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Identification of two pigments as possible antiviral agents from the sponge *Amphimedon* sp.

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Abstract

As part of our project searching for antiviral molecules, a lipophilic extract of a sponge *Amphimedon* sp. collected at Iriomote Island was found to show antiviral activity against HCV. After bioassay-guided chromatographic separation, two pigments from the extract, mytiloxanthin (**1**) and chlorophyllone a (**2**), were identified as possible antiviral agents.

Introduction

Diseases caused by viruses are prevalent worldwide, and even apart from COVID-19, there is great demand for new antiviral molecules and treatment therapies. Hepatitis C virus (HCV) is a causative agent of hepatitis C, which can lead over time to liver cirrhosis and hepatocellular carcinoma. In our collaboration with researchers at the Advanced Institute of Science and Technology (AIST), Yamanashi University, The University of Tokyo, Waseda University, and Hoshi Pharmaceutical University, we carried out antiviral screening on extracts prepared from coral reef organisms collected mainly in Okinawa Prefecture, Japan. To find possible antiviral molecules against HCV, our collaborators employed two assay systems: a fluorescent assay targeting helicase activity of NS3 protein involved in viral replication,[1] and replicon assay using a genetically engineered virus-like material. As results, we characterized biological activities of manoalide,[2] cholesterol sulfate,[3] psammplin A,[4] halisulfate 3 and suvanine,[5] polybrominated biphenylethers (PBDEs),[6] and aromatic sulfates[7] as possible candidates of antiviral agents.

During the project, a small library consisted of 84 extracts was evaluated. One of the extracts, tagged C-29EA as a lipophilic extract of the sponge *Amphimedon* sp., collected at Iriomote Island, was found to be active against replicon assay. The biological activity of the extract was characterized as inhibiting both viral helicase and protease,[8] and we report the chemical characterization of the extract in this note.

After bioassay-guided separation, two known pigments, a carotenoid mytiloxanthin (**1**) and a phoephorbide named chlorophyllone a (**2**), were isolated as possible antiviral molecules after identification by spectroscopic analyses and comparison with reported data. A brief description of the identification of two compounds **1** and **2** is presented here. This note is based on the Master's thesis of the first author, who separated and characterized the chemical constituents of C-29EA.

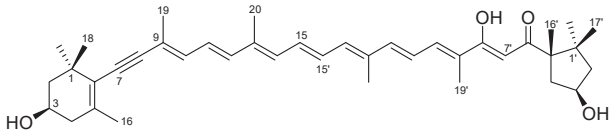

Figure 1. Structure of mytiloxanthin (**1**)

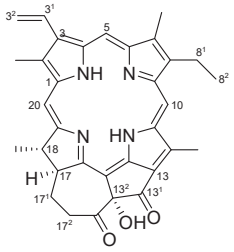

Figure 2. Structure of chlorophyllone a (**2**)

Results and Discussion

An extract library was prepared from 54 specimens, consisted mainly of sponges collected in 2003 in the Yaeyama region of Okinawa, including Aragusuku, Iriomote, Kohama, and Ishigaki islands. Both EtOAc and MeOH soluble extracts were prepared from each specimen. Excluding the smaller amount extracts, a total of 84 extracts were screened for HCV replicon assay by our collaborators. One extract, tagged C-29EA, was found to be active and its mode of action was characterized to inhibit both RNA helicase and protease.[8]

As the original amount of extract was small, additional specimen was collected in 2012 and a lipophilic extract was prepared. With bioassay-guided fractionation on silica gel followed by repeated HPLC, pigment molecules mytiloxanthin (**1**) and a phoephorbide chlorophyllone a (**2**) were obtained in small quantities.

The molecular formula of **1** was found to be $C_{40}H_{54}O_4$ by ESIMS. Its ^{1}H NMR spectrum showing a number of olefinic signals around δ 6.3-6.7 suggested the molecule as a carotenoid with red color due to absorption at 485 nm. Two characteristic signals: one at δ 16.3 due to internal hydrogen bonding and the other at δ 5.85 as a sharp olefinic singlet, were observed. After database search and spectral analysis on HSQC, compound **1** was identified as mytiloxanthin.[9,10]

ESIMS data indicated the molecular formula of compound **2** as $C_{33}H_{32}N_4O_3$. The ^{1}H NMR showed a highly-shielded signal at δ -1.88 indicating the presence of a large aromatic ring. The green coloration of the compound, together with low-field signals at δ 9.59, 9.52 and 8.66 suggested **2** as a chlorophyll-like molecule. After database search and comparison with reported compounds,[11,12] compound **2** was identified as chlorophyllone a.

In the replicon assay mytiloxanthin (**1**) showed antiviral activity (EC_{50} <0.4 μg/mL) and weak cytotoxicity (CC_{50} >12.5 μg/mL), while chlorophyllone a (**2**) also showed antiviral activity (EC_{50} <0.4 μg/mL) with moderate cytotoxicity (CC_{50} >1.3 μg/mL). However, due to the small amount of the material available and possible decomposition during storage, we did not conduct further experiments.

