Bioorganic & Medicinal Chemistry 18 (2010) 7607–7610

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Binding of diarrheic shellfish poisoning toxins to okadaic acid binding proteins purified from the sponge *Halichondria okadai*

Keiichi Konoki [a,*], Kaori Saito [a], Hiroki Matsuura [a], Naoyuki Sugiyama [b,†], Yuko Cho [a], Mari Yotsu-Yamashita [a], Kazuo Tachibana [b]

[a] Graduate School of Agricultural Science, Tohoku University, Tsutsumidori-Amamiyamachi 1-1, Aoba-ku, Sendai 981-8555, Japan
[b] Department of Chemistry, School of Science, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

ARTICLE INFO

Article history:
Received 22 June 2010
Revised 20 August 2010
Accepted 21 August 2010
Available online 27 August 2010

Keywords:
Okadaic acid
Okadaic acid binding protein
Binding
Cytotoxicity

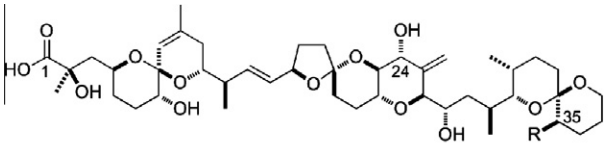
ABSTRACT

Okadaic acid (OA) and dinophysistoxin-1 (DTX1) cause diarrheic shellfish poisoning. This article examines the biochemical interactions of the two toxins with novel okadaic acid binding proteins (OABPs) 2.1 and 2.3, originally isolated from the marine sponge *Halichondria okadai*. First, recombinant OABPs 2.1 and 2.3 were expressed in *Escherichia coli* BL21 (DE3) cells. Binding assays using [24-^{3}H]OA and the recombinant OABP 2.1 or 2.3 demonstrated the dissociation constant K_d of 1.30 ± 0.56 nM and 1.54 ± 0.35 nM, respectively. Binding of [24-^{3}H]okadaic acid to recombinant OABP2.1 was almost equally replaced with OA and DTX1. OA-induced cytotoxicity in mouse leukemia P388 cells was inhibited in the presence of the recombinant OABPs 2.1 and 2.3 with an EC_{50} of 92 ± 8.4 nM and 87 ± 13 nM, respectively. These results suggest that the blockage of OA-induced cytotoxicity by OABPs 2.1 and 2.3 may be involved in regulating symbiotic relationships present in the sponge *H. okadai*.

1. Introduction

Okadaic acid (OA, Fig. 1) was originally isolated from the marine sponge *Halichondria okadai* in 1981.[1] Although the dinoflagellate *Prorocentrum lima* was subsequently discovered to produce OA,[2] evidence that *P. lima* is a symbiotic species for *H. okadai* has yet to be provided. OA is a well-known secondary metabolites due to its inhibitory activity towards serine/threonine protein phosphatases 1 (PP1) and 2A (PP2A). OA binds to PP1 and PP2A with a K_d of 150 nM and 30 pM, respectively,[3] and exhibits cancer-promoting activity.[4] As protein phosphatases are highly conserved enzymes,[5] indispensable for many physiologically important signal transduction processes,[6] questions have been raised about why *H. okadai* accumulates OA and whether protein phosphatases in *H. okadai* are targeted by OA.

Previously, we identified okadaic acid binding proteins from *H. okadai*.[7] A 37 kDa protein, OABP1, was partially cloned to share an 88% identity with rabbit PP2Aβ in the cloned region.[8] This protein showed protein phosphatase activity when examined with *p*-nitrophenyl phosphate (*p*NPP)[3] as a substrate. However, OABP2, which was initially thought to be a single protein, did not hydrolyze *p*NPP, although OA bound to OABP2 with a K_d of 0.97 nM.[8] LC/MS analysis revealed that OABP2 was composed of three similar-sized proteins: OABP2.1, OABP2.2, and OABP2.3. Molecular cloning together with peptide mapping by LC/MS/MS demonstrated that OABP2.1 was 96% homologous to OABP2.2 and 66% homologous to OABP2.3; neither of them showed similarity to protein phosphatases.



