forms between the helicase and protease domains of NS3. Indeed, a novel small-molecule binding site at the interface between these two domains was reported recently [41]. Furthermore, the inhibitory specificity of hal3 and suvanine against HCV NS3 helicase might be explained by structural differences between HCV and DENV NS3 helicases. A specific beta-strand tethers the *C* terminus of the helicase domain to the protease domain of HCV NS3, maintaining it in a compact conformation that differs from the extended conformation of DENV NS3 helicase [42]. As only HCV NS3 helicase forms an interface between the helicase and protease domains, the specificity of hal3 and suvanine for HCV NS3 helicase would be explained by the binding of hal3 and suvanine to the interface of HCV NS3.

Figure 6. Effects of hal3 (**A**) and suvanine (**B**) on DENV NS3 helicase activity, assessed using a gel-based helicase assay. Fluorescence-labeled ssRNA and dsRNA were applied to lanes *1* and *2*, respectively. The dsRNA was incubated with NS3 in the presence of increasing concentrations of inhibitor (lanes *3–7*, 0–100 μM). Lanes *8* and *9* contain the control reaction mixtures in the absence of NS3, and in the presence of 15 mM ATP-gamma-S as an inhibition control, respectively.
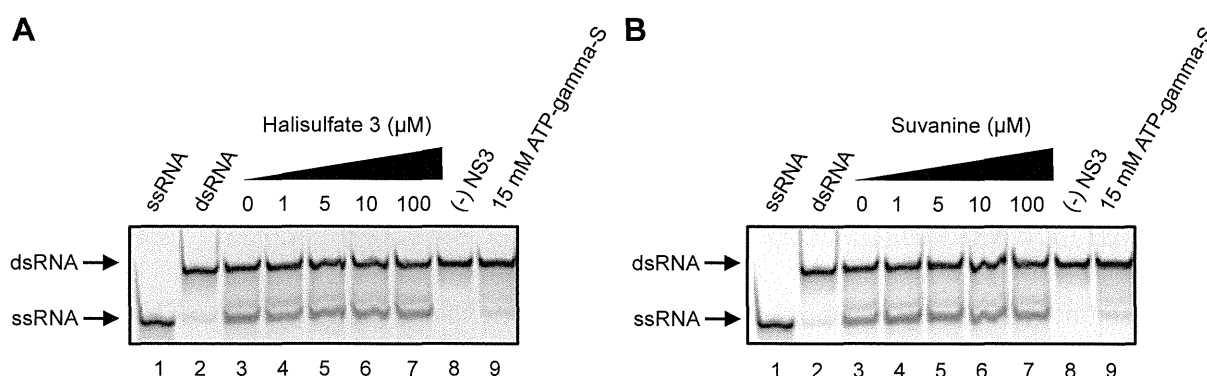
3. Experimental Section

3.1. Preparation of Extracts from Marine Organisms

Specimens of marine organisms were collected by scuba diving in Okinawa, Japan, and Sorong, Indonesia, and kept frozen until use. The specimens were chopped into small pieces, and soaked in acetone for 20 h followed by methanol for 6 h. The acetone and methanol solutions were then combined and concentrated, and residual materials were separated into ethyl acetate and aqueous layers; each layer was then dried to obtain residues.

3.2. Screening for HCV NS3 Helicase Inhibitors

The PET-based fluorescence helicase assay was performed as described previously [30]. The dsRNA substrate was prepared by annealing the 5′ BODIPY FL-labeled fluorescence strand (5′-CUAUUACCUCCACCCUCAUAACCUUUUUUUUUUUUU-3′) to the quencher strand (5′-GGUUAUGAGGGUGGAGGUAAUAG-3′) at a 1:2 molar ratio. The dsRNA substrate contains the 3′-overhang that is necessary for the NS3 helicase to bind RNA prior to duplex unwinding. The capture strand (5′-CTATTACCTCCACCCTCATAACC-3′), which is complementary to the quencher strand, prevents the unwound duplexes from reannealing. None of the capture, quencher, or fluorescence

strands are self-complementary. The fluorescence strand was purchased from J-Bio 21 Corporation (Tokyo, Japan), and was labeled with BODIPY FL at the 5′-end via an aminohexylphosphate linker with a six-carbon spacer. The quencher and capture strands were purchased from Japan Bio Services (Saitama, Japan). The reaction mixture contained 25 mM MOPS-NaOH (pH 6.5), 3 mM $MgCl_2$, 2 mM dithiothreitol, 4 U RNasin (Promega, Madison, WI, USA), 50 nM dsRNA substrate, 100 nM capture strand, 5 mM ATP, a marine organism extract, and 240 nM NS3 in a total reaction volume of 20 μL. Each marine organism extract diluted in DMSO was added to the reaction mixture at a final concentration of 17.5–32.5 μg/mL. The full-length HCV NS3 protein with serine protease and NTPase/helicase activities was expressed and purified as described previously [43].

The reaction was started by the addition of HCV NS3 helicase, and was performed at 37 °C for 30 min using a LightCycler 1.5 (Roche Diagnostics, Basel, Switzerland). The fluorescence intensity was recorded every 5 s from 0 to 5 min, and then every 30 s from 5 to 30 min. Helicase activity was calculated as the initial reaction velocity relative to control (in the absence of a marine extract, but presence of DMSO vehicle). The IC_{50} was calculated using KaleidaGraph (Synergy Software, Reading, PA, USA) by fitting plots of % activity *vs.* [*I*] using Equation (1) unless otherwise noted [44]:

$$\% \text{ Activity} = \frac{100}{1 + \left([I]/IC_{50} \right)^h}$$

(1)

where *h* is the Hill coefficient, and [*I*] is the inhibitor concentration.

3.3. Gel-Based HCV NS3 Helicase Assay

A gel-based helicase assay was performed on HCV NS3 helicases using an Alexa Fluor 488-labeled dsRNA strand and capture strand with the same nucleic acid sequences described in Section 3.2. The dsRNA substrate was prepared by annealing the 5′ Alexa Fluor 488-labeled strand (5′-CUAUUACCUCCACCCUCAUAACCUUUUUUUUUUUUU-3′) to the complementary strand (5′-GGUUAUGAGGGUGGAGGUAAUAG-3′) at a 1:2 molar ratio. The same capture strand described in Section 3.2 was used. All nucleic acid strands were purchased from Japan Bio Services (Saitama, Japan). The reaction mixture for HCV NS3 helicase contained the same components as described in Section 3.2, with increasing concentrations of hal3 or suvanine in a reaction volume of 20 μL. The reaction was started by the addition of HCV NS3 helicase, and performed at 37 °C for 60 min using a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). The reaction was stopped by the addition of 5 μL of helicase termination buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM EDTA, 30% glycerol, 0.06% bromophenol blue, and 0.12% Orange G. The inhibition of NS3 helicase was analyzed on a 20% native Tris/borate/EDTA (TBE) polyacrylamide gel, and labeled RNAs were visualized using Typhoon 9210 (GE Healthcare, Waukesha, WI, USA). The helicase activity was calculated as the ratio of the signal intensity derived from ssRNA in the sample containing inhibitor to that in the control sample containing DMSO vehicle instead of inhibitor.

3.4. ATPase Assay

NS3 ATPase activity was determined directly by monitoring [γ-^{32}P] ATP hydrolysis by thin-layer chromatography, as described previously [45,46]. The reaction mixture contained 25 mM MOPS-NaOH

(pH 7.0), 1 mM dithiothreitol, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 1 mM [γ-^{32}P] ATP (Muromachi Yakuhin, Tokyo, Japan), 300 nM NS3, 0.1 μg/μL poly (U) ssRNA (Sigma-Aldrich, St. Louis, MO, USA), and increasing concentrations of hal3 or suvanine in a volume of 10 μL. The reaction was conducted at 37 °C for 10 min, and stopped by the addition of 10 mM EDTA. Two microliters of each reaction mixture was then spotted onto a polyethyleneimine cellulose sheet (Merck, Darmstadt, Germany) and developed in 0.75 M LiCl/1 M formic acid solution for 20 min. The cellulose sheet was dried, and the released [γ-^{32}P] phosphoric acid was visualized using an Image Reader FLA-9000 and quantified using Multi Gauge software V 3.11 (Fujifilm, Tokyo, Japan). ATPase activity was calculated as the ratio of the signal intensity derived from the released Pi in the sample containing inhibitor to that in the control sample containing DMSO vehicle instead of inhibitor.

