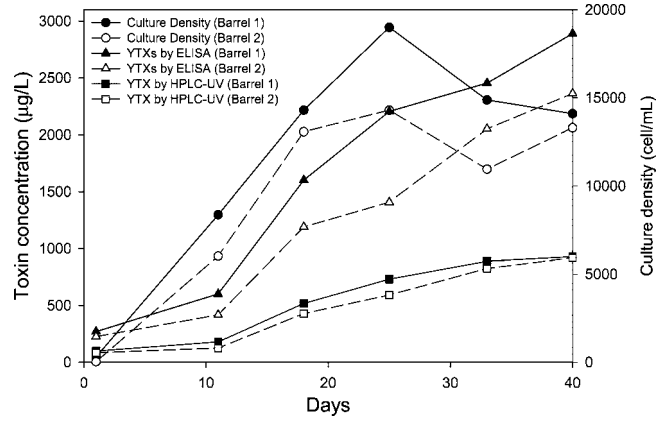

Figure 2. Growth and production of yessotoxin(s) in two representative barrels during large-scale culture of CAWD129. Data for barrel 1 are shown with solid symbols and solid lines, whereas data for barrel 2 are shown with open symbols and dotted lines. Concentrations of total yessotoxins measured by ELISA are shown with triangles, and concentrations of yessotoxin (**1**) measured by HPLC are shown with squares, using the scale on the left-hand side of the graph. Culture densities are shown with circles using the scale on the right-hand side of the graph.

Table 1. Cell Quota of Yessotoxins (by ELISA) and Yessotoxin (by HPLC-UV) with Time for Two Representative Barrels from the Bulk Culture of *P. reticulatum* CAWD129

	cell quota (pg/cell) in barrel 1		cell quota (pg/cell) in barrel 2	
day	YTXs (ELISA)	YTX (HPLC-UV)	YTXs (ELISA)	YTX (HPLC-UV)
1	871	319	6457	2376
11	72	21	69	21
18	112	36	91	33
25	116	38	98	41
33	165	60	187	75
40	205	66	178	69

A range of *P. reticulatum* strains, including CAWD40 used in the previous large-scale production of yessotoxin (*22*) and five strains from Westport, New Zealand (including CAWD129 and CAWD130) (*20*), were grown in small-scale cultures and their profiles of yessotoxins assessed by LC-UV-MS^3 analysis. What was required was a strain producing high levels of **1**, with a metabolite profile that did not include other yessotoxins eluting close to **1** that might interfere with purification of **1**, as occurred with CAWD40 in the previous study (*22*). CAWD129 was chosen for bulk culture. Bulk cultures of CAWD129 reached ca. 1.5×10^4 cells/mL at day 25 and were harvested at day 43 (**Figure 2**). Growth rates were 0.3–0.5 division per day during the exponential phase. The cell quota of yessotoxins and **1** was anomalously high on day 1 (**Table 1**), presumably due to cell damage and release of toxins during inoculation. However, the apparent cell quotas of yessotoxins and **1** were much lower by day 11, after which they slowly increased throughout the culture period (**Table 1**). Some of this increase may be due to release of yessotoxins from the cells into the medium (*3*), but no attempt was made to determine the toxin content within the

cells because the present experiment was aimed at maximizing the total isolatable yield of **1** from the culture. The ratio of concentration of yessotoxins measured by ELISA to **1** determined from UV-absorbance chromatograms obtained during LC-UV-MS^3 analyses remained fairly constant, at an average of 2.9 (standard deviation = 0.3) for both barrels throughout the culture period. Because the ELISA method detects a wide range of yessotoxins (*9, 29, 30*), this suggests that production of both yessotoxin analogues and **1** may be a constitutive trait of CAWD129, rather than analogue production occurring in a particular growth phase or due to degradation of **1** itself. Similar ratios have been observed for other strains of *P. reticulatum* from Norway and New Zealand (*7, 20, 25, 30*). At harvest (day 43), analyses of a composite sample from all culture vessels indicated the culture contained 1100 μg/L (249 mg in total) of **1** by LC-UV-MS^3 (UV chromatogram, 235 nm) and 3670 μg/L (830 mg in total) of yessotoxin analogues by ELISA.