Okadaic Acid (OA): R=H
Dinophysistoxin 1 (DTX1): R=Me

Figure 1. Structures of OA and DTX1.

In order to evaluate the physiological roles of OA and OABP2, it is important to characterize OA binding in vitro to each component of OABP2. Therefore, we describe herein the preparation of recombinant OABPs 2.1 and 2.3 and demonstrate that OA-induced toxicity in mouse leukemia P388 cells is inhibited in a dose-dependent manner by both OABP2.1 and OABP2.3, due to the comparatively high affinity for binding that OA to OABPs 2.1 and 2.3 possess.

Abbreviations: DSP, diarrheic shellfish poisoning; DTX1, dinophysistoxin 1; OA, okadaic acid; OABP, okadaic acid binding proteins; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; IPTG, isopropyl β-D-1-thiogalactopyranoside.

* Corresponding author. Tel./fax: +81 22 717 8819.
E-mail address: konoki@biochem.tohoku.ac.jp (K. Konoki).
† Present address: Institute for Advanced Biosciences, Keio University, 403-1 Nipponkoku, Daihoji, Tsuruoka, Yamagata 997-0017, Japan.

0968-0896/$ - see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2010.08.043

2. Results

2.1. Cloning and expression of OABPs 2.1 and 2.3

The amino acid sequence of OABP2.1, which was deduced from the cDNA sequence cloned in this study, was identical to that which was previously cloned;[8] however, the amino acid sequence of OABP2.3 contained the substitutions of Leu and Ile at the positions of 28 and 133 with Ser and Met, respectively. The effect of this was marginal, as acidic or basic residues were not involved in any of these mutations. Cloning of OABP2.2 was not achieved. Presumably, cDNA for OABP2.2 failed to amplify due to the homology of OABP2.2 mRNA with OABP2.1 mRNA, and the predominance of mRNA for OABP2.1 over that of OABP2.2, as well.

The recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) cells after induction for 5 h with IPTG at either 30 °C or 37 °C. After purification with nickel-nitrilotriacetic acid resin, 4.2 mg of recombinant OABP2.1 (recOABP2.1) and 6.0 mg of recombinant OABP2.3 (recOABP2.3) were obtained from 250 mL of bacterial culture. SDS–PAGE analysis revealed the presence of a single band for each recombinant protein (Fig. 2).

2.2. Binding assays and cytotoxicity assays

Binding assays using [24-^{3}H]OA were carried out to compare the binding affinity of OA to the recombinant OABPs 2.1 and 2.3. As shown in Figure 3A, [24-^{3}H]OA bound to both recOABPs 2.1 and 2.3 in a dose-dependent manner, where the amount of nonspecific binding measured in the presence of excess OA was negligible compared with that of the total binding (data not shown). The recombinant proteins were kept stable for 2 weeks at 4 °C and were able to provide reproducible results in terms of binding to [24-^{3}H]OA. In addition, their binding capacity was not diminished after storage at −25 °C for 6 months. Saturation binding curves were subjected to nonlinear fitting to Hill's equation (Fig. 3A) and the dissociation constants for OABPs 2.1 and 2.3 were calculated to be 1.30 ± 0.56 nM (N = 6) and 1.54 ± 0.35 nM (N = 4), respectively. OA and DTX1 were then tested to find out whether the small difference in their structures would affect binding affinity to OABP2.1. As shown in Figure 3B, [24-^{3}H]OA binding was blocked in a dose-dependent manner almost equally by OA and DTX1.

We next examined OA cytotoxicity in mouse leukemia P388 cells in the presence of OABPs 2.1 and 2.3. OA at 100 nM proved fatal to the cultured cells, despite OA-induced cytotoxicity being inhibited in a dose-dependent manner by recOABP2.1 (Fig. 4). The EC_{50} values for recOABP2.1 and recOABP2.3 were calculated to be 92 ± 8.4 nM and 87 ± 13 nM, respectively. In the absence of OA, neither recOABP2.1 nor recOABP2.3 appeared to alter the cell viability. As mitomycin is one of the well-known anti-tumor compounds, showing its activity though various signal transduction processes, we examined mitomycin whether its toxicity was blocked by recOABPs 2.1 and 2.3. However, mitomycin-induced cytotoxicity in mouse leukemia P388 cells was not affected by the presence of recOABP2.1.