3.5. RNA Binding Assay

NS3 RNA binding activity was determined by gel mobility shift assay, as described previously [45,46]. The ssRNA (5′-UGAGGUAGUAGGUUGUAUAGU-3′) synthesized by Gene Design (Osaka, Japan) was labeled at the 5′-end with [γ-^{32}P] ATP (Muromachi Yakuhin, Tokyo, Japan) using T4 polynucleotide kinase (Toyobo, Osaka, Japan) at 37 °C for 60 min, and purified using the phenol-chloroform extraction method. The reaction mixture contained 30 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 20 U RNasin Plus (Promega), 300 nM NS3, 0.1 nM ^{32}P-labeled ssRNA, and increasing concentrations of inhibitor in a volume of 20 μL. The reaction was performed at room temperature for 15 min. An equal volume of a dye solution containing 0.025% bromophenol blue and 10% glycerol in 0.5× TBE was then added to each reaction mixture, and samples were loaded onto a 6% native-polyacrylamide gel. The labeled RNA bands were visualized using an Image Reader FLA-9000 and quantified using Multi Gauge software V 3.11 (Fujifilm, Tokyo, Japan). RNA binding activity was calculated as the ratio of the signal intensity derived from the NS3-ssRNA complex in the sample containing hal3 or suvanine to that in the control sample containing DMSO vehicle rather than inhibitor.

3.6. Serine Protease Assay

A fluorescence NS3 serine protease assay, based on fluorescence resonance energy transfer, was conducted using reagents provided in a SensoLyte™ 520 HCV protease assay kit (AnaSpec, San Jose, CA, USA), as described previously [30]. Briefly, NS3 protein with a two-fold excess of the NS4A cofactor peptide Pep4AK was prepared in 1× assay buffer provided with the kit. HCV NS3/4A protease was mixed with increasing concentrations of inhibitor, and incubated at 37 °C for 15 min. The reaction was started by the addition of 5-FAM/QXL 520 substrate in a 20 μL total reaction volume containing 240 nM HCV NS3/4A protease and increasing concentrations of hal3 or suvanine. Reactions were then incubated at 37 °C for 120 min on a LightCycler 1.5 (Roche Diagnostics, Basel, Switzerland), and the fluorescence intensity was recorded every min for 120 min. NS3 serine protease activity was calculated as the initial reaction velocity in the sample containing inhibitor relative to the control sample containing DMSO vehicle rather than inhibitor.

3.7. Gel-Based DENV NS3 Helicase Assay

A gel-based helicase assay was performed using DENV NS3 helicases, and the Alexa Fluor 488-labeled dsRNA strand and capture strand with the same nucleic acid sequences described in the Section 3.3. DENV NS3 helicase requires a single stranded 3′ overhang to unwind dsRNA substrates in the 3′ to 5′ direction [39]; therefore, the substrate specificities of the DENV and HCV NS3 helicases are the same. The reaction mixture contained 50 mM Tris-HCl (pH 7.4), 1 mM DTT, 0.5% Tween 20, 0.25 μg/mL BSA, 2 mM $MgCl_2$, 4 U RNasin (Promega), 5 mM ATP, 50 nM dsRNA substrate, 300 nM capture strand, an inhibitor, and 240 nM DENV NS3 in a total volume of 20 μL. DENV NS3 helicase was prepared as described previously [47]. The reaction was started by the addition of DENV NS3 helicase, and was performed at 37 °C for 60 min using a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). The reaction was then stopped by the addition of 5 μL helicase termination buffer that contained 10 mM Tris-HCl (pH 7.5), 50 mM EDTA, 30% glycerol, 0.06% bromophenol blue, and 0.12% Orange G. The inhibition of DENV NS3 helicase was analyzed on a 20% native TBE polyacrylamide gel, and the labeled RNAs were visualized using Typhoon 9210 (GE Healthcare, Waukesha, WI, USA). The helicase activity was calculated as the ratio of the signal intensity from ssRNA in the sample containing inhibitor to that in the control sample containing DMSO vehicle instead of inhibitor.

4. Conclusions

This study demonstrated that hal3 and suvanine isolated from a marine sponge inhibited NS3 helicase by suppressing the ATPase, RNA binding, and serine protease activities. Moreover, DENV NS3 helicase, which shares a catalytic core consisting mainly of ATPase and RNA binding activity sites with HCV NS3 helicase, was not inhibited by hal3 or suvanine. Therefore, it can be concluded that hal3 and suvanine inhibit HCV NS3 helicase specifically through interaction with an allosteric site of NS3 rather than the catalytic core, leading to the inhibition of all NS3 activities, presumably by inducing conformational changes. As such, it is possible that hal3 and suvanine are less likely to inhibit other cellular helicases that share a similar catalytic core to HCV NS3 helicase. This provides potentially useful information on advanced drug design strategies to identify novel NS3 helicase inhibitors that are expected to be more specific and less toxic. Experiments to address whether resistant HCV mutants emerge with the use of these compounds are underway in our laboratory.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Ghany, M.G.; Nelson, D.R.; Strader, D.B.; Thomas, D.L.; Seeff, L.B. An update on treatment of genotype 1 chronic hepatitis C virus infection: 2011 practice guideline by the American Association for the Study of Liver Diseases. *Hepatology* **2011**, *54*, 1433–1444.

2. Liang, T.J.; Ghany, M.G. Current and future therapies for hepatitis C virus infection. *N. Engl. J. Med.* **2013**, *368*, 1907–1917.

3. Sarrazin, C.; Hézode, C.; Zeuzem, S.; Pawlotsky, J.-M. Antiviral strategies in hepatitis C virus infection. *J. Hepatol.* **2012**, *56*, S88–S100.

4. Scheel, T.K.H.; Rice, C.M. Understanding the hepatitis C virus life cycle paves the way for highly effective therapies. *Nat. Med.* **2013**, *19*, 837–849.

5. Lam, A.M.I.; Frick, D.N. Hepatitis C virus subgenomic replicon requires an active NS3 RNA helicase. *J. Virol.* **2006**, *80*, 404–411.

6. Kwong, A.D.; Rao, B.G.; Jeang, K.-T. Viral and cellular RNA helicases as antiviral targets. *Nat. Rev. Drug Discov.* **2005**, *4*, 845–853.

7. Bartenschlager, R.; Penin, F.; Lohmann, V.; André, P. Assembly of infectious hepatitis C virus particles. *Trends Microbiol.* **2011**, *19*, 95–103.

8. Bartenschlager, R.; Lohmann, V.; Penin, F. The molecular and structural basis of advanced antiviral therapy for hepatitis C virus infection. *Nat. Rev. Microbiol.* **2013**, *11*, 482–496.

9. Lohmann, V.; Körner, F.; Koch, J.-O.; Herian, U.; Theilmann, L.; Bartenschlager, R. Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **1999**, *285*, 110–113.

10. Gallinari, P.; Brennan, D.; Nardi, C.; Brunetti, M.; Tomei, L.; Steinkühler, C.; de Francesco, R. Multiple enzymatic activities associated with recombinant NS3 protein of hepatitis C virus. *J. Virol.* **1998**, *72*, 6758–6769.

11. Kim, D.W.; Gwack, Y.; Han, J.H.; Choe, J. *C*-terminal domain of the hepatitis C virus NS3 protein contains an RNA helicase activity. *Biochem. Biophys. Res. Commun.* **1995**, *215*, 160–166.

12. Tai, C.-L.; Chi, W.-K.; Chen, D.-S.; Hwang, L.-H. The helicase activity associated with hepatitis C virus nonstructural protein 3 (NS3). *J. Virol.* **1996**, *70*, 8477–8484.

13. Gwack, Y.; Kim, D.W.; Han, J.H.; Choe, J. Characterization of RNA binding activity and RNA helicase activity of the hepatitis C virus NS3 protein. *Biochem. Biophys. Res. Commun.* **1996**, *225*, 654–659.

