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Interaction between the Marine Sponge Cyclic Peptide Theonellamide A and Sterols in Lipid Bilayers As Viewed by Surface Plasmon Resonance and Solid-State ^{2}H Nuclear Magnetic Resonance

Rafael Atillo Espiritu,[†] Nobuaki Matsumori,*[,†] Michio Murata,[†,‡] Shinichi Nishimura,[§] Hideaki Kakeya,[§] Shigeki Matsunaga,[∥] and Minoru Yoshida[⊥]

[†]Department of Chemistry, Graduate School of Science, Osaka University, Toyonaka, Osaka 560-0043, Japan

[‡]Lipid Active Structure Project, ERATO, Japan Science and Technology Agency, Toyonaka, Osaka 560-0043, Japan

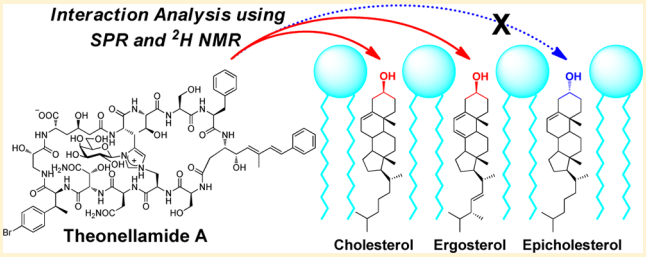
[§]Division of Bioinformatics and Chemical Genomics, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan

[∥]Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

[⊥]RIKEN Advanced Science Institute, Wako, Saitama 351-0198, Japan

Supporting Information

ABSTRACT: Theonellamides (TNMs) are members of a distinctive family of antifungal and cytotoxic bicyclic dodecapeptides isolated from the marine sponge *Theonella* sp. Recently, it has been shown that TNMs recognize 3β-hydroxysterol-containing membranes, induce glucan overproduction, and damage cellular membranes. However, to date, the detailed mode of sterol binding at a molecular level has not been determined. In this study, to gain insight into the mechanism of sterol recognition of TNM in lipid bilayers, surface plasmon resonance (SPR) experiments and solid-state deuterium nuclear magnetic resonance (^{2}H NMR) measurements were performed on theonellamide A (TNM-A). SPR results revealed that the incorporation of 10 mol % cholesterol or ergosterol into 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) membranes significantly enhances the affinity of the peptide for the membrane, particularly in the initial binding to the membrane surface. These findings, together with the fact that binding of TNM-A to epicholesterol (3α-cholesterol)-containing liposomes and pure POPC liposomes was comparably weak, confirmed the preference of the peptide for the 3β-hydroxysterol-containing membranes. To further establish the formation of the complex of TNM-A with 3β-hydroxysterols in lipid bilayers, solid-state ^{2}H NMR measurements were conducted using deuterium-labeled cholesterol, ergosterol, or epicholesterol. The ^{2}H NMR spectra showed that TNM-A significantly inhibits the fast rotational motion of cholesterol and ergosterol, but not epicholesterol, therefore verifying the direct complexation between TNM-A and 3β-hydroxysterols in lipid bilayers. This study demonstrates that TNM-A directly recognizes the 3β-OH moiety of sterols, which greatly facilitates its binding to bilayer membranes.



Marine sponges are a rich source of natural products, largely because of their inclination to produce various structurally unique bioactive secondary metabolites.[1] It is believed that, in some cases, these bioactive compounds are actually produced by a diverse and abundant microbial community, which sometimes comprises half of the animals' body weight, rather than the sponge itself.[2−4] Species belonging to the order Lithistida, such as the genus *Theonella*, have been the source of diverse natural products with promising therapeutic potential,[5] e.g., the antifungal theopalauamide,[6] theonegramide, and theonellamides (TNMs),[7−9] HIV entry inhibitor koshikamide F and H,[10] anti-inflammatory solomonamide A,[11] antipsoriatic perthamide C and E,[12] cytotoxic polytheonamides and theopapuamide,[13,14] and rare sterol ligands for human nuclear receptors theonellasterols and conicasterols.[15]

TNMs (Figure 1), isolated from *Theonella* sp., belong to a family of unique bicyclic dodecapeptides first reported in 1989 by Matsunaga et al.[8] Since then, five additional congeners have been isolated.[8,9] These compounds possess a common bis-macrocyclic structure encompassing some unusual amino acids and a bridge of a histidinoalanine moiety. In some of these, a sugar moiety is attached to the bridge yet plays no significant role in the biological activity.[8,9] Structurally related peptides, theopalauamide[6] and theonegramide,[7] have also been isolated from sponges of the same genus. It has previously been reported that TNM-F induces the formation of extraordinarily

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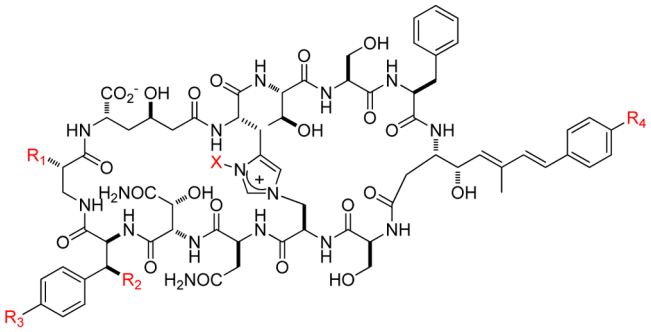


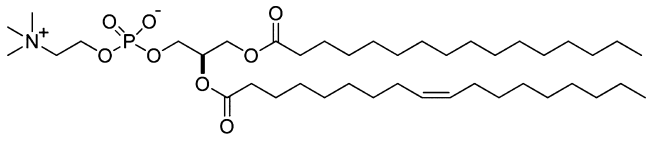
	R_1	R_2	R_3	R_4	X
Theonellamide A	OH	Me	Br	H	β-D-Gal
Theonellamide B	OH	Me	Br	Br	H
Theonellamide C	H	H	H	Br	H
Theonellamide D	H	H	Br	Br	β-L-Ara
Theonellamide E	H	H	Br	Br	β-D-Gal
Theonellamide F	H	H	Br	Br	H

Figure 1. Structures of theonellamides (TNMs).

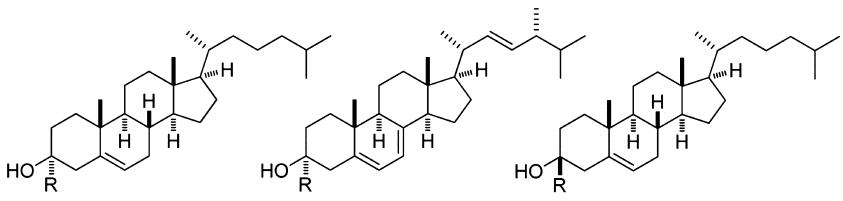
large vacuoles in 3Y1 rat fibroblasts, and thus, TNM-F is postulated to affect cellular autophagy and inhibit organelle degradation as well as cause the breakdown of their proteins.[16] In addition, by using TNM-A-conjugated gel beads, glutamate dehydrogenase and 17β-hydroxysteroid dehydrogenase IV were identified as its binding proteins from rabbit liver tissues, and in vitro assays revealed that TNM-F activates glutamate dehydrogenase leading to the amination of α-ketoglutarate, although its potency is lower than that of the known activator adenosine diphosphate.[17]

Recently, a series of systematic experiments have provided substantial insights into the mode of action of TNMs and the structurally related theopalauamide.[18,19] Budding yeast genomics, using a collection of molecularly barcoded ORF libraries, revealed that theopalauamide was less effective toward mutated cells in the ergosterol biosynthesis pathway.[19] Indeed, theopalauamide permeabilizes liposomes containing ergosterol.[19] Chemical genomic analysis using a fission yeast ORF collection suggested a mechanistic link between TNMs and 1,3-β-D-glucan synthesis, and an overproduction of 1,3-β-D-glucan was observed following the treatment of yeast cells with TNM-F in a Rho1-dependent manner.[18] Furthermore, in vitro binding assays, using a fluorescently labeled TNM derivative, demonstrated that it specifically binds to 3β-hydroxysterols such as cholesterol and ergosterol (Figure 2).[18] Sterol binding is required for the TNM-induced 1,3-β-D-glucan synthesis and subsequent loss of membrane integrity. Judging from the phenotypic changes in yeasts, the membrane action of TNM-F is apparently distinct from that of polyene antifungals such as amphotericin B, which is also known to bind sterols in membranes; e.g., TNM-F caused fragmentation, instead of enlargement, of vacuoles and exhibited a time-dependent toxicity, as opposed to amphotericin B, which had acute fungicidal activity. Thus, TNMs represent a novel class of sterol-binding compounds whose mode of action is different from that of polyene antibiotics and therefore are expected to be a new tool for exploring the function and localization of sterols in cells. However, as is the case with amphotericin B and other polyene antibiotics, how TNMs recognize the sterols in lipid bilayers is yet to be determined. Therefore, a detailed analysis of the bimolecular interaction between TNMs and sterols is indispensable in understanding the mode of action of TNMs.

