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Bioorganic Studies Utilizing Rationally Designed Synthetic Molecules: Absolute Configuration of Ciguatoxin and Development of Immunoassay Systems

Hiroki Oguri

Division of Chemistry, Graduate School of Science, Hokkaido University, Sapporo 060-0810

Division of Innovative Research, Non-traditional Science, Creative Research Initiative "Sousei" (CRIS), Hokkaido University, Sapporo 001-0021

Received March 30, 2007; E-mail: oguri@sci.hokudai.ac.jp

Ciguatoxins are the major causative toxins in ciguatera seafood poisoning. The limited availability of ciguatoxins has precluded structural elucidation and development of a reliable and specific immunoassay for detecting the toxins in contaminated fish. To seek a solution for the longstanding problem of ciguatera, we addressed the synthetic challenge by utilizing rationally designed model compounds of ciguatoxins. The C2 configuration and entire absolute configuration of ciguatoxin were successfully elucidated with minutest amounts of natural toxins, using an approach which combined partial structure synthesis, microscale chemical transformations, and the CD exciton chirality method. On the basis of the absolute configuration, the partial structures of ciguatoxins were designed and synthesized as haptens for the preparation of anti-ciguatoxin antibodies. Monoclonal antibodies (mAbs) against both ends of ciguatoxin CTX3C were prepared by immunization of mice with protein conjugates of synthetic haptens of the ABCDE-ring and the IJKLM-ring, in place of the natural toxin. Haptenic groups with surface areas larger than 400 Å^2 were required to produce mAbs, which could bind strongly to CTX3C itself. A direct sandwich enzyme-linked immunosorbent assay (ELISA) using these mAbs was shown 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. In order to make the sandwich immunoassay protocol a general method for detecting other ciguatoxins congeners, the preparation of mAbs for the left-end of ciguatoxin was investigated. Expeditious synthesis of the left end of ciguatoxin with installation of the 3-butene-1,2-diol side-chain of the A-ring as well as surface plasmon resonance (SPR) analysis of the antibody–hapten interactions are also described.

1. Determination of Absolute Configuration of Ciguatoxin

1.1 Ciguatoxins. Ciguatera is a human intoxication syndrome caused by the ingestion of a variety of reef fish.[1] The disease is characterized by severe neurological, gastrointestinal, and cardiovascular disorders. Globally, 20000–60000 people are estimated to suffer annually from this type of poisoning, making it one of the largest scale food poisonings of nonbacterial origin. The causative toxins of ciguatera, known as ciguatoxins, are produced by the marine dinoflagellate *Gambierdiscus toxicus* and accumulate in various types of reef fish throughout the food chain.[2] Efforts for isolation and structural characterization of ciguatoxins have faced difficulties for a long time, mainly due to limited supply of ciguatoxins from natural sources as well as complexity of their chemical structures. Approximately 4000 kg of moray eels were collected in French Polynesian waters, and 124 kg of viscera was extracted to provide no more than 0.35 mg of pure ciguatoxin (**1**, CTX1B). In 1989, on the basis of extensive NMR study, Murata, Yasumoto, and co-workers successfully established the whole structures including the relative stereochemistry of the causative principles, ciguatoxin (**1**) and CTX4B (**2**),[3] which are huge ladder-shaped polycyclic ethers with the 13 ether rings ranging from five- to nine-membered (Fig. 1). In subsequent studies, the Yasumoto's group have characterized other congeners including CTX4A (**3**),[4] CTX3C (**4**),[5] and 51-hydroxy CTX3C (**5**).[6] Interestingly, the CTX3C (**4**) isolated from the cultured *G. toxicus* not only lacks the side-chain of **1**, but contains an oxocene E-ring instead of the oxepene ring. The restricted availability of ciguatoxins has precluded further studies for structural elucidation, and the absolute configuration and stereochemistry of the C2 hydroxy group of **1** have remained unidentified. In 1989, Hirama's group initiated collaborative investigations with the Yasumoto's group with the aim of elucidating the absolute configuration of ciguatoxins.[7]

1.2 Elucidation of the C2 Configuration of Ciguatoxin. The conventional method for determining the absolute configuration of natural and synthetic molecules has been X-ray analysis and optical rotation measurements, which generally require several milligrams of sample. Over the past decade,

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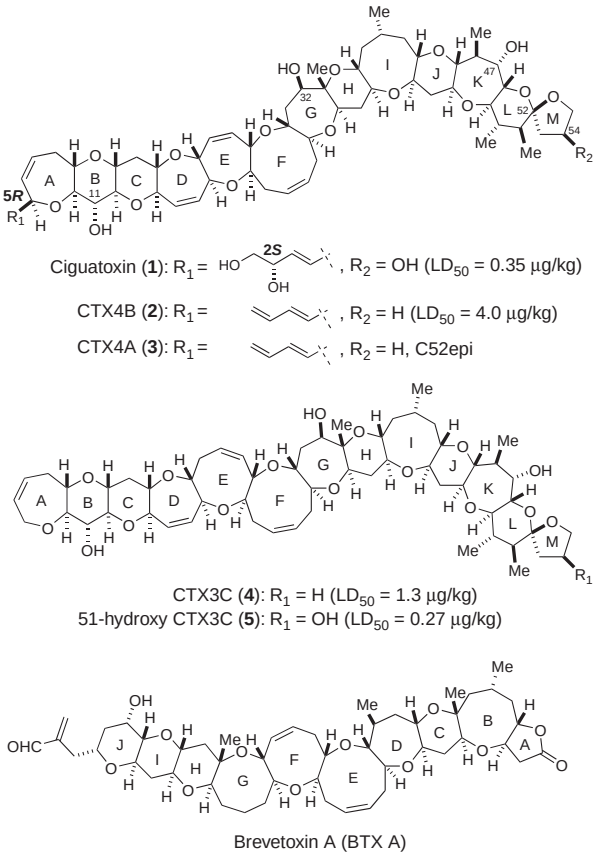

Fig. 1. Structures of ciguatoxins and brevetoxin A.

the modified Mosher method has been commonly used as a sensitive and versatile spectroscopic means for elucidating the absolute configuration of sub-milligram amounts of compounds containing secondary hydroxy (amine) groups.[8] This method relies on microscale esterification with MTPA chloride, followed by separation of the products and NMR analysis. Due to the extremely limited availability of ciguatoxins as well as the presence of multiple hydroxy groups, application of the modified Mosher method for elucidation of the absolute configuration of **1** and **2** is expected to be difficult. Accordingly, we planned to synthesize a diastereomeric pair of AB-ring fragments as fully functionalized model compounds of the left end of ciguatoxin and sought to develop a feasible approach for elucidation of the C2 configuration and the whole absolute configuration with a minimum amount of natural samples.

As illustrated in Fig. 2, we chose the chiral compound **7** as a versatile starting material for synthesizing the AB-ring model **17**, since manipulation of either the benzyloxymethyl or the allyl group of **7** would lead to each enantiomer of the target compound **17**.[9] Compound **7** was prepared readily and stereoselectively from methyl α-D-glucopyranoside **6** according to Kishi's method.[10] Based on an assumption that ciguatoxin has a 5*R* configuration, our synthesis commenced with **7**. The A-ring skeleton was stereoselectively constructed based on (*Z*)-γ-alkoxyallylstannane-aldehyde cyclization (**8** $\rightarrow$ **9**),[11] and the internal double bond was then incorporated by regioselective elimination (**10** $\rightarrow$ **11**). Installation of the acid-sensitive 3-butene-1,2-diol side-chain of the A-ring was achieved by Wittig-type olefination of aldehyde **12** with **13** under

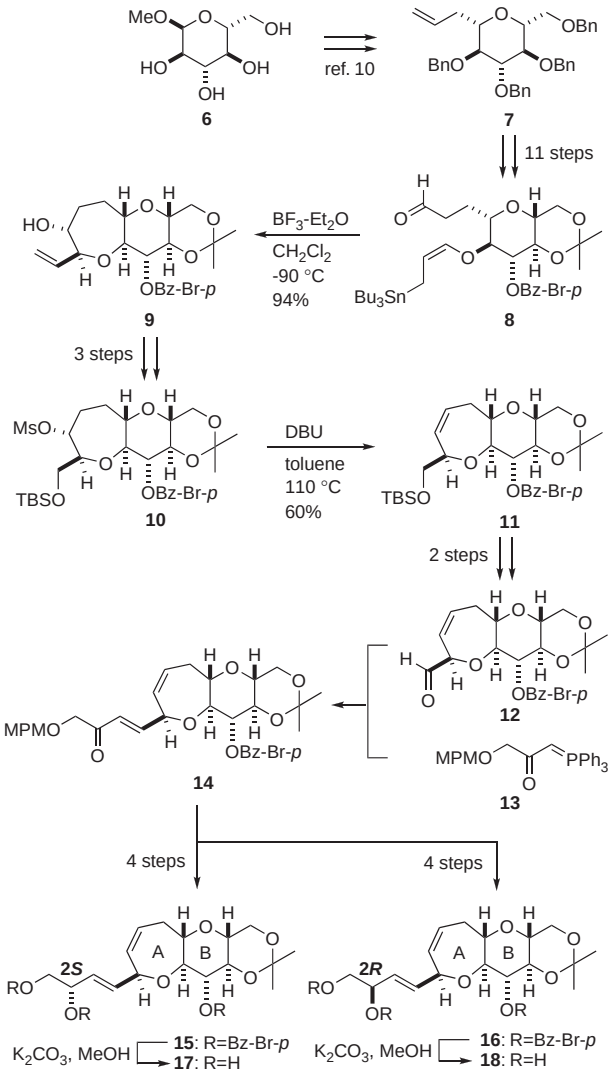

Fig. 2. Stereocontrolled synthesis of the AB-ring fragments of ciguatoxin.

neutral conditions to afford enone **14**. Although asymmetric reduction of the resulting enone may have been possible, we adopted a convenient method to secure a pair of C2 diastereomers for elucidating the C2 configuration: the enone was reduced with $NaBH_4$–$CeCl_3$ to yield a separable 1:1 diastereomeric mixture. Subsequent protecting group manipulation produced the diastereomers of the AB-ring models (**17** and **18**) as well as their tris-*p*-bromobenzoates (**15** and **16**).