14. Gwack, Y.; Kim, D.W.; Han, J.H.; Choe, J. DNA helicase activity of the hepatitis C virus nonstructural protein 3. *Eur. J. Biochem.* **1997**, *250*, 47–54.

15. Kolykhalov, A.A.; Mihalik, K.; Feinstone, S.M.; Rice, C.M. Hepatitis C virus-encoded enzymatic activities and conserved RNA elements in the 3′ nontranslated region are essential for virus replication *in vivo*. *J. Virol.* **2000**, *74*, 2046–2051.

16. Hall, M.C.; Matson, S.W. Helicase motifs: The engine that powers DNA unwinding. *Mol. Microbiol.* **1999**, *34*, 867–877.

17. Gorbalenya, A.E.; Koonin, E.V. Helicases: Amino acid sequence comparisons and structure-function relationships. *Curr. Opin. Struct. Biol.* **1993**, *3*, 419–429.

18. Jankowsky, E. *RNA Helicases*; Royal Society of Chemistry: London, UK, 2010; pp. 168–188.

19. Frick, D.N.; Lam, A.M.I. Understanding helicases as a means of virus control. *Curr. Pharm. Des.* **2006**, *12*, 1315–1338.

20. Borowski, P.; Deinert, J.; Schalinski, S.; Bretner, M.; Ginalski, K.; Kulikowski, T.; Shugar, D. Halogenated benzimidazoles and benzotriazoles as inhibitors of the NTPase/helicase activities of hepatitis C and related viruses. *Eur. J. Biochem.* **2003**, *270*, 1645–1653.

21. Zhang, N.; Chen, H.-M.; Koch, V.; Schmitz, H.; Liao, C.-L.; Bretner, M.; Bhadti, V.S.; Fattom, A.I.; Naso, R.B.; Hosmane, R.S.; *et al.* Ring-expanded ("fat") nucleoside and nucleotide analogues exhibit potent *in vitro* activity against *Flaviviridae* NTPases/helicases, including those of the West Nile virus, hepatitis C virus, and Japanese encephalitis virus. *J. Med. Chem.* **2003**, *46*, 4149–4164.

22. Borowski, P.; Heising, M.V.; Miranda, I.B.; Liao, C.-L.; Choe, J.; Baier, A. Viral NS3 helicase activity is inhibited by peptides reproducing the Arg-rich conserved motif of the enzyme (motif VI). *Biochem. Pharmacol.* **2008**, *76*, 28–38.

23. Frick, D.N.; Ginzburg, O.; Lam, A.M.I. A method to simultaneously monitor hepatitis C virus NS3 helicase and protease activities. *Methods Mol. Biol.* **2010**, *587*, 223–233.

24. Dahl, G.; Sandström, A.; Akerblom, E.; Danielson, U.H. Effects on protease inhibition by modifying of helicase residues in hepatitis C virus nonstructural protein 3. *FEBS J.* **2007**, *274*, 5979–5986.

25. Frick, D.N. The hepatitis C virus NS3 protein: A model RNA helicase and potential drug target. *Curr. Issues Mol. Biol.* **2007**, *9*, 1–20.

26. Belon, C.A.; Frick, D.N. Helicase inhibitors as specifically targeted antiviral therapy for hepatitis C. *Future Virol.* **2009**, *4*, 277–293.

27. Mayer, A.M.S.; Glaser, K.B.; Cuevas, C.; Jacobs, R.S.; Kem, W.; Little, R.D.; McIntosh, J.M.; Newman, D.J.; Potts, B.C.; Shuster, D.E. The odyssey of marine pharmaceuticals: A current pipeline perspective. *Trends Pharmacol. Sci.* **2010**, *31*, 255–265.

28. Gerwick, W.H.; Moore, B.S. Lessons from the past and charting the future of marine natural products drug discovery and chemical biology. *Chem. Biol.* **2012**, *19*, 85–98.

29. Newman, D.J.; Cragg, G.M. Natural products as sources of new drugs over the 30 years from 1981 to 2010. *J. Nat. Prod.* **2012**, *75*, 311–335.

30. Furuta, A.; Salam, K.A.; Akimitsu, N.; Tanaka, J.; Tani, H.; Yamashita, A.; Moriishi, K.; Nakakoshi, M.; Tsubuki, M.; Sekiguchi, Y.; *et al.* Cholesterol sulfate as a potential inhibitor of hepatitis C virus NS3 helicase. *J. Enzym. Inhib. Med. Chem.* **2013**, doi:10.3109/14756366. 2013.766607.

31. Müller, E.L.; Faulkner, D.J. Absolute configuration of halisulfate 3 from the sponge *Ircinia* sp. *Tetrahedron* **1997**, *53*, 5373–5378.

32. Manes, L.V.; Crews, P.; Kernan, M.R.; Faulkner, D.J.; Fronczek, F.R.; Gandour, R.D. Chemistry and revised structure of suvanine. *J. Org. Chem.* **1988**, *53*, 570–575.

33. Kernan, M.R.; Faulkner, D.J. Sesterterpene sulfates from a sponge of the family Halichondriidae. *J. Org. Chem.* **1988**, *53*, 4574–4578.

34. Manes, L.V.; Naylor, S.; Crews, P.; Bakus, G.J. Suvanine, a novel sesterterpene from an *Ircinia* marine sponge. *J. Org. Chem.* **1985**, *50*, 284–286.

35. Kimura, J.; Ishizuka, E.; Nakao, Y.; Yoshida, W.Y.; Scheuer, P.J.; Kelly-Borges, M. Isolation of 1-methylherbipoline salts of halisulfate-1 and of suvanine as serine protease inhibitors from a marine sponge, *Coscinoderma mathewsi. J. Nat. Prod.* **1998**, *61*, 248–250.

36. Di Leva, F.S.; Festa, C.; D'Amore, C.; de Marino, S.; Renga, B.; D'Auria, M.V.; Novellino, E.; Limongelli, V.; Zampella, A.; Fiorucci, S. Binding mechanism of the farnesoid X receptor marine antagonist suvanine reveals a strategy to forestall drug modulation on nuclear receptors. Design, synthesis, and biological evaluation of novel ligands. *J. Med. Chem.* **2013**, *56*, 4701–4717.

37. Cassiano, C.; Monti, M.C.; Festa, C.; Zampella, A.; Riccio, R.; Casapullo, A. Chemical proteomics reveals heat shock protein 60 to be the main cellular target of the marine bioactive sesterterpene suvanine. *ChemBioChem* **2012**, *13*, 1953–1958.

38. Singleton, M.R.; Dillingham, M.S.; Wigley, D.B. Structure and mechanism of helicases and nucleic acid translocases. *Annu. Rev. Biochem.* **2007**, *76*, 23–50.

39. Wang, C.-C.; Huang, Z.-S.; Chiang, P.-L.; Chen, C.-T.; Wu, H.-N. Analysis of the nucleoside triphosphatase, RNA triphosphatase, and unwinding activities of the helicase domain of dengue virus NS3 protein. *FEBS Lett.* **2009**, *583*, 691–696.

40. Ndjomou, J.; Kolli, R.; Mukherjee, S.; Shadrick, W.R.; Hanson, A.M.; Sweeney, N.L.; Bartczak, D.; Li, K.; Frankowski, K.J.; Schoenen, F.J.; *et al.* Fluorescent primuline derivatives inhibit hepatitis C virus NS3-catalyzed RNA unwinding, peptide hydrolysis and viral replicase formation. *Antivir. Res.* **2012**, *96*, 245–255.

41. Saalau-Bethell, S.M.; Woodhead, A.J.; Chessari, G.; Carr, M.G.; Coyle, J.; Graham, B.; Hiscock, S.D.; Murray, C.W.; Pathuri, P.; Rich, S.J.; *et al.* Discovery of an allosteric mechanism for the regulation of HCV NS3 protein function. *Nat. Chem. Biol.* **2012**, *8*, 920–925.

42. Ding, S.C.; Kohlway, A.S.; Pyle, A.M. Unmasking the active helicase conformation of nonstructural protein 3 from hepatitis C virus. *J. Virol.* **2011**, *85*, 4343–4353.

