

Rafael A. Espiritu^{a,*}

Membrane permeabilizing action of amphidinol 3 and theonellamide A in raft-forming lipid mixtures

DOI 10.1515/znc-2016-0043

Received March 7, 2016; revised March 7, 2016; accepted April 11, 2016

Abstract: Amphidinol 3 (AM3) and theonellamide A (TNM-A) are potent antifungal compounds produced by the dinoflagellate *Amphidinium klebsii* and the sponge *Theonella* spp., respectively. Both of these metabolites have been demonstrated to interact with membrane lipids ultimately resulting in a compromised bilayer integrity. In this report, the activity of AM3 and TNM-A in ternary lipid mixtures composed of 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC):brain sphingomyelin:cholesterol at a mole ratio of 1:1:1 or 3:1:1 exhibiting lipid rafts coexistence is presented. It was found that AM3 has a more extensive membrane permeabilizing activity compared with TNM-A in these membrane mimics, which was almost complete at 15 μ M. The extent of their activity nevertheless is similar to the previously reported binary system of POPC and cholesterol, suggesting that phase separation has neither beneficial nor detrimental effects in their ability to disrupt the lipid bilayer.

Keywords: amphidinol 3; calcein leakage; lipid rafts; theonellamide A.

1 Introduction

Marine organisms are among the most prolific, and yet still underexplored, sources of novel compounds. The number of new molecules being reported from these sources has increased steadily over the years, to about a thousand each year [1]. Owing to the distinctive environment where these marine natural products are being biosynthesized, a considerable proportion of these has unique structures and properties, usually rendering them with promising bioactivities [2]. In fact, several drug leads of marine origin have

either been approved for the treatment of certain medical conditions or are already in various stages of clinical trials [3]. Among the many marine organisms, dinoflagellates and sponges offer great potential to obtain new structures largely because the former are implicated in algal blooms common around the world, while the latter has been traditionally the dominant source of unique marine secondary metabolites. For instance, a recent review of the 2014 literature showed that dinoflagellates and sponges account for 22.5% of the 1378 new marine-derived bioactive compounds reported during this period [1].

Amphidinols (AMs) are a class of polyhydroxy, polyene, antifungal, and hemolytic metabolites first reported in 1991 from the dinoflagellate *Amphidinium klebsii* found in Japan. Currently, 19 AM homologs have been reported from the same genus [4–12], in addition to the numerous congeners of this bioactive molecule that have also been isolated [13–17], including karlotoxins from the harmful algal bloom-implicated dinoflagellate *Karlodinium* sp. [18]. The defining characteristic of this family of compounds is the presence of two tetrahydropyran rings separating the polyol and polyene regions of the molecule. The antifungal activity of AMs is believed to arise from its ability to permeabilize the membrane, and among the homologues, amphidinol 3 (AM3) (Figure 1) has showed significantly greater antifungal effect. Investigation into the dye leakage capabilities of AM3 in binary lipid membrane mimics showed its absolute dependence on the presence of sterol exhibiting the correct 3 β -OH stereochemistry [19–21]. Furthermore, AM3 activity has been found to be independent of membrane thickness, and coupled with results showing a hairpin configuration of the molecule in the membrane with the polyhydroxy region remaining on the surface while the polyene part penetrating the membrane interior [22], has led to a proposal on the formation of toroidal pores by the compound. However, we have also recently reported that AM3 does not disrupt phospholipid bilayer packing, as what would be expected for toroidal pore formers, so a barrel stave-like mechanism remains a distinct possibility [20].

Theonellamides (TNMs) are potent antifungal and cytotoxic metabolites isolated from the marine sponge *Theonella* spp. These are bicyclic dodecapeptides

^aPresent address: Department of Chemistry, De La Salle University, 2401 Taft Avenue, Manila 0922, Philippines

*Corresponding author: Rafael A. Espiritu, Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan, E-mail: rafael.espiritu@dlsu.edu.ph