As an initial attempt to establish the C2 configuration, we used a circular dichroic exciton chirality method for acyclic *vic*-dibenzoates, which is capable of detecting CD curves even for very small samples (microgram order).[12] Murata and Yasumoto have conducted *p*-bromobenzoylation of ciguatoxin (**1**), which possesses six hydroxy groups. By treating 30 μg of **1** with excess *p*-bromobenzoyl chloride, Et_3N, and 4-dimethylaminopyridine (DMAP) in CH_2Cl_2, tetrakis-*p*-bromobenzoate (ca. 7 μg based on the UV absorption) was isolated as a major product. The number of benzoyl groups was determined by FAB-MS spectroscopy, and the tetrakis-*p*-bromobenzoate exhibited a positive split CD curve, which is characteristic of *vic*-di-*p*-bromobenzoates. On the basis of the CD exciton

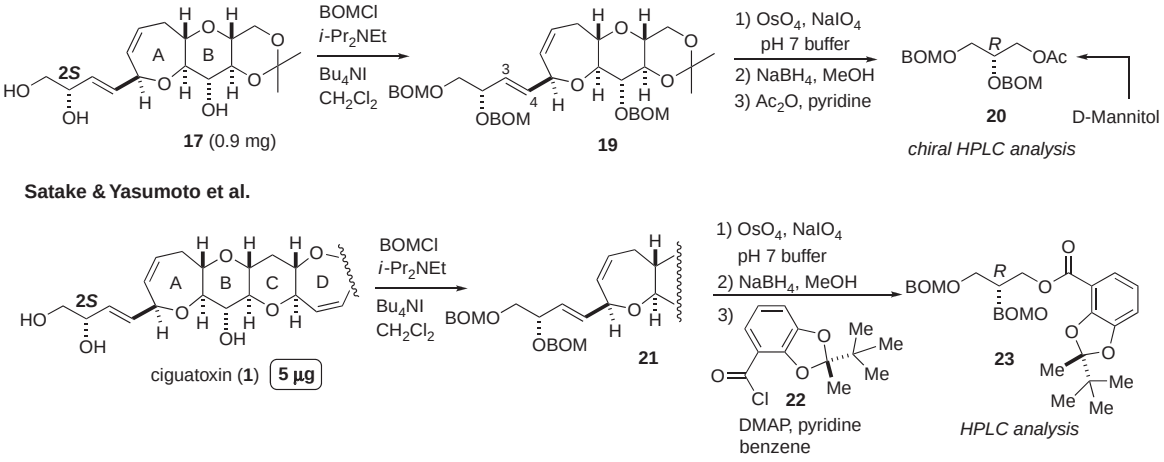

Fig. 3. Chemical transformation of AB-ring fragment **17** and ciguatoxin for elucidation of C2 stereochemistry.

chirality data of the tetrakis-*p*-bromobenzoates as well as bis-[13] and tris-*p*-bromobenzoates (**15** and **16**) of the AB-ring fragments, 2*S* configuration was proposed.[14] However, further confirmation was required due to the difficulty in assigning the position of tetrakis-*p*-bromobenzoates of ciguatoxin.

In order to establish a conclusive approach for elucidating the C2 configuration, we then planned to chemically transform the 3-butene-1,2-diol side-chain into a chiral glycerol derivative for HPLC comparison with an authentic glycerol sample derived from D-mannitol (Fig. 3).[9,14] For this purpose, we developed a microscale transformation sequence by use of the synthetic model **17**, which possesses all the functionalities of the left end of **1**. The hydroxy groups of **17** were protected as (benzyloxy)methyl (BOM) ethers, and then the C3,C4-double bond of **19** was oxidatively cleaved by treatment with OsO_4–$NaIO_4$. Reduction of resulting aldehyde with $NaBH_4$ and subsequent acetylation afforded **20**, which was subjected to chiral HPLC analysis for comparison with authentic samples. Thus, we established a protocol for elucidating the C2 stereochemistry based on micro-scale chemical transformations.

On the basis of the protocol mentioned above, Satake and Yasumoto have carried out a chemical transformation of ciguatoxin (**1**), starting only with 5 µg of sample (Fig. 3). The final acylation step was modified by use of a chiral acid chloride **22**,[15] yielding fluorescent **23**. Comparison of **23** with authentic samples using two HPLC systems (reverse and normal phase) demonstrated that the retention times of derivative **23** of **1** agreed well with those of authentic **23** having a 2*R* configuration derived from the 2*S*-standard. Consequently, the C2 configuration of **1** was unambiguously determined to be *S*.[16]

1.3 Absolute Configuration of Ciguatoxin. Since the relative configuration of the polycyclic ether core (C5–C54) of ciguatoxin (**1**) was determined based on extensive ^{1}H NMR NOE experiments,[3] the assignment of a single stereogenic center in the core, such as C5, is sufficient for elucidation of the entire absolute configuration. CTX4B (**2**), which possesses a 1,3-butadiene on A-ring (rather than the 3-butene-1,2-diol side-chain of ciguatoxin (**1**)), was used for the initial attempt, which was based on the CD exciton chirality method. Although **2** exhibited a characteristic UV absorption maximum at 222 nm (ε 27000), the Cotton effect at the corresponding wavelength was obscure. In 1991, Hirama and co-workers synthesized the AB-ring model **24** possessing 5*S* configuration, which lacks the C11 hydroxy group (Fig. 4a). Based on a comparison of their CD spectra, 5*R* configuration was proposed for CTX4B (**2**).[17] However, uncertainty remained due to the small Cotton effects observed.

To provide definitive evidence of the absolute configuration of the cyclic ether core, we renewed our efforts, assuming that C11-*p*-bromobenzoylated CTX4B would exhibit a distinct Cotton effect due to the close proximity of the *p*-bromobenzoate chromophore to the 1,3-butadiene. In this context, we designed and synthesized the AB-ring models **26** and **27**, as shown in Fig. 4a. The CD spectrum of C11-*p*-bromobenzoylated AB-ring model **26** clearly exhibits a split Cotton effect with a positive followed by a negative extremum (Fig. 4b), which is caused by the expected interaction between the 1,3-diene and *p*-bromobenzoate (MeOH, λ_{ext} 242 nm, $\Delta\varepsilon$ +25; λ_{ext} 225 nm, $\Delta\varepsilon$ −14).[16] These results prompted collaborative research with Satake and Yasumoto using the scarce natural CTX4A (**3**), which possesses a terminal 1,3-butadiene at C5 and an epimeric cyclic ketal at C52 (Fig. 4c). The reaction of **3** (100 µg) with *p*-bromobenzoyl chloride for 24 h in the presence of Et_3N and DMAP afforded tris-*p*-bromobenzoate **28**, which was purified by HPLC and identified by FAB-MS [MH^+ m/z 1607, 1609, 1611, 1613]. As shown in Fig. 4b, the CD spectrum of the tris-*p*-bromobenzoate **28** (MeOH, λ_{ext} 246 nm, $\Delta\varepsilon$ +32; λ_{ext} 230 nm, $\Delta\varepsilon$ −28) was virtually identical to that of **26**, which implies that the split Cotton effect was mainly due to coupling between the 1,3-diene and 11-α benzoate. The contribution due to chiral exciton coupling between the C11-α and C32-β benzoate chromophores should be insignificant, because of their large separation (approximately 13 Å). Similarly, coupling between the C32-β and C47-α benzoate chromophores was expected to be small, because the two chromophores are approximately 10 Å apart. On the basis of these results, the configuration of C5 in **2** and **3** was concluded to be *R*. Since **1** is an oxidized metabolite of **2** and has been confirmed to have the same relative configurations at C5 and C11, **1** must have 5*R* configuration.[16]

Thus, the absolute configuration of **1** was determined unambiguously as depicted in Fig. 1. The goals were successfully

