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Communication

Cytotoxic Metabolites from the Okinawan Ascidian *Diplosoma virens*

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Abstract: The unstable isomeric compounds 5-hydroxy-7-prop-2-en-(*E*)-ylidene-7,7a-dihydro-2H-cyclopenta[b]pyran-6-one (**1**) and 5-hydroxy-7-prop-2-en-(*Z*)-ylidene-7,7a-dihydro-2H-cyclopenta[b]pyran-6-one (**2**), previously described as antimicrobial metabolites from the sponge *Ulosa* sp., were isolated and identified as major components of the ascidian *Diplosoma virens*. In this paper, full spectral data for **2** and complete ^{13}C-NMR data for **1**, based on 2D NMR measurements, are provided for the first time. Compounds **1** and **2** showed cytotoxity against HCT116 cells (human colorectal cancer cells) by triggering apoptotic cell death.

Keywords: *Diplosoma virens*, HCT116, apoptosis, NMR

Introduction

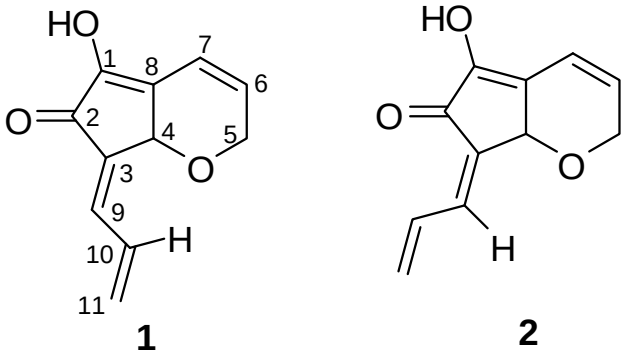
Marine organisms containing symbiotic microorganisms, such as ascidians, sponges and soft corals, are a rich source of bioactive compounds [1, 2a-b]. Ascidians belong to the family Didemnidae, which frequently possess prokaryotic algae (cyanobacteria, *Prochloron* spp., etc.) [3-5], and have yielded structurally unique and pharmacologically interesting compounds such as didemnenones, enterocins, paterallazoles, varacins and virenamides [6-10]. In our search for bioactive compounds from Okinawan marine organisms [11-14], we investigated the ascidian *Diplosoma virens*, which inhabits the coast of Hateruma Island. From this organism, we isolated compounds **1** and **2**, which are major constituents of its acetone extract. Antimicrobial metabolites **1** and **2**, previously isolated from the Caribbean sponge *Uloma* sp. in 1978 [15], were characterized mainly by spectral analysis of their derivatives, including compounds such as acetates, methyl ethers and phenyltriazolinedione adducts. The physical properties of **2** and the ^{13}C-NMR data for **1** have not been previously described in the literature [15].

In this study, we confirmed the structure of **2** by interpreting its 2D-NMR data, and we provided full spectral data for **2** and **1**. In addition, the compounds' cytotoxity against HCT116 cells (human colorectal cancer cells) via apoptotic cell death was demonstrated.

Results and Discussion

A specimen of *Diplosoma virens* (72 g, wet weight) was collected by hand from the coast of Hateruma Island, Okinawa, and extracted with acetone. The acetone extract was analyzed using ^{1}H-NMR, and two major compounds were observed. The acetone extract was partitioned between aqueous MeOH and hexanes. The aqueous MeOH phase was concentrated *in vacuo* and then partitioned between CH_2Cl_2 and water. Purification of the CH_2Cl_2 extract by silica gel column chromatography followed by reversed-phase (C8) HPLC (H_2O/MeOH) yielded compounds **1** (0.13%) and **2** (0.086%). When a solution of **1** or **2** in CH_2Cl_2 or MeOH was concentrated, a large portion of the resulting residue was no longer soluble in the organic solvents.

Figure 1. Structures of compounds **1** and **2**.



Compound **1** was identified by performing comprehensive spectral analyses (Figure 1 and Table 1) and by comparing resulting spectral data with those in the literature [15].

