Isolation and Structure Elucidation of New and Unusual Saxitoxin Analogues from Mussels

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Chemical analyses of plankton and highly toxic mussel samples collected in eastern Canada during an intense bloom of the dinoflagellate *Alexandrium tamarense* established the presence of a complex mixture of paralytic shellfish poisoning (PSP) toxins. Application of a newly developed technique, hydrophilic interaction liquid chromatography−mass spectrometry, confirmed the identities of the known toxins and revealed the presence in the mussels of five saxitoxin analogues (M1−M5) that were not present in the plankton. Four of these compounds were isolated and their structures established by tandem mass spectrometry, 1D- and 2D-NMR spectroscopy, and chemical interconversion experiments. One of these was found to be 11β-hydroxysaxitoxin (M2), while the other three were found to be new saxitoxin analogues, namely, 11β-hydroxy-*N*-sulfocarbamoylsaxitoxin (M1), 11,11-dihydroxy-*N*-sulfocarbamoylsaxitoxin (M3), and 11,11-dihydroxysaxitoxin (M4). Compound M5 remains unidentified because of insufficient material for characterization.

Paralytic shellfish poisoning (PSP) is a severe and occasionally fatal neurological illness caused by consumption of shellfish contaminated with potent neurotoxins. Such toxins are produced by "red tide" dinoflagellates belonging to *Alexandrium*, *Pyrodinium*, and *Gymnodinium* genera, subsequently accumulated in wild and cultured shellfish, and finally transmitted to humans through the food chain.[1]

Structurally, PSP toxins (Figure 1) are based on the parent compound, saxitoxin (STX), which possesses some interesting structural features, namely, a tetrahydropurine moiety with a five-membered ring fused at an angular position and a ketone hydrate stabilized by two neighboring electron-withdrawing guanidinium groups. Several derivatives of STX are formed by addition of hydroxyl or hydroxysulfate groups at the C11 position, *N*-hydroxylation at the N1 position, *N*-sulfation at the N21 position, or decarbamoylation to a hydroxyl function at the C17 position.

Due to differences in charge state and substitutions on the basic STX structure, various PSP toxins bind with different affinities to site 1 of sodium channels, resulting in different toxicities: the carbamoyl toxins are the most toxic and the *N*-sulfocarbamoyl derivatives are the least toxic. However, the latter can be converted into their highly toxic carbamoyl counterparts at low pH, such as in the stomach.[2]

Both toxicity-based assays and instrumental methods are used for routine monitoring of PSP toxins in shellfish.[3] The AOAC official mouse bioassay is used widely for providing a single integrated response from all the toxins.[4] However, this approach suffers from low sensitivity, poor reproducibility, interferences from other components in the extract, and lack of information on the specific toxins present. Chemical analysis methods are required for confirmation of positive results and for determining a detailed toxin profile of samples containing variable levels of individual toxins. The most common technique for the analysis of PSP toxins is ion-pair liquid chromatography coupled with postcolumn oxidation and fluorescence detection (LC-ox-FLD).[5,6] It is based on rapid conversion of PSP toxins into fluorescent derivatives under alkaline oxidative conditions. More comprehensive approaches based on liquid chromatography with mass spectrometry (LC-MS) detection have been developed.[6,7] Recently, our group proposed a new analytical method based on the combination of hydrophilic interaction liquid chromatography and tandem mass spectrometry (HILIC-MS/MS).[8,9] It allows the selective and sensitive detection and the accurate quantitation of all saxitoxin-related compounds in a single 30 min analysis, with no need for further confirmatory analyses.

In early June 2000, an intense bloom ($>7 \times 10^5$ cells/L) of the dinoflagellate *Alexandrium tamarense*, a known producer of PSP toxins, occurred in southeast Nova Scotia (Canada). This was shown to be responsible for enhanced mortality of farmed Atlantic salmon in aquaculture cages.[10] LC-ox-FLD analyses of the plankton showed a range of PSP toxins to be present. In decreasing order of relative abundance, the toxins C2, GTX4, NEO, GTX5, GTX3, GTX1, STX, C1, and GTX2 dominated the plankton toxin profile.

During investigation of this event, samples of wild blue mussels (a mixture of *Mytilus edulis* and *M. trossulus*) were collected from the vicinity of the salmon cages. The mussel samples showed very high toxicity (up to 67 000 μg saxitoxin equivalents per kg tissue) by the AOAC mouse bioassay. Their toxin profiles determined by LC-ox-FLD appeared very similar to those of the natural plankton samples, which was not surprising, as they had consumed this same plankton material. The newly developed HILIC-MS/MS method was used in chemical investigation of both plankton and mussel samples. These analyses unequivocally confirmed the LC-ox-FLD results but also indicated the presence in mussels of small amounts of five saxitoxin-related compounds not detected in the plankton. They were named M1−M5. This work reports on the isolation and structure elucidation of four of these compounds (Figure 2) based on MS/MS spectra, 1D- and 2D-NMR measurements, and chemical interconversion experiments.

