Pyrrolactams from Marine Sponge *Stylissa massa* Collected from Myanmar and Their Anti-Vpr Activities

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A new brominated pyrrolactam *stylissaol A* (1) together with four known analogues, 2-bromoaldisine, aldisine, spongiacidin D, and Z-hymenialdisine, were isolated from the EtOAc extract of marine sponge *Stylissa massa* collected in Myanmar. The absolute configuration at C-10 of 1 was determined as Z-hymenialdisine, were isolated from the EtOAc extract of marine sponge *Stylissa massa* collected in Myanmar. The absolute configuration at C-10 of 1 was determined as

**Key words** *Stylissa massa*; pyrrolactam; stylissaol A; TREx-HeLa-Viral protein R (Vpr)

**Introduction**

Viral Protein R (Vpr) is a small basic protein (14kDa), and is well conserved in human immunodeficiency virus (HIV)-1, HIV-2, and simian immunodeficiency virus (SIV). Vpr was reported as a promising drug target for a comprehensive AIDS therapy. 1, 2 In order to find Vpr inhibitors, we have established an assay system where we developed tetracycline regulated TREx-HeLa-Vpr cells and studied the inhibition effect of compounds on Vpr by monitoring the proliferation rate of cells.3 By using this assay, we identified a number of natural small molecules including isopimarane diterpenoids, 4 quassinoids, 5 iridoid and bis-iridoid glucosides, 6 xanthone, 7 and triterpenoid 8 from medicinal plants of Myanmar as potent Vpr inhibitors. 9

Interestingly, as infectious microorganisms evolve and develop resistance to existing pharmaceuticals, marine sponges provide novel leads against bacterial, fungal, and viral diseases, although the availability of more sensitive and different bioassay systems to date will be needed for drug discovery against these diseases. Myanmar is a country with a rich source of not only medicinal plants, but also marine sponges. However, there is no report regarding natural products from sponges of Myanmar origin, except for our previous study. 9

In our previous pioneer study, we identified two new pyrrolactams from a Myanmar marine sponge, *Clathria prolifera*. 9 In a continuous work, we found that an EtOAc extract of *Stylissa massa* collected in Myanmar exhibited potent Vpr inhibitory activity against the established cell line, TREx-HeLa-Vpr. *S. massa* is in a Demospongiae class and belongs to the family Scopalinidae. It is widely distributed in Andaman Sea Coral Coast, Indian Ocean, and South China Sea. A number of constituents such as cycloheptapeptides, 10, 11 bromopyrrole alkaloids, 12 and amphiamecane diterpenes 13 have been discovered from *S. massa* and they were reported as antiproliferative, protein kinase USP7, and nitric oxide (NO) inhibitors. 10, 11, 12

Herein, the isolation, structural elucidation, and the anti-Vpr activities of the active EtOAc extract and isolated chemical constituents are described.

**Results and Discussion**

Screening of the extracts of *S. massa* sponge collected in Myanmar against TREx-HeLa-Vpr cells revealed that the EtOAc extract possessed the anti-Vpr activity, with an effective dose of 10 µM and its potency was comparable to that of positive control damnacanthal.