Analysis of the ^{13}C-NMR and HRFABMS data [*m/z* 191.0691 $(M + H)^+$, Δ -1.7 mmu] for compound **2** provided a molecular formula of $C_{11}H_{10}O_3$, which suggested seven degrees of unsaturation. The IR absorption bands at 1680 and 3250 cm^{-1} indicated the presence of carbonyl and hydroxyl groups. The spectral data of compound **2** showed close similarities to those of **1**. The ^{1}H- and ^{13}C-NMR data analysis indicated the presence of a carbonyl carbon (δ_C 187.8), a *cis* double bond [δ_C 134.2, 118.4; δ_H 6.17 (1H, ddd), 6.77 (1H, ddd)], a tetrasubstituted double bond (δ_C 128.3, 147.5), a conjugated diene [δ_C 131.6 (s), 136.6 (d); δ_H 6.65 (1H, br d) and δ_C 132.1 (d), 127.3 (t); δ_H 7.76 (1H, ddd), 5.57 (1H, dd), 5.61 (1H, dd)], an oxygenated methine [δ_C 71.9; δ_H 4.79 (1H, s)] and an oxygenated methylene [δ_C 67.2; δ_H 4.48 (1H, ddd), 4.57 (1H, ddd)].

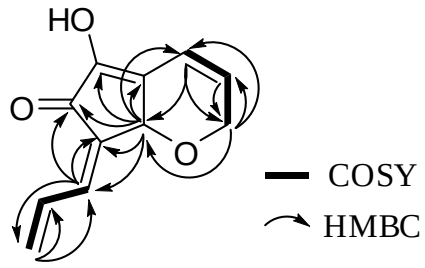
Table 1. ^{1}H- and ^{13}C-NMR data for compounds **1** and **2**[a].

	1		**2**	
C no.	δ_C	δ_H **(mult, *J* in Hz)**	δ_C	δ_H **(mult, *J* in Hz)**
1	147.2		147.5	
2	187.9		187.8	
3	132.6		131.6	
4	71.3	5.01 (s)	71.9	4.79 (s)
5α	67.1	4.50 (ddd, 18.5, 4.2, 2.0)	67.2	4.48 (ddd, 18.3, 4.2, 1.7)
5β		4.61 (ddd, 18.5, 2.4, 2.4)		4.57 (ddd, 18.3, 2.4, 2.4)
6	134.0	6.17 (ddd, 10.0, 4.2, 2.4)	134.2	6.17 (ddd, 10.0, 4.2, 2.4)
7	118.4	6.78 (br d, 10.0)	118.4	6.77 (ddd, 10.0, 2.4, 1.7)
8	129.1		128.3	
9	133.6	7.04 (br d, 11.7)	136.6	6.65 (br d, 11.5)
10	132.4	6.89 (ddd, 16.8, 11.7, 10.0)	132.1	7.76 (ddd, 17.1, 11.5, 10.0)
11α	127.9	5.64 (br d, 10.0)	127.3	5.57 (dd, 10.0, 1.7)
11β		5.71 (br d, 16.8)		5.61 (dd, 17.1, 1.7)
OH		6.08 (br s)		6.12 (br s)

[a] ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) were recorded in $CDCl_3$.

Extensive analysis of ^{1}H-^{1}H COSY demonstrated two isolated spin systems, C5-C7 and C9-C11 (Figure 2). The connectivity of the aforementioned partial structures was established from the HMBC correlations of H-4/C-1, H-4/C-2, H-9/C-2, H-4/C-3, H-9/C-3, H_2-5/C-4, H-7/C-4, H-6/C-5, H-7/C-5, H-5/C-6, H-5/C-7, H-4/C-7, H-4/C-8, H-4/C-9, H-11/C-9, H-11/C-10 and H-9/C-11 as shown in Figure 2, to describe the entire carbon framework of **2**.

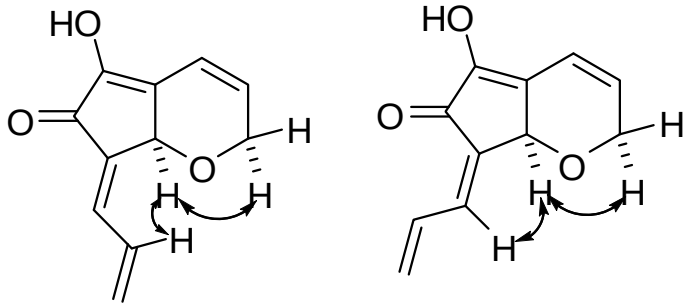
Figure 2. Planar structure of **2** based on COSY and HMBC correlations.



