Structure and Biological Activity of 8-Deoxyheronamide C from a Marine-Derived *Streptomyces* sp.: Heronamides Target Saturated Hydrocarbon Chains in Lipid Membranes

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S *Supporting Information*

ABSTRACT: Polyene macrolactams are a class of microbial metabolites, many of which show potent biological activities with unidentified modes of action. Here we report that 8-deoxyheronamide C, a new 20-membered polyene macrolactam from a marine-derived actinomycete *Streptomyces* sp., is a unique membrane binder. 8-Deoxyheronamide C showed a characteristic sensitivity profile against fission yeast sterol mutant cells, indicating that the metabolite targets cell membranes. We detected tight physical interaction between heronamides including 8-deoxyheronamide C and heronamide C and saturated hydrocarbon chains in lipid membranes using surface plasmon resonance experiments. We further show that heronamides induced abnormal cell wall morphology in fission yeast probably by perturbing the structure of membrane microdomains. This work will accelerate the biological and medical investigation of polyene macrolactams.

Nature seems to have facilitated survival of living things possessing defensive mechanisms against competitive organisms; production of antibiotics is one such survival method.[1−4] Polyene macrolactams are a class of bioactive metabolites produced by actinomycetes (Figure S1). They exhibit a variety of biological activities, including antimicrobial[5] and antitumor activities,[6,7] modulation of Bcl-xL function,[8] and inhibition of leukocyte adhesion.[9] There have been no reports, however, on the identification of their target molecules.

The cell membrane is a common target of antibiotics, and a variety of chemical classes are known to bind membrane lipids, e.g., polyene macrolides, peptides, lipopeptides, and saponins, some of which have been utilized as medicine. Small molecules that exhibit characteristic phenotypes upon binding to membrane lipids could be used as chemical tools to dissect the structure and function of cell membranes. Most antibiotics, however, quickly damage the cell membrane and are not suitable for a chemical genetics approach. To obtain novel membrane-targeting natural products, we focused on a traditional chemical genetic interaction (Figure S2); mutant yeast cells lacking ergosterol biosynthetic genes, as represented by *erg2* and *erg3*, are tolerant to lipid-binding antibiotics such as

amphotericin B (AmB) and syringomycin E.[10,11] We screened microbial culture extracts and identified a marine-derived actinomycete *Streptomyces* sp., whose culture extract showed less toxicity to the fission yeast cells lacking *erg2* gene than to wild-type cells (Figure S3). Bioassay-guided fractionation afforded 8-deoxyheronamide C (8-dHC, **1**), a new 20-membered polyene macrolactam as an active constituent (Chart 1). Metabolite **1** is a putative biosynthetic precursor

Chart 1. Chemical Structures of Heronamides
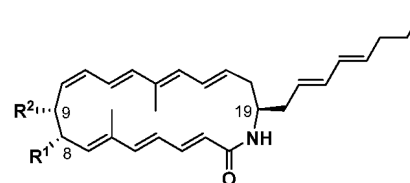
8-deoxyheronamide C (8-dHC, **1**): R[1] = H, R[2] = OH
heronamide C (**2**): R[1] = R[2] = OH
heronamide C diacetate (**4**): R[1] = R[2] = OAc
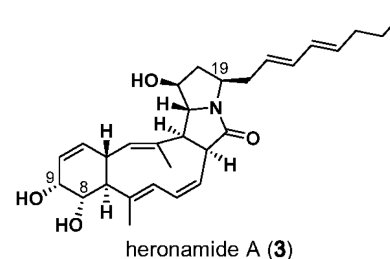
heronamide A (**3**)

of the polyene macrolactam heronamide C (**2**).[12] In fact, the microbial culture also contained **2** and heronamide A (**3**), both of which were originally reported from an Australian marine-derived actinomycete (Figure S4).[12]

The chemical structure of **1** was deduced by spectroscopic analysis and a chemical conversion experiment inspired by the proposed biosynthetic pathway.[12] The planar structure of **1** was determined as an 8-deoxy congener of heronamide C (**2**) by MS and NMR experiments (Supplementary method; Figures