Harvesting of the toxins was accomplished by filtration under low pressure, during which the toxin burden is released into the water and collected by solid-phase extraction with hydrophobic HP-20 resin. This procedure has the advantage of leaving behind cellular debris and much of the organic components of the cells, thereby achieving a significant purification at the harvesting step. This strategy has been successfully applied to large-scale extractions of a number of algal toxins including gymnodimine (*31, 32*), pectenotoxins (*33*), and okadaic acid analogues (*33–35*), as well as yessotoxins from *P. reticulatum* (*22, 25*) (T. Yasumoto, personal communication). Yessotoxins were extracted from the resin with organic solvent and then purified by conventional reverse-phase flash chromatography as previously (*22*). In the present experiment, yessotoxin was obtained conveniently in high yield and purity with two functioning flash chromatography procedures; the second flash column eluted too quickly and did not lead to separation. Purification was thus markedly easier than in the previous preparation (*22*) due to the absence of yessotoxin analogues eluting in close vicinity to **1** (*23*) apart from trace amounts of nor-ring-A-yessotoxin (*m/z* 1085) (**Figure 3**) (*23*). The retention time, UV spectrum (inset, **Figure 4**), mass spectra (**Figures 4D** and **5**), and HR-MS of the yessotoxin were consistent with **1**. Furthermore, NMR assignments, coupling constants, and integrals of NMR resonances obtained for **1** were essentially identical to those previously determined (*22*) and were entirely consistent with structure **1**, confirming the identity. No significant impurities were observed by NMR or LC-UV-MS^3. This established the purity of the yessotoxin and indicated that no degradation had occurred during the isolation and weighing of the sample.

LC-UV-MS^3 analysis of the crude extract recovered from the HP-20 resin revealed the presence of numerous minor yessotoxin analogues in addition to **1** (**Figure 3**). These corresponded to analogues previously reported (*22*), although only a portion of these have been identified (*20–24, 28*). An unidentified early eluting compound (**2**) was observed with the same molecular mass (*m/z* 1141 for $[M - H]^-$) as **1** (**Figure 3C**) and possessing a similar mass spectrum (**Figure 4**) and fragmentation pattern (**Figure 5**), with ca. 1% abundance relative to **1** as judged by LC-UV-MS^3 (**Figure 3C**). Although this compound partitioned preferentially into water during the butanol extraction, fractions enriched in **2** were obtained during isolation of **1** from the butanolic extract. These fractions were combined and purified by preparative HPLC to give apparently pure **2** as a colorless solid. HR-MS of **2** was consistent with $C_{54}H_{77}O_{22}S_2^-$. The MS^3 spectrum (**Figure 5**) indicated that **2** probably contained the same disulfated ring system (rings A−K) as **1**, from C-1 to C-40. Thus, the difference between **1** and **2** lay in the side chain attached at C-40. The UV absorbance spectrum of **2** was similar to that of **1** but was significantly broader (**Figure 4**), suggesting a minor modification to the dienyl chromophore. A subsequent more detailed LC-UV-MS^3 analysis of the purified specimen of **2** (**Figure 4**) revealed the presence of at least four closely eluting monohydroxylated yessotoxins [*m/z* 1157 (**Figure 4C**), hydroxylated on their ring and side-chain moieties as judged from MS^3 spectra] totaling ca. 20% of the total material. Because they individually comprised only a small proportion of the material, they did not interfere with NMR analysis.

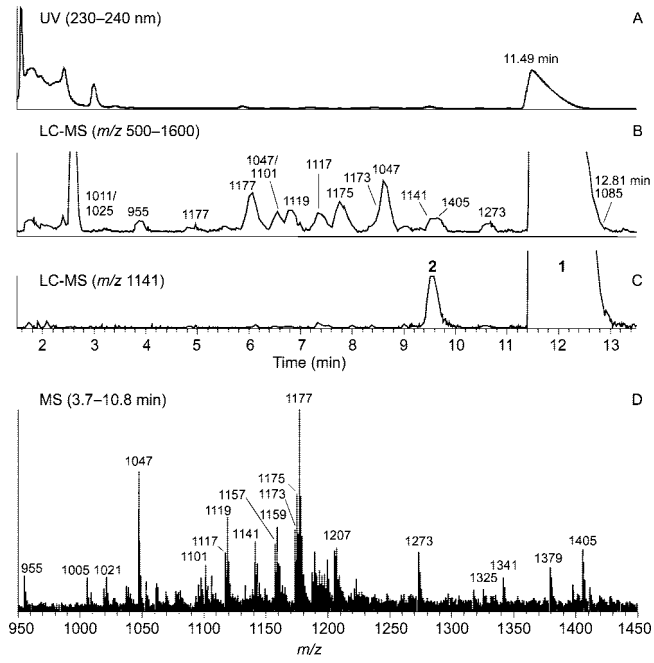

Figure 3. LC-MS chromatogram and spectra of the crude *P. reticulatum* extract obtained from the HP-20 resin, showing (**A**) UV absorbance chromatogram at 230–240 nm, (**B**) base peak chromatogram for *m/z* 500–1600, (**C**) selected ion chromatogram at *m/z* 1141, and (**D**) mass spectrum extracted from the LC-MS chromatogram between 3.7 and 10.8 min.