2

The chemical shift of H-10 in **2** (δ_H 7.76) was at lower field than in **1** (δ_H 6.89) owing to the magnetic anisotropy effect of the carbonyl group and the chemical shift of H-9 in **2** (δ_H 6.65) was at higher field than in **1** (δ_H 7.04), suggesting a *Z* configuration for the C-3, 9 double bond of **2**. This was confirmed by an NOEDS experiment, in which NOE was observed between H-9 and H-4 (Figure 3).

Figure 3. Selected NOEs of compounds **1** and **2**.



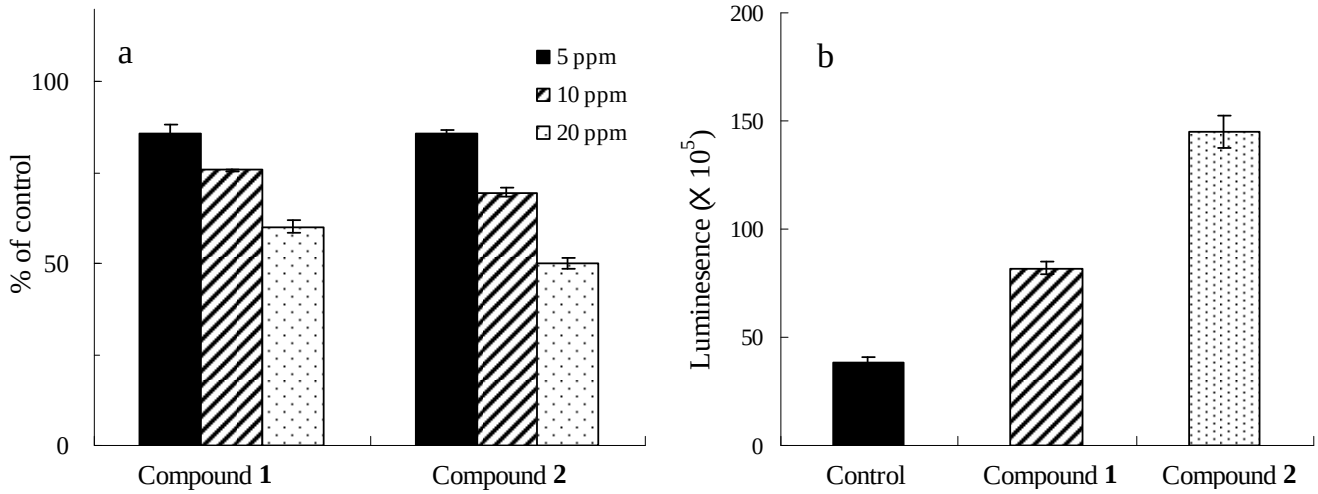
1 **2**

Biological Activities

Compounds **1** and **2** showed cytotoxity against HCT116 cells (human colorectal cancer cells) in a dose dependent manner (Figure 4a). Necrosis is a form of traumatic cell death that results from acute cellular injury. In contrast, apoptosis is a form of programmed cell death involving a biochemical cascade that includes caspases and cysteine proteases. Caspase 3/7 exists downstream in the caspase cascade.

To examine the type of cell death induced by these compounds at 20 ppm, caspase 3/7 activity was measured in HCT116 cells in the presence of compounds **1** and **2**. Caspase 3/7 activity in cells treated with compounds **1** and **2** was expressed as percent induced, compared to control cells not treated with the compound (Figure 4b). The caspase 3/7 induction associated with compounds **1** and **2** were 53.6% and 73.6%, respectively, indicating that these compounds induce apoptotic cell death by activating caspases through the mitochondrial/cytochrome C stress pathway that begins with the release of cytochrome C from mitochondria [16-18].

Figure 4. Cytotoxicity and caspase 3/7 activity of compounds **1** and **2** against HCT116 cells. (a) Cytotoxicity of compounds **1** and **2**; (b) caspase 3/7 induction due to compounds **1** and **2**.



