SPECIAL GUEST EDITOR SECTION

Determination of Toxins Involved in Ciguatera Fish Poisoning in the Pacific by LC/MS

KENTARO YOGI
University of the Ryukyus, Faculty of Medicine, Department of Health Sciences, 207 Uehara, Nishihara, Okinawa 903-0215, Japan
SATSUKI SAKUGAWA
Okinawa Prefectural Institute of Health and Environment, 2085 Aza-Ozato, Ozato, Nanjo, Okinawa 901-1202, Japan
NAOMASA OSHIRO
National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan
TSUYOSHI IKEHARA
Nagasaki University, Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki, Nagasaki 852-8523, Japan
KIMINORI SUGIYAMA
Central Research Laboratory Nippon Suisan Kaisha, Ltd, 559-6 Kitanomachi, Hachiouji-shi, Tokyo 192-0606, Japan
TAKESHI YASUMOTO[1]
Japan Food Research Laboratories, 6-11-10 Nagayama, Tama-shi, Tokyo 206-0025; National Research Institute of Fisheries Science, 2-12-4 Fukuura, Kanazawa-ku, Yokohama, Kanagawa 236-8648, Japan

Ciguatera fish poisoning is the most extensive and difficult to control of the seafood poisonings. To facilitate monitoring of fish toxicity, toxin profiles were investigated by an LC/MS/MS method using 14 reference toxins on eight representative species of fish collected in four different areas of the Pacific. Snappers and groupers from Okinawa contained ciguatoxin-1B (CTX1B) and two deoxy congeners at variable but species-specific ratios, while red snapper, *Lutjanus bohar*, from Minamitorishima, and amberjack, *Seriola dumerili,* from Hawaii, contained both CTX1B-type and CTX3C-type toxins. Spotted knifejaw, *Oplegnathus punctatus*, from Okinawan waters, contained mainly CTX4A and CTX4B, but the same species caught at Miyazaki was contaminated primarily with the CTX3C-type toxins. Otherwise, the toxin profiles were consistently species-specific in fish collected from various locations around Okinawa over 20 years. The LC/MS/MS and mouse bioassay results agreed well, indicating the LC/MS/MS method is a promising alternative to the mouse bioassay. Pure CTX1B and CTX3C were prepared for use in future LC/MS/MS analysis.

Ciguatera fish poisoning (CFP) is an illness resulting from eating fish that contain toxins collectively named ciguatoxins (CTXs; 1). CFP is prevalent in tropical or subtropical areas and affects several tens of thousands of people annually. Thus, the illness is suggested to be the largest foodborne disease of a natural toxin origin. In the Pacific, the benthic dinoflagellate *Gambierdiscus toxicus* was identified as the primary source of the toxins that accumulated in fish via the food chain (2). In view of the multiple fish species involved, the diverse symptoms of patients, and wide genetic variations among the *Gambierdiscus* spp., the divergence of the toxins was assumed. Adding to the complexity, toxins undergo structural modifications, mainly in an oxidative manner, in fish. Thus, as many as 23 toxin analogs were identified from fish and *G. toxicus* collected in French Polynesia (1). They are separable based on skeletal structures into two types, CTX1B type and CTX3C type (Figure 1). Previously, using 14 reference toxins, we carried out LC/MS/MS analysis on fish from waters around Japan and reported that the toxin profiles in the fish were essentially species-specific (3). We also inferred by comparing the toxin profiles between Okinawa Archipelago and Miyazaki, Kyushu Island, that the toxin profiles of *G. toxicus* must be different in the two areas. The information is not only important for implementation of monitoring but also interesting from an ecotoxicological point of view.

The present study was undertaken to further prove the species-specific and the region-specific toxin profiles with an increased number of fish, and to prove compatibility of mouse bioassays (MBAs) with LC/MS/MS so that possible contributions of other toxins to MBA results could be ruled out. Since the paucity of reference toxins severely hampered the validation of our method, preparation of toxin standards was initiated. The most laborious part of this study, preparation of CTX1B and CTX3C, was successful, providing sufficient amounts for use in our future validation studies.

