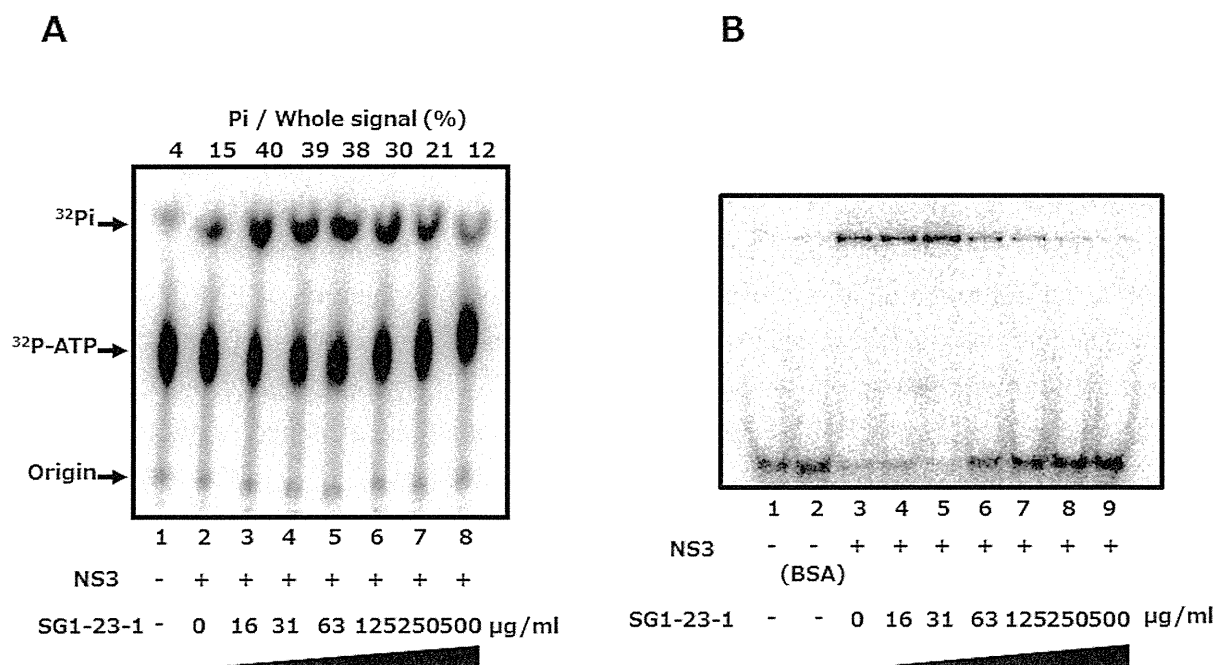


was carried out between 16 and 500  $\mu\text{g}$  of SG1-23-1 per milliliter. ATPase activity was slightly increased at 16  $\mu\text{g}$  SG1-23-1 per milliliter and slightly decreased at 500  $\mu\text{g}$  SG1-23-1 per milliliter (Figure 4A). However, the helicase activity was decreased to less than 10% in the presence of 50  $\mu\text{g}$  of SG1-23-1 per milliliter (Figure 3A,B). Next, we examined the effect of SG1-23-1 on the binding of NS3 helicase to single-strand RNA (ssRNA). A gel-mobility shift assay was employed to estimate the binding activity of NS3 to 21 mer of ssRNA. The binding of NS3 to ssRNA was inhibited with SG1-23-1 in a dose-dependent manner (Figure 4B). These results suggest that SG1-23-1 contains the compound that inhibits RNA binding to NS3 helicase.

**Figure 4.** Effect of SG1-23-1 on ATPase and RNA-binding activities of NS3 helicase. **(A)** The reaction mixtures were incubated with  $[\gamma\text{-}^{32}\text{P}]$  ATP as described in Materials and Methods. The reaction mixtures were subjected to thin-layer chromatography. The start positions and migrated positions of ATP and free phosphoric acid are indicated as “Origin”, “ $^{32}\text{P-ATP}$ ”, and “ $^{32}\text{P-Pi}$ ”, respectively, on the left side of this figure. The data represent three independent experiments. **(B)** Gel mobility shift assay for RNA-binding activity of NS3 helicase. The reaction was carried out at the indicated concentration of SG1-23-1. The reaction mixture was subjected to gel mobility shift assay. The data represent three independent experiments.



### 2.3. Effect of SG1-23-1 on HCV RNA Replication in HCV 1b Replicon Cells

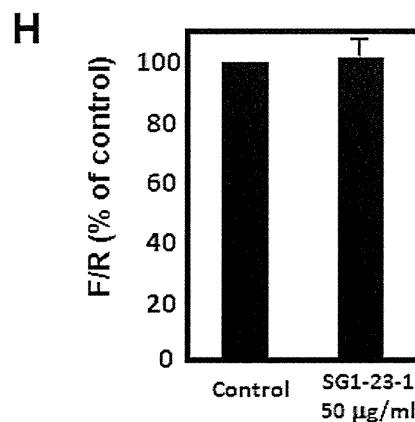
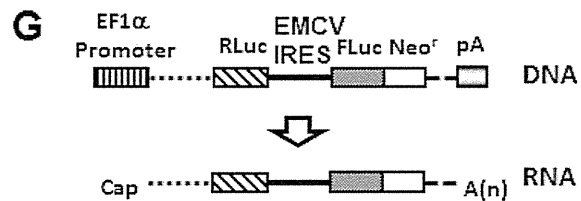
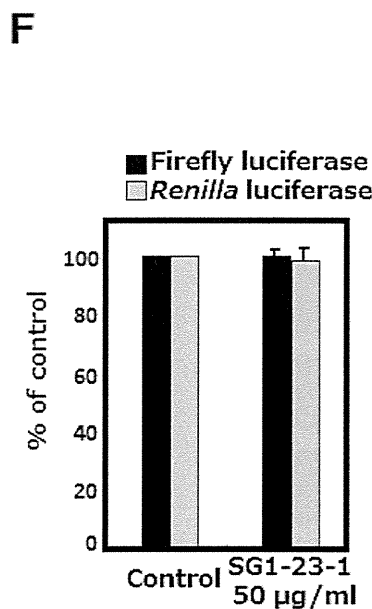
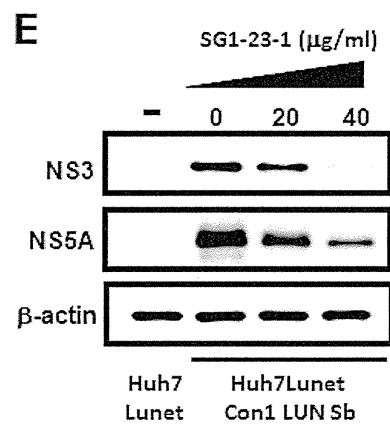
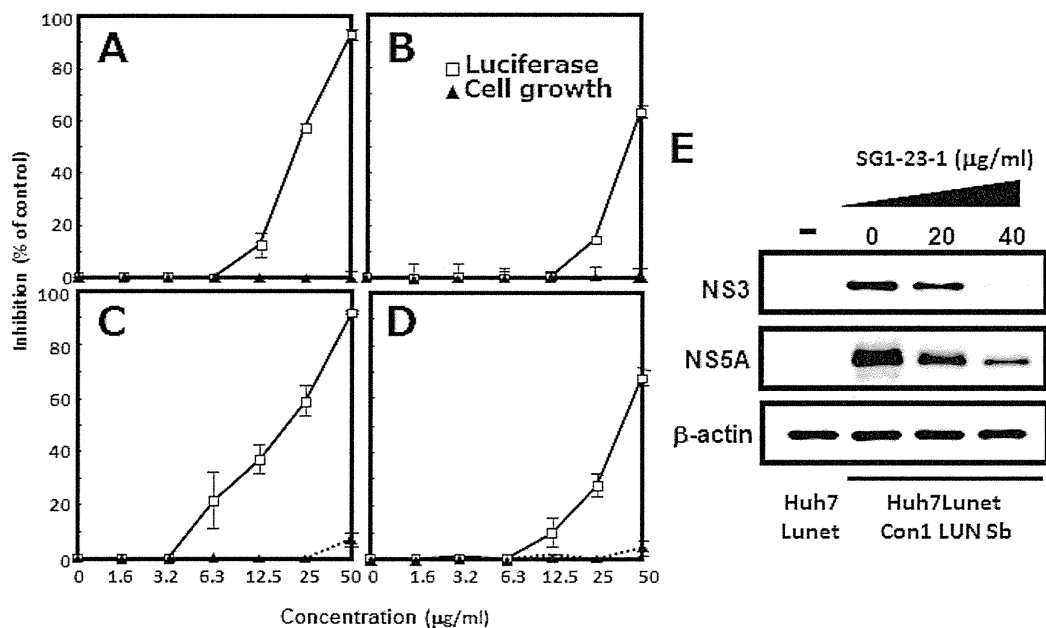
We investigated the effect of SG1-23-1 on both viral replication and growth of the replicon cell lines. The cell lines possess viral subgenomic RNAs derived from three genotype 1b strains (strains N [33], Con1 [34], and O [35]) or a full genomic RNA derived from the O strain [35]. Each cell line was treated with various concentrations of SG1-23-1. The treated cells were harvested 72 h post-treatment. Treatment with SG1-23-1 suppressed HCV RNA replications of all cell lines in a dose-dependent manner irrespectively of full- and sub-genome replicons; it exhibited no effect below 25  $\mu\text{g/ml}$  and

little effect on cellular viability at the highest concentration, 50  $\mu\text{g/mL}$  (Figure 5C,D). Both HCV NS3 and NS5A were decreased at the protein level in a dose-dependent manner, corresponding to the viral replication, but beta-actin was not changed in the cell line harboring subgenome replicon RNA of the Con1 strain (Figure 5E).

The inhibitory effect of SG1-23-1 on HCV replication is summarized in Table 2. The inhibitory effects on the HCV replication of the subgenome replicon derived from Con1, O, and N strains were  $22.9 \pm 0.4$ ,  $19.9 \pm 1.8$ , and  $44.2 \pm 1.5$   $\mu\text{g/mL}$ , respectively, as  $\text{EC}_{50}$ ; and  $48.1 \pm 1.5$ ,  $48.5 \pm 0.3$ , and  $>50$   $\mu\text{g/mL}$ , respectively, as  $\text{EC}_{90}$ . Treatment with SG1-23-1 inhibited the replication of the subgenome replicon of the O strain ( $\text{EC}_{50}$ :  $19.9 \pm 1.8$   $\mu\text{g/mL}$ ;  $\text{EC}_{90}$ :  $48.5 \pm 0.3$   $\mu\text{g/mL}$ ) at a more potent level than the replication of the full genomic replicon of the O strain ( $\text{EC}_{50}$ :  $39.5 \pm 0.8$   $\mu\text{g/mL}$ ;  $\text{EC}_{90}$ :  $>50$   $\mu\text{g/mL}$ ). When luciferase of firefly or *Renilla* was expressed under the control of the EF promoter, neither showed a significant change in activity in the presence of SG1-23-1 (Figure 5F). The replicon RNA of HCV is composed of the 5'-UTR of HCV, indicator genes (luciferase and drug-resistant genes), encephalomyocarditis virus (EMCV) IRES, the viral genes encoding complete or nonstructural proteins, and the 3'-UTR of HCV in that order [33–35]. The replicon RNA replicated autonomously in several HCV replication-permissive cell lines derived from several hepatoma cell lines. Nonstructural proteins in replicon cells were polycistronically translated through EMCV IRES. The cap-dependent translated mRNA, including *Renilla* luciferase, EMCV IRES, and the firefly luciferase/neomycin-resistant gene in that order, was constructed to examine the effect of the extract on EMCV-IRES-dependent translation (Figure 5G). When the expression of the mRNA was transcribed by an EF promoter of the transfected plasmid in the presence of SG1-23-1, the ratio of firefly luciferase activity to *Renilla* luciferase activity was not changed, suggesting that treatment with SG1-23-1 exhibited no effect on EMCV-IRES-dependent translation (Figure 5H). Thus, the inhibitory effect of SG1-23-1 on the luciferase activity must correspond to the replication efficiency of the replicon RNA but not to the inhibition of luciferase activity or the inhibition of EMCV-IRES-dependent translation. The inhibitory effect of the extract on the viral replication is similar to that of the extract on the helicase activity with respect to the values of  $\text{IC}_{50}$  and  $\text{EC}_{50}$  (Figure 3A and Table 2). These results suggest that treatment with SG1-23-1 inhibits HCV replication in a manner similar to that of the inhibitory effect on NS3 helicase activity.