Results and Discussion

Preparative work aimed at isolation in pure form of the presumably new compounds M1−M5 was carried out using whole mussel tissues (50 g). A combination of Biogel P2 column chromatography with FIA-MS detection was used in the isolation procedure. Biogel P-2 stationary phase is known to have a slight anionic charge; thus an ion exchange occurs according to the charge of the toxins: the first batch of fractions eluted contains mainly the neutral toxins (C1−C4), the second batch contains the monocharged toxins (GTX1−6 and their decarbamoyl derivatives), and the third batch contains the bicharged toxins (STX, NEO, and their decarbamoyl derivatives). M1 and M5 were contained in the GTX

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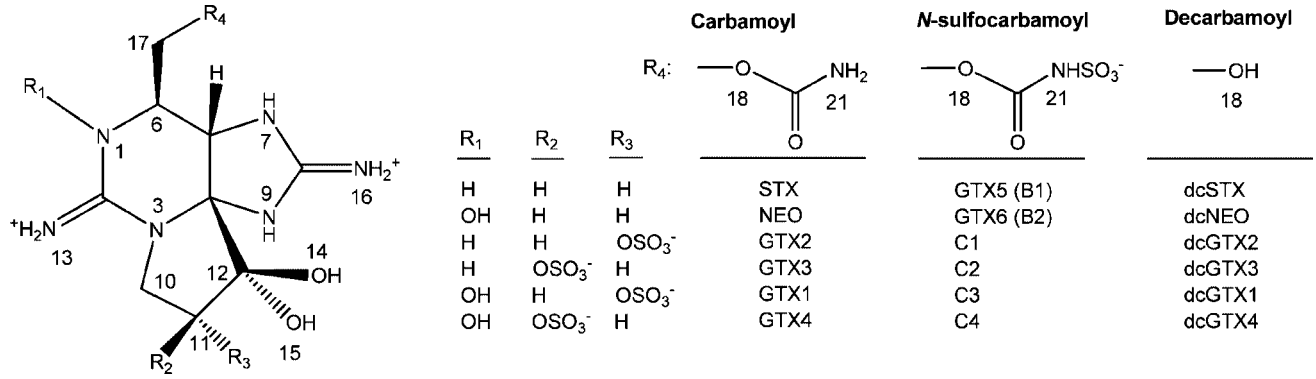

Figure 1. Structures of several known paralytic shellfish poisoning (PSP) toxins.

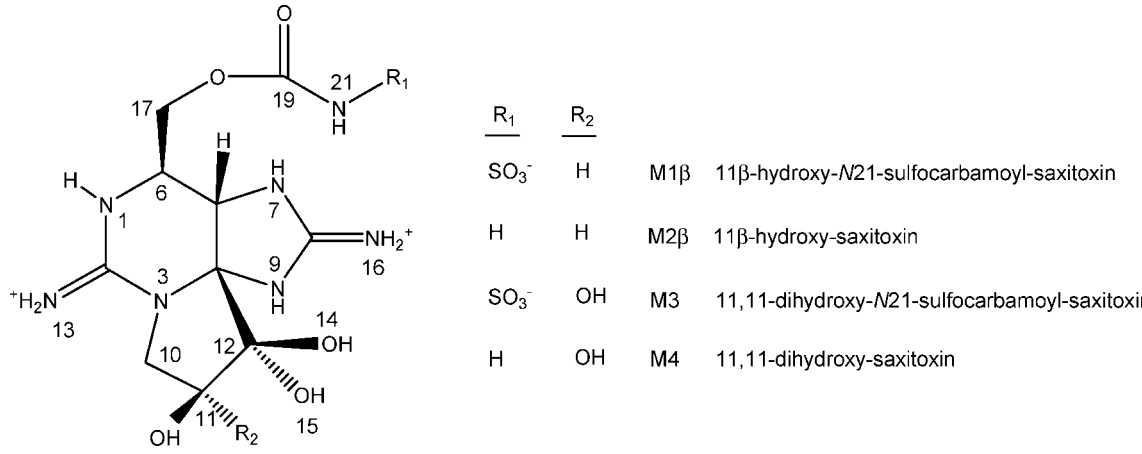

Figure 2. Structures of M1−M4.

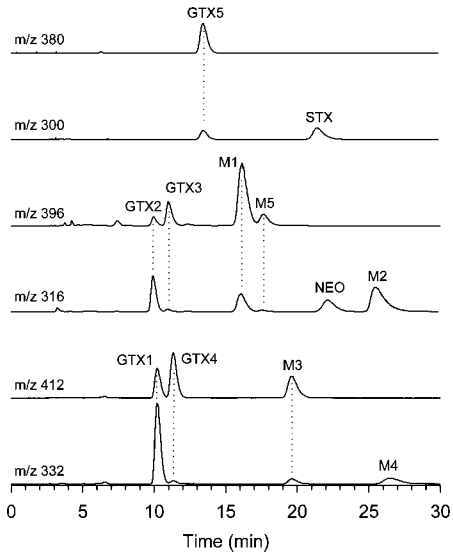

Figure 3. HILIC-MS analysis in SIM positive ion mode of the mussel extract after an initial Biogel P-2 column clean up. For LC conditions see the Experimental Section.

fraction, thus suggesting they possessed one net charge, while M2, M3, and M4 eluted within the bicharged compounds. However, the separation of the three groups of toxins was not complete after the ion exchange chromatography since each fraction contained small amounts of toxins from other groups. Figure 3 shows the HILIC-MS analysis in selected ion monitoring (SIM) mode of an extract that had been taken through an initial Biogel P2 column, which eliminated the neutral toxins. The SIM acquisition mode proved to be suitable for monitoring the toxins during the purification procedure, due to elimination of signals from other coextractives. The protonated ions and the main fragment ions formed in-source were selected as diagnostic and/or confirmatory ions of each toxin (Table 1).

Fractions containing M1−M5 were combined and further separated by semipreparative HILIC-MS, which provided submilligram amounts of the pure toxins. The basis of HILIC separation is the combination of a polar-bonded stationary phase and a mobile phase containing a high percentage of organic modifier plus a low concentration of volatile buffer. The separation mechanism involves partitioning of sample components between the mobile phase and a stagnant aqueous phase that forms at the stationary phase surface. Electrostatic interactions play a major role in separations of the PSP toxins, as supported by the observed retention times, which are low for neutral C toxins, intermediate for gonyautoxins, and high for double-charged toxins (Table 1). The effect of the hydroxysulfate function at the C11α or β position on the charge states of individual functional groups is critical for separation of epimeric pairs (GTX1/4 or GTX2/3). Molecular modeling showed that when the hydroxysulfate group at C11 is α-oriented (GTX1 and GTX2), an intramolecular interaction with the guanidinium function at C-8 is established that reduces the number of positively charged functions on the molecule available for interaction with the stationary phase. On the contrary, both guanidinium groups are available for interaction with the stationary phase when the hydroxysulfate group is at the C11β position (GTX3 and GTX4).

