Three Novel Hydroxybenzoate Saxitoxin Analogues Isolated from the Dinoflagellate *Gymnodinium catenatum*

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Received March 1, 2003

In a recent survey of paralytic shellfish poisoning (PSP) toxins in *Gymnodinium catenatum* Graham extracts, using LC with postcolumn oxidation and fluorescence detection, three novel saxitoxin analogues were revealed in isolates from several locations, including Australian waters. We have named them as *G. catenatum* toxins, GC1 (**1**), GC2 (**2**), and GC3 (**3**). The compounds were isolated from a culture of the Australian strain by LC-MS-guided fractionation employing a C18-silica column and hydrophilic interaction chromatography. The unusual structures of these novel compounds were characterized by low- and high-resolution MS, MS/MS, and NMR spectroscopy. GC3 (**3**) was found to be the 4-hydroxybenzoate ester derivative of decarbamoylsaxitoxin, while GC1 (**1**) and GC2 (**2**) are the epimeric 11-hydroxysulfate derivatives of GC3 (**3**).

Introduction

Blooms of neurotoxic *Gymnodinium catenatum* Graham (Dinophyceae) have been identified in coastal waters off all continents (*1*). This species contains a wide range of PSP[1] toxins including N-sulfocarbamoyl-11-hydroxysulfate toxins (C1−4), 11-hydroxysulfate toxins (GTX1−6), decarbamoyl-11-hydroxysulfate toxins (dcGTX1−4), decarbamoylsaxitoxin (dcSTX), neosaxitoxin, and deoxydecarbamoyl toxins (doGTX2−3, doSTX) (Figure 1) (*2*−*4*). Two novel STX analogues were recently discovered in *G. catenatum* isolates from Australian, Spanish, Portuguese, Uruguayan, and Chinese waters (*4*) using LC-FLD. These compounds eluted late in the C toxin chromatogram and were tentatively labeled as C5 and C6. Further investigations revealed that the compounds were not C toxins but they did test positive for sodium channel blocking activity. Additional tests also revealed the presence of a further related compound. This study reports on the structure elucidation of the three new compounds, which we have named GC toxins, GC1 (**1**), GC2 (**2**), and GC3 (**3**).

Experimental Procedures

Algal Source and Cultivation. *G. catenatum* strain GCDE09 was isolated from a planktonic bloom in the Derwent Estuary, Tasmania, Australia, as a chain of four vegetative cells in June 1987 and maintained in the CSIRO Collection of Living Microalgae (http://www.marine.csiro.au/microalgae/). Fifty liters was cultured in GSe medium (*5*) at 20 °C and 100 μmol photons PAR m^{-2} s^{-1} (cool white fluorescent lamps) with a 12:12 light:dark cycle. Cells were harvested in late logarithmic phase by filtration onto 47 mm diameter glass fiber filters (GFC, Whatman, Kent, U.K.).

Analytical Chromatography. LC-FLD was performed using a Waters (Milford, MA) model 600 HPLC unit in combination with a PCX 5100 Postcolumn Reactor (Pickering, Mountain View, CA) and a Linear LC305 spectrofluorometric detector (Alltech Associates, Deerfield, IL) (*6*). GC toxins were eluted isocratically at 0.25 μL min^{-1} on a 5 μm, 250 mm × 2.1 mm Alltech Alltima C-18 (Alltech, IL) column with 10 mM heptafluorobutyric acid:CH_3CN (82:18, v/v), and the fluorescent derivatives were monitored using $\lambda_{excitation}$ = 330 nm and $\lambda_{emission}$ = 390 nm. LC-MS analyses were performed using an Agilent (Palo Alto, CA) model 1100 LC system connected to Perkin-Elmer-SCIEX (Concord, Ontario, Canada) API-165 single quadrupole MS and API4000 triple quadrupole systems equipped with ionspray sources. Analyses were conducted in positive ion mode. Analytical separations were carried out on a 250 mm × 2 mm i.d. column packed with 5 μm TosoHaas TSK-GEL Amide-80 material with 0.2 mL min^{-1} of mobile phase composed of CH_3-

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[1] Abbreviations: C toxin, N-sulfocarbamoyl-11-hydroxysulfate toxin; COSY, correlated spectroscopy; E.COSY, exclusive correlation spectroscopy; GC toxins, *Gymnodinium catenatum* toxins; GTX, gonyautoxin; HILIC-MS, hydrophilic interaction chromatography mass spectroscopy; HMBC, heteronuclear multiple bond connectivities; HMQC, heteronuclear multiple quantum correlation; LC-FLD, liquid chromatography with postcolumn oxidation and fluorescence detection; PSP, paralytic shellfish poisoning; STX, saxitoxin; TOCSY, total correlation spectroscopy.