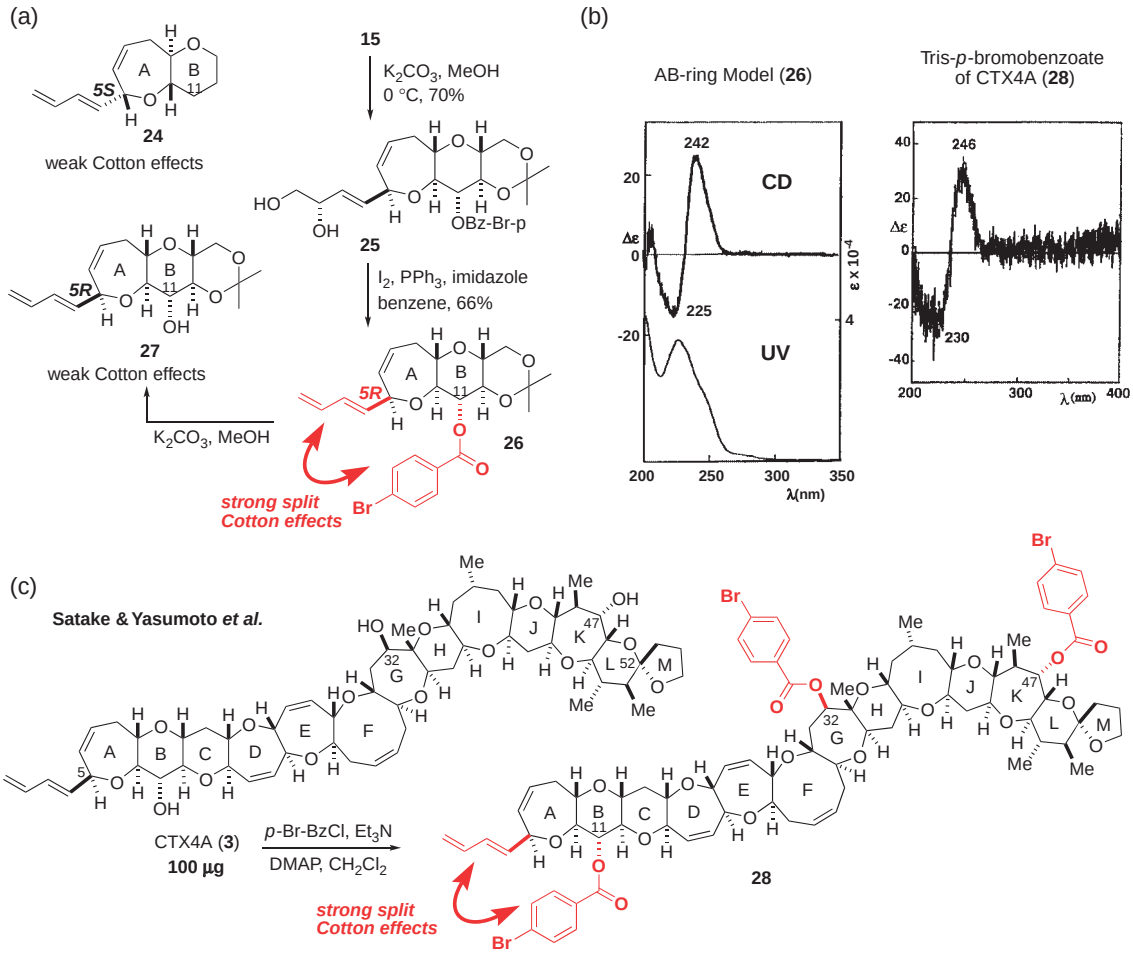

Fig. 4. Elucidation of the whole absolute configuration of ciguatoxins. (a) Synthesis of AB-ring fragments of CTX4A. (b) CD and UV spectra of synthetic **26** and CD spectrum of CTX4A tris-*p*-bromobenzoate **28**. (c) *p*-Bromobenzoylation of CTX4A.

achieved using very small amounts of the toxins by using a combined approach, which included synthesis of partial structures, carefully controlled microscale chemical transformations, and use of the CD exciton chirality method based on the interaction of 1,3-diene and C11-*p*-bromobenzoate.[18]

The elucidation of the whole structure of ciguatoxin has prompted many synthetic studies of ciguatoxins in the organic chemistry community.[19] In 2001, the Hirama group accomplished the total synthesis of CTX3C (**4**), and the absolute stereochemistry of the ladder-shaped cyclic ether core was unambiguously confirmed.[20] Furthermore, the 2*S* configuration has been also confirmed based on total synthesis of ciguatoxin (**1**), achieved by Hirama and co-workers in 2006.[21]

As for the absolute configurations of the ladder-shaped polycyclic ether marine toxins, it is interesting to note that the pentacyclic 8/9/7/6/8 partial structure (BCDEF-ring) of brevetoxin A (BTX A),[22] the causative toxin of red tide, closely resembles the EFGHI-ring of CTX3C (**4**) (Fig. 1). In addition, these partial structures have an identical absolute configuration. Thus, the biosynthetic mechanisms of the polycyclic ether marine toxins that account for the construction of the ladder-shaped cyclic ether skeleton with *syn*/*trans* stereochemical uniformity at the ring junctions are of great interest.[23]

2. Synthetic Approach toward Preparation of Anti-Ciguatoxin Antibodies and Development of Immunoassay System for Detection of Contaminated Toxins

2.1 Anti-Ciguatoxin Antibodies for Development of Immunoassay Systems. The spread of ciguatera inflicts tremendous damage on public health, fishery resources development, and economic income in tropical and subtropical regions.[1] A major problem in terms of disease prevention is that fish contaminated with ciguatoxins look, smell, and taste normal. In addition, because ciguatoxins are insensitive to temperature, they are not deactivated by either cooking or freezing. Ciguatoxin (**1**) and its congener CTX3C (**4**) exhibit potent toxicity in mammals [acute toxicity in mice: medium lethal dose (LD_{50}) 0.15–4 µg kg^{-1}].[24] In addition to the traditional mouse bioassay of fish lipid extracts,[25] several other methods have recently been developed for detection of ciguatoxins, including assays based on cytotoxicity,[26] radio ligand binding,[24,26] high-performance liquid chromatography,[27] and mass spectrometry.[28,29] However, antibody-based immunoassay remains the most desirable method for accurate, sensitive, routine, and portable use. In the anti-ciguatoxin antibodies prepared by Hokama and co-workers using the scarce natural toxins,[30,31] the mAb exhibited cross-reactivity to another marine toxin,

okadaic acid.[32] The extremely low content of ciguatoxin in fish has hampered the further development of anti-ciguatoxin antibodies. We therefore planned to use synthetic haptens to solve the problem of antibody development. In this account, we describe the preparation of monoclonal antibodies (mAbs) against the right and left ends of CTX3C by immunizing mice with protein conjugates of synthetic fragments. Furthermore, we established a direct sandwich ELISA for specific and reliable detection of CTX3C. As well as the preparation of anti-CTX3C antibodies, synthetic approaches for the production of antibodies against ciguatoxin and the development of immunoassays using surface plasmon resonance (SPR) are also described.

2.2 Hapten Design. In an attempt to prepare antibodies that bind specifically to one end of CTX3C, we designed haptens on the basis of antibody antigen interactions. X-ray structural analysis of antibody–hapten complexes has shown that relatively small haptens are buried deep in the antigen-combining site, with surface areas of 200–400 Å^2.[33] Since the surface areas of the tri-, tetra-, and pentacyclic parts of the left end (ABC, ABCD, and ABCDE) were calculated to be 253, 318, and 398 Å^2, respectively (Fig. 5), the ABC-ring was expected to be of sufficient size to allow the preparation of specific antibodies for CTX3C.

2.3 Attempts to Prepare Anti-CTX3C Antibodies Using ABC- and ABCD-Ring Fragments as Haptens. Taking into account the size of the molecular surface, we initially designed ABC-ring fragment **32** as a hapten for production of antibodies, which would bind to the left end of CTX3C (Fig. 6).[34] The ABC-ring fragment **32** was synthesized from the AB-ring moiety **29**, which is readily available from tri-*O*-acetyl-D-glucal.[35] One carbon homologation of the primary alcohol of **29** and conjugate addition of the secondary alcohol to ethyl propiolate gave **30**. SmI_2-mediated cyclization of **30** furnished a *trans*-fused tricyclic ether **31** in 94% yield in a stereo-controlled manner.[36] Removal of the benzyl group and subsequent hydrolysis liberated hapten **32**. Hapten **32** was conjugated to keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) via an activated ester methods to yield

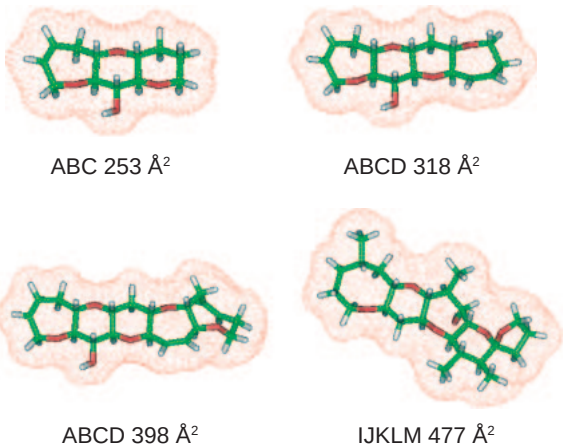

Fig. 5. Energy-minimized structures of tri-, tetra-, and pentacyclic ether parts of CTX3C [carbon frameworks (green), oxygen (red), hydrogen (white)] and water-accessible surface areas (Connolly surface shown as red dots) were calculated by Macromodel Ver 6.0, (MM2*).