![Fig. 1. The Structures of Isolated Pyrrolactam Alkaloids (1–5) from *S. massa* Sponge](image_url)
HRESIMS in conjugation with $^{13}$C-NMR. The UV spectrum showed the absorption maximum at 272 nm, suggesting the presence of the pyrrole chromophore in conjugation with the carbonyl group. The Fourier transform (FT)IR spectrum of 1 showed absorption bands at 1680 and 1626 cm$^{-1}$ due to the presence of the carbonyl and amide groups, respectively. The $^1$H-NMR spectrum of 1 showed signals corresponding to one olefinic proton [$\delta_H$ 6.20 (d, $J = 2.5$ Hz, H-2)], one oxygenated methine [$\delta_H$ 4.65 (m, H-10)], two methylene protons, two exchangeable NH protons [$\delta_H$ 7.72 (t, $J = 4.5$ Hz, NH-7), 11.89, s (NH-1)], and one exchangeable hydroxy proton [$\delta_H$ 5.12 (d, $J = 5.7$ Hz, OH-10)] (Table 1). The $^{13}$C-NMR spectrum of 1 in combination with $^1$H-detected heteronuclear multiple quantum coherence (HMQC) spectrum displayed eight carbon signals, including one amide carbon [$\delta_C$ 162.1 (C-6)], four sp$^2$ olefinic carbons [$\delta_C$ 103.2 (C-3), 112.4 (C-2), 123.2 (C-5), 130.7 (C-4)], one oxygenated methine carbon [$\delta_C$ 65.0 (C-10)], and two methylene carbons [$\delta_C$ 35.7 (C-8), 36.0 (C-9)] (Table 1). Its $^1$H- and $^{13}$C-NMR spectroscopic data were very similar to those of axinellin B, a pyrrolactam alkaloid isolated from the Chinese marine sponge Axinella sp. $^{17}$ The significant difference is the position of the bromo group. In axinellin B, an olefinic methine proton ($\delta_H$ 6.21) was appeared as singlet due to the presence of bromo group at C-2 of the pyrrole ring, $^{18}$ whereas the olefinic methine proton in 1 was appeared as doublet [$\delta_H$ 6.20 (d, $J = 2.5$ Hz)]. This appearance was consistent with that found in 3-bromoaldisine. $^{18}$ Thus, the bromo group was placed at C-3 on the pyrrole ring. The $^1$H $^1$H correlation spectroscopy (COSY) correlation between H-2 and NH-1 further supported this assumption (Fig. 2). The heteronuclear multiple bond connectivity (HMBC) correlations from NH-1 to C-2, from H-2 to C-3/C-4, H-10 to C-5/C-8, and from H-9 to C-6 assisted the connection between the pyrrole ring and lactam ring at C-4 and C-5, indicating that 1 is a pyrrolactam alkaloid. The absolute configuration at C-10 was determined on the basis of the comparison of the experimental and calculated electronic circular dichroism (ECD) data. The experimental ECD spectrum of 1 exhibited the negative Cotton effects (CE) at 280 nm and positive CE at 272 nm, which were in accordance with the calculated ECD data for the model compound of 1 with 10R configuration, using the time-dependent density functional theory (TDDFT) method $^{19}$ (Fig. 3). Thus, the absolute structure of 1 was elucidated as shown in Fig. 1 and 1 was named stylissaol A.

The structures of the known compounds 2–5 were elucidated by the analyses of their experimental NMR spectroscopic data as well as by comparing with the reported data. The secondary metabolites of S. massa native to the marine biosphere of Myanmar are not different from those found in China, India, Japan and Australia. Pyrrolactam derivatives found in S. massa reportedly possessed various biological activities such as anti-cancer, $^{18,20,21}$ antibacterial, $^{22}$ anti-inflammatory, $^{23}$ and neuroprotective activities. $^{13}$ In the present study, we evaluated the Vpr inhibitory activities of the isolated brominated pyrrolactams (1–2, 4, 5) and pyrrolactam (3) against Vpr-transfected and tetracycline regulated HeLa (TREx-HeLa-Vpr) cells. Of the tested compounds, 2-bromoaldisine (2) was the strongest inhibitor exhibiting anti-Vpr activity at 10 $\mu$M [Cell proliferation (%): 134] and its potency was comparable to that of the positive control damnacanthal [Cell proliferation (%): 158]. In contrast, non-brominated pyr-
rolactam, aldisine (3), did not display the Vpr activity. Thus, the bromine substituent at C-2 of the pyrrole ring may be important for activity. However, even though bromine is present at C-2, the additional amino-imidazolinone or the hydantoin ring linking to the pyrrolactams led cytotoxicity to TREx-HeLa-Vpr cells [Cell proliferation (%) at 10 μM: 22 (4), 86 (5)] (Fig. 4).

Conclusion
Biochemical studies of Myanmar marine sponge, S. massa was investigated for the first time. The EtOAc extract was found to be active against TREx-HeLa-Vpr cells with an effective dose of 10 μg/mL. The isolation of the active EtOAc extract afforded a new brominated pyrrolactam, stylissaol A (1), together with four known analogues. The 2-bromoaldisine (2) was identified as the most active Vpr inhibitor. The position of bromine group at C-2 of the pyrrole ring might be important for brominated pyrrolactam in inhibiting the effect of Vpr where favoring the proliferation of TREx-HeLa-Vpr cells.

Experimental
General Experimental Procedures Optical rotations were recorded on a Jasco P2100 polarimeter. CD measurements were carried out on a Jasco J-805 spectropolarimeter. UV spectra were measured on a NANODROP 2000c spectrophotometer (Thermo Scientific, MA, U.S.A.). FTIR spectra were recorded as KBr pellets on a Jasco FT/IR-460 Plus spectrometer. NMR spectra were recorded at 400 MHz (1H-NMR) and 100 MHz (13C-NMR) on a Jeol JNM-ECX400 spectrometer. The chemical shifts were calibrated to the residual proton and carbon resonances of the deuterated solvent used for each compound measurement (δH 2.50 ppm and δC 39.5 ppm for dimethyl sulfoxide (DMSO)-d6). The HRESIMS was recorded on a Shimadzu LCMS-IT-TOF spectrometer. Open column chromatography was performed with Diaion HP-20 resin, normal-phase silica gel (silica gel 60N, spherical, neutral, 40–50 μm, Kanto Chemical Co., Inc., Japan) and Cosmosil 75C18-OPN (Nacalai Tesque Inc., Kyoto, Japan). TLC was performed on precoated silica gel 60F254 and RP-18F254 plates (Merck, 0.25 or 0.50 mm thickness). An SH-1200 Microplate Reader® (Corona, Hitachinaka, Japan) was used to measure the absorbance in the bioassays. The cell line, TREx-HeLa-Vpr was maintained in our laboratory. Cell culture flasks and 48-well plates were purchased from Corning Inc. (Corning, NY, U.S.A.).