10.1021/tx034037j CCC: $25.00
Published on Web 07/29/2003

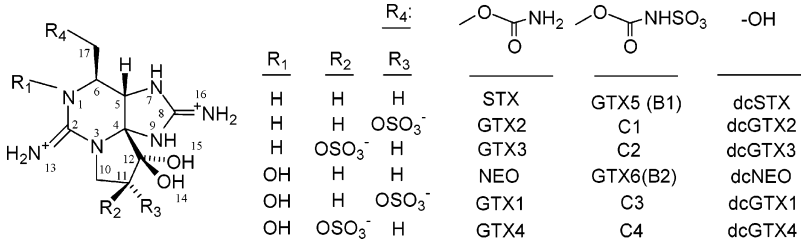

Figure 1. Structures of previously reported PSP toxins.

CN:H_2O (62:38, v/v) containing 2 mM ammonium formate and 3.5 mM formic acid (*7*).

Isolation. Cells were extracted in 0.05 N acetic acid using ultrasonication for 20 s in an ice bath. This was repeated three times, and the extracts were combined and stored frozen at −20 °C. Preliminary fractionation of the *G. catenatum* extract was performed using a 5 cm × 1.5 cm C-18 column (Waters 150 Å bulk packing). The column was eluted using a stepwise gradient from 0 to 100% (v/v) MeOH in 20% steps (30 mL each). Fractions containing **1**−**3** (LC-FLD) were combined and rechromatographed on a 1 cm × 15 cm C-18 column. The resulting fractions containing **1**−**3** were combined and dissolved in ACN:H_2O (8:2, v/v) and further separated by semipreparative hydrophilic interaction chromatography (HILIC, TosoHaas TSK-GEL Amide-80, ACN:H_2O (66.5:34.5, v/v) containing 2 mM ammonium formate and 3.5 mM formic acid).

MS and NMR Spectral Measurements. High-resolution Fourier transform electrospray mass spectrometry was used to measure accurate masses by infusion of the 80% (v/v) methanol fraction collected from the C18 column separation into the ESI source (Analytica of Branford, Branford, CT) of a BioApex 47e Fourier transform ion cyclotron resonance mass spectrometer (FTMS) (Bruker Daltonics, Billerica, MA) at a flow rate of 80 μL/h. A total of 16 transients in the time domain were summed prior to Fourier transform and data analysis. To ensure the accuracy of the mass measurement, the sample was mixed with an internal calibrant (10 mM sodium trifluoroacetate), and a new spectrum was obtained. The ions at *m/z* 473 were isolated in the analyzer cell and subjected to collisionally activated dissociation using sustained off-resonance irradiation excitation. Measurements of exact mass were also obtained by LC-MS using a Shimadzu LC600 isocratic pump and an Agilent 1050 autosampler connected to a Micromass Autospec oa-TOF (Micromass, Manchester, U.K.) hybrid sector/time-of-flight mass spectrometer equipped with an electrospray interface. Separation of components was carried out using the column and mobile phase noted above. Calibrant (5 mg mL^{-1} PPG in CH_3CN) was added to the column effluent via a tee junction with an ICSO SFC-500 micro flow pump at a rate of 0.02 mL min^{-1}. The combined column effluent and calibrant were then split, with 10% going to the mass spectrometer.

NMR experiments were carried out with a Bruker DRX-500 (Bruker, Canada). Measurements were performed on samples dissolved in either 0.1 M CD_3COOD in D_2O or 0.1 M CD_3COOD in H_2O:D_2O (9:1, v/v) and maintained at 20 or 5 °C. Experiments were referred to CD_2HCOOD at δH 2.03 ppm. Standard Bruker pulse sequences were used for ^{1}H, ^{13}C, COSY, E.COSY, TOCSY, HMQC, and HMBC experiments with solvent signal suppression where appropriate (see Claridge (*8*) for detailed descriptions of each NMR experiment).

Results and Discussion

Detection by LC-FLD. *G. catenatum* strain GCDE09 contains 12 of the known PSP toxins with C1−C4 representing on average 70−90% of the known toxin content on a molar basis (*3*, *4*). Three additional peaks were detected by LC-FLD (Figure 2a). Like the known PSP toxins, these compounds only fluoresce following postcolumn oxidation and exhibited a fluorescence emission maximum at 390 nm. Although the contribution of **1**−**3** to total toxin composition has not been quantified, these peaks represented approximately 30% of the total fluorescence response.