In general, interactions between bioactive molecules and cell membranes play a key role in the regulation of several cellular processes such as signal transduction, ion channel formation, and the action of antimicrobial and cytotoxic peptides. Among a wide range of techniques employed to investigate these interactions,[20,21] surface plasmon resonance (SPR) has been proven to be an excellent tool for examining the binding of biomolecules to membranes.[20,22] This technique was successfully employed to characterize the interaction of cationic amphiphilic drugs and antimicrobial peptides with artificial membranes.[23−25] Recently, using SPR, we have also evaluated the binding of amphotericin B[26] and amphidinol 3[27] to palmitoyloleoylphosphatidylcholine [POPC (Figure 2)] liposomes and demonstrated that the affinity of these molecules for the vesicles is significantly enhanced by incorporating cholesterol or ergosterol into the lipid bilayer. Solid-state deuterium NMR (^{2}H NMR) is an invaluable tool in the study of lipid, and sterol dynamics in model membranes, e.g., the membrane perturbing effects, induced by antimicrobial peptides, can be directly observed by the change in quadrupole splitting of deuterated acyl chain segments of phospholipids.[28]



1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC)



Cholesterol : R = H Ergosterol : R = H Epicholesterol : R = H
3-*d*-Cholesterol : R = D 3-*d*-Ergosterol : R = D 3-*d*-Epicholesterol : R = D

Figure 2. Chemical structures of the lipids used in this study.

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More recently, ^{2}H NMR was used to reveal the dynamics of sterols [cholesterol and ergosterol deuterated at C3 (Figure 2)] affected by the presence of amphotericin B.[29] This study has shown that amphotericin B inhibits the fast rotational motion of ergosterol in membranes, thus unequivocally demonstrating the direct amphotericin B−ergosterol interaction in lipid bilayers.

Hence, in this study, we investigate the interaction between TNM-A and POPC liposomes containing cholesterol, ergosterol, or epicholesterol (Figure 2) with SPR to understand its mode of interaction. Then, we observed the direct interaction between TNM-A and sterols incorporated into lipid bilayers using solid-state ^{2}H NMR.

MATERIALS AND METHODS

Materials. Theonellamide A (TNM-A) was isolated as reported previously.[9] 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) was purchased from NOF Corp. Cholesterol was from Nacalai Tesque; ergosterol was obtained from Tokyo Kasei, and epicholesterol was obtained from Steraloids, Inc. 3-*d*-Cholesterol, 3-*d*-ergosterol, and 3-*d*-epicholesterol (Figure 2) were synthesized as previously reported.[29,30] 1-Ethyl-3-[3-(dimethylamino)propyl]-carbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), 10 mM acetate buffer (pH 5.0), 1 M ethanolamine (pH 8.5), 50 mM sodium hydroxide (NaOH), 0.5% (w/v) sodium dodecyl sulfate (SDS), and 10× PBS buffer (pH 7.4) were purchased from GE Healthcare (Uppsala, Sweden). Dodecylamine and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Polycarbonate filters was purchased from AVESTIN Inc. All the other chemicals were standard and analytical quality reagents.

Preparation of Liposomes for SPR Analysis. Large unilamellar vesicles (LUVs) were prepared as follows. POPC (10 mg) with or without 10 mol % (~0.5 mg) sterol (cholesterol, ergosterol, or epicholesterol) were dissolved together in chloroform in a round-bottom flask. The solvent was evaporated, and the resulting lipid film was further dried in vacuo overnight. It was then hydrated with 1 mL of PBS buffer [10 mM phosphate buffer (pH 7.4), 2.7 mM potassium chloride, and 137 mM sodium chloride]. The mixture was vortexed, sonicated, and subjected to three cycles of freezing (−80 °C), thawing (60 °C), and vortexing (5 s) to form multilamellar vesicles (MLVs). The MLV suspension was passed through double 100 nm polycarbonate filters 19 times with LiposoFast-Basic (AVESTIN Inc.) at room temperature to form LUVs. The lipid concentration of the LUVs was determined using a phospholipid C-Test (Wako Pure Chemical Industries Ltd.). The LUVs were then diluted with the same PBS buffer to produce a suspension with a final lipid concentration of 0.5 mM for injection into the SPR instrument.

Surface Plasmon Resonance Experiments. TNM-A (1 mg, 0.57 μmol) was first dissolved in DMSO (1 mL) and stored as a 0.57 mM stock solution; 50 μL of the TNM-A stock solution was diluted to 28 μM with 950 μL of PBS buffer. This solution was further diluted with PBS buffer containing 5% DMSO to give 10, 15, and 20 μM TNM-A solutions. We ensured that all these solutions, together with the running buffer, had the same DMSO concentration.

The SPR experiments were performed at 25 °C using a dodecylamine-modified CM5 sensor chip mounted on a Biacore T200 system (GE Healthcare), and the running buffer was PBS containing 5% DMSO (pH 7.4). The unmodified CM5 sensor chip was first washed three times with a 50 mM NaOH/2-propanol solution [3:2 (v/v)] at a flow rate of 20 μL/min for 2 min. Dodecylamine was immobilized in one of the flow cells (fc2) of the CM5 chip with an amino coupling method while the other flow cell (fc1) was left untouched to serve as the control lane. This chip was activated by injecting a solution of 390 mM EDC and 100 mM *N*-hydroxysuccinimide [1:1 (v/v), 70 μL] and then dodecylamine (1 mg/mL) in 10 mM acetate buffer containing 10% DMSO (pH 5.0) at a flow rate of 10 μL/min and a contact time of 7 min. The remaining *N*-hydroxysuccinimide ester groups on the sensor chip were deactivated by converting them to amide groups with an injection of 1 M ethanolamine hydrochloride (pH 8.5). Thus, the obtained modified sensor chip was then washed with 10% DMSO to remove nonspecifically bound molecules.

For the immobilization of liposomes on the sensor surface, the dodecylamine-modified sensor chip was first conditioned by an injection of running buffer at a rate of 10 μL/min for 5 min. The liposome suspension (0.5 mM) was then injected at a flow rate of 2 μL/min for 40 min, followed by the injection of 50 mM NaOH at a rate of 20 μL/min for 2 min, three times to generate a stable sensorgram, which indicated the formation of a stable liposome layer on the sensor surface.

TNM-A solutions, at concentrations of 10, 15, and 20 μM, were then injected at a flow rate of 10 μL/min; its association was observed for 300 s. Then running buffer was injected at the same flow rate for 300 s, and the dissociation of TNM-A from the surface was monitored. A new liposome surface was prepared for each injection of the TNM-A solution. To remove the liposomes from the sensor surface, injections of 0.5% (w/v) SDS, followed by a 50 mM NaOH/2-propanol solution [3:2 (v/v)] both at a flow rate of 20 μL/min for 2 min, were conducted twice to ensure a return to the original baseline.

Data Analysis. The sensorgrams obtained from the peptide−lipid bilayer interaction were analyzed by curve fitting[31,32] with Biacore T200 Evaluation version 1.0. Global fitting, which is normally done by simultaneously fitting sensorgrams corresponding to different analyte concentrations, was found to be incompatible with TNM-A as in the case with amphotericin B.[26] This is probably because concentration-dependent aggregate formation of TNM-A in the aqueous phase affects its membrane binding, thus leading to the inconsistency in the kinetic parameters at different TNM-A concentrations. Thus, we evaluated the kinetic parameters not by global fitting but by local fitting to the sensorgrams from 20 μM TNM-A. The sensorgrams were fit to a two-state reaction model,[26] in which interaction between the peptide and the lipid bilayers is assumed to occur in two steps (Scheme 1). The first

Scheme 1

$$A + B \underset{k_{d1}}{\overset{k_{a1}}{\rightleftharpoons}} AB \underset{k_{d2}}{\overset{k_{a2}}{\rightleftharpoons}} ABx$$

step involves the binding of TNM-A (A) to membrane lipids (B) in a parallel and stoichiometric manner, identical to the simple bimolecular or Langmuir model if the second step does not proceed. The second step involves a conformational or morphological change of the complex (AB) to a second complex (ABx).