43. Tani, H.; Akimitsu, N.; Fujita, O.; Matsuda, Y.; Miyata, R.; Tsuneda, S.; Igarashi, M.; Sekiguchi, Y.; Noda, N. High-throughput screening assay of hepatitis C virus helicase inhibitors using fluorescence-quenching phenomenon. *Biochem. Biophys. Res. Commun.* **2009**, *379*, 1054–1059.

44. Copeland, R.A. *Evaluation of Enzyme Inhibitors in Drug Discovery*; John Wiley & Sons: New York, NY, USA, 2005; pp. 111–140.

45. Salam, K.A.; Furuta, A.; Noda, N.; Tsuneda, S.; Sekiguchi, Y.; Yamashita, A.; Moriishi, K.; Nakakoshi, M.; Tsubuki, M.; Tani, H.; *et al.* Inhibition of hepatitis C virus NS3 helicase by manoalide. *J. Nat. Prod.* **2012**, *75*, 650–654.

46. Salam, K.A.; Furuta, A.; Noda, N.; Tsuneda, S.; Sekiguchi, Y.; Yamashita, A.; Moriishi, K.; Nakakoshi, M.; Tsubuki, M.; Tani, H.; *et al.* Psammaplin A inhibits hepatitis C virus NS3 helicase. *J. Nat. Med.* **2013**, *67*, 765–772.

47. Takahashi, H.; Takahashi, C.; Moreland, N.J.; Chang, Y.-T.; Sawasaki, T.; Ryo, A.; Vasudevan, S.G.; Suzuki, Y.; Yamamoto, N. Establishment of a robust dengue virus NS3-NS5 binding assay for identification of protein-protein interaction inhibitors. *Antivir. Res.* **2012**, *96*, 305–314.

molecules

Article

PBDE: Structure-Activity Studies for the Inhibition of Hepatitis C Virus NS3 Helicase

Kazi Abdus Salam [1], Atsushi Furuta [2,3], Naohiro Noda [2,3], Satoshi Tsuneda [2], Yuji Sekiguchi [3], Atsuya Yamashita [4], Kohji Moriishi [4], Masamichi Nakakoshi [5], Hidenori Tani [6], Sona Rani Roy [7], Junichi Tanaka [7], Masayoshi Tsubuki [8,*] and Nobuyoshi Akimitsu [1,*]

[1] Radioisotope Center, The University of Tokyo, 2-11-16 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan; E-Mail: salam_bio26@yahoo.com

[2] Department of Life Science and Medical Bioscience, Waseda University, 2-2 Wakamatsu-cho, Shinjuku-ku, Tokyo 162-8480, Japan; E-Mails: atsushi.5961@ruri.waseda.jp (A.F.); stsuneda@waseda.jp (S.T.)

[3] Biomedical Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Higashi, Tsukuba, Ibaraki 305-8566, Japan;
E-Mails: noda-naohiro@aist.go.jp (N.N.); y.sekiguchi@aist.go.jp (Y.S.)

[4] Department of Microbiology, Graduate School of Medicine and Engineering, University of Yamanashi, 1110 Shimokato, Chuo-shi, Yamanashi 409-3898, Japan;
E-Mails: atsuyay@yamanashi.ac.jp (A.Y.); kmoriishi@yamanashi.ac.jp (K.M.)

[5] Faculty of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan; E-Mail: nakakoshi@phar.toho-u.ac.jp

[6] Research Institute for Environmental Management Technology, National Institute of Advanced Industrial Science and Technology (AIST), 16-1, Onogawa, Tsukuba, Ibaraki 305-8569, Japan; E-Mail: h.tani@aist.go.jp

[7] Department of Chemistry, Biology and Marine Science, University of the Ryukyus, Nishihara, Okinawa 903-0213, Japan; E-Mails: sonarroy@gmail.com (S.R.R.); jtanaka@sci.u-ryukyu.ac.jp (J.T.)

[8] Institute of Medical Chemistry, Hoshi University, Ebara 2-4-41, Shinagawa-ku, Tokyo 142-8501, Japan

* Authors to whom correspondence should be addressed; E-Mails: tsubuki@hoshi.ac.jp (M.T.); akimitsu@ric.u-tokyo.ac.jp (N.A.); Tel.: +81-3-5498-5793 (M.T.); Fax: +81-3-3787-0036 (M.T.); Tel.: +81-3-5841-2877 (N.A.); Fax: +81-3-5841-3049 (N.A.).

Abstract: The helicase portion of the hepatitis C virus nonstructural protein 3 (NS3) is considered one of the most validated targets for developing direct acting antiviral agents. We isolated polybrominated diphenyl ether (PBDE) **1** from a marine sponge as an NS3 helicase inhibitor. In this study, we evaluated the inhibitory effects of PBDE (**1**) on the essential activities of NS3 protein such as RNA helicase, ATPase, and RNA binding activities. The structure-activity relationship analysis of PBDE (**1**) against the HCV ATPase revealed that the biphenyl ring, bromine, and phenolic hydroxyl group on the benzene backbone might be a basic scaffold for the inhibitory potency.

Keywords: hepatitis C virus; NS3 RNA helicase; marine sponge; polybrominated diphenyl ether

1. Introduction

Hepatitis C virus (HCV) is one of the major causative agents for hepatitis C, which has caused an epidemic of liver fibrosis, cirrhosis, and hepatocellular carcinoma [1]. HCV infects more than 150 million people worldwide, and over 350,000 people die from HCV-related liver diseases each year [2]. The virus is undetectable for long periods of time, even decades, and replicates slowly without major complications. Therefore, most infected people are unaware they carry the virus. HCV is only transmitted via blood and blood products, while sexual and mother-to-child transmission is much less likely than for HIV infection [2].

The recently approved new treatment regimen for HCV infection is the combination of pegylated interferon and ribavirin with either telaprevir or boceprevir for genotype 1 infected patients. However, the emergence of viral resistance to the drugs as well as side effects, such as anemia, neutropenia, dysgeusia, rash, and anorectal discomfort, are the main concerns [3–6]. Despite intensive studies for the development of new antiviral drugs, HCV is still a major threat to human health. Therefore, there is an urgent need to develop new antiviral drugs with fewer side effects and the highest antiviral efficacy.

HCV is a single-stranded, positive-sense RNA virus in the *Flaviviridae* family [1,7]. Seven genotypes and more than 50 subtypes of HCV have been described [8]. The viral genome is 9.6 kb in length and contains one main open reading frame encoding an approximately 3,000 amino acid single polyprotein, flanked by a 5'-non-translated region (NTR) and a 3'-NTR. Once translation initiated by an internal ribosome entry site present at the 5'-NTR, host and viral proteases cleave the product into 10 individual viral mature proteins [9]. The structural proteins (envelope glycoproteins; E1 and E2) are responsible for receptor binding, thereby facilitating viral entry into the hepatocyte. The core protein (C) forms the viral nucleocapsid [10]. The nonstructural proteins p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B are involved in viral replication and packaging of the HCV genome. NS3 is a multifunctional protein that plays an important role in the viral life cycle. It has a serine protease (NS3/4A) activity at the N-terminal to cleave all downstream junctions, and helicase activity at the C-terminal to separate double-stranded RNA in a reaction fueled by ATP hydrolysis during replication of viral genomic RNA [11,12]. Although the precise role of the helicase activity in the viral life cycle

is not well understood, a fully functional helicase is essential for HCV RNA replication. The helicase portion of NS3 is thus a valid target for the development of direct acting antiviral therapy.

The development of antiviral agents for the treatment of HCV infection has been focused on small molecule inhibitors of HCV infection that can act directly on viral targets or other host target proteins critical to HCV replication. The first two approved direct acting antiviral agents, telaprevir and boceprevir, are inhibitors of the NS3/4A protease activity [13]. However, very few compounds that inhibit the NS3 helicase function have been reported, and to the best of our knowledge, no helicase inhibitors have entered clinical trials. Thus there is still a great need in HCV research to develop novel NS3 helicase inhibitors.

The aim of this project was to identify a possible NS3 helicase inhibitor from marine natural products. In this study, we successfully obtained from marine sponge and identified hydroxylated polybrominated diphenyl ether OH-PBDE-47 (**1**), as a helicase inhibitor through a high-throughput screening method based on fluorescence resonance energy transfer (FRET). We also evaluated several commercially available compounds that are structurally related to PBDE (**1**) for the study of structure-activity relationships.