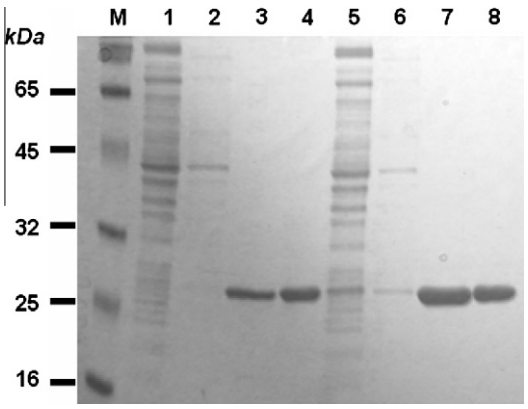


Figure 2. SDS–PAGE analysis of purified recOABP2.1 and recOABP2.3. M represents a lane for the protein size marker. HisLink™ Protein Purification Resin was used for the purification of recOABP2.1 (lanes 1–4) and recOABP2.3 (lanes 5–8). The flow-through fractions are shown in lanes 1 and 5, wash fractions are in lanes 2 and 6, elution fractions are in lanes 3 and 7, and dialyzed fractions are in lanes 4 and 8.

3. Discussion

Marine sponges have been identified as sources for highly potent secondary metabolites, which are generally thought to be produced by symbiotic species.[9–11] Whereas numerous studies have focused on the isolation, structure determination, total synthesis, and/or pharmacology of these secondary metabolites, environmental roles have not been elucidated for most of them. Toxicity of the secondary metabolites could be fatal to the toxin-accumulating species. Self-defense systems that suppress toxicity of the accumulated secondary metabolites seem to be present in some species, such as the pufferfish *Fugu pardalis*[12] and the bivalve *Mya arenaria*,[13] which accumulate tetrodotoxin and saxitoxin, respectively. Due to a single amino acid mutation in the toxin binding site on voltage-sensitive sodium channels[14] expressed in each species, binding affinity to the receptor decreases about a 1000-fold.[15,16] Furthermore, puffer fish saxitoxin tetrodotoxin binding proteins (PSTBP)[17] that are not homologous to the voltage-sensitive sodium channels have been identified in puffer fish. Similar to PSTBP, OABP2 has low homology with serine/threonine protein phosphatases PP1 and PP2A. Therefore, it was initially thought to participate in detoxification by competing for OA molecule binding with protein phosphatases.

OABP2 was revealed to be a mixture of OABPs 2.1, 2.2, and 2.3.[8] Among them, OABP2.1 and OABP2.3 were successfully expressed in *E. coli* in the present study. OA exhibited a K_d of 1.30 ± 0.56 nM and 1.54 ± 0.35 nM for recOABP2.1 and recOABP2.3, respectively. OABP2.1 and OABP2.3 proved 66% identical in amino acid sequence; we expect that conserved residues are important for binding of OA to recOABPs 2.1 and 2.3. The highly specific binding was also supported by the fact that mitomycin-induced cytotoxicity in mouse leukemia P388 cells was not blocked by recOABPs 2.1 and 2.3. However, cytotoxicity exhibited by OA in mouse leukemia P388 cells was inhibited by OABPs 2.1 and 2.3 at relatively higher concentrations (Fig. 4). Although OABPs 2.1 and 2.3 did not appear to show cytotoxicity, the binding site of OA could be hindered by nonspecific binding to the cell surface.