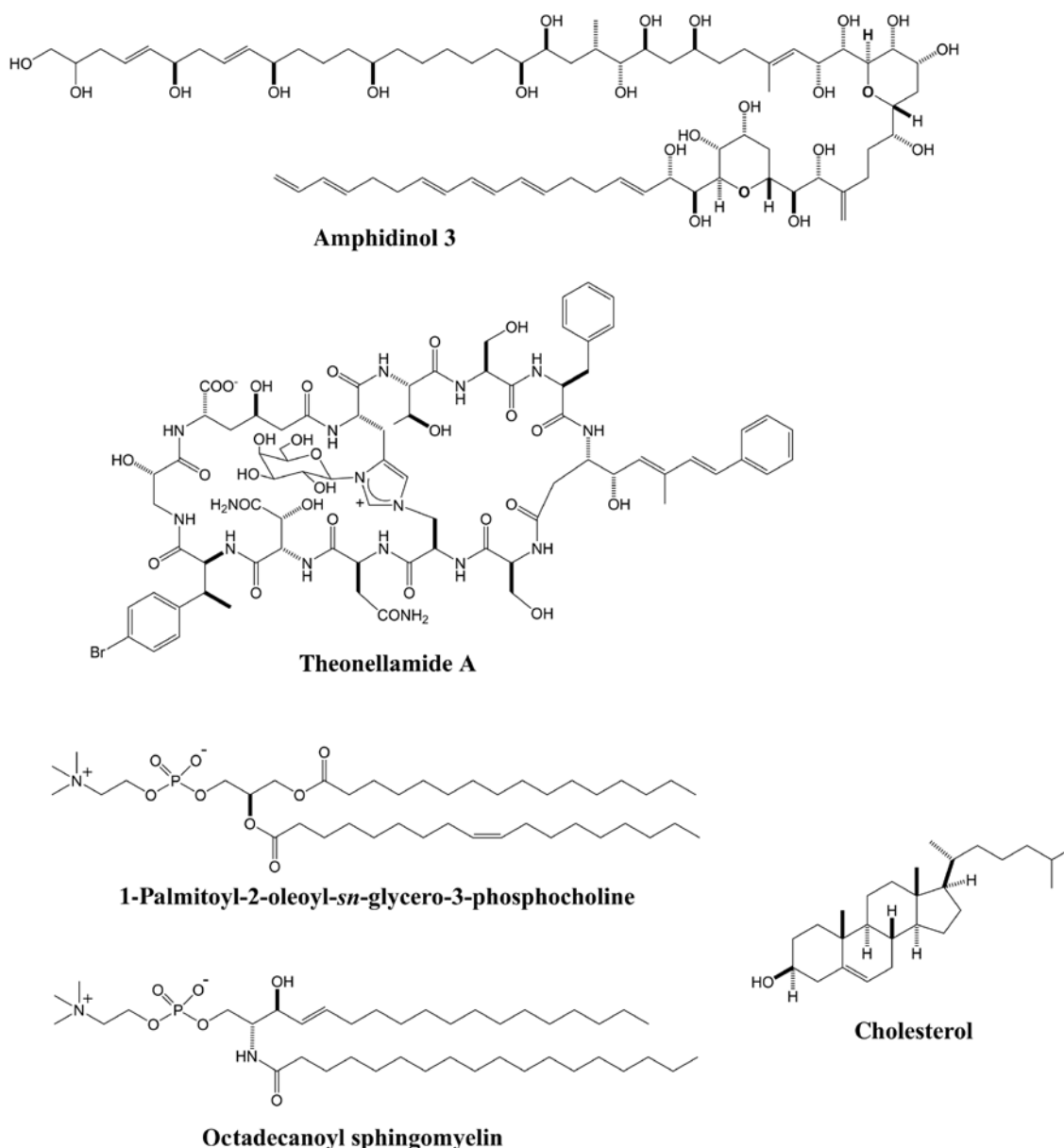


Figure 1: Chemical structures of amphidinol 3, theonellamide A, and lipids used in this study. Octadecanoyl sphingomyelin is the predominant species of BSM from porcine.

bridged by a unique histidinoalanine residue, first reported in 1989 with seven homologues known so far [23–25]. It was previously reported that TNMs specifically recognize β -OH sterols ultimately resulting in membrane damage via a mechanism different from the other common antifungal agents [26]. Moreover, these peptides preferentially partition into the liquid disordered domains as well as result in phase separation in biomembrane mimics [27]. Recently, we have also demonstrated the existence of a direct interaction between sterols in membranes and theonellamide A (TNM-A) (Figure 1), where it recognizes the hydroxyl moiety in the

shallow region of the membrane [28]. However, unlike AM3, TNM-A does not seem to form pore structures in membranes composed of binary lipid mixtures, apparent from the very weak dye leakage observed, although the peptide induces disruption of the tight packing of the lipid bilayer [29].