**Figure 5.** Effect of SG1-23-1 on viral replication in replicon cell lines. (A–D) Huh7 Lunet/Con1 LUN Sb #26 (A), Huh7 rep Feo (B), Huh7#94/ORN3-5B#24 (C), and OR6 (D) cell lines were incubated in medium containing various concentrations of SG1-23-1. Luciferase and cytotoxicity assays were carried out as described in Materials and Methods. Error bars indicate standard deviation. The data represent three independent experiments. (E) Protein extract was prepared from Huh7 Lunet/Con1 LUN Sb #26 cells treated for 72 h with an indicated concentration of SG1-23-1 and then was subjected to Western blotting using antibodies to NS3, NS5A, and beta-actin. (F) Huh7 cell line transfected with pEF Fluc IN vector or pEF Rluc IN was established in the presence of G418. Both cell lines were incubated without (control) and with 50  $\mu\text{g/mL}$  SG1-23-1. Firefly or *Renilla* luciferase activity was measured 72 h post-treatment. Luciferase activity was normalized with protein concentration. Error bars indicate standard deviation. The data represent three

independent experiments. (G) Schematic structure of the plasmid, pEF RLuc EMCV IRES Feo. The bicistronic gene is transcribed under the control of elongation factor 1 $\alpha$  (EF1 $\alpha$ ) promoter. The upstream cistron encoding *Renilla* luciferase (RLuc) is translated by a cap-dependent mechanism. The downstream cistron encodes the fusion protein (Feo), which consists of the firefly luciferase (Fluc) and neomycin phosphotransferase (Neo<sup>r</sup>), and is translated under the control of the EMCV IRES. (H) Huh7 cell line transfected with pEF RLuc EMCV IRES Feo was established in the presence of G418. The cells were incubated for 72 h without (control) and with 50  $\mu$ g/mL of SG1-23-1. Firefly or *Renilla* luciferase activity was measured by the method described in Materials and Methods and was normalized by the protein concentration. F/R: Relative ratio of Firefly luciferase activity to *Renilla* luciferase activity. F/R is presented as a percentage of the control condition. Error bars indicate standard deviation. The data represent three independent experiments.



**Table 2.** Anti-HCV activity of SG1-23-1 in different replicon cell lines of genotype 1b.

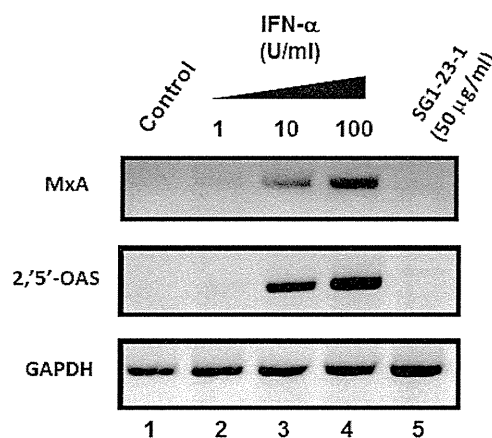
| Replicon Cell Line             | Virus Strain<br>(Genotype 1b) | EC <sub>50</sub> <sup>a</sup><br>(µg/mL) | EC <sub>90</sub> <sup>b</sup><br>(µg/mL) | CC <sub>50</sub> <sup>c</sup><br>(µg/mL) |
|--------------------------------|-------------------------------|--|--|--|
| <u>Subgenome</u>               |                               |  |  |  |
| Huh7 Lunet/<br>Con1 LUN Sb #26 | Con1                          | 22.9 ± 0.4                               | 48.1 ± 1.5                               | >50                                      |
| Huh7 rep Feo                   | N                             | 44.2 ± 1.5                               | >50                                      | >50                                      |
| Hu7#94/ORN3-5B#24              | O                             | 19.9 ± 1.8                               | 48.8 ± 0.3                               | >50                                      |
| <u>Full genome</u>             |                               |  |  |  |
| OR6                            | O                             | 39.5 ± 0.8                               | >50                                      | >50                                      |

All data represent means ± standard deviation for three independent experiments; <sup>a</sup> Fifty percent effective concentration based on the inhibition of HCV replication; <sup>b</sup> Ninety percent effective concentration based on the inhibition of HCV replication; <sup>c</sup> Fifty percent cytotoxicity concentration based on the reduction of cell viability.

#### 2.4. Effect of SG1-23-1 on the Interferon (IFN) Signaling Pathway

It has been reported that the HCV replication in cultured cells is potently inhibited by interferon (IFN) [36,37]. We examined whether or not treatment with SG1-23-1 induces interferon from replicon cells. The replicon cells were treated with various concentrations of interferon-alpha 2b or 50 µg of SG1-23-1 per milliliter. The treated cells were harvested at 72 h post-treatment. The interferon-inducible genes, MxA and 2',5'-OAS, were induced with IFN-alpha 2b but not with SG1-23-1 (Figure 6). These results suggest that the inhibitory effect of SG1-23-1 on the replication of the HCV replicon is independent of the IFN signaling pathway.

**Figure 6.** Effect of SG1-23-1 on interferon signaling pathway. Huh7 Lunet/Con1 LUN Sb #26 cells were treated without (lane 1) or with 1, 10, or 100 U/mL IFNα-2b (lanes 2–4), and 50 µg/mL SG1-23-1 (lane 5) for 48 h. The mRNAs of MxA, 2',5'-OAS, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control were detected by reverse-transcription polymerase chain reaction (RT-PCR). Error bars indicate standard deviation. The data represent three independent experiments.



Treatment with SG1-23-1 suppressed the helicase activity of NS3 in a dose-dependent manner and exhibited an  $IC_{50}$  of 11.7  $\mu\text{g/mL}$ . Interestingly, treatment with SG1-23-1 inhibited the RNA binding activity of the helicase but not the ATPase activity of NS3. Treatment with SG1-23-1 inhibited the luciferase activity corresponding to the HCV replication in the replicon cell lines, but not the enzymatic activity of luciferase or the translational activity of EMCV IRES, suggesting that treatment with SG1-23-1 decreases HCV replication. Figure 4 shows that the viral proteins NS3 and NS5A in replicon cells were decreased by treatment with SG1-23-1, supporting the notion that SG1-23-1 inhibits HCV replication but not the enzymatic activity of luciferase. The inhibition of cell growth would not contribute to the inhibition of HCV replication by SG1-23-1 (Figure 3 and Table 2). Treatment with SG1-23-1 did not induce the interferon-stimulated genes in the replicon cell lines (Figure 6), suggesting that inhibition of HCV replication by treatment with SG1-23-1 is not due to interferon induction or interferon signaling. The extract SG1-23-1 inhibited the HCV replicon with an  $EC_{50}$  of 22 to 44  $\mu\text{g/mL}$ , which is similar to the value of  $IC_{50}$ . These results suggest that the anti-HCV compound(s) included in *A. polycladia* can suppress viral replication by inhibiting NS3 helicase activity.

### 3. Experimental Section

#### 3.1. Preparation of Extracts from Marine Organisms

All marine organisms used in this study were collected by hand during scuba diving off Shimoji, Okinawa, Chibishi, Kuro, Kume, and Tokashiki Islands in Okinawa Prefecture, Japan. In the case of OK-99-tagged extract, a specimen was soaked in ethanol. The ethanol-soluble fraction was concentrated, and the resulting aqueous material was suspended in ethyl acetate (EtOAc). The organic fraction was used for screening.

Each specimen from Kume was soaked in ethanol. The ethanol-soluble fraction was concentrated. The resulting material was suspended in EtOAc. The EtOAc-soluble fraction was used for screening and tagged with SG1 and the last digit of "1". The water layer was concentrated to dryness and suspended in methanol (MeOH). The MeOH-soluble fraction was used for screening and tagged with SG1 and the last digit of "2".

Each specimen from Tokashiki was extracted three times with acetone. After removal of acetone from the solution, the residual material was suspended in EtOAc. The EtOAc-soluble fraction was used for screening and tagged with SG3.

All samples were dried and then solubilized in dimethyl sulfoxide (DMSO) before testing.

#### 3.2. High-Throughput Screening of NS3 Helicase Inhibitors

A continuous fluorescence assay based on photoinduced electron transfer (PET) was described previously [38] and was slightly modified with regard to the reaction mixture. A schematic diagram of the PET assay for HCV NS3 helicase activity is shown in Figure 1. The double-strand RNA was prepared as a substrate by annealing, at a 1:2 molar ratio, a 5' BODIPY FL-labeled 37-mer (5'-CUAUUACCUCCACCCUCAUAACCUUUUUUUUUUUUUU-3') to a 23-mer (GGUUAUGAG GGUGGAGGUAUAG). When unwound by HCV NS3 helicase, the unlabeled ssRNA was captured by a DNA capture strand (5'-CTATTACCTCCACCCTCATAACC-3'). A fluorescent-dye-labeled

oligonucleotide was purchased from J-Bio 21 Corporation. BODIPY FL was attached to the 5'-end via an aminoethylphosphate linker with a six-carbon spacer. Unlabeled oligonucleotides were purchased from Japan Bio Services Co., Ltd. The PET NS3 helicase assay was carried out in 22  $\mu$ L of 25 mM MOPS-NaOH (pH 6.5) containing 3 mM  $MgCl_2$ , 2 mM dithiothreitol (DTT), 4 U RNasin, 50 nM of the double-strand RNA described above, 100 nM DNA capture strand, 5 mM ATP, and the extract (25  $\mu$ g/mL) and 240 nM HCV NS3 helicase. The reaction was started by the addition of HCV NS3 helicase. The reaction mixture was incubated at 37 °C for 30 min. The fluorescence intensity was recorded every 5 s until 5 min post-reaction, and then every 30 s between 5 and 30 min post-reaction by using a LightCycler 1.5 (Roche Diagnostics, Tokyo, Japan). The initial reaction velocity was calculated and represented as NS3 helicase activity.

