

Award Accounts

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Structural Features of Dinoflagellate Toxins Underlying Biological Activity as Viewed by NMR

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Marine dinoflagellates are a rich source of structurally and biologically intriguing secondary metabolites. Among those, maitotoxin may be one of the best known examples, which is the largest natural product known to date and possesses the most potent toxicity known amongst non-proteinaceous compounds. Structural studies of maitotoxin are reviewed with a particular focus on the configuration and mode of action of this unique toxin. In addition, there are many marine natural products which bind to biomembranes to exert their biological activities. To gain a better understanding of their mode of action, conformation, and bimolecular interaction occurring in biomembranes are particularly important. Recent results from NMR studies of membrane systems in the authors' group are reviewed briefly.

1. Introduction

Bioactive marine natural products, particularly polyether compounds such as okadaic acid (**1**), brevetoxins (BTXB, **2**), ciguatoxins (CTX1B, **3**), (Chart 1) and maitotoxin (**4**) (Chart 2), attract considerable attention due to their complicated structures and powerful biological activities. These compounds are all polyketide metabolites, which are characterized by a long carbon skeleton encompassing polycyclic ethers, hydroxy groups, and olefins. Their names with "toxin" imply that they were found in association with food poisonings or massive fish fatality; e.g., ciguatoxin (**3**) and maitotoxin (**4**), for ciguatera,[1,2] okadaic acid (**1**) for shellfish poisoning,[3,4] and brevetoxins (**2**) for the Florida coastal fish kill.[5] Progenitors of these toxins are marine unicellular eukaryotic algae, chiefly dinoflagellates. From these organisms, toxic metabolites with high molecular weight have been isolated and characterized in the last decade, during which rapid progress in NMR hardware and pulse technology has greatly accelerated the structure determination of complicated natural products. Without these, the structural determination of compounds with molecular weight over 1000 Da, such as those described in this review, would have never been accomplished. Besides considerable progress in NMR and related technologies, innovation in mass spectrometry has also brought breakthroughs in this field. Particularly, innovation of ionization methods such as MALDI (Matrix-Assisted Laser Desorption Ionization) and ESI (ElectroSpray Ionization) has greatly facilitated mass measurements of natural products over 2000 Da. The technological progress has made it possible to determine the active conformation of natural products and drugs, which may lead to a deeper understanding of their mechanism of actions and the designing of better drugs.

In this review, the authors describe NMR-based methods and strategies adopted for structure elucidation of bioactive compounds from marine microalgae, particularly focusing on their conformation and interaction, possibly relevant to biological activities. The results described here are largely the outcome of collaborations where the authors' group sometimes participated and sometimes not. It would also be intriguing and informative to see how the structures of these complicated compounds were determined since new methods were devised to solve problems inherent to larger molecules in the course of the structural studies. The authors hope that the review may also be helpful for the configuration/conformation analysis of natural products with lower molecular weight.

2. Structure and Mode of Action of Maitotoxin

2.1 Structure of Maitotoxin. MTX attracts considerable attention for various reasons. Firstly, MTX has extremely strong biological activities. Its toxicity against mice by intraperitoneal injection is $50\,\mathrm{ng\,kg^{-1}}$, which is the most potent of all the known non-proteinaceous toxins.[6] Secondly, the toxin shows the diverse pharmacological actions as described later, most of which are ascribable to stimulation of calcium influx into cells. Thirdly, its chemical structure belongs to ladder-shaped polyethers (LSPs), which are characteristic metabolites of dinoflagellates. Finally, the toxin possesses a molecular weight of 3422 Da ($C_{164}H_{256}O_{68}S_2Na_2$, as the disodium salt), and is the largest non-biopolymer natural product reported so far. The structure of MTX reported in 1993[7] is composed of 32 ether rings, 28 hydroxy groups, and 2 sulfate esters, all of which reside on a long C_{142} chain. Uneven distribution of polar functionalities in half of the molecule gives it amphiphilic nature (Chart 2).

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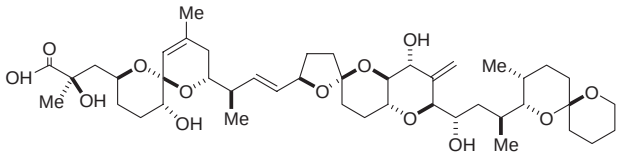
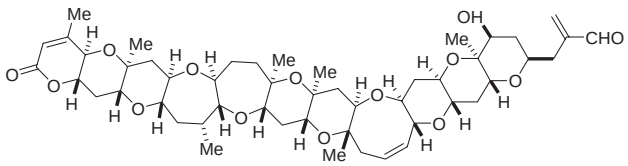
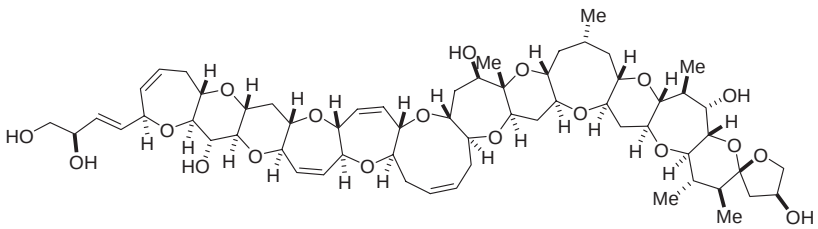


Okadaic acid **1**

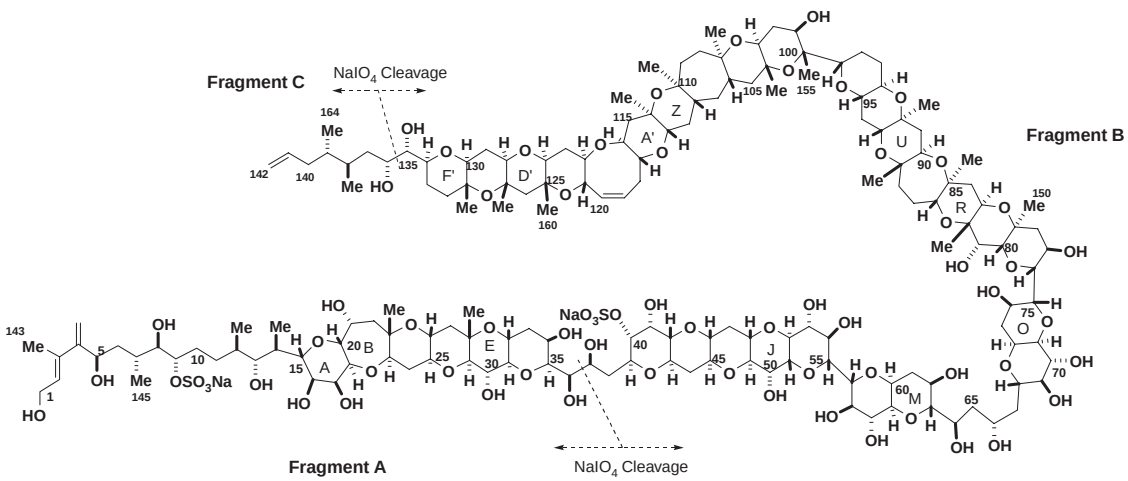


Brevetoxin B (BTXB) **2**



Ciguatoxin (CTX1B) **3**

Chart 1.



