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CYTOTOXIC COMPOUNDS FROM A TWO-SPONGE ASSOCIATION

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ABSTRACT.—Investigation of the cytotoxic constituents of a two-sponge association (*Poecillastra* sp. and *Jaspis* sp.) led to the isolation of pectenotoxin II [**1**] and psammaplin A as the active compounds. In an in vitro cell culture assay, **1** displayed very potent cytotoxic activities against human lung (A-549), colon (HT-29), and breast (MCF-7) cancer cell lines. Pectenotoxin II also exhibited selective cytotoxicity against several cell lines representing ovarian, renal, lung, colon, CNS, melanoma, and breast cancer, with differences in LC_{50} values between sensitive and resistant cell lines of 100-fold or more.

In our search for natural cytotoxic compounds, twenty-one marine sponges collected off Cheju and Komun islands, Korea, were screened for brine shrimp lethality (1), and significant brine shrimp (*Artemia salina*) lethality was detected (BST LC_{50} 61 μg/ml) in the crude extract of a two-sponge association. A cross-section of this sample showed two layers of morphologically distinct sponges. The thin and dirty yellow outer layer was identified as *Poecillastra* sp. (Pachastrellidae), the surface of which was very rough. The light-grey inner layer was identified as *Jaspis* sp. (Jaspidae), the surface of which was smooth. This two-sponge association appears to be consistent as these sponges were always found in associated form regardless of collection site or collection period.

Bioactivity-directed fractionation of the sponges (liquid/liquid partition followed by reversed-phase cc) afforded pectenotoxin II [**1**] (BST LC_{50}<0.1 μg/ml) and psammaplin A (BST LC_{50} 4 μg/ml) as the active constituents responsible for the brine shrimp lethality. Along with these two bioactive compounds, a nucleoside, 2′,3′-didehydro-2′,3′-dideoxyuridine, was also isolated.

Since it is known that there is a good correlation between brine shrimp lethality and cytotoxicity (and antitumor activity) (1), **1** was assayed for cytotoxicity against three human solid tumor cell lines. Very potent cytotoxic effects against human colon (HT-29), lung (A-549), and breast (MCF-7) carcinomas were observed. The in vitro cytotoxicity of **1** against these three human tumor cell

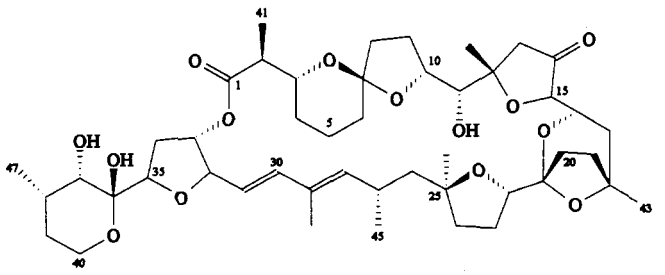

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TABLE 1. Cytotoxicity of Pectenotoxin II [**1**] Against Human Tumor Cell Lines.[a]

Panel	Cell line	LC_{50} (Molar)
Non-Small Cell Lung Cancer	EKVX	6.20×10^{-8}
	HOP-62	2.51×10^{-8}
	HOP-92	8.07×10^{-7}
Colon Cancer	COLO 205	7.82×10^{-9}
	HCT-116	7.33×10^{-8}
CNS Cancer	SF-268	1.54×10^{-7}
	SF-295	7.77×10^{-8}
Melanoma	MALME-3M	1.23×10^{-7}
	M14	4.73×10^{-6}
	SK-MEL-28	4.23×10^{-8}
	SK-MEL-5	5.52×10^{-8}
Ovarian Cancer	IGROV1	6.73×10^{-8}
	OVCAR-3	6.16×10^{-8}
Renal Cancer	A498	3.41×10^{-8}
	CAKI-1	1.91×10^{-8}
	RXF-393	3.00×10^{-8}
	TK-10	7.25×10^{-8}
	UO-31	5.63×10^{-8}
Breast Cancer	HS 578T	7.79×10^{-8}
	BT-549	6.27×10^{-8}