Lipid rafts are ordered membrane microdomains believed to play a critical role in the segregation, lateral separation, and order in cell membranes [30, 31]. These structures are enriched in sphingolipids with saturated acyl chains and cholesterol, brought about by the preferential interaction between these two lipids, as well certain

proteins that convey upon rafts important biological functions.

In this short communication, the membrane disrupting capabilities of AM3 and TNM-A in raft-forming ternary lipid mixtures containing 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine (POPC), brain sphingomyelin (BSM), and cholesterol is reported, and compared with the previously published data on activity in membranes that do not exhibit phase separation.

2 Materials and methods

2.1 Materials

AM3 was isolated as reported previously [20] while TNM-A was a kind gift from Dr. Shinichi Nishimura of Kyoto University. POPC and BSM were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol was obtained from Nacalai Tesque (Kyoto, Japan), while calcein was from Dojindo Laboratories (Kumamoto, Japan). Lipids were used as received and all other chemicals and reagents were standard and analytical quality reagents.

2.2 Preparation of calcein-entrapped liposomes

Large unilamellar vesicles (LUV) exhibiting phase separation were prepared as follows [20]: POPC (5.2 or 7.7 mg), BSM (5 or 2.5 mg), and cholesterol (2.6 or 1.3 mg) were mixed in a round bottom flask with chloroform to prepare mixtures of POPC:BSM:cholesterol at mole ratio of 1:1:1 or 3:1:1. The solution was vigorously mixed, after which the solvent was removed *in vacuo* to afford a thin lipid film, which was further dried overnight under vacuum. The dried lipid film was then rehydrated with 1 mL of 60 mM calcein (in 10 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA and 150 mM NaCl). It was then subjected to two cycles of vortexing (1 min) and warming up (65°C), followed by five cycles of freezing (−20°C) and thawing (65°C) to obtain a suspension of multilamellar vesicles. Afterwards, the suspension was passed through a polycarbonate membrane filter (pore size, 200 nm) 19 times using a Liposofast extruder (Avestin Inc., Ottawa, Canada) to afford LUVs. Unencapsulated calcein was separated from the LUVs by passing the suspension through a Sepharose 4B column equilibrated and eluted with the same Tris-HCl buffer. The phospholipid concentration in

the suspension was determined using the Phospholipid C-Test Wako (Wako Pure Chemical Industries Ltd., Osaka, Japan) following the manufacturer's protocol. Resulting stock solution was stored at 4°C.

2.3 Calcein leakage measurement

Fluorescence measurements of the released calcein were carried out in a JASCO FP 6500 spectrofluorometer (JASCO Corp., Tokyo, Japan) using 490 and 517 nm as the excitation and emission wavelengths, respectively. Briefly, 20 μ L of the LUV suspension was mixed with 960 μ L of Tris-HCl buffer, then 20 μ L of either AM3 or TNM-A was added to obtain the desired metabolite concentration. Fluorescence recordings were measured at 25°C and observed for a total of 5 min. Final lipid concentration in all measurements was 27 μ M. To obtain the condition of 100% leakage, 20 μ L of 10% Triton X-100 (v/v) in the same Tris-HCl buffer was added to the suspension.

3 Results and discussion

AM3 and TNM-A are two unique marine natural products that have been shown previously to act via a mechanism that involves direct interaction with membrane lipids resulting to a loss of membrane integrity [19–22, 26–29]. AM3 shows an absolute dependence in the presence of sterols in order to permeabilize the membrane, which most likely involves the formation of pores. On the other hand, TNM-A does not seem to form distinct membrane pores, although it disrupts the tight packing of the phospholipid bilayer. In these previous investigations of the membrane permeabilizing action by these compounds, only binary lipid mixtures of phospholipids and cholesterol were used. Furthermore, under the conditions where these experiments were performed, such binary lipid mixtures would result in membranes with no phase separation, i.e. it will be only in the liquid-disordered (l_d) phase [32].

