Isolation and Structure of Prorocentrolide B, a Fast-Acting Toxin from *Prorocentrum maculosum*

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A new toxin, prorocentrolide B (**1**), has been isolated following bioassay-guided fractionation of a BuOH extract of the tropical dinoflagellate, *Prorocentrum maculosum* Faust. This compound produces a rapid toxic response in the mouse bioassay, a type of activity not accounted for by other diarrhetic shellfish poisoning toxins produced by *P. maculosum*. The structure **1** was established by NMR and MS and is similar to prorocentrolide (**2**), a toxin from a strain of *Prorocentrum lima*. NMR data and the modeling program ConGen have been used to establish the relative stereochemistry of some individual ether ring systems and the hexahydroisoquinoline ring.

Various species of marine dinoflagellates belonging to the genus *Prorocentrum* are known to produce diarrhetic shellfish poisoning (DSP) toxins.[1−3] These toxins are a group of lipid-soluble compounds that inhibit phosphatases PP1 and PP2A, and induce powerful, and usually deleterious, biological effects in eukaryotic cells.[4] In addition to the symptoms caused by these lipid-soluble polyether toxins, which include okadaic acid, DTX-1, and DTX-2, there are occasional reports that different toxicity has been found in the more polar BuOH-soluble fractions from the same DSP toxin-producing strains of *Prorocentrum* spp. For example, in 1984, Tindall and co-workers reported that both the Et_2O- and BuOH-soluble fractions of the MeOH extract of Caribbean *P. concavum* caused toxic response in a mouse bioassay.[5] Interestingly, the toxins in these two fractions appeared to be quite different: the lipid-soluble toxins from the ether fraction killed mice in hours, whereas the more polar toxins from the BuOH fraction killed mice in minutes. Furthermore, these polar "fast-acting toxins" were reported to display an "all or nothing" effect.[5] Since then, there have been no reports of the chemical characterization of such toxins, though very recently three groups of related compounds, all displaying similar "fast-acting" toxicity, have been reported.[6−8] In only one of these cases has a phytoplankton source (*Gymnodinium mikimoitoi*) been identified.[7] Consequently, we undertook a chemical investigation of the dinoflagellate *P. maculosum* Faust (Prorocentraceae) [formerly *P. concavum* [9]] with the aim of identifying the chemical nature of the compound responsible for this toxicity. This investigation has resulted in the isolation of the known lipid-soluble DSP toxins,[9] as well as a new class of sulfated H_2O-soluble DSP toxin derivatives.[10] Although none of these compounds displayed any "fast acting" toxicity, polar fractions of the same organism caused rapid death in mice, as originally reported by Tindall and co-workers.[5] In this paper, we report the isolation and structure elucidation of prorocentrolide B, a new toxin from *P. maculosum*, which causes the characteristic "fast acting" symptoms.

Results and Discussion

The BuOH-soluble fraction of a MeOH extract of *P. maculosum* cells (302 g wet wt) displayed activity similar to that described by Tindall *et al.* in the mouse bioassay.[5] This extract was purified by sequential chromatography steps (see Experimental Section), to yield the pure toxin **1** as a colorless solid (1.5 mg). The presence of sulfonate in the compound was suggested by the strong band at 1256 cm^{-1} in the IR spectrum,[11] and this was confirmed by mass spectrometry. Liquid secondary ion mass spectrometry (LSIMS) in the positive ion mode gave a weak protonated molecular ion $(MH)^+$ at *m/z* 1076, in addition to a stronger peak at *m/z* 996 due to the loss of sulfate $(MH - SO_3)^+$, and a base peak at *m/z* 978 due to the loss of H_2O from the latter ion $(MH - SO_3 - H_2O)^+$. The peaks at *m/z* 1184 and *m/z* 1104 were identified as the thioglycerol adducts of the $(MH)^+$ and $(MH-SO_3)^+$ ions, respectively. LSIMS in the negative ion mode gave a single peak at *m/z* 1074 identified as $(M - H)^-$, which confirmed the molecular weight of 1075. The electrospray mass spectrum was consistent with the LSIMS data and also showed a peak at *m/z* 1074 in the negative ion mode $(M - H)^-$, while in the positive ion mode peaks were observed at *m/z* 978 $(MH - SO_3 - H_2O)^+$, 996 $(MH - SO_3)^+$, 1058 $(MH - H_2O)^+$, 1076 $(MH)^+$, and 1098 $(M + Na)^+$. Accurate mass measurement of the sodiated form was made using LSIMS (with added NaI) and established the elemental formula to be $C_{56}H_{85}NO_{17}SNa$. Additional accurate mass measurement using electrospray ionization confirmed that the ion at *m/z* 996 is the desulfated form of the molecular species, having the elemental formula $C_{56}H_{86}NO_{14}$, and that the ion at *m/z* 978 is due to loss of H_2O from the desulfated ion of *m/z* 996 to give $C_{56}H_{84}NO_{13}$. Finally, further proof of the sulfate group was provided by the MS/MS spectrum of the $(M + 2Na - H)^+$ ion, which showed characteristic