PBDEs have been found to exhibit antibacterial, antifungal, and antimicroalgal activities [14–19]. They inhibit a wide range of enzymes that are relevant to anticancer drug discovery such as inosine monophosphate dehydrogenase, guanosine monophosphate synthetase, and 15-lipoxygenase [20]. PBDEs have also been shown to exhibit inhibitory activities against the assembly of microtubule protein, the maturation of starfish oocytes [21] and Tie2 kinase [22]. In this research, we found a novel activity of PBDE in the specific inhibition of HCV NS3 helicase activity.

2. Results and Discussion

To screen potential NS3 helicase inhibitors from extracts of marine organisms, we used a high-throughput fluorescence helicase assay based on FRET [23]. Out of 41 extracts isolated (Table 1), PBDE (**1**) (Figure 1) exhibited the strongest inhibition (37%) of NS3 helicase activity.

Table 1. Inhibitory effects of extracts from marine organisms on hepatitis C virus (HCV) nonstructural protein 3 (NS3) helicase activity.

| No.	Sample ID	FRET (%) [a]	Possibly Contained Molecule	Species	Location
1	PM-35-1	85	misakinolide	sponge (*Theonella* sp.)	Tokashiki Island, Okinawa
2	PM-35-2	94		sponge (*Theonella* sp.)	Tokashiki Island, Okinawa
3	PM-36-1	79		gorgonian (*Euplexaura* sp.)	Tokashiki Island, Okinawa
4	PM-36-2	114		gorgonian (*Euplexaura* sp.)	Tokashiki Island, Okinawa
5	PM-37-1	93	briarane diterpenes	gorgonian (*Junceella fragilis*)	Tokashiki Island, Okinawa

Table 1. *Cont.*

No.	Sample ID	FRET (%) [a]	Possibly Contained Molecule	Species	Location
6	PM-37-2	115		gorgonian (*Junceella fragilis*)	Tokashiki Island, Okinawa
7	PM-38-1	92	hippuristanol	gorgonian (*Isis hippuris*)	Tokashiki Island, Okinawa
8	PM-38-2	112		gorgonian (*Isis hippuris*)	Tokashiki Island, Okinawa
9	PM-39-2	94		sponge (*Petrosia* sp.)	Tokashiki Island, Okinawa
10	SR-1-1	37	PBDE	sponge (*Dysidea granulosa*)	Yonaguni Island, Okinawa
11	SR-2-2	92		sponge (*Jaspis* sp.)	Yonaguni Island, Okinawa
12	SR-3-1	75	petrosynol/petrosynone	sponge (*Petrosia* sp.)	Yonaguni Island, Okinawa
13	SR-4-1	68	strongylophorines	sponge (*Strongylophora* sp.)	Yonaguni Island, Okinawa
14	SR-4-2	86		sponge (*Strongylophora* sp.)	Yonaguni Island, Okinawa
15	SR-6-1	98	sesquiterpenes	soft coral (*Clavularia* sp.)	Yonaguni Island, Okinawa
16	SR-8-1	98		soft coral (*Parerythropodium* sp.)	Yonaguni Island, Okinawa
17	SR-8-2	84		Yonaguni Island, Okinawa	Yonaguni Island, Okinawa
18	SR-10-1	112	polyketide peroxides	sponge (*Plakortis* sp.)	Yonaguni Island, Okinawa
19	SR-11-1	65		sponge (unidentified)	Yonaguni Island, Okinawa
20	SR-12-2	135		sponge (unidentified)	Yonaguni Island, Okinawa
21	SR-13-2	109		sponge (*Pseudoceratina purpurea*)	Yonaguni Island, Okinawa
22	SR-14-1	64	swinholide	sponge (*Theonella swinhoei*)	Yonaguni Island, Okinawa
23	SR-15-1	61		sponge (unidentified)	Yonaguni Island, Okinawa
24	SR-16-1	92		sponge (unidentified)	Yonaguni Island, Okinawa
25	SR-17-2	87		sponge (unidentified)	Yonaguni Island, Okinawa
26	SR-19-2	131		sponge (*Hyrtios* sp.)	Yonaguni Island, Okinawa

Table 1. *Cont.*

No.	Sample ID	FRET (%) [a]	Possibly Contained Molecule	Species	Location
27	SR-21-1	155	xestospongin	sponge (*Xestospongia* sp.)	Yonaguni Island, Okinawa
28	SR-21-2	156		sponge (*Xestospongia* sp.)	Yonaguni Island, Okinawa
29	SR-23-1	73	avarol	sponge (*Dysidea arenaria*)	Yonaguni Island, Okinawa
30	SR-23-2	82		sponge (*Dysidea arenaria*)	Yonaguni Island, Okinawa
31	SR-24-1	123	isocyanosesquiterpenes	sponge (*Theonella* sp.)	Yonaguni Island, Okinawa
32	SR-26-2	102		sponge (unidentified)	Yonaguni Island, Okinawa
33	SR-27-1	188		sponge (*Leucetta* sp.)	Yonaguni Island, Okinawa
34	SR-27-2	171		sponge (*Leucetta* sp.)	Yonaguni Island, Okinawa
35	SR-28-1	107		sponge (unidentified)	Yonaguni Island, Okinawa
36	SR-29-2	145		sponge (*Aaptos* sp.)	Yonaguni Island, Okinawa
37	SR-30-2	215	agelasine	sponge (*Agelas* sp.)	Yonaguni Island, Okinawa
38	SR-31-1	100	hippuristanol	gorgonian (*Isis hippuris*)	Yonaguni Island, Okinawa
39	SR-33-2	88		sponge (unidentified)	Yonaguni Island, Okinawa
40	SR-34-1	125		zoanthus (*Palythoa* sp.)	Yonaguni Island, Okinawa
41	SR-34-2	124	palytoxin	zoanthus (*Palythoa* sp.)	Yonaguni Island, Okinawa

[a] NS3 activity in the presence of marine organisms extract is expressed as a percentage of the control in the absence of extract (100%).

Figure 1. Chemical structure of PBDE (**1**).

1
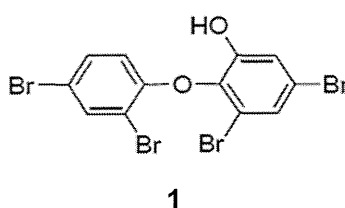
NS3 helicase hydrolyzes ATP as an energy source to drive the unwinding of dsRNA or dsDNA. Therefore, we measured the inhibitory effects of PBDE (**1**) on the ATPase activity of the helicase portion of NS3. A radioisotope labeling ATPase assay showed that PBDE (**1**) inhibited the hydrolytic release of inorganic phosphate from ATP with an IC_{50} of 80 μM (Figure 2A,B).

Figure 2. PBDE (**1**) inhibits NS3 ATPase activity. (**A**) Radioisotope labeling ATPase assay with NS3 (300 nM) and various concentrations of PBDE. Lane 1 shows the negative control reaction. Lanes 2–3 show the reaction mixture containing only NS3 and DMSO. Lanes 4–17 show hydrolytic reactions with NS3 (300 nM) in the presence of PBDE as indicated. (**B**) Graphical representation of the inhibition results. The relative ATPase activity for control reactions was considered as 100%. The average values are presented with error bars from duplicate assays.
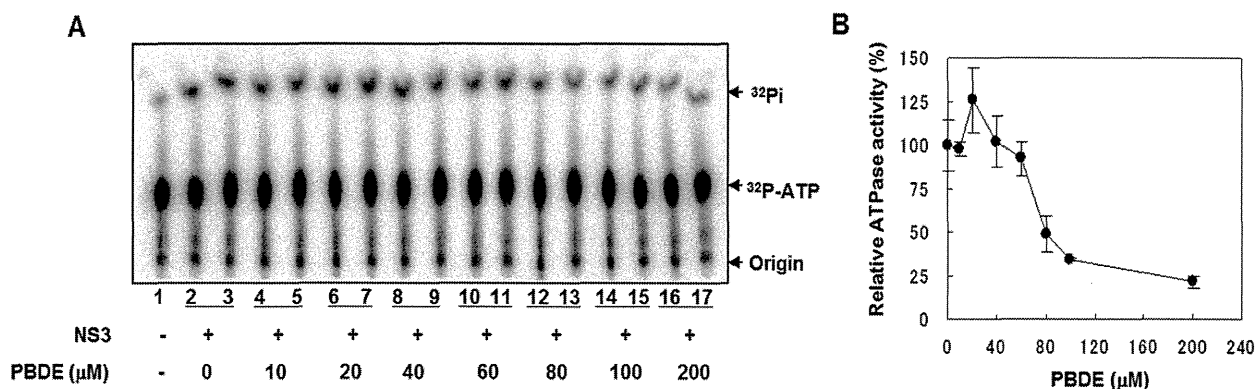
To examine the specificity of PBDE (**1**) for the inhibition of ATPase activity, we evaluated the ATP hydrolytic effect on bacterial alkaline phosphatase. PBDE (**1**) exhibited no inhibition (Figure 3), indicating that the inhibitory activity of PBDE (**1**) is specific to NS3.