Ternary mixture of phospholipids, sphingomyelin (or a mixture of saturated and unsaturated phospholipids), and cholesterol has been the canonical model systems to mimic lipid rafts, and has been used extensively to investigate the membrane-disrupting mechanism of action of small molecules, such as antimicrobial peptides [32–34]. In this study, the mole ratio of POPC:BSM:cholesterol used (1:1:1 and 3:1:1) was previously shown to exhibit the coexistence of both the liquid-ordered (l_o) and liquid-disordered (l_d) domains [32].

Corresponding liposomes were prepared with an encapsulated calcein, at a dye concentration of 60 mM. At this concentration, calcein fluorescence is quenched, and upon its leakage into the extracellular *milieu*, for instance, after pore formation, an increase in fluorescence will be observed. Figure 2 shows the % leakage activity of AM3 and TNM-A in raft-forming membrane mimics. AM3 permeabilizing activity is comparable in both lipid systems tested and was almost complete at the highest concentration used (15 μ M). This extent of membrane damage is very similar to its activity in binary lipid mixtures consisting of POPC and cholesterol or ergosterol, as we have reported earlier [20]. These results imply that the presence of l_o-l_d phase separation has neither detrimental nor beneficial effect on the membrane disrupting capabilities of AM3. These findings are different from those observed with other membrane-active compounds such as antimicrobial peptides, whose membrane-disrupting effect is greatly increased in raft-forming membrane due to the preferential accumulation of the peptide in the l_d phase leading to more drastic perturbation and subsequent dye leakage [33, 34], although there is no way from the current data to infer on which phase AM3 is colocalized. In addition, the presence of phase separation in membranes necessitates the existence of innate defects found along the interface of such phases, and these have been implicated in better binding and activity observed for certain pore-forming toxins [35]. In contrast, AM3 activity seems unaffected by these packing defects. Nevertheless, since AM3 action is primarily dependent on the presence of sterol, it is possible that the compound is able to recognize cholesterol

in both phases, even though it is found deeper in raft domains [36], leading to pore formation. This ability to bind to raft domains has been reported earlier for certain pore-forming protein toxins, where depletion of membrane cholesterol significantly reduced raft binding and pore formation [37].

On the other hand, TNM-A shows a very weak dye leakage potential at all concentrations tested, and these activities were comparable to the previous report using binary lipid mixtures of POPC and cholesterol [28]. These data appear to be consistent with our proposal that TNM-A indeed does not form distinct membrane pores [29]. It has been reported earlier that TNM-A recognizes cholesterol in and preferentially partitions into the l_d domain [27], which intuitively should result in faster membrane accumulation compared with lipid mixtures exhibiting no phase separation. In addition, we have demonstrated before, through surface plasmon resonance experiments that rate constants for TNM-A binding to ergosterol- or cholesterol-containing POPC membranes were about 10 times larger than in sterol free ones, which results in faster and greater accumulation in the former [28]. However, considering that the TNM-A activity is comparable on both types of lipid bilayers [28] and observed on the same time course suggests that the rate of peptide accumulation may not be significantly enhanced by domain formation.

Even though no significant difference was observed in the activity of AM3 in the two raft-forming membrane mimics, a closer look at the time course fluorescence in these systems may provide additional insights into initial association rates of the compound. Figure 3 shows the

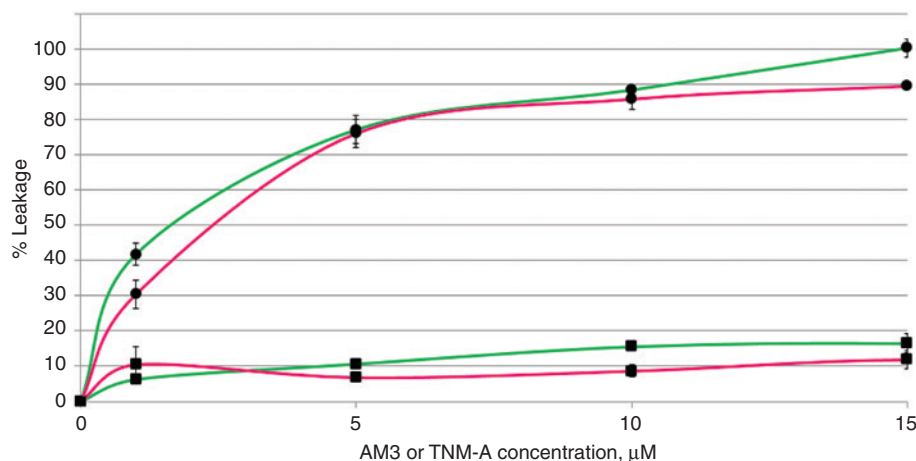


Figure 2: Calcein leakage from POPC:BSM:cholesterol liposomes after the addition of various concentration of AM3 (circles) or TNM-A (square).