In this model, rate equations are given by

$$d[A]/dt = -k_{a1}[A][B] + k_{d1}[AB] \quad (1)$$

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$$d[AB]/dt = (k_{a1}[A][B] - k_{d1}[AB]) - (k_{a2}[AB] - k_{d2}[ABx]) \quad (2)$$

$$d[ABx]/dt = k_{a2}[AB] - k_{d2}[ABx] \quad (3)$$

$$K_{A1} = k_{a1}/k_{d1};\ K_{A2} = k_{a2}/k_{d2};\ K_A = K_{A1}K_{A2} \quad (4)$$

where k_{a1} and k_{d1} correspond to the association and dissociation rate constants for the liposome surface of free TNM-A, respectively, while k_{a2} and k_{d2} correspond to the distribution rate constants from AB to ABx forms and vice versa, respectively. K_{A1}, K_{A2}, and K_A represent the affinity constants for the first, second, and overall equilibrium steps, respectively. A detailed method for determining the kinetic parameters on the basis of this model is given in the literature.[24,26,32]

Sample Preparation for Solid-State ^{2}H NMR. For the measurement of ^{2}H NMR spectra of the deuterated sterols in POPC membranes, 2.6 μmol of 3-*d*-sterol, 46 μmol of POPC, and TNM-A (0 or 2.6 μmol) were dissolved in a $CHCl_3$/MeOH solution [2:1 (v/v)] in a round-bottom flask. The solvent was removed in vacuo and further dried overnight. The lipid film was then rehydrated with 1 mL of milli-Q water. After being sonicated and vortexed for a few minutes, the lipid suspension was subjected to three cycles of freezing (−80 °C) and thawing (40 °C) to make MLVs. The vesicle suspension was lyophilized overnight, rehydrated with deuterium-depleted water [50% (w/w)], and homogenized by being vortexed, frozen, and thawed. It was then transferred to a 5 mm glass tube (Wilmad) and sealed with epoxy glue.

^{2}H NMR Measurements. All the ^{2}H NMR spectra were recorded on a 400 MHz ECA400 (JEOL, Tokyo, Japan) or a 300 MHz CMX300 (Chemagnetics, Agilent, Palo Alto, CA) spectrometer. Spectra were collected at 30 °C using a 5 mm ^{2}H static probe (Doty Scientific Inc., Columbia, SC, or Otsuka Electronics, Osaka, Japan) following a quadrupolar echo sequence.[29] The 90° pulse width was 2 μs; the interpulse delay was 30 μs, and the repetition rate was 0.5 s. The sweep width was 200 kHz, and the number of scans was around 400000.

RESULTS

Binding of TNM-A to POPC Liposomes Measured by SPR. A dodecylamine-modified CM5 sensor chip was utilized to evaluate the interaction of TNM-A with POPC/sterol (10 mol %) and pure POPC liposomes as we previously reported for amphotericin B.[26] The liposomes were captured by the modified lane with an immobilization level of around 12700 ± 800 RU.[26,27] The response from the control lane was subtracted from that in the liposome-immobilized lane. Figure 3 shows typical sensorgrams representing the binding of TNM-A to various POPC liposomes. Interaction of the peptide with the membranes showed clear concentration dependence, regardless of the presence or absence of sterol, indicating that the amount of membrane-bound peptide is directly proportional to the peptide concentration in the bulk solution. Moreover, as evidenced by the RU increase, the presence of cholesterol and ergosterol significantly enhanced the binding of the peptide to POPC liposomes by approximately 3-fold compared with that of pure POPC liposomes, while the binding of the peptide to epicholesterol (3α-cholesterol)-containing liposomes was comparable to that of pure liposomes. These observations clearly demonstrate the preference of the peptide for 3β-hydroxysterols, which is consistent with the previous findings obtained with fluorescently labeled TNM.[18]

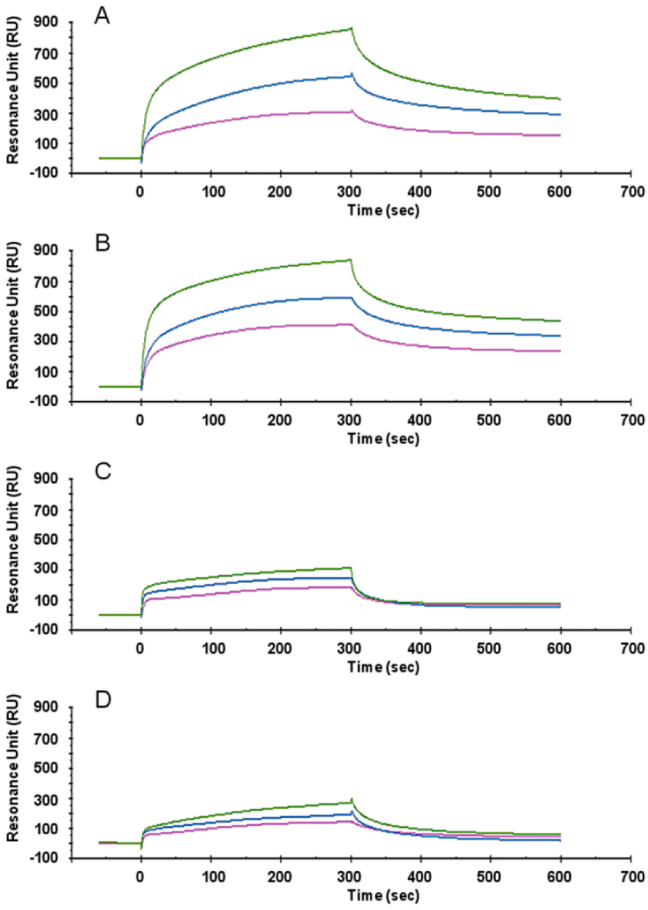

Figure 3. SPR sensorgrams for binding of TNM-A to liposomes captured on a dodecylamine-modified CM5 sensor chip: (A) 10 mol % cholesterol-containing POPC liposomes, (B) 10 mol % ergosterol-containing POPC liposomes, (C) 10 mol % epicholesterol-containing POPC liposome, and (D) pure POPC liposomes. Sensorgrams correspond to 20 (green), 15 (blue), and 10 μM TNM-A (violet).

Next we analyzed the kinetics of binding of TNM-A to lipid membranes by fitting the experimental curves to those calculated from a theoretical model. On the basis of the possible mechanism of action of antimicrobial and membrane-active peptides,[20,24] the sensorgrams could be fit to a two-state reaction model (Scheme 1). This model assumes that the interaction between the peptide and membrane lipids occurs via a two-step process: the first step involving the actual binding of TNM-A to the membrane surface and the second step probably corresponding to a conformational or morphological change to form a more stable membrane complex. The two-state analysis was first applied to well-known membrane peptides melittin and magainin[24,25] and then successfully used for antifungal natural products amphotericin B[26] and amphidinol 3.[27] For these compounds, the two-state model reproduced the experimental SPR sensorgrams better than the conventional Langmuir model, which is consistent with the mechanisms of their membrane activities. Figure 4 shows the curve fitting of the experimental sensorgrams to the two-state reaction model (Scheme 1 and eqs 1−4) together with the components of the theoretical curve, demonstrating that theoretical curves using the two-state model excellently reproduced the experimental sensorgrams. As is evident from

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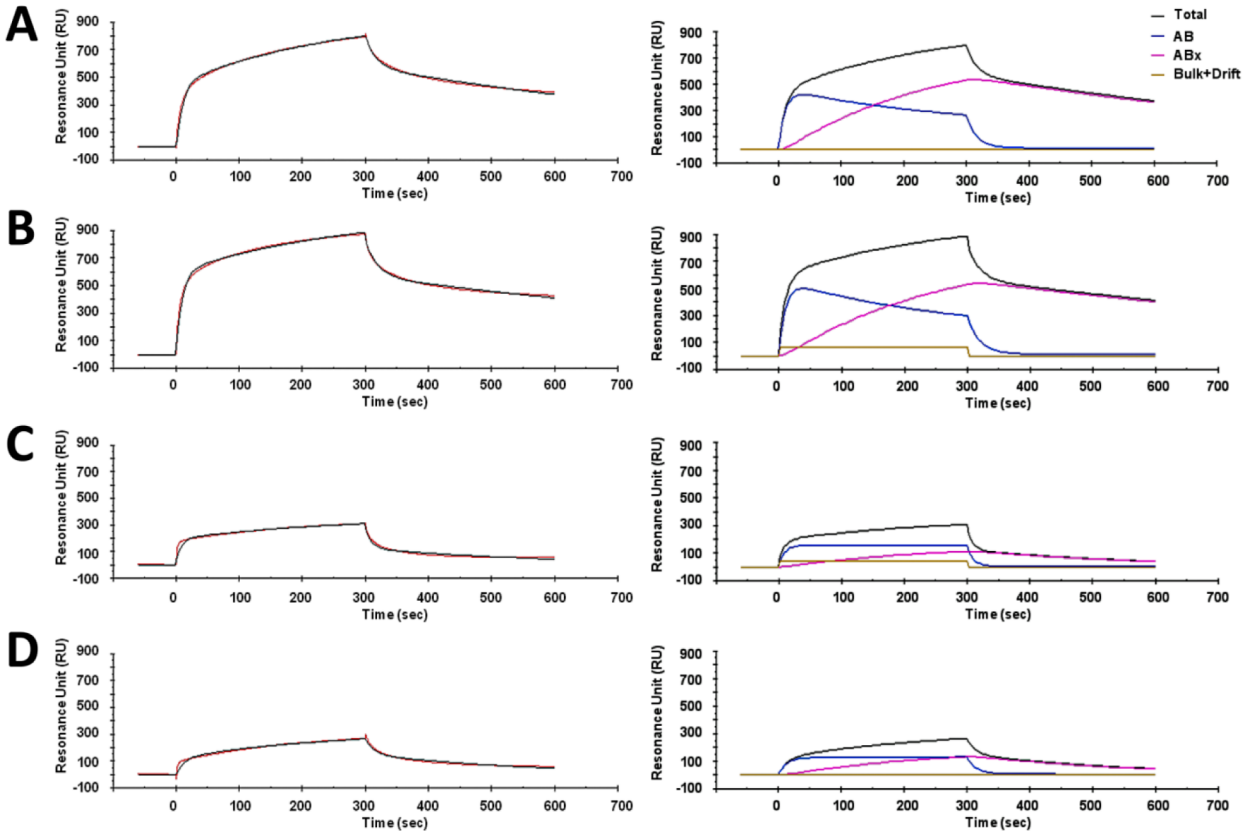