S5 and S6; Table S1). The relative stereochemistry of **1** was deduced by interpretation of the NOESY spectrum (Figure S7). NOESY analysis was also conducted for **2**, revealing that **1** and **2** have the same relative stereochemistry at C9 and C19. This was supported by the characteristic Cotton effect in their CD spectra (Figure S7), simultaneously indicating that the absolute stereochemistry was shared between metabolites **1** and **2**. Next we investigated the biosynthetic mechanisms. A biosynthetic study of a closely related macrolactam metabolite, BE-14106 (Figure S1), revealed that the C8 hydroxyl group was introduced by cytochrome P450 monooxygenase following formation of the macrolactam ring.[13] In fact, an 8-deoxy congener of BE-14106 has been reported.[14] It is likely that **1** is a biosynthetic precursor of **2**, itself being proposed to undergo epoxidation and cyclization to afford **3** (Figure S4).[12] Based on our previous report on the stereochemistry of **3**,[15] we predicted that **2** possessed a stereochemistry of 8S, 9R, 19R. Interestingly, it was discovered that **2** spontaneously converted to **3** in DMSO, confirming that the absolute stereochemistry is conserved between metabolites **2** and **3** (Figure S8). In combination, these results allowed us to conclude that the absolute configuration of **1** is 9S, 19R.

Metabolite **1** inhibited the growth of wild-type fission yeast cells with a minimum inhibitory concentration (MIC) of 5.8 μM. Growth inhibitory activities were also examined against cells mutant for ergosterol biosynthesis: cells lacking *erg2*, *erg31* and *erg32*, *erg5* or *sts1/erg4* gene (Figure S2). Interestingly, all mutant cells were tolerant to **1**; growth of mutant cells was observed even in the presence of 46 μM of the metabolite (Figure 1a). Next we examined the structure–activity relation-
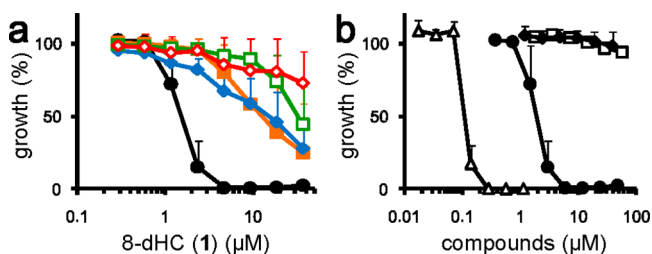

Figure 1. Sensitivity profile of heronamides to yeast sterol mutant cells. (a) Tolerance of ergosterol mutant cells to 8-dHC (**1**). Growth of wild-type (black), Δ*erg2* (red), Δ*erg31*, Δ*erg32* (blue), Δ*sts1/erg4* (orange), and Δ*erg5* (green) cells were measured in the presence of various concentration of 8-dHC (**1**). (b) Effect of heronamides on wild-type fission yeast cells. Cells were incubated with compound **1** (circle), **2** (triangle), **3** (square), or **4** (diamond) for 24 h, and cell growth was measured. Data represent the mean of three independent experiments. Error bars indicate SD.

ship (SAR) of heronamides. Metabolite **2** exhibited more potent growth inhibitory activity (MIC, 0.28 μM) than metabolite **1**, whereas acetylated heronamide C (**4**) lost growth inhibitory activity, thus implying the importance of the hydroxyl groups (Figure 1b). Metabolite **3** exhibited no growth inhibition (Figure 1b), suggesting that the 20-membered macrolactam ring was also essential for the biological activity. Metabolite **2** showed almost no specificity; MIC values for *erg* mutants were 0.28 μM, which was identical to the activity displayed against the wild-type cells (Figure S9).

Yeast ergosterol mutants have been reported to show tolerance to membrane-targeting antibiotics such as AmB,[10,11] indicating that metabolite **1** targets cell membrane. Mutant cells

were still sensitive to metabolite **2**, nonspecificity however does not preclude the possibility that metabolite **2** targets the cell membrane. For example, *erg* mutant cells were not tolerant to amphidinol 3 that specifically recognizes membrane sterols (Figure S10).[16] These results suggested that heronamides **1** and **2** might be unique membrane binders.