Experimental

General experimental conditions. NMR data were obtained on a Bruker Avance III spectrometer. ESIMS spectra were obtained on a Jeol JMS-T100LP instrument. Solvents used were reagent grade without distillation.

Extraction and Isolation. A specimen of a light blue standing sponge *Amphimedon* sp. was collected at Funauki Bay, Iriomote Island, July

2012 and kept frozen until extraction. The specimen was identified by Dr. Nicole J. de Voogd, of the Netherlands Centre for Biodiversity, and deposited under the code RMNH POR 6100 at Naturalis.

The sponge material (2.6 kg, wet) was extracted with acetone (13 L) for three times. After filtration, the acetone solution was concentrated under vacuum, and the resulting combined residue was partitioned between EtOAc (500 mL) and water. The EtOAc layer was concentrated to give a dark oil (15.8 g), which was separated on a silica gel flash column with stepwise elution using hexane, 50% CH_2Cl_2 in hexane, CH_2Cl_2, EtOAc (10, 35, 50, 75, and 100%) in hexane, and MeOH to give nine fractions. The fifth fraction (4.56 g) was separated on a silica gel column (hexane, 50% EtOAc in hexane, and MeOH) to give four subfractions. The active third subfraction (91.5 mg) was further separated on reversed phase gradient HPLC (70-100% MeOH, Cosmosil 5C18-ARII) to give eight fractions including the active sixth (13.0 mg) and seventh (23.1 mg) fractions. The sixth fraction (13.0 mg) was successively separated on HPLC, first with Cosmosil 5C18-ARII with a gradient from 5-10% water in 75% MeOH in MeCN to 0%, second with Develosil C30-UG-5 using 50% MeOH in MeCN, third with Phenomenex Gemini 5μ C18 110A using MeCN) to give 0.3 mg of mytiloxanthin (**1**). The seventh fraction was separated first with a gradient elution on Cosmosil 5C18-ARII using 5-10% water in 75% MeOH in MeCN to 0%, second with Cosmosil Cholester with MeCN, and finally with Imtakt Cadenza CD-C18 using MeCN to give 0.7 mg of chlorophyllone a (**2**).

Mytiloxanthin (1). A red solid, ESIMS *m/z* 621.4081 ($[M+Na]^+$, calcd for $C_{40}H_{54}NaO_4$, 621.4088), 599.4132 ($[M+H]^+$, calcd for $C_{40}H_{55}O_4$ 599.4100); UV-VIS ($CHCl_3$) 481 nm (Δε 4.9), 301 nm (Δε 4.2); ^{1}H NMR ($CDCl_3$) δ 0.85 (3H, s, H-16′), 1.14 (3H, s, H-16), 1.19 (3H, s, H-17′), 1.20 (3H, s, H-17), 1.35 (3H, s, H-18′), 1.71 (dd, *J* = 13.6, 4.8 Hz, H-2′), 1.84 (brd, *J* = 12.4 Hz, H-2), 1.92 (3H, s, H-18), 1.97 (6H, H-20, 19′), 1.99 (3H, s, H-20), 2.01 (3H, s, H-19), 2.43 (brdd, *J* = 17.8, 5.0 Hz, H-4), 2.88 (dd, *J* = 14.4, 8.5 Hz, H-4′), 3.99 (m, H-3), 4.52 (m, H-3′), 5.85 (1H, s, H-7′), 6-7 (totally 9H, m, H-10, 11, 12, 14, 15, 11′, 12′, 14′, 15′), 16.2 (s, OH-8′).

Chlorophyllone a (2). A dark green solid, ESIMS *m/z* 555.2360 ($[M+Na]^+$, calcd for $C_{33}H_{32}NaN_4O_3$ 555.2498), 533.2533 ($[M+H]^+$, calcd for $C_{33}H_{33}N_4O_3$ 533.2552); UV-VIS ($CHCl_3$) 669 (Δε 4.35), 612 (3.5), 537 (3.6), 507 (3.7), 416 nm (4.6) ^{1}H NMR ($CDCl_3$) δ -1.88 (1H, br, NH-21), 0.65 (1H, br, NH-23), 1.71 (3H, t, *J* = 7.6 Hz, H-8^2), 2.19 (3H, d, *J* = 7.4 Hz, H-18^1), 2.28 (1H, m, H-17^{1b}), 2.81 (1H, ddd, *J* = 4.3, 3.8, 3.1 Hz, H-17^{2b}), 2.89 (1H, m, H-17^{1a}), 3.27 (3H, s, H-7^1), 3.43 (3H, s, H-2^1), 3.69 (2H, m, H-8^1), 3.72 (3H, s, H-12^1), 4.36 (1H, m, H-17^{2a}), 4.91 (1H, dt, *J* = 12.8, 3.6 Hz, H-17), 6.21 (1H, dd, *J* = 10.1, 1.4 Hz, H-3^{2Z}), 6.31 (1H, dd, *J* = 16.4, 1.4 Hz, H-3^{2E}), 8.04 (1H, dd, *J* = 11.5, 6.2 Hz, H-3^1), 8.66 (1H, s, H-20), 9.52 (1H, s, H-5), 9.59 (1H, s, H-10).

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