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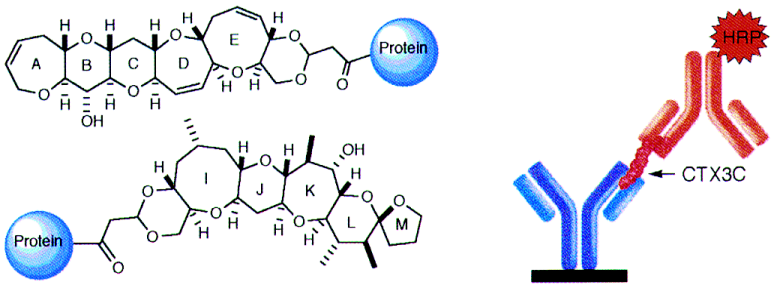

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Synthesis-Based Approach toward Direct Sandwich Immunoassay for Ciguatoxin CTX3C

Hiroki Oguri,[†] Masahiro Hirama,*[,†] Takeshi Tsumuraya,[‡] Ikuo Fujii,*[,‡] Megumi Maruyama,[†] Hisatoshi Uehara,[†] and Yoko Nagumo[†]

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Abstract: Ciguatoxins are the major causative toxins of ciguatera seafood poisoning. Limited availability of ciguatoxins has hampered the development of a reliable and specific immunoassay for detecting these toxins in contaminated fish. Monoclonal antibodies (mAbs) specific against both ends of ciguatoxin CTX3C were prepared by immunization of mice with protein conjugates of rationally designed synthetic haptens, **3** and **4**, in place of the natural toxin. Haptenic groups that possess a surface area larger than 400 Å^2 were required to produce mAbs that can bind strongly to CTX3C itself. A direct sandwich enzyme-linked immunosorbent assay (ELISA) using these mAbs was established to detect CTX3C at the ppb level with no cross-reactivity against other related marine toxins, including brevetoxin A, brevetoxin B, okadaic acid, or maitotoxin.

Introduction

Ciguatera is a human intoxication caused by the ingestion of a variety of reef fish.[1,2] The disease is characterized by severe neurological, gastrointestinal, and cardiovascular disorders. The causative toxins of ciguatera, known as ciguatoxins, are produced by the marine dinoflagellate *Gambierdiscus toxicus* and accumulate in various kinds of reef fish through the food chain. The spread of ciguatera strongly impacts public health, the development of fishery resources, and economic income in tropical and subtropical regions. A major problem in avoiding the disease is that fish contaminated with ciguatoxins look, smell, and taste normal. In addition, neither cooking nor freezing deactivates the heat-stable ciguatoxins.

Ciguatoxin (**1**)[3] and its congener, CTX3C (**2**),[4] exhibit a potent toxicity to mammals [acute toxicity in mice: medium lethal dose (LD_{50}) 0.15−4 μg/kg][5] and are structurally classified as ladder-like polyethers (Figure 1).[6,7] In addition to the traditional mouse bioassay of fish lipid extracts,[8] several other methods have recently been developed to detect ciguatoxins, including assays based on cytotoxicity,[9] radio ligand binding,[5,9] high performance liquid chromatography,[10] and mass spectrometry.[11,12] However, antibody-based immunoassays remain the most desirable method for accurate, sensitive, routine, and portable use. While Hokama and co-workers prepared anti-ciguatoxin antibodies using the scarce natural toxins,[13,14] the mAb exhibited cross-reactivity to another marine toxin, okadaic acid.[15] The extremely low content of ciguatoxin in fish has hampered the further development of anti-ciguatoxin antibodies.

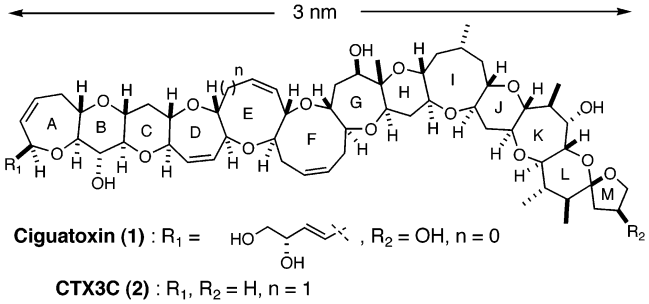

Figure 1. Structures of ciguatoxin (**1**) and CTX3C (**2**).

[†] Tohoku University
[‡] Biomolecular Engineering Research Institute.