Detailed analyses of one- and two-dimensional NMR spectra, including ^{1}H,^{13}C, DEPT135, 1D-TOCSY, COSY, TOCSY, g-HSQC, g-HMBC, SELNOESY, and NOESY data determined in CD_3OD, afforded complete ^{1}H and ^{13}C assignments for **1** and furanoyessotoxin (**2**) (**Table 2**; see also the Supporting Information). Typically, ^{1}H−^{1}H connectivities were established in COSY and TOCSY experiments, and ^{1}H−^{13}C connectivities were identified in g-HSQC and g-HMBC experiments. Stereochemistries of structurally significant skeletal portions (e.g., the side-chain structure of **2**) were established in SELNOESY and NOESY experiments. Resolution in the ^{1}H axis in the g-HSQC spectra of **1** was such that stereochemistries of axially and equatorially oriented methylene protons attached to six-membered rings (but not seven- or eight-membered rings) could be distinguished (*22, 36*). Typically, axial methylene protons exhibited resolvable ^2J (geminal) and $^3J_{ax-ax}$ couplings (ca. 10–14 Hz), whereas the smaller $^3J_{eq-ax}$ or $^3J_{eq-eq}$ couplings (ca. 3–5 Hz) exhibited poorly resolved or overlapping signals. Axial

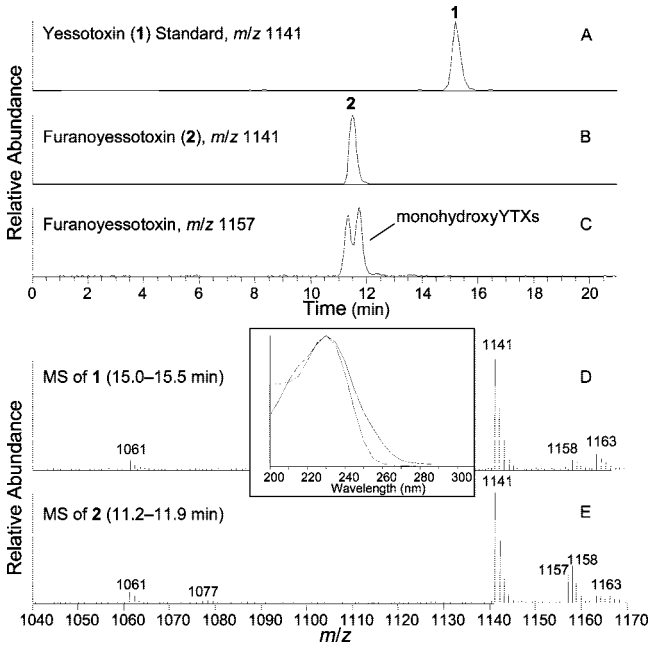

Figure 4. Selected ion LC-MS analyses of (**A**) authentic yessotoxin (**1**) at *m*/*z* 1141, (**B**) furanoyessotoxin (**2**) at *m*/*z* 1141, and (**C**) *m*/*z* 1157. Mass spectra taken across the full widths of LC-MS peaks are shown for (**D**) yessotoxin (**1**) and (**E**) furanoyessotoxin (**2**). Ions at *m*/*z* 1061, 1141, 1158, and 1163 for **1** correspond to $[M - H - SO_3]^-$, $[M - H]^-$, $[M - 2H + NH_4]^-$, and $[M - 2H + Na]^-$, respectively. The corresponding ions for **2** can be seen at *m*/*z* 1061, 1141, 1158, and 1163 in panel **E**, together with some of the corresponding ions from the contaminating hydroxyyessotoxins (at *m*/*z* 1077 and 1157). (Inset) UV absorbance spectra of yessotoxin (**1**) (dashed line) and furanoyessotoxin (**2**) (solid line) acquired by means of a diode array detector during LC-MS analysis after background subtraction.