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Table 1. ^{1}H and ^{13}C Assignments for Prorocentrolide B (**1**)[a,b]

C no.	δ^{13}C (mult)	δ^1H	C no.	δ^{13}C (mult)	δ^1H
1	172.3 (s)		29	135.5 (d)	5.68
2	40.0 (t)	2.58	30	71.3 (d)	4.21
		2.80	31	76.4 (d)	3.58
3	79.4 (d)	4.46	32	33.9 (t)	1.58
4	87.8 (d)	4.42			2.33
5	76.8 (d)	4.29	33	70.1 (d)	3.75
6	85.6 (d)	3.43	34	78.4 (d)	2.95
7	68.2 (d)	3.47	35	75.3 (d)	3.11
8	40.0 (t)	1.32	36	32.1 (t)	1.23
		1.58			2.23
9	40.4 (d)	2.70	37	35.9 (t)	1.11
10	153.2 (s)				1.79
11	26.6 (t)	1.57	38	73.0 (d)	4.05
		2.14	39	131.7 (d)	5.60
12	31.2 (t)	1.40	40	133.1 (d)	6.21
		1.90	41	132.8* (s)	
13	74.6 (d)	3.20	42	132.5 (d)	5.43
14	71.0 (d)	3.18	43	24.0 (t)	2.38
15	37.3 (t)	1.58			2.38
		2.08	44	36.8** (t)	2.39
16	68.0 (d)	3.88			2.62
17	73.8 (d)	3.97	45	175.7 (s)	
18	34.3 (t)	2.10	46	42.3 (s)	
		2.70	47	35.9 (d)	1.69
19	132.9* (s)		48	26.2 (t)	1.40
20	126.5 (d)	5.18			1.93
21	57.3 (d)	3.05	49	50.1 (t)	3.52
22	137.8 (s)				3.71
23	131.2 (d)	5.38	50	32.3 (t)	1.70
24	36.9** (t)	1.55			2.60
		1.98	51	22.0 (q)	1.17
25	29.9 (d)	1.45	52	110.5 (t)	4.75
26	44.6 (t)	1.23			5.03
		1.73	53	15.0 (q)	1.44
27	73.0 (d)	5.47	54	17.7 (q)	0.80
28	133.5 (d)	5.61	55	12.8 (q)	1.67
			56	29.0 (q)	1.30

[a] Recorded in CD_3OH, chemical shift values are reported as δ values (ppm) relative to CD_2HOH at $\delta_H = 3.30$ and $^{13}CD_3OH$ at $\delta_C = 49.0$, at 500.13 MHz for ^{1}H and 125.77 MHz for ^{13}C. For δ_C ppm, (s) = C, (d) = CH, (t) = CH_2, (q) = CH_3. [b] Two ^{13}C-NMR chemical shifts marked with * or ** are interchangeable.

fragment ions at *m/z* 126 and 143, corresponding to $(SO_3Na_2)^+$ and $(HOSO_3Na_2)^+$ ions. In the presence of KI, these ions were shifted predictably to *m/z* 158 and *m/z* 175, corresponding to the equivalent potassiated forms.