(1) Scheuer, P. J.; Takahashi, W.; Tsutsumi, J.; Yoshida, T. *Science* **1967**, *155*, 1267.
(2) For recent reviews on ciguatera, see: (a) Scheuer, P. J. *Tetrahedron* **1994**, *50*, 3. (b) Lewis, R. J. *Toxicon* **2001**, *39*, 97.
(3) (a) Murata, M.; Legrand, A.-M.; Ishibashi, Y.; Fukui, M.; Yasumoto, T. *J. Am. Chem. Soc.* **1989**, *111*, 8929. (b) Murata, M.; Legrand, A.-M.; Ishibashi, Y.; Fukui, M.; Yasumoto, T. *J. Am. Chem. Soc.* **1990**, *112*, 4380. (c) Murata, M.; Legrand, A.-M.; Scheuer, P. J.; Yasumoto, T. *Tetrahedron Lett.* **1992**, *33*, 525. (d) Satake, M.; Morohashi, A.; Oguri, H.; Oishi, T.; Hirama, M.; Harada, N.; Yasumoto, T. *J. Am. Chem. Soc.* **1997**, *119*, 11325.
(4) Satake, M.; Murata, M.; Yasumoto, T. *Tetrahedron Lett.* **1993**, *34*, 1975.
(5) Dechraoui, M.-Y.; Naar, J.; Pauillac S.; Legrand, A.-M. *Toxicon* **1999**, *37*, 125.
(6) For congeners of ciguatoxins, see: (a) Satake, M.; Fukui, M.; Legrand, A.-M.; Cruchet, P.; Yasumoto, T. *Tetrahedron Lett.* **1998**, *39*, 1197. (b) Lewis, R. J.; Vernoux, J.-P.; Brereton, I. M. *J. Am. Chem. Soc.* **1998**, *120*, 5914 and references therein.
(7) For reviews on marine polycyclic ether toxins, see: (a) Yasumoto, T.; Murata, M. *Chem. Rev.* **1993**, *93*, 1897. (b) Murata, M.; Yasumoto, T. *Nat. Prod. Rep.* **2000**, *17*, 293. (c) Yasumoto, T. *Chem. Rec.* **2001**, *1*, 228 and ref 2.
(8) Lewis, R. J.; Sellin, M. *Toxicon* **1993**, *31*, 1333.
(9) Manger, R. L.; Leja, L. S.; Lee, S. Y.; Hungerford, J. M.; Hokama, Y.; Dickey, R. W.; Granade, H. R.; Lewis, R.; Yasumoto, T.; Wekell, M. M. *J. AOAC Int.* **1995**, *78*, 521.
(10) Yasumoto, T.; Fukui, M.; Sasaki, K.; Sugiyama, K. *J. AOAC Int.* **1995**, *78*, 574.
(11) (a) Lewis, R. J.; Jones, A. *Toxicon* **1997**, *35*, 159. (b) Lewis, R. J.; Jones, A.; Vernoux, J.-P. *Anal. Chem.* **1999**, *71*, 247.
(12) Yasumoto, T.; Igarashi, T.; Legrand, A.-M.; Cruchet, P.; Chinain, M.; Fujita, T.; Naoki, H. *J. Am. Chem. Soc.* **2000**, *122*, 4988.

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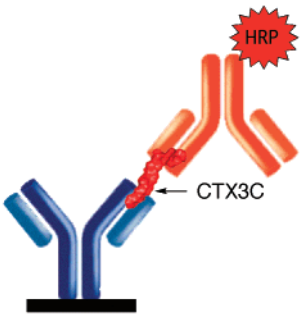


Figure 2. Schematic diagram of the direct sandwich ELISA for CTX3C (red). Specific antibody 10C9 (blue) against the left end of CTX3C is immobilized, and 3D11 (orange) against the right end is conjugated with horseradish peroxidase (HRP).

We therefore planned to use synthetic haptens to solve the problem of antibody development. We recently succeeded in the total synthesis of CTX3C based on a highly convergent strategy[16] that unified the ABCDE and the HIJKLM ring fragments. In this report, we describe the preparation of monoclonal antibodies (mAbs) against the right and left wings of CTX3C by immunizing with protein conjugates of synthetic fragments. Furthermore, we have established a direct sandwich ELISA for specific and reliable detection of CTX3C (Figure 2).