DTX1[18] and OA[19] are the primary toxins causative for diarrheic shellfish poisoning (DSP); they are accumulated in bivalves when toxic algal blooms occur. DTX1, produced by the benthic dinoflagellate *Dinophysis* sp.,[20] is 35*S*-methyl okadaic acid (Fig. 1). In fact, recognition of these two DSP toxins by OABP2.1 could not be distinguished, as they have similar K_d values (Fig. 3B). Acute toxicity tests in mice have been periodically performed by public health authorities to prevent outbreaks of food poisoning. However, in the future, alternative approaches that do not harm animals are preferred. State-of-the-art technologies have already been developed for this purpose, but their availability is limited, as they require HPLC/MS instrumentation[21,22] or a monoclonal antibody to OA.[23] The recombinant OABP2 can be easily prepared in large amounts and stored at −25 °C for over 6 months without losing the OA's binding affinity. This property of OABP2 is suitable for developing an alternative method to quantify OA.

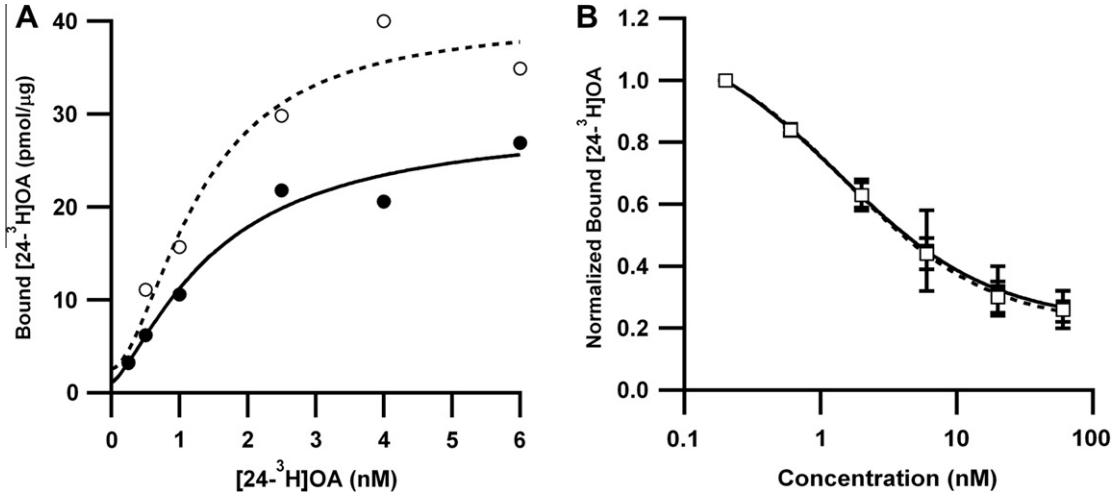


Figure 3. Binding of [24-^{3}H]OA to recOABPs 2.1 and 2.3. (A) Dose-dependency of [24-^{3}H]OA binding to recOABP2.1 (closed circles with straight line) or recOABP2.3 (open circles with dotted line). (B) Replacement of [24-^{3}H]OA binding to recOABP2.1 with various concentrations of OA (closed squares with straight line) or DTX1 (open squares with dotted line).

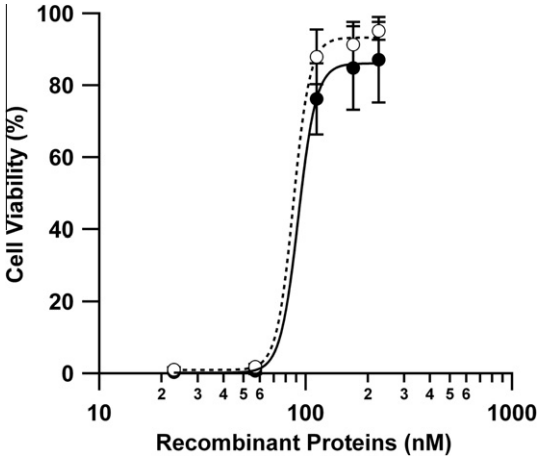


Figure 4. Blockade of OA-induced cytotoxicity by recOABPs 2.1 and 2.3. Mouse leukemia P388 cells at a concentration of 5.0×10^4 cells/mL were incubated with 100 nM OA for 2 days in the presence of various concentrations of recOABP2.1 (closed circles with straight line) and recOABP2.3 (open circles with dotted line). Cell viability at indicated concentration of the recombinant protein was then measured using WST-8.