To investigate the affinity between heronamides and lipid membranes, we conducted surface plasmon resonance (SPR) experiments. We used a Biacore CM3 chip, in which liposomes were immobilized on dodecylamine attached via condensation through the amide bond (Supplementary methods). Using this system, we successfully detected the significantly higher affinity of AmB to 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) liposomes containing 20 mol % ergosterol, compared with POPC liposomes alone (Figure S11). In analogy with sterol-targeting molecules, e.g., AmB[17] and theonellamide A (TNM-A),[18] we initially expected that **1** would show higher affinity to sterol-containing liposomes. Contrary to our expectations, however, this was not the case for heronamides: both **1** and **2** displayed only weak binding to POPC membranes, which were not influenced by the presence of ergosterol (Figures 2a, S12).

The type of acyl groups present in phospholipids is critical to the nature of the membrane, e.g., fluidity. For this reason we examined liposomes consisting of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) or 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC); DOPC has two unsaturated acyl chains, whereas DMPC has two saturated ones. When DOPC liposomes containing ergosterol were immobilized on the sensor chip, only weak binding of **1** was observed, which was comparable to that of POPC membranes (Figure S12). In contrast, liposomes consisting of DMPC exhibited drastically increased binding affinity to **1**. Although the increase in the resonance unit was only several-fold, the resonance unit linearly increased during the association phase, and almost no dissociation was observed (Figure 2a). This indicated that **1** binds to the DMPC liposomes, in an irreversible manner.

Phospholipids of fission yeast contain primarily 18:1 fatty acyls.[19] In contrast, the predominant type of sphingolipid was reported to be t18:0/26:0, consisting of phytosphingosine and a long saturated fatty acid. To examine the possibility that sphingolipids are targeted by **1**, we tested the binding to sphingomyelin (SM) liposomes. As expected, irreversible binding of **1** to SM liposomes was observed, and its affinity was comparable to DMPC (Figure 2a). It is worth noting that inclusion of ergosterol or cholesterol did not affect the affinity pattern of **1** toward lipid membranes (Figures S12 and S13). All experiments were conducted at 30 °C. At this temperature DMPC membranes display a liquid disordered phase, whereas DMPC membranes containing cholesterol are a mixture of liquid disordered and ordered phases. SM membranes are in a gel phase. This indicates that the membrane fluidity is unlikely to affect the affinity of **1**. Rather, **1** seems to favor lipid molecules possessing saturated hydrocarbon chains.