Results and Discussion

Hapten Design. To prepare antibodies that bind specifically to either end of CTX3C, we designed haptens on the basis of antibody−antigen interactions. X-ray structural analyses of antibody−hapten complexes have shown that relatively small haptens are buried deep in the antigen-combining site, with surface areas of 200−400 Å^2.[17] Since the surface areas of the tri-, tetra-, and pentacyclic parts of the left wing (ABC, ABCD, and ABCDE) were calculated to be 253, 318, and 398 Å^2, respectively (Figure 3), the ABC ring was expected to be of sufficient size to allow the preparation of specific antibodies for CTX3C. In fact, although the antibodies elicited by the ABC ring hapten−protein conjugate bound strongly to the hapten, they did not bind to CTX3C itself.[18,19] Given that the antibody may bind to the ABC ring as well as to the alkyl linker moiety, we anticipated that haptens larger than the ABC ring were required to induce mAbs to recognize CTX3C. We therefore designed hapten **3**, which consists of the pentacyclic skeleton (ABCDE ring, surface area 398 Å^2) and a cyclic acetal attached to a linker (Figure 4). Since the maximum buried surface area of haptens in antibody−hapten complexes has been reported to be approximately 400 Å^2, we predicted that hapten **3** would be large enough to occupy the antigen-combining site while leaving the acetal and the linker moiety free from antibody−antigen interactions. In addition, hapten **3** was designed to elicit antibodies that would bind to the left end of CTX3C in an orientation appropriate for the direct sandwich immunoassay. For this purpose, the carboxylic acid linker was attached to the E ring via a cyclic acetal in place of the central F ring. Similarly, hapten **4**, which consists of a pentacyclic skeleton (IJKLM ring, surface area 477 Å^2) and a cyclic acetal with a linker, was designed to induce antibodies that could bind to the right end of CTX3C.[20]

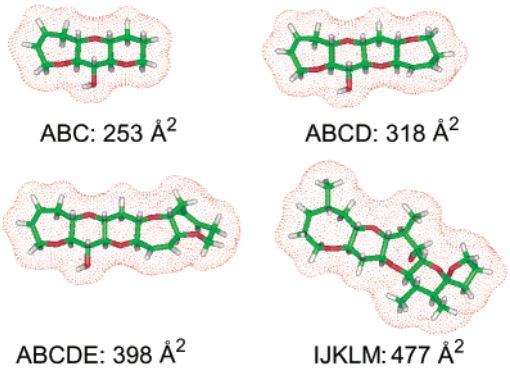


Figure 3. Energy-minimized structures of tri-, tetra-, and pentacyclic ether parts of CTX3C [carbon frameworks (green), oxygen (red), hydrogen (white)] and water accessible surface areas (Connolly surface shown as red dots) were calculated by Macromodel Ver 6.0 (MM2*).

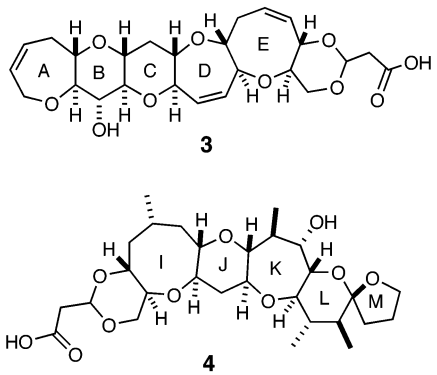
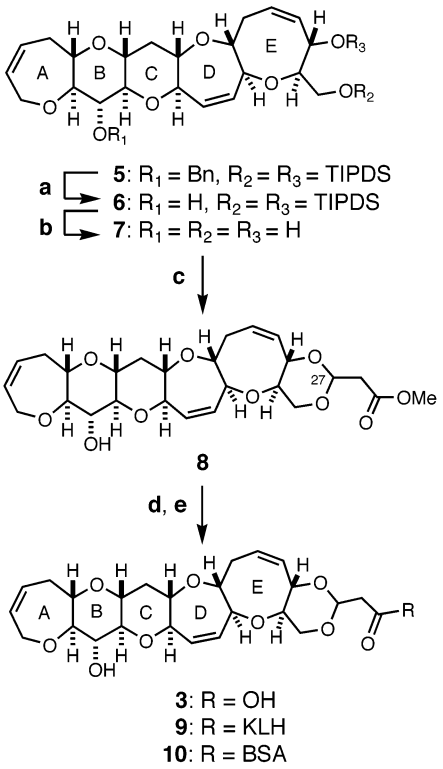


Figure 4. Structures of synthetic haptens (**3**, **4**) used to elicit specific anti-CTX3C antibodies.