### 3.3. ATPase Assay

NS3 ATPase activity was determined by the method of Gallinari *et al.* [39] with slight modifications. The reaction was carried out at 37 °C for 10 min in 10  $\mu$ L of the reaction mixture containing 25 mM MOPS-NaOH (pH 7.0), 1 mM DTT, 5 mM  $MgCl_2$ , 5 mM  $CaCl_2$ , 1 mM [ $\gamma$ - $^{32}P$ ] ATP (Muromachi, Tokyo, Japan), 300 nM NS3, and 0.1  $\mu$ g poly (U) per microliter and an indicated concentration of SG1-23-1, and then was terminated by the addition of 15 microliters of 10 mM EDTA. Two microliters of the reaction mixture were spotted onto a polyethyleneimine cellulose sheet (Merck, Darmstadt, Germany) and then developed in 0.75 M LiCl/1 M formic acid solution at room temperature for 20 min. The sheet was air-dried completely and then exposed to an image plate. Radioactive bands were visualized with an Image Reader FLA-9000 and quantified by Multi Gauge V 3.11 software (version 3.11; Fujifilm: Tokyo, Japan, 2008).

### 3.4. RNA Helicase Assay

NS3 RNA helicase assay was carried out by the method of Gallinari *et al.* [39] with slight modifications. The substrate for annealing two complementary RNA oligonucleotides, 5'-AGAGAGAGAGGUUGAGAGAGAGAGAGUUUGAGAGAGAGAGAG-3' (40-mer, template strand) and 5'-CAAACUCUCUCUCUCUCAACAAAAA-3' (26-mer, release strand) was purchased from Shanghai GenePharma Co., Ltd. The release strand was labeled at the 5'-end with [ $\gamma$ - $^{32}P$ ] ATP (Muromachi, Tokyo, Japan) using the T4 polynucleotide kinase (Toyobo, Osaka, Japan) at 37 °C for 60 min and then purified by phenol chloroform extraction. The template and the labeled release strands were annealed at a molar ratio of 3:1 (template: release), denatured at 80 °C for 5 min, and slowly renatured at 23 °C for 30 min in an annealing buffer consisting of 20 mM Tris-HCl (pH 8), 0.5 M NaCl, and 1 mM EDTA. The partial duplex RNA substrate was purified on a G-50 micro column (GE Healthcare, Uppsala, Sweden) and stored at -20 °C in H<sub>2</sub>O containing 0.25 U of RNasin Plus (Promega, Madison, WI, USA) per microliter.

SG1-23-1 extract was added at various concentrations to a helicase reaction mixture consisting of 25 mM MOPS-NaOH (pH 7.0), 2.5 mM DTT, 2.5 U of RNasin Plus (Promega), 100  $\mu$ g of BSA per milliliter, and 3 mM  $MgCl_2$ . The mixture was supplemented with 300 nM NS3 protein and 5 fM  $^{32}P$ -labeled partial duplex RNA substrate. It was then preincubated at 23 °C for 15 min. After adding ATP at a final concentration of 5 mM, the reaction mixture (20  $\mu$ L) was incubated at 37 °C for 30 min

and stopped by adding 5  $\mu$ L helicase termination buffer consisting of 0.1 M Tris-HCl (pH 7.5), 20 mM EDTA, 0.5% SDS, 0.1% Nonidet P-40, 0.1% bromophenol blue, 0.1% xylene cyanol, and 25% glycerol. The terminated reaction mixture was subjected to native TBE 10% polyacrylamide gel electrophoresis. The radioactive RNAs in the gel were visualized with an Image Reader FLA-9000 (Fujifilm) and quantified by Multi Gauge V 3.11 software.

### 3.5. RNA Binding Assay

RNA binding to NS3 helicase was analyzed by gel mobility shift assay [40]. First, let-7 single-strand RNA (5'-UGAGGUAGUAGGUUGUAUAGU-3') was incubated with [ $\gamma$ - $^{32}$ P] ATP (Muromachi, Tokyo, Japan) and T4 polynucleotide kinase (Toyobo) at 37 °C for 60 min for labeling at the 5'-end of the single-strand RNA. The reaction mixture was subjected to phenol chloroform extraction for purification of labeled RNA. The reaction was carried out at room temperature for 15 min in 20  $\mu$ L of the mixture consisting of 30 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM MgCl<sub>2</sub>, 1 mM DTT, 1 unit of RNasin Plus (Promega) per microliter, 300 nM NS3, 5 fmol let-7-labeled ssRNA, and an indicated concentration of SG1-23-1. The reaction was stopped by adding an equal volume of dye solution consisting of 0.025% bromophenol blue, 10% glycerol, and 0.5 $\times$  Tris/borate/EDTA (TBE). The resulting mixture was subjected to native 6% polyacrylamide gel electrophoresis (acrylamide: bis acrylamide = 19:1). The radioactive RNA was visualized with the Image Reader FLA-9000 and quantified by Multi Gauge V 3.11 software.

### 3.6. Cell Lines

The following Huh-7-derived cell lines used in this study were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 0.5 mg/mL G418: The Lunet/Con1 LUN Sb #26 cell line, which harbors the subgenomic replicon RNA of the Con1 strain (genotype 1b) [34]; the Huh7/ORN3-5B #24 cell line, which harbors the subgenomic replicon RNA of the O strain (genotype 1b) [35]; the Huh7 Rep Feo cell line, which harbors the subgenomic replicon RNA of the N strain (genotype 1b) [33]; and the OR6 cell line, which harbors the full genomic RNA of the O strain (genotype 1b) [35].

### 3.7. Determination of Luciferase Activity in HCV Replicon Cells

HCV replicon cells were seeded at  $2 \times 10^4$  cells per well in a 48-well plate 24 h before treatment. The extract SG1-23-1 was added to the culture medium at various concentrations. The treated cells were harvested 72 h post-treatment and lysed in cell culture lysis reagent (Promega) or *Renilla* luciferase assay lysis buffer (Promega). Luciferase activity in the harvested cells was estimated with a luciferase assay system (Promega) or a *Renilla* luciferase assay system (Promega). The resulting luminescence was detected by the Luminescencer-JNR AB-2100 (ATTO, Tokyo, Japan) and corresponded to the expression level of the HCV replicon.

### 3.8. Determination of Cytotoxicity in HCV Replicon Cells

HCV replicon cells were seeded at a density of  $1 \times 10^4$  cells per well in a 96-well plate and incubated at 37 °C for 24 h. The extract fraction of the sample code SG1-23-1 was added to the culture medium at various concentrations. These cells were treated with an indicated concentration of the extract fraction and then were harvested 72 h post-treatment. Cell viability was measured by dimethylthiazol carboxymethoxy-phenylsulfophenyl tetrazolium (MTS) assay using a CellTiter 96 aqueous one-solution cell proliferation assay kit (Promega).

### 3.9. Effects on Activities of Luciferase and Internal Ribosome Entry Site (IRES)

The plasmid pEF Fluc IN and pEF Rluc EMCV IRES Feo were described previously [41]. The firefly luciferase gene was replaced with the *Renilla* luciferase gene in the plasmid pEF Fluc IN. The resulting plasmid was designated as pEF RlucIN in this study. The Huh7 cells were transfected with the pEF Fluc IN, pEF Rluc IN, or pEF Rluc EMCV IRES Feo and then were established in a medium containing 0.25 mg/mL G418 as described previously [41]. These cell lines were seeded at  $2 \times 10^4$  cells per well in a 48-well plate 24 h before treatment, treated with 50 µg/mL extract SG1-23-1, and then harvested at 72 h post-treatment. Activities of firefly and *Renilla* luciferases in pEF Rluc EMCV IRES Feo were measured with the dual luciferase reporter assay system (Promega). Total protein concentration was measured using the BCA Protein Assay Reagent Kit (Thermo Scientific, Rockford, IL, USA) to normalize luciferase activity.

### 3.10. Western Blotting

The cells were lysed in lysis buffer containing Cell Culture Lysis Reagent (Promega). The cell lysate was subjected to SDS-10% polyacrylamide gel (SDS-PAGE). The proteins in the gel were transferred onto a polyvinylidene fluoride (PVDF) membrane. The resulting membrane was incubated with the primary antibodies at 4 °C overnight and then was washed three times with PBS containing 0.02% Tween 20 (PBS-T). The resulting membrane was reacted with a horseradish peroxidase-labeled anti-IgG antibody at room temperature for 2 h and then was washed three times with PBS-T. The reacted proteins were visualized with ImmunoStar LD (Wako Pure Chemical, Osaka, Japan). The antibodies to NS3 (Abcam, Cambridge, UK), NS5A (ViroGen, Watertown, MA, USA) and beta-actin were purchased from New England Biolabs (Beverly, MA, USA) and were used as the primary antibodies in this study.

### 3.11. Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

The previously described method of RT-PCR [41] was slightly modified, as described below. Total RNA was isolated from cultured cells with the RNAqueous-4PCR kit (Ambion, Austin, TX, USA) and then was reverse-transcribed with a Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The transcribed mRNA was amplified with PCR using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) and an appropriate primer pair. Primer sequences targeting the genes encoding 2',5'-oligoadenylate synthetase (2',5'-OAS), myxovirus resistance protein A (MxA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described previously [41].



#### 4. Conclusions

In conclusion, we showed that the ethyl acetate extract from *Alloeocomatella polycladia* significantly inhibits HCV replication by suppressing viral helicase activity. The purification of an inhibitory compound from the extract of *Alloeocomatella polycladia* will be required in order to improve the efficacy of chemical modification of the compound(s).

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# Inhibition of Both Protease and Helicase Activities of Hepatitis C Virus NS3 by an Ethyl Acetate Extract of Marine Sponge *Amphimedon* sp.

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## Abstract

Combination therapy with ribavirin, interferon, and viral protease inhibitors could be expected to elicit a high level of sustained virologic response in patients infected with hepatitis C virus (HCV). However, several severe side effects of this combination therapy have been encountered in clinical trials. In order to develop more effective and safer anti-HCV compounds, we employed the replicon systems derived from several strains of HCV to screen 84 extracts from 54 organisms that were gathered from the sea surrounding Okinawa Prefecture, Japan. The ethyl acetate-soluble extract that was prepared from marine sponge *Amphimedon* sp. showed the highest inhibitory effect on viral replication, with EC<sub>50</sub> values of 1.5 and 24.9 μg/ml in sub-genomic replicon cell lines derived from genotypes 1b and 2a, respectively. But the extract had no effect on interferon-inducing signaling or cytotoxicity. Treatment with the extract inhibited virus production by 30% relative to the control in the JFH1-Huh7 cell culture system. The *in vitro* enzymological assays revealed that treatment with the extract suppressed both helicase and protease activities of NS3 with IC<sub>50</sub> values of 18.9 and 10.9 μg/ml, respectively. Treatment with the extract of *Amphimedon* sp. inhibited RNA-binding ability but not ATPase activity. These results suggest that the novel compound(s) included in *Amphimedon* sp. can target the protease and helicase activities of HCV NS3.

