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Structural Elucidation of Ciguatoxin Congeners by Fast-Atom Bombardment Tandem Mass Spectroscopy

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Many tropical species of fish when ingested may cause an intoxication known as ciguatera.[1] The causative toxins originate in an epiphytic dinoflagellate *Gambierdiscus toxicus* and enter the food chain via herbivorous to carnivorous fish. Toxins labeled as CTX4A,[2] CTX4B,[3] and CTX3C[4] were isolated from *G. toxicus* and their structures elucidated. These dinoflagellate toxins undergo changes in the fish, yielding the principal toxin, ciguatoxin (CTX),[1,3,5] and a number of congeners. Many of the congeners remained unidentified because of the extreme difficulty in obtaining enough material for NMR studies. Recently, 50 toxic fractions were isolated from *G. toxicus* and from three species of fish collected in French Polynesia.[6] Despite our best effort, many of the fractions remained mixtures in microgram amounts. We undertook structural elucidation with these small and impure samples by new strategies.

For years we have used fast-atom bombardment tandem mass spectrometry (FAB/MS/MS),[7] in combination with NMR, for structural studies of polyether compounds: e.g., yessotoxin,[8a] maitotoxin,[8b] brevetoxins B2 through B4,[8c,d,e] and azaspiracid.[8f] An ionic charge located near the termini of the molecules facilitated charge-remote fragmentations induced by high-energy (8 kV) collision activation.[9] Though CTX congeners possess no charge group, we found that their Na-adduct ions could provide comparable information. When further support for structural assignment was needed, we prepared 2-sulfobenzoates.[10] To assign the configuration of spiro carbons, we followed acid-catalyzed rearrangement of spiroketal rings by liquid chromatography coupled with mass spectrometry (LC/MS).[11]

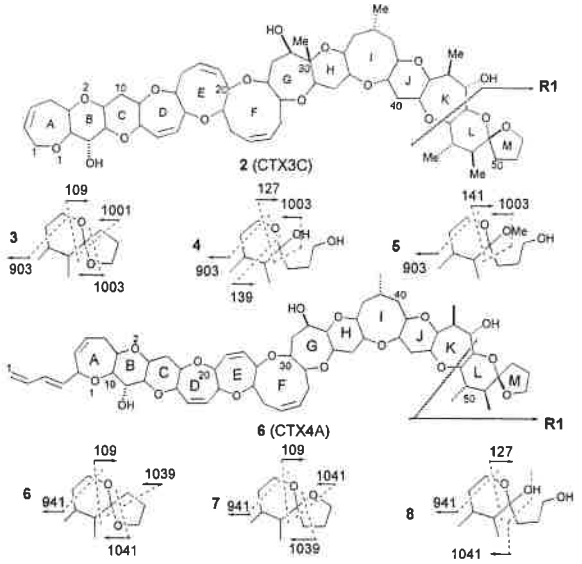

Figure 1. Structures and characteristic fragments of congeners found in *G. toxicus*. The mass units indicated are those of Na-adduct ions.

In FAB/MS/MS spectra of two known structures, CTX ($C_{60}H_{86}O_{19}$, **1**) and CTX3C (**2**) fragment ions were clearly related to the size and alignment of ether rings, in analogy with charge-remote fragmentation of charged polyethers (Supporting Information). Shifts of ions in the spectrum of **2** measured after replacing the hydroxy protons with deuterium verified the proposed fragmentation mechanism. Fragmentation at C5/O1 and C8/C9 in **1** gave rise to a stable molecular species, 3,5,7-heptatriene-1,2-diol (*m/z* 163), providing structural information for this segment (Figure 2). Ions at *m/z* 1075 and 1073 in **1** indicated that a hydroxyl group exist at C54 but not at C53. Likewise ions at *m/z* 1003, 1001, and 109 were indicative for spiro rings L/M in **2** (Figure 1). As structural modifications in the fish had occurred mainly in the termini of the molecules, structures of most congeners could be deduced by simply comparing the spectra with those of **1** or **2.** Coexistence of toxins in one fraction posed little difficulty, as they could be analyzed one by one by selecting each $[M + Na]^+$ ion. Parent ions differing by 2 μ could be utilized.