Synthesis of Protein Conjugates and Production of Antibodies. Hapten **3** was synthesized as a diasteromeric acetal mixture from the ABCDE ring fragment **5**, a key intermediate in the total synthesis of CTX3C,[16,21] and conjugated to keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA), via an activated ester method (Scheme 1). In a similar fashion, protein conjugates **13** and **14** were synthesized from the IJKLM ring fragment **11**[16,22] (Scheme 2). Balb/c mice were immunized

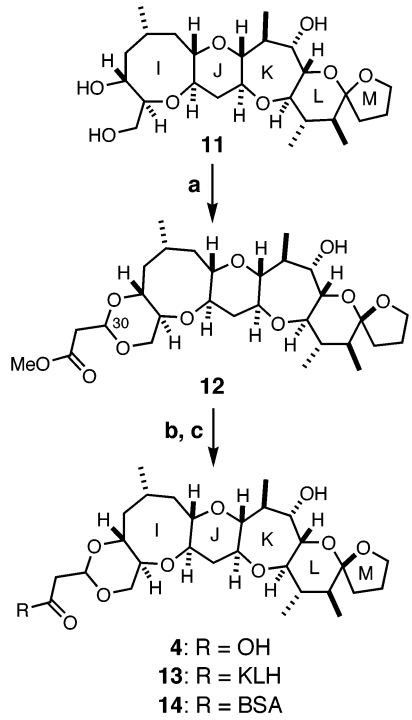
(13) (a) Hokama, Y.; Banner, A. H.; Boylan, D. *Toxicon* **1977**, *15*, 317. (b) Hokama, Y.; Abad, M. A.; Kimura, L. H. *Toxicon* **1983**, *21*, 817. (c) Hokama, Y. *Toxicon* **1985**, *23*, 939. (d) Hokama, Y. *J. Clin. Lab. Anal.* **1990**, *4*, 231. (e) Hokama, Y.; Asahina, A. Y.; Hong, T. W. P.; Shang, E. S.; Miyahara, J. T. *J. Clin. Lab. Anal.* **1990**, *4*, 363. (f) Hokama, Y.; Asahina, A. Y.; Shang, E. S.; Hong, T. W. P.; Shirai, J. L. R. *J. Clin. Lab. Anal.* **1993**, *7*, 26. (g) Ganal, C. A.; Asahina, A.; Hokama, Y.; Miyahara, J. T. *J. Clin. Lab. Anal.* **1993**, *7*, 41 and references therein.
(14) For reviews, see: (a) Hokama, Y. *J. Toxicol. Toxin. Rev.* **1991**, *10*, 1. (b) Hokama, Y. *Food Addit. Contam.* **1993**, *10*, 71. (c) Hokama, Y. *Food Addit. Contam.* **1993**, *10*, 83.
(15) (a) Hokama, Y.; Hong, T. W. P.; Isobe, M.; Ichikawa, Y.; Yasumoto, T. *J. Clin. Lab. Anal.* **1992**, *6*, 54. (b) Hokama, Y.; Takenaka, W. E.; Nishimura, K. L.; Ebesu, J. S. M. *J. AOAC Int.* **1998**, *81*, 727.
(16) (a) Hirama, M.; Oishi, T.; Uehara, H.; Inoue, M.; Maruyama, M.; Oguri, H.; Satake, M. *Science* **2001**, *294*, 1904. (b) Inoue, M.; Uehara, H.; Maruyama, M.; Hirama, M. *Org. Lett.* **2002**, *4*, 4551.
(17) (a) Arevalo, J. H.; Taussig, M. J.; Wilson, I. A. *Nature* **1993**, *365*, 859. (b) Jeffery, P. D.; Schildbach, J. F.; Chang, C.-Y. Y.; Kussie, P. H.; Margolies, M. N.; Sheriff, S. *J. Mol. Biol.* **1995**, *248*, 344. (c) Tanaka, F.; Kinoshita, K.; Tanimura, R.; Fujii, I. *J. Am. Chem. Soc.* **1996**, *118*, 2332. (d) Romesberg, F. E.; Spiller, B.; Schultz, P. G.; Raymond, C. S. *Science* **1998**, *279*, 1929. (d) Xu, J.; Deng, Q.; Chen, J.; Houk, K. N.; Bartek, J.; Hilvert, D.; Wilson, I. A. *Science* **1999**, *286*, 2345 and references therein.
(18) Oguri, H.; Tanaka, S.; Hishiyama, S.; Oishi, T.; Hirama, M.; Tsumuraya, T.; Tomioka, Y.; Mizugaki, M. *Synthesis* **1999**, *Special Issue*, 1431.
(19) Nagumo, Y. Ph. D Thesis, Tohoku University, 2002.
(20) Pauillac, S.; Sasaki, M.; Inoue, M.; Naar, J.; Branaa, P.; Chinain, M.; Tachibana, K.; Legrand, A.-M. *Toxicon* **2000**, *38*, 669.
(21) (a) Maruyama, M.; Maeda, K.; Oishi, T.; Oguri, H.; Hirama, M. *Heterocycles* **2001**, *54*, 93. (b) Maruyama, M.; Inoue, M.; Oishi, T.; Oguri, H.; Ogasawara, N.; Shindo, Y.; Hirama, M. *Tetrahedron* **2002**, *58*, 1835.