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## Introduction

Hepatitis C virus (HCV) is an enveloped RNA virus of the genus *Hepacivirus* of the *Flaviviridae* family. More than 170 million patients persistently infected with HCV have been reported worldwide, leading to liver diseases including steatosis, cirrhosis, and hepatocellular carcinoma [1,2]. The genome of HCV is characterized as a single positive-strand RNA with a nucleotide length of 9.6 kb, flanked by 5' and 3'-untranslated regions (UTRs). The genomic RNA encodes a large polyprotein consisting of approximately 3,000 amino acids [3], which is translated under the control of an internal ribosome entry site (IRES) located within the 5'-UTR of the genomic RNA [4]. The translated polyprotein is cleaved by host and viral proteases, resulting in 10 mature viral

proteins [3]. The structural proteins, consisting of core, E1, and E2, are located in the N-terminal quarter of the polyprotein, followed by viroporin p7, which has not yet been classified into a structural or nonstructural protein. Further cleavage of the remaining portion by viral proteases produces six nonstructural proteins—NS2, NS3, NS4A, NS4B, NS5A, and NS5B—which form a viral replication complex with various host factors. The viral protease NS2 cleaves its own C-terminal between NS2 and NS3. After that, NS3 cleaves the C-terminal ends of NS3 and NS4A and then forms a complex with NS4A. The NS3/4A complex becomes a fully active form to cleave the C-terminal parts of the polyprotein, including nonstructural proteins. NS3 also possesses

RNA helicase activity to unwind the double-stranded RNA during the synthesis of genomic RNA [5,6].

Although the previous standard therapy, combining pegylated interferon with ribavirin, was effective in only about half of patients infected with genotype 1, the most common genotype worldwide [7–9], recent biotechnological advances have led to the development of a novel therapy using anti-HCV agents that directly target HCV proteins or host factors required for HCV replication and have improved the sustained virologic response (SVR) [10–12]. Telaprevir and boceprevir, which are categorized as advanced NS3/4A protease inhibitors, were recently approved for the treatment of chronic hepatitis C patients infected with genotype 1 [13,14]. The triple combination therapy with pegylated interferon, ribavirin, and telaprevir improved SVR by 77% in patients infected with genotype 1 [15]. However, this therapy exhibits side effects including rash, severe cutaneous eruption, influenza-like symptoms, cytopenias, depression, and anemia [7,16,17]. Furthermore, the possibility of the emergence of drug-resistant viruses is a serious problem with therapies that use antiviral compounds [18,19].

Recent technical advances in the determination of molecular structures and the synthesis of chemical compounds have led to the development of various drugs based on natural products, especially drugs identified from terrestrial plants and microbes [20–22]. Marine organisms, including plants and animals, were recently established as representative of a natural resource library for drug development. Potent biological activity is often found in products isolated from marine organisms because of their novel molecular structures [23,24]. Trabectedin (Yondelis), cytarabine (Ara-C), and eribulin (Halaven), which are known as antitumor drugs, were developed from compounds found in marine organisms [25].

In this study, we screened 84 extracts prepared from 54 marine organisms by using replicon cell lines derived from HCV genotype 1b and attempted to identify the extract that inhibits HCV RNA replication. A marine organism may produce anti-HCV agent(s) that could inhibit the protease and helicase activities of NS3.

## Results

### Effect of the Extract from Marine Sponge and Tunicate on HCV Replication

We prepared methanol (MeOH)- and ethyl acetate (EtOAc)-soluble extracts from 54 marine organisms in order to test which of these extracts could best suppress HCV replication. Each extract was added at 25 µg/ml to the culture supernatant of HCV replicon cell lines derived from O and Con1 strains of genotype 1b, which produce the luciferase/neomycin hybrid protein depending on RNA replication. Luciferase activity and cell viability were measured 72 h after treatment with the extracts (Table 1). The extracts exhibiting more than 85% cell viability and lower than 15% luciferase activity were selected as arbitrary candidates for the extract including anti-HCV compounds. The EtOAc-extract prepared from sample C-29 (C-29EA) was selected as a candidate in both cell lines. Thus, the anti-HCV activity of extract C-29EA was tested.

The EtOAc-soluble extract C-29EA was prepared from the marine sponge *Amphimedon* sp. (Fig. 1A), which inhabits the sea surrounding Okinawa Prefecture, Japan. HCV replication was inhibited in a dose-dependent manner but did not exhibit cytotoxicity when replicon cells were treated with C-29EA (Fig. 1B). The extract C-29EA exhibited EC<sub>50</sub> values of 1.5 µg/ml (Table 2). Furthermore, treatment with C-29EA suppressed the HCV replication derived from the genotype 2a strain JFH1 with an EC<sub>50</sub> of 24.9 µg/ml, irrespective of cell viability (Fig. 2A and

Table 2). Extract C-29EA also inhibited the production of infectious viral particles, viral RNA, and core protein from JFH1-infected cells in the supernatant (Fig. 2B and C). These results suggest that the marine sponge *Amphimedon* sp. possesses anti-HCV agents.

### Effect of Extract C-29EA on IRES-dependent Translation

Extract C-29EA had the most potent inhibitory activity against HCV replication. The viral replication (Fig. 1B and 2A) and viral proteins (Fig. 3A and B) in replicon cell lines derived from genotype 1b strain Con1 and 2a strain JFH1 were decreased 72 h after treatment in a dose-dependent manner. HCV protein has been translated based on the positive-sense viral RNA in an IRES-dependent manner. The replicon RNA of HCV is composed of the 5'-UTR of HCV, indicator genes (a luciferase-fused drug-resistant gene), encephalomyocarditis virus (EMCV) IRES, the viral genes encoding complete or nonstructural proteins, and the 3'-UTR of HCV, in that order [26]. The replicon RNA replicated autonomously in several HCV replication-permissive cell lines derived from several hepatoma cell lines. Nonstructural proteins in replicon cells were polycistronically translated through EMCV IRES. The cap-dependent translated mRNA, including *Renilla* luciferase, EMCV IRES, and the firefly luciferase/neomycin-resistant gene, in that order, was constructed to examine the effect of the extract on EMCV-IRES-dependent translation (Fig. 3C). When the mRNA expression was transcribed by an EF promoter of the transfected plasmid in the presence of C-29EA, the ratio of firefly luciferase activity to *Renilla* luciferase activity was not changed (Fig. 3C). This suggested that treatment with C-29EA exhibited no effect on EMCV-IRES-dependent translation. Furthermore, treatment with C-29EA did not significantly affect the activity of HCV IRES that was used instead of EMCV IRES in the system described above (Fig. 3D). Thus, these results suggest that treatment with C-29EA exhibits no effect on EMCV- or HCV-IRES-dependent translation.

### Effect of C-29EA on the Interferon Signaling Pathway

It has been well known that HCV replication in cultured cells is potently inhibited by interferon [27,28]. We examined whether or not treatment with C-29EA elicits an interferon-inducible gene from replicon cells. The replicon cells were treated with various concentrations of interferon-alpha 2b or 15 µg of C-29EA per milliliter. The treated cells were harvested at 72 h post-treatment. The interferon-inducible gene 2', 5'-OAS, was induced with IFN-alpha 2b but not with a 10-times EC<sub>50</sub> concentration of C-29EA (Fig. 4). These results suggest that the inhibitory effect of C-29EA on the replication of the HCV replicon is independent of the IFN signaling pathway.

### Effect of C-29EA on the NS3 Helicase Activity

We previously established an assay system for unwinding HCV activity based on photoinduced electron transfer (PET) [29,30]. The fluorescent dye (BODIPY FL) is attached to the cytosine at the 5'-end of the fluorescent strand and quenched by the guanine base at the 3'-end of the complementary strand via PET. When helicase unwinds the double-strand RNA substrate, the fluorescence of the dye emits a bright light upon the release of the dye from the guanine base. The capture strand, which is complementary to the complementary strand, prevents the reannealing of the unwound duplex. Treatment with C-29EA inhibited the helicase activity in a dose-dependent manner, with an IC<sub>50</sub> value of 18.9 µg/ml (Fig. 5A). We confirmed the effect of C-29EA on NS3 helicase unwinding activity by the RNA helicase assay using <sup>32</sup>P-labeled double-stranded RNA (dsRNA) as a substrate. Treatment