Toxins identified in *G. toxicus* are shown in Figure 1. Three known toxins, CTX3C (**2**), CTX4A (**6**), and CTX4B (52-*epi*CTX4A, **7**), were confirmed by comparing MS/MS and LC/MS data with those of reference toxins. A congener eluted ahead of **2** was shown to be a stereoisomer of **2** by FAB/MS/MS. In the presence of HCl, both the isomer and **2** resulted in the same equilibrated mixture, implying that rearrangement of the spiro rings had occurred. The isomer was thus assigned the structure of 49-*epi*CTX3C (**3**). Structures of M-*seco*-CTX3C (**4**), M-*seco*-CTX3C methyl acetal (**5**), and M-*seco*-CTX4A (**8**) were deduced by FAB/MS/MS. The carbon chain C49−C52 in **5** had no branching, because the FAB/MS/MS spectrum of its 52-*O*-sulfobenzoate showed no indication of bond cleavage between oxygen-

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(9) A JMS-HX110/HX110 mass spectrometer was used for FAB/MS/MS experiments. A JASCO Gulliver pump coupled to a QUATRO II mass spectrometer, a Capcel pak C_{18} column (1.5 × 150 mm), and MeOH/MeCN/H_2O (8:1:1) were used for LC/MS.

(10) A mixture of a sample (1−5 μg), 2-sulfobenzoic anhydride (1 mg), and pyridine (100 μL) was left overnight at room temperature. After decomposing excess reagent with 2 mL of cold water, the sulfobenzoate was extracted with 2 mL of $CHCl_3$.
(11) A sample (<1 μg) was dissolved in 90 μL of $CHCl_3$ and mixed with 10 μL of 1 N HCl in dioxane. After 90 min the solution was evaporated under an N_2 stream and the residue was dissolved in the HPLC mobile phase. MM2 calculations on spiro rings L/M indicate that 52*R*/54*S*OH configuration in **1** is more stable (minimum energy 336.59 kJ/mol) than 52*S*/54*S*OH configuration in **9** (minimum energy 351.88 kJ/mol). The same is true with rings L/M in **2** (49*R*, 306.14 kJ/mol) and **3** (49*S*, 321.85 kJ/mol).

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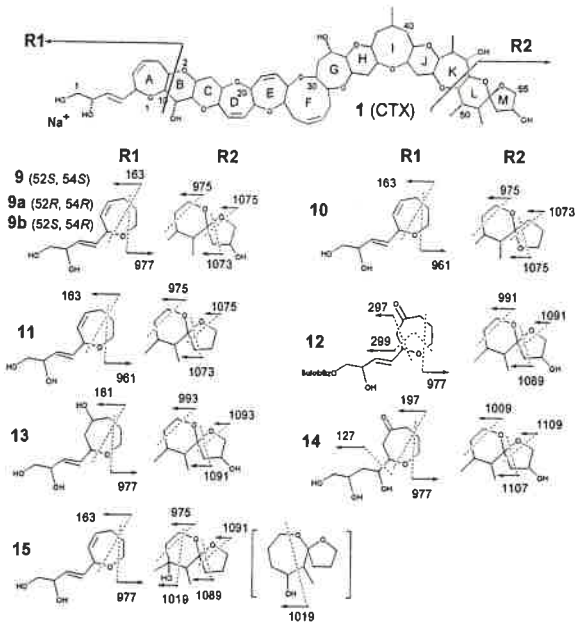

Figure 2. Structures and characteristic fragments of CTX congeners in fish. CTX4A (**6**) is not shown. The fragments of the R1 segment of **12** are those of sulfobenzoate. The mass units indicated are those of Na-adduct ions.

bearing C49 and putative tertiary carbon C50. By analogy C49−C52 in **4** and C52−C55 in **8** were presumed to be straight chains. The toxins **2** through **8** were all detected by LC/MS in extracts of *G. toxicus*, implying that they are genuine products of the organism and are precursors of other congeners present in fish.