Experimental

Reference CTXs

As in the previous study, the following 14 reference toxins were used: CTX4A, CTX4B, M-*seco*-ciugatoxin-4A/B (M-*seco*-CTX4A/B), 52-*epi*-54-deoxyciguatoxin-1B (52-*epi*-54-deoxyCTX1B), 54-deoxyciguatoxin-1B (54-deoxyCTX1B), CTX1B, CTX3C, M-*seco*-ciguatoxin-3C (M-*seco*-CTX3C), 49-*epi*ciguatoxin-3C (49-*epi*CTX3C), 2-hydroxyciguatoxin-3C (2-hydroxyCTX3C), 2,3-dihydroxyciguatoxin-3C (2,3-dihydroxyCTX3C), 51-hydroxyciguatoxin-3C (51-hydroxy CTX3C), M-*seco*-ciguatoxin-3C methyl acetal (Figure 1), and gambierol. The chromatographic and spectral data to support

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[1] Corresponding author's e-mail: yasumotot@jfrl.or.jp

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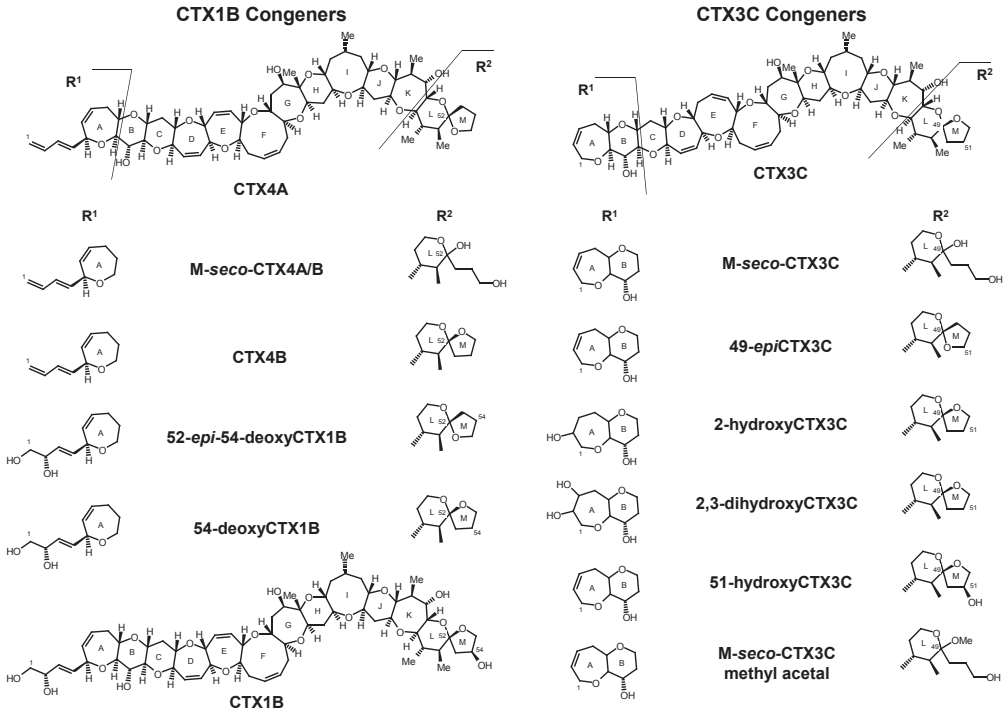

Figure 1. Structures of reference ciguatoxins. Note that CTX1B is also referred to as P-CTX-1 (Pacific ciguatoxin 1) in some publications.

the structures were presented in the previous articles and the references therein (1, 2).

LC/MS/MS

The LC/MS/MS analysis was carried out using an Agilent (Santa Clara, CA) 1200 Series LC coupled to an Agilent 6460 Triple Quadrupole MS instrument according to the method reported in detail previously (3). The chromatogram for 14 reference toxins obtained with a Zorbax Eclipse Plus C18 column (2.1 × 50 mm id, 1.8 µm particle size) is shown in Figure 2. The flow rate was 0.4 mL/min with a gradient starting at 73% B, increased to 90% B in 11 min, and held for 4 min [A = 5 mM ammonium formate and 0.1% formic acid in water, B = methanol (MeOH)]. Solvents and other chemicals were purchased from Wako Pure Chemical Industries, Ltd (Tokyo, Japan). For LC/MS/MS analysis, 5 µL each of sample solutions in MeOH was injected.