**Table 1.** Effect of marine organism extracts on HCV replication and cell viability.

| No. | Sample | Luciferase activity (% of control) |      | Cell viability (% of control) |      | Phylum     | Specimen                         | Extract | Site |
|-----|--------|------------------------------------|------|-------------------------------|------|------------|----------------------------------|---------|------|
|     |        | O                                  | Con1 | O                             | Con1 |            |                                  |         |      |
| 1   | A-1    | 10                                 | 111  | 105                           | 104  | Sponge     | <i>Unidentified</i>              | MeOH    | A    |
| 2   | A-2    | 82                                 | 209  | 91                            | 132  | Soft coral | <i>Briareum</i>                  | MeOH    | A    |
| 3   | A-3    | 87                                 | 177  | 54                            | 110  | Tunicate   | <i>unidentified</i>              | MeOH    | A    |
| 4   | A-4    | 82                                 | 186  | 84                            | 100  | Sponge     | <i>Liosina</i>                   | MeOH    | A    |
| 5   | B-5    | 110                                | 165  | 86                            | 110  | Sponge     | <i>unidentified</i>              | MeOH    | B    |
| 6   | B-6    | 70                                 | 149  | 103                           | 119  | Sponge     | <i>Xestospongia</i>              | MeOH    | B    |
| 7   | B-7    | 89                                 | 191  | 111                           | 144  | Sponge     | <i>Epipolasis</i>                | MeOH    | B    |
| 8   | B-8    | 89                                 | 182  | 115                           | 132  | Sponge     | <i>unidentified</i>              | MeOH    | B    |
| 9   | B-9    | 57                                 | 72   | 92                            | 124  | Sponge     | <i>Strongylophora</i>            | MeOH    | B    |
| 10  | B-10   | 106                                | 182  | 73                            | 96   | Sponge     | <i>Stylotella aurantium</i>      | MeOH    | B    |
| 11  | C-12   | 96                                 | 162  | 114                           | 98   | Sponge     | <i>Epipolasis</i>                | MeOH    | B    |
| 12  | C-13   | 123                                | 141  | 91                            | 103  | Sponge     | <i>unidentified</i>              | MeOH    | B    |
| 13  | C-14   | 89                                 | 175  | 77                            | 100  | Sponge     | <i>Hippospongia</i>              | MeOH    | B    |
| 14  | C-16   | 80                                 | 177  | 108                           | 88   | Sponge     | <i>unidentified</i>              | MeOH    | B    |
| 15  | C-18   | 119                                | 170  | 93                            | 94   | Sponge     | <i>unidentified</i>              | MeOH    | B    |
| 16  | C-19   | 0                                  | 0    | 0                             | 4    | Sponge     | <i>unidentified</i>              | MeOH    | B    |
| 17  | C-20   | 101                                | 158  | 61                            | 106  | Sponge     | <i>Xestospongia testudinaria</i> | MeOH    | B    |
| 18  | C-21   | 85                                 | 161  | 83                            | 102  | Sponge     | <i>unidentified</i>              | MeOH    | B    |
| 19  | C-22   | 109                                | 88   | 38                            | 89   | Sponge     | <i>unidentified</i>              | MeOH    | B    |
| 20  | C-23   | 94                                 | 156  | 32                            | 90   | Sponge     | <i>unidentified</i>              | MeOH    | B    |
| 21  | C-24   | 118                                | 86   | 42                            | 94   | Sponge     | <i>Theonella</i>                 | MeOH    | B    |
| 22  | C-25   | 82                                 | 111  | 91                            | 106  | Sponge     | <i>unidentified</i>              | MeOH    | B    |
| 23  | C-27   | 0                                  | 0    | 15                            | 2    | Sponge     | <i>unidentified</i>              | MeOH    | B    |
| 24  | C-28   | 90                                 | 166  | 30                            | 90   | Sponge     | <i>Petrosia</i>                  | MeOH    | B    |
| 25  | C-29   | 65                                 | 151  | 29                            | 101  | Sponge     | <i>Amphimedon</i>                | MeOH    | B    |
| 26  | D-31   | 81                                 | 127  | 55                            | 91   | Tunicate   | <i>unidentified</i>              | MeOH    | C    |
| 27  | D-32   | 80                                 | 141  | 47                            | 93   | Sponge     | <i>unidentified</i>              | MeOH    | C    |
| 28  | D-33   | 88                                 | 153  | 72                            | 90   | Gorgonian  | <i>Junceella fragilis</i>        | MeOH    | C    |
| 29  | E-35   | 114                                | 156  | 40                            | 118  | Sponge     | <i>Phyllospongia sp.</i>         | MeOH    | C    |
| 30  | E-36   | 80                                 | 125  | 69                            | 116  | Tunicate   | <i>Didemnum molle</i>            | MeOH    | C    |
| 31  | E-37   | 88                                 | 129  | 54                            | 108  | Sponge     | <i>Xestospongia sp.</i>          | MeOH    | C    |
| 32  | E-38   | 70                                 | 153  | 35                            | 112  | Sponge     | <i>unidentified</i>              | MeOH    | C    |
| 33  | F-40   | 119                                | 170  | 38                            | 104  | Sponge     | <i>unidentified</i>              | MeOH    | C    |
| 34  | F-41   | 88                                 | 166  | 48                            | 101  | Soft coral | <i>unidentified</i>              | MeOH    | C    |
| 35  | G-42   | 113                                | 157  | 31                            | 126  | Sponge     | <i>unidentified</i>              | MeOH    | D    |
| 36  | H-43   | 83                                 | 0    | 39                            | 5    | Sponge     | <i>unidentified</i>              | MeOH    | D    |
| 37  | J-44   | 62                                 | 183  | 27                            | 105  | Sponge     | <i>Cinachyra</i>                 | MeOH    | D    |
| 38  | J-45   | 96                                 | 140  | 47                            | 103  | Sponge     | <i>Liosina</i>                   | MeOH    | D    |
| 39  | J-46   | 83                                 | 149  | 77                            | 102  | Sponge     | <i>unidentified</i>              | MeOH    | D    |
| 40  | J-47   | 94                                 | 37   | 40                            | 111  | Sponge     | <i>unidentified</i>              | MeOH    | D    |
| 41  | J-48   | 24                                 | 16   | 53                            | 70   | Sponge     | <i>Stylotella</i>                | MeOH    | D    |
| 42  | J-49   | 78                                 | 123  | 55                            | 105  | Sponge     | <i>unidentified</i>              | MeOH    | D    |
| 43  | J-50   | 93                                 | 138  | 51                            | 108  | Sponge     | <i>unidentified</i>              | MeOH    | D    |
| 44  | J-51   | 103                                | 73   | 41                            | 115  | Sponge     | <i>unidentified</i>              | MeOH    | D    |
| 45  | J-52   | 162                                | 237  | 113                           | 131  | Sponge     | <i>unidentified</i>              | MeOH    | D    |
| 46  | J-53   | 51                                 | 90   | 93                            | 122  | Tunicate   | <i>Didemnum</i>                  | MeOH    | D    |
| 47  | J-54   | 42                                 | 90   | 113                           | 124  | Sponge     | <i>unidentified</i>              | MeOH    | D    |

Table 1. Cont.

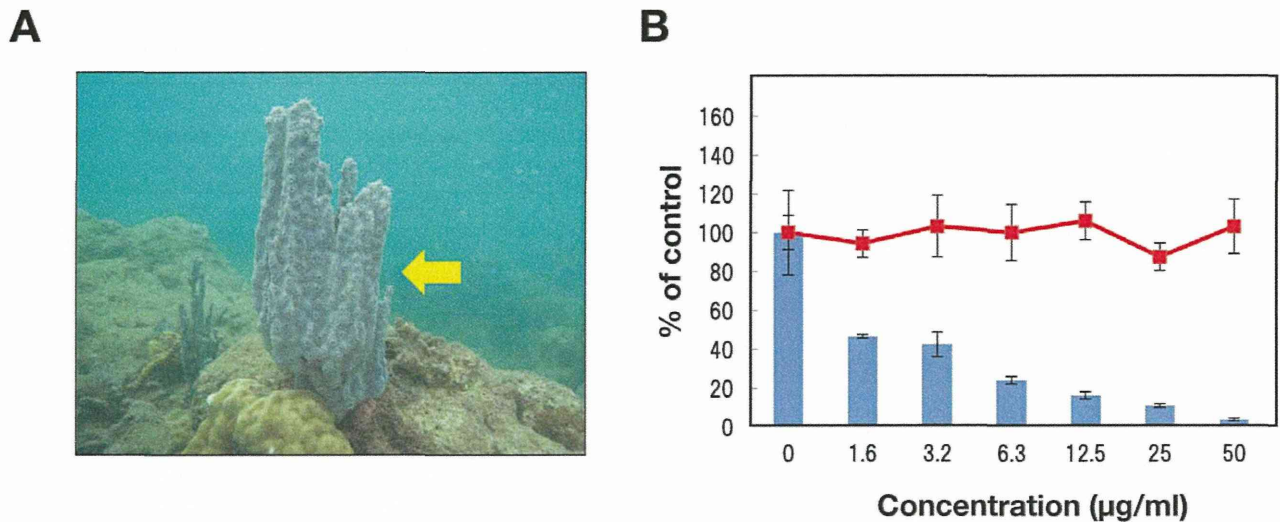
| No. | Sample | Luciferase activity (% of control) |      | Cell viability (% of control) |      | Phylum     | Specimen                         | Extract | Site |
|-----|--------|------------------------------------|------|-------------------------------|------|------------|----------------------------------|---------|------|
|     |        | O                                  | Con1 | O                             | Con1 |            |                                  |         |      |
| 48  | J-55   | 88                                 | 133  | 131                           | 110  | Jellyfish  | unidentified                     | MeOH    | D    |
| 49  | J-56   | 28                                 | 51   | 113                           | 103  | Sponge     | unidentified                     | MeOH    | D    |
| 50  | J-57   | 8                                  | 63   | 94                            | 85   | Tunicate   | <i>Pseudodistoma kanoko</i>      | MeOH    | D    |
| 51  | J-58   | 0                                  | 2    | 48                            | 65   | Sponge     | unidentified                     | MeOH    | D    |
| 52  | J-59   | 0                                  | 2    | 45                            | 71   | Sponge     | unidentified                     | MeOH    | D    |
| 53  | J-60   | 98                                 | 134  | 122                           | 95   | Annelid    | unidentified                     | MeOH    | D    |
| 54  | A-2    | 0                                  | 1    | 6                             | 15   | Soft coral | <i>Briareum</i>                  | EtOAc   | A    |
| 55  | A-3    | 0                                  | 0    | 6                             | 9    | Tunicate   | unidentified                     | EtOAc   | A    |
| 56  | A-4    | 22                                 | 36   | 74                            | 76   | Sponge     | <i>Liosina</i>                   | EtOAc   | A    |
| 57  | B-5    | 33                                 | 107  | 69                            | 93   | Sponge     | unidentified                     | EtOAc   | B    |
| 58  | B-6    | 0                                  | 0    | 5                             | 8    | Sponge     | <i>Xestospongia</i>              | EtOAc   | B    |
| 59  | B-7    | 0                                  | 0    | 5                             | 9    | Sponge     | <i>Epipolasis</i>                | EtOAc   | B    |
| 60  | B-8    | 0                                  | 0    | 2                             | 46   | Sponge     | unidentified                     | EtOAc   | B    |
| 61  | B-9    | 0                                  | 0    | 8                             | 14   | Sponge     | <i>Strongylophora</i>            | EtOAc   | B    |
| 62  | B-10   | 0                                  | 0    | 3                             | 8    | Sponge     | <i>Stylotella aurantium</i>      | EtOAc   | B    |
| 63  | C-12   | 0                                  | 0    | 4                             | 14   | Sponge     | <i>Epipolasis</i>                | EtOAc   | B    |
| 64  | C-13   | 0                                  | 0    | 4                             | 5    | Sponge     | unidentified                     | EtOAc   | B    |
| 65  | C-14   | 48                                 | 119  | 82                            | 102  | Sponge     | <i>Hippospongia</i>              | EtOAc   | B    |
| 66  | C-15   | 0                                  | 0    | 8                             | 11   | Sponge     | unidentified                     | EtOAc   | B    |
| 67  | C-18   | 0                                  | 0    | 4                             | 3    | Sponge     | unidentified                     | EtOAc   | B    |
| 68  | C-19   | 23                                 | 76   | 63                            | 109  | Sponge     | unidentified                     | EtOAc   | B    |
| 69  | C-20   | 34                                 | 32   | 63                            | 112  | Sponge     | <i>Xestospongia testudinaria</i> | EtOAc   | B    |
| 70  | C-21   | 1                                  | 0    | 52                            | 12   | Sponge     | unidentified                     | EtOAc   | B    |
| 71  | C-22   | 76                                 | 34   | 74                            | 110  | Sponge     | unidentified                     | EtOAc   | B    |
| 72  | C-24   | 0                                  | 0    | 20                            | 7    | Sponge     | <i>Theonella</i>                 | EtOAc   | B    |
| 73  | C-26   | 41                                 | 43   | 80                            | 110  | Sponge     | unidentified                     | EtOAc   | B    |
| 74  | C-27   | 1                                  | 0    | 35                            | 40   | Sponge     | unidentified                     | EtOAc   | B    |
| 75  | C-28   | 68                                 | 62   | 82                            | 115  | Sponge     | <i>Petrosia</i>                  | EtOAc   | B    |
| 76  | C-29   | 10                                 | 11   | 93                            | 88   | Sponge     | <i>Amphimedon</i>                | EtOAc   | B    |
| 77  | D-31   | 20                                 | 71   | 85                            | 120  | Tunicate   | <i>Eudistoma</i>                 | EtOAc   | C    |
| 78  | D-33   | 0                                  | 0    | 5                             | 7    | Gorgonian  | <i>Junceella fragilis</i>        | EtOAc   | C    |
| 79  | E-35   | 0                                  | 0    | 4                             | 5    | Sponge     | <i>Phyllospongia sp.</i>         | EtOAc   | C    |
| 80  | E-36   | 71                                 | 83   | 75                            | 100  | Tunicate   | <i>Didemnum molle</i>            | EtOAc   | C    |
| 81  | F-40   | 72                                 | 110  | 87                            | 130  | Sponge     | unidentified                     | EtOAc   | C    |
| 82  | F-41   | 8                                  | 33   | 73                            | 104  | Soft coral | unidentified                     | EtOAc   | C    |
| 83  | H-43   | 0                                  | 197  | 4                             | 119  | Sponge     | unidentified                     | EtOAc   | D    |
| 84  | J-46   | 113                                | 58   | 103                           | 126  | Sponge     | unidentified                     | EtOAc   | D    |