Identification of M2. The electrospray ionization (ESI) mass spectrum of M2 showed an $[M + H]^+$ ion at *m/z* 316. The MS/MS product ion spectrum of *m/z* 316 (Figure 4c) contained ions at *m/z* 298 (loss of H_2O), *m/z* 237 (loss of $NH_2COOH + H_2O$), *m/z* 220 (loss of $NH_2COOH + H_2O + NH_3$), and *m/z* 196 (loss of $NH_2COOH + NHCO + NH_3$). This spectrum and the compound's retention time matched exactly with those of an authentic sample

Table 1. Ions and Transitions Selected in Selected Ion Monitoring (SIM) and Selected Reaction Monitoring (SRM) Experiments, Respectively

		SIM[a]		SRM[a]	
toxin	t_R (min)[b]	$[M + H]^+$ ion (*m/z*)	in source fragment ion (*m/z*)	transition #1 (*m/z* > *m/z*)	transition #2 (*m/z* > *m/z*)
STX	20.3	**300**	282	**300 > 282**	300 > 204
NEO	21.0	**316**	298	**316 > 298**	316 > 220
GTX2	9.6	396	**316**	**396 > 316**	316 > 298
GTX3	10.7	**396**	316	**396 > 298**	396 > 316
GTX1	9.8	412	**332**	**412 > 332**	412 > 314
GTX4	10.9	**412**	332	**412 > 314**	412 > 332
GTX5 (B1)	13.1	**380**	300	**380 > 300**	300 > 282
GTX6 (B2)	14.6	**396**	316	**396 > 316**	396 > 298
C1	7.2	476	**396**	**396 > 316**	396 > 298
C2	8.0	476	**396**	**396 > 298**	396 > 316
C3	7.9	492	**412**	**412 > 332**	412 > 314
C4	8.8	492	**412**	**412 > 314**	412 > 332
dcSTX	21.1	**257**	239	**257 > 239**	
dcNEO	20.8	**273**	255	**273 > 255**	
dcGTX2	10.2	53	**273**	**353 > 273**	273 > 255
dcGTX3	11.3	**353**	273	**353 > 255**	353 > 273
dcGTX1	10.1	369	**289**	**369 > 289**	369 > 271
dcGTX4	11.4	**369**	289	**369 > 271**	369 > 289
M1	15.5	**396**	316	**396 > 316**	396 > 298
M2 (11β-OH-STX)	25.1	**316**	298	**316 > 298**	316 > 220
M3	19.7	**412**	332	**412 > 314**	412 > 332
M4	26.6	**332**	314	**332 > 314**	
M5	17.0	**396**	316	**396 > 316**	396 > 298

[a] The most intense ions and transitions are boldfaced. They were used as diagnostic ions and transitions in SIM and SRM experiments, respectively. [b] Retention times (t_R) are referenced to the following chromatographic conditions: 5 μm of TSK-Gel Amide-80 (2 × 250 mm i.d.) column; mobile phase 65% B isocratic with eluent A being water and B acetonitrile/water (95:5), both containing 2.0 mM ammonium formate and 3.6 mM formic acid (pH 3.5); flow rate 0.2 mL/min.

of 11β-hydroxy-STX. Therefore, M2 was identified as 11β-hydroxy-STX. NMR data for M2 are reported in Table 2.

Identification of M1. ESIMS of M1 showed an $[M + H]^+$ ion at *m/z* 396 and an $[M - H]^-$ ion at *m/z* 394, indicating a molecular weight of 395 for the free base. High-resolution ESIMS data were consistent with an elemental composition of $C_{10}H_{17}N_7O_8S$ ($[M + H]^+$ 396.0940 ± 0.0002 ($n = 6$), calc 396.0938, $\Delta = 0.5$ ppm), confirming M1 to be an isomer of GTX2 and GTX3. The MS/MS product ion spectrum of the $[M + H]^+$ ion (Figure 4a) showed prominent ions at *m/z* 316 (loss of SO_3), *m/z* 298 (loss of $SO_3 + H_2O$), and *m/z* 148. This fragmentation pattern was similar to that observed for GTX5, strongly suggesting the presence of an *N*21-sulfocarbamate group. This was also supported by the MS/MS spectrum of the $[M - H]^-$ ion, which showed fragment ions at *m/z* 122 ($O{=}C{=}N\text{-}SO_3^-$), *m/z* 97 ($HOSO_3^-$), and *m/z* 80 (SO_3^-), as does GTX5. The positive MS/MS spectrum of the in-source fragment ion at *m/z* 316 (Figure 4b) from M1 had prominent ions at *m/z* 298 (loss of H_2O) and *m/z* 237 (loss of $NH_2COOH + H_2O$) and was identical with that of M2 (Figure 4c), suggesting that M1 was the *N*21-sulfocarbamoyl derivative of 11-hydroxysaxitoxin.