Fish Specimens

The snappers, *Lutjanus bohar* (36 specimens) and *L. monostigma* (five specimens); the groupers, *Epinephelus fuscoguttatus* (11 specimens), *Variola louti* (17 specimens), *Plectropomus laevis* (six specimens), and *Anyperodon leucogrammicus* (two specimens); and the spotted knifejaws, *Oplegnathus punctatus*

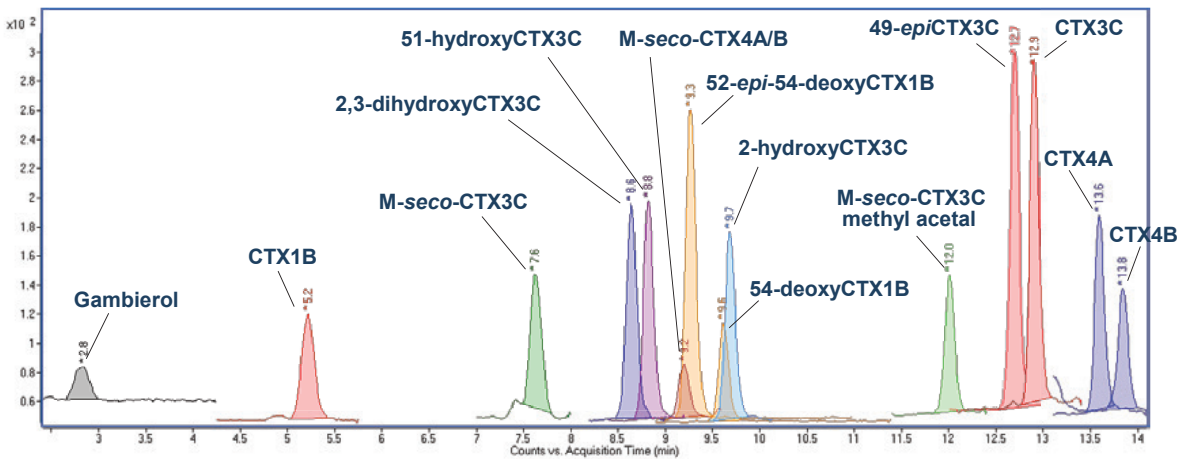

Figure 2. Chromatogram for 14 reference toxins. The structure of gambierol is omitted.

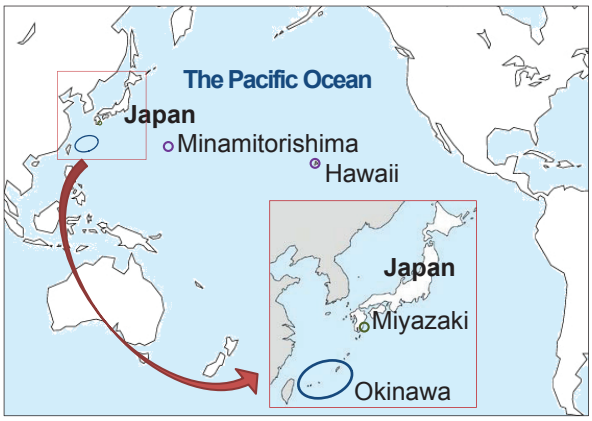

Figure 3. Catch locations of fish specimens.

(three specimens) were collected in Okinawa Prefecture, the Ryukyu Islands. Of these, three specimens of *L. bohar* and one specimen of *V. louti* were collected in 1990, and the others during the last 10 years. These fish are the most frequently implicated species in CFP in Okinawa (4). Additionally, an acetone extract prepared from the viscera of the amberjacks, *Seriola dumerili*, implicated in CFP in Hawaii, was kindly supplied by Y. Hokama, University of Hawaii. The effect of autoclaving was tested using *L. monostigma* and *L. bohar*. The geographical locations of the fish analyzed in this study as well as in the previous study are shown in the map presented as Figure 3. To test the method performance at low toxin levels, the flesh of *L. monostigma* and *V. louti* judged as negative by MBA were used.

Preparation of Test Solutions for LC/MS Analysis

Because of the necessity to compare MBA and LC/MS results, crude extracts were prepared following the manual guide for MBA (5). Further cleanup of the extract was performed using Florisil and primary secondary amine (PSA) cartridges (GL Sciences Inc., Tokyo, Japan; Figure 4). Muscle samples were taken from the dorsal, caudal, and ventral parts to compare the toxin contents. The efficacy of cleanup on Florisil and PSA cartridges was examined using crude extracts of MBA-negative *L. bohar* spiked with two different doses of CTX1B (0.16 and 0.32 μg/kg).