Figure 3. Effect of PBDE (**1**) on the ATPase activity of bacterial alkaline phosphatase. The assay was conducted in the absence (DMSO) or presence of PBDE (**1**) (at the highest concentration tested, 200 μM). The data are expressed as the mean of three replicates with error bars representing standard deviation.
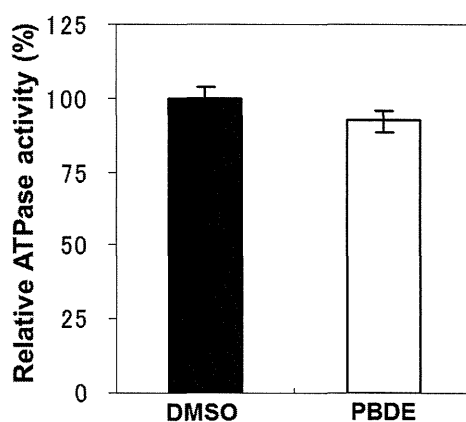
The binding of NS3 to ssRNA is required to initiate the unwinding activity of dsRNA during viral replication. We employed a gel mobility shift assay to characterize the inhibition of NS3 binding to RNA. PBDE (**1**) inhibited RNA binding of NS3 in a dose-dependent manner with an IC$_{50}$ of 68 μM (Figure 4A,B). Previous reports indicate that poly(U) RNA enhances the ATPase activity of NS3 [24]. Because PBDE (**1**) inhibits the RNA binding ability of NS3, we speculated that inhibition of NS3 ATPase activity by PBDE (**1**) could be mediated through the inhibition of poly(U) RNA binding.

Therefore, we next performed ATPase assays including poly(U) RNA to determine the effects of poly(U) with PBDE (**1**) near to its IC_{50} concentration. We found that PBDE (**1**) was significantly active in both the presence and absence of poly(U) (Figure 5A,B), suggesting that poly(U) has no effect on the ATPase inhibition mediated by PBDE (**1**). These results are consistent with our previous data (Figure 2B).

Figure 4. PBDE (**1**) inhibits NS3 RNA binding. (**A**) Gel mobility shift assay to characterize the inhibition of NS3 binding to [γ-^{32}P] labeled ssRNA. RNA only control (lane 1), 300 nM BSA instead of NS3 control (lane 2), NS3 protein (300 nM) and DMSO control (lane 3), and NS3 protein with increasing concentrations of PBDE (**1**) (lanes 4–8). (**B**) Graphical representation of the RNA binding inhibition shown in panel A.
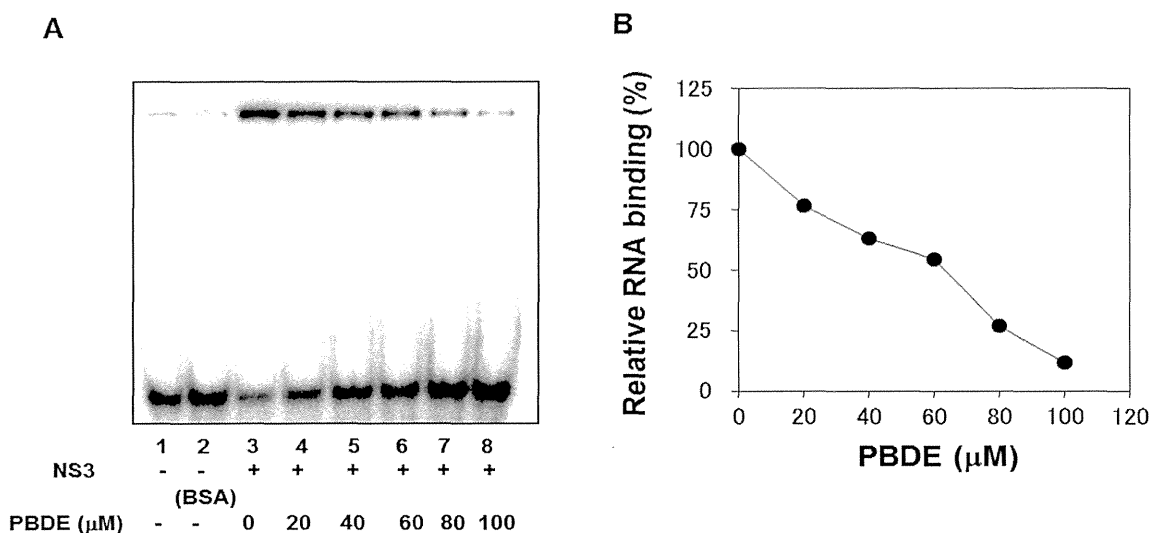
Figure 5. Effect of poly(U) RNA on NS3 ATPase activity. (**A**) ATPase assay with hydrolytic reaction buffer containing NS3 (600 nM), 1 mM [γ-^{32}P] ATP, poly(U) RNA and PBDE (0.1 mM) as indicated. (**B**) Graphical representation of data presented in (A). The solid and white bars represent NS3 and poly(U) ATPase reactions performed with DMSO and PBDE (**1**), respectively. The assay was performed in triplicate and data are presented as mean ± standard deviation. * $p > 0.05$ and ** $p > 0.01$ from Student t-test.
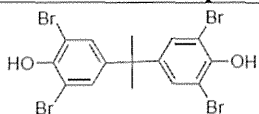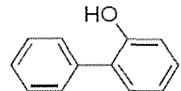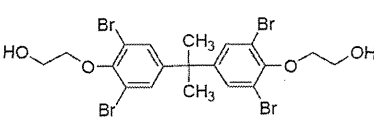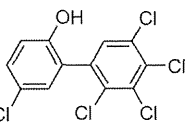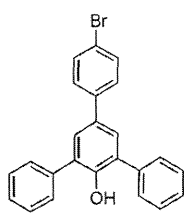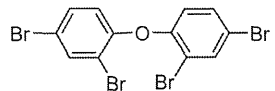
Figure 5. *Cont.*