Full structure elucidation of M1 was carried out on the basis of ^1H and ^{13}C NMR spectra and 2D-NMR experiments, including ^1H COSY, TOCSY, and $^1H/^{13}C$ HSQC in D_2O/0.1 M acetic acid-d_4 solution (pH 2.0). They showed two isolated ^1H spin systems (A: δ_H 4.79, 3.83, 4.11, 4.40; B: δ_H 3.26, 3.99, 4.42) in the molecule corresponding to CH5−CH6−$CH_2$17 and $CH_2$10−CH11 moieties, respectively. ^1H and ^{13}C chemical shifts and $^1H-^1H$ coupling constants of M1 (Table 3) were compared with literature data for GTX5[11] and 11α- and 11β-hydroxysaxitoxin[12] obtained from samples in D_2O at unspecified pH (Table 2). We also recorded ^1H and ^{13}C spectra from a sample of 11β-OH STX at pH 2.0 and a ^1H spectrum from an authentic sample of GTX5 at pH 3.75 (Table 2). The δ_C at pH 2.0 for M1 were closely similar to those for 11β-OH STX, slightly displaced to high field for all positions except C6 (shielded in M1 by 0.4 ppm) and C17 (deshielded by 0.4 ppm). C19 was shielded by only 0.2 ppm in M1 compared to 11β-OH STX. The δ_H differences between 11β-OH STX and M1 were all less than 0.08 ppm, but again the only deshielding in M1 compared to 11β-OH STX occurred at the H17 resonances.

J_{HH} values (Tables 2 and 3) were the same within error for M1 and 11β-OH STX, but differed from those of 11α-OH STX. In particular, couplings involving protons at positions 10 and 11 indicated that the stereochemistry at C11 was identical in 11β-OH STX and M1. The combined NMR and MS results suggested that M1 had the same structure as 11β-OH STX except for the *N*-sulfocarbamate function. Changes in chemical shifts of carbons at positions 6 and 17 for M1 compared to 11β-OH STX suggested that the carbamoyl group was the point of sulfation. The magnitudes and signs of these changes (see above) agreed with those deduced from a comparison of the reported NMR data of STX and GTX5[11] (Table 2), in which substitution of a sulfate at the carbamoyl group produced shielding at C19 by 0.5 ppm, deshielding at C17 by 0.4 ppm, and shielding by 0.2 ppm at C6.

Additional chemical evidence unequivocally demonstrated the proposed structure as 11β-hydroxy-*N*21-sulfocarbamoylsaxitoxin. HILIC-MS analysis showed the disappearance of M1 upon heating in 0.1 M HCl at 100 °C for 15 min and the appearance of a compound with a mass spectrum and retention time that matched those of 11β-OH STX.

Identification of M3. The structure of M3 was also deduced from MS and NMR evidence. ESI mass spectra of M3 showed an $[M + H]^+$ ion at *m/z* 412 and an $[M - H]^-$ at *m/z* 410, indicating a molecular weight of 411 for the free base. High-resolution ESIMS data were consistent with an elemental composition $C_{10}H_{17}N_7O_9S$ ($[M + H]^+$ 412.0893 ± 0.0006 ($n = 6$), calc 412.0887, $\Delta = 1.6$ ppm).

The MS/MS product ion spectrum of the $[M + H]^+$ ion of M3 (Figure 4d) paralleled that of M1 (Figure 4a), showing prominent ions at *m/z* 332 (loss of SO_3), *m/z* 314 (loss of $SO_3 + H_2O$), and *m/z* 164. Additional ions not present in the spectrum of M1 were observed at *m/z* 296 (loss of $SO_3 + 2H_2O$) and *m/z* 235 (loss of $HO_3SNHCOOH + 2H_2O$), in the positive MS/MS spectrum of the in-source fragment ion at *m/z* 332 from M3 (Figure 4e), suggesting the presence of an additional hydroxyl function in M3 compared with M1. As for M1, the presence of an *N*21-sulfocarbamate group was also supported by the MS/MS spectrum of the $[M - H]^-$ ion, which showed fragment ions at *m/z* 122 ($O{=}C{=}N\text{-}SO_3^-$), *m/z* 97 ($HOSO_3^-$), and *m/z* 80 (SO_3^-).

Combined analyses of ^1H, ^{13}C, and 2D NMR spectra (^1H COSY, TOCSY, and $^1H/^{13}C$ HSQC) of M3 (Table 3) showed again two ^1H spin systems: one (δ_H 4.83, 3.85, 4.11, 4.43) corresponding closely in δ_H, J_{HH}, and δ_C of directly bonded carbons, to system CH5−CH6−$CH_2$17 in M1, the other (δ_H 3.64, 3.88) consisting of a geminal pair ($CH_2$10) with no further coupling, unlike M1. The absence of a ^1H resonance for H11 and the lack of vicinal couplings for H10a,b (Table 3), taken with the unequivocal molecular formula, provided strong evidence that C11 in M3 bore two OH groups. The structure M3 was fully supported by ^{13}C NMR data. Particularly, signals at δ 97.1 and 97.3 clearly pointed to the presence in the molecule of an additional carbon bearing two hydroxyl functions, besides C12. δ_C for C2 to C8 were all within 0.4 ppm of those for the corresponding positions in M1β, and for C17 and C19 they were coincident. In contrast, C10 in M3 was further deshielded by 6.1 ppm and C12 was shielded by 0.9 ppm, in agreement with the additional OH group at C11. The *N*-sulfocarbamate group of M3 was indicated for the same reasons as for