Initially, when the NMR spectra of **1** were recorded using CD_3OD or CD_3OD/C_6D_6 as a solvent, some of the data were of poor quality with many broad peaks, and it was suspected that some peaks were missing or greatly diminished in intensity. The poor spectral quality was surmised to be due to isotope exchange with the solvent, and indeed the problem was resolved by running the protiated sample in CD_3OH. The ^{13}C-NMR spectrum (Table 1) now displayed 56 distinct resonances, in agreement with the molecular formula. The ^{13}C- and ^{13}C- DEPT-NMR data identified 7 quaternary carbons, 27 methines, 17 methylenes, and 5 methyl groups, and HMQC located the resonances of the corresponding protons. The resonances at δ 172.3 and δ 175.7 indicated two carbonyl or imino carbons, while the signals at δ 110.5 (CH_2) and δ 153.2 (quaternary C) established the presence of a terminal double bond. Ten additional resonances in the range δ 126.5−137.8 indicated another five double bonds. Taken together, these data account for 8 of the 15 degrees of unsaturation indicated by the molecular formula. From this it was concluded that the molecule contained 7 rings.

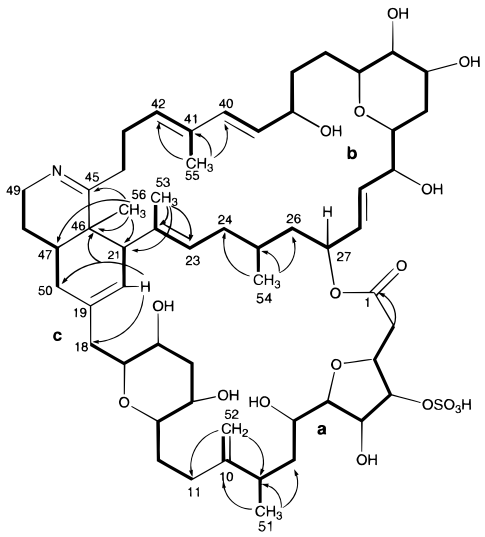
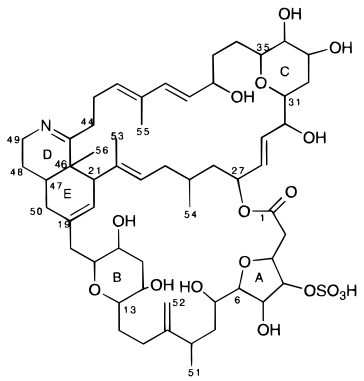


Figure 1. Carbon connectivity of prorocentrolide B (**1**). Arrows denote HMBC correlation from H to C. Bold lines are used to denote each partial structure.

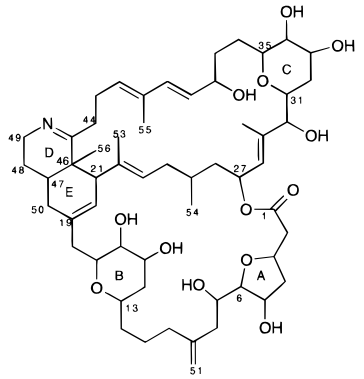
Three significant partial structures of **1** were established by analysis of the TOCSY, COSY and HMBC spectral data (Figure 1). Partial structure **a** corresponds to C-1 to C-10, plus C-51 and C-52. The protons at positions 2−9 plus 51 and 52 are all in the same TOCSY-defined ^{1}H spin system; the connectivity of these protons was determined by COSY. The linkages of C-1 to C-2 and C-9 to C-10 were determined by HMBC. Partial structure **b** corresponds to C-22 to C-40 plus C-53 and C-54, in which the protons at positions 23−40 plus 53 and 54 are in the same ^{1}H spin system. The HMBC data were used to establish the positions of C-53 (C-22 methyl) and C-54 (C-25 methyl).