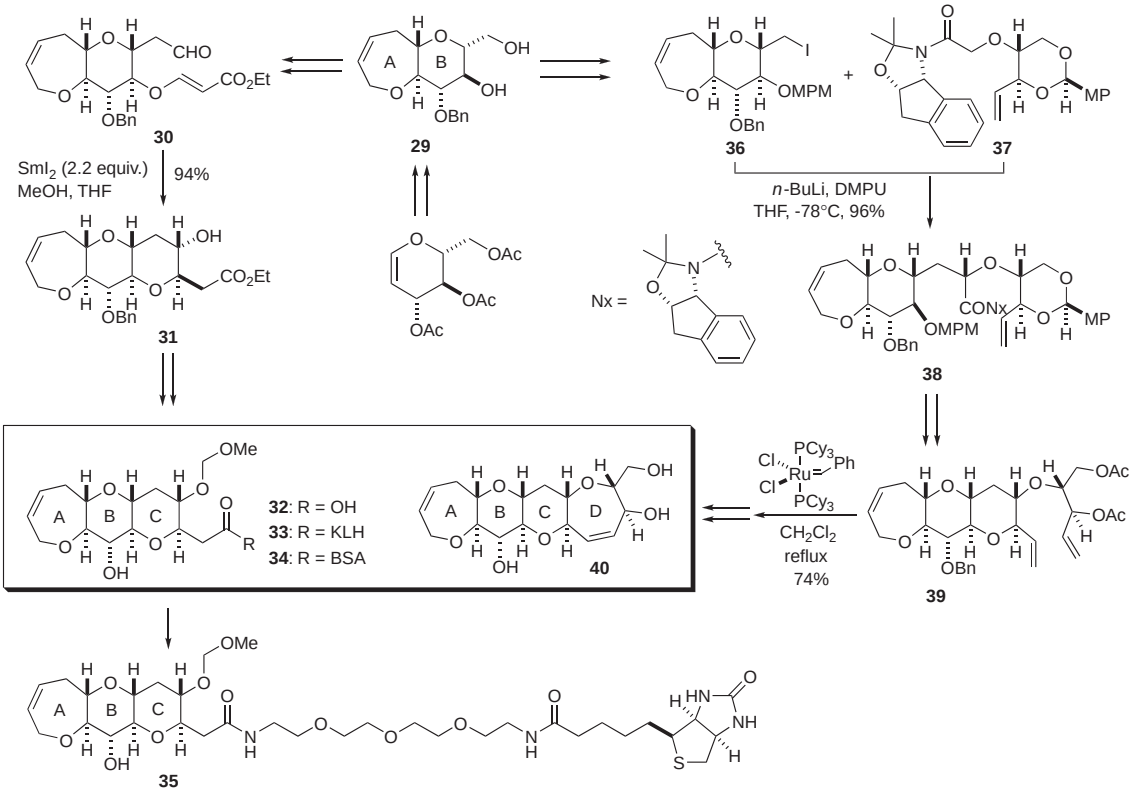

Fig. 6. Stereocontrolled synthesis of the ABC-ring and ABCD-ring fragments (**32** and **40**) and their protein conjugates (**33** and **34**), and biotin conjugate **35** for preparation of anti-CTX3C antibodies.

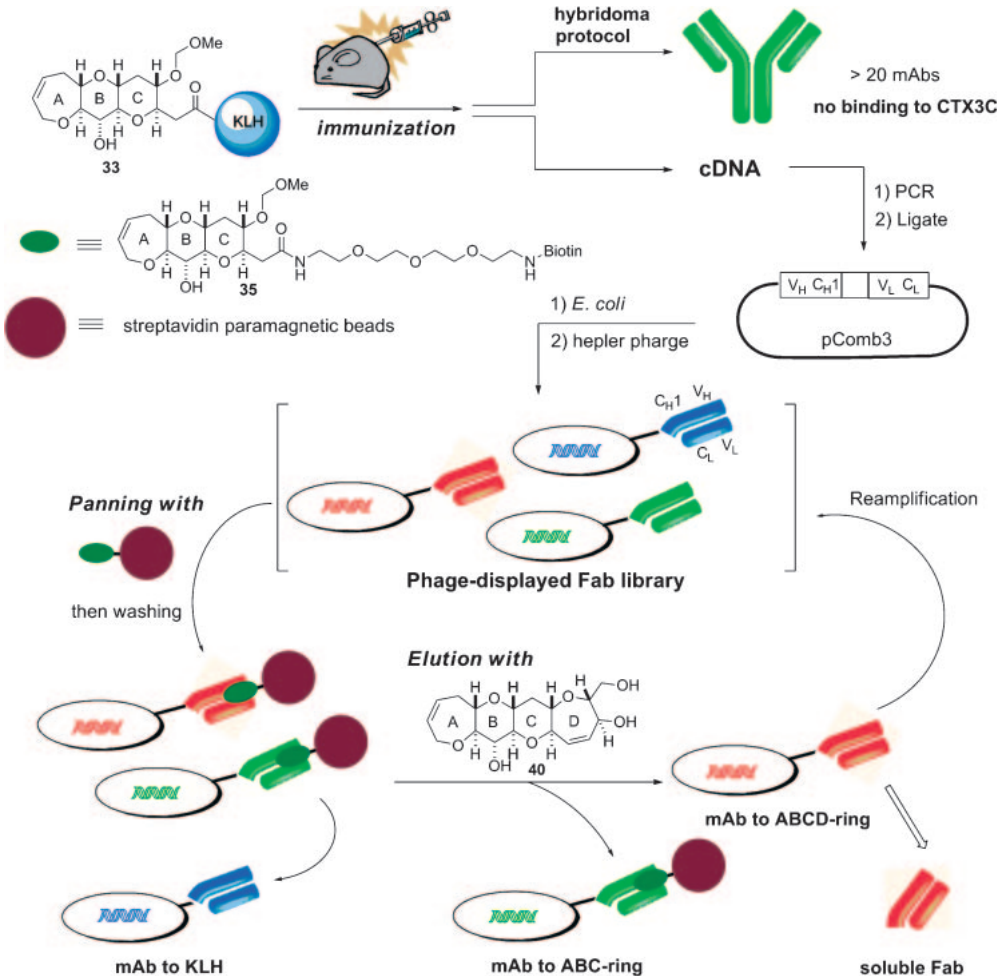

Fig. 7. Preparation of anti-CTX3C antibodies based on hybridoma protocol and screening of phage-displayed rFab libraries using synthetic ABC- and ABCD-ring fragments and their conjugates.

hapten–protein conjugates **33** and **34**, respectively. In a similar fashion, the water-soluble biotin conjugate **35** was synthesized by condensation of **32** with EZ-Link™ Biotin-LC-PEO-amine.[37]

In collaboration with Tsumuraya and Fujii, BALB/c mice were immunized intraperitoneally with KLH conjugate **33** with RIBI adjuvant (Fig. 7). The spleens were taken from the mice, and the cells were fused with myeloma cells according to the standard hybridoma protocol. Positive clones were screened against both the BSA conjugate **34** and the free ABC-ring fragment **32**. After considerable efforts, we prepared more than 20 mAbs that bind to either **34** or **32**. However, none of the mAbs bound to CTX3C itself.

These preliminary results suggested that the antibody may bind to the ABC-ring skeleton as well as the linker moiety (Fig. 8a), and we proposed that a possible epitope for molecular recognition of the left end of CTX3C by an antibody should be larger than the tricyclic ABC-ring skeleton (surface area: approximately 250 Å^2). This prompted the question of how much of the surface area of the polycyclic ether skeleton is required for an epitope. To our knowledge, there has not yet been a systematic study to determine the required size of epitopes for non-protein ladder-like molecules, such as CTX3C. For this reason, we next attempted to prepare antibodies to the tetracyclic ABCD-ring skeleton (surface area: approximately 320 Å^2), with the expectation that a study of the binding of such antibodies to CTX3C would provide an indication of the required epitope size (Fig. 8b).

For the preparation of antibodies for the ABCD-ring skeleton, triol **40** was designed as a hapten with a moderate aqueous solubility (Fig. 6). The convergent synthesis of **40** was achieved by a combination of asymmetric alkylation (**36** + **37** → **38**) using (1*R*,2*S*)-1-amino-2-indanol derivative as a chiral auxiliary, and a ring-closing metathesis reaction of **39**.[38] With **40** in hand, however, we struggled to undertake experiments for preparation of mAbs according to the hybridoma protocol by immunization with a protein conjugate linked with **40** at this stage. As an alternative, a rational protocol to screen antibodies that bind to the free ABCD-ring hapten **40**, making use of phage display technology, was devised (Fig. 7).[37] In collaboration with Tomioka and Mizugaki, a recombinant Fab library was constructed from the spleen of Bulb/c mice immunized with ABC–KLH **33**. Total RNA was isolated from the spleen, and the first-strand complementary DNA was synthesized. The Fd [comprising heavy-chain variable region (V_H) and heavy-chain constant region 1 domains (C_H1)] and κ [comprising light-chain variable region (V_L) and light-chain constant region domains (C_L)] gene fragments were amplified