B
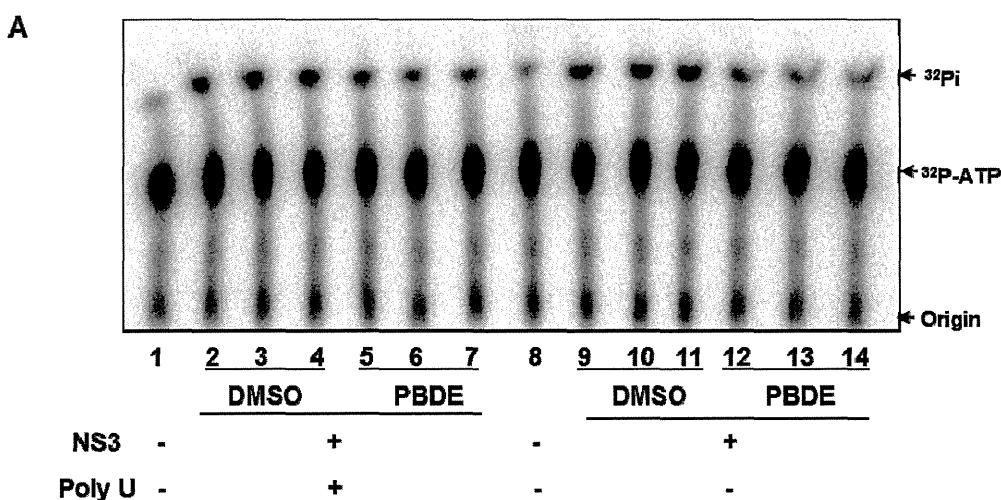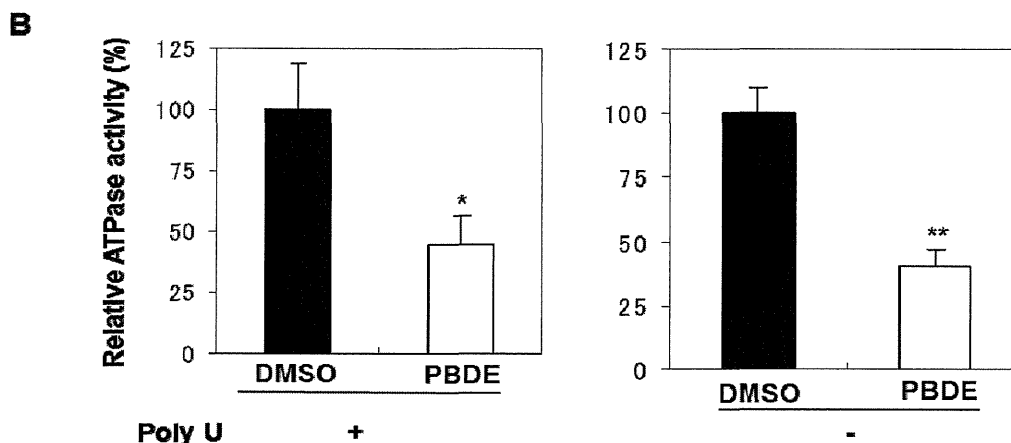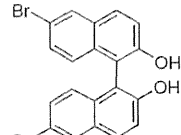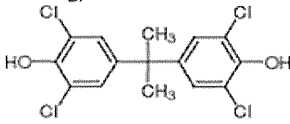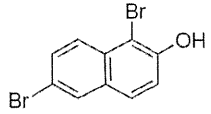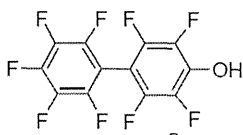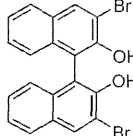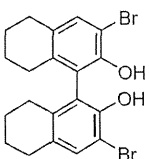
To clarify the structure-activity relationships of PBDE for inhibition of the ATPase activity of the NS3 protein, commercially available and natural phenol derivatives were examined (Table 2). We first investigated whether the hydroxyl group of PBDE (**1**) is required for ATPase activity. Substituting a methoxy group [25] (*i.e.*, PBDE methyl ether **2**) and a hydrogen (*i.e.*, deoxy PBDE **17**), for a phenolic hydroxyl group in PBDE led to a complete loss of the inhibitory activity. These findings indicated that the phenolic hydroxyl group has important effects on the inhibitory activity. Triclosan (**4**), which is structurally very close to PBDE, showed moderate levels of inhibition, indicating that bromine substituents on benzene rings can be replaced by chlorine substituents.

Table 2. Inhibition of the ATPase activity of the NS3 protein by PBDE (**1**) and its structurally related compounds.

Compound No.	Chemical Structure (PBDE/related compounds)	NS3 ATPase Inhibition IC_{50} (μM)
1		80
2		>200
3		94
4		150
5		>200

Table 2. *Cont.*

Compound No.	Chemical Structure (PBDE/related compounds)	NS3 ATPase Inhibition IC$_{50}$ (µM)
6		120
7		>200
8		54
9		94
10		>200
11		>200
12		>200
13		>200
14		>200
15		54
16		>200
17		>200

Next, we took into consideration the size of the structural motif [biphenyl (compounds **3,7,11,15**) compared to phenyl (compound **5**) and fused ring (compound **10**)]. Interestingly, the inhibitory activity of bromophene **3**, a biphenyl derivative possessing bromine and phenolic hydroxyl groups, remained at the same level as that of PBDE (**1**). While *o*-hydroxybiphenyl **7** showed loss of the inhibitory activity, hydroxyl-pentachlorobiphenyl **15** displayed the most potent inhibitory activity of all the analogs in this study. Notably, an additional halogen substituent on the benzene ring led to a nearly two-fold increase in activity over bromophene **3**.

Furthermore, hydroxynonafluorobiphenyl **11** was also inactive. These findings suggested that both halogen, such as bromine and chlorine, and phenolic hydroxyl groups on benzene rings would be crucial for the inhibition of the ATPase activity of the NS3 protein. Unfortunately, tribromophenol **5** and dibromonaphthalenol **10** did not exhibit the inhibitory activity, indicating that the molecular frame could affect the activity level. Tetrahalobisphenols A, (compounds **6** and **9**), showed the same level of inhibition as that of bromophene **3**. Replacement of methoxy groups in tetrabromobisphenol A (**6**) with 2-hydroxyethoxy groups, *i.e.*, the bisphenol A hydroxyethyl ether **14**, brought about loss of activity. Dibromobinaphthol **8**, a dimer of bromonaphthalenol **10**, displayed the most potent activity, whereas isomeric bromobinaphthol **12** and tetrahydrobromobinaphthol **13** showed no activity. These findings indicated that the distance between halogen and phenolic hydroxyl groups has important effects on the inhibitory activity. 4-Bromophenyl-2,6-diphenylphenol **16** did not show inhibitory activity likely because of the steric hindrance around the phenolic hydroxyl group.

The log P is a measure of the lipophilicity of an organic compound, and can be defined as the ratio of the concentration of the unionized compound at equilibrium between organic and aqueous phases. Studies have shown that many biological phenomena can be correlated with this parameter, such that structure-activity relationships may be deduced. The relationship between the IC_{50} and log P of PBDE and its structurally related compounds **1–17** is shown in Figure 6. Biphenyl ethers **1** and **4**, biphenyls **3** and **15**, tetrahalobisphenols A **6** and **9**, and binaphthol **8** with a log P of over approximately 5 were located at the upper left, indicating the inhibitory activity. The inhibitory potency of halogenated phenols on the ATPase might increase with growing lipophilicity. Therefore, we have identified PBDE (**1**) and related compounds, hydroxypentachlorobiphenyl and dibromobinaphthol, as potent inhibitors of the HCV ATPase.

Figure 6. The relationships between Log P and IC_{50} values of the compounds.
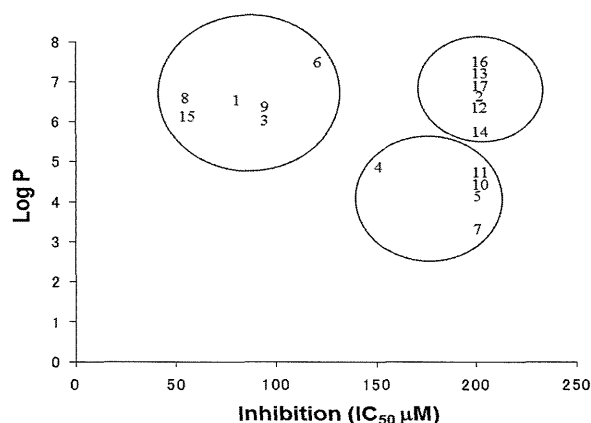
Finally, we examined the effects of PBDE (**1**) on HCV replication. As shown in Figure 7, PBDE (**1**) suppressed HCV replication in a dose-dependent manner (EC_{50} = 3.3 μM) without cytotoxic effect (CC_{50} > 5 μM).