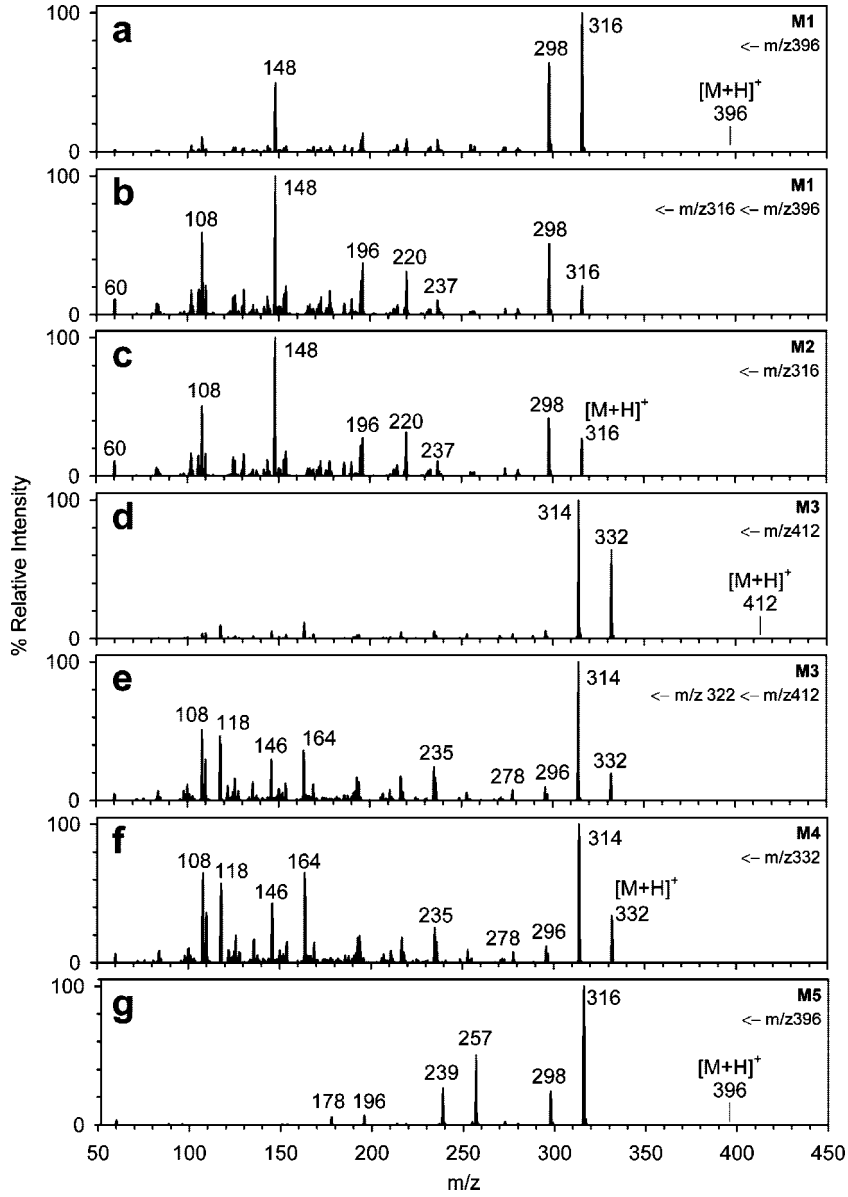

Figure 4. Product ion mass spectra acquired in the positive ion mode: (a) M1 $[M + H]^+$, *m/z* 396; (b) M1 $[M + H - SO_3]^+$, *m/z* 316; (c) M2 $[M + H]^+$, *m/z* 316; (d) M3 $[M + H]^+$, *m/z* 412; (e) M3 $[M + H - SO_3]^+$, *m/z* 332; (f) M4 $[M + H]^+$, *m/z* 332; (g) M5 $[M + H]^+$, *m/z* 396.

M1. On the basis of these data, it was concluded that M3 is 11,11-dihydroxy-*N*21-sulfocarbamoylsaxitoxin.

Identification of M4. The ESI mass spectra of M4 showed an $[M + H]^+$ at *m/z* 332 and an $[M - H]^-$ at *m/z* 330, indicating a molecular weight of 331 for the free base. High-resolution ESIMS data were consistent with an elemental composition of $C_{10}H_{17}N_7O_7$ ($[M + H]^+$ 332.1325, calc 332.1319, $\Delta = 1.8$ ppm).

The MS/MS product ion spectrum of the $[M + H]^+$ ion (Figure 4f) was identical to that of the in-source fragment ion at *m/z* 332 from M3 (Figure 4e) in both fragmentation pattern and ion ratio. The ^{1}H NMR spectrum of M4 (Table 3) closely resembled that of M3 in both δ_H and J_{HH}, except for H17a and H17b resonances, which were further shielded in M4 by 0.09 and 0.12 ppm, respectively. Lack of a ^{1}H resonance for H11 and of vicinal couplings for H10a,b suggested that C11 in M4 bore two OH groups, as in M3. Although the small amounts of M4 available hampered acquisition of ^{13}C NMR data, the whole of the above MS/MS and ^{1}H NMR results pointed to M4 being the carbamate analogue of M3, namely, 11,11-dihydroxysaxitoxin. This structural assignment was further substantiated by a desulfation reaction of M3, which provided a product with the same retention time and MS/MS spectrum as M4. On the basis of these data, it was concluded that M4 is 11,11-dihydroxysaxitoxin.

Identification of M5. M5 eluted 1.5 min later than M1 on the HILIC column, and its mass spectrum showed an $[M + H]^+$ ion at *m/z* 396, the same as that of M1. High-resolution ESIMS data were consistent with an elemental composition of $C_{10}H_{17}N_7O_8S$ ($[M + H]^+$ 396.0941 ± 0.0004 ($n = 4$), calc 396.0938, $\Delta = 0.8$ ppm), confirming M5 to be an isomer of M1 and GTX2/3. On the basis of HILIC behavior of the epimeric pairs GTX2/3 and GTX1/4, we initially hypothesized that M5 might be an epimeric analogue of M1, i.e., 11α-hydroxy-*N*21-sulfocarbamoylsaxitoxin. However, its product ion mass spectrum (Figure 4g) was very different from that of M1, suggesting a significant structure modification. In addition, it was realized that the separation of compounds with 11α- and 11β-hydroxy substituents is not optimal on the HILIC column, as demonstrated by the poor separation of 11α- and 11β-hydroxysaxitoxins (data not shown). Unfortunately, quantities of M5 were insufficient for NMR investigation, and thus it remains unidentified.