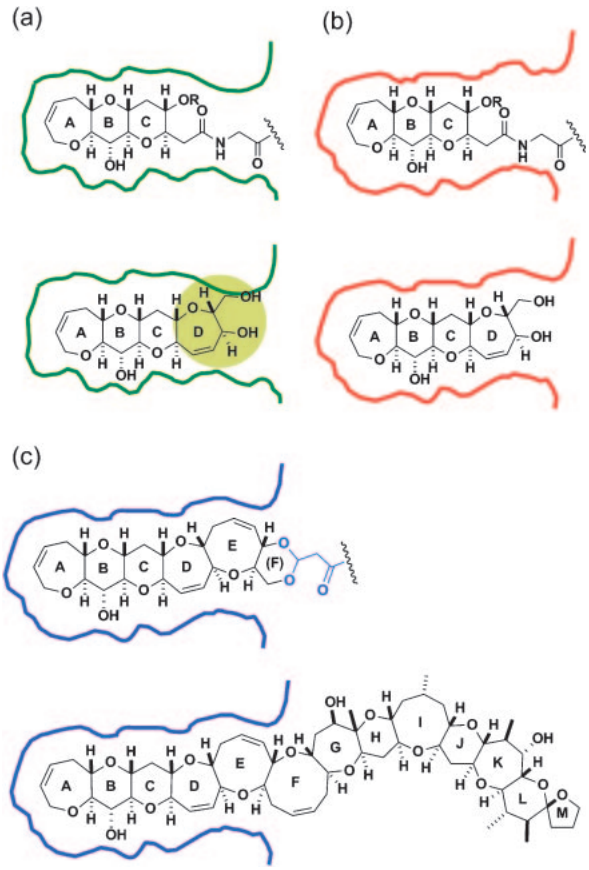

Fig. 8. Working hypothesis for hapten–antibody interactions. Antibodies against (a) ABC-ring fragment, (b) ABCD-ring fragment, and (c) ABCDE-ring fragment.

by PCR using the primers for mouse immunoglobulin containing specific restriction enzyme sites [*Sac*I and *Xba*I for light-chain (LC), *Xho*I and *Spe*I for heavy-chain (HC)] for cloning into a vector.[39] In a collaborative investigation with Tsumoto and co-workers, the PCR products of the HC and LC gene segments were purified and ligated with the phage display vector pComb3. The constructed recombinant Fab (rFab) library was displayed on phage surfaces. Phages that bound to soluble ABC–PEG–Biotin (**35**) were selected and captured on streptavidin-linked magnetic beads. The beads were then incubated with the free ABCD-ring fragment **40** to elute the desired antibodies. Antibodies that bound to the ABCD-ring skeleton were selected and expressed as soluble rFabs. A competitive inhibition ELISA study showed that three rFabs bound to the free ABCD-ring fragment **40** with K_d values in the range of 2.4×10^{-5} to 5.0×10^{-5} M (Table 1). Thus, we generated a rational protocol for the selection of phage-displayed antibodies that bind to a free small molecule via elution with a designed synthetic hapten.[37] Moreover, this protocol, which allowed antibody production with minimum consumption of the target complex compound **40**, was suitable from a practical standpoint, since, at this stage, the synthesis of the ABCD-ring fragment **40** was a far more difficult and laborious task than that of the ABC-ring fragment **32**. We further demonstrated that rFab 1C49 exhibited the highest affinity for the free **40** and also bound to CTX3C (Fig. 9). The reduced binding affinity

Table 1. Dissociation Constants (K_d)[a] for Binding of rFabs to Synthetic Fragments **32** and **40**

Antibody (rFab)	K_d/M	
	ABC (**32**)	ABCD (**40**)
1C49	8.6×10^{-8}	2.4×10^{-5}
1C2	4.0×10^{-8}	3.7×10^{-5}
3C32	3.0×10^{-8}	5.0×10^{-5}

a) K_ds were determined by competitive ELISA.

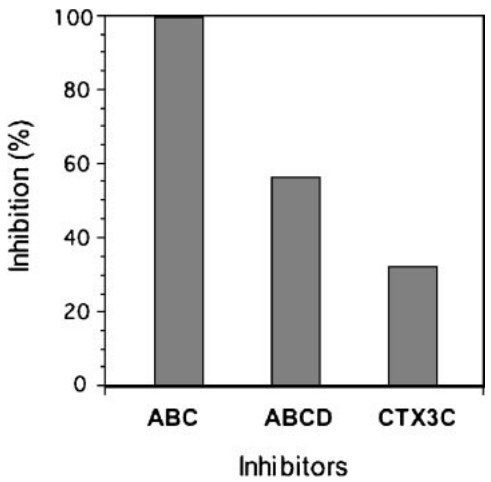

Fig. 9. Competitive inhibition of binding of 1C49 to ABC-BSA by synthetic fragments ABC (**32**), ABCD (**40**), and CTX3C (**4**). Each inhibitor was used at 50 μM.

of 1C49 for CTX3C compared to the ABCD-ring fragment is presumably due to the absence of the E-ring fused to the D-ring. Thus, it is likely that the possible epitope for the left side of CTX3C should be as large as the pentacyclic ABCDE-ring skeleton (surface area: approximately 400 Å^2).

2.4 Preparation of Anti-CTX3C Antibodies for Both Ends of CTX3C Using ABCDE- and IJKLM-Ring Fragments as Haptens. We next designed hapten **42**, which consists of a pentacyclic skeleton (ABCDE-ring, surface area: 398 Å^2) and a cyclic acetal attached to a linker (Fig. 10). 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 **42** would be large enough to occupy the antigen-combining site while leaving the acetal and the linker moiety free from antibody–antigen interactions, as illustrated in Fig. 8c. In addition, hapten **42** was designed to elicit antibodies that would bind to the left end of CTX3C in an orientation appropriate for direct sandwich immunoassay. For this purpose, a carboxylic acid linker was attached to the E-ring via a cyclic acetal in place of the central F-ring. Similarly, hapten **46**, which consisted of a pentacyclic skeleton (IJKLM-ring, surface area: 477 Å^2, Fig. 5) and a cyclic acetal with a linker, was designed to induce antibodies that could bind to the right end of CTX3C.[40]

Hapten **42** was synthesized as a diastereomeric acetal mixture from the ABCDE-ring fragment **41**, a key intermediate in the total synthesis of CTX3C,[41] and conjugated to keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) via an

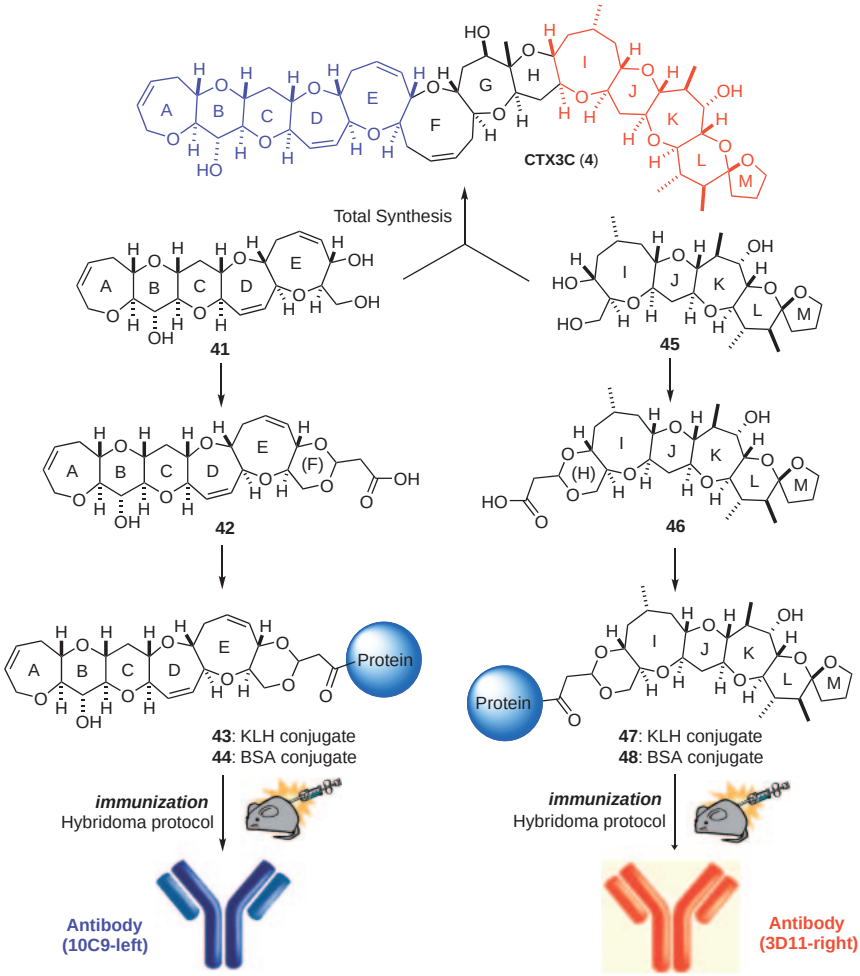

Fig. 10. Preparation of anti-CTX3C antibodies using synthetic fragments as haptens.