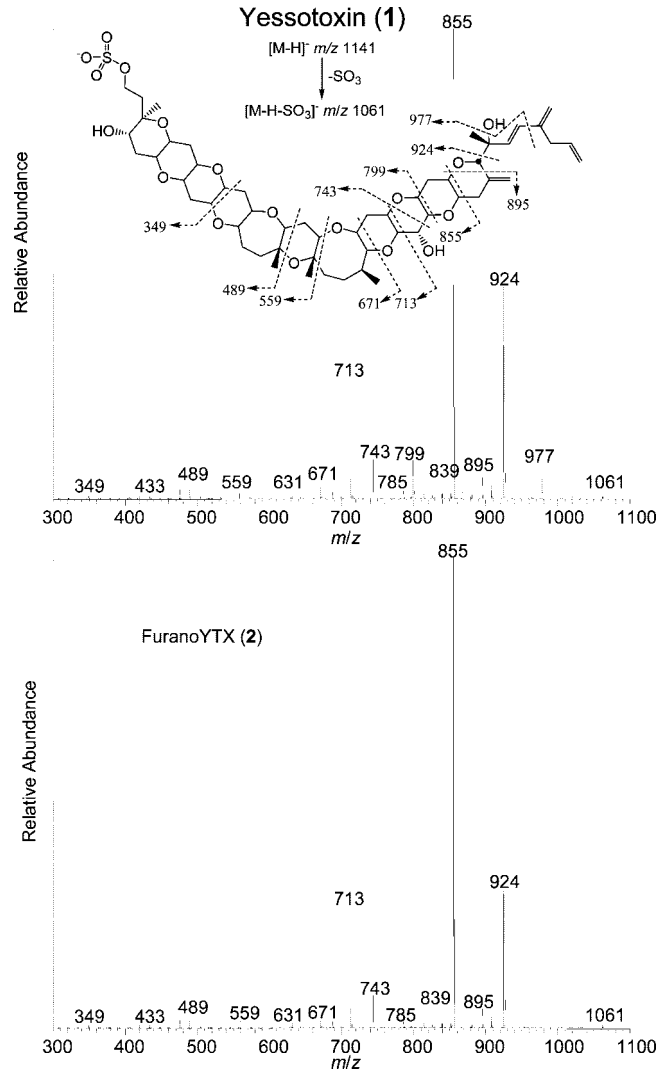

Figure 5. MS^3 spectra ($[M - H]^-$ (*m*/*z* 1141) → $[M - H - SO_3]^-$ (*m*/*z* 1061) → MS^3 spectrum) of yessotoxin (**1**) (top) and furanoyessotoxin (**2**) (bottom) acquired under identical conditions during LC-MS^3 analysis, together with assigned fragmentation pathway for **1**.

protons therefore typically appear triplet-like, whereas equatorial protons appear doublet-like in spectral cross sections. Axial protons usually appeared at lower chemical shift than their corresponding equatorial protons. The resonances of the axial and equatorial protons attached to six-membered rings of furanoyessotoxin (**2**) were achieved by analogy with assignments established for **1**.

Notable features of the ^{1}H NMR data obtained for furanoyessotoxin (**2**), compared to those of **1**, were the absence of the H-55 (4.97 and 5.04 ppm) and H-47 (5.05 and 5.08 ppm) methylene signals, the appearance of furan ring proton resonances at 6.56 ppm (d, $J = 1.8$ Hz, H-45), 7.41 ppm (br m, H-46), and 7.47 ppm (br s, H-55), and the position of the trans-coupled olefinic proton signals at 6.04 ppm (d, $J = 16$ Hz, H-42) and 6.45 ppm (d, $J = 16$ Hz, H-43), the resonances of which were shifted downfield appreciably from the corresponding olefinic proton signals of a conventional yessotoxin side chain [5.82 ppm (d, $J = 14.8$ Hz, H-42) and 6.29 ppm (d, $J = 14.8$ Hz, H-43)].

In accord with the HR-MS data, the ^{13}C and DEPT135 NMR spectra of **2** revealed the presence of 54 carbon resonances (6 methyl, 15 methylene, 26 methine, and 7 quaternary), compared to 55 carbon resonances for **1** (6 methyl, 18 methylene, 24 methine, and 7 quaternary). Further evaluation of NMR data revealed the carbon and proton resonances associated with the A−K ring structures of **2** were essentially identical to those of **1**, with differences essentially confined to resonances associated with the side chain attached at C-40.