Scheme 1. Synthesis of **3**, **9**, and **10**[a]



[a] Reagents and conditions: (a) DDQ, CH_2Cl_2, H_2O, 42%; (b) TBAF, THF, 98%; (c) TsOH, $(MeO)_2CHCH_2CO_2Me$, CH_2Cl_2, 57%, **8** (27*R*:27*S* = 2:1); (d) LiOH·H_2O, *t*BuOH/H_2O = 4/1; (e) EDC·HCl, *N*-hydroxysuccinimide (NHS), DMF, then KLH or BSA in phosphate buffer (pH 7.4). TIPDS, 1,3-(1,1,3,3)-tetraisopropyldisiloxanyldiene.

Scheme 2. Synthesis of **4**, **13**, and **14**[a]



[a] Reagents and conditions: (a) TsOH, $(MeO)_2CHCH_2CO_2Me$, CH_2Cl_2, **12** (30*R*:30*S* = 1:3), 94%; (b) LiOH·H_2O, *t*BuOH/H_2O = 4/1; (c) EDC·HCl, NHS, DMF, then KLH or BSA in phosphate buffer (pH 7.4).

with KLH conjugates **9** and **13** to elicit six mAbs for the left wing and three mAbs for the right wing of CTX3C, respectively. MAb 10C9 for the left wing and mAb 3D11 for the right wing were examined for binding affinities to the hapten and to CTX3C itself using a competitive ELISA.[23,24]

As shown in Table 1, mAb 10C9 exhibited a high affinity (K_d = 0.8 nM) for ABCDE ring fragment **7**. More importantly, mAb 10C9 binds tightly to CTX3C itself with a comparable K_d value of 2.8 nM, and it shows no cross-reactivity with the other wing IJKLM ring **11**[25] or the structurally related marine toxins[7] (Figure 5), including brevetoxin A (BTX A),[26] brevetoxin B (BTX B),[27] okadaic acid (OA),[28] or maitotoxin (MTX).[29] Furthermore, we determined that the K_d values of 10C9 for ABCD ring **15**[30] and ABC ring **16**[31] were 1.8 and 74 μM, respectively, while that for **7** was 0.8 nM (Table 1). Therefore, as the number of ether rings in the synthetic fragments decreased, the K_d values of 10C9 increased by a factor of 100 or 1000. This result strongly suggests that 10C9 recognizes the pentacyclic ABCDE ring skeleton of CTX3C and that the surface area of CTX3C for molecular recognition of 10C9 agrees

(22) (a) Oishi, T.; Nagumo, Y.; Shoji, M.; Le Brazidec, J.-Y.; Uehara, H.; Hirama, M. *Chem. Commun.* **1999**, 2035. (b) Oishi, T.; Uehara, H.; Nagumo, Y.; Shoji, M.; Le Brazidec, J.-Y.; Kosaka, M.; Hirama, M. *Chem. Commun.* **2001**, 381. (c) Uehara, H.; Oishi, T.; Inoue, M.; Shoji, M.; Nagumo, Y.; Kosaka, M.; Le Brazidec, J.-Y.; Hirama, M. *Tetrahedron* **2002**, *58*, 6493.

(23) (a) Friguet, B.; Chaffotte, A. F.; Djavadi-Ohaniance, L.; Goldberg, M. E. *J. Immunol. Methods* **1985**, *77*, 305. (b) Suga, H.; Ersoy, O.; Tsumuraya, T.; Lee, J.; Sinskey, A. J.; Masamune, S. *J. Am. Chem. Soc.* **1994**, *116*, 487.

(24) Dissociation constants of the antibodies with CTX3C (analyte) were not determined by surface plasmon resonance (SPR) for two reasons: (1) Determination of K_d values by using SPR (BIAcore 2000) required several measurements at different concentrations of analyte. Therefore, the sensor chip should be washed out in every run to peel the analyte off from the immobilized proteins. However, in our case, the interaction of the antibodies with CTX3C was too strong to regenerate the sensor chip. Washing the sensor chip under harsh conditions resulted in denaturing the immobilized antibodies. (2) SPR signals of interactions of the immobilized antibodies with small analytes were extremely weak; this is generally observed for interactions of proteins with small molecules (M.W. <1000). Alternatively, small molecules can be conjugated with a protein such as BSA to make the SPR intensity strong. However, the obtained K_d values do not show those for the free small molecules. We believe that the K_d values obtained by competitive ELISA experiments should be useful for comparison of binding selectivity of the small molecules and suitable for the issue in this work.