Heronamides exhibited growth inhibition of fission yeast cells; its potency was dependent on the number of hydroxyl groups and presence of a 20-membered macrolactam ring (Figure 1b). We subsequently investigated the SAR of heronamides in SPR experiments. Metabolite **2**, that showed growth inhibitory activity against wild-type cells 10 times more potent than the deoxy congener **1**, exhibited 8.4 times higher affinity to DMPC membranes (Figure 2b). In contrast, protection of the hydroxyl groups eliminated not only the
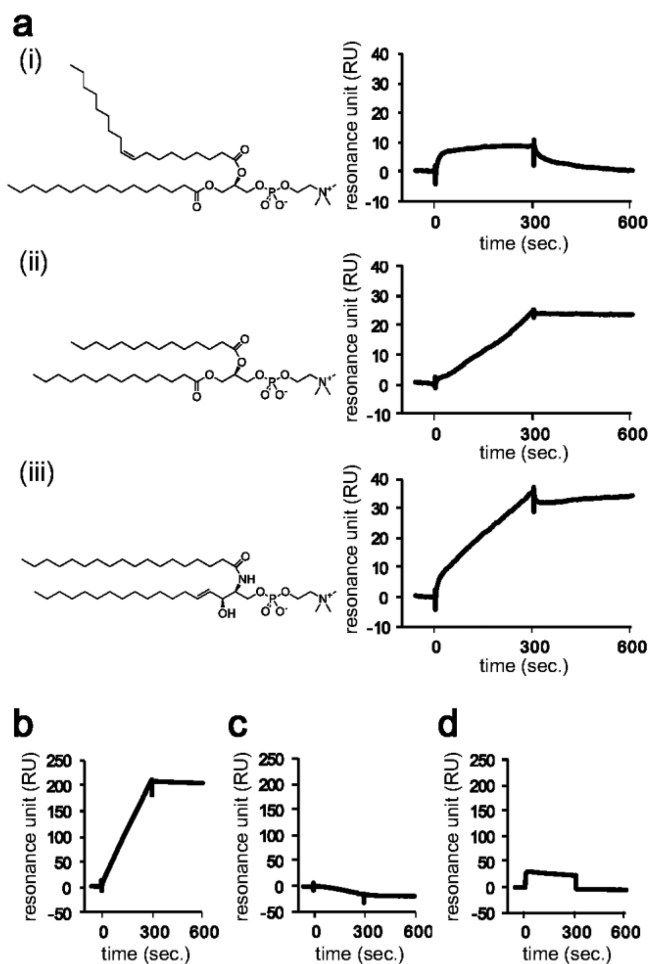
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Figure 2. Affinity of heronamides to liposomes. (a) Chemical structures of the phospholipids used for the SPR experiments and sensorgrams for binding of 8-dHC (**1**) to liposomes. 20 mol % ergosterol-containing (i) POPC and (ii) DMPC liposomes and (iii) 20 mol % cholesterol-containing SM liposomes were captured on a dodecylamine-modified CM3 sensor chip. (b–d) Sensorgrams for binding of heronamides to 20 mol % ergosterol-containing DMPC liposomes. The affinity of (b) heronamide C (**2**), (c) heronamide C diacetate (**4**), and (d) heronamide A (**3**) are compared. Similar results were obtained for more than three experiments, and the characteristic sensorgrams are shown. Elution of 20 μM heronamides was started at time 0 and maintained for 300 s. Flow rate was 10 μL/min.
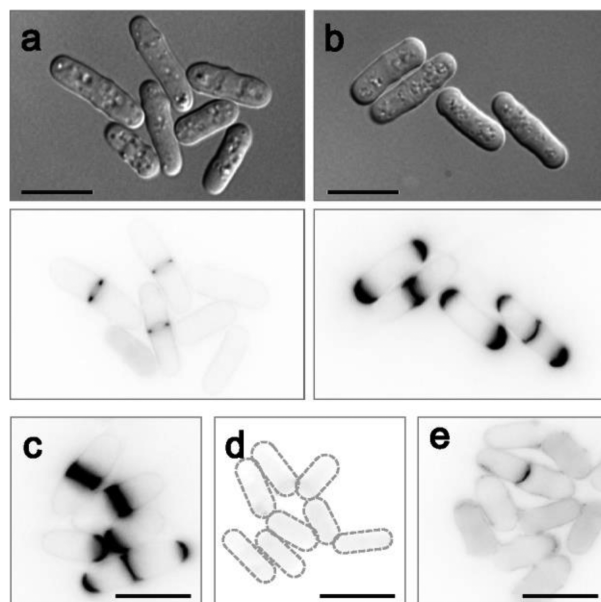

Figure 3. Cell wall abnormalities induced by heronamides. (a–c) Abnormal cell wall morphology induced by heronamides. Wild-type cells were exposed to DMSO (a, 1% v/v, solvent for compounds), 8-dHC (**1**) (b, 10 μM), or heronamide C (**2**) (c, 1 μM) for 2 h. (d–e) Involvement of Rho1 and Bgs1 in the action of 8-dHC (**1**). Cells expressing dominant-negative Rho1 protein, Rho1T20N (d) or bgs1 temperature-sensitive mutant cells with lowered glucan synthase activity (e) were treated with 8-dHC (**1**) (10 μM). In all experiments, cells were fixed with formalin and stained with calcofluor white (Cfw) to visualize cell wall material. In a–b, differential interference contrast (DIC; upper) and Cfw (lower) images are shown. DIC images for (c–e) are included in Figures S15 and S16. Scale bars indicate 10 μm.

growth inhibition but also the affinity to lipid membranes (Figure 2c). In addition, metabolite **3**, an inactive congener, showed a box-type sensorgram, indicating its weak and nonspecific binding to the lipid membrane (Figure 2d).