Maitotoxin (MTX) **4**

Chart 2.

For the structure elucidation of MTX, most of the 2D NMR measurements feasible were recorded, which resulted in structural assignments of some partial structures.[8] However, overlap and poor resolution of ^{1}H NMR signals (Fig. 1) hampered the elucidation of the ^{1}H–^{1}H connectivity, particularly for the middle portion of the molecule. In order to reduce the molecule size, periodate degradation was carried out to cleave six C–C bonds at vicinal-diol sites, among which cleavages at C36/C37 and C135/136 split the molecule into three pieces, fragment A, fragment B, and fragment C (Chart 2).[6–8] The structure of fragment-A was readily elucidated on the basis of conventional 2D NMR methods.[8] The most difficult problem laid in fragment B, which prompted us to adopt a CID MS/MS (Collision-Induced Dissociation Tandem Mass Spectrometry) method. Polycyclic ethers are known to undergo a characteristic fragmentation due to the double C–O bond cleavages at particular positions,[9] hence resulting in unambiguous confirmation of the structure.[6]

The 2D NOESY spectra for the most congested area could not be assigned due to heavily overlapping signals. Thus, 3D NMR (^{13}C × ^{1}H × ^{1}H) measurements of MTX were attempted to obtain better separation of ^{1}H NMR signals. To prepare a ^{13}C-enriched sample, the dinoflagellate *Gambierdiscus toxicus* was grown in media containing 0.01% $Na_2{}^{13}CO_3$. Using ^{13}C-enriched MTX (9 mg, 4% ^{13}C), 3D NOESY–HMQC[10] was measured at 600 MHz (JEOL A600 spectrometer) to fur-

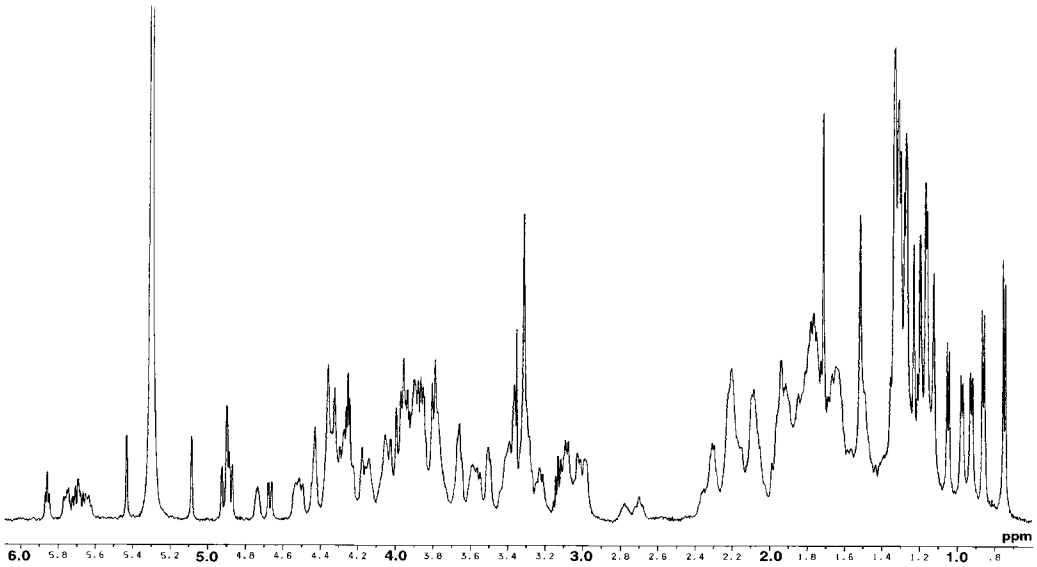


Fig. 1. ^{1}H NMR of maitotoxin (600 MHz).

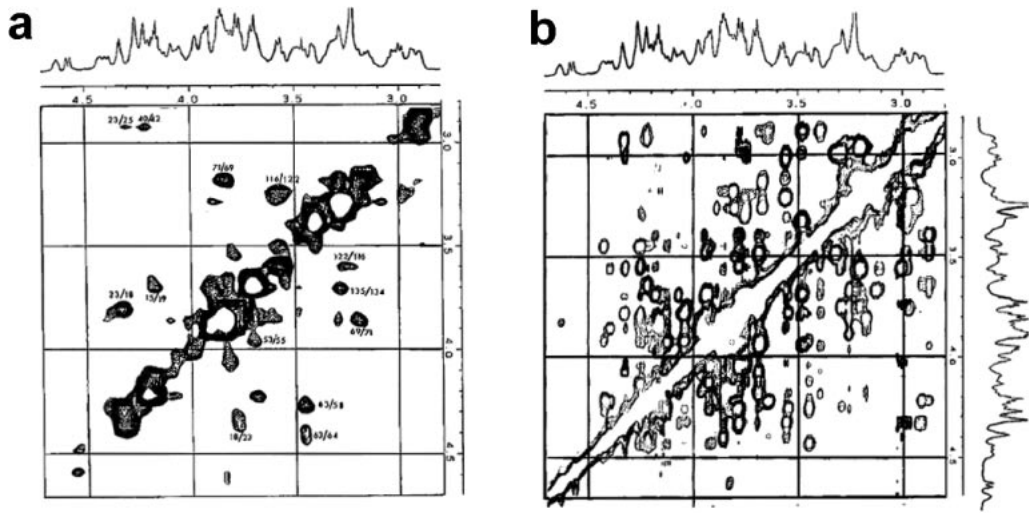


Fig. 2. One of the ^{1}H–^{1}H planes (**a**) in 3D NOESY–HMQC spectrum of MTX at δ_C 77.5 in comparison with conventional 2D NOESY (**b**).

nish a 3D spectrum (F1 128, F2 128, and F3 512). The NOESY plane in Fig. 2 from 128 two-dimensional sheets in the 3D spectrum gave rise to fewer cross peaks with no overlap while a conventional 2D NOESY spectrum for the same area showed more than 500 cross peaks. The relative configuration of the polycyclic ethereal portions was largely determined by these NOE data, which resulted in a proposed partial stereostructure of MTX.[6] Configuration of acyclic moieties such as C1–C14, C36–C38, C64–C67, and C135–C142 could not be elucidated by NMR data alone. Plausible diastereomers of acyclic moieties deduced from *J*-based configuration analysis (see Section **3** for details) and NOE data were synthesized and compared with natural MTX in their ^{13}C and ^{1}H NMR chemical shifts; e.g., two diastereomers for the stereochemistry of the C1–C14 chain were considered equally plausible, judging from this *J*-based method. Thus, both of them were synthesized, and comparing each one with MTX in ^{13}C chemical shifts led to the unambiguous assignments of stereochemistry for C5, C7, C8, C9, C12, C13, and C14.[11,12] The configuration of the other acyclic parts, including the absolute stereochemistry of the whole molecule, was determined in a similar manner independently by Kishi's[13–15] and Sasaki's[11,16–18] groups.