(25) We have also checked the two-step binding assay using SPR (BIAcore 2000): When CTX3C (100 nM) was applied to a 10C9-immobilized sensor chip, a very weak resonance (~40 RU) was observed. When 100 nM of antibody 3D11 was consecutively applied to the sensor chip, a large resonance (~900 RU) appeared. Thus, the two-step binding assay using SPR also demonstrated that the two antibodies recognize the different epitopes as shown by ELISA (Table 1).

(26) (a) Shimizu, Y.; Chou, H. N.; Bando, H.; Van Duyne, G.; Clardy, J. *J. Am. Chem. Soc.* **1986**, *108*, 514. (b) Pawlak, J.; Tempesta, M. S.; Golik, J.; Zagorski, M. G.; Lee, M. S.; Nakanishi, K.; Iwashita, T.; Gross, M. L.; Tomer, K. B. *J. Am. Chem. Soc.* **1987**, *109*, 1144.

(27) (a) Lin, Y.-Y.; Risk, M.; Ray, S. M.; Van Engen, D.; Clardy, J.; Golik, J.; James, J. C.; Nakanishi, K. *J. Am. Chem. Soc.* **1981**, *103*, 6773. (b) Lee, M. S.; Repata, D. J.; Nakanishi, K.; Zagorksi, M. G. *J. Am. Chem. Soc.* **1986**, *108*, 7855.

(28) Tachibana, K.; Scheuer, P. J.; Tsukitani, Y.; Kikuchi, H.; Van Engen, D.; Clardy, J.; Gopichand, Y.; Schmitz, F. J. *J. Am. Chem. Soc.* **1981**, *103*, 2469.

(29) (a) Murata, M.; Iwashita, T.; Yokoyama, A.; Sasaki, M.; Yasumoto, T. *J. Am. Chem. Soc.* **1992**, *114*, 6594. (b) Murata, M.; Naoki, H.; Matsunaga, S.; Satake, M.; Yasumoto, T. *J. Am. Chem. Soc.* **1994**, *116*, 7098. (c) Satake, M.; Ishida, S.; Yasumoto, T.; Murata, M.; Utsumi, H.; Hinomoto, T. *J. Am. Chem. Soc.* **1995**, *117*, 7019. (d) Zheng, W.; DeMattei, J. A.; Wu, J.-P.; Duan, J. J.-W.; Cook, L. R.; Oinuma, H.; Kishi, Y. *J. Am. Chem. Soc.* **1996**, *118*, 7946. (e) Sasaki, M.; Matsumori, N.; Maruyama, T.; Nonomura, T.; Murata, M.; Tachibana, K.; Yasumoto, T. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1672. (f) Nonomura, T.; Sasaki, M.; Matsumori, N.; Murata, M.; Tachibana, K.; Yasumoto, T. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 1675. (g) Cook, L. R.; Oinuma, H.; Semones, M. A.; Kishi, Y. *J. Am. Chem. Soc.* **1997**, *119*, 7928.

(30) Oishi, T.; Tanaka, S.; Ogasawara, Y.; Maeda, K.; Oguri, H.; Hirama, M. *Synlett* **2001**, 952.

(31) Nagumo, Y.; Oguri, H.; Shindo, Y.; Sasaki, S.; Oishi, T.; Hirama, M.; Tomioka, Y.; Mizugaki, M.; Tsumuraya, T. *Bioorg., Med. Chem. Lett.* **2001**, *15*, 2037.

Table 1. Dissociation Constants (K_ds) for the Binding of Marine Toxins and Synthetic Fragments to mAbs. K_ds Were Determined by Competitive ELISA as Described in Ref 23

	K_d (nM)			K_d (μM)					
mAb	A–E (**7**)	I–M (**11**)	CTX3C	BTX A	BTX B	OA	MTX	A–D (**15**)	A–C (**16**)
10C9	0.8	*a*	2.8	*a*	*a*	*a*	*a*	1.8	73.6
3D11	*a*	8.6	122	43	*a*	*a*	*a*	ND[b]	ND[b]

[a] No inhibition was observed at the maximum concentrations of the inhibitors. The maximum concentrations for **7**, **11**, BTX A, BTX B, OA, and MTX were 250, 250, 100, 100, 100, and 25 μM, respectively. (b) Not determined.