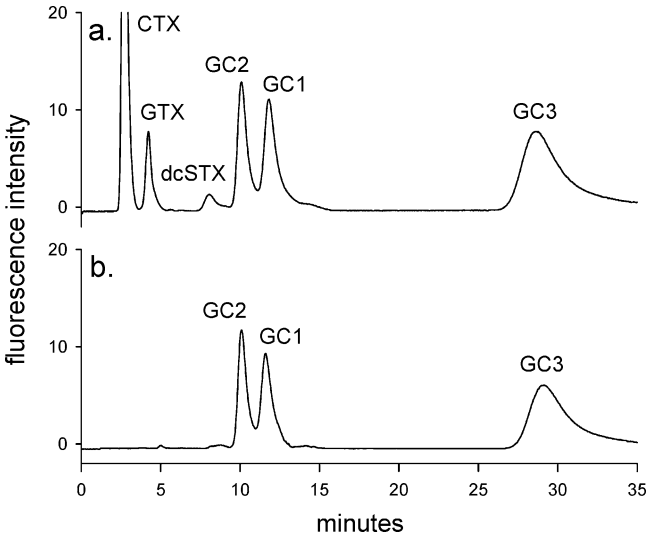

Figure 2. LC-FLD chromatograms of (a) crude *G. catenatum* extract and (b) amphipathic toxins GC1−GC3 (**1**−**3**), eluted with 80% methanol on a C-18 chromatography column.

Isolation and MS. Compounds **1**−**3** were extracted as minor components from the acetic acid extract of a 50 L culture of *G. catenatum*. The amphipathic nature of **1**−**3** allowed separation of these new toxins from known PSP toxins using C-18 chromatography. The known toxins eluted from a C-18 column with distilled water whereas **1**−**3** eluted with 80% v/v MeOH (Figure 2b). Partial purification was achieved by LC-FLD-guided fractionation on two successive C-18 columns. Only limited separation of **1** and **2** could be achieved using C18 chromatography, and after several weeks, fractions containing mostly **2** were found to revert to mostly **1**, indicating that the two compounds may be epimers.

Further purification was achieved using HILIC-MS. Signals were observed at *m/z* 377, 473, and 393, and these ions were plotted as mass chromatograms (Figure 3). The background-subtracted mass spectra acquired at the peak maxima (Figure 4) indicated that the molecular weights of **1** and **2** were both 472, while that of **3** was 376. The ion at *m/z* 393 in the spectra of **1** and **2** is due to loss of SO_3 from the $[M + H]^+$ ions. This fragmentation indicated that **1** and **2** were closely related to the 11-hydroxysulfate-STX epimers, GTX2 and GTX3. The differences in the spectra of **1** and **2** offer some clues as to their relative stereochemistries, and this is discussed later.

Accurate mass measurements with high-resolution mass spectrometry (Table 1) allowed the molecular

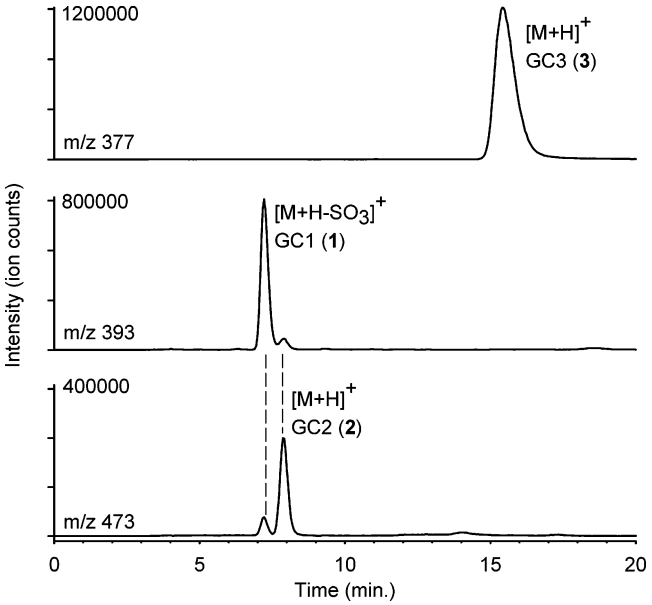

Figure 3. HILIC-MS selected ion chromatograms of toxins **1**−**3** in a *G. catenatum* extract.