2.2 Mode of Action of Maitotoxin. On the basis of the stereostructure of maitotoxin (**4**) constructed by NOEs and spin coupling constants (see Section **3.2**), one possible conformation was deduced as depicted in Fig. 3, where the hydrophobic polyether chain is long enough to span a lipid bilayer membrane and the hydrophilic portion shows more flexible orientation.[19] This conformation, particularly the membrane-integral polyether chain, may account for its extremely potent toxicity as described later (Section **4.1**).

The pharmacology of MTX has been providing intriguing research targets. In addition to its high mammalian toxicity, the toxin possesses very potent cytotoxicity and pharmacological activities. Since Takahashi et al. first reported that MTX stimulated calcium influx into cells,[20] a great number of phar-

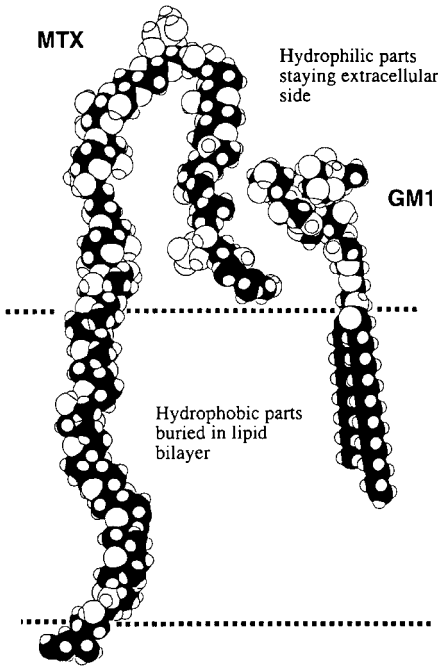


Fig. 3. One possible 3D structure of maitotoxin deduced from NMR data.[19]

macological and biochemical studies have been reported using MTX as a calcium channel agonist.[21] To date, more than 130 papers have been published about biological actions of MTX. According to a review by Gusovsky and Daly,[21] 26 cell lines and 25 tissue preparations have been examined for MTX actions. MTX elicits Ca^{2+} influx in virtually all of the cells and tissues,[21] synaptosomes,[22] and even erythrocyte ghosts,[19,23] the last of which are a vacant vesicle made up by the cell membrane. The toxin, however, induces no Ca^{2+} entry into artificial phospholipid vesicles,[24] which indicates the presence of target molecule(s) for MTX on the erythrocyte surface other than usual membrane lipids. The elevation of intracellular calcium concentrations induced by MTX leads to secondary events such as phosphoinositide breakdown,[25] arachidonic acid release,[26] muscle contraction,[27] secretion of dopamine,[28] norepinephrine,[29] and insulin,[30] and acrosome reaction in sperm.[31] MTX-induced Ca^{2+} influx usually occurs concomitantly with depolarization of the membrane potential.[32] In some excitable cells, MTX-induced Ca^{2+} current at concentrations below 1 nM has been markedly inhibited by organic and inorganic blockers against voltage-sensitive Ca^{2+} channels (VSCC).[33] However, in other cells, the response of MTX is not effectively blocked by VSCC antagonists, but is reduced by other agents, such as a nonselective cation channel blocker, SK&F 96365.[34] Unlike VSCCs, channels activated by MTX are reportedly rather non-selective, and pass various cations such as Rb^{+}, Na^{+}, Mn^{2+}, Ba^{2+}, and Sr^{2+}.[35,36] These findings suggest that the VSCCs are not the primary target of MTX but are activated by depolarization caused by ion influx through a primary target.

Electrophysiological studies based on the current–voltage relationship have revealed that channels activated by MTX are voltage-independent, which is almost in direct proportion with reversal potential of nearly zero.[37] It is important to address that MTX-induced membrane depolarization was mostly dependent on extracellular calcium ion. In contrast, Nishio et al. revealed that the primary action of MTX is caused by Na^{+} influx in cells,[35] which suggests that calcium is not essential for ion entry across biomembranes by MTX, whereas being necessary to elicit physiological changes in cells. Recently, it has been reported that large current is induced after initial sodium and/or calcium uptake through nonselective cation channels, and fluorescent markers such as ethidium (MW 314 Da), YO-PRO-1 (MW 375 Da), and POP-3 (MW 715 Da) permeate through the pores.[37–39] The latter process has been referred to formation of cytolytic/oncotic pores (COP). Consequently, lactate dehydrogenase (MW 140 kDa) was released from the cell, causing necrotic cell death. However, the mechanism linking the initial activation of nonselective cation channels and the following appearance of large pores has not been elucidated.

The complete chemical structure of MTX (Fig. 3)[19] implies somewhat similarity between MTX and membrane associated components such as glycolipids. The hydrophobic polyether portion from rings R through F′ in MTX penetrates into the plasma membrane as suggested for brevetoxin B (**2**),[40,41] whereas the polyhydroxy groups residing on rings A through Q along with two anionic sulfate esters stay on the extracellular side. The entire polarity distribution infers that MTX can build molecular assemblage to form a pore-like structure across biological membranes. The steep increases in calcium influx with respect to the concentrations of MTX imply that four or more molecules form a self-assemblage. However, unlike polyene antibiotics such as amphotericin B, a certain membrane component other than lipids or sterols is thought to be necessary for MTX activity.[19,42]

Despite the more than two decades that have passed since MTX was isolated from the dinoflagellate *G. toxicus*, the target molecule for MTX has not been identified. Due to the amphiphilic nature of maitotoxin, nonspecific binding to membrane proteins is extremely high. Binding studies to characterize the receptor(s) using tritiated MTX have not been successful. Small molecules that specifically inhibit MTX-induced calcium influx have been extensively sought from natural and unnatural sources. Interestingly, brevetoxin B (**2**) and synthetic molecules mimicking the hydrophobic and hydrophilic part of MTX, respectively, were found to inhibit MTX-induced Ca^{2+} influx in rat glioma C6 cells,[23] providing a working hypothesis that MTX exhibits multi-point recognition of the receptor protein.[19] Efficient inhibitors with a smaller molecular size will be necessary to identify target molecule(s) of this unprecedented toxin.