LC-FLD Analyses. Responses of individual solutions of the new compounds in the LC-ox-FLD method were investigated. M1

Table 2. NMR Data (δ_C, δ_H, J_{HH}) for the Following Known PSP Toxins: STX, GTX5, 11α-OH STX, and 11β-OH STX (namely M2)

	STX[11a]		GTX5[11a]		GTX5[b,c]	11α-OH STX[12a]	11β-OH STX[12a]	11β-OH STX[b,d]	
C	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
2	156.6		154.8					156.4	
4	83.0		83.1					82.6	
5	57.5	4.70, d(1.3)	57.6	4.71, d(1.3)	4.74, d(1.3)	4.84, s	4.80, d(1.1)	58.2	4.80, d (1.2)
6	53.6	3.84, ddd(1.3, 6.0, 9.0)	53.4	3.85, ddd(1.3, 6.0, 9.5)	3.87, ddd(1.1, 4.9, 9.9)	4.08, dd(6.0, 6.4)	3.81, ddd(5.5, 9.6, 1.1)	53.7	3.85, ddd(5.4, 9.5, 1.2)
8	158.4		156.7					nd	
10a	43.5	3.57, ddd (8.0, 10.0, 11.0)	43.2	3.57, m	3.61, m	3.70, d (11.8)	3.26, dd(7.3, 10.6)	49.2	3.30, dd (7.2, 10.3)
10b		3.82, ddd (2.5, 10.0, 11.0)		3.77, m	3.79, m	3.84, dd (5.0, 11.5)	4.01, dd (8.4, 10.3)		4.05, dd(8.2, 10.3)
11	33.5	2.37, m	33.5	2.37 m	2.33 m	4.28 d(5.0)	4.44, dd(8.1, 7.0)	71.2	4.49, dd(8.2, 7.2)
12	99.1		99.1					98.4	
17a	63.8	4.02, dd(6.0, 12.0)	64.2	4.09, dd(6.0, 12.0)	4.10, dd(4.9, 11.9)	4.18,dd(11.6, 6.3)	4.02, dd(12.0, 5.5)	63.7	4.07, dd(11.7, 5.2)
17b		4.25, dd(9.0, 12.0)		4.37, dd(9.5, 12.0)	4.40, dd(9.7, 11.9)	4.38, dd(6.0, 12.4)	4.25, dd(9.2, 11.4)		4.36, dd(9.5, 11.7)
19	159.3		158.8					158.4	

[a] Chemical shifts of ^{1}H resonances in refs 11 and 12 were referred to dissolved $CHCl_3$ in D_2O, at unspecified pH. To convert to an internal C^1HD_2COOD reference, 0.37 ppm was added to δ_H values. [b] ^{13}C resonances were referred to internal dioxane at 67.6 ppm. ^{1}H NMR spectra recorded at 500.13 MHz. ^{13}C NMR spectra recorded at 125.77 MHz. ^{1}H reference to CHD_2COOD at $\delta_H = 2.03$. ^{13}C reference to dioxane at $\delta_C = 67.6$. Multiplicity s = singlet, d = doublet, dd = double doublet, ddd = double double doublet, m = multiplet, b = broad, nd = not detected, *interchangeable. J_{HH} in Hz (error ± 0.3 Hz). [c] Sample provided by NRC CRMP, dissolved in D_2O/0.1 M CD_3COOD, pH 3.75. [d] Sample provided by NRC CRMP, dissolved in D_2O/DCl, pH 2.0.

Table 3. NMR Data (δ_C, δ_H, J_{HH}) for New PSP Toxins M1, M3, and M4

	M1[a,b]		M3[a,c]		M4[a,d]
pos.	δ_C	δ_H, J_{HH}	δ_C	δ_H, J_{HH}	δ_H, J_{HH}
2	156.1		156.5		
4	82.3		81.9		
5	58.1	4.79, d *1.0*	58.5	4.83, d *0.7*	4.86
6	53.3	3.83, ddd *5.1, 9.9, 1.0*	53.3	3.85, ddd *5.1, 9.7, 0.7*	3.81, bdd *5.1, 9.7*
8	154.3		154.2		
10a	49.0	3.26, dd *6.8, 10.5*	55.1	3.64, d *10.4*	3.60, d *10.3*
10b		3.99, dd *8.2, 10.5*		3.88, d *10.4*	3.87, d *10.3*
11	71.0	4.42, dd *8.2, 6.8*	97.1*		
12	98.2		97.3*		
17a	64.1	4.11, dd *5.1, 11.8*	64.1	4.11, dd *5.1, 11.8*	4.02, dd *5.2, 11.7*
17b		4.40, dd *9.9, 11.8*		4.43, dd *9.7, 11.8*	4.31, dd *9.6, 11.7*
19	158.2		158.2		

[a] ^{1}H NMR spectra recorded at 500.13 MHz. ^{13}C NMR spectra recorded at 125.77 MHz. ^{1}H reference to CHD_2COOD at $\delta_H = 2.03$. ^{13}C reference to dioxane at $\delta_C = 67.6$. Multiplicity s = singlet, d = doublet, dd = double doublet, ddd = double double doublet, b = broad, *interchangeable. J_{HH} in Hz (error ± 0.3 Hz). [b] Sample dissolved in D_2O/0.1 M CD_3COOD, pH 2.0. Assignments based on ^{1}H/^{1}H COSY, TOCSY, and ^{1}H/^{13}C HSQC spectra. [c] Sample dissolved in 9/1 H_2O/D_2O/0.1 M CD_3COOD, pH 3.9. Assignments based on ^{1}H/^{1}H COSY, TOCSY, and ^{1}H/^{13}C HSQC spectra from earlier sample dissolved in D_2O/0.1 M CD_3COOD, pH 2.0. [d] Sample dissolved in H_2O/0.1 M CH_3COOH, pH 4.6. Temperature 20 °C.

gave a poor molar response, estimated at ca. 100-fold lower than STX, while no response at all was observed for M3 and M4. The reasons for the low fluorescence signal are not understood at this time. These results do provide an explanation for why the new compounds had not been detected before and show the key role played by the newly developed HILIC-MS method in highlighting the presence of the new compounds.