Full NMR structural assignment (**Table 2**) revealed the presence of a furan-containing side chain (**Figure 1**). When considered alongside the MS data for **2**, this observation is consistent with the incorporation of an oxygenated furan ring combined with the loss of a methylene group (54 carbon atoms in **2** versus 55 for **1**), resulting in the same molecular mass for **2** (*m*/*z* 1141.4 $[M - H]^-$) as for **1** in mass spectra obtained during LC-UV-MS^3. The equatorial orientation of H-40 (3.90 ppm), and consequently the axial orientation of the side chain with respect to ring K, was confirmed by an NOE correlation between H-40 and H-53*Z* (4.79 ppm). This and other structurally significant NOE correlations observed for H-38 and side-chain protons of **2** are shown in **Figure 6**. This evidence, combined with NOE correlations between H-53*Z* (4.79 ppm) and H-42 (6.04 ppm), and an HMBC correlation between H-43 (6.45 ppm) and C-40 (85.1 ppm), also confirmed that the position of the 42/43 olefinic bond was identical to that in a conventional yessotoxin side-chain.

COSY correlations in the side chain were observed between H-42 (6.04 ppm) and H-43 (6.45 ppm) and between H-45 (6.56 ppm) and H-46 (7.41 ppm). Strong TOCSY (^4J) correlations were observed between H-55 (7.47 ppm) and both H-45 (6.56 ppm) and H-46 (7.41 ppm) in the furan ring. Weak TOCSY correlations ($^4J/^5J$) were observed from the furanyl H-45 proton (6.56 ppm) to both H-42 and H-43 (6.04 and 6.45 ppm,

Table 2. ^{1}H and ^{13}C NMR Assignments for **1** (*22*) and **2** in CD_3OD[a]

	yessotoxin (**1**)		furanoyessotoxin (**2**)			yessotoxin (**1**)		furanoyessotoxin (**2**)	
atom	^{13}C	^{1}H	^{13}C	^{1}H	atom	^{13}C	^{1}H	^{13}C	^{1}H
1	65.1	4.19 (2H)	65.0	4.22 (2H)	29	40.0	1.52_{ax}, 2.27_{eq}	40.0	1.53, 2.29
2	40.3	1.94, 2.16	40.4	1.93, 2.22	30	73.3	3.60	73.3	3.60
3	76.6		76.6		31	79.7	3.16	79.7	3.18[d]
4	78.64	4.21	78.8	4.21	32	73.9	3.84	73.9	3.85
5	32.8	1.72_{ax}, 2.57_{eq}	32.8	1.73, 2.57	33	76.8		76.8	
6	78.54	3.05	78.5[c]	3.06[c]	34	73.3	3.77	73.3	3.77 (dd, $J = 3.8$, 12.5 Hz)
7	70.7	3.31	70.7	3.33[d]	35	31.7	1.48_{ax}, 2.09_{eq}	31.7	1.50, 2.10
8	36.5	1.39_{ax}, 2.17_{eq}	36.5	1.40, 2.18	36	73.2	4.04	73.2	4.08
9	78.33	3.14	78.31[b]	3.14[b]	37	73.0	3.40	73.0	3.40
10	78.39	3.13	78.39[b]	3.14[b]	38	39.0	2.43_{eq}, 2.71_{ax}	39.0	2.44_{eq} (dd, $J = 4.5$, 12.4 Hz), 2.75_{ax}
11	36.2	1.40_{ax}, 2.25_{eq}	36.2	1.38, 2.26	39	143.2		143.3	
12	77.7	3.02	77.6	3.04	40	85.2	3.87	85.1	3.90
13	78.15	3.08	78.1[c]	3.05[c]	41	78.39		78.44	
14	38.0	1.42_{ax}, 2.30_{eq}	38.0	1.43, 2.31	42	136.7	5.82	136.2	6.04 (d, $J = 16$ Hz)
15	81.2	3.32	81.2	3.33[d]	43	130.7	6.29	118.9	6.45 (d, $J = 16$ Hz)
16	82.2	3.21	82.2	3.22	44	145.5		125.5	
17	30.3	1.79, 1.94	30.3	1.79, 1.94	45	37.8	2.96 (2H)	108.6	6.56 (d, $J = 1.8$ Hz)
18	41.1	1.78, 1.82	41.1	1.80 (2H)	46	137.6	5.87	144.7	7.41 (br m)
19	78.45		78.44		47	116.5	5.05, 5.08		
20	82.5	3.41	82.5	3.43	48	16.2	1.27	16.1	1.27
21	33.2	1.74_{ax}, 1.92_{eq}	33.1	1.75, 1.93	49	23.8	1.25	23.9	1.25
22	87.4	3.49	87.4	3.49 (dd, $J = 4.6$, 11.8Hz)	50	20.7	1.15	20.7	1.15
23	77.1		77.0		51	22.3	1.02	22.3	1.02 (d, $J = 6.2$ Hz)
24	47.0	1.49, 1.76	47.0	1.50, 1.76	52	15.4	1.20	15.3	1.20
25	32.8	1.49, 1.70	32.8	1.48, 1.70	53	115.6	4.79, 5.01	115.5	4.79 (*Z*), 5.01(*E*)
26	40.9	1.70	40.9	1.70	54	26.2	1.38	26.1	1.43
27	89.5	2.76	89.5	2.78	55	116.5	4.97, 5.04	141.6	7.47 (br s)
28	84.1	3.29	84.1	3.31[d]					