3. Configuration Analysis of Acyclic Structures

3.1 *J*-Based Configuration Analysis. Natural products with complicated molecular architectures have been isolated and their structures have been elucidated mainly on the basis of NMR spectrometry in the last two decades. Among those, the configurations of acyclic or macrocyclic structural moieties have been determined chiefly by X-ray crystallography and/or laborious synthetic studies. Otherwise, assignments of configuration of acyclic structures are usually difficult even with current NMR-based methodologies; with modern NOE-based

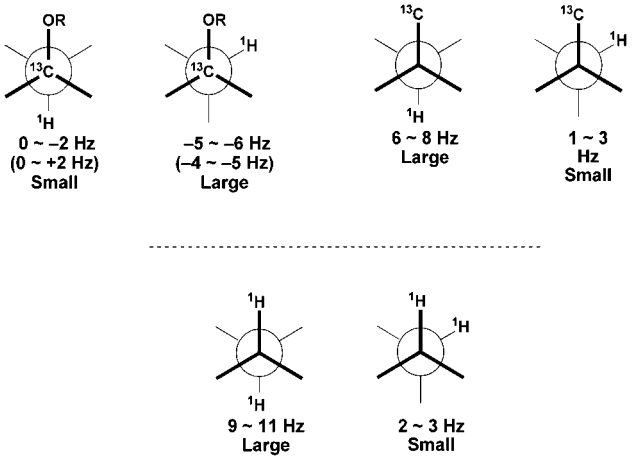


Fig. 4. Carbon–proton spin–spin coupling constants for anti and gauche rotamers.

techniques,[42–45] it is still very difficult to unambiguously assign the stereochemistry of highly flexible carbon chains because of the presence of multiple conformers, where minor populations sometimes give rise to disproportionately large NOEs. The spin-coupling constant is often utilized for conformational analysis of biomolecules since it is observed as a weighted average of those from each conformer. For configuration assignments, interproton J values ($^{3}J_{H,H}$) alone are inadequate because two H/H-gauche rotamers cannot be distinguished. Additional carbon–proton coupling $^{2,3}J_{C,H}$ greatly expands the utility of the coupling constants. Due to progress in two-dimensional NMR techniques,[46] $^{2,3}J_{C,H}$ values in complicated natural products have become measurable. These conditions have led to the invention of a new method called "J-based configuration analysis" for assigning the relative configuration of acyclic structures in natural products.[12,47,48] Vicinal carbon–proton spin-coupling constants ($^{3}J_{C,H}$) show Karplus-type dihedral dependence[49–51] (Fig. 4); applications of $^{2,3}J_{C,H}$ to conformational analyses and/or stereospecific assignments of methylene protons have been reported for proteins,[52–54] carbohydrates,[55,56] and polynucleotides.[57,58] In addition, geminal carbon–proton coupling constants ($^{2}J_{C,H}$) also provide stereochemical information;[59] when an oxygen atom on a carbon atom is gauche to its geminal proton, $^{2}J_{C,H}$ becomes large (in minus sign), and when it is anti, the value becomes small (Fig. 4). Our first assumption was that, in the systems in Fig. 5, conformations can be represented by three staggered rotamers. This seems to be quite reasonable for acyclic carbon chains with hydroxy and methyl substitutions, which, unlike bulky substituents, usually cause no significant deviations from the anti or gauche orientation[6,60–62] $^{3}J_{H,H}$ can be precisely determined (0.1–0.2 Hz) by using decoupling difference spectra or E. COSY-type experiments.[63] Among 1,2-disubstituted ethylene moieties, four conformers, **A-1**, **A-2**, **B-1**, and **B-2**, can be identified using $^{3}J_{H,H}$, $^{2}J_{C,H}$, and $^{3}J_{C,H}$, while the two rotamers **A-3** and **B-3** with an H/H-anti orientation cannot be distinguished (Fig. 5). For these anti conformers, NOE-type experiments may be a practical way to assign their configuration. To determine the relative configuration of two methine carbons separated by a methylene group, the pair of diastereotopic methylene protons must be assigned stereospecifically. The

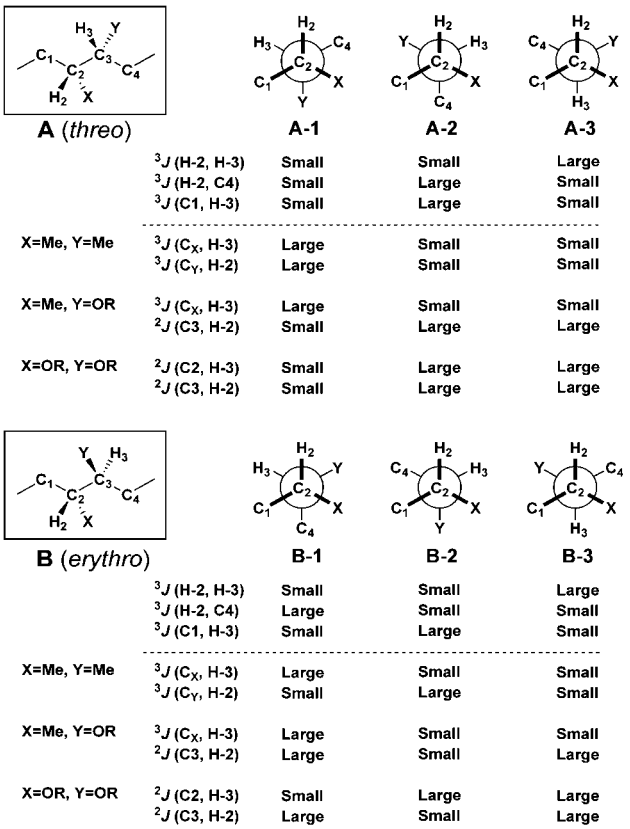


Fig. 5. Rotamers and size of spin–spin coupling constants $^{2,3}J_{C,H}$ and $^{3}J_{H,H}$ used for J-based configuration analysis.

method for assigning these methylene protons with respect to the adjacent methine is similar to that for vicinal methines as described in the following section. Basic strategy for assignment of configuration[12] is summarized in Fig. 6.

3.2 Application of *J*-Based Configuration Analysis to Maitotoxin and Amphidinol. The aforementioned 2D and 3D NMR experiments coupled with CID MS/MS data resulted in the elucidation of not only the entire planar structure but also the relative configuration for the cyclic parts of MTX. However, the configuration of acyclic moieties and the absolute stereochemistry remained unassigned. Thus, J-based configuration analysis was applied to the acyclic sections, where NOEs sometimes caused confusing results owing to the coexistence of multiple conformers. The ^{13}C-enriched MTX prepared for the 3D NMR[10] was used for measurements of J values. Geminal and vicinal C–H coupling constants ($^{2,3}J_{C,H}$) were determined by hetero half-filtered TOCSY (HETLOC),[62] where $^{2,3}J_{C,H}$ was measured as dislocation of an ^{1}H–^{1}H cross peak, and by phase-sensitive HMBC spectra,[64] where the relative values of $^{2,3}J_{C,H}$ were obtained from the intensity of cross peaks. $^{3}J_{H,H}$ values were determined by E. COSY.[65] As an application of this strategy, the dihedral analysis around an oxygen-bearing carbon such as C5, C8, and C9 is briefly reviewed.[12,16] To correlate the stereochemistry between C5 and C7 using J values, stereospecific assignment for two methylene protons on C6 was necessary. As depicted in Fig. 7, $^{2}J_{C,H}$ and $^{3}J_{H,H}$ values allow the assignment for methylene protons on C6. The diastereomeric relationship between C6–C7 can then be established by a similar manner, thereby