Toxicity. The very limited amounts of pure M1, M3, and M4 isolated from mussels prevented us from evaluating their toxicological properties. However, on the basis of structure−activity relationship (SAR) studies for other saxitoxin analogues,[2,13] we can speculate that the specific toxicities of M1, M3, and M4 will be relatively low. SAR suggests that hydrogen bonding with the hydroxyl group at C12α and C12β and ion pairing between guanidinium groups and an anionic site on the channel are probably major factors involved in receptor binding. Thus, pK_a values at the two guanidinium groups and ketone hydrate at C12 play a key role in binding affinity. Substitution at C11α, C11β, or N1 seems to cause a ca. 3-fold reduction in the toxicity, but sulfation at N21 results in a remarkable 15-fold decrease (e.g., intraperitoneal toxicity in mice of 2483 MU/μmol for STX versus 791 MU/μmol for 11α/β-OH STX and 160 MU/μmol for GTX5).

Conclusions. Three new saxitoxin analogues, M1, M3, and M4, were isolated from Canadian mussels during an intense bloom of *A. tamarense*. The novel structures represent significant additions to the PSP toxins class. Particularly, vicinal *gem*-diols contained in M3 and M4 constitute a very unusual structural feature, the only example in natural products being, to the best of our knowledge, the tannin geraniin.[14] The new compounds, as well as M2, appear to be metabolites and/or degradation products formed in shellfish, as they were not observed in the toxin profile of the plankton consumed by the mussels. This sheds new light on the fate of PSP toxins as they enter the food web.

The poor response of M1, M3, and M4 in the LC postcolumn oxidation-FLD method partially explains their late discovery and points to the key role played by the newly developed HILIC-MS method in detection of such compounds. Thus, besides the structural novelty of the new compounds, the finding reported herein unequivocally demonstrates the power of HILIC-MS to detect the presence of new saxitoxin analogues together with known derivatives. The new technique showed great potential both in monitoring the fate of the toxins during isolation procedures and in addressing structural studies. The complementary use of HILIC, MS, and NMR appears to be a powerful combination for the structural investigation of polar marine biotoxins.

Experimental Section

Chemicals and Samples. All organic solvents were of distilled-in-glass grade (Caledon Laboratories, Georgetown, ON, Canada). Water was distilled and passed through a MilliQ water purification system (Millipore Ltd., Bedford, MA) to 18 MΩ quality or better. Formic acid (90%, laboratory grade) and ammonium formate (AR grade) were purchased from Fisher Scientific (Fair Lawn, NJ). Certified reference materials for PSP toxins were provided by the NRC Certified Reference Materials Program (Institute for Marine Biosciences, Halifax, NS, Canada).

Wild mussels (a mixture of *Mytilus edulis* and *M. trossulus*) were collected in June 2000 from one sampling site located along the Eastern Canada coasts. The shellfish tissues were found to be highly toxic (up to 67 000 μg saxitoxin equivalents per kg tissue) by the official mouse bioassay method for PSP toxins.[4]

Extraction and Isolation. The mussel tissues (50 g, wet weight) were placed into 150 mL plastic centrifuge tube and homogenized with Polytron at 11 000 rpm. The homogenate was added to 50 mL of the extraction solvent (0.1 M acetic acid) and blended at medium speed for 1 h. The resulting mixture was centrifuged at 3000 rpm for 10 min to produce a pellet, which was extracted twice more in order to ensure the recovery of all the toxins. The supernatants were combined and the volume was made up to 160 mL. A 100 μL aliquot of the crude extract was filtered through a 0.45 μm filter and directly analyzed by HILIC-MS/MS. The crude extract was then partitioned against dichloromethane (2 × 260 mL), and the aqueous layers were combined and

freeze-dried. The residue was dissolved in 10 mL of 0.1 M AcOH and loaded onto a low-pressure column, 2.7 × 90 cm (Pharmacia, Sweden), with the stationary phase Biogel P-2 gel (fine, 45−90 μm wet) (Bio-Rad Laboratories, CA). Acetic acid (0.1 M) was used as mobile phase, and the flow rate was kept constant at 0.5 mL/min; 10 mL fractions were collected automatically overnight. All the fractions were analyzed by flow injection analysis (FIA)-MS at 0.05 mL/min in SIM mode (Table 1) to check the appearance of the toxins. Fractions containing M1−M4 were combined and reloaded onto a Biogel P-2 column, in order to increase the purity. The resulting fractions were combined and further separated by semipreparative HILIC using a 5 μm TSK-Gel Amide-80 (7.8 × 250 mm i.d.) column maintained at 20 °C and eluted isocratically at 4 mL/min with 65% B, where eluent A was water and B was acetonitrile/water (95:5), both containing 2.0 mM ammonium formate and 3.6 mM formic acid (pH 3.5). A sample injection volume of 10 μL was used in most cases. A mass spectrometer was used as the detector. This procedure led to the isolation in pure form of M1β (0.2 mg), M2 (0.1 mg), M3 (0.1 mg), M4 (0.1 mg), and M5 (0.03 mg).

MS Experiments. MS experiments were performed using a HP1090 liquid chromatograph (Hewlett-Packard Co., CA) coupled either to a PE-SCIEX API 165 single quadrupole (Concord, ON, Canada) or a SCIEX API III+ triple quadrupole mass spectrometer, equipped with pneumatically assisted electrospray (Ionspray) ionization sources. The LC equipment included a solvent reservoir, in-line degasser, binary pump, refrigerated autosampler, and temperature-controlled column oven. The HILIC-MS analyses were carried out using a 5 μm Amide-80 (250 × 2 mm i.d.) column (Tosoh Bioscience LLC, Montgomeryville, PA) maintained at 20 °C and eluted isocratically at 0.2 mL/min with 65% B, where eluent A was water and B was acetonitrile/water (95:5), both containing 2.0 mM ammonium formate and 3.6 mM formic acid (pH 3.5). A postcolumn split was employed to deliver approximately 20 μL/min to the ion spray interface. A sample injection volume of 5 μL was used in most cases.