[a] ax, axial; eq, equatorial; br m, multiplet; br s, broad singlet; d, doublet; dd, doublet of doublets. [b] Interchangeable assignments. [c] Assignments based on the corresponding yessotoxin atom resonances. [d] 2-D spectra proton chemical shift values (not resolvable in 1-D spectra).

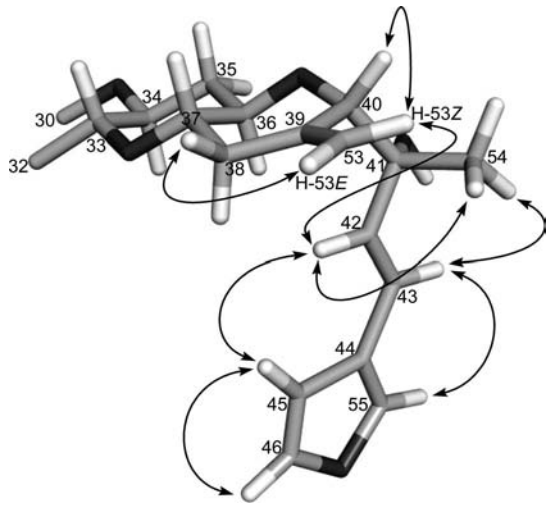

Figure 6. NMR NOE interactions observed for side-chain and selected K-ring protons of furanoyessotoxin (**2**).

respectively), but no corresponding correlations were observed for the furanyl H-55 proton.

The structure of the side chain, point of attachment of the furan ring, preference in solution for a transoid (rather than cisoid) double-bond conformation, and the planar structure of the furanyl-containing side chain were confirmed by NOESY correlations (**Figure 6**). Attachment of the furanyl ring via its 3-position (C-44) (β-substitution) was further confirmed through g-HMBC correlations from both the H-42 and H-43 to the signal at 125.5 ppm (C-44). Absence of the 125.5 ppm carbon signal in g-HSQC and DEPT135 spectra confirmed it as a quaternary carbon. NMR assignments reported herein confirm the identity and point of attachment of the side-chain furan ring of **2** and are consistent with NMR chemical shifts for the β-substituted furanyl moiety of a novel furanoterpene (*37*).

The isolation method developed here is rapid and convenient and yielded large quantities (193 mg) of high-purity yessotoxin (**1**) with high recovery (77%). The yessotoxin obtained is suitable for toxicological and immunochemical studies as well as preparation of derivatives for structure–activity studies. The isolation of **2** and the identification of yessotoxin analogues in *P. reticulatum* strain CAWD129 confirm the ability of this species to produce a range of yessotoxins. Toxicological assessment of **2** was not performed due to its contamination with ca. 20% of closely eluting hydroxyyessotoxins, which have proven to be difficult to remove.

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Supporting Information Available: ^{1}H NMR spectra of **1** and **2** in CD_3OD; TOCSY NMR spectrum of **2**, including a slice showing TOCSY correlations to H-45; HMBC NMR spectrum of **2**, including a table of structurally important HMBC correlations in the furanyl ring and side chain; structures and NMR tables comparing chemical shifts for furanoyessotoxin (**2**) and a furanoterpene containing an analogous β-substituted furanyl ring (*37*). This information is available free of charge via the Internet at http://pubs.acs.org.

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