There are a total of 54 marine organisms, while 84 extracts were prepared from them with ethyl acetate and/or methanol. Aragusuku, Iriomote, Kohama, and Ishigaki islands are indicated by A, B, C, and D, respectively, in the collection-site column (right end). EtOAc: Ethyl acetate; MeOH: Methanol.  
doi:10.1371/journal.pone.0048685.t001

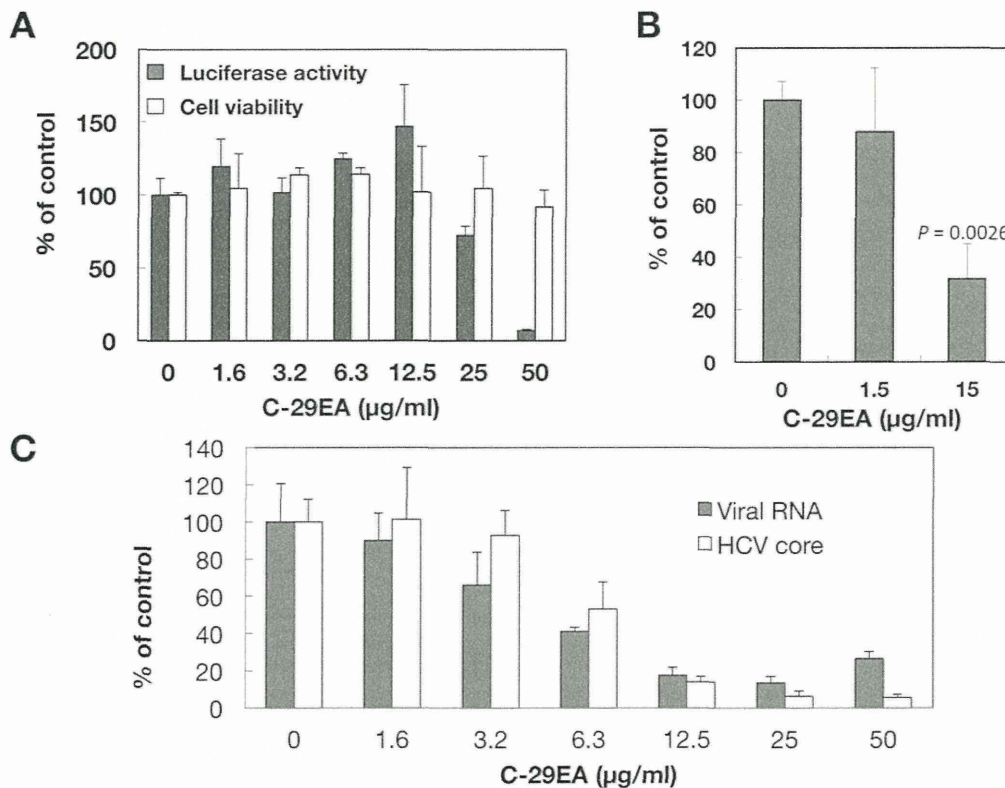
with C-29EA inhibited dsRNA dissociation at a concentration of 16 µg/ml and above (Fig. 5B).

The unwinding ability of HCV helicase depends on ATP binding, ATP hydrolysis, and RNA binding [30,31]. We examined the effect of C-29EA on the ATPase activity of NS3. The ratio of free phosphate (<sup>32</sup>P-Pi) to ATP (<sup>32</sup>P-ATP) was determined in the presence of C-29EA. The reaction was carried out between 16 and 250 µg of C-29EA per milliliter. The ATPase activity of NS3 helicase was not inhibited (Fig. 6A), although the helicase activity

was decreased to less than 20% in the presence of 50 µg of C-29EA per milliliter (Fig. 5A). Next, we examined the effect of C-29EA on the binding of NS3 helicase to single-strand RNA (ssRNA). A gel-mobility shift assay was employed to estimate the binding activity of NS3 to the 21-mer of ssRNA. The binding of NS3 to ssRNA was inhibited by C-29EA in a dose-dependent manner (Fig. 6 B and C). These results suggest that treatment with C-29EA inhibits the helicase activity of NS3 by suppressing RNA binding.



**Figure 1. Effect of the extract prepared from a marine sponge on viral replication in the replicon cell line derived from viral genotype 1b.** (A) *Amphimedon* sp. belongs to a marine sponge. The ethyl acetate fraction prepared from the marine organism was designated C-29EA in this study. (B) The Huh7 cell line, including the subgenomic replicon RNA of genotype 1b strain Con1, was incubated in medium containing various concentrations of C-29EA or DMSO (0). Luciferase and cytotoxicity assays were carried out as described in Materials and Methods. Error bars indicate standard deviation. The data represent three independent experiments. doi:10.1371/journal.pone.0048685.g001



**Figure 2. Effect of C-29EA extract on viral replication in the replicon cell line derived from viral genotype 2a.** (A) The Huh7 cell line, including the subgenomic replicon RNA of genotype 2a strain JFH1, was incubated in medium containing various concentrations of C-29EA or DMSO (0). Luciferase and cytotoxicity assays were carried out as described in Materials and Methods. (B) The Huh7 OK1 cell line infected with HCVcc JFH1 was incubated with various concentrations of C-29EA or DMSO (0). The virus titers were determined by a focus-forming assay. The significance of differences in the means was determined by Student's *t*-test. (C) Amounts of viral RNA and core protein were estimated by qRT-PCR and ELISA, respectively. Error bars indicate standard deviation. The data represent three independent experiments. Treatment with DMSO corresponds to '0'. doi:10.1371/journal.pone.0048685.g002



**Table 2.** Effect of C29EA on HCV replication.

| HCV strain (genotype) | EC <sub>50</sub> (μg/ml) <sup>a</sup> | CC <sub>50</sub> (μg/ml) <sup>b</sup> | SI <sup>c</sup> |
|-----------------------|---------------------------------------|---------------------------------------|-----------------|
| Con 1 (1b)            | 1.5                                   | >50                                   | >33.3           |
| JFH1 (2a)             | 24.9                                  | >50                                   | >2.3            |

<sup>a</sup>: Fifty percent effective concentration based on the inhibition of HCV replication.

<sup>b</sup>: Fifty percent cytotoxicity concentration based on the reduction of cell viability.

<sup>c</sup>: SI, selectivity index (CC<sub>50</sub>/EC<sub>50</sub>).

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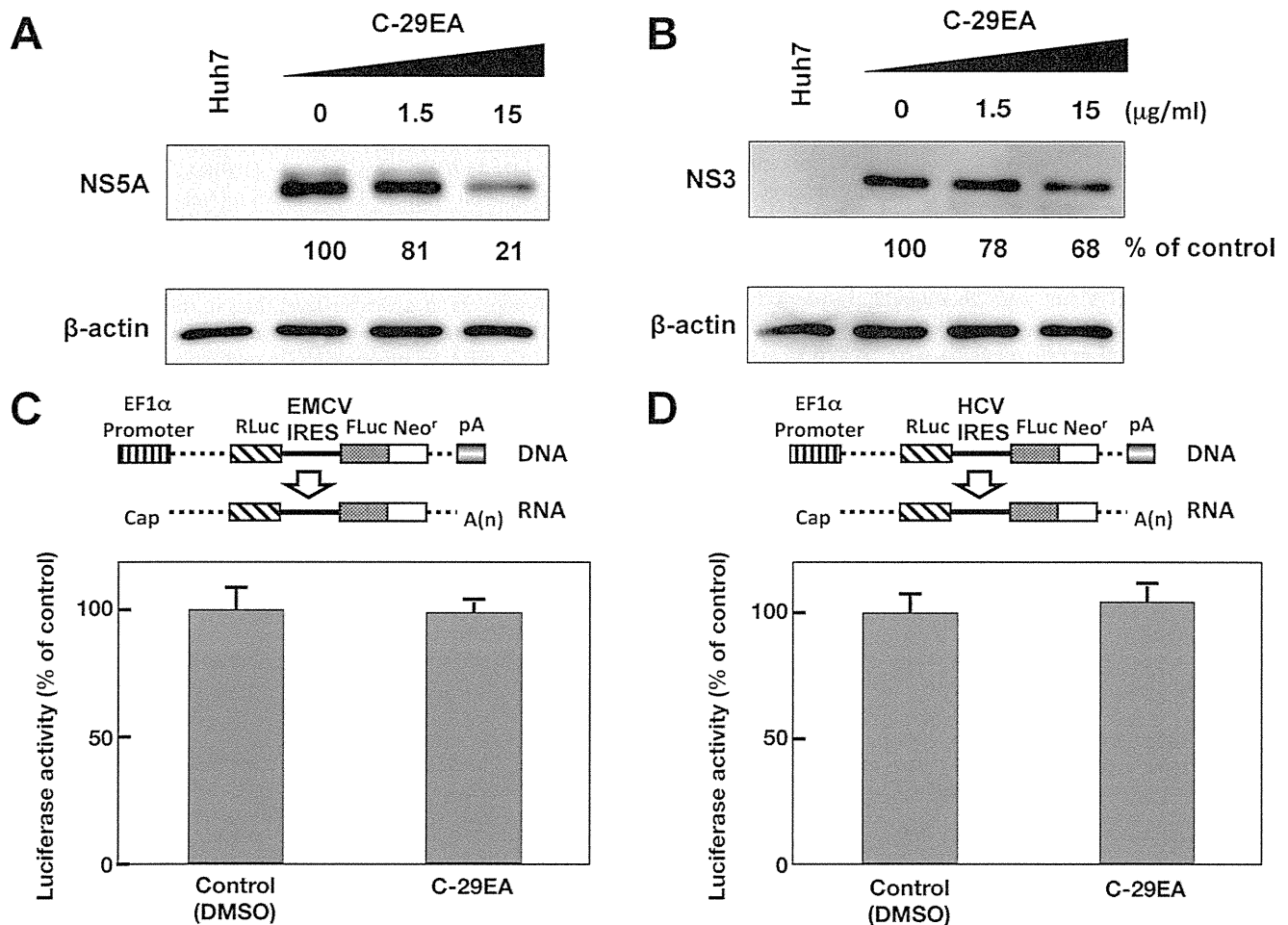
### Effect of C-29EA on NS3 Protease Activity

Serine protease and helicase domains are respectively located on the N-terminal and C-terminal portions of NS3 [32]. Thus, we examined the effect of C-29EA on NS3 protease activity by using

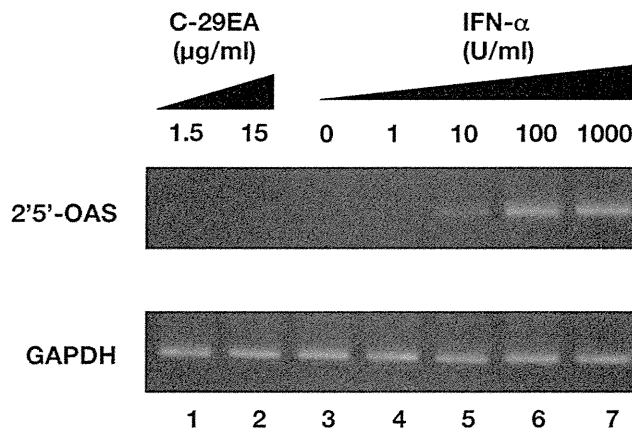
an NS3 protease assay based on FRET. NS3/4A serine protease was mixed with various concentrations of C-29EA. The initial velocity at each concentration of C-29EA was calculated during a 120 min reaction. The initial velocity in the absence of C-29EA represented 100% of relative protease activity. C-29EA decreased the serine protease activity in a dose-dependent manner (Fig. 7). The IC<sub>50</sub> of C-29EA was 10.9 μg/ml, which is similar to the value estimated by helicase assay. These results suggest that C-29EA includes the compound(s) inhibiting the protease activity of NS3 in addition to the helicase activity.