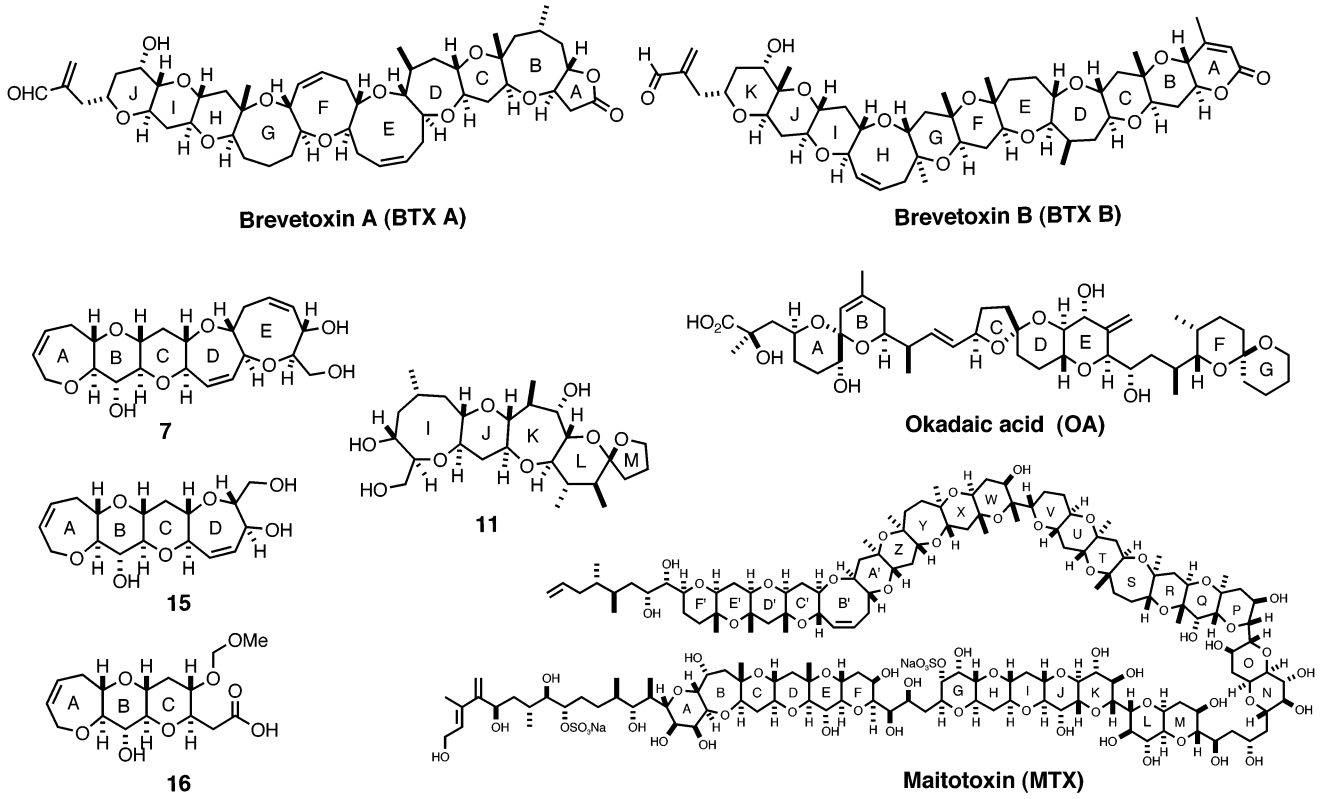


Figure 5. Structures of marine toxins [brevetoxin A (BTX A), brevetoxin B (BTX B), okadaic acid (OA), or maitotoxin (MTX)] and synthetic fragments of CTX3C.

with our prediction of approximately 400 Å^2. Similarly, mAb 3D11 for the right wing was also found to bind strongly to CTX3C itself (K_d = 122 nM) and did not cross-react with ABCDE ring **7**[25] or other marine toxins. Thus, for the first time, we have successfully produced two specific mAbs that bind to CTX3C with high affinity.

Direct Sandwich ELISA. Using the two mAbs for right and left ends of CTX3C, we then attempted to develop a direct sandwich ELISA for specific and reliable detection of CTX3C (Figure 2). For this assay, we used mAb 10C9 to capture CTX3C and mAb 3D11 as a detector. Wells of a microtiter plate were directly coated with 10C9 mAb, while 3D11 was conjugated with horseradish peroxidase (HRP). Following a conventional sandwich ELISA protocol using *o*-phenylenediamine (OPD) as a colorimetric substrate, CTX3C was detected in a dose-dependent manner (Figure 6). This protocol could detect down to ppb levels of CTX3C [detection limits: ~5 ng/mL (5 nM)]. None of the other marine toxins (BTX A, BTX B, OA, and MTX) or synthetic fragments (ABCDE ring **7** and IJKLM ring **11**) tested gave any detectable signals at a concentration of 20 μM.