The partial structure **c** corresponds to C-11 to C-21 and C-41 to C-50, plus C-55 and C-56, in which four ^{1}H spin systems were found. These four spin systems comprise, respectively, H-11 to H-18; H-20 to H-21; H-42 to H-44 plus H-55, and H-47 to H-50; the connectivities in each system were established by TOCSY and COSY. The long-range ^{1}H coupling of H-21 (δ 3.05)/H-18 (δ 2.10, 2.70) and H-21(δ3.05)/H-50 (δ 2.60, 1.70), as well as the COSY correlation of H-20/H-21, and HMBC of H-20/C-18 and H-20/C-50 established the carbon connectivity of C-18/C-19/C-20/C-21, and C-19/C-50. The connectivity of C-50/C-47/C-48/C-49 was established by TOCSY and COSY. The NMR chemical shift data for position 49 (δ_C 50.1; δ_H 3.52, 3.71) were consistent with those for the C-49 methylene group in **2** and the C-33 methylene group in spirolides B and D,[8] which are both linked to a nitrogen. Furthermore, the existence of long-range coupling between H-49 and H-44 suggested the insertion of an imine group between C-49 and C-44, thus establishing the carbon connectivity sequence C-41/C-42/C-43/C-44/C-45/N/C-49/C48/C-47/C-50. Finally, the HMBC of C-45/H-56, C-46/H-56, C-47/H-56, C-21/H-56, C-46/H-20, and C-50/H-20 established the hexahydroisoquinoline ring.

These three partial structures **a**−**c** were linked by the HMBC data (Figure 1). The correlations between C-11/H-52 and C-9/H-52 revealed that partial structure **a** is connected to partial structure **c** through C-10/C-11. In addition, the HMBC correlations of C-21/H-53, C-22/H-53, C-23/H-53 and C-40/H-55, C-41/H-55, C-42/H-55 indicate that partial structure **b** is also connected to



Prorocentrolide B (**1**)



Prorocentrolide (**2**)

partial structure **c**, this time through C-21/C-22 and C-40/C-41. This left C-1 and C-27 as the only two remaining carbons not connected, but the downfield shift of the oxymethine H-27 (δ 5.47) suggested that partial structure **a** is connected to partial structure **b** through an ester oxygen linkage (C-1/O/C-27).

The structure depicted in Figure 1 is composed of two macrocyclic rings containing 17 carbons bearing oxygen. It remained to distinguish the hydroxyl- and ether-bearing carbons based on the deuterium-induced upfield ^{13}C chemical shifts observed upon changing the NMR solvent from CD_3OH to CD_3OD. Typically, isotopically induced upfield shifts ($\Delta\delta$ 0.09−0.15 ppm) are reported for carbons bearing a hydroxyl group,[12,13] and shifts of this magnitude were observed for eight carbons: C-5, C-7, C-14, C-16, C-30, C-33, C-34, and C-38. The remaining eight oxygen-bearing carbons showed shifts of less than 0.04 ppm. Of these, C-27 has been identified as bearing an ester oxygen, one must be linked to a sulfate, and hence the final six oxygen-bearing carbons (C-3/C-6, C-13/C-17, and C-31/C-35) were assigned to ether linkages to form one furan and two pyran ring systems. These assignments, and in particular those for the cyclic ether systems, were confirmed by the NOESY and ROESY data (see below), although no HMBC were observed across these ether linkages or across the C-1/C27 ester bridge, perhaps because of the high degree of coupling of protons at these positions. Thus, the sulfate group can be assigned to the remaining oxygen-bearing carbon at C-4, and this was confirmed by preparation of the desulfated product and comparison of its ^1H-NMR data with those of **1**. Only the resonances for H-3, H-4, and H-5 were affected compared to the corresponding resonances in the sulfated toxin, and showed upfield shifts of 0.11, 0.64, and

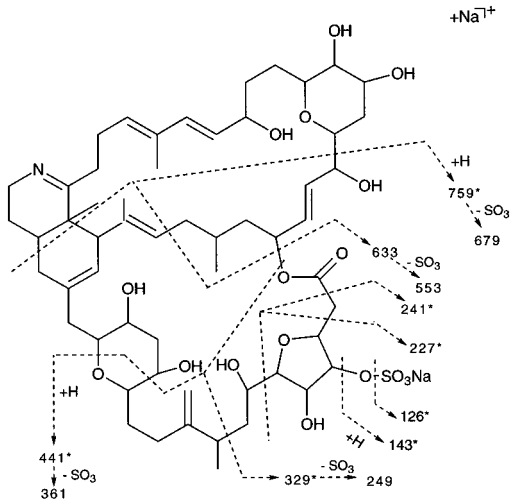


Figure 2. Major peaks in the MS/MS (fragment ion) spectrum of the $[M + 2\ Na - H]^+$ ion of prorocentrolide B (**1**). Asterisks denote where the corresponding ions, shifted by 32 Daltons, were observed in the MS/MS spectrum of the $[M + 2K - H]^+$ ion.