Conclusions

In this study we isolated compounds **1** and **2**, constituents of the sponge *Uloma* sp. [15], as major components of the ascidian *Diplosoma virens*, and we confirmed the structure of **2** by interpreting its 2D-NMR and MS data, thus providing full spectral data for **2** and **1**. Compounds **1** and **2** showed weak cytotoxity against HCT116 cells (human colorectal cancer cells) by triggering apoptotic cell death. C_{11} cyclopentenones (didemnenones) [6] and the related compounds (nakienone and terpiodiene) [19, 20] have been isolated from ascidians, cyanobacteria and a sponge, respectively. Isolation of a series of the C_{11} compounds, including compounds **1** and **2**, from unrelated marine organisms supports the potential microbial origin of these compounds. Studies on the origin of compounds **1** and **2** and their bioactivities are under way in our laboratory.

Experimental

General

Optical rotations were measured on a JASCO P-1020 polarimeter. UV spectra of the methanol solutions were measured on a JASCO V-550 spectrophotometer. IR spectra were recorded as dry films on a JASCO FT/IR-300 spectrometer. The ^{1}H-, ^{13}C-, and 2D-NMR spectra were recorded on a JEOL lambda 400 or a JEOL α-500 spectrometer, and ^{1}H- and ^{13}C- chemical shifts were referenced to the solvent peaks [δ_H 7.24 and δ_C 77.0 in $CDCl_3$]. Mass spectra were measured on a Waters Quattro *micro* API triple quadruple mass analyzer. Open column chromatography was performed on Kieselgel 60 (70-230 mesh, Merck). Preparative HPLC was run on a Waters 600 multi solvent system using a reversed-phase column (YMC-Pack C8, 20 x 250 mm I. D., YMC). All solvents used were reagent grade.

Animal Collection

The small yellowish green ascidian was collected at low tide from the coast of Hateruma Island, Okinawa (Japan) in April 2006, and identified as *Diplosoma virens* by Professor Euichi Hirose, University of the Ryukyus, Japan. The identified ascidian was kept frozen at -80˚C until used. A voucher specimen was deposited at the University of the Ryukyus (Specimen no. 070222).

Extraction and Purification

The ascidian (72 g, wet weight) was exhaustively extracted with acetone (100 mL, 3 times) and filtered. The filtrates were combined and concentrated *in vacuo* to remove acetone. The resulting residue was first partitioned between MeOH/H_2O (9:1, 100 mL) and hexane (100 mL). The aqueous MeOH phase was concentrated *in vacuo*, and then the resulting mixture was partitioned between CH_2Cl_2 (100 mL) and water (100 mL). The CH_2Cl_2 extract was column chromatographed on Si gel eluting with CH_2Cl_2 (100 mL), CH_2Cl_2: EtOAc (1:1 v/v, 100 mL), EtOAc (200 mL) and MeOH (200 mL). One twentieth of the CH_2Cl_2 eluate which contained compounds **1** and **2** was concentrated, and then the residue was separated by reversed-phase HPLC (YMC-Pack C8) using a linear gradient of water (eluent A) and acetonitrile (eluent B), (0 min, 30% B; 1 min, 30% B; 10 min, 40 % B and 25 min, 60 % B; flow rate, 15 mL min^{-1}) to furnish compounds **1** (t_R = 12.8 min, 4.8 mg) and **2** (t_R = 11.7 min, 3.1 mg).

5-Hydroxy-7-prop-2-en-(E)-ylidene-7,7a-dihydro-2H-cyclopenta[b]pyran-6-one (**1**). Pale yellow powder; $[\alpha]^{21}_D$ +4.1 (*c* 0.15, MeOH); UV (MeOH) λ_{max} (log ε) 249 (4.4) and 347 (4.2) nm; FT/IR (film) ν_{max} 3250, 1680, 1620, 1420, 1360, 1220, 1150, 1080 and 770 cm^{-1}; ^{1}H- and ^{13}C- NMR ($CDCl_3$) data, see Table 1; for UV, IR and ^{1}H−NMR data refer to [15]; LRESIMS *m/z* 191 $(M+H)^+$, *m/z* 225 $(M+Na)^+$ and *m/z* 189 $(M-H)^-$; HRFABMS *m/z* $(M+H)^+$ 191.0752 (calcd for $C_{11}H_{11}O_3$, 191.0708).