Figure 4. Curve fitting of the SPR sensorgrams to the two-state reaction model (left) and components of the fitting curves (right): (A) 10 mol % cholesterol-containing POPC liposomes, (B) 10 mol % ergosterol-containing POPC liposomes, (C) 10 mol % epicholesterol-containing POPC liposomes, and (D) pure POPC liposomes. Experimental RU values were recorded for 20 μM TNM-A. Red and black traces in the left panels depict experimental and theoretical curves, respectively. Blue, pink, and yellow traces in the right panels represent contributions from the AB complex, the ABx complex, and the bulk effect of the solvent, respectively, to the total component (black line).

Table 1. Kinetic Parameters for the Binding of TNM-A to Liposomes Obtained from the Two-State Reaction Model[a]

	POPC/cholesterol (10 mol %)	POPC/ergosterol (10 mol %)	POPC/epicholesterol (10 mol %)	POPC
k_{a1} ($\times 10^3$ M^{-1} s^{-1})	1.6 ± 0.3	2.5 ± 0.2	0.22 ± 0.09	0.15 ± 0.05
k_{d1} ($\times 10^{-2}$ s^{-1})	5.3 ± 0.5	4.73 ± 0.06	7.5 ± 3.7	7.7 ± 1.1
k_{a2} ($\times 10^{-3}$ s^{-1})	6.6 ± 0.4	5.7 ± 0.2	3.4 ± 0.7	6.1 ± 0.4
k_{d2} ($\times 10^{-3}$ s^{-1})	1.8 ± 0.2	1.2 ± 0.1	2.4 ± 1.3	4.7 ± 0.3
K_{A1} ($\times 10^3$ M^{-1})	30 ± 4	52 ± 4	3.0 ± 0.5	1.8 ± 0.4
K_{A2}	3.6 ± 0.6	4.8 ± 0.4	1.6 ± 0.5	1.3 ± 0.1
K_A ($\times 10^3$ M^{-1})	109 ± 32	250 ± 26	4.9 ± 1.9	2.4 ± 0.5

[a]The concentration of TNM-A was 20 μM.

the right panels of Figure 4, progression of the second step is much slower than that of the first step in all four cases, thus showing that the second step is rate-limiting. We also tried to fit the sensorgrams to the Langmuir or 1:1 bimolecular interaction model but could not reproduce the theoretical curve (Figure S1 of the Supporting Information), thus supporting the notion that the binding of TNM-A to the lipid bilayers occurs via two different processes. Table 1 lists the kinetic and affinity constants obtained from the fitting, showing that the presence of 3β-hydroxysterol, cholesterol, or ergosterol significantly enhanced the affinity (K_A) of TNM-A for the membranes compared with those of epicholesterol (3α-hydroxysterol)-containing and pure POPC liposomes. This enhanced affinity of the peptide for the 3β-hydroxysterol-containing membranes is mainly due to the first binding step, because the rate constants (k_{a1}) are ~10 times larger in cholesterol- and ergosterol-containing liposomes than in epicholesterol-containing and pure POPC liposomes. In contrast, the second rate constants (k_{a2} and K_{A2}) were approximately the same for all systems tested, thus indicating that the second process is less affected by the presence of 3β-hydroxysterol.

In these measurements, the binding of TNM-A to POPC liposomes was very strong, especially with cholesterol- or ergosterol-containing membranes, such that regeneration of the liposome surface via repeated washing with NaOH was not successful (Figure S2 of the Supporting Information). Thus, the liposomes once used for analysis had to be removed, and then fresh liposomes were immobilized on the sensor chip for the next analysis, which resulted in the relatively large standard deviations shown in Table 1. Nevertheless, we can safely compare the kinetic data because the differences in the kinetic

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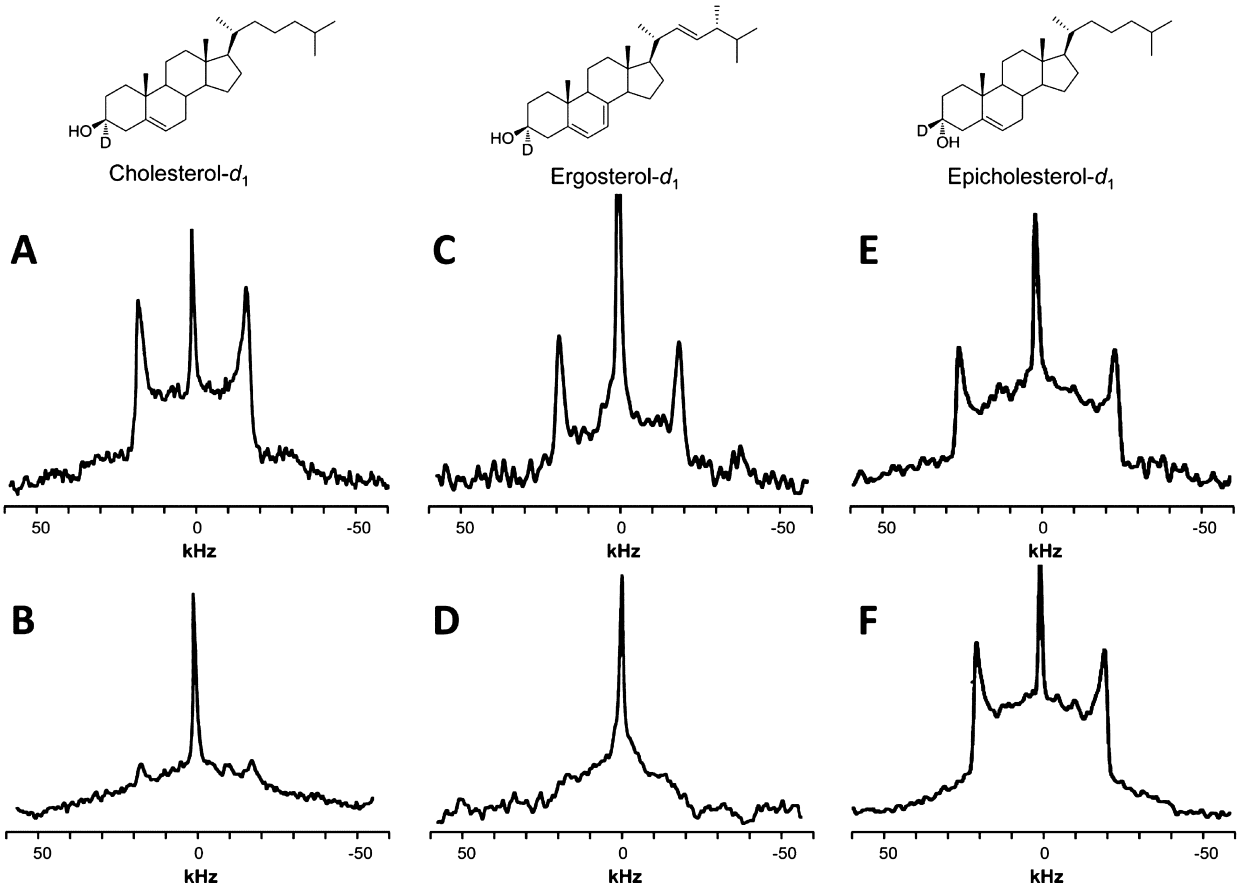

Figure 5. ^{2}H NMR spectra of 3-*d*-sterol incorporated into POPC bilayers in the absence (A, C, and E) and presence (B, D, and F) of TNM-A. 3-*d*-Cholesterol (A and B), 3-*d*-ergosterol (C and D), and 3-*d*-epicholesterol (E and F) were used. 3-*d*-Sterol:TNM-A:POPC molar ratios of 1:0:18 (A, C, and E) and 1:1:18 (B, D, and F) were used. Isotropic signals are mostly from residual deuterium water.

constants between membrane systems significantly exceed the deviations.