4. Conclusion

Sponges are filter-feeders that take in various planktons and bacteria, giving rise to the accumulation of secondary metabolites, such as OA. The physiological roles of OA and the presence of OA-binding proteins in *Lubomirskia baicalensis* and *Suberites domuncula* were discussed previously in literature.[24,25] However, our study is the first time that the binding thermodynamics between the target molecules and OA has been evaluated. Upon in vitro observation of the binding and cytotoxicity exhibited by OA, we clearly demonstrated that the high affinity binding of OA to OABPs 2.1 and 2.3 leads to the inhibition of OA-derived cytotoxicity. Thus, we speculate that OABP2 may play a role in regulating the cytotoxicity of OA, so as to maintain the symbiotic relationship present in the sponge *H. okadai*.

5. Experimental

5.1. Materials

H. okadai was collected in Kanagawa Prefecture, Japan, on March 27, 2008. [24-^{3}H]OA was kindly provided by Dr. Takeshi Yasumoto. Mouse leukemia P388 cells were given by Professor Michio Murata, Osaka University. Isogen and Isogen LS were purchased from Nippon Gene (Tokyo, Japan). ReverTraAce and restriction enzymes were purchased from Toyobo (Osaka, Japan). Phu™ DNA Polymerase was purchased from New England Biolabs, Inc. (Ipswich, MA). PfuUltra® II Fusion HS DNA Polymerase was purchased from Stratagene (La Jolla, CA). *p*ET21a plasmid vector and BugBuster Protein Extraction Reagent were purchased from Novagen, Inc. (Madison, WI). PureYield Plasmid Miniprep System and HisLink™ Protein Purification Resin were purchased from Promega (Madison, WI). BioDesign Dialysis Tubing MWCO 8000 was purchased from Biodesign, Inc. (Carmel, NY). Nick™ Column was purchased from GE Healthcare (Waukesha, WI). WST-8 was purchased from Dojin (Kumamoto, Japan). Other chemicals were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). Gene-specific primers were synthesized at Operon Biotechnologies (Tokyo, Japan).

5.2. Subcloning of OABP2.1 and OABP2.3 in *p*ET21a

The previous methods were modified as follows: total RNA was extracted from 100 mg of freshly frozen *H. okadai* with Isogen (Nippon Gene, Tokyo, Japan) and was successively extracted with Isogen LS (Nippon Gene, Tokyo, Japan). The total RNA was reverse-transcribed to cDNA with ReverTraAce (Toyobo, Osaka, Japan). The cDNAs for OABPs 2.1 and 2.3 were obtained by PCR with PfuUltra® II Fusion HS DNA Polymerase (Stratagene, La Jolla, CA) or Phu™ DNA Polymerase (New England Biolabs, Inc., Ipswich, MA). The forward primers designed for OABP2.1 and OABP2.3 were ATTTTgggATCCgCTAATTTAAaggAgCCATCA and gAAgTgggATCCgCAgTATCAggAgAACCCTCg, respectively, and the reverse primers for OABPs 2.1 and 2.3 were TTgAATCTCgAgATCCACAAgTgCTCCCCTCAC and TAATTTCTCgAgATCCACCAgTggTCCTCTTAT, respectively. The PCR reaction condition was 2 min at 94 °C, 30 cycles of 10 s at 98 °C, 30 s at 55 °C and 1 min at 68 °C and held at 4 °C. The PCR fragment and *p*ET21a vector (Novagen, Inc., Madison, WI) were digested with BamHI and XhoI overnight at 37 °C and were purified after running agarose gel electrophoresis. A ligation reaction was performed at 16 °C overnight and transformed directly into chemically competent DH5α cells; then the cells are cultured overnight on LB agar containing ampicillin. Resultant colonies were inoculated in 3 mL of LB media containing ampicillin. The plasmid was extracted with PureYield Plasmid Miniprep System (Promega,

Madison, WI). The universal T7 promoter and T7 terminator primers were used for sequencing.