5-Hydroxy-7-prop-2-en-(Z)-ylidene-7,7a-dihydro-2H-cyclopenta[b]pyran-6-one (**2**). Pale yellow powder; $[\alpha]^{21}_D$ +11 (*c* 0.32, MeOH); UV (MeOH) λ_{max} 249 (log ε) (4.0), 346 (3.9) nm; FT/IR (film) ν_{max} 3250, 1680, 1620, 1420, 1350, 1220, 1080 and 780 cm^{-1}; ^{1}H- and ^{13}C-NMR ($CDCl_3$) data, see Table 1; LRESIMS *m/z* 191 $(M+H)^+$, *m/z* 225 $(M+Na)^+$ and *m/z* 189 $(M-H)^-$; HRFABMS *m/z* $(M+H)^+$ 191.0691 (calcd for $C_{11}H_{11}O_3$, 191.0708).

Biological Assay

Cells: HCT-116 cells (human colorectal cancer cells) were cultured in DMEM medium (including 10% FBS, 100 U/mL penicillin, and 100 ng/mL streptomycin) at 37˚C in a 5% CO_2 atmosphere.

Cell viability: The MTT assay was used to examine the cell viability. Briefly, HCT116 cells were seeded at a density of 2.5 x 10^4 cells/mL on 96-well plates and cultured for 17 hrs with or without the test compound. After incubation, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, 0.5 mg/mL) was added to each well, the samples were again incubated for 3 hrs, and then they

were removed from suspension. Extraction with DMSO (100 μL), measured at 570 nm, provided the reference for readings at 630 nm with a microplate reader (BIO-RAD Model 550, BIO-RAD, USA).

Caspase activity: HCT116 cells were plated at a density of 2.5×10^4 cells on 96-well plates, which were incubated for 17 hrs with or without the test compound. Caspase activation in cultured cells was measured using the commercially available caspase-3/7 assay kit (Promega, USA), following the protocol supplied by the manufacturer. Each cultured cell in the well was incubated at room temperature for 2 hrs 30 min with 100 μL of proluminescent substrate containing Z-DEVD (Caspase-Glo™ 3/7), provided with the kit. Following caspase cleavage, a substrate reacts with luciferase and releases light in the presence of ATP and oxygen. The luminescence of the reaction products was measured with a CL-detector (CLD-110, Tohoku Electronic Co.).

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References

1. Davidson, B. S. Ascidians: producers of amino acid-derived metabolites. *Chem. Rev.* **1993**, *93*, 1771-1791.
2. (a) Faulkner, D. J. Marine Natural Products. *Nat. Prod. Rep.* **2002**, *19*, 1-48, and previous reports in this series; (b) Blunt, J. W.; Copp, B. R.; Munro, M. H. G.; Northcote, P. T. and Prinsep, M. R. J. Marine Natural Products. *Nat. Prod. Rep.* **2006**, *23*, 26-78, and previous reports in this series.
3. Lewin, R. A. Prochlorophyta as a proposed new division of algae. *Nature* **1976**, *261*, 697-698.
4. Withers, N.; Vidaver, W.; Lewin, R. A. Pigment Composition, Photosynthesis and Fine Structure of a Non-Blue-Green Prokaryotic Algal Symbiont (Prochloron Sp.) in a Didemnid Ascidian from Hawaiian Waters. *Phycologia* **1978**, *17*, 167-171.
5. Lewin, R. A.; Cheng, L. *Prochrolon: A microbial Enigma*. Chapman & Hall: New York, **1989**; p. 129.
6. Lindquist, N.; Fenical, W.; Sesin, D. F.; Ireland, C. M.; Duyne, G. D. V.; Forsyth, C. J.; Clardy, J. Isolation and structure determination of the didemnenones, novel cytotoxic metabolites from tunicates. *J. Am. Chem. Soc.* **1988**, *110*, 1308-1309.
7. Zabriskie, T. M.; Mayne, C. L.; Ireland, C. M. Patellazole C: a novel cytotoxic macrolide from *Lissoclinum patella*. *J. Am. Chem. Soc.* **1988**, *110*, 7919-7920.
8. Coreley, D. G.; Moore, R. E.; Paul, V. J. Patellazole B: a novel cytotoxic thiazole-containing macrolide from the marine tunicate *Lissoclinum patella*. *J. Am. Chem. Soc.* **1988**, *110*, 7920-7922.
9. Davidson, B. S.; Molinski, T. F.; Barrows, L. R.; Ireland, C. M. Varacin: a novel benzopentathiepin from *Lissoclinum vareau* that is cytotoxic toward a human colon tumor. *J. Am. Chem. Soc.* **1991**, *113*, 4709-4710.
10. Carroll, A. R.; Feng, Y.; Bowden, B. F.; Coll, J. C. Studies of Australian Ascidians. 5. Virenamides A-C, New Cytotoxic Linear Peptides from the Colonial Didemnid Ascidian *Diplosoma virens. J. Org. Chem.* **1996**, *12*, 4059-4061.