0.06 ppm, respectively. Collectively these data supported structure **1** for prorocentrolide B.

The fragmentation in the MS/MS spectra of quasi molecular ions of **1** is not easily predictable due to the presence of the two macrocyclic rings within the structure. Nevertheless, additional proof of structure **1** was obtained by examining the disodiated form of the molecule (i.e., $[M - H + 2Na]^+$) in a series of LSIMS−MS/MS experiments. The dominant MS/MS fragmentation involves localization of charge at the sulfate group and cleavage of the macrocycle as depicted in Figure 2. In most cases the corresponding ions, shifted by 32 Da, were also observed in the MS/MS spectrum of the $[M - H + 2K]^+$ ion. The most intense high mass fragment ion at m/z 759 is most likely formed by cleavage of the macrocycle giving a neutral fragment containing the nitrogen. Consistent with this, another fragment ion 80 Da lower and corresponding to the loss of SO_3 confirms the presence of sulfate in the macrocyclic fragment ion at m/z 759. This fragmentation scheme is supported further by the MS/MS spectrum of the $[M + H]^+$ ion, which shows a major fragment ion at m/z 362 that represents cleavage at the same positions, but with the charge (in this case H^+ rather than Na^+) retained at the nitrogen atom.

As the structural details of **1** began to emerge, it became apparent that the new compound was related to prorocentrolide (**2**), previously isolated from a strain of *P. lima*.[14] No stereochemical details have been reported for prorocentrolide (**2**), and details of the relative stereochemistry of **1** obtained from NOESY and ROESY NMR experiments are shown in Figure 3. All the double bonds, except C-19/C-20, possess the *E* geometry based on the correlations depicted in Figure 3. In ring A, NOESY cross peaks of H-2a (δ 2.58)/H-6, H-4/H-6, H-5/H-6, and H-4/H-5 indicate that H-3, the C-4 sulfate, the OH group at C-5, and the methine at C-7 are on the same side of this five-membered ring. Similarly, cross peaks of H-12/H-14, H-14/H16, and H-13/H-18 establish that H-12, H-14, H16, and H-17 are on the same side of ring B, and those between H-30/H-33, H-30/H-35, H-33/H-35, and H-34/H-36 show that H-30, H-33, H-35, and the OH group at C-34 are on the

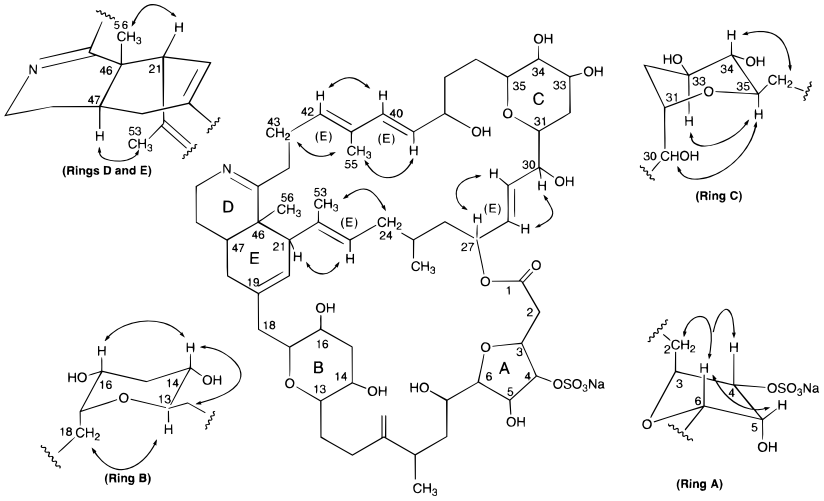


Figure 3. The relative stereochemistry of the 5- and 6-membered rings of prorocentrolide B (**1**). The arrows denote NOE correlations.