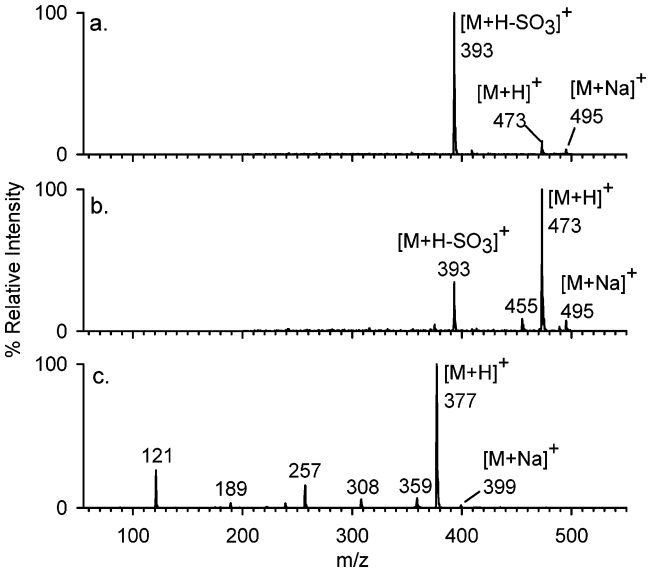

Figure 4. Full scan mass spectra of GC1 (a), GC2 (b), and GC3 (c) acquired from HILIC-MS analyses.

formulas of the new compounds to be assigned as $C_{16}H_{20}N_6O_9S$ for **1** and **2** and $C_{16}H_{20}N_6O_5$ for **3**. Tandem mass spectrometry was also used to reveal structural information on the compounds (Figure 5). The fragmentation pattern of **3** included ions at *m/z* 179, 204, and 221, providing clear evidence of a basic STX (**4**) skeleton since these ions are also present in the STX fragment ion spectrum (*9*). The loss of water from the $[M + H]^+$ ion of **3** to give *m/z* 359 is also characteristic of the STX structure. All of the evidence indicated a modification of the carbamate side chain of STX. A key ion in the spectrum of **3** is *m/z* 121, which was eventually assigned as $HO{-}C_6H_4C{\equiv}O^+$. A further key ion is the corresponding ion at *m/z* 257, due to $[M + H - 122]^+$. All of this indicated that the carbamate side chain of STX has been replaced with a hydroxbenzoate moiety.

NMR Spectroscopy. Preparative isolation using a wide bore HILIC column and repeated injections of material cleaned on a C18-silica column provided sufficient **1**, and a larger quantity of **3**, for NMR spectroscopy. The most obvious features of the ^{1}H NMR spectra were two aromatic doublet signals each integrating for 2H, at 6.95 and 7.95 ppm, consistent with the hydroxybenzoate moiety (Table 2). The coupling pattern located the hydroxy function on the aromatic ring *para* to the carbonyl. Most ^{1}H and ^{13}C assignments for **3** (Table 2) were postulated by comparison to STX (*10*) and confirmed by TOCSY, E.COSY, HMQC, and HMBC experiments (*8*). Four additional carbon signals were assigned to the six carbons of the hydroxybenzoate moiety by direct comparison to the carbon signals of methyl 4-hydroxybenzoate (*11*). HMQC experiments located directly bonded ^{1}H/^{13}C pairs. HMBC experiments independently proved the remaining ^{13}C assignments and several key connectivities, including the substitution of the hydroxybenzoate moiety at C17 (HMBC of H17 and H2′/6′ to C19). For **1**, the coupling of the two H10 protons and the sole remaining H11 proton located the position of substitution as C11. The ^{1}H NMR spectrum of **1** showed that δ_H and J_{HH} for H10a, H10b, and H11 closely resembled those for GTX2 (**5**) (H10a and H10b resonances separated by 0.18 ppm, (J(10b,11) = 5 Hz) and were distinctly different from those of GTX3 (**6**) (H10a and H10b resonances