5.3. Expression of recombinant OABPs 2.1 and 2.3

The *p*ET21a/OABP2.1 and *p*ET21a/OABP2.3 plasmids were transformed in chemically competent BL21 (DE3) cells. The transformed bacterial colony was inoculated in 3.0 mL of LB media containing ampicillin overnight then the suspension was transferred to 250 mL of ampicillin-free LB media. The mixture was incubated until OD_{600} reached 0.5–1.0. After the mixture was cooled on ice for 30 min, 250 μM of IPTG were added. Incubation was carried out at 30 °C for another 5 h. The bacterial cells were harvested by centrifugation at 2000*g* for 15 min and were either sonicated on ice three times for 1 min in Binding Buffer containing 100 mM Hepes (pH 7.4), 150 mM NaCl, and 10 mM imidazole, or were subjected to treatment with BugBuster Protein Extraction Reagent (Novagen, Inc., Madison, WI) for 15 min at room temperature. The mixture was centrifuged at 10,000*g* for 20 min and loaded onto 1.0 mL of HisLink™ Protein Purification Resin (Promega, Madison, WI) manually packed in a Poly-Prep Chromatography Column (Bio-Rad Laboratories, Hercules, CA). The column was washed with Wash Buffer containing 100 mM Hepes (pH 7.4), 500 mM NaCl, and 20 mM imidazole, and the recombinant protein was eluted with Elution Buffer containing 100 mM Hepes (pH 8.2), 300 mM NaCl, and 300 mM imidazole. The eluate was placed into BioDesign Dialysis Tubing MWCO 8000 (BioDesign Inc., Carmel, NY) and dialyzed against a buffer containing 20 mM Tris-HCl (pH 7.4) and 150 mM NaCl. The purification profile for flow-through fractions, wash fractions, elution fractions, and dialyzed fractions were monitored with 15% SDS–PAGE electrophoresis.

5.4. Binding assays

The recombinant protein was dissolved in 100 μL of 20 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, 1 mM EDTA, 0.1 mg/mL BSA, and 0.01% Tween 20 and incubated with 0.5–5 nM [24-^{3}H]OA for 3 h.[26] The mixture was loaded onto the NICK™ column (GE Healthcare, Waukesha, WI)[17] and eluted with 800 μL of 20 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, 1.0 mM EDTA, and 0.01% Tween 20. The recovered sample was mixed with 8.0 mL of scintillation cocktail, and its radioactivity was quantified with a liquid scintillation counter. Nonspecific binding was assessed in the presence of 100-fold excess of unlabeled OA.

5.5. Cytotoxicity assays

Mouse leukemia P388 cells were grown for 2 days in RPMI 1640 medium containing fetal bovine serum, penicillin and streptomycin. Aliquots of 100 μL of the cells at 5.0×10^4 cells/mL were mixed with 100 nM OA and various concentrations of recOABP2.1 or recOABP2.3. After 2 days, 5 μL of WST-8 (Dojin, Kumamoto, Japan) was added to the mixture for 4 h and the relative cell viability was measured by absorbance at 450 nm using Microplate Reader (Bio-Rad Laboratories, Hercules, CA).

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research (C) (No. 21603003) from MEXT, Japan as well as by The Naito Science and Engineering Foundation and the Novartis Foundation. The authors thank Dr. Seketsu Fukuzawa of The University of Tokyo for many helpful discussions.