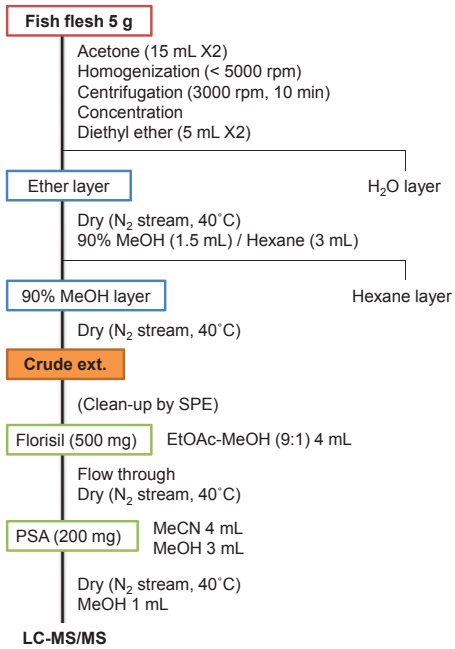

Figure 4. Preparation of test solutions for LC/MS/MS analysis; EtOAc = ethyl acetate and MeCN = acetonitrile.

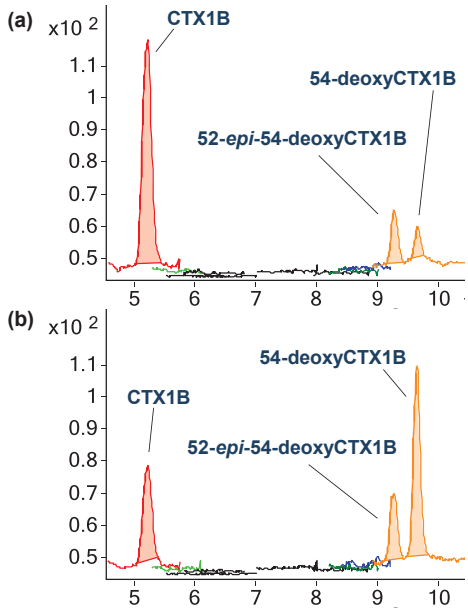

Figure 5. Toxin profiles of MBA-negative fish (a) *L. monostigma* and (b) *V. louti*.

Large-Scale Preparation of Toxins

Fish were autoclaved before separating muscles from other organs and tissues. In preliminary tests, loss of toxins during autoclaving was indicated to be insignificant. Only muscles were used for extraction. The viscera were kept separately for future use. In addition to the fish collected in Okinawa, crude extracts accumulated over the past years from fish caught in other parts of the Pacific were also used. Purification of the toxins was carried out basically following the methods in the literature (1). The ^{1}H NMR spectra were recorded at Riken NMR Facility (Yokohama, Japan) with a Bruker AV800 (Rheinstetten, Germany) spectrometer equipped with a cryoprobe. The samples were dissolved in 300 μL of CD_3OD (99.95%, Aldrich, St. Louis, MO), placed in a tube of 5 mmΦ, and measured at 10°C without spinning.

Assessment of Bioactivities to Convert LC/MS Data to Mouse Lethality

The MBAs were carried out following the method officially recommended in Japan (5). The ion-influx activity through voltage-sensitive sodium channels (VSSCs) was measured using radioactive ^{14}C-guanidine (NEN, Boston, MA; 6). The potency to bind VSSCs was measured using radioactive ^{3}H PbTx-3 (Chiral Corp., Miami, FL) following the published

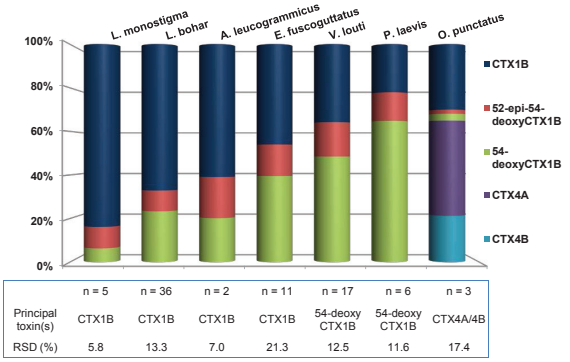

Figure 6. Toxin profiles of representative ciguatera fish in Okinawa.

method (7). The neuroblastoma-2A assay also followed the published method (8). Further details of measurements were reported in 1994 in the Master of Science degree thesis by K. Sugiyama, Faculty of Agriculture of the Graduate School of Tohoku University, Sendai, Japan.