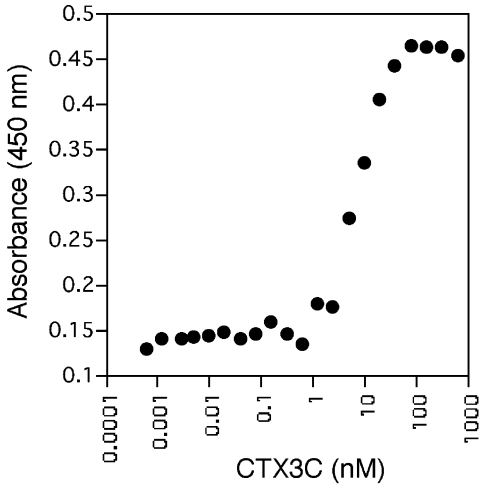


Figure 6. Direct sandwich ELISA for CTX3C.

Thus, the direct sandwich ELISA provides a sensitive analytical method to detect CTX3C through the simultaneous binding of two antibodies to each end. This method should be of enormous help in detecting contamination of seafood with CTX3C.[32]

Conclusion

We have successfully prepared specific mAbs against both ends of CTX3C by immunization with protein conjugates of rationally designed synthetic haptens in place of the scarce natural toxins. The haptenic groups required a surface area larger than 400 Å^2 to induce mAbs which can strongly bind to CTX3C. Furthermore, we have established a direct sandwich ELISA protocol for sensitive and specific detection of a nonprotein molecule, CTX3C. This sandwich immunoassay can detect down to ppb levels of CTX3C without cross-reactivity with other related marine toxins.

Finally, we wish to emphasize that organic synthesis can play a key role in developing a specific detection system for natural marine toxin. To minimize further outbreaks of ciguatera seafood poisoning, it is vital to be able to detect other principal congeners of ciguatoxins because several of the congeners are typically present within a single fish. The structural difference between the congeners mainly arises from the substituents on the terminal A and L rings.[6,12] Thus, syntheses of such ABCDE and IJKLM ring fragments are necessary for preparing mAbs that can differentiate between these terminal structures. Our strategy described herein is expected to be generally applicable to all ciguatoxin congeners.

Methods

Production of mAbs. Balb/c mice were immunized intraperitoneally with KLH-conjugate (**9** or **13**: 100 μg/mouse) mixed with RIBI adjuvant (RIBI Immunol. Res. Inc.) on days, 1, 14, 31, and 53. Three days after the last boost, the spleen was taken from the mice, and the cells were fused with P3X63-Ag8.653 myeloma cells using a somatic hybridizer as described previously.[33] A BSA conjugate (**10** or **14**) was used to screen antibodies in the ELISA. All positive hybridomas were subcloned twice according to the standard protocols. All cell lines were individually grown to 200 mL, and the supernatant was purified by anti-mouse IgG+IgM affinity chromatography (NGK Industries). The purified antibodies were judged to be homogeneous (>95%) by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Direct Sandwich ELISA. The mAb 3D11 (1 mg) was conjugated with HRP using an EZ-Link Plus Activated Peroxidase Kit (Pierce). Each well of a 96-well ELISA plate (Coster) was coated with 50 μL of mAb 10C9 (4.3 μg/mL) in PBS overnight at 4 °C. After the plate was washed, a serially diluted solution of CTX3C (50 μl) in PBS was added to the ELISA plates and incubated for 1 h. Next, the supernatant was removed and the plates were washed. Each well was incubated for 1 h with a solution (50 μL) of HRP-linked mAb 3D11 (1 μg/mL) in PBS containing 5% Tween-20. After washing, each well was treated with 100 μL of a solution of 1,2-phenylenediamine (0.4 mg/mL) in 0.1 M sodium citrate, pH 5.0 containing 0.1% hydrogen peroxide. The yellow color was allowed to develop for approximately 5 min, and the reaction was terminated by the addition of 2 N H_2SO_4 (50 μL). The absorbance at 450 nm was measured using a microtiter plate reader (BIO-RAD, Benchmark 170-6850).

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Supporting Information Available: Experimental procedures for the preparation of protein conjugates. This material is available free of charge via the Internet at http://pubs.acs.org.

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(32) Samples of ciguateric fish contaminated with CTX3C are not currently available, and studies of our sandwich assay to actual biological samples will be reported in due course.

(33) Kurihara, S.; Tsumuraya, T.; Suzuki, K.; Kuroda, M.; Liu, L.; Takaoka, Y.; Fujii, I. *Chem. Eur. J.* **2000**, *6*, 1659.