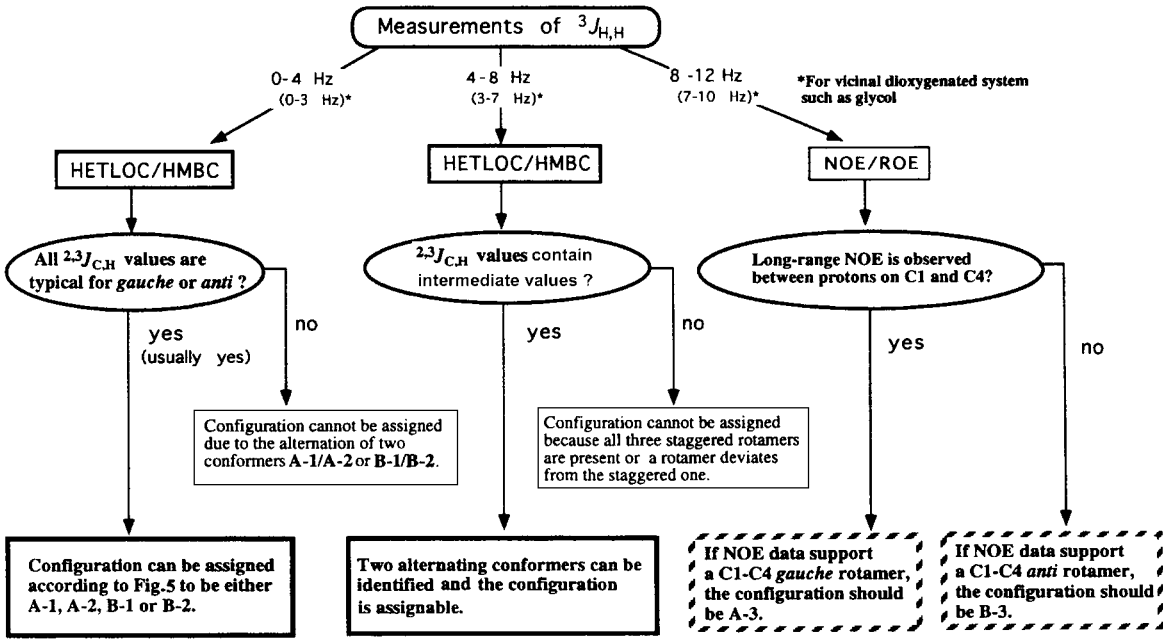


Fig. 6. Determination of *threo* (**A-1**–**A-3**) or *erythro* (**B-1**–**B-3**) configuration using *J*-based configuration analysis.

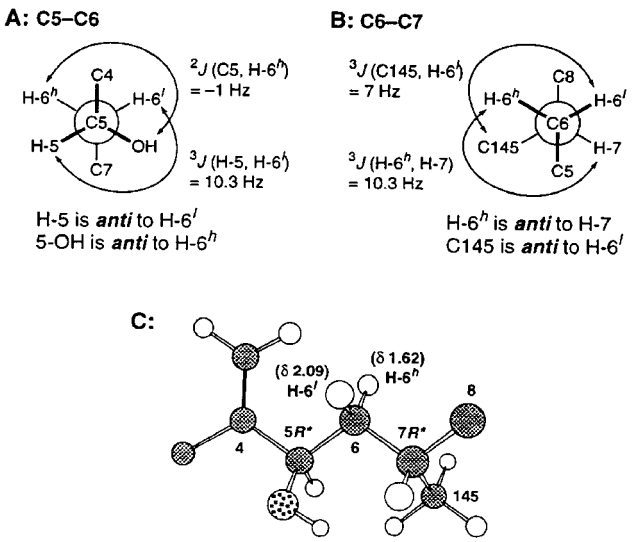


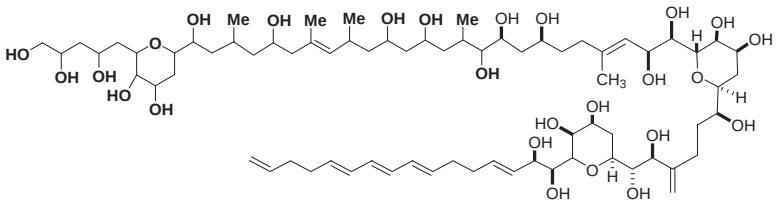
Fig. 7. Configuration analysis of C5–C7 of MTX by *J*-based configuration analysis.

resulting in the configurational and conformational assignment between C5 and C7 (Fig. 7).[16] Based on this strategy, the relative stereochemistry of asymmetric carbons in the side chain of MTX was deduced to be 5*S**, 7*R**, 8*R**, and 9*S**.[16] For the other terminus, similar analysis led to the configuration assignment of 134*S**, 135*S**, 136*R**, and 138*R**.[13,18]

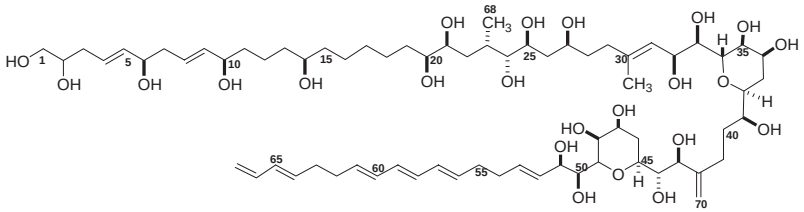
Among the polyketide metabolites of dinoflagellate origin, amphidinols (AMs) are unique compounds since they are primarily composed of a linear polyhydroxy–polyene backbone. The first member of this group was isolated by Yasumoto's group[66] from *Amphidinium klebsii* as a potent antifungal substance. A series of homologues[67,68] have since been found in the same genus, including lueteophanols,[69] amphezonol,[70] linguishuiols,[71] and karatungiols.[72] These long-chain polyhydroxy compounds may be one of the most challenging targets for stereostructural elucidation since chiral centers are scattered over a flexible acyclic structure. Thus, *J*-based configuration analysis was applied to AM3 (Chart 3, **6**).[63,73] To facilitate NMR measurements[62,74] for determining $^{2,3}J_{C,H}$, a ^{13}C-enriched sample of AM3 (25% ^{13}C, 8 mg) was prepared by culturing the algae in the presence of 12 mM $NaH^{13}CO_3$. The stereochemical assignment was accomplished as follows; (a) the *J*-based method was used for acyclic parts with 1,2- and 1,3-chiral centers, C20–C27, C32–C34, C38–C39, C44–C45, and C50–C51; (b) NOE analysis combined with the *J* analysis was used for two ether cycles and their linkage C39–C44; (c) a modified Mosher's method[75] and chromatographic/NMR comparison were used for degradation products to determine the absolute stereochemistry at C2, C6, C10, C14, C23, and C39.