Mole ratio of POPC:BSM:cholesterol used were 1:1:1 (green) and 3:1:1 (pink). Error bars represent standard deviation of three independent trials.

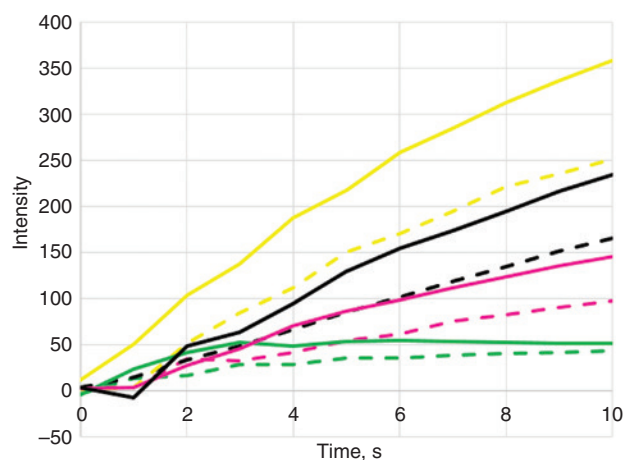


Figure 3: Time course plots of calcein fluorescence within 10 s after the addition of AM3 to POPC:BSM:cholesterol liposomes. Concentration of AM3 used were 15 μM (yellow), 10 μM (black), 5 μM (pink), and 1 μM (green). Mole ratio of POPC:BSM:cholesterol used were 1:1:1 (dashed lines) and 3:1:1 (solid lines). Lines show the average of three independent trials.

average time course plots of calcein fluorescence (three independent trials) within 10 s after AM3 addition. In all concentration tested, dye fluorescence was stronger in the 3:1:1 ternary lipid mixture. Since dye fluorescence is directly related to amount of released dye, a higher fluorescence indicates a more substantial disruption of the bilayer integrity. It is therefore reasonable to suggest that more AM3 are membrane bound during the first few moments of contact between AM3 and the 3:1:1 membrane, and concomitantly a relatively faster rate of accumulation in this system. A possible explanation for this is that since the l_o phase predominates in the 1:1:1 system [33], AM3 will have an easier time interacting with cholesterol in the 3:1:1 membrane mixture due the greater area of l_d phase, which is loosely packed, present in this system.

4 Conclusions

To summarize, AM3 showed a significantly greater dye leakage activity in ternary lipid mixtures exhibiting phase separation than TNM-A, which was comparable to the compounds' previously reported activity in binary mixtures without l_o – l_d segregation [20, 28]. This demonstrates that for AM3, at least, its membrane permeabilizing capability is not dependent on whether lipid rafts form or not. It also appears that AM3 has a better initial interaction with ternary lipid mixtures where l_o does not predominate. Notwithstanding, further experiments will have to

be performed to analyze the interaction of both AM3 and TNM-A in raft-forming lipid bilayers, such as the kinetics involved in the binding of the compounds to these systems as well as whether or not cholesterol is also recognized in these kinds of membrane mimics.

Acknowledgments: The author wishes to express his gratitude to Prof. Michio Murata (Osaka University) and Prof. Nobuaki Matsumori (Kyushu University) for critical discussion, and to Dr. Shinichi Nishimura (Kyoto University) for providing the TNM-A sample.