Results and Discussion

The chromatogram for 14 standard toxins is shown in Figure 2. The solutions for injection were 1 ng/mL. Test solutions prepared from different parts of the body suggested an even distribution of CTX1B: dorsal part, 0.84; caudal part, 1.06; and ventral part, 0.98 mg/kg flesh. The same trend was observed for 52-*epi*-54-deoxyCTX1B and 54-deoxyCTX1B. Recoveries of CTX1B spiked at two different doses (0.8 ng and 1.6 ng) into a crude extract of an MBA-negative fish were 90.3% at the lower dose and 78.6% at the higher dose ($n = 2$). The use of Florisil and PSA cartridges for cleanup seemed justifiable. The LODs (S/N > 3) and LOQs (S/N > 10) of CTXs were determined to be 0.25 and 1.0 pg on column, respectively, in the previous study (3). For further validation of the method for low level samples, three specimens of fish judged as marginal or negative by MBA were tested. As shown in Figure 5, toxins were clearly detected. The calculated CTX1B contents were 0.181 µg/kg flesh in *L. monostigma* and 0.079 µg/kg flesh in *V. louti*. Judging

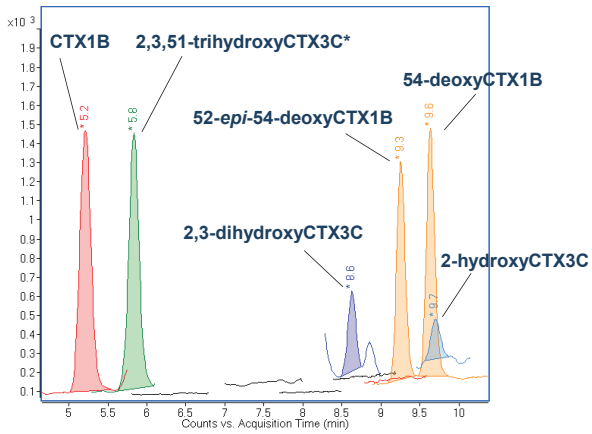

Figure 7. Toxin profile of the amberjack, *Seriola dumerili*, from Hawaii; *2,3,51-trihydroxyCTX3C was deduced from [M + Na]$^+$ (*m/z* 1095) and Rt in (2).

Table 1. Bioactivities of CTXs

Toxins	Mouse (i.p.), pmole/kg	Ion influx[a] ED50[b], pmole/L	Receptor binding[c] Ki[d], pmole/L	Neuro-2A[e] ED50, pmole/L
CTX1B	320	260	49	2.6
52-*epi*-54-deoxyCTX1B	640	150	21	25.0
51-hydroxyCTX3C	190	250	28	8.5
CTX3C	1200	390	87	20.0
CTX4A	1300	1010	73	150.0
CTX4B	3400	5400	340	220.0

[a] Radioactive guanidine was used to measure ion influx.

[b] ED50 = Median effective dose.

[c] Radioactive PbTx-3 was used.

[d] Ki = Inhibition constant.

[e] Neuroblastoma 2A cells were used for cytotoxicity.

from the intensities of the ion peaks, detection of CTX1B at one order lower levels seems possible by changing the dilution of the sample solution or adding one step of cartridge treatment. With adequate amounts of CTX1B and CTX3C at hand, as will be mentioned later, the toxin levels for the most sensitive detection and quantification will be investigated in the future.

The toxin profiles of seven representative fish species from Okinawa are shown in Figure 6. CTX1B, 54-deoxyCTX1B, and 52-*epi*-54-deoxyCTX1B were dominant in snappers and groupers, and the relative abundance of toxins was in support of the species-specific profiles observed previously. Interestingly, the specimens of *L. bohar* and *V. louti* collected in 1990 produced