Table 2. Dissociation Constants (K_d)[a] for Binding of Marine Toxins and Synthetic Fragments to mAbs

mAb	K_d/nM			K_d/μM					
	A–E (**41**)	I–M (**44**)	CTX3C	BTX A	BTX B	OA	MTX	A–D (**40**)	A–C (**32**)
10C9	0.8	—[b]	2.8	—[b]	—[b]	—[b]	—[b]	1.8	74
3D11	—[b]	8.6	122	43	—[b]	—[b]	—[b]	ND[c]	ND[c]

a) K_ds were determined by competitive ELISA. b) No inhibition was observed at the maximum concentrations of the inhibitors. The maximum concentrations for **44**, **47**, BTX A, BTX B, OA, and MTX are 250, 250, 100, 100, 100, and 25 μM, respectively. c) Not determined.

activated ester method (Fig. 10). In a similar fashion, protein conjugates **47** and **48** were synthesized from the IJKLM-ring fragment **45**.[42] In collaboration with Tsumuraya and Fujii, Balb/c mice were immunized with KLH conjugates **43** and **47**, resulting in the formation of six mAbs for the left end and three mAbs for the right end of CTX3C, respectively. MAb 10C9 (left end) and mAb 3D11 (right end) were examined for their binding affinity to the hapten and to CTX3C itself using a competitive ELISA.[43]

As shown in Table 2, mAb 10C9 exhibited a high affinity (K_d = 0.8 nM) for the ABCDE-ring fragment **41**. More importantly, mAb 10C9 bound tightly to CTX3C itself with a comparable K_d value of 2.8 nM, and it showed no cross-reactivity with the other IJKLM-ring **45** or with structurally related marine toxins (Fig. 11) including brevetoxin A (BTX A),[22] brevetoxin B (BTX B),[44] okadaic acid (OA),[45] and maitotoxin (MTX).[46] Furthermore, we determined that the K_d values of 10C9 for the ABCD-ring **40** and the ABC-ring **32** were 1.8 and 74 μM, respectively, and that for the ABCDE-ring **41** was 0.8 nM (Table 2). 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 should recognize the pentacyclic ABCDE-ring skeleton of CTX3C and that the surface area of CTX3C required for molecular recognition by 10C9 is in line with our prediction of approximately 400 Å^2. Similarly, mAb 3D11 (right end) was also found to bind strongly to CTX3C itself (K_d = 122 nM) and did not cross-react with the ABCDE-ring

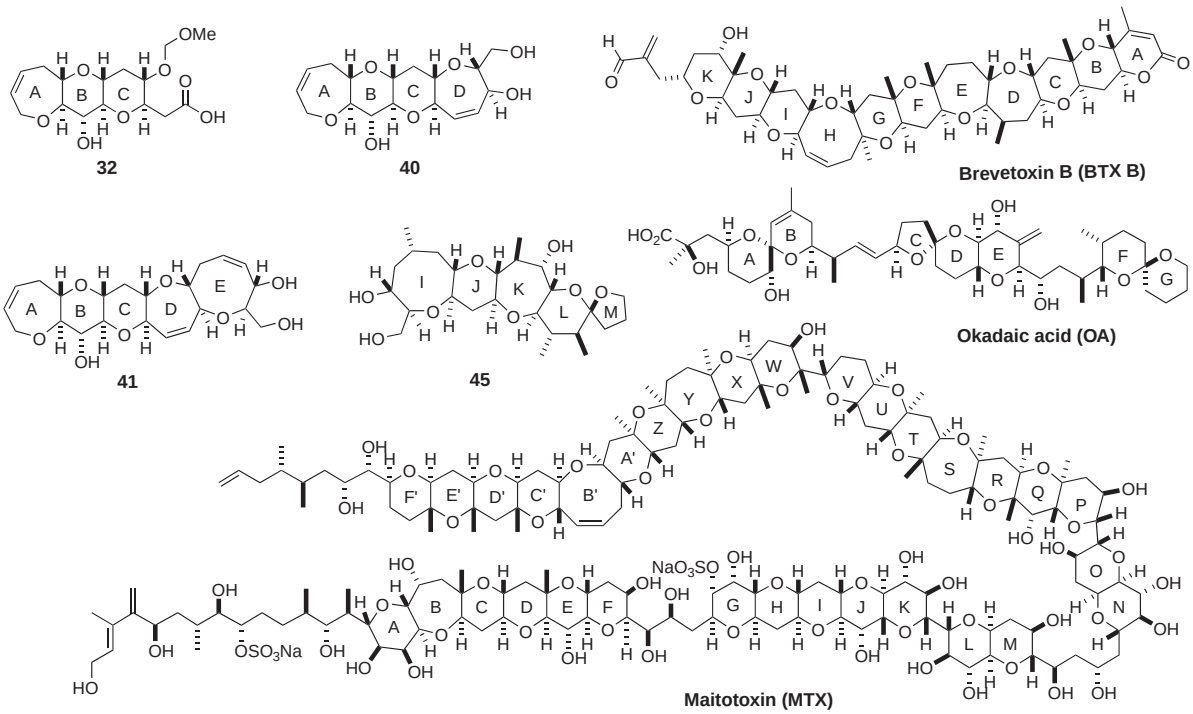

Fig. 11. Structures of marine toxins [brevetoxin A (BTX A), brevetoxin B (BTX B), okadaic acid (OA), and maitotoxin (MTX)], and synthetic fragments of CTX3C.

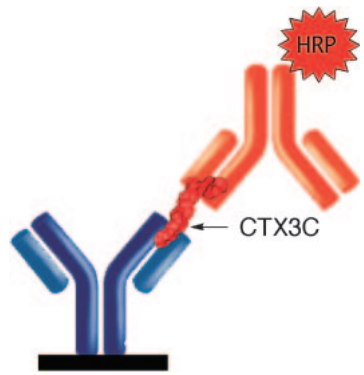

Fig. 12. Schematic diagram of direct sandwich ELISA for CTX3C (red). Specific antibody 10C9 (blue) against the left end of CTX3C is immobilized, and 3D11 (orange) against the right end is conjugated with horseradish peroxidase (HRP).

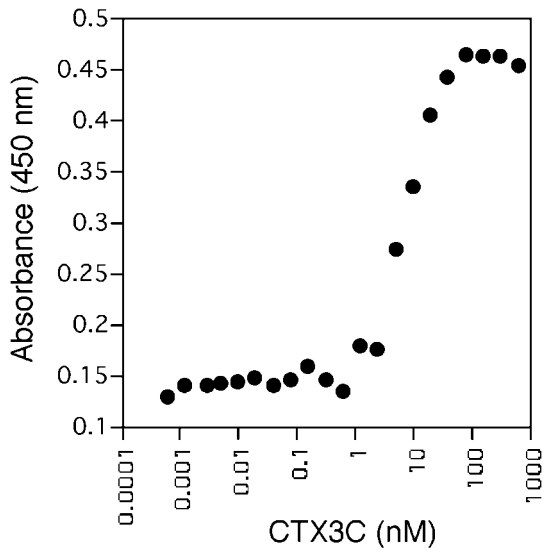

Fig. 13. Direct sandwich ELISA for CTX3C.

41 or with other marine toxins. Thus, for the first time, we have successfully produced two specific mAbs that bind to CTX3C with high affinity.[47] These results form the basis of a useful strategy for designing a hapten for the production of antibodies against large non-protein molecules, especially for scarce marine toxins.

2.5 Direct Sandwich ELISA. Using the two mAbs for the right and left ends of CTX3C, we then attempted to develop a direct sandwich ELISA for specific and reliable detection of CTX3C (Fig. 12).[47] 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, and 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 (Fig. 13). This protocol could detect CTX3C down to ppb levels [detection limit: $\approx$5 ng mL^{-1} (5 nM)]. None of the other marine toxins (BTX A, BTX B, OA, and MTX) or synthetic fragments (ABCDE-ring **41** and IJKLM-ring **45**) tested gave any detectable signal at a concentration of 20 µM. 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 significant use in detecting contamination of seafood with CTX3C.

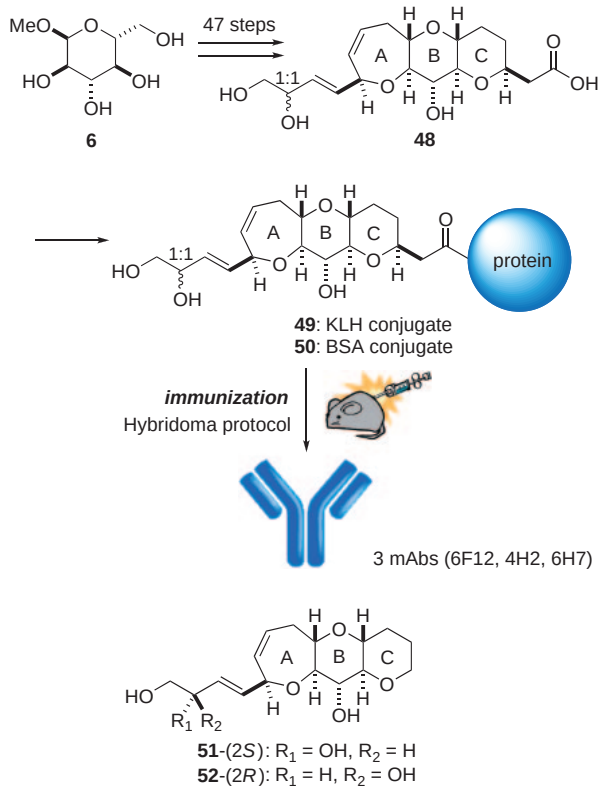

Fig. 14. Attempts to prepare anti-ciguatoxin antibodies using the ABC-ring fragment as a hapten.

2.6 Attempts to Prepare Anti-Ciguatoxin Antibodies. 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 variations between the congeners are mainly due to the presence of different substituents on the terminal A and L rings.[2] Thus, we developed some further synthetic approaches to the preparation of anti-ciguatoxin antibodies.