[a]Cytotoxicity was measured at the National Cancer Institute, Bethesda, MD. LC_{50} values $>1.00\times10^{-5}$ M were determined for CCRF-CEM, HL-60 (TB), K-562, MOLT-4, RPMI-8226, SR, A549/ATCC, NCI-H226, NCI-H23, NCI-H322M, NCI-H460, NCI-H522, HCC-2998, HCT-15, HT-29, KM12, SW-620, SF-539, SNB-19, U251, LOX IMVI, SK-MEL-2, UACC-257, UACC-62, OVCAR-4, OVCAR-5, OVCAR-8, SK-OV-3, 786-0, ACHN, PC-3, DU-145, MCF-7, MCF7/ADR-RES, MDA-MB-231/ATCC, MDA-MB-435, MDA-N, and T-47D.

lines was much higher than that of adriamycin, but its mouse toxicity was reported to be about 50 times higher than that of adriamycin [LD_{50} of pectenotoxin II: 0.26 mg/kg, ip (2); LD_{50} of adriamycin: 13-14 mg/kg, ip]. Pectenotoxin II was submitted to the National Cancer Institute (NCI) for selectivity determination against 60 human tumor cell lines. Human tumor cell line panels included leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, and breast cancer. In the NCI evaluation, **1** exhibited selective cytotoxicity for several cell lines of ovarian, renal, lung, colon, CNS, melanoma, and breast cancer with differences in LC_{50} values between sensitive and resistant cell lines of 100-fold or more (Table 1). No selective cytotoxicity was observed against any leukemia or prostate cancer cell lines. We have tested **1** in a DNA-cleavage assay (3) and the rat plasma membrane assay (4). The lack of activity of **1** in the DNA-cleavage and rat plasma membrane assays indicated that it does not block DNA synthesis or reduction-oxidation processes in the cell membrane.

Pectenotoxin II [**1**] was previously isolated from the digestive glands of the scallop *Patinopecten yessoensis* as a suspected DSP (Diarrhetic Shellfish Poisoning) toxin (2,5). It was later shown that the dinoflagellates *Dinophysis fortii* and *D. acuminata* actually produce the pectenotoxins and transmit these to shellfish (6). Accordingly, it may be assumed that **1** in the sponges *Poecillastra* sp. and (or) *Jaspis* sp. originates from dinoflagellates which were consumed by the sponge. At present, a total of seven pectenotoxin analogues has been reported from the scallop. However, **1** was the only analogue detected in the dinoflagellate *Dinophysis fortii* which is thought to transmit the toxin to the scallop. Accordingly, other congeners of **1** are suspected to be

oxidation products of **1** in the hepatopancreas of the shellfish (7). Despite our efforts to detect other congeners, only **1** could be isolated from the sponges, supporting the above hypothesis.

Psammaplin A was tested against the murine leukemia (P-388) and human lung carcinoma (A-549) cell lines and showed moderate cytotoxic effects against both these tumor cell lines (Table 2). Psammaplin A was previously isolated from a *Psammaplysilla* sp. of the order Verongida (8). Bromotyrosine derivatives such as psammaplin A have served as important chemotaxonomic markers, since these compounds are known to be exclusive to the Verongida (9). Since it is unusual for a bromotyrosine derivative to be isolated from a non-Verongida sponge, the frozen sponge specimen was carefully re-examined by Dr. P.R. Bergquist of New Zealand, who concluded that these sponges are clearly not verongid. To our knowledge, discorhabdin C from *Latrunculia* sp. (10) and another bromotyrosine derivative from the Caribbean sponge *Iotrochota birotulata* (11) are the only other exceptions to the generalization that bromotyrosine-derived metabolites occur only in the order Verongida.

The nucleoside, 2′,3′-didehydro-2′,3′-dideoxyuridine, was previously isolated from *Aplysina* sp. (12). Synthetic analogues, such as dde Tyd (2′,3′-didehydro-2′,3′-dideoxythymidine) and dde Cyd (2′,3′-didehydro-2′,3′-dideoxycytidine) have been shown to possess antiviral effects against HIV, leukemia, and sarcoma viruses (13–15). Even though 2′,3′-didehydro-2′,3′-dideoxyuridine bears a close structural similarity to dde Tyd and dde Cyd, it did not show measurable antiviral activity against HSV-1 or HSV-2 in our virus-induced CPE (cytopathic effect) inhibition assay.

TABLE 2. Cytotoxicity (LC_{50} values in μg/ml) of Psammaplin A Against P-388 and A-549 Cell Lines.