Table 1. Accurate Mass Measurements on the New Compounds[a]

observed *m/z*	formula	expected mass	error (Δ, ppm)
	A		
473.1081	$[C_{16}H_{21}N_6O_9S]^+$	473.1091	2.1
495.0897	$[C_{16}H_{20}N_6O_9SNa]^+$	495.0904	1.4
455.0982	$[C_{16}H_{21}N_6O_9S-H_2O]^+$	455.0979	0.7
393.1511	$[C_{16}H_{21}N_6O_9S-SO_3]^+$	393.1517	1.5
	B		
473.1114	$[C_{16}H_{21}N_6O_9S]^+$	473.1091	4.8
393.1532	$[C_{16}H_{21}N_6O_6]^+$	393.1523	2.3
	C		
473.1101	$[C_{16}H_{21}N_6O_9S]^+$	473.1091	2.1
	D		
377.1585	$[C_{16}H_{21}N_6O_5]^+$	377.1573	3.1

[a] A, FTMS results from infusion of **1** and **2**; B, LC-HRMS result on **1**; C, LC-HRMS result on **2**; and D, LC-HRMS result on **3**.

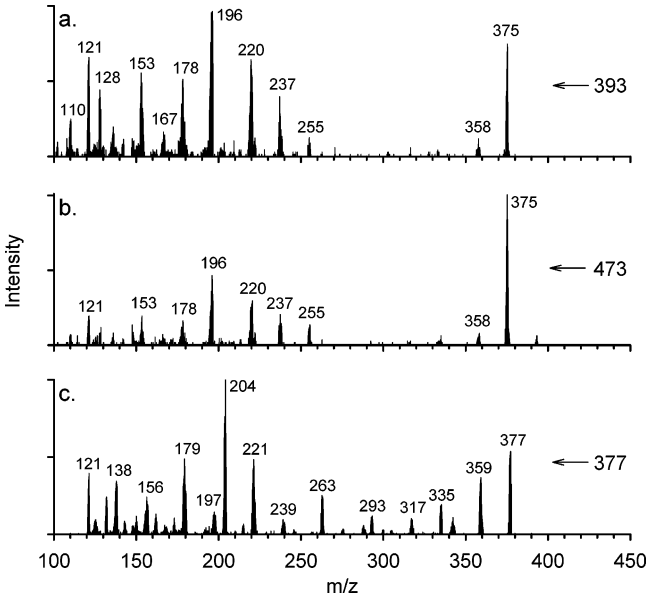

Figure 5. MS/MS spectra of *m/z* 393 of GC1 (a), *m/z* 473 of GC2 (b), and *m/z* 377 of GC3 (c).

Table 2. NMR Data for GC1 (1) and GC3 (3)[a]

	GC1	GC3	
C no.	δH (a,b)	δC	δH (a,b)
2		155.6	
4		82.0	
5	4.91*	57.0	4.82
6	4.02	52.7	3.97
8		157.4	
10	4.17, 3.96	42.3	3.79, 3.54
11	4.79*	32.4	2.37, 2.33
12		98.1	
17	4.61, 4.29	62.5	4.64, 4.25
19		167.7	
1′		120.4	
2′,6′	7.96	132.1	7.95
3′,5′	6.93	115.4	6.95
4′		161.1	
NH	8.45		8.33

[a] Solvent H_2O/D_2O (9:1) plus 0.1 M CD_3COOD. Reference: ^1H to CHD_2COOH = 2.03 ppm; ^{13}C to $^{13}CD_3COOH$ = 20.0 ppm. Spectra of GC1 (**1**) were recorded at 20 °C except for peaks marked with an *; these were obtained from spectra at 5 °C as they were obscured by the water resonance at 20 °C. Spectra of GC3 were recorded at 5 (^1H) or 9 °C (^{13}C). Coupling constants J(H,H) (Hz, error ± 0.5 Hz): GC1 (**1**): J(6,17a) 8.8, J(6,17b) 5.0, J(17a,17b) 11.7, J(2′,3′ and 5′,6′) 8.7, J(10a,10b) 12.1, J(10b,11) 5.2. GC3 (**3**): J(5,6) 0.9, J(6,17a) 9.3, J(6,17b) 5.0, J(17a,17b) 11.7, J(2′,3′ and 5′,6′) 8.8, J(10a,10b) 10.2, J(11a,11b) 11.0. Other couplings in GC3 (**3**) involving H10a,b and H11a,b were obscured by exchange at C11. The connectivity was confirmed by E.COSY.

separated by 0.61 ppm, (J(10a,11) = 7 Hz, J(10b,11) = 9 Hz) (*12*), indicating that the sulfate group in **1** had the same α-orientation as in GTX2 (**5**).

