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Abstract of Thesis

Name (Kimberly Cornelio)	
Title	Elucidation of the structure based mechanism of the sterol-dependent membrane disrupting activity of the marine sponge derived peptide Theonellamide A (海綿由来ペプチド・セオネラミドAの構造に基づくステロール依存的膜撹乱活性の機構解明)

Abstract of Thesis

Sponges comprise of a large and diverse group of invertebrates under the phylum Porifera, which are considered ecologically important members of the marine benthic communities. Marine sponges have been an abundant source of new, diverse, and highly bioactive compounds. The structural diversity of compounds isolated from marine sponges provide novel leads against bacterial, viral, fungal, parasitic and cancer diseases. Bioactive metabolites isolated from sponges include terpenoids, alkaloids, macrolides, polyethers, nucleoside derivatives, and peptides to name a few.

Theonellamide-A (TNM-A) belongs to the family of antifungal bicyclic dodecapeptides known as theonellamides, isolated from the marine sponge *Theonella* sp. Although the detailed mechanism of action of TNMs is still unclear, it was recently revealed that the peptides have preferential interaction with 3β-hydroxysterols in the plasma membrane. Moreover, surface plasmon resonance measurements have indicated that its affinity to POPC liposomes is greatly enhanced in the presence of 3β-hydroxysterols and this preferential binding is revealed to be due to the peptide's direct interaction with 3β-hydroxysterols based on solid state ^{2}H NMR studies. In order to propose a mechanism underlying TNM-A's membrane activity, several aspects that could affect its membrane action were examined in this study such as its behavior in solution, interaction with 3β-hydroxysterols, and interaction with sterol-free and sterol-containing membranes.

The affinity and interaction of TNM-A with 3β-hydroxysterols was assessed by solution ^{1}H NMR titration measurements. However, due to the poor solubility of Chol in the NMR solvent of TNM-A (4:1 DMSO-d_6/H_2O), a polar Chol derivative 25-hydroxycholesterol (25-HC) was used for peptide/ 3β-hydroxysterol interaction studies. First, the suitability of 25-HC as a Chol substitute was examined using solid state NMR techniques. Through ^{2}H solid state NMR measurements, it was confirmed that TNM-A also exhibits direct interaction with 3*d*-25-HC, similarly to 3*d*-Chol and 3*d*-ergosterol. Moreover, results from ^{31}P solid state NMR indicated that TNM-A's interaction with 25-HC-containing POPC membranes also caused similar membrane perturbations as it did with chol-containing membranes as evidenced by the appearance of an isotropic peak within the powder pattern spectra characteristic of lamellar membranes upon incorporation of TNM-A to POPC/25-HC membranes. Altogether, these results indicate that 25-HC can be used as a Chol substitute in the examination of TNM-A/3β-hydroxysterol interactions. Results from ^{1}H NMR titration measurements indicated that TNM-A has moderate affinity to 3β-hydroxysterols in solution with the K_d value of about 37 μM-49 μM. Moreover, peptide protons only incurred very minor chemical shift changes ($\Delta\delta$) upon addition of 25-HC, suggesting that the electronic environment of the peptide protons remained relatively unchanged during the peptide/sterol interaction.

Interestingly, most of the TNM-A protons incurring the greatest Δδ after addition of 25-HC were confined in one region of TNM-A's sequence involving residues Iser, β-MeBrPhe, OHAsn, Asn, Apoa and sAla which points to a possible sterol interaction site of the peptide. It is highly possible that this is a consequence of the moderate affinity between TNM-A and 25-HC where the peptide/sterol complex may be exhibiting a fast association/dissociation exchange beyond the timescale of NOESY NMR detection limits. The tendency of TNM-A to form micellar aggregates in solution and in the presence of 25-HC was also examined by the pyrene 1:3 ratio method. Results indicated that TNM-A forms micelle-like assemblies in aqueous media above the peptide concentrations of ~186 μM. However, in the presence of 25-HC in solution, the formation of micelle-like assemblies by TNM-A was observed at the higher peptide concentration of ~ 299 μM. This result not only confirms the interaction of TNM-A with 25-HC but also suggest that the interaction of TNM-A/3β-hydroxysterols interaction in solution is stronger than TNM-A/TNM-A interactions.