Interaction between TNM-A and Sterol As Viewed by Solid-State ^{2}H NMR. Although the SPR experiments described above clearly show the preferential binding of TNM-A to 3β-hydroxysterol-containing membranes, it does not necessarily indicate that TNM-A directly interacts with 3β-hydroxysterol in lipid bilayers; it is also possible to assume that the presence of 3β-hydroxysterols changes the physicochemical properties of the membrane and consequently enhances the membrane affinity of TNM-A. Hence, to gain decisive evidence of the direct binding of TNM-A to membrane sterols, we conducted solid-state ^{2}H NMR measurements using 3-*d*-sterols (Figure 2) incorporated into POPC liposomes. Sterol molecules in lipid bilayers undergo fast lateral diffusion, which can be regarded as an axial rotation in NMR, and quadrupolar splitting observed in the ^{2}H NMR spectra depends both on the tilt angle of the C–^{2}H bond with respect to the rotation axis and the wobbling of the molecule.[29] Figure 5 shows the spectra of the 3-*d*-sterols incorporated into POPC bilayers in the absence and presence of TNM-A. In the absence of TNM-A (Figure 5A), 3-*d*-cholesterol exhibits a characteristic Pake doublet indicative of fast rotational motion of the sterol in the POPC bilayers. However, upon addition of the peptide, a stark attenuation of the splitting signal is observed (Figure 5B), indicating that the molecular rotation falls into an intermediate motional speed with correlation times of 10^{-4} to 10^{-5} s. A similar change in the splitting pattern was observed with 3-*d*-ergosterol (Figure 5C,D). On the other hand, in 3-*d*-epicholesterol-containing POPC liposomes (Figure 5E,F), the characteristic splitting pattern hardly changed, although the splitting value was slightly reduced by the presence of TNM-A, which may be attributable to a morphological change in the liposome induced by TNM-A that will be discussed shortly. Therefore, this demonstrates that the fast rotational motion of both 3-*d*-cholesterol and 3-*d*-ergosterol slows via direct interaction with TNM-A in lipid bilayers, which is indicative of considerable intermolecular interaction. Conversely, no such inhibition with 3-*d*-epicholesterol means that the fast rotational motion occurs even in the presence of TNM-A, indicative of a weaker intermolecular interaction between these two molecules. In addition, these observations strongly support the fact that the presence of either cholesterol or ergosterol, but not epicholesterol, significantly enhances the affinity of TNM-A for POPC liposomes as was shown explicitly in the SPR experiments (Figure 3 and Table 1). These results unequivocally prove the direct interaction between TNM-A and 3β-hydroxysterols in lipid bilayers.

DISCUSSION

Although we have reported that fluorescently labeled TNMs bind to 3β-sterols,[18] the detailed mode of interaction is yet to be clarified. In this study, to improve our understanding of the sterol recognition mechanism exhibited by the peptide, we scrutinized the interaction between TNM-A and sterol-containing liposomes using SPR and solid-state ^{2}H NMR and

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demonstrated for the first time the direct interaction using TNM-A as an intact TNM. Here it should be mentioned that, although the concentrations of TNM-A are different by 2 orders of magnitude between the two experiments (20 μM and 2.6 mM), the molar ratios between TNM-A and lipid molecules are almost identical (1:25 and 1:19 for SPR and ^{2}H NMR, respectivley), thus rationalizing the concomitant use of both methods.

As described in the introductory section, the presence or absence of a sugar moiety in TNM molecules hardly affects the activity based on comparable results obtained from biological tests.[8,9] Theopalauamide, which is structurally closely related to TNM-A and has a sugar moiety, recognizes sterol molecules as is the case in TNM-F lacking the sugar moiety;[19] therefore, it is reasonable to consider that these results obtained for TNM-A essentially hold true for TNM-F and other TNM congeners.

Using SPR with a dodecylamine-modified CM5 sensor chip that was devised to minimize nonspecific hydrophobic interactions, we successfully demonstrated that the presence of 3β-hydroxysterols, as seen in cholesterol and ergosterol, significantly increases the affinity of the peptide for POPC membranes (Figures 3 and 4 and Table 1). In contrast, TNM-A had a lower affinity for epicholesterol-containing or pure POPC liposomes. The kinetic parameters listed in Table 1 further revealed that the 3β-sterols markedly promote the initial binding of TNM-A to the membrane surfaces (k_{a1}). The membrane affinity is enhanced by direct interaction between the peptide and the sterols that was explicitly shown on the basis of characteristic spectral changes in solid-state ^{2}H NMR. These findings, together with the fact that the alcohol groups of sterols are located close to the membrane interface,[33−35] imply that TNM-A has a direct interaction with the hydroxy moieties of 3β-sterols at the initial binding of TNM-A to the membrane surface, resulting in the greater level of accumulation of the peptide in the shallow area of the membrane. The direct recognition of the sterol hydroxy groups at the membrane surface is consistent with the relatively small difference (a factor of approximately 2) in the affinity between cholesterol and ergosterol (Table 1), indicating that TNM-A does not strictly recognize the difference in the steroid skeleton or side chain structure. It is not so far-fetched to assume that intermolecular hydrogen bonds play a crucial functional role in the interaction between TNM-A and the sterol hydroxy group.

In a previous report,[18] it was demonstrated that calcein, a membrane-impermeable fluorescent dye, was able to enter yeast cells in the presence of TNM-F (11 μM), indicating that membrane integrity is compromised by the peptide. Although the presence of 3β-hydroxysterols had a weaker effect on the second step of the binding process (K_{A2} in Table 1), the greater level of accumulation of TNM-A on the membrane surface, in the presence of cholesterol or ergosterol, results in an increased level of formation of the second complex (ABx) (Figure 4A,B). This presumably corresponds to the relocation of the peptide from the surface-binding form to a more stable membrane-complex form. We have recently observed through microscope and ^{31}P NMR experiments that TNM-A induced a microscopic morphological change in liposome features such as the occurrence of high-curvature regions (to be published in due course), which is consistent with the reduced quadrupole splitting value of epicholesterol by the presence of TNM-A (Figure 5E,F). Therefore, the second step may correspond to deformation of membrane morphology through the binding of the peptide. Namely, the accumulated TNM-A in the 3β-sterol-containing membranes could change the membrane morphology and integrity, ultimately resulting in membrane damage as previously reported for yeast cells.[18] In fact, TNM-induced membrane damage as well as cytotoxicity is reported to be time-dependent,[18] consistent with the slow progression of the second process as shown in Figure 4.

As described in the introductory section, the membrane action of TNM-F is apparently different from that of amphotericin B, a representative polyene antibiotic forming sterol-dependent pores in fungal membranes; e.g., TNM-F exhibited a time-dependent toxicity, while amphotericin B has acute fungicidal activity.[18] In our experiments using artificial membranes, distinct pore formation by TNM-A could not be observed (Figure S3 of the Supporting Information), although it induced hemolysis (Table S1 of the Supporting Information). Here it may be meaningful to compare the SPR kinetic parameters of TNM-A with those of amphotericin B. We previously reported that sterols, particularly ergosterol, prominently promote not only the initial surface binding step of amphotericin B but also the subsequent reorientation process presumably corresponding to pore formation.[26] In particular, the second step of amphotericin B hardly proceeds without sterols being present, thus suggesting that the sterol is involved in the pore complex. In contrast, as clearly shown in Table 1, the second step of TNM-A binding proceeds without sterols and is not significantly accelerated by the presence of sterols. In addition, amphotericin B shows a more than 10-fold difference in the K_A values between cholesterol and ergosterol, which is largely attributed to the second reorientation step, while TNM-A exhibits a smaller difference (a factor of ∼2) as shown in Table 1. More recently, we have found that amphotericin B does not interact with epiergosterol (manuscript in preparation). Taken together, this means that amphotericin B recognizes not only the 3-hydroxy group but also steroid rings and side chains, the latter of which would provide ergosterol selectivity and promote the second reorientation process corresponding to pore formation, while TNM-A mostly recognizes the sterol hydroxy group in the initial binding process. This difference is consistent with the observation that TNM-A is unlikely to form distinctive pores as does amphotericin B. Rather, as mentioned previously, it is more plausible that the accumulation of TNM-A in the sterol-containing membrane disturbs and damages the bilayer morphology and integrity, which would correspond to the second process in TNM-A binding. Here it should be mentioned that the weaker sterol dependency in the second process of the binding of TNM to the membrane does not indicate the lack of the interaction between TNM-A and sterol in the second step, because ^{2}H NMR spectra demonstrate the persistent interaction in the membrane, but rather suggests that TNM-A induces the second step irrespective of the presence or absence of the interaction with the sterol molecules.