Stereochemistry. The fact that GC1 (**1**) and GC2 (**2**) interconverted (see above) supported their assignment as an epimeric pair. It is known that phytoplankton produce the *β*-epimer of the known 11-sulfated toxins (*3*). Over time, the sulfate group epimerizes to the more stable α-form (*13*). This epimerization is enhanced by elevated temperatures and higher pH (*14*). For all of the α/*β* epimeric pairs known from the PSP group, these being dcGTX1/dcGTX4, dcGTX2/dcGTX3, GTX1/GTX4, GTX2/GTX3, C1/C2, and C3/C4, the 11α-epimer is eluted from the HILIC column before the 11*β*-epimer (*7*). This supports the NMR evidence that the early eluting peak (Figure 3), designated GC1 (**1**), is the 11α-epimer and that the later eluting peak, designated GC2 (**2**), is the *β*-form. The reverse pattern is observed when using the reversed phase LC-FLD method (Figure 2), further supporting the epimer designation of the new toxins. Comparison of the fragmentation pattern for GTX2/GTX3 (**5**/**6**) and GC1/GC2 (**1**/**2**) also provides evidence for the configurations of the epimers. At identical orifice voltages, GTX2 (**5**) fragments readily with the loss of SO_3 to give a strong $[M + H - SO_3]^+$ ion, whereas GTX3 (**6**) gives a much stronger $[M + H]^+$ ion (*7*, *15*). This pattern indicates that the 11α-epimer is more vulnerable to fragmentation and loss of the 11-sulfate group. This same pattern of sulfate loss is displayed by the GC toxins (Figure 5), with GC1 (**1**) giving a relatively strong $[M + H - SO_3]^+$ ion and GC2 (**2**) giving a stronger $[M + H]^+$ ion, thus supporting GC1 (**1**) being the 11α-epimer and GC2 (**2**) being the 11*β*-epimer. The deduced structures of GC1−3 are shown in Figure 6.

GC1 and GC2 have been recognized previously as potential STX analogues by Negri et al. (*4*). Strains of *G. catenatum* from Australia, Portugal, Spain, Uruguay, and Hong Kong all contained GC1 (**1**) and GC2 (**2**)

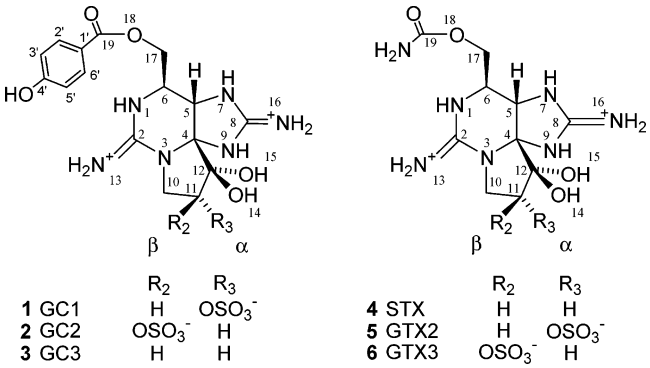

Figure 6. Structures of *G. catenatum* compounds (**1**−**3**), STX (**4**), and GTXs 2 (**5**) and 3 (**6**). The numbering system for the structures follows that of Hall et al. (*17*).

(previously designated C6 and C5, respectively). It has been observed that when extracts of *G. catenatum* are applied to an aging column using the LC-FLD technique, GC1 and GC2 are not always resolved. It is possible that the single peak observed by Oshima et al. (*3*) in another *G. catenatum* isolate, which he indicated to be a nontoxic but fluorescent compound, may have been a combination of GC1 and GC2. Onodera et al. (*16*) isolated five novel STX analogues containing an acetyl moiety on the C17 side chain. These toxins were from extracts of the cyanobacteria *Lyngbya wollei* and were found to be an order of magnitude less toxic than STX. It remains unclear whether the acetyl and hydroxybenzoate analogues share similar biosynthetic pathways. The toxicities of GC1−3 have not yet been established, but preliminary investigations show that these toxins do bind to rat brain sodium channels, demonstrating a biological activity indicative of the known PSP toxins (L. Llewellyn, unpublished results). The phenol group of GC1−3 results in compounds more hydrophobic than any other STX analogue previously described. It is unknown how this modification will affect the potential oral toxicity of this group of compounds, as oral toxicity is dependent on the rate of transport across the gut wall as well as strong binding of the toxins to the sodium channel. Work is continuing on the evaluation of the toxicity of GC1−3.

Acknowledgment. The technical assistance of Shelia Crain (NRC), Bill Hardstaff (NRC), and Dion Frampton (CSIRO) is greatly appreciated.

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TX034037J