### Combination Antiviral Activity of C-29EA and Interferon-alpha

Treatment with C-29EA may potentiate inhibitory action of interferon-alpha, since it inhibited the protease and helicase activities of NS3 but not induce the interferon response as described above. Then, we examined effect of treatment using both interferon and C-29EA on HCV replication. The replication



**Figure 3. Effect of C-29EA on expression of viral proteins in replicon cell lines.** The Huh7 replicon cell lines derived from genotype 1b (A) and 2a (B) were incubated with C-29EA at 37°C for 72 h. The treated cells were harvested and then subjected to Western blotting. Treatment with DMSO corresponds to '0'. The bicistronic gene is transcribed under the control of the elongation factor 1α (EF1α) promoter. The upstream cistron encoding *Renilla* luciferase (RLuc) is translated by a cap-dependent mechanism. The downstream cistron encodes the fusion protein (Feo), which consists of the firefly luciferase (FLuc) and neomycin phosphotransferase (Neo<sup>r</sup>), and is translated under the control of the EMCV IRES (C) or HCV IRES (D). The Huh7 cell line transfected with the plasmid (each above the panel in C and D) was established in the presence of G418. The cells were incubated for 72 h without (control) and with 15 μg/ml of C-29EA. Firefly or *Renilla* luciferase activity was measured by the method described in Materials and Methods and was normalized by the protein concentration. F/R: relative ratio of firefly luciferase activity to *Renilla* luciferase activity. F/R is presented as a percentage of the control condition. Error bars indicate standard deviation. The data represent three independent experiments. doi:10.1371/journal.pone.0048685.g003



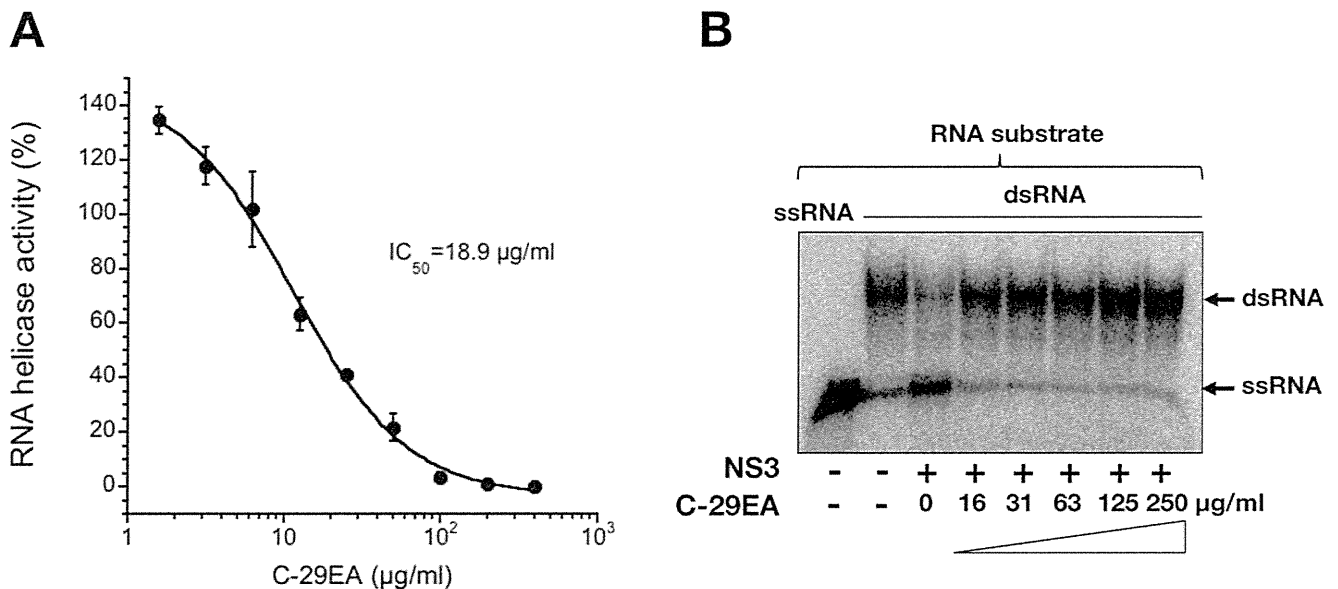
**Figure 4. Effect of C-29EA on interferon signaling pathway.** The Huh7 replicon cell line of genotype 1b was treated without (lane 3) or with 1, 10, 100, or 1000 U/mL interferon-alpha 2b (lanes 4–7), and 1.5 or 15 μg/ml C-29EA (lanes 1–2) for 48 h. Treatment with DMSO corresponds to '0'. The mRNAs of 2', 5'-OAS, and GAPDH as an internal control were detected by RT-PCR. Error bars indicate standard deviation. The data represent three independent experiments. doi:10.1371/journal.pone.0048685.g004

of replicon was decreased in the presence of C-29EA or interferon-alpha and further decreased by combination treatment using interferon-alpha and C-29EA (Fig. 8A). Furthermore, we employed the isobologram method [33] to determine whether antiviral effect of the combination treatment exhibits additive or synergistic.  $EC_{90}$  values of interferon-alpha and C-29EA were estimated at 10.7 U/ml and 26.4 μg/ml, respectively, in the absence of each other.  $EC_{90}$  values of C-29EA in the presence of 0, 2.5 and 5 U/ml interferon-alpha were plotted to generate an isobole. Figure 8B shows that the isobole exhibits concave

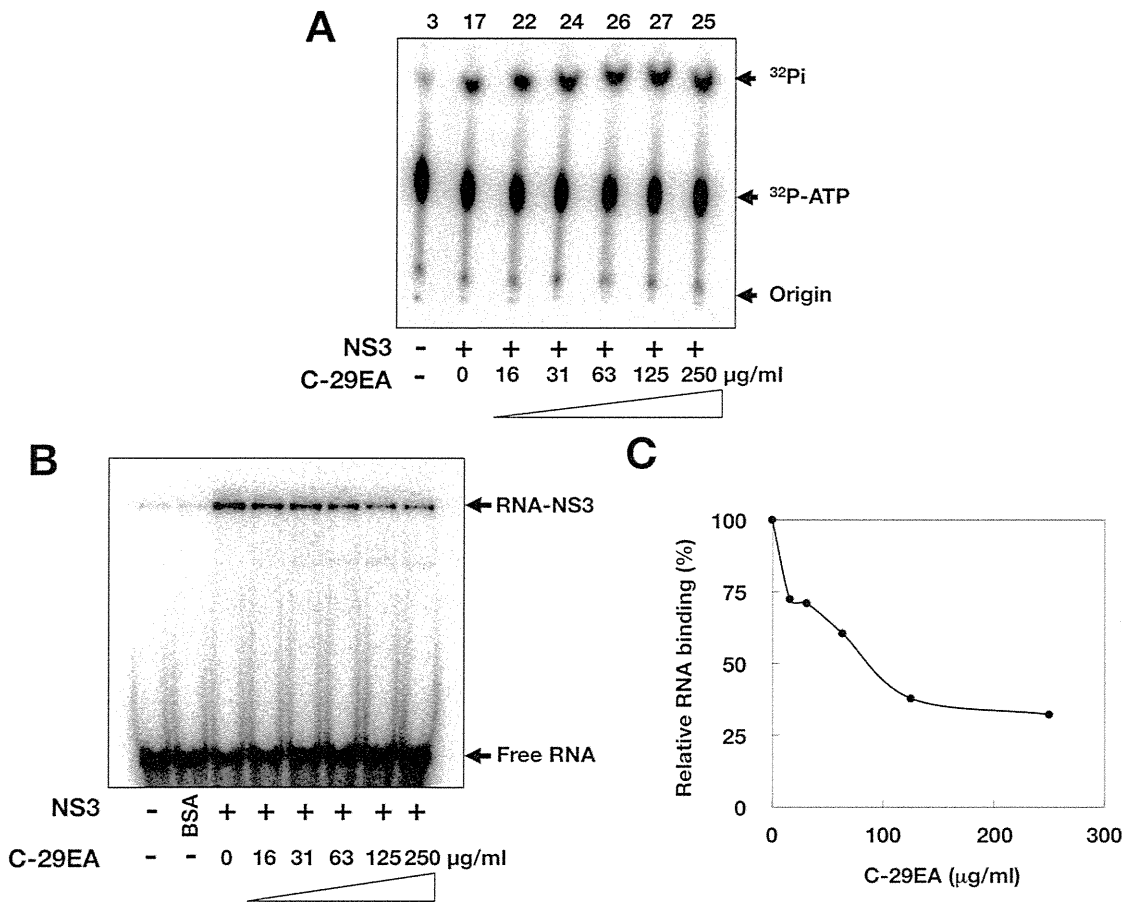
curvilinear, representing synergy but not additivity. These results suggest that combination treatment of interferon-alpha and C-29EA exhibits synergistic inhibition of HCV replication.

## Discussion

Several natural products have been reported as anti-viral agents against HCV replication. Silbinin, epigallocatechin 3-gallate, and proanthocyanidins, which were prepared from milk thistle, green tea, and blueberry leaves, respectively, have exhibited inhibitory activity against HCV replication in cultured cells [34–37]. In our previous report, we identified manoalide as an anti-HCV agent from a marine sponge extract by high-throughput screening targeting NS3 helicase activity [38]. Manoalide inhibited ATPase, RNA binding, and NS3 helicase activity in enzymological assays. The EtOAc extract of the marine feather star also suppressed HCV replication in HCV replicon cell lines derived from genotype 1b, and it inhibited the RNA-binding activity but not the ATPase activity of NS3 helicase [30]. In this study, we screened 84 extracts of marine organisms for their ability to inhibit HCV replication in replicon cell lines and HCV cell culture system. Among these extracts, C-29EA, which was extracted from *Amphimedon* sp., most strongly inhibited HCV replication regardless of cytotoxicity. We previously reported that the EtOAc extract (SG1-23-1) of the feather star *Alloecomatella polycladia* inhibited HCV replication with an  $EC_{50}$  of 22.9 to 44.2 μg/ml in HCV replicon cells derived from genotype 1b [30]. Treatment with C-29EA potently inhibited HCV replication with an  $EC_{50}$  of 1.5 μg/ml and with an SI of more than 33.3 in the replicon cell line derived from genotype 1b, regardless of cytotoxicity (Fig. 1B and Table 2). However, C-29EA exhibited an  $EC_{50}$  of 24.9 μg/ml in a replicon cell line derived from genotype 2a at a weaker level than in the replicon cell line derived from genotype 1b (Figs. 1 and 2), suggesting that the ability of C-29EA to suppress HCV replication is dependent on the viral genotype or strain.