The propensity of TNM-A to form self-aggregates in aqueous environment was also assessed by diffusion ordered spectroscopy (DOSY NMR). Results indicate that TNM-A has the propensity to form oligomeric structures in aqueous environment, with an approximate aggregation number of 2 and 9, based on peptide concentration. Moreover, the lowest concentration at which the peptide forms micelles/aggregates (~186 μM) agrees well with the results obtained from DOSY measurements (~200 μM) when minimum aggregation was detected. The effect of TNM-A in the membrane morphology of artificial membranes were also assessed through differential interference and confocal fluorescence microscopy using POPC or POPC/Cho GUVs with pure TNM-A or with 10 mol% of the fluorescent TNM derivative TNM-DCCH. Microscopy images indicate that TNM-A binds faster to Cho-containing GUVs than to sterol-free ones. More importantly, the fluorescence images revealed that TNM-A can disrupt membrane bilayers by altering membrane curvature but only to Cho-containing liposomes. In addition, TNM-A's membrane association and localization were also assessed by ^{1}H NMR paramagnetic quenching measurements using phospholipid assemblies such as SDS-d_{25} micelles and Cho-free and Cho-containing DMPC-d_{54}/DHPC-d_{22} (1:2) bicelles. Data suggest that TNM-A binds more abundantly to Cho-containing membranes and inefficiently associates with Cho-free ones. Results also indicate that when TNM-A interacts with model membranes it remains surface bound and does not penetrate, regardless of the presence or absence of Cho. However, it should be noted that when TNM-A binds Cho-containing membranes, it resides in a relatively deeper region close to the depth of the carbonyl moiety of phospholipid. On the other hand, when TNM-A associates with Cho-free membranes, it resides mostly near the most hydrated region of the membrane where the ionic headgroup of phospholipids resides. All these results obtained in this study were organized to propose a mechanism to explain the membrane activity of TNM-A.

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論文審査の結果の要旨及び担当者

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論文審査の結果の要旨

Cornelio 氏は、海洋生物の産生する活性化合物について、それらの膜作用の分子機構解明を目指して学位論文研究を行った。具体的には、海綿から単離された環状ペプチド・セオネラミド A(TMNA)の作用機構に関する研究を行なった。TNMA は顕著な抗真菌活性を有するが、その活性発現には膜中のステロールが必須であることから TNMA の生物活性が新しい作用機構の基づいていると考えられ、興味が持たれた。膜脂質と相互作用することによって膜の透過性を増大させる物質は、学術研究および応用研究の両面において大いに注目されている。したがって、過去に膨大な研究例があり、その作用様式は形式的に樽板型モデルやトロイダル型モデルなどに分類されているものの精密な会合体構造や会合様式を解明した例は、両新媒性 α ヘリックスを主体とする抗菌ペプチドなどに限られており、大部分の天然物について未解明である。

Cornelio 氏は、TNMA の脂質二重膜上での動的挙動を固体・液体 NMR を利用して精密に解析した。まず、DOSY 法を用いて TNMA の拡散係数を測定し、その会合体形成を評価した結果、溶液中では TNMA が 2－7 量体を形成することを明らかにした。これは TNMA の生物活性に特徴的な長時間にわたる膜変形作用に関係する可能性が高い。また、ステロール誘導体と TNMA の結合親和力について溶液 NMR を用いて解析し、ステロールとの存在比に依存して TNMA のシグナルの化学シフト値が変化することを利用してその結合定数を見積もった。また、常磁性金属であるマンガン塩を NMR 試料中に徐々に加えていくという手法によって、バイセルやミセルの中に取り込まれた TNMA と溶液中に存在するものの割合を調べ、さらにバイセルの脂質二重膜部分に結合した TNMA の深度を推定した。その結果、TNMA がステロール依存的に二重膜外膜の比較的浅いところに結合し、ステロール含有膜の曲率を大きく増加させることを突き止めた。これらの知見から Cornelio 氏は、環状ペプチドの 3 カ所に疎水的側鎖が置換する特異な構造によって、TNMA の膜透過化作用が発現しており、その作用機構は従来の抗菌ペプチド機構とは異なることを示した。これらの知見は、膜作動性天然物の作用機構研究に新たな観点をもたらすとともに、実験手法の確立という点でも顕著な成果であると認められた。

このように Cornelio 氏の研究は、特徴的な膜透過化作用を有する生物活性天然物の研究を推進し、貴重な新知見を得ると同時に実験手法の有効性を示した点において顕著な学術的意義を有するものと考える。以上のように、本論文は博士（理学）の学位論文として十分価値あるものと認める。