same side of ring C. The ring junction of the hexahydroisoquinoline ring system was determined to be *trans* by the NOESY cross peaks between H-56 (δ 1.30) and H-21 (δ 3.05), as well as a correlation between H-53 (δ 1.44) and H-47 (δ 1.69). These stereochemical conclusions received further support from modeling of the complete molecule using SYBYL software (version 6.1, Tripos Inc., St. Louis, MO), including ConGen routines[15] in which approximate H−H distances derived from the strengths of NOESY cross peaks are used as constraints for molecular dynamics at high temperature followed by annealing and minimization. The absolute stereochemistry of the rings and the relative configuration of one ring with respect to the others remain undetermined. Molecular modeling also demonstrated that considerable conformational flexibility occurs at all sp^3 carbons in chains joining the 5- and 6-membered rings, which hinders further definition of configuration from NOE constraints and H−H coupling information.[16]

With the identification of **1** as a fast-acting toxin from *P. maculosum*, it is likely that this compound or a related derivative—perhaps prorocentrolide itself—accounts for the similar toxicity originally reported[5] in polar extracts of *P. concavum*. As yet we do not know the full distribution of this family of toxins, but it is reasonable to conclude from the structural data that toxin **1** from *P. maculosum* shares a common biosynthetic pathway with prorocentrolide (**2**) from *P. lima*. As a result of this observation we examined the polar extracts of two DSP toxin-producing strains of *P. lima* obtained from various localities in eastern Canada. In both cases we isolated and identified sulfated H_2O-soluble DSP toxin derivatives similar to DTX-4,[17] but no prorocentrolide derivatives were observed. At the moment it is too early to speculate that cold water strains of *Prorocentrum* species do not produce prorocentrolide derivatives, and further chemical investigations of both cold-water and warm-water strains are necessary. The toxicological and pharmacological effects of these "fast-acting" toxins are not yet understood, though we have shown that they are not phosphatase inhibitors,[18] unlike their co-metabolites, the DSP toxins. Interestingly, the other recently characterized marine compounds reported to elicit a similar toxic response in the mouse bioassay, namely the pinnatoxins,[6] gymnodimine,[7] and the spirolides,[8] although not related to the prorocentrolide group, all contain a cyclic imine moiety, suggesting that this functionality may be important for biological activity. Further work is planned to investigate the toxicology and distribution of this class of phycotoxins.

Experimental Section

General Experimental Procedures. Si gel was used for vacuum liquid chromatography and flash liquid chromatography; Sephadex LH-20, for gel permeation chromatography; and C18, for reversed-phase chromatography. TLC plates were eluted with BuOH/HOAc/H_2O (4:1:5, BuOH layer) and visualized by acid and heat. All solvents used were HPLC grade purchased from Anachemia. H_2O was purified using a Waters Millipore system. UV spectra were recorded in CH_3CN, and the IR spectra were obtained from small amounts of dried solid using a Bomem DA3.02 FTIR spectrometer equipped with a Spectrascope IR microscope. Samples (ca. 1 mg) were dissolved in CD_3OD, CD_3OH or a CD_3OD/C_6D_6 (2:1) mixture in 5-mm tubes (Wilmad 520pp), and NMR spectra were recorded at 20 °C with a Bruker AMX-500 spectrometer at 500.14 MHz (^1H) using a 5-mm inverse-geometry probe, or at 125.77 MHz (^{13}C) using a 5-mm standard-geometry probe. Spectra were obtained under the following conditions: ^1H one-pulse, spectral width (SW) 7576 Hz, 40° pulse, acquisition time (AQ) 2.16 s, delay after acquisition (D) 2 s, processed with 0.2 Hz line broadening (LB) or with Lorentz-Gauss resolution enhancement and zero-filling. ^{13}C {^1H}-decoupled SW 31250 Hz, 40° pulse, D 0.1 s, AQ 1.05 s, Waltz decoupling, processing with zero filling and LB 1.0 Hz; ^{13}C DEPT 90° and 135° conditions were similar except that D was 2 s; ^1H 2D double-quantum filtered COSY, TOCSY, ROESY, and NOESY SW 4032 Hz, D 2 s, 512 × 512 or 256 increments, 90° shifted sine-bell-squared apodization, zero-filled to 1024 in both dimensions during processing, mixing times 150 ms (TOCSY), 200 ms to 300 ms (ROESY and NOESY); ROESY spin lock field $\gamma B_1/2\pi = 1500$ Hz; $^1H/^{13}C$ HMQC and HMBC SW 4032 Hz (^1H), 19 483 Hz or 25 154 Hz (^{13}C), 512 × 246 to 1024 increments, processing as for ^1H 2D above, HMBC long-range delay of 60 ms and 90 ms. Solvent suppression, where necessary, was by presaturation. Chemical shifts were referred to the solvent resonances (CHD_2OD at δ_H 3.30 ppm, CD_3OD at δ_C 49.0