4. Activity-Relevant Conformation and Bimolecular Interactions with Membrane Proteins and Lipids

The structure studies of maitotoxin as described in Section **2** have unveiled that the toxin is largely composed of a ladder-shaped polyether (LSP) skeleton. To gain a better understanding of the mode action of LSP toxins, the general mechanism involved in interaction between membrane proteins and polycyclic ethers may provide a key. As often seen for antimicrobial peptides, amphiphiles possessing membrane affinity often act as an ion channel but the molecular modes of action for agents other than peptides/proteins are hardly known. Thus, membrane-disrupting action by non-peptidic compounds poses a scientific challenge. Interestingly, both LSP-type and polyenepolyol-type natural products are found in dinoflagellate toxins and commonly increase ion permeability of biomembranes although they possess different mechanisms; LSPs often bind to and open ion channel proteins while polyenepolyol compounds usually interact with membrane lipids. In this section, one from each of the two classes, yessotoxin (Chart 4, **9**) and amphidinol (Chart 3, **5** and **6**), has been selected to discuss their mode of action.

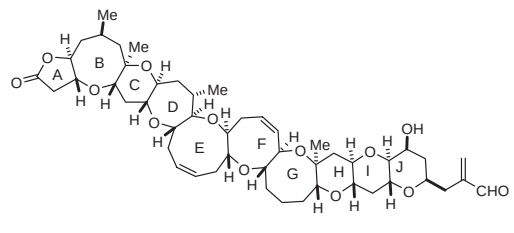


Amphidinol 2 (AM2) **5**

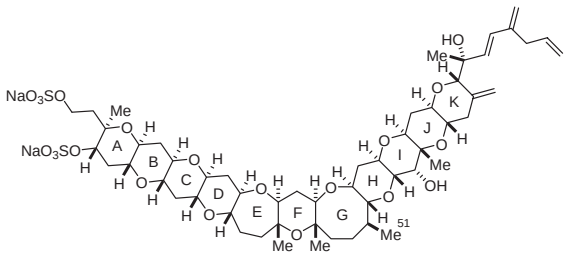


Amphidinol 3 (AM3) **6**

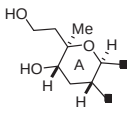
Chart 3.



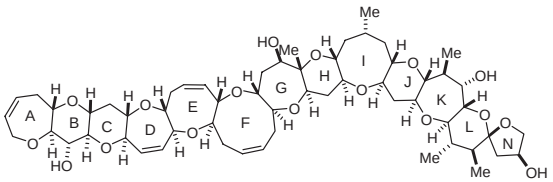
Brevetoxin A **7**
(Voltage-sensitive sodium channel)



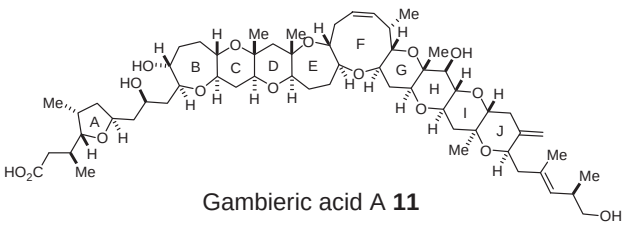
Yessotoxin **9**



Ds-yessotoxin (dsYTX) **10**



Ciguatoxin 3C **8**
(Voltage-sensitive sodium channel)



Gambieric acid A **11**

Chart 4.

4.1 Desulfated-Yessotoxin as an LSP Model. Ladder-shaped polyether compounds (LSPs) comprise continuous trans-fused cyclic ethers and possess potent toxicity. Brevetoxins (BTXB **2** and BTXA **7**), the first members of LSPs, have been extensively studied by Nakanishi's[76] and Shimizu's groups.[77] Besides these toxins, ciguatoxin,[78–81] maitotoxin, prymnesins,[82,83] and yessotoxins (YTX **9**)[84–86] have been found in association with fish-killing red tides or toxic seafood. More than 50 compounds belonging to LSP have been reported so far. These LSPs elicit a broad spectrum of biological activities; e.g., brevetoxin A (**7**) and ciguatoxins are potent ichthyotoxins ($LD_{50} = 0.25$–$4\,\mu g\,kg^{-1}$), and gymnocin-A exhibits cytotoxicity against P388 mouse leukemia cells ($ED_{50} = 1.3\,\mu g\,mL^{-1}$). Gambieric acid A (**11**) is not an ichthyotoxin but an antifungal, which is 2000 times more potent than amphotericin B, and yessotoxin (**9**) induces apoptosis via mitochondrial signal transduction pathways.[87] Although the number of known LSP members is increasing, molecular mode-of-action studies have lagged as described earlier. Brevetoxins (BTXB **2** and BTXA **7**) and ciguatoxins (CTX1B **3** and CTX3C **8**) are rare examples in that their molecular target has been identified; the toxins share a common binding site on voltage sensitive sodium channels.[40,41,88,89] These toxins show extremely high affinity to the channel with dissociation constants of nanomolar–subnanomolar concentrations. Diverse biological activities of LSPs suggest that they bind to specific molecular targets upon exerting their toxicity, although sharing similar structural features. These observations may imply a notion that there is a general and weakly interacting motif of proteins for LSP[90–92] and, when a given LSP exactly matches the binding portion of a target protein, probably due to an arrangement of polyether oxygen atoms, its powerful activity is manifested. Thus, this general motif should provide a clue to understand their potent biological activity. As a model LSP, yessotoxin (**9**),

which was obtained from dinoflagellate cultures, and its desulfated analogue (dsYTX, **10**) are focused on for examining interaction with membrane proteins and peptides; YTX and dsYTX are shown to interact with the transmembrane part of glycophorin A (GpA) to induce the dissociation of oligomers of the protein by SDS-PAGE.[93]

Glycophorin A, a heavily glycosylated membrane protein occurring in erythrocyte membranes, is known to form a dimer[94–97] in membrane environments by interaction mainly between each of α-helix portions at GXXXG residues called "glycine zipper." In addition, GpA tends to aggregate in water, even in the presence of detergents. It is reported that GpA is migrated on SDS-PAGE as dimers or oligomers, which can be dissociated into monomers by peptides corresponding to the transmembrane domain.[98] These peptides (GpA-TM) are reported to take an α-helix structure in SDS micelles.[99] The dissociation can, thus, be accounted for by direct binding of GpA-TM to the transmembrane portion of GpA. This method was utilized to determine whether dsYTX binds to a membrane-integral α-helix peptide. As shown in Fig. 8, addition of dsYTX markedly reduced oligomers and increased dimers and monomers.[93] Appearance of a monomer band in the presence of dsYTX, as is the case with GpA-TM, is attributable to the binding of dsYTX to an α-helical domain of GpA. This dsYTX binding is further supported by PFO (perfluorooctanoic acid)-PAGE as shown in Fig. 8c; PFO is known to stabilize oligomers of hydrophobic peptides.[93,100] In the presence of dsYTX, the oligomers of GpA-TM were mostly dissociated into dimers.[93] These observations again suggest that dsYTX binds to the hydrophobic α-helix, and prevents aggregations of GpA-TMs. Brevetoxin B (**2**) was subjected to the same experiment, indicating efficacy in GpA dissociating activity similar to dsYTX while salinomycin as a polyether compound without a ladder-shaped skeleton showed only marginal activitiy.[93]