Figure 2 shows congeners identified in fish with the same backbone as **1**. CTX4A (**6**) found in *G. toxicus* was also identified in fish. A congener eluting after **1** was assigned the structure of 52-*epi*CTX (**9**), because it was indistinguishable from **1** by FAB/MS/MS and was changed to **1** by HCl. Two congeners coded **9a** and **9b**, respectively, differed from both **1** and **9** in retention times but were identical with **1** and **9** by FAB/MS/MS. When treated with HCl, **9b** was changed to **9a** but not to **1**. Thus, **9a** and **9b** should be 54-epimers of **1** and **9**, respectively.[11] 52-*epi*-54-DeoxyCTX (**10**) deduced from the spectrum was confirmed by comparison with a reference toxin.[12] 54-DeoxyCTX (**11**) was identified, because it was identical with **10** by FAB/MS/MS and was converted to **10** by HCl. The spectrum of 7-oxoCTX (**12**) did not reveal whether an extra oxygen atom was in a keto, epoxy, or hydroxy function. It was also not clear whether the oxygen resided in the side-chain or ring A. In the spectrum of 1-*O*-sulfobenzoate of **12**, ions arising from stable 3-ene-5-one (*m/z* 299) and 3,5-diene (*m/z* 297) species pointed to a 7-oxo structure. The position of 7-OH in 7-hydroxyCTX (**13**) was inferred from the Na-adduct ion of 3,5,7-octatriene-1,2,7-triol generated by cleavages at C5/O1 and C8/C9. The positions of 4-OH and 7-C=O in 4-hydroxy-7-oxoCTX (**14**) were inferred from the spectrum of its $[M + Na]^+$ ion and were further confirmed by the spectrum of its 1-*O*-sulfobenzoate. Bond cleavages facilitated by 4-OH and 7-C=O were more clearly observed. A regioisomer of **1** was indicated by FAB/MS/MS and was assigned the structure of 54-deoxy-50-hydroxyCTX (**15**) based on a characteristic ion at *m/z* 1019 generated by bond cleavage between oxygen-bearing C50 and tertiary carbon C51. An alternative structure with oxepane ring L was possible, as shown in parentheses, but was ruled out because none of the precursor toxins has an oxepane ring L.

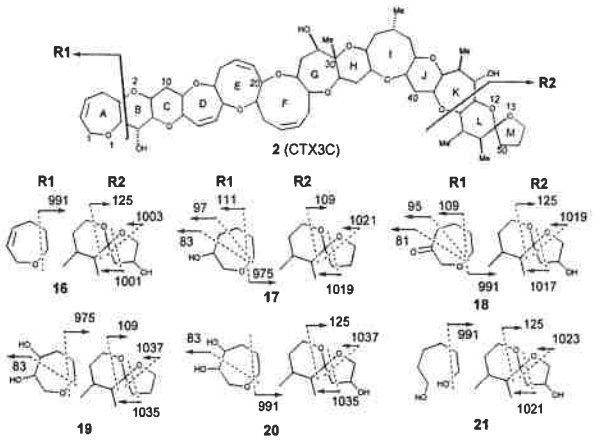

Figure 3. Structures and characteristic fragments of CTX3C congeners in fish. 49-*epi*CTX3C (**3**) is not shown. The mass units of ions are those of Na-adduct ions.

CTX3C and its congeners found in fish are shown in Figure 3. 49-*epi*CTX3C (**3**) detected in *G. toxicus* also occurred in fish. 51-HydroxyCTX3C (**16**) was confirmed by FAB/MS/MS and LC/MS comparison with a reference toxin.[13] The position of extra oxygen atoms in 2,3-dihydro-2-hydroxyCTX3C (**17**) and 2,3-dihydro-51-hydroxy-2-oxoCTX3C (**18**) was deduced from the Na-adduct ion corresponding to ethane-1,2-diol (*m/z* 83) or ethylene-1,2-diol (*m/z* 81) produced by cleavages at C2/C3 and C6/O1. 2,3-Dihydro-2,3-dihyroxyCTX3C (**19**) was confirmed by comparing the spectral and LC/MS data with those of a reference toxin.[13] The FAB/MS/MS spectrum of 2,3-dihydro-2,3,51-trihydroxyCTX3C (**20**) could not eliminate the possibility of a 2,4-diol structure. In the spectrum of 2-*O*-sulfobenzoate of **20**, ions generated by bond cleavage at C2/C3 supported a 2,3-vicinal diol structure. The spectrum of A-*seco*-2,3-dihydro-51-hydroxyCTX3C (**21**) clearly showed the presence of 51-OH, but did not provide information for the side-chain C1−C4. As none of the precursor toxins has branching between C1 and C4, a straight chain was assumed.

Working with samples of only a few micrograms and of insufficient purity, we successfully identified 7 known and 16 new congeners. FAB/MS/MS of $[M + Na]^+$ ions was a very informative technique because fragmentations that were charge-remote from both termini could be observed. Two nearby oxygen atoms facilitated formation of Na-adduct ions. Wide applicability of the method to natural products is expected.

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Supporting Information Available: FAB/MS/MS spectra of reference toxins **1** and **2**, all congeners and the sulfobenzoates of **5**, **12**, **14**, and **20** with assigned fragments and LC/MS data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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