ppm). HRLSIMS and LRLSIMS and electrospray mass spectra were recorded using a Fisons Instruments ZAB-EQ mass spectrometer. LSIMS spectra used a matrix of 1:1 (v/v) glycerol/thioglycerol with 1% TFA in the positive ion mode or triethylamine in the negative ion mode. Electrospray mass spectra used a mobile phase of 1:1 MeOH-H_2O with 1% HOAc in the positive ion mode or 1:1 MeCN-H_2O with 0.1% NH_4OH in the negative ion mode. Accurate mass measurements were made at a mass resolution of 7000 (10% valley definition) using polyethylene glycol as a calibrant. Some spectra were recorded using an AutoSpec EQFPD mass spectrometer at a resolution of greater than 5000 (10% valley definition) using polyethylene glycol as calibrant. MS/MS data were obtained using an AutoSpec oaTOF using Xe collision gas at a collision energy of 800 eV.

Culture Conditions for *Prorocentrum maculosum*. The marine dinoflagellate *P. maculosum* was obtained from the collection of R. W. Dickey, U.S. Food and Drug Administration, Dauphin Island, and brought into culture as previously reported,[3] and procedures already described for large-scale culture were followed.[9] Cultures (50 L) were grown in mechanically agitated fiberglass tanks equipped with light and temperature control, and the pH was maintained at 7.9 by CO_2 sparging. Cells were harvested by initial sedimentation in a conical container, followed by centrifugation.

Bioassays. The biological assays were performed using female mice (CD-1, Charles River Canada, Inc.), essentially following the AOAC standardized procedures for paralytic shellfish poisoning (PSP) toxin testing. Each mouse was injected intraperitoneally with an extract or fraction (1 mL) dissolved in 1% Tween 80 and was then observed for up to 3 h. Pure toxin or toxin-containing fractions induced characteristic symptoms within 3−10 min after injection, depending on the doses used, and death followed shortly thereafter. There was a critical dose dependency, below which surviving mice recovered completely.

Isolation of Prorocentrolide B (1). Cultures of *P. maculosum* (302 g wet wt) were extracted with MeOH, and the extract was partitioned between hexane and 70% aqueous MeOH. The MeOH layer was diluted to 25% aqueous MeOH and extracted successively with Et_2O and BuOH. Mouse bioassay data indicated that DSP toxins were present in the ether fraction, and this was confirmed by comparative TLC with authentic standards. The BuOH fraction, which displayed the characteristic "fast-acting" toxic properties described above, was purified by gel permeation (Sephadex LH-20 open column). The purified toxic fraction was chromatographed on a reversed-phase C18 open column, eluting with 80% aqueous CH_3OH. Finally, semi-preparative HPLC (Capcell C18 column, eluting with 30% aqueous EtOH) was used to isolate the toxic components, yielding the toxin prorocentrolide B (**1**) as white solid (1.5 mg) , mp: 199−201 °C; $[\alpha]^{25}_D$ + 76.7 (*c* 0.18, MeOH); UV (CH_3CN) λ_{max} 235 nm; IR (crystal) υ max 3407, 1715, 1646, 1606, 1256, 1069 cm^{-1}; HRL-SIMS *m/z* $[M + Na]^+$ 1098.5397 (calcd for $C_{56}H_{85}NO_{17}$-SNa, 1098.5436, Δ = 3.9 ppm); HR electrospray MS *m/z* $[M + H - SO_3Na]^+$ 996.6069 (calcd for $C_{56}H_{86}NO_{14}$, 996.6048, Δ = 2.1 ppm); *m/z* $[M + H - (SO_3Na + H_2O)]^+$ 978.5955 (calcd for $C_{56}H_{84}NO_{13}$, 978.5943, Δ = 1.2 ppm); ^1H NMR and ^{13}C NMR data, see Table 1.

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