11. Ueda, K.; Hu, Y. Haterumalide B: A new cytotoxic macrolide from an Okinawan ascidian *Lissoclinum* sp.. *Tetrahedron Lett.* **1999**, *40*, 6305-6308.
12. Takada, N.; Sato, H.; Suenaga, K.; Arimoto, H.; Yamada, K.; Ueda, K.; Uemura, D. Isolation and structures of haterumalides NA, NB, NC, ND, and NE, novel macrolides from an Okinawan Sponge *Ircinia* sp.. *Tetrahedron Lett*. **1999**, *40*, 6309-6312.
13. Kokubo, S.; Yogi, K.; Uddin, M. J.; Inuzuka, T.; Suenaga, K.; Ueda, K.; Uemura, D. Kohamaic Acids A and B, Novel Cytotoxic Sesterterpenic Acids, from the Marine Sponge *Ircinia* sp. *Chem. Lett*. **2001**, *2*, 176-177.
14. Uddin, M. J.; Kokubo, S.; Ueda, K.; Suenaga, K.; Uemura, D. Haterumaimides F-I, Four New Cytotoxic Diterpene Alkaloids from an Ascidian *Lissoclinum* Species. *J. Nat. Prod*. **2001**, *64*, 1169-1173.
15. Wratten, S. J.; Faulkner, D. J. Antimicrobial metabolites from the marine sponge *uloma* sp.. *Tetrahedron Lett.* **1978**, *19*, 961-964.
16. Li, P.; Nijhawan, D.; Budihardjo, I.; Srinivasula, S. M.; Ahmad, M.; Alnemri, E. S.; Wang, X. Cytochrome c and dATP-Dependent Formation of Apaf-1/Caspase-9 Complex Initiates an Apoptotic Protease Cascade. *Cell* **1997**, *91*, 479-489.
17. Scaffidi, C.; Fulda, S.; Srinivasan, A.; Friesen, C.; Li, F.; Tomaselli, K. J.; Debatin, K. M.; Krammer, P. H.; Peter, M. E. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J*. **1998**, *17*, 1675-1687.
18. Kuwana, T.; Smith, J. J.; Muzio, M.; Dixit, V.; Newmeyer, D. D.; Kornbluth, S. Apoptosis Induction by Caspase-8 Is Amplified through the Mitochondrial Release of Cytochrome *c*. *J. Biol. Chem*. **1998**, *273*, 16589-16594.
19. Nagle, D. G.; Gerwick, W. H. Nakienones A-C and nakitriol, new cytotoxic cyclic C_{11} metabolites from an okinawan cyanobacterial (*Synechocystis* sp.) overgrowth of coral. *Tetrahedron. Lett.* **1995**, *36*, 849-852.
20. Teruya, T.; Nakagawa, S.; Koyama, T.; Suenaga, K.; Uemura, D. Terpiodiene: A Novel Tricyclic Alcohol from the Okinawan Sponge *Terpios hoshinota*. *Chem. Lett*. **2002**, 38-39.

Sample Availability: Samples of the compounds are available from authors.