As shown in Fig. 14, ABC-ring fragment **49**, having a 3-butene-1,2-diol and a carboxylic acid for conjugation, was designed as a hapten. Although hapten **49** was synthesized as a C2 epimeric mixture (1:1), the hapten was conjugated with carrier protein and subjected to immunization in mice.[48] Using the hybridoma protocol, three mAbs, designated as 4H2, 6F12, and 6H7, were obtained. In order to evaluate the binding selectivity of these mAbs to the C2 stereoisomers, epimers (2*S*)-**52** and (2*R*)-**53** were synthesized, separated and used as competitive inhibitors. The binding of mAb 6H7 to the BSA conjugate **51** was inhibited by (2*R*)-**53** more effectively than by (2*S*)-**52**. In contrast, 4H2 and 6F12 selectively bound to (2*S*)-**52**, but not to (2*R*)-**53**. Thus, each mAb was capable of distinguishing the C2 configuration of the epimeric ABC-ring fragment, although the affinities for **52** and **53** were not high.[48] As part of our further efforts to develop efficient analytical methods for the interaction of antibodies with haptens and to elucidate an antigenic determinant, we next plan to investigate the interaction of hapten–BSA conjugates with mAbs by surface plasmon resonance (SPR) spectroscopy.

2.7 Concise Synthesis of the ABC-Ring Fragment of Ciguatoxin. As illustrated in Fig. 14, the previous synthesis of the ciguatoxin ABC-ring fragment **49** was lengthy and laborious (47 steps from **6**).[48] We thus established a more concise and convergent route to ABC-ring fragment **61** possessing 2*S* configuration (Fig. 15).[34] The second-generation synthesis of **61** began with commercially available tri-*O*-acetyl-D-glucal. Protecting group manipulation and stereoselective allylation using Spilling's protocol[49] gave the *C*-glycoside **54**. The acid-sensitive A-ring, containing a (2*S*)-3-butene-1,2-diol side-

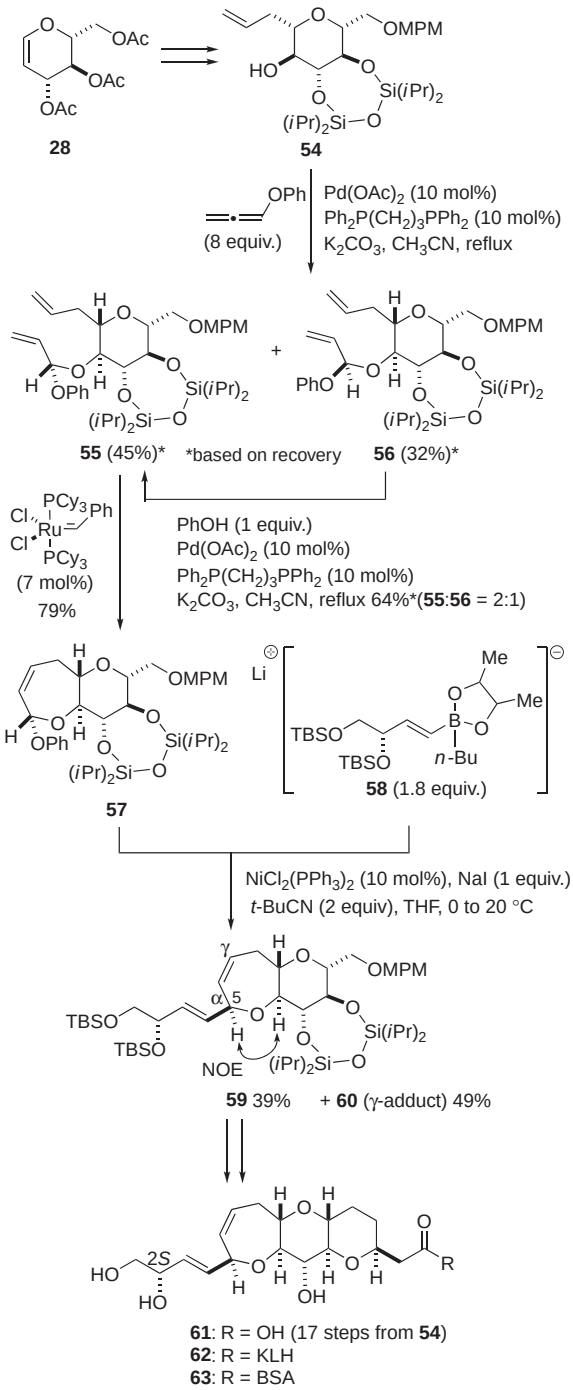

Fig. 15. Concise synthesis of ciguatoxin ABC-ring fragment **61** and its protein conjugates (**62** and **63**) based on transition-metal-catalyzed sequential reactions.

chain, was constructed from **54** via a transition metal ([Pd], [Ru], and [Ni])-catalyzed sequential process.[50] Palladium-catalyzed acetalization of secondary alcohol **54** with 1-phenoxy-1,2-propadiene according to the Rutjes's procedure[51] yielded a mixture of allylic acetals, **55** and **56**, in 45 and 32% yields, respectively. After separation, the undesired isomer **56** was epimerized by treatment with palladium catalyst and phenol to produce the desired **55**. A ring-closing metathesis reaction of diene **55** afforded the seven-membered allylic acetal **57** in 79% yield. Our initial attempt to install a 3-butene-1,2-diol side-chain involved palladium-catalyzed cross coupling of a seven-membered allylic acetal with a vinyl zinc reagent. However, a test Negishi reaction, employing a model compound with different protecting groups, resulted in the exclusive formation of the undesired γ-adduct in 70% yield.[50] This problem was solved by applying Kobayashi's nickel-catalyzed reaction.[52] Coupling of **57** with borate **58** was conducted in the presence of $NiCl_2(PPh_3)_2$ (10 mol %), NaI (1 equiv), and *t*-BuCN (2 equiv) in THF. The reaction proceeded smoothly below room temperature to give **59** and the regioisomer **60** in 39 and 49% yields, respectively. The C5 stereochemistry of **59** was confirmed by NOE experiments. Thus, we succeeded in the synthesis of AB-ring moiety **59** of ciguatoxin in only three steps from the alcohol **54**. Construction of the C-ring and the synthesis of **61** were achieved by intramolecular conjugate addition according to our reported protocol.[48] The concise synthetic route to **61** from tri-*O*-acetyl-D-glucal involves only 17 steps and accelerated the preparation of synthetic haptens for antibody development. For the SPR study analyzing the interaction of mAbs with hapten–BSA conjugates, the ABC-ring fragments of ciguatoxin and CTX3C, (2*S*)-**61** and **32**, respectively, were conjugated with BSA. The resulting hapten–BSA conjugates **63** and **34** were purified by gel filtration (Amersham Pharmacia, PD-10 column), and from MALDI-TOF-MS analysis, the average number of haptens attached to BSA in the conjugates was determined to be 14 and 15 for **63** and **34**, respectively.[34]

2.8 Surface Plasmon Resonance Study of Interaction of Hapten–BSA Conjugates with mAbs. To develop a rapid screening system for antibodies using SPR spectroscopy in place of the ELISA protocol, BSA conjugate **63** was immobilized on a sensor chip [CM5 sensor chips (BIACORE)], via its amine group and the interactions of **63** with the mAbs 4H2 and 6H7 were analyzed using BIACORE 2000 instruments.[34] The mAbs, diluted to a final concentration of 1.9 mg mL^{-1} in HBS buffer [10 mM Hepes (pH 7.4), 150 mM NaCl, 3 mM EDTA], were passed over the coated sensor tip at a constant flow of 20 mL min^{-1} at 25 °C (Fig. 16). MAb 4H2 bound to the immobilized **63** in a dose-dependent manner, whereas 6H7 did not bind at all. These affinities of the mAbs with **63** are in agreement with the previous results obtained using ELISA: 4H2 binds to (2*S*)-**52** and 6H7 binds to (2*R*)-**53**, but not to **52**.[48] Thus, the SPR assay is an effective replacement for the ELISA method for screening of antibodies that bind to the synthetic fragments.

Since epitope analysis of antibodies can be performed using SPR by passing various conjugates over the immobilized antibodies, we next examined the interactions of immobilized 4H2 with the BSA conjugates **63** and **34**. As shown in Fig. 17, 4H2

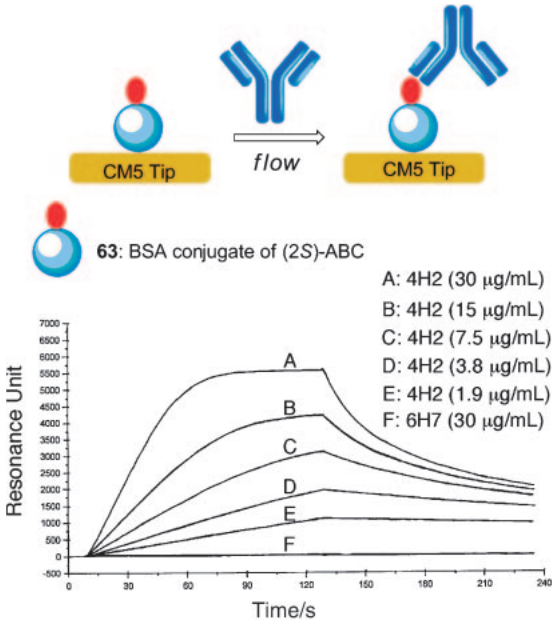

Fig. 16. Sensorgrams obtained by passing mAbs [4H2 (A–E) and 6H7 (F)] over immobilized **63**; [HBS buffer, 25 °C, flow rate: 20 mL min^{-1}].