References and notes

1. Tachibana, K.; Scheuer, P. J.; Tsukitani, Y.; Kikuchi, H.; Vanengen, D.; Clardy, J.; Gopichand, Y.; Schmitz, F. J. *J. Am. Chem. Soc.* **1981**, *103*, 2469.
2. Murakami, Y.; Oshima, Y.; Yasumoto, T. *Bull. Jpn. Soc. Sci. Fish.* **1982**, *48*, 69.
3. Takai, A.; Mieskes, G. *Biochem. J.* **1991**, *275*, 233.
4. Fujiki, H.; Suganuma, M. *Adv. Cancer Res.* **1993**, *61*, 143.
5. Cohen, P. T. W.; Brewis, N. D.; Hughes, V.; Mann, D. J. *FEBS Lett.* **1990**, *268*, 355.
6. Cohen, P. T. W. *J. Cell Sci.* **2002**, *115*, 241.
7. Konoki, K.; Sugiyama, N.; Murata, M.; Tachibana, K.; Hatanaka, Y. *Tetrahedron* **2000**, *56*, 9003.
8. Sugiyama, N.; Konoki, K.; Tachibana, K. *Biochemistry* **2007**, *46*, 11410.
9. Lee, Y. K.; Lee, J. H.; Lee, H. K. *J. Microbiol.* **2001**, *39*, 254.
10. Piel, J. *Nat. Prod. Rep.* **2004**, *21*, 519.
11. Taylor, M. W.; Radax, R.; Steger, D.; Wagner, M. *Microbiol. Mol. Biol. Rev.* **2007**, *71*, 295.
12. Yotsu-Yamashita, M.; Nishimoto, K.; Nitanai, Y.; Isemura, M.; Sugimoto, A.; Yasumoto, T. *Biochem. Biophys. Res. Commun.* **2000**, *267*, 403.
13. Bricelj, V. M.; Connell, L.; Konoki, K.; MacQuarrie, S. P.; Scheuer, T.; Catterall, W. A.; Trainer, V. L. *Nature* **2005**, *434*, 763.
14. Catterall, W. A.; Goldin, A. L.; Waxman, S. G. *Pharmacol. Rev.* **2005**, *57*, 397.
15. Venkatesh, B.; Lu, S. Q.; Dandona, N.; See, S. L.; Brenner, S.; Soong, T. W. *Curr. Biol.* **2005**, *15*, 2069.
16. Maruta, S.; Yamaoka, K.; Yotsu-Yamashita, M. *Toxicon* **2008**, *51*, 381.
17. Yotsu-Yamashita, M.; Sugimoto, A.; Terakawa, T.; Shoji, Y.; Miyazawa, T.; Yasumoto, T. *Eur. J. Biochem.* **2001**, *268*, 5937.
18. Murata, M.; Shimatani, M.; Sugitani, H.; Oshima, Y.; Yasumoto, T. *Bull. Jpn. Soc. Sci. Fish.* **1982**, *48*, 549.
19. Kumagai, M.; Yanagi, T.; Murata, M.; Yasumoto, T.; Kat, M.; Lassus, P.; Rodriguezvazquez, J. A. *Agric. Biol. Chem.* **1986**, *50*, 2853.
20. Yasumoto, T.; Oshima, Y.; Sugawara, W.; Fukuyo, Y.; Oguri, H.; Igarashi, T.; Fujita, N. *Bull. Jpn. Soc. Sci. Fish.* **1980**, *46*, 1405.
21. Suzuki, T.; Yasumoto, T. *J. Chromatogr. A* **2000**, *874*, 199.
22. Suzuki, T.; Kamiyama, T.; Okumura, Y.; Ishihara, K.; Matsushima, R.; Kaneniwa, M. *Fish. Sci.* **2009**, *75*, 1039.
23. Matsuura, S.; Hamano, Y.; Kita, H.; Takagaki, Y. *Jpn. J. Toxicol. Environ. Health* **1994**, *40*, 365.
24. Muller, W. E. G.; Belikov, S. I.; Kaluzhnaya, O. V.; Perovic-Ottstadt, S.; Fattorusso, E.; Ushijima, H.; Krasko, A.; Schroder, H. C. *FEBS J.* **2007**, *274*, 23.
25. Schroder, H. C.; Breter, H. J.; Fattorusso, E.; Ushijima, H.; Wiens, M.; Steffen, R.; Batel, R.; Muller, W. E. G. *Appl. Environ. Microbiol.* **2006**, *72*, 4907.
26. Suganuma, M.; Suttajit, M.; Suguri, H.; Ojika, M.; Yamada, K.; Fujiki, H. *FEBS Lett.* **1989**, *250*, 615.