In conclusion, we have demonstrated using SPR that the presence of cholesterol or ergosterol significantly enhances the affinity of TNM-A for POPC liposomes and the peptide exhibits a preference for sterols with a 3β configuration. This enhanced affinity is caused by direct interaction between the peptide and 3β-hydroxysterols that is explicitly shown by characteristic spectral patterns in solid-state ^{2}H NMR. These results are consistent with the selective binding of fluorescently labeled TNM to 3β-hydroxysterols.[18] Our kinetic data show that 3β-hydroxysterols accelerate the initial binding of TNM-A to the membrane surface, thus suggesting that TNM-A directly

recognizes the 3β-OH moiety upon binding to the membrane surface. Although the presence of 3β-hydroxysterols had a weaker effect on the kinetic constants of the second process corresponding to some deformation of bilayer morphology, the accumulation of TNM-A in the sterol-containing membranes eventually promotes the second process, thus causing the damage to the integrity of the membrane as reported previously.[18] However, the nature of these membrane effects as well as that of the molecular recognition is still a subject of further investigation. To this end, further microscopic and NMR-based experiments will be necessary to observe changes in membrane morphology caused by the peptide and to determine the peptide's structure complexed with sterols in bilayer systems; such studies are currently underway in our group.

■ ASSOCIATED CONTENT

Supporting Information

Fitting of experimental sensorgrams corresponding to 20 μM TNM-A to the Langmuir 1:1 bimolecular interaction model, regeneration of the liposome surface by 50 mM NaOH washing, and experimental details and results of the calcein leakage assay using POPC liposomes as well as the hemolysis assay using human erythrocytes. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: +81-6-6850-5790. E-mail: matsmori@chem.sci.osaka-u.ac.jp.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

LUV, large unilamellar vesicle; MLV, multilamellar vesicle; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; RU, resonance unit; SPR, surface plasmon resonance; TNMs, theonellamides; TNM-A, theonellamide A; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride.

■ REFERENCES

(1) Faulkner, D. J. (2002) Marine Natural Products. *Nat. Prod. Rep. 19*, 1−48.

(2) Tabares, P., Pimentel-Elardo, S. M., Schirmeister, T., Hünig, T., and Hentschel, U. (2011) Anti-protease and immunomodulatory activities of bacteria associated with Caribbean sponges. *Mar. Biotechnol. 13*, 883−892.

(3) Proksch, P., Putz, A., Ortlepp, S., Kjer, J., and Bayer, M. (2010) Bioactive natural products from marine sponges and fungal endophytes. *Phytochem. Rev. 9*, 475−489.

(4) Taylor, M. W., Radax, R., Steger, D., and Wagner, M. (2007) Sponge-associated microorganisms: Evolution, ecology, and biotechnological potential. *Microbiol. Mol. Biol. 71*, 295−347.

(5) Wright, A. E. (2010) The Lithistida: Important sources of compounds useful in biomedical research. *Curr. Opin. Biotechnol. 21*, 801−807.

(6) Schmidt, E. W., Bewley, C. A., and Faulkner, D. J. (1998) Theopalauamide, a bicyclic glycopeptide from filamentous bacterial symbionts of the lithistid sponge *Theonella swinhoei* from Palau and Mozambique. *J. Org. Chem. 63*, 1254−1258.

(7) Bewley, C. A., and Faulkner, D. J. (1994) Theonegramide, an antifungal glycopeptide from the Philippine lithistid sponge *Theonella swinhoei*. *J. Org. Chem. 59*, 4849−4852.

(8) Matsunaga, S., Fusetani, N., Hashimoto, K., and Wälchli, M. (1989) Theonellamide F. A novel antifungal bicyclic peptide from a marine sponge *Theonella* sp. *J. Am. Chem. Soc. 111*, 2582−2588.

(9) Matsunaga, S., and Fusetani, N. (1995) Theonellamides A-E, cytotoxic bicyclic peptides, from a marine sponge *Theonella* sp. *J. Org. Chem. 60*, 1177−1181.

(10) Plaza, A., Bifulco, G., Masullo, M., Lloyd, J. R., Keffer, J. L., Colin, P. L., Hooper, J. N. A., Bell, L. J., and Bewley, C. A. (2010) Mutremdamide A and koshikamides C-H, peptide inhibitors of HIV-1 entry from different *Theonella* species. *J. Org. Chem. 75*, 4344−4355.

(11) Festa, C., De Marino, S., Sepe, V., D'Auria, M. V., Bifulco, G., Debitus, C., Bucci, M., Vellecco, V., and Zampella, A. (2011) Solomonamides A and B, new anti-inflammatory peptides from *Theonella swinhoei*. *Org. Lett. 13*, 1532−1535.

(12) Festa, C., De Marino, S., Sepe, V., D'Auria, M. V., Bifulco, G., Andrés, R., Terencio, M. C., Payá, M., Debitus, C., and Zampella, A. (2011) Perthamides C-F, potent human antipsoriatic cyclopeptides. *Tetrahedron 67*, 7780−7786.

(13) Hamada, T., Matsunaga, S., Yano, G., and Fusetani, N. (2005) Polytheonamides A and B, highly cytotoxic, linear polypeptides with unprecedented structural features, from the marine sponge, *Theonella swinhoei*. *J. Am. Chem. Soc. 127*, 110−118.

(14) Ratnayake, A. S., Bugni, T. S., Feng, X., Harper, M. K., Skalicky, J. J., Mohammed, K. A., Andjelic, C. D., Barrows, L. R., and Ireland, C. M. (2006) Theopapuamide, a cyclic depsipeptide from a Papua New Guinea lithistid sponge *Theonella swinhoei*. *J. Nat. Prod. 69*, 1582−1586.

(15) De Marino, S., Ummarino, R., D'Auria, M. V., Chini, M. G., Bifulco, G., Renga, B., D'Amore, C., Fiorucci, S., Debitus, C., and Zampella, A. (2011) Theonellasterols and conicasterols from *Theonella swinhoei*. Novel marine natural ligands for human nuclear receptors. *J. Med. Chem. 54*, 3065−3075.

(16) Wada, S., Matsunaga, S., Fusetani, N., and Watabe, S. (1999) Theonellamide F, a bicyclic peptide marine toxin, induces formation of vacuoles in 3Y1 rat embryonic fibroblast. *Mar. Biotechnol. 1*, 337−341.

(17) Wada, S., Matsunaga, S., Fusetani, N., and Watabe, S. (2000) Interaction of cytotoxic bicyclic peptides, theonellamides A and F, with glutamate dehydrogenase and 17β-hydroxysteroid dehydrogenase IV. *Mar. Biotechnol. 2*, 285−292.

(18) Nishimura, S., Arita, Y., Honda, M., Iwamoto, K., Matsuyama, A., Shirai, A., Kawasaki, H., Kakeya, H., Kobayashi, T., Matsunaga, S., and Yoshida, M. (2010) Marine antifungal theonellamides target 3β-hydroxysterol to activate Rho1 signaling. *Nat. Chem. Biol. 6*, 519−526.

(19) Ho, C. H., Magtanong, L., Barker, S. L., Gresham, D., Nishimura, S., Natarajan, P., Koh, J. L. Y., Porter, J., Gray, C. A., Andersen, R. J., Giaever, G., Nislow, C., Andrews, B., Botstein, D., Graham, T. R., Yoshida, M., and Boone, B. (2009) A molecular barcoded yeast ORF library enables mode-of-action analysis of bioactive compounds. *Nat. Biotechnol. 27*, 369−377.

(20) Mozsolits, H., and Aguilar, M. (2002) Surface plasmon resonance spectroscopy: An emerging tool for the study of peptide-membrane interactions. *Biopolymers 66*, 3−18.

(21) Seddon, A. M., Casey, D., Law, R. V., Gee, A., Templer, R. H., and Ces, O. (2009) Drug interactions with lipid membranes. *Chem. Soc. Rev. 38*, 2509−2519.