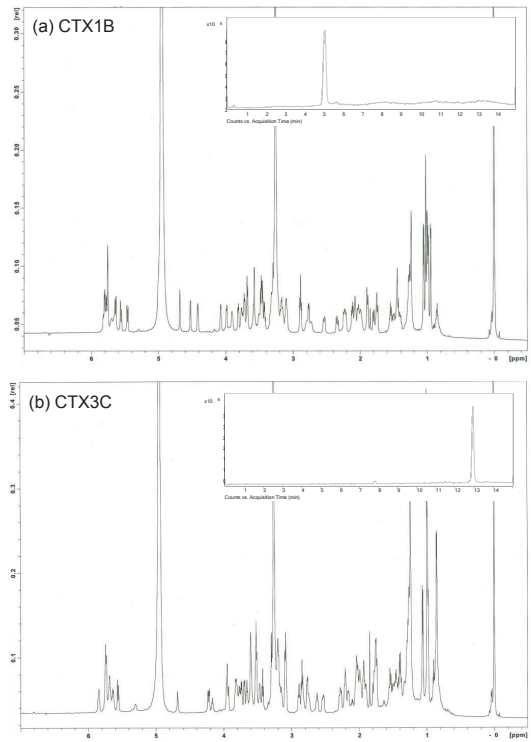

Figure 8. LC/MS chromatograms (insets) and ^{1}H NMR spectra of purified (a) CTX1B and (b) CTX3C.

toxin profiles similar to those of the latest catch. Hence, an unchanged toxin profile during the past 20 years was suggested. The species-specific toxin profiles revealed in the previous study were further supported in this study with increased numbers of fish. Perceivably, the inherent enzymes to metabolize the toxins are species-specific in fish. Hence, the species-specific toxin profiles are produced. If that is the case, most likely to be so from the present results, toxin profiles could be predicted by testing fewer specimens than used presently. The consistency of toxin profiles in fish collected from the wide area of Okinawa during the past 20 years would be helpful when applying ELISA kits or biosensors such as surface plasmon resonance detectors for monitoring. The limited toxin profiles are also interesting from the ecological point of view, because the organism producing CTXs in Okinawa likely belongs to only one genetic type. That is in contrast to the phylogenetic diversity of *Gambierdiscus* spp. found in Japan (9). Prominently, *O. punctatus* differed from the snappers and groupers in dominantly containing CTX4A and CTX4B, less oxidized precursors of CTX1B congeners. Reportedly, *O. punctatus* feeds on sea urchins and marine snails, suggesting these grazers as the first link in the food chain. The occurrence of CFP in Minamitorishima after eating turban shells and subsequent detection of CTX-like toxins in the same species support the above hypothesis (10, 11). Previously, we showed that CTX3C, 49-*epi*CTX3C, 51-hydroxyCTX3C (main), and M-*seco*-CTX3C were the major toxins in *O. punctatus* caught on the Miyazaki coast. It is suggested, therefore, that *G. toxicus* in the two areas belongs to two different genetic types.

The amberjack, *S. dumerili,* from Hawaii resembled *L. bohar* from Minamitorishima used in the previous study in containing both CTX1B-type and CTX3C-type toxins (Figure 7). Because the fish has a migratory behavior, the toxin profiles could vary depending on the place of catch.

MBA was treated as a reference method to evaluate the safety of fish, and for this reason it is critical to increase our knowledge of CTX profiles as well as the relative potencies of congeners to determine if LC/MS can predict total potency. One challenge in researching the CTXs, however, is the requirement of both MBA and LC/MS for sufficient quantities of sample. Fortunately a variety of different microscale assays can be used to study the relative potencies of the CTXs, and it is remarkable that the results roughly parallel the MBA values among these methods, as shown in Table 1. Some of the discrepancies seen may have resulted from the inaccuracy of weighing small samples.

In the present study, good agreement was observed between the MBA and LC/MS/MS data using the individual congener's mouse intraperitoneal (i.p.) potencies given in Table 1, further demonstrating the potential of the LC/MS method as an alternative to MBA. To meet the very stringent hazard advisory levels of 0.01 μg/kg CTX1B equivalents recently published by the U.S. Food and Drug Administration (12), however, additional optimization of the current LC/MS/MS method seems desirable.

The low toxin concentrations coupled with the absence of CTX3C-type toxins posed a great obstacle in using Okinawan fish as the toxin sources; however, this difficulty was overcome by using crude material retrieved from past studies. Two important toxins, CTX1B and CTX3C, were produced at around 120 μg yields. Their purities were confirmed by LC/MS and ^{1}H NMR spectrometry data (Figure 8). A parallel effort to purify other toxins, i.e., CTX4A, CTX4B, and 51-hydroxyCTX3C, is expected to produce these toxins, though in smaller amounts. They will be useful in future validation studies and to promote the use of the LC/MS method.

Acknowledgments

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