Full scan spectra were collected in the mass range *m/z* 250−550. MS/MS product ion spectra on the API-III$^+$ were acquired at a collision energy of 30 V using either the protonated or in-source fragment ions as precursor ions. Argon was used as collision gas in the second radio frequency only quadrupole. Selected ion monitoring (SIM) and selected reaction monitoring (SRM) detection were carried out by selecting ions and transitions reported in Table 1, respectively. Ion dwell times were adjusted to give a total cycle time of 1 s.

High-resolution ESIMS (positive and negative ion modes) were performed on a VG Autospec sector instrument operating at 5000 resolution.

LC-ox-FLD Experiments. The postcolumn LC-FLD analyses were performed according to the method described by Oshima[5] with further modifications. The system used a Develosil-C8 (250 × 4 mm i.d.) column (Nomura Chemical, Japan), a 10 μL injection volume, and three different elution conditions for the separation of selected groups of PSP toxins. In all cases, the flow rate was maintained at 0.8 mL/min and the elution conditions were 2 mM 1-heptanesulfonic acid in 30 mM ammonium phosphate buffer (pH 7.1) with 5% CH_3CN for the bicharged toxins (STX, NEO, and their decarbamoyl derivatives); 2 mM 1-heptanesulfonic acid in 10 mM ammonium phosphate buffer (pH 7.1) for monocharged toxins (GTX1−6 and their decarbamoyl derivatives); and 1 mM tetrabutyl ammonium phosphate solution adjusted to pH 6.0 with acetic acid for the neutral toxins (C1−C4). The eluate was mixed continuously with 7 mM periodic acid in 50 mM sodium phosphate buffer (pH 7.9) at 0.4 mL/min, it was then passed through a Teflon mixing coil (0.5 mm × 10 m) maintained at 65 °C, and it was finally mixed with 0.5 M acetic acid at 0.4 mL/min just before detection. The Hitachi F-1000 fluorimeter was set at 330 nm excitation and 390 nm emission. Individual solutions of pure M1, M3, and M4 were injected under the conditions of elution of both mono- and bicharged toxins. Their response at different reaction coil temperatures was investigated.

Reactions. Chemical interconversion of M1 into M2 and of M3 into M4 was accomplished by a desulfation reaction. An aliquot of purified M1 (or M3) was lyophilized and reconstituted with 50 μL of 0.1 M HCl. The solution was kept at 100 °C for 15 min. A 10-fold dilution was carried out before HILIC-MS analyses. SIM and SRM experiments were carried out by selected ions and transitions reported in Table 1.

NMR Experiments. NMR spectra were measured on a Bruker DRX-500 spectrometer in D_2O/0.1 M CD_3COOD solution (pH 2.0), in 9:1 H_2O/D_2O/0.1 M CD_3COOD solution (pH 3.9), or in H_2O/0.1 M CH_3COOH solution (pH 4.6). ^1H NMR spectra were recorded at 500.13 MHz (^1H reference to CHD_2COOD or CH_3COOH at $\delta_H = 2.03$). ^{13}C NMR spectra were recorded at 125.77 MHz (external ^{13}C reference to dioxane at $\delta_C = 67.6$). Two-dimensional ^1H-COSY, TOCSY, and $^1H/^{13}C$ HSQC NMR were carried out to unambiguously assign signals. The correlation time (mixing time) in the TOCSY experiments was 160 ms (optimized for long-range). For HSQC experiments, the $^1J(CH)$ was assumed to be 135 Hz. NMR data for the known PSP toxins STX, GTX5, 11α-OH STX, and 11β-OH STX (M2) are reported in Table 2.

M1. ESIMS yielded *m/z* 396 $[M + H]^+$ in positive ion mode and *m/z* 394 $[M - H]^-$ in negative ion mode. HRESIMS (positive ion mode) yielded *m/z* 396.0940 ± 0.0002 ($n = 6$) (calcd for $C_{10}H_{17}N_7O_8S$ $[M + H]^+$ 396.0938, $\Delta = 0.5$ ppm). ^1H and ^{13}C NMR data (D_2O/0.1 M CD_3COOD, pH 2.0) are reported in Table 3.

M2. ESIMS yielded *m/z* 316 $[M + H]^+$ in positive ion mode and *m/z* 314 $[M - H]^-$ in negative ion mode. ^1H and ^{13}C NMR data (D_2O/DCl, pH 2.0) are reported in Table 2.

M3. ESIMS yielded *m/z* 412 $[M + H]^+$ in positive ion mode and *m/z* 410 $[M - H]^-$ in negative ion mode. HRESIMS (positive ion mode) yielded *m/z* 412.0893 ± 0.0006 ($n = 6$) (calcd for $C_{10}H_{17}N_7O_9S$ $[M + H]^+$ 412.0887, $\Delta = 1.6$ ppm). ^1H and ^{13}C NMR data (9:1 H_2O/D_2O/0.1 M CD_3COOD, pH 3.9) are reported in Table 3.

M4. ESIMS yielded *m/z* 332 $[M + H]^+$ in positive ion mode and *m/z* 330 $[M - H]^-$ in negative ion mode. HRESIMS (positive ion mode) yielded *m/z* 332.1325 ± 0.0006 ($n = 6$) (calcd for $C_{10}H_{17}N_7O_7$ $[M + H]^+$ 332.1319, $\Delta = 1.8$ ppm). ^1H NMR data (H_2O/0.1 M CHD_2COOD, pH 4.6) are reported in Table 3.

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Supporting Information Available: ^1H, ^{13}C, and $^1H/^{13}C$ HSQC NMR spectra of compounds M1−M4. This material is available free of charge via the Internet at http://pubs.acs.org.

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