Animal Material
The marine sponge, S. massa, was collected from Than-kyune-nge Island, Kawthaung, Tanintharyi Region, Myanmar in March 2017. The sponge was identified by Lecturer Dr. Aung Aung Aye (Department of Marine Sciences, Myeik University) and was air dried. A voucher sample (No. KS-3) is maintained at Department of Marine Sciences, Myeik University, Myanmar.

Extraction and Isolation
The dried powder of S. massa (1 kg) were percolated in MeOH (5L × 90 min × 3) at room temperature, and the solvent was evaporated under reduced pressure to give 100 g of a MeOH extract. The MeOH extract (100 g) was subjected for further chemical isolation and identification.

The EtOAc extract (3 g) was subjected to silica gel column chromatography, eluted with 1L each of CHCl3–MeOH (30:1, 20:1, 15:1, 10:1, 5:1, 3:1) to give six fractions [fr. 1 (388 mg), fr. 2 (385 mg), fr. 3 (127 mg), fr. 4 (350 mg), fr. 5 (650 mg), fr. 6 (700 mg)]. Recrystallization of fr. 1 (388 mg) in MeOH afforded 2 (100 mg) as colorless crystals. Compound 3 (50 mg) was directly obtained as precipitated form from fr. 2 (385 mg). Fraction 3 (127 mg) was rechromatographed
on Cosmolis 75 CI8-OPN using an isocratic solvent system of MeOH–H₂O (2:3) to afford three subfractions [3-1: 100 mg; 3-2: 65 mg; 3-3: 45 mg]. Purification of subfraction 3:1 (100 mg) by normal-phase column chromatography using CHCl₃–MeOH (7:1) solvent system gave precipitate of 3:1 (100 mg) by normal-phase column chromatography using MeOH (10:1) solvent afforded 3:1 (100 mg). Compound 5 (4 mg, Rf = 0.51) was obtained by purification of subfraction 3:1 (65 mg) by reverse-phase TLC using a MeCN–H₂O (1:2) solvent system. Purification of subfraction 3:1 (45 mg) by normal phase preparative TLC using a CHCl₃–MeOH (10:1) solvent afforded 1 (2 mg, Rf = 0.30).

**Styliassol A (1):** amorphous powder; [α]₂⁰° −18.3 (c 0.1, MeOH); UV (MeOH) λ_max (log ε) 210 (2.5), 230 (2.7), 272 nm (2.9); FTIR (KBr) ν_max 3278, 2926, 1680, 1626, 1483, 1438, 1025 cm⁻¹; CD (c 4.08 × 10⁻⁴ M, MeOH) [θ]₂₃⁰ −16509.41, [θ]₁₇₂ +7736.14. 

**ECD Calculation** The eight conformers with low-energy of 1 were produced by the DFT/TDDFT calculations, Merck Molecular Force Field (MMFF94) using Win notchor program. All DFT/TDDFT calculations were conducted using the Gaussian 16 program. Firstly, each low-energy conformer obtained by MMFF calculations was subjected to geometry optimization by the DFT method at the B3LYP/6-31G(d) level. Each optimized conformer was then subjected to a frequency calculation at the B3LYP/6-31G(d) level in order to estimate the thermal free energy (ΔG) and check for the presence of imaginary frequencies. On the basis of the estimated thermal energy, the abundance ratio of each conformer was calculated by the Boltzmann distribution. Finally, three conformers of 1 (in total, occupying approximately 99%) were selected for ECD calculations. The ECD spectra of all conformers were calculated using the TDDFT method at the B3LYP/6-31G(d) level with PCM in methanol, and the weighted-average spectra were compared with the experimental ECD spectra recorded in methanol.

**In Vitro Anti-Vpr Activity** We used the cell line TREx-HeLa-Vpr, which was established in our lab.³ The in vitro anti-Vpr activities of the extracts and the isolated compounds were determined according to the procedure described previously.³,⁵,³³

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**Conflict of Interest** The authors declare no conflict of interest.

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**Supplementary Materials** The online version of this article contains supplementary materials.

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