In order to know what types of structural moieties other than

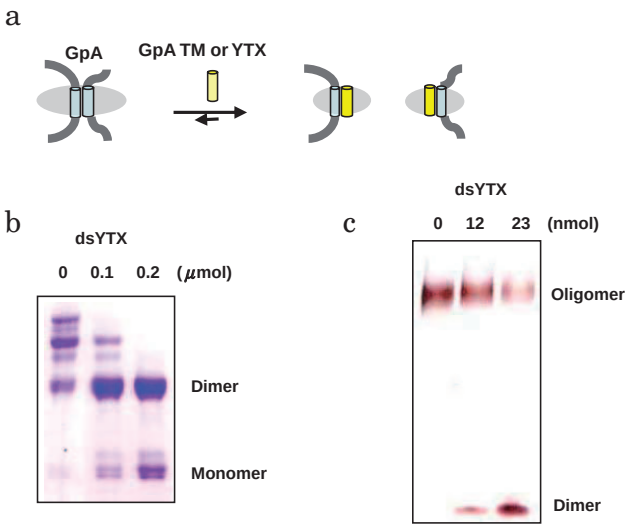


Fig. 8. SDS-PAGE (**b**) and PFO-PAGE (**c**) experiments to examine the interaction between ds-YTX and glycophorin-A.

LSPs dissociate oligomers of GpA, more than 20 compounds were subjected to the SDS-PAGE experiments. Among polyether compounds tested, polyethylene glycol (average molecular weight around 1000), or 18-crown-6 did not induce dissociation of oligomeric GpA. On the other hand, amphipathic polyether compounds such as dicyclohexyl-18-crown-6 and Triton X-100 elicited the dissociation with about 10 times higher doses than dsYTX.[93] To further confirm the interaction between YTX and GpA, saturation transfer difference NMR experiments[101] were carried out, and revealed the interaction with the whole molecule of YTX, particularly with the polyene side chain and sulfate groups.[102] These results strongly suggest that polyether functionality with moderate hydrophobicity possesses affinity for a transmembrane α-helix motif, which may partly account for the powerful biological activities. On the other hand, efficacy was similar among the LSPs tested, which implied that the bilateral arrangement of ether oxygen atoms grossly enhances the interaction with the transmembrane α-helix peptide of glycophorin A.

It has been reported that binding of a brevetoxin B derivative to VSSCs was competitively inhibited by the addition of not only ciguatoxin (CTX3C **8**) ($K_i = 0.15$ nM) but also gambieric acid A (Chart 4, **11**) ($K_i = 0.11$ μM) and gambierol ($K_i = 1.4$ μM), while their affinities are 10^4–10^6 times lower than ciguatoxin.[90] These findings suggest a close relationship between the size of the polycyclic region and inhibitory activity. On the other hand, yessotoxin (**9**) did not inhibit the binding although the number of rings is the same as brevetoxin B (**2**, undecacyclic). In addition, Bourdelais et al.[103] reported that the K_i value of brevenal (pentacyclic) is 1.85 μM, which is comparable to that of gambierol (heptacyclic). These observations imply that the affinity of LPSs to membrane integral α-helices depends not only on the number (and length) of ether cycles but on the ring size and pendant functionalities.

LSPs are very unique compounds in terms of both structural and physicochemical properties. In particular, these compounds have a relatively rigid skeleton usually with one hinge portion consisting of 7-, 8-, or 9-membered ring(s). Affinity between LSPs and peptides should be based upon hydrogen bonding and/or electrostatic interactions. Tetrahydropyran has a large electric dipole moment. In LSP, however, these moments are arranged alternately in opposite directions, which greatly reduce the overall dipole of a molecule, and thus lead to poor water solubility as compared with polyethylene glycol derivative comprising linear polyether functionalities. The presence of flexible 7-, 8-, or 9-membered rings in LSPs may disturb this regular arrangement of dipole moments, which results in higher solubility of the natural LPSs. Under membrane environments, these local dipoles may be attracted to polarized bonds of peptides. In particular, if these functionalities of LSP and peptides face each other on one side, a certain attractive force may be generated (Fig. 9). It is hypothesized that one of these is C–H–O hydrogen bonding[92] as seen in the dimerization of GpA.[97] Another possibility lies in the electric dipole interaction between polar functionalities of peptides and ether bonds of LSP.

4.2 Amphidinol as a Large Amphiphilic Compound. Amphidinols (e.g. AM2 and AM3, Chart 3),[66–68,73,104–106] a family of polyhydroxy–polyene compounds isolated from

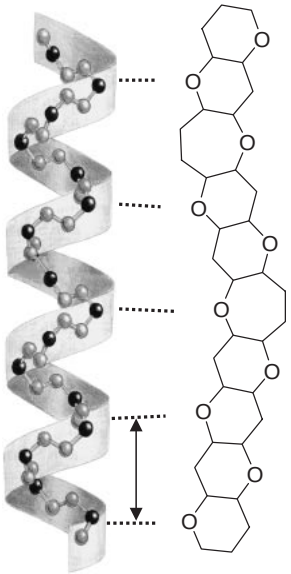


Fig. 9. Possible interaction between α-helix peptide and ladder-shaped polyether. The average distance of ether oxygen atoms on one side matches the α-helix pitch.

the dinoflagellate *Amphidinium* species, showed potent antifungal activity, which significantly exceed that of commercial drugs such as amphotericin B. AMs enhance the permeability of the biological membrane in the presence of sterol[104,107] by directly interacting with membrane lipids. Among known antifungal agents, AMs are very unique in lacking nitrogenous functionalities usually present in synthetic drugs, or macrocyclic structures commonly found in polyene-macrolide antibiotics. Therefore, a hitherto unknown mechanism should be involved in their antifungal action, making AMs an intriguing model to gain a better understanding of the mechanism of membrane disruption. The structure of AMs is characterized by a long amphiphilic carbon chain encompassing multiple hydroxy and olefin groups.[66,67] As membrane permeabilizing agents implicated in native immune systems, a great number of antimicrobial peptides have been reported, most of which are short, helical, and amphiphilic peptides.[108–112] In addition to peptides, polyene antibiotics such as amphotericin B have been a target of pharmaceutical research. Membrane pores or lesions formed by AM3 (**6**) have revealed features similar to those of amphotericin B, implying that the activity is due to the molecular assemblage formed in the biomembrane.[113–115] On the other hand, the size of pores/lesions in erythrocyte membranes were estimated to be 2.0–2.9 nm in diameter, which is significantly larger than that of amphotericin B, 0.8 nm.[116]