References

- Blunt JW, Copp BR, Keyzers RA, Munro MH, Prinsep M. Marine natural products. *Nat Prod Rep* 2016;33:382–431.
- Yiwen H, Guping H, Jianchen Y, Yongcheng L, Shengping C, Jie Y, et al. Statistical research on the bioactivity of new marine natural products discovered during the 28 years from 1985 to 2012. *Mar Drugs* 2015;13:202–21.
- Montaser R, Luesch H. Marine natural products: a new wave of drugs? *Future Med Chem* 2011;3:1475–89.
- Satake M, Murata M, Yasumoto T, Fujita T, Naoki H. Amphidinol, a polyhydroxy-polyene antifungal agent with an unprecedented structure, from a marine dinoflagellate, *Amphidinium klebsii*. *J Am Chem Soc* 1991;113:9859–61.
- Paul GK, Matsumori N, Murata M, Tachibana K. Isolation and chemical structure of amphidinol 2, a potent haemolytic compound from marine dinoflagellate *Amphidinium klebsii*. *Tetrahedron Lett* 1995;36:6279–82.
- Paul GK, Matsumori N, Konoki K, Murata M, Tachibana K. Chemical structures of amphidinols 5 and 6 isolated from marine dinoflagellate *Amphidinium klebsii* and their cholesterol-dependent membrane disruption. *J Mar Biotechnol* 1997;5:124–8.
- Murata M, Matsuoka S, Matsumori N, Paul GK, Tachibana K. Absolute configuration of amphidinol 3, the first complete structure determination from amphidinol homologues: application of a new configuration analysis based on carbon-hydrogen spin-coupling constants. *J Am Chem Soc* 1999;121:870–71.
- Morsy N, Matsuoka S, Houdai T, Matsumori N, Adachi S, Murata M, et al. Isolation and structure elucidation of a new amphidinol with a truncated polyhydroxyl chain from *Amphidinium klebsii*. *Tetrahedron* 2005;61:8606–10.
- Echigoya R, Rhodes L, Oshima Y, Satake M. The structures of five new antifungal and haemolytic amphidinol analogs from *Amphidinium carterae* Collected in New Zealand. *Harmful Algae* 2005;4:383–9.
- Morsy N, Houdai T, Matsuoka S, Matsumori N, Adachi S, Oishi T, et al. Structures of new amphidinols with truncated polyhydroxyl chain and their membrane-permeabilizing activities. *Bioorg Med Chem* 2006;14:6548–54.
- Meng Y, Van Wagoner RM, Misner I, Tomas C, Wright JL. Structure and biosynthesis of amphidinol 17, a haemolytic compound from *Amphidinium carterae*. *J Nat Prod* 2010;73:409–15.