**Figure 5. Effect of C-29EA on unwinding activity of NS3 helicase.** (A) NS3 helicase activity was measured by PET assay. The reactions were carried out in the absence or presence of C-29EA. Helicase activity in the absence of C-29EA was defined as 100% helicase activity. Treatment with DMSO corresponds to '0'. The data are presented as the mean  $\pm$  standard deviation for three replicates. (B) The unwinding activity of NS3 helicase was measured by an RNA unwinding assay using radioisotope-labeled RNA. The heat-denatured single-strand RNA (26-mer) and the partial duplex RNA substrate were applied to lanes 1 and 2, respectively. The duplex RNA was reacted with NS3 (300 nM) in the presence of C-29EA (lanes 4–9, 16–250 μg/ml). The resulting samples were subjected to native polyacrylamide gel electrophoresis. Treatment with DMSO corresponds to '0'. doi:10.1371/journal.pone.0048685.g005



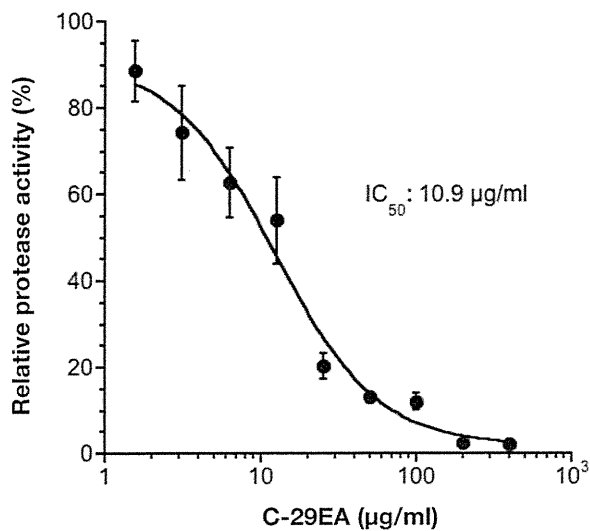
**Figure 6. Effect of C-29EA on ATPase and RNA-binding activities of NS3 helicase.** (A) The reaction mixtures were incubated with [ $\gamma$ - $^{32}$ P] ATP as described in Materials and Methods. The reaction mixtures were subjected to thin-layer chromatography. The start positions and migrated positions of ATP and free phosphoric acid are indicated as 'Origin', ' $^{32}$ P-ATP', and ' $^{32}$ P-Pi', respectively, on the right side of the figure. The data represent three independent experiments. Treatment with DMSO corresponds to '0'. (B) Gel mobility shift assay for RNA-binding activity of NS3 helicase. The reaction was carried out with 0.5 nM labeled ssRNA at the indicated concentrations of C-29EA or DMSO. The reaction mixture was subjected to gel mobility shift assay. (C) The relative RNA-binding ability was calculated with band densities in each lane and presented as a percentage of RNA-NS3 in the total density. The data represent three independent experiments. Treatment with DMSO corresponds to '0'. doi:10.1371/journal.pone.0048685.g006

HCV NS3 is well known to play a crucial role in viral replication through helicase and protease activities [5,39]. The N-terminal third of NS3 is responsible for serine protease activity in order to process the C-terminal portion of polyprotein containing viral nonstructural proteins [32]. The remaining portion of NS3 exhibits ATPase and RNA-binding activities responsible for helicase activity, which is involved in unwinding double-stranded RNA during replication of genomic viral RNA [40–42]. A negative-strand RNA is synthesized based on a viral genome (positive strand) after viral particles in the infected cells are uncoated, and is then used itself as a template to synthesize a positive-stranded RNA, which is translated or packaged into viral particles. Thus, both helicase and protease activities of NS3 are critical for HCV replication and could be targeted for the development of antiviral agents against HCV.

NS3 helicase activity was inhibited by treatment with C-29EA in a dose-dependent manner with an  $IC_{50}$  of 18.9  $\mu$ g/ml (Fig. 5A). RNA-binding activity, but not ATPase activity, was inhibited by treatment with C-29EA (Fig. 6). Treatment with C-29EA did not significantly affect the HCV-IRES activity and did not induce interferon-stimulated gene 2',5'-OAS (Figs. 3 and 4). Furthermore, the serine protease activity of NS3 was inhibited by using C-

29EA with an  $IC_{50}$  of 10.9  $\mu$ g/ml (Fig. 7). These results suggest that *Amphimedon* sp. includes the unknown compound(s) that could suppress NS3 enzymatic activity to inhibit HCV replication. Although the mechanism by which treatment with C-29EA could inhibit HCV replication has not yet been revealed, the unknown compound(s) may be associated with the inhibition of NS3 protease and helicase, leading to the suppression of HCV replication. However, other effects of extract C-29EA on HCV replication could not be excluded in this study.

The compound 1-N, 4-N-bis [4-(1H-benzimidazol-2-yl)phenyl] benzene-1,4-dicarboxamide, which is designated as (BIP) $_2$ B, was reported to be a potent and selective inhibitor of HCV NS3 helicase [43]. This compound competitively decreases the binding ability of HCV NS3 helicase to nucleic acids. The compound (BIP) $_2$ B inhibited RNA-induced stimulation of ATPase, although it did not directly affect the ATP hydrolysis activity of NS3 helicase. Thus, (BIP) $_2$ B could not affect ATPase activity without RNA or with a high concentration of RNA. Treatment with C-29EA inhibited helicase activity and viral replication but not ATPase activity (Figs. 1B, 2, 5, and 6). This extract suppressed the binding of RNA to helicase but exhibited no suppression of ATPase by NS3 helicase. Thus, the inhibitory action of extract C-29EA seems

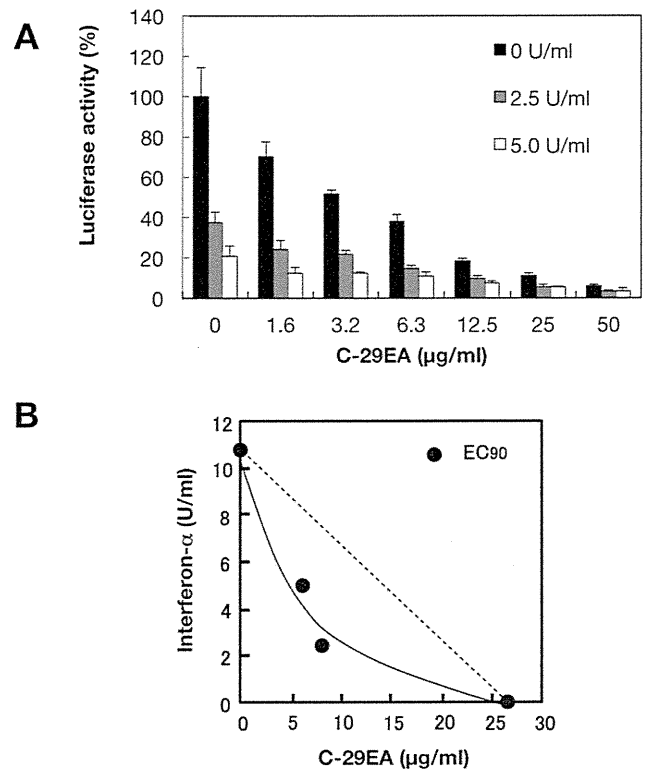


**Figure 7. Effect of C-29EA on the activity of NS3 serine protease.** NS3/4A serine protease was mixed with various concentrations of C-29EA or DMSO (0) in the reaction mixture and then incubated at 37°C for 120 min. The initial velocity at each concentration of C-29EA was calculated during 120 min reaction. The initial velocity in the absence of C-29EA was defined as 100% of relative protease activity. The data are presented as the mean  $\pm$  standard deviation for three replicates.

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different from that of (BIP)<sub>2</sub>B. The quinolone derivative QU663 was reported to inhibit the unwinding activity of NS3 helicase by binding to an RNA-binding groove irrespective of its own ATPase activity [44]. The compound QU663 may competitively bind the RNA-binding site of NS3 but not affect ATPase activity, resulting in the inhibition of unwinding activity. In this study, treatment with C-29EA inhibited the RNA-binding activities of NS3 helicase but did not affect ATPase activity (Fig. 6). Furthermore, treatment with C-29EA suppressed the viral replication of HCV in an HCV cell culture system derived from several virus strains (Figs. 1 and 2, Table 2). The mechanism of C-29EA on the inhibition of NS3 helicase may be similar to that of compound QU663.

It is unknown whether one or several molecules included in C-29EA are critical for the inhibition of protease and helicase activities. The serine protease NS3/4A is one of the viral factors targeted for development into antiviral agents. Improvements in HCV therapy over the past several years have resulted in FDA approval of telaprevir (VX-950) [15,45] and boceprevir (SCH503034) [46,47]. Several studies suggest that the activities of NS3/4A protease and helicase in the full-length molecule enhance each other [48,49]. The NS3/4A protease has formed a complex with macrocyclic acylsulfonamide inhibitors [50,51]. Schiering et al. recently reported the structure of full-length NS3/4A in complex with a macrocyclic acylsulfonamide protease inhibitor [52], although the structure of full-length HCV NS3/4A in complex with a protease inhibitor has not been reported. The inhibitor binds to the active site of the protease, while the P4-capping and P2 moieties of the inhibitor are exposed toward the helicase interface and interact with both protease and helicase residues [52]. An unknown compound included in C-29EA might interact with both protease and helicase domains of NS3 to inhibit their activities. However, our data in this study have not excluded the possibility that several compounds included in C-29EA are related to the inhibition of protease and helicase of NS3/4A.



**Figure 8. Effect of C-29EA on the antiviral activity of interferon-alpha.** (A) The Huh7 cell line, including the subgenomic replicon RNA of genotype 1b strain Con1, was incubated in medium containing various concentrations of C-29EA or DMSO (0) in the presence or the absence of interferon-alpha. Luciferase assay were carried out as described in Materials and Methods. Error bars indicate standard deviation. The data represent three independent experiments. (B) Isobole plots of 90% inhibition of HCV replication. The broken line indicates the additive effect in the isobologram.

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In conclusion, we showed that the EtOAc extract from *Amphimedon* sp. significantly inhibits HCV replication by suppressing viral helicase and protease activities. The purification of an inhibitory compound from the extract of *Amphimedon* sp. will be necessary in order to improve its efficacy by chemical modification.

## Materials and Methods

### Preparation of Extracts from Marine Organisms

All marine organisms used in this study were hand-collected by scuba diving off islands in Okinawa Prefecture, Japan. No specific permits were required for the described field studies. We do not have to obtain a local government permit to collect invertebrates except for stony corals and marine organisms for fisheries, which we did not collect in this study. The areas where we collected are not privately-owned or protected in any way. We did not collect any invertebrates listed in the red data book issued by Ministry of Environment, Japan. The sponges, tunicates, and soft corals used in this study are not listed at all. Hence, no specific permits are required for this collection in the same way as the previous report of Aratake et al. [53].

The sponge from which C-29EA was extracted was identified as *Amphimedon* sp. and deposited at Naturalis under the code RMNH POR 6100. Each specimen was soaked in acetone. The acetone-extract fraction prepared from each specimen was concentrated.