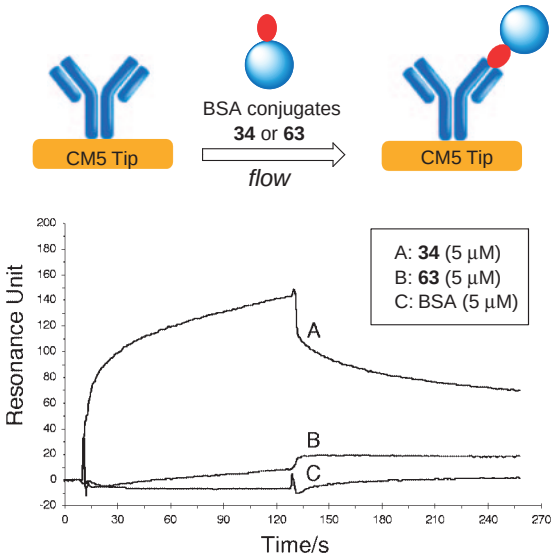

Fig. 17. Sensorgrams obtained by passing **34**, **63**, and BSA over immobilized 4H2; [HBS buffer, 25 °C, flow rate: 30 mL min^{-1}].

bound to **34**, but showed only negligible binding to **63**, which lacks the A-ring side-chain. Thus, the side-chain of **61** is an essential antigenic determinant for 4H2.[34]

Kinetic analysis of interaction between immobilized 4H2 with **34** gave the association rate constant (k_a) to be 5.8×10^3 M^{-1} s^{-1} and the dissociation rate constant (k_d) to be 3.5×10^{-3} s^{-1}. In the present system, association is rapid, whereas dissociation is slow: the equilibrium dissociation constant (K_d) is calculated to be 6.0×10^{-7} M.[34] The affinity is too weak for application to immunoassay.

2.9 Synthesis of the ABCD-Ring Fragment of Ciguatoxin

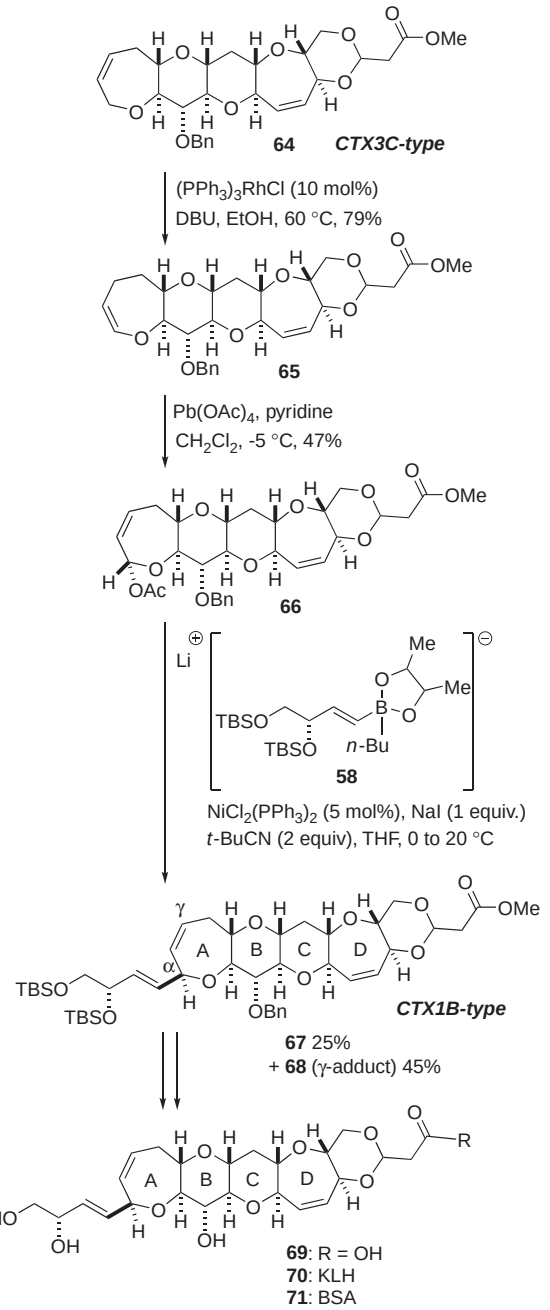

Fig. 18. Installation of 3-butene-1,2-diol side-chain into the ABCD-ring fragment of CTX3C, and preparation of hapten–protein conjugates **70** and **71** for preparation of anti-ciguatoxin antibodies.

by Installation of a 1-Butene-3,4-diol Side-Chain into the A-Ring of a CTX3C-Type Polycyclic Ether Core. We next designed and synthesized ABCD-ring fragment **69** as a hapten with a larger molecular size, which was expected to be suitable for antibody development through immunization in mice (Fig. 18). Several synthetic efforts to construct the A-ring of **1** have been reported, but most of these syntheses are linear and lengthy.[14,53,54] Connecting the side-chain fragment with the core at C4–C5 appeared to be the most convergent approach. This approach would facilitate the total synthesis of **1**, as well as supplying synthetic haptens for antibody development, because it involves the installation of a labile system in the later stages of synthesis.

Isomerization of allylic ether **64** into cyclic enol ether **65** was achieved in 79% yield using Willkinson's catalyst in the presence of DBU, without affecting the allyl ether of the D-ring (Fig. 18).[55] Upon treatment of the resulting enol ether **65** with $Pb(OAc)_4$ and pyridine, oxidation proceeded at 5 °C to produce the allylic acetate **66** in a stereo- and regio-controlled fashion. Nickel-catalyzed coupling of **66** with alkenylborate **58** yielded α-adduct **67** (25%) and regioisomer **68** (45%). After removal of the protecting groups from the hydroxy groups, hapten **69** was conjugated with carrier proteins.

3. Conclusion

The extremely limited availability of ciguatoxins has hampered their structural elucidation and delayed the preparation of anti-ciguatoxin antibodies for detecting these toxins. As one approach to addressing the longstanding problems of ciguatera seafood poisonings, we sought to explore a synthetic approach, in which collections of rationally designed model compounds of ciguatoxins were extensively utilized in place of scarce natural toxins. Custom-made methods for elucidating the C2 configuration and the absolute configuration of ciguatoxin using synthetic AB-ring fragments were developed, and the absolute configuration of ciguatoxin was unambiguously elucidated as **1**-(2*S*,5*R*) with extremely limited amounts of the natural toxin. Furthermore, we devised a reliable and specific protocol for detection of CTX3C at the ppb level using a direct sandwich immunoassay based on mAbs, which bind to the right and left ends of CTX3C. Rational design of synthetic haptens was critical in the production of antibodies that could bind to CTX3C itself with high affinity ($K_d \approx$ nM) and could be utilized in the direct sandwich immunoassay format. This approach should also be applicable to other ciguatoxin congeners[56] and related non-protein toxins. Thus, we have shown that the use of organic synthesis can significantly contribute to the structural elucidation of the scarce marine natural products and also to the development of reliable immunoassays for the detection of seafood toxins.

The research described in this account was performed by a remarkable group of co-workers, to whom I am indebted. First of all, I would like to express my sincere appreciation to Prof. Masahiro Hirama (Tohoku University) for his guidance, encouragement, and invaluable discussion throughout the study. I would like to acknowledge Prof. Tohru Oishi (Osaka University) and Prof. Masayuki Inoue (Tohoku University) for stimulating discussions and for their cooperation in the synthetic studies, to Emeritus Prof. Takashi Yasumoto (Tohoku University) and Prof. Masayuki Satake (the University of Tokyo) for insightful suggestions and their studies using natural ciguatoxins, to Prof. Ikuo Fujii and Prof. Takeshi Tsumuraya (Osaka Prefecture University) for valuable discussions and collaboration in the development of antibodies and immunoassays, to Prof. Kouhei Tsumoto (the University of Tokyo) and Prof. Yoshihisa Tomioka (Josai International University) for their helpful guidance in the experiments with the phage-displayed antibodies, and to Dr. Takeshi Sato (Cell Science & Technology Institute) for collaborating in the development

of sandwich immunoassay kits. I am also indebted many former graduate students particularly Shin-ichiro Tanaka, Yoko Nagumo, Hisatoshi Uehara, Mugumi Maruyama, Shin-ya Sasaki, and Yumi Shindo and the other members of Prof. Hirama's laboratory at Tohoku University. Their enthusiasm, creativity, and spirit have made it possible for us to undertake these exciting and rewarding synthetic challenges.

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Hiroki Oguri was born in 1970 in Tokyo, Japan. He recieved B. Science (1993) and Ph.D. (1998) degrees from Tohoku University under the supervision of Profs. Masahiro Hirama and Tohru Oishi, and was then appointed as an assistant professor in the research group of Prof. M. Hirama (1998–2003). In 2003–2004, he worked with Prof. Stuart L. Schreiber at the Department of Chemistry and Chemical Biology, Harvard University as a visiting scientist; then, in 2004, he joined the Division of Chemistry, Graduate School of Science, Hokkaido University as an associate professor in the research group of Prof. Hideaki Oikawa. Since October 2006, he has also been serving as an investigator at the Division of Innovative Research, Non-traditional Science, Creative Research Initiative "Sousei" (CRIS), Hokkaido University. He was honored with the Inoue Research Award for Young Scientists (2000), the Young Scientist's Research Award in Natural Product Chemistry (2001), and the Chemical Society of Japan Award for Young Chemists (2005). His current research interests include the development of a programmable synthetic process for generating a systematic collection of natural product-like molecules with rich skeletal and stereochemical diversity and the modulation of cellular functions using designed molecules.