12. Nuzzo G, Cutignano A, Sardo A, Fontana A. Antifungal amphidinol 18 and its 7-sulfate derivative from the marine dinoflagellate *Amphidinium carterae*. *J Nat Prod* 2014;77:1524–7.
13. Doi Y, Ishibashi M, Nakamichi H, Kosaka T, Ishikawa T, Kobayashi J, et al. A new polyhydroxyl compound from symbiotic marine dinoflagellate *Amphidinium sp.* *J Org Chem* 1997;62:3820–3.
14. Huang X, Zhao D, Guo Y, Wu H, Trivellone E, Cimino G. Ling-shuiols A and B, two new polyhydroxy compounds from the chinese marine dinoflagellate *Amphidinium sp.* *Tetrahedron Lett* 2004;45:5501–4.
15. Washida K, Koyama T, Yamada K, Kita M, Uemura D. Karatungliols A and B, two novel antimicrobial polyol compounds, from the symbiotic marine dinoflagellate *Amphidinium sp.* *Tetrahedron Lett* 2006;47:2521–5.
16. Sugahara K, Kitamura Y, Murata M, Satake M, Tachibana K. Prorocentrol, a polyoxy linear carbon chain compound isolated from the toxic dinoflagellate *Prorocentrum hoffmannianum*. *J Org Chem* 2011;76:3131–8.
17. Inuzuka T, Yamada K, Uemura D. Amdigenols E and G, long carbon-chain polyol compounds, isolated from the marine dinoflagellate *Amphidinium sp.* *Tetrahedron Lett* 2014;55:6319–23.
18. Waters AL, Oh J, Place AR, Hamann MT. Stereochemical studies of the karlotoxin class using NMR spectroscopy and DP-4 chemical-shift analysis: insights into their mechanism of action. *Angew Chem* 2015;54:15705–10.
19. Morsy N, Houdai T, Konoki K, Matsumori N, Oishi T, Murata M. Effects of membrane constituents on membrane-permeabilizing activity of amphidinols. *Bioorg Med Chem* 2008;16:3084–90.
20. Espiritu RA, Matsumori N, Tsuda M, Murata M. Direct and stereospecific interaction of amphidinol 3 with sterol in lipid bilayers. *Biochemistry* 2014;53:3287–93.
21. Swasono RT, Mouri R, Morsy N, Matsumori N, Oishi T, Murata M. Sterol effect on interaction between amphidinol 3 and liposomal membrane as evidenced by surface plasmon resonance. *Bioorg Med Chem Lett* 2010;20:2215–8.
22. Houdai T, Matsumori N, Murata M. Structure of membrane-bound amphidinol 3 in isotropic small bicelles. *Org Lett* 2008;10:4191–4.
23. Matsunaga S, Fusetani N, Hashimoto K, Walchli M, Theonellamide F. A novel antifungal bicyclic peptide from a marine sponge *Theonella sp.* *J Am Chem Soc* 1989;111:2582–8.
24. Matsunaga S, Fusetani N. Theonellamide A-E, cytotoxic bicyclic peptides, from a marine sponge *Theonella sp.* *J Org Chem* 1995;60:1177–81.
25. Youssef DT, Shaala LA, Mohamed GA, Badr JM, Bamanie FH, Ibrahim SR. Theonellamide G, a potent antifungal and cytotoxic bicyclic glycopeptide from the red sea marine sponge *Theonella swinhoei*. *Mar Drugs* 2014;12:1911–23.
26. Nishimura S, Arita Y, Honda M, Iwamoto K, Matsuyama A, Shirai A, et al. Marine antifungal theonellamides target $\beta\beta$ -hydroxysterols to activate rho1 signalling. *Nat Chem Biol* 2010;6:519–26.
27. Arita Y, Nishimura S, Ishitsuka R, Kishimoto T, Ikenouchi J, Ishii K, et al. Targeting cholesterol in a liquid-disordered environment by theonellamides modulate cell membrane order and cell shape. *Chem Biol* 2015;22:1–7.
28. Espiritu RA, Matsumori N, Murata M, Nishimura S, Kakeya H, Matsunaga S, et al. Interaction between the marine sponge cyclic peptide theonellamide a and sterols in lipid bilayers as viewed by surface plasmon resonance and solid-state ^2H nuclear magnetic resonance. *Biochemistry* 2013;52:2410–18.
29. Espiritu RA, Cornelio K, Kinoshita M, Matsumori N, Murata M, Nishimura S, et al. Marine sponge cyclic peptide theonellamide A disrupts lipid bilayer integrity without forming distinct membrane pores. *Biochim Biophys Acta* 2016;1858:1373–9.
30. Simons K, Ikonen E. Functional rafts in cell membranes. *Nature* 1997;387:569–72.
31. Lingwood D, Simons K. Lipid rafts as a membrane-organizing principle. *Science* 2010;327:46–50.
32. de Almeida RF, Fedorov A, Prieto M. Sphingomyelin/phosphatidylcholine/cholesterol phase diagram: boundaries and composition of lipid rafts. *Biophys J* 2003;85:2406–16.
33. McHenry AJ, Sciacca MF, Brender JR, Ramamoorthy A. Does cholesterol suppress the antimicrobial peptide induced disruption of lipid raft containing membranes? *Biochim Biophys Acta* 2012;1818:3019–24.
34. Losada-Perez P, Khorshid M, Hermans C, Robijns T, Peeters M, Jimenez-Monroy KL, et al. Melittin disruption of raft and non-raft-forming biomimetic membranes: a study by quartz crystal microbalance with dissipation monitoring. *Colloids Surf B Biointerfaces* 2014;123:938–44.
35. Ros U, Edwards MA, Epand RF, Lanio ME, Schreier S, Yip CM, et al. The sticholysin family of pore-forming toxins induces the mixing of lipids in membrane domains. *Biochim Biophys Acta* 2013;1828:2757–62.
36. Rog T, Pasenkiewicz-Gierula M. Cholesterol-sphingomyelin interactions: a molecular dynamics simulation study. *Biophys J* 2006;91:3756–67.
37. Nagahama M, Hayashi S, Morimitsu S, Sakurai J. Biological activities and pore formation of *Clostridium perfringens* beta toxin in hl 60 cells. *J Biol Chem* 2003;278:36934–41.