SDS micelles are reported to adequately mimic the amphipathic nature of a bilayer membrane even for a conformationally sensitive peptide such as gramicidin A.[114] Thus, the conformation of membrane-bound AM was deduced on the basis of NMR data obtained in SDS micelles. Signal assignment was effected by DQF-COSY, TOCSY, and NOESY (Fig. 10) experiments.[115] From the structures calculated for AM3 by Molecular Dynamics with NOE constrains obtained from the data in SDS micelles, five low energy conformations were retained for AM3 (Fig. 11).[115] The models appear well ordered

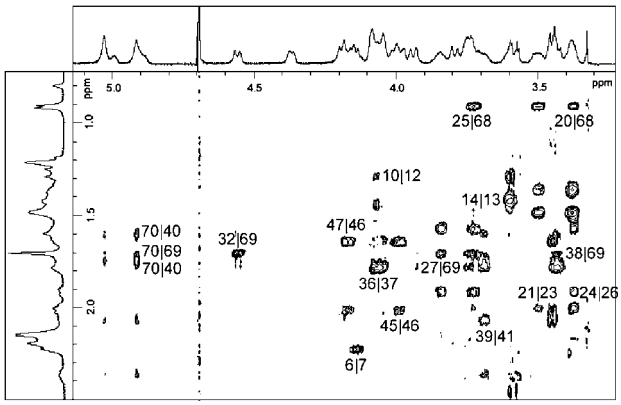


Fig. 10. Partial NOESY spectrum of AM3 with SDS-d_{25} in D_2O. High-resolution ^1H NMR data are shown with micelle-bound AM3, and utilized for conformational elucidation.

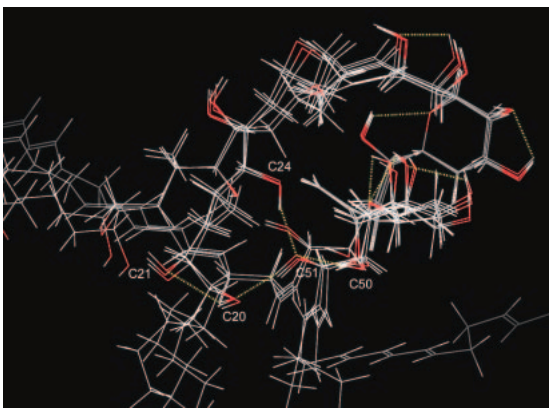


Fig. 11. Superimposition of five lowest energy structures calculated for AM3 from NOE data. The picture shows ensemble of conformations in SDS micelles. The dotted lines between hydroxy groups express the hydrogen-bond network involving O^H20–H^O51, H^O24–O^H51.

except for the two side chains. A hydrogen-bond network, which stabilizes a hairpin conformation of AM3 in the central region, is formed around a C51–OH group. To determine the position of AM3 molecules relative to the surface and interior of the SDS micelles, spin–lattice relaxation time T_1 was determined in the presence and absence of paramagnetic Mn^{2+}.[115] T_1 relaxation data for some isolated and assignable protons were obtained in SDS-d_{25}; T_{1M} values are related to the distance between the proton of interest and Mn^{2+}. Figure 12 shows the relative distance of AM3 protons from the surface of micelles, which indicates that methylene proton (H1), methine protons (H27, H34, and H49), methyl groups (H68 and H69) and an olefin proton (H53) reside near the surface while the other olefin protons (H57, H60, and H67) inside the micelle. In other words, the hydrophilic region of the molecule is predominantly present at the surface, whereas the hydrophobic polyene region penetrates the interior of the micelle; the overall configuration of membrane interaction by AM3 mimics that of MTX to a certain extent (Fig. 3). All these findings together with previous results[116] may lead to a hypothetical molecular mode of action for AMs: (a) binding to the bi-

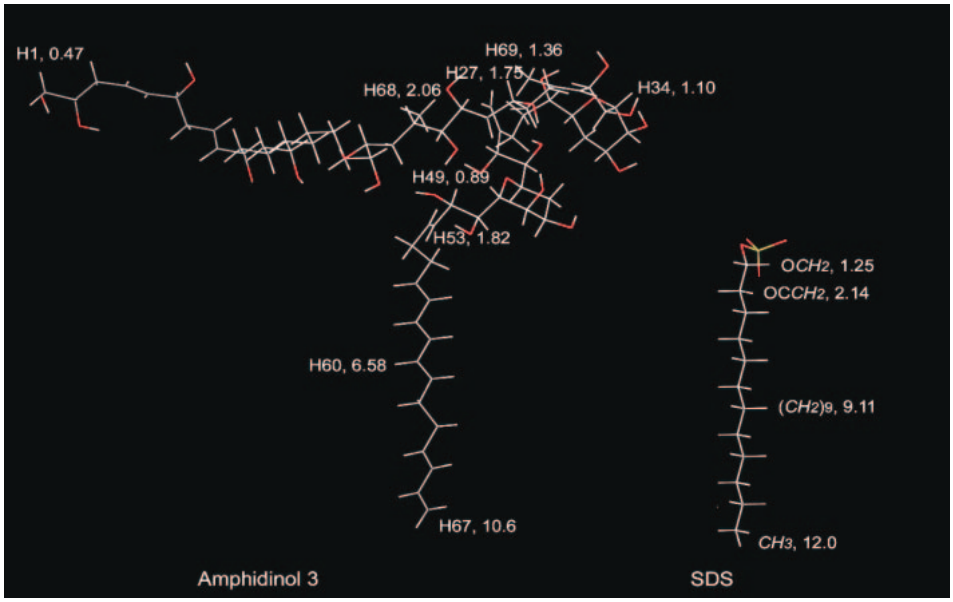


Fig. 12. Hypothetical illustration of the relative positions of AM3 and SDS in micelle with respect to the depth from surface. Figures show the proton number and paramagnetic contribution to ^{1}H relaxation time T_{1M} (s) of AM3 in SDS-d_{25} micelles and pure SDS micelles.

layer membrane containing sterols chiefly with the polyolefin region (the C52 moiety) and the membrane affinity is heavily dependent on the length of the polyolefinic chain; (b) the center region (the C20–C51 moiety) takes a hairpin-shaped conformation, which is stabilized by a hydrogen-bond network in amphipathic environments, and (c) the flexible polyhydroxy region (the C1–C20 moiety) acts as a lining of ion-permeable ion channels.[115,116]

5. Conclusion

Dinoflagellates are unique organisms for their diverse secondary metabolites in terms of both structures and biological activities. In this review, maitotoxin, yessotoxin, and amphidinols have been selected to discuss their conformation and interactions in membranes, which may be able to provide some insights into the structural basis of their potent biological activities. In particular, the NMR-based structure elucidation of natural products and drugs upon binding their targets and/or biomembranes is essential in understanding true active conformation and in development of drug leads. However, lack of reliable analytical methods has prevented elucidation of the structural basis underlying their potent biological activities. In addition to X-ray co-crystallography approaches, solid-state NMR and solution-state NMR with bicelle/micelle systems seem promising, and thus strategies are extensively explored in biophysical and biochemical research groups including the authors'.[117–122]

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