(22) Hall, D. (2001) Use of optical biosensors for the study of mechanistically concerted surface adsorption processes. *Anal. Biochem. 288*, 109−125.
(23) Nussio, M. R., Sykes, M. J., Miners, J. O., and Shapter, J. G. (2007) Characterisation of the binding of cationic amphiphilic drugs to phospholipid bilayers using surface plasmon resonance. *ChemMedChem 2*, 366−373.
(24) Papo, N., and Shai, Y. (2003) Exploring peptide membrane interaction using surface plasmon resonance: Differentiation between pore formation versus membrane disruption by lytic peptides. *Biochemistry 42*, 458−466.
(25) Hall, K., Mozsolits, H., and Aguilar, M. (2003) Surface plasmon resonance analysis of antimicrobial peptide-membrane interactions: Affinity & mechanism of action. *Lett. Pept. Sci. 10*, 475−485.
(26) Mouri, R., Konoki, K., Matsumori, N., Oishi, T., and Murata, M. (2008) Complex formation of amphotericin B in sterol-containing membranes as evidenced by surface plasmon resonance. *Biochemistry 47*, 7807−7815.
(27) Swasono, R. T., Mouri, R., Morsy, N., Matsumori, N., Oishi, T., and Murata, M. (2010) Sterol effect on interaction between amphidinol 3 and liposomal membrane as evidenced by surface plasmon resonance. *Bioorg. Med. Chem. Lett. 20*, 2215−2218.
(28) Strandgerg, E., and Ulrich, A. S. (2004) NMR Methods for Studying Membrane-Active Antimicrobial Peptide. *Concepts Magn. Reson., Part A 23*, 89−120.
(29) Matsumori, N., Tahara, K., Yamamoto, H., Morooka, A., Doi, M., Oishi, T., and Murata, M. (2009) Direct interaction between amphotericin B and ergosterol in lipid bilayers as revealed by ^{2}H NMR spectroscopy. *J. Am. Chem. Soc. 131*, 11855−11860.
(30) Murari, R., Murari, M. P., and Baumann, W. J. (1986) Sterol orientations in phosphatidylcholine liposomes as determined by deuterium NMR. *Biochemistry 25*, 1062−1067.
(31) Khalifa, M. B., Choulier, L., Lortat-Jacob, H., Altschuh, D., and Vernet, T. (2001) BIACORE data processing: An evaluation of the global fitting procedure. *Anal. Biochem. 293*, 194−203.
(32) Morton, T. A., Myszka, D. G., and Chaiken, I. M. (1995) Interpreting complex binding kinetics from optical biosensors: A comparison of analysis by linearization, the integrated rate equation, and numerical integration. *Anal. Biochem. 227*, 176−185.
(33) Kessel, A., Ben-Tal, N., and May, S. (2001) Interactions of cholesterol with lipid bilayers: The preferred configurations and fluctuations. *Biophys. J. 81*, 643−658.
(34) Róg, T., and Pasenkiewicz-Gierula, M. (2003) Effects of epicholesterol on the phosphatidylcholine bilayer: A molecular simulation study. *Biophys. J. 84*, 1818−1826.
(35) Róg, T., Pasenkiewicz-Gierula, M., Vattulainen, I., and Karttunen, M. (2009) Ordering effects of cholesterol and its analogues. *Biochim. Biophys. Acta 1788*, 97−121.

Supporting Information

Interaction between the Marine Sponge Cyclic Peptide Theonellamide A and Sterols in Lipid Bilayers as Viewed by Surface Plasmon Resonance and Solid State ^{2}H NMR

Rafael Atillo Espiritu,[†] *Nobuaki Matsumori,*[*,†] *Michio Murata,*[†,‡] *Shinichi Nishimura,*[§] *Hideaki Kakeya,*[§] *Shigeki Matsunaga,*[‖] *and Minoru Yoshida*[⊥]

[†]Department of Chemistry, Graduate School of Science, Osaka University, Osaka, Japan

[‡]Lipid Active Structure Project, ERATO, Japan Science and Technology Agency, Osaka, Japan

[§]Division of Bioinformatics and Chemical Genomics, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan

[‖]Graduate School of Agricultural and Life Sciences, The University of Tokyo, Tokyo, Japan

[⊥]RIKEN Advanced Science Institute, Saitama, Japan

Contents

1. SPR sensorgrams

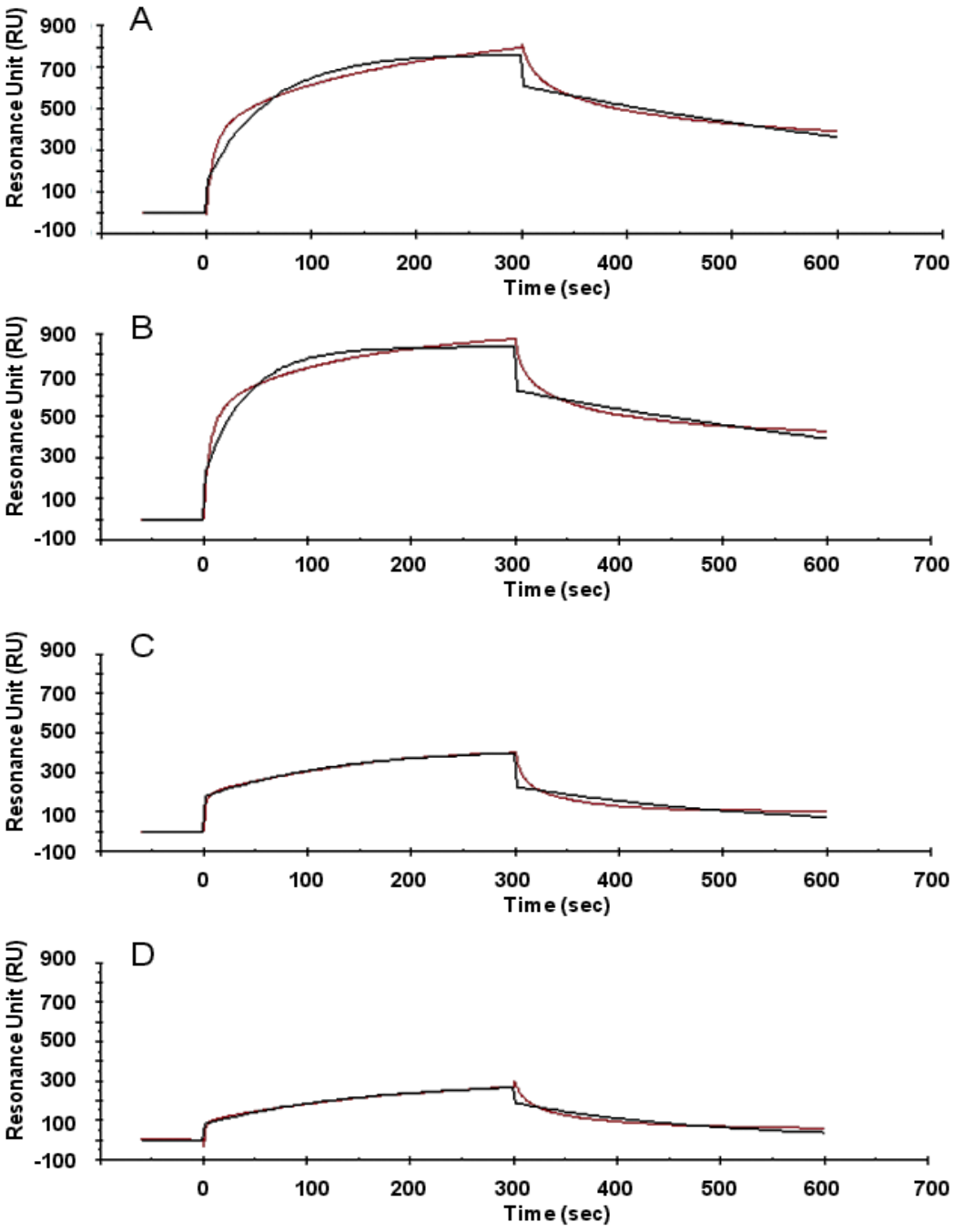

Figure S1. Curve fitting of the SPR sensorgrams using the Langmuir or 1:1 bimolecular interaction model for TNM-A (20 μM) binding to POPC liposomes captured on a modified CM5 sensor chip. (A) 10 mol% cholesterol-

containing POPC liposomes, (B) 10 mol% ergosterol-containing POPC liposomes, (C) 10 mol% epicholesterol-containing POPC liposomes, and (D) pure POPC liposomes. Red and black traces correspond to experimental and theoretical curves respectively.