Finally, we carried out morphological analysis to investigate the consequence of heronamide binding to yeast cell membranes. We were unable to detect significant damage in bright-field images following treatment of cells with **1** or **2**. Such damage would include formation of large vacuoles, a characteristic effect of polyene antibiotics that target membrane ergosterols in yeast (Figure S14). This result supported the data obtained in the SPR experiments; response curves of heronamides were seldom affected by the inclusion of ergosterol (Figures 2, S12, and S13). Instead, metabolite-treated cells exhibited slightly abnormal septa. Detailed analysis revealed that heronamides **1** and **2** induced accumulation of cell wall material at both cell tips and septa (Figure 3a–c).

Importantly, *css1* temperature-sensitive mutant cells are known to display similar morphological changes when placed under restriction temperature (Figure S15); *css1* gene encodes sphingolipid-phospholipase C.[20] It is likely that heronamides and *css1* mutation have a similar effect on the cell membrane by perturbing the function of sphingolipids. Interestingly, similar cell wall defect is induced by TNM-A (Figure S15). We have reported that TNMs induce accumulation of 1,3-β-glucan by binding to membrane ergosterol, and the phenomenon is dependent on Bgs1, a putative 1,3-β-glucan synthase catalytic subunit, and a small GTPase Rho1.[21] This was also the case for cell wall abnormalities induced by heronamides. Neither cells expressing dominant-negative mutant Rho1 protein (Rho1T20N) nor *bgs1* mutant cells possessing low glucane synthase activity showed abnormal accumulation of cell wall material after treating cells with heronamides (Figures 3d–e, S16).

Lipid rafts are tightly packed sphingolipid and sterol-rich liquid-ordered membrane domains.[22] In this study, heronamides have been revealed to favor lipid molecules with saturated hydrocarbon chains, e.g., DMPC and SM. TNMs are known to bind membrane sterols, whereas the *css1* gene product regulates sphingolipid metabolism. It is likely that cell wall biosynthesis requires functional lipid domains consisting of sphingolipids and sterols, which were perturbed by treatment with heronamides or TNMs or mutation of the *css1* gene (Figure S17). In fact, Bgs1 is a membrane protein containing 13 potential transmembrane helices and was shown to be insoluble in nonionic detergents,[23] one of the characteristics of lipid-raft

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proteins. It is noted that heronamide treatment or *css1* mutation slowly induced accumulation of cell wall material (Figure S15), whereas TNM-A induced a similar phenomenon quickly, suggesting that the role of sphingolipids and sterols in membrane microdomains can be distinguished using a chemical genetics approach.

Not only sphingolipid mutant but also ergosterol mutant cells become less sensitive to syringomycin E, whose channel forming activity is regulated by sphingolipids in artificial model membranes.[24] This suggests that modulation of sterol species affects the activity of membrane sphingolipids. This can be the cause for the tolerance of ergosterol mutant cells to 8-dHC. However, modulation of sphingolipid activity might be moderate, which is not effective enough for cells to obtain tolerance to heronamide C that has a higher affinity to lipids. Alternatively, we cannot exclude the possibility that ergosterol contributes to the action of 8-dHC in cell membranes. The role of the hydroxyl group at C8 for membrane affinity is under investigation.[25]

In summary, we present the structures, biological activities, and membrane affinities of heronamides. Heronamides are 20-membered macrolactam metabolites. Their molecular size suggests that heronamides can act as pseudosterols. Cholesterol, e.g., has a preference for interaction with lipids that have fully saturated hydrocarbon chains when compared with lipids that have unsaturated chains.[26] In this context, the membrane affinity of other polyene macrolactams, whose ring size varies from 18 to 26, is of interest. Finally, introducing a hydroxyl group at C8 to 8-deoxyheronamide C (**1**) was shown to produce a highly active metabolite, suggesting the post-polyketide synthase hydroxylation enzyme responsible for the hydroxylation has proved evolutionarily favorable to the organism.

ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details, structure elucidation, and biological and biophysical evaluations. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(25) Chemical structures of heronamides imply that hydrophobic ring and tail are inserted into the lipid membranes, whereas hydroxyl groups are located in the shallow area of the membranes. The presence of hydroxyl groups might stabilize the position of heronamides probably by hydrogen bonding with lipid head groups, which can explain the difference of the potency of metabolites **1** and **2**. Alternatively, hydroxyl groups could accelerate membrane binding or insertion through unrevealed mechanisms.
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