Figure 7. Effect of PBDE (**1**) on viral replication. The subgenomic replicon RNA of genotype 1b N strain was incubated in medium containing various concentrations of PBDE (**1**) or DMSO. Luciferase and cytotoxicity assays were carried out as described in Experimental section. Error bars indicate standard deviation. The data represent three independent experiments.
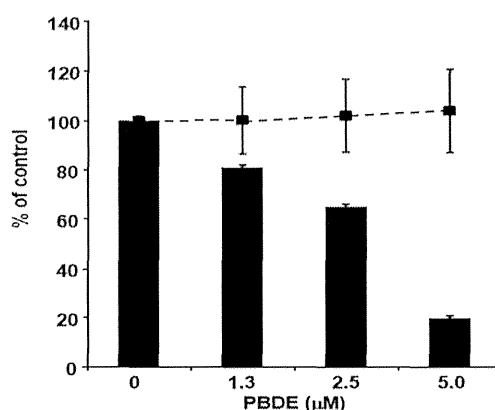
3. Experimental

3.1. Chemicals and Reagents

The γ-^{32}P-ATP isotope was purchased from Muromachi Yakuhin (Tokyo, Japan). Oligonucleotides were synthesized by Gene Design Inc. (Osaka, Japan). Bacterial alkaline phosphatase (BAPC75) was purchased from Takara Bio (Otsu, Japan). 6-hydroxy-2,2',4,4'-tetrabromodiphenyl ether (PBDE, **1**) was isolated from a marine sponge, and compound **2** was obtained by methylation of compound **1** with trimethylsilyldiazomethane. Bromophene **3**, triclosan (**4**), 2,4,6-tribromophenol (**5**), 3,3',5,5'-tetrabromobisphenol A (**6**), *O*-hydroxybiphenyl (**7**), 1,6-dibromo-2-naphthol (**10**), and 4,4'-isopropylidenebis[2-(2,6-dibromophenoxy)ethanol] (**14**) were purchased from Wako Pure Chemical (Osaka, Japan). Poly(U) RNA, 2,3,5,6-tetrafluoro-4-(pentafluorophenyl)phenol (**11**), (*R*)-(+)-3-3'-dibromo-1,1'-bi-2-naphthol (**12**), (*R*)-(+)-3,3'-dibromo-5,5',6,6',7,7',8,8'-octahydro-1,1'-bi-2,2'-naphthalenediol (**13**), and 4-(4-bromophenyl)-2,6-diphenylphenol (**16**) were obtained from Sigma-Aldrich (St. Louis, MO, USA). (*R*)-(−)-6,6'-dibromo-1,1'-bi-2-naphthol (**8**) and tetrachlorobisphenol A (**9**) were purchased from TCI (Tokyo, Japan). 2-Hydroxy-2',3',4',5,5'-pentachlorobiphenyl (**15**) and 2,2',4,4'-tetrabromodiphenyl ether (**17**) were obtained from AccuStandard (New Haven, CT, USA).

3.2. Extraction of PBDE

The specimens used in this study were collected from marine organisms near Okinawa Islands, Japan (Table 1). Extractions were performed three times with either ethanol or acetone, and the ethyl-soluble portions (PM/SR-*-1) were obtained after concentration and partition. The aqueous layer

was concentrated and methanol-soluble portions (PM/SR-*-2) were obtained by washing the residue and concentration.

3.3. Screening for HCV NS3 Helicase Inhibitors

The fluorescence helicase assay based on FRET was performed as described in our previous study [23]. The dsRNA substrate was prepared by annealing the 5' Alexa Fluor 488 labeled fluorescence strand (5'-UAGUACCGCCACCCUCAGAACCUUUUUUUUUUUUU-3') to the 3' BHQ1 labeled quencher strand (5'-GGUUCUGAGGGUGGCCUACUA-3') at a 1:2 molar ratio. The dsRNA substrate has the 3'-overhang that is necessary for NS3 helicase to bind RNA prior to the duplex unwinding. The capture strand (5'-TAGTACCGCCACCCTCAGAACC-3'), which is complementary to the quencher strand, prevents the unwound duplexes from reannealing. None of the above three strands is self-complementary. The fluorescence and quencher strands were purchased from Japan Bio Services (Saitama, Japan). The capture strand was purchased from Tsukuba Oligo Service (Ibaraki, Japan). The reaction mixture contained 25 mM MOPS-NaOH (pH 6.5), 3 mM MgCl$_2$, 2 mM dithiothreitol, 4 U of RNasin (Promega, WI, USA), 50 nM dsRNA substrate, 100 nM capture strand, 5 mM ATP, an extract from a marine organism, and 240 nM NS3 in a total volume of 20 µL. Each extract from a marine organism diluted with DMSO was added to the reaction mixture at a final concentration in the range of 17.5–32.5 µg/mL. The full-length HCV NS3 protein with serine protease and ATPase/helicase was expressed and purified as described previously [23].

The reaction was started by adding HCV NS3 helicase and performed at 37 °C for 30 min using a LightCycler 1.5 (Roche Diagnostics, Basel, Switzerland). Fluorescence intensity was recorded every 5 s from 0 to 5 min, and then every 30 s from 5 to 30 min. Helicase activity was calculated as the initial reaction velocity relative to that of the control without a sample but with DMSO.

3.4. ATP Hydrolysis (ATPase) Assay

Following our previous report [26], unless otherwise stated, the standard assay reaction (10 µL) contained the following components: 25 mM MOPS-NaOH (pH 7.0), 1 mM DTT, 5 mM MgCl$_2$, 5 mM CaCl$_2$, 1 mM [γ-^{32}P] ATP, 300 nM NS3, and 0.1 µg/µL poly(U) with serial dilution of the tested compounds in DMSO. Samples were incubated at 37 °C for 10 min, and the reaction was terminated by adding 15 µL of stop solution (10 mM EDTA). A small portion (2 µL) of reaction mixture was spotted on a PEI-cellulose TLC plate (Merck Millipore, Darmstadt, Germany) and developed by ascending chromatography in 0.75 M LiCl/1 M formic acid solution for 25 min. The TLC plate was then air-dried, and applied to autoradiography measured by an Image Reader FLA-9000 and quantified by Multi Gauge V3.11 software (Fujifilm, Tokyo, Japan). For the bacterial alkaline phosphatase assay, the buffer provided with the kit (Takara Bio, Otsu, Japan) was used, and then subjected to the assay as described above.

3.5. Gel Mobility Shift Assay (GMSA)

GMSA was performed with slight modification as described previously [26]. In brief, [γ-^{32}P] ATP-labeled single-stranded RNA (0.4 nM) was incubated in a buffer containing 30 mM Tris-HCl pH 7.5,

100 mM NaCl, 2 mM MgCl$_2$, 1 mM DTT, 20 U of RNasin plus (Promega) in the presence of 300 nM NS3 protein with serial dilution of PBDE in DMSO at room temperature for 15 min in a final reaction volume of 20 μL. The protein-RNA complexes were loaded onto a 6% native-PAGE (acrylamide:bis = 19:1) and after electrophoresis in TBE buffer, the labeled RNA bands were visualized and quantified with an Image Reader FLA-9000 (Fujifilm) and Multi Gauge V3.11 software (Fujifilm), respectively.

3.6. HCV Replication Assay

The cell lines harboring the subgenomic replicon RNAs of genotype 1b strain N [27] were seeded at 2×10^4 cells per well in a 48-well plate 24 h before treatment. The cells were treated with PBDE at various concentrations for 72 h and lysed in cell culture lysis reagent (Promega). A luciferase assay system (Promega) was used to determine the luciferase activity, and the luminescence was measured using Luminescencer-JNR AB-2100 (ATTO, Tokyo, Japan), corresponding to the expression level of the HCV replicon.

3.7. Toxicity Assay

MTS assay was carried out to determine cytotoxicity using a CellTiter 96 aqueous one-solution cell proliferation assay kit (Promega) according to the manufacturer's instructions.

4. Conclusions

In conclusion, the present study showed that PBDE (**1**) isolated from a marine sponge inhibited NS3 helicase through suppression of the ATPase and RNA binding activities. Moreover, PBDE (**1**) did not inhibit bacterial alkaline phosphatase, suggesting that PBDE (**1**) is specific for NS3 inhibition. Structure-activity relationships demonstrated that the biphenyl ring, bromine, and phenolic hydroxyl group on the benzene backbone might be crucial groups essential for the inhibitory potency.

Author Contributions

Conceived and designed the experiments: M.T., N.A., H.T., N.N., S.T., Y.S. Performed the experiments: K.A.S., A.F., A.Y. Analyzed the data: K.A.S., M.T., N.A., K.M., M.N. Wrote the paper: K.A.S., M.T., N.A. Collected and identified the marine sponge: J.T., S.R.R.

Conflicts of Interest

The authors declare no conflict of interest.