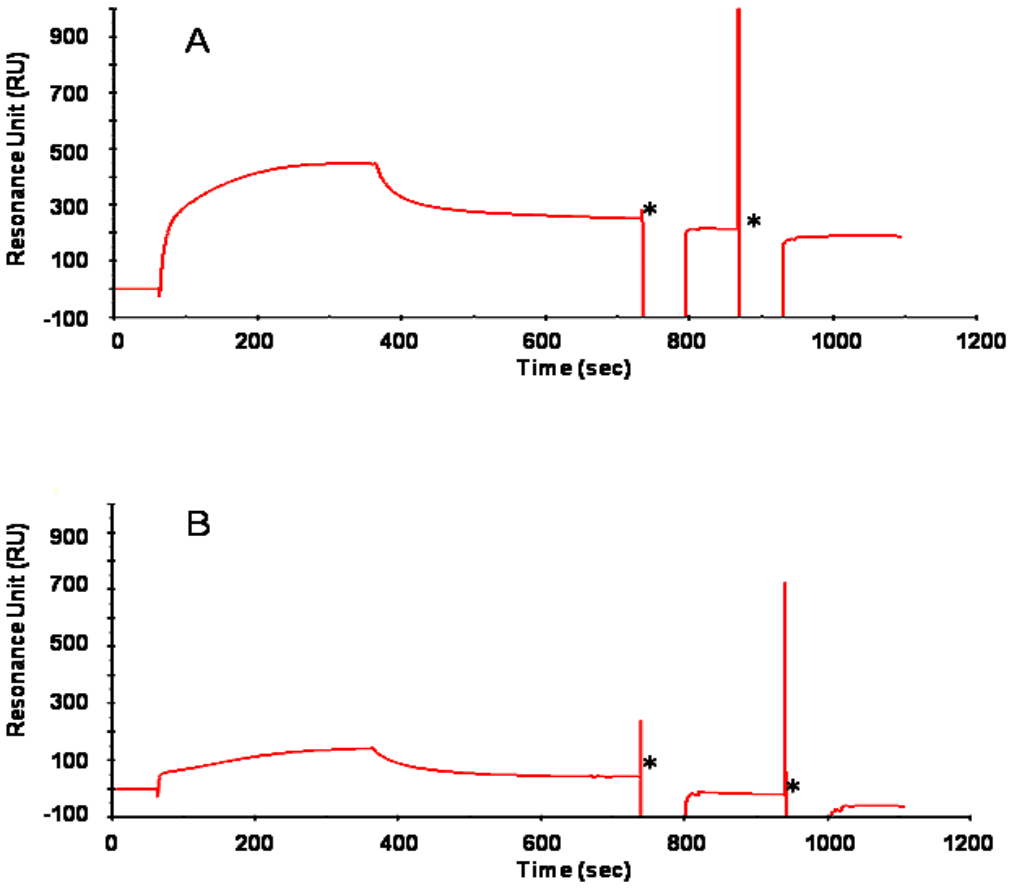

Figure S2. Regeneration of (A) 10 mol% ergosterol-containing POPC liposome and (B) pure POPC liposome surface with 50 mM NaOH. TNM-A bound to ergosterol-containing POPC liposomes were hardly removed after NaOH washing such that new liposome surface was immobilized for each TNM-A injection. Asterisk denote the point when NaOH was injected.

2. Calcein leakage assay

a. Preparation of calcein-entrapped POPC liposomes

Large unilamellar vesicles were prepared as follows. POPC or sphingomyelin (10 mg, Avanti Polar Lipids, Inc., Alabaster, AL) with or without sterol (10 mol%) was dissolved in chloroform in a round-bottom flask. The solvent was removed under vacuum for about 2 hours at 30 ºC and further dried *in vacuo* overnight. The lipid film thus obtained was rehydrated with 1 mL of 60 mM calcein in Tris-HCl containing 1 mM EDTA and 150 mM NaCl (pH 7.4) and subjected to two cycles of vortexing (1 min) and warming-up (65 ºC) followed by five cycles of freezing (-20 ºC) and thawing (65 ºC) to obtain multilamellar vesicles (MLVs). 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 prepare large unilamellar vesicles (LUVs) of homogenous size. Excess calcein was removed by passing the suspension through a Sepharose 4B column (GE Healthcare BioSciences AB, Uppsala, Sweden) with Tris-HCl containing 1 mM EDTA and 150 mM NaCl (pH 7.4). The lipid concentration in the LUV fraction was quantified using Phospholipid C-Test Wako (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Resulting stock solution was stored at 4 ºC under nitrogen gas.

b. Dye leakage measurement

Measurement of calcein leakage was performed on a JASCO FP 6500 spectrofluorometer (JASCO Corp., Tokyo, Japan) with an excitation wavelength

of 490 nm (slit 1.5 nm) and an emission wavelength of 517 nm (slit 5 nm). To monitor calcein leakage, 20 µL of the LUV suspension was diluted to 980 µL with the same Tris buffer. A 20 µL aliquot of TNM-A in MeOH:DMSO (1:1 v/v) was then added. Subsequently, 20 µL of 10% (v/v) Triton X-100 (Nacalai Tesque Inc., Kyoto, Japan) was added to obtain the condition of 100% leakage. All measurements were carried out at room temperature with a final lipid concentration of 27 µM.

c. Results

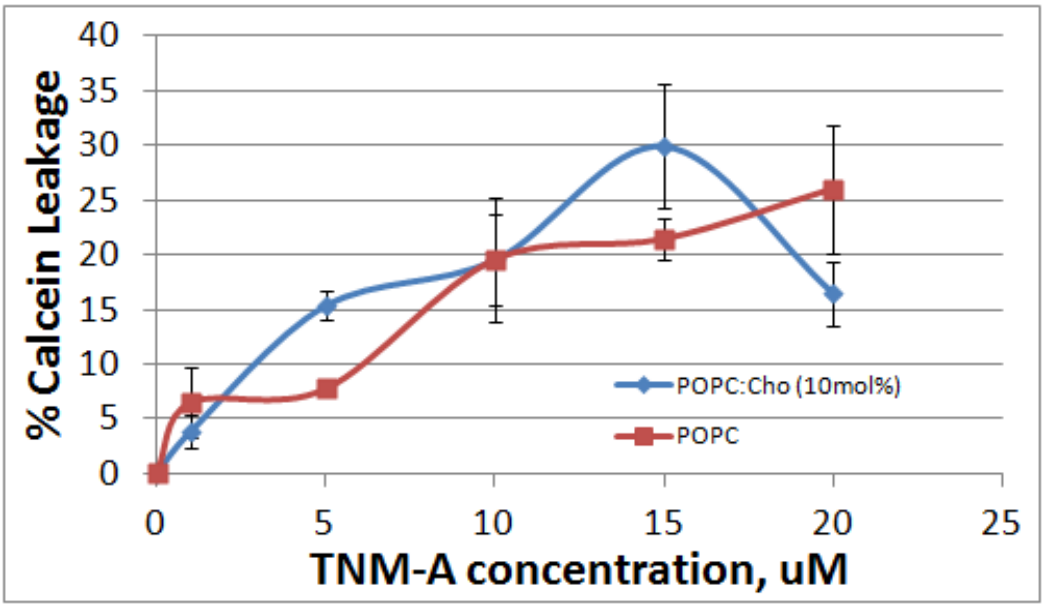

Figure S3. TNM-A induced calcein leakage from POPC liposomes.

3. Hemolysis Assay

a. Procedure

Human red blood cells (2 mL) were suspended in PBS buffer containing 8.10 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 137 mM NaCl and 2.68 mM KCl (pH 7.4) and immediately separated from the plasma by centrifugation at 2000 rpm for 5 minutes, repeated twice. The sedimented cells were re-suspended in the same PBS buffer to yield 1% hematocrit. Various concentrations of TNM-A in DMSO:MeOH (1:1 v/v, 10 μL) was then added to the red blood cell suspension (1% hematocrit, 190 μL) and incubated for 18 hours at 37 ºC. Afterwards, the mixtures were centrifuged at 2000 rpm for 5 minutes and the supernatant (50 μL) was transferred to a 96-well microplate and subjected to colorimetric measurements at 450 nm to determine the absorbance (A_{S450}) using an Emax® Precision Microplate Reader (Molecular Devices Corp., Sunnyvale, CA). Condition for 100% hemolysis was obtained by incubating the blood suspension in water (A_{T450}). The percentage of released hemoglobin from ertthrocytes was computed as (A_{S450}/A_{T450}) x 100. The concentration that caused 50% hemolysis (EC_{50}) was determined from the dose-dependent curve.

b. Results

Table S1. EC_{50} values for TNM-A-induced hemolysis in human erythrocytes.

	EC_{50}
Theonellamide-A	0.70 μM
Amphidinol 3	0.58 μM
Amphotericin B	1.62 μM