Cell Line	Psammaplin A	5-Fluorouracil[a]
P-388 ..	0.64	0.2
A-549 ..	6.43	—

[a]5-Fluorouracil was tested as a positive control.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—^{1}H and ^{13}C-nmr spectra were recorded on a Varian Unity 500 instrument at 500 and 125 MHz, respectively. Chemical shifts were reported relative to the residual solvent peaks (Me_2CO-d_6 2.04 ppm, 29.8 ppm; MeOH-d_4 3.30 ppm, 49 ppm) or TMS peak. Lrfabms measurement was performed on a Finnigan MAT 400 instrument with NBA matrix at the Department of Medicinal Chemistry and Pharmacognosy, Purdue University, West Lafayette, IN. Lreims (22 eV) measurement was performed on a Hewlett-Packard HP5985-B instrument at the College of Pharmacy, Seoul National University. RP-18 $F_{254}S$ (Merck) was used for tlc.

SPONGE MATERIAL.—The two-sponge association was collected by hand using scuba (15–30 m) during November 1991, off Komun island, Korea. Both sponges were attached tightly to each other as if they were one sponge. The thin and dirty yellow outer layer, 6–15 mm thick, was identified as *Poecillastra* sp. by Sim and Kim (16). The surface of the sponge was very rough because of a protruding long spicule bundle. The light grey inner layer, 30–40 mm thick, was identified as *Jaspis* sp. (16), the surface of which was smooth and completely covered by a *Poecillastra* sp. The large oxea protruding from the under part of *Poecillastra* sp. and the large oxea from the upper part of *Jaspis* sp. were like clasped hands. They were easily detached from one another. *Poecillastra* sp. was an encrusting sponge, with a size of up to 150×100 mm, possessing various spicules; large oxea (1800 μm), thin oxea (2500 μm), style (2100 μm), calthrop, microxea (200 μm), and metaster (15 μm). *Jaspis* sp. was large in mass; oxea (1300 μm), style (1000 μm), and oxyaster (17–40 μm). This two-sponge association is similar to *P. wondoensis* and *J. wondoensis* in its spicules, but different in their arrangement of skeleton. A voucher specimen of this two-sponge association (registry No. por. 20) was deposited at the Natural History Museum, Han Nam University, Korea.

EXTRACTION AND ISOLATION.—The sponges (ca. 5 kg, wet wt) were extracted with MeOH-CH_2Cl_2 (1:1, 2 liters×3) at room temperature. After evaporation of solvent *in vacuo*, the residue (191.3 g) was partitioned between H_2O and CH_2Cl_2. The aqueous portion was subsequently extracted with *n*-BuOH. The CH_2Cl_2 portion was evaporated *in vacuo* and the resulting residue was

partitioned between 90% aqueous MeOH and *n*-hexane. Only the 90% aqueous MeOH fraction (5.3 g) was active in the brine shrimp lethality assay (BST LC_{50} 7 μg/ml). This fraction was divided into three portions and each portion was chromatographed on a C_{18} reversed-phase vacuum flash column (Europrep 60-60, Knauer) with the eluting solvent system H_2O-MeOH (1:3→1:5→1:9→100% MeOH), followed by EtOAc and CH_2Cl_2. A total of eight fractions was collected. Fraction No. 2 (F-2) was the most active (BST LC_{50} 0.1 μg/ml) in the brine shrimp lethality assay. The neighboring fractions No. 1 (F-1, 2g) (BST LC_{50} 0.4 μg/ml) and No. 3 (F-3, 170 mg) (BST LC_{50} 0.2 μg/ml) also displayed significant activities. The most active fraction, No. 2 (F-2, 360 mg), was subsequently chromatographed on a C_{18} reversed-phase prep. hplc column (Whatman Partisil 10 ODS, 10 μm, 250×22 mm i.d.) with a solvent system of H_2O-MeCN-MeOH (10:3:40) and uv detection (254 nm, flow rate 2.5 ml/min). A total of eight fractions was collected. Brine shrimp toxicity was detected in fractions 3–6. Fraction 5 (80 mg) was the major band and was collected at a R_t of 42 min; and was subsequently purified on a C_{18} reversed-phase semi-prep. hplc column (Alltech Econosil 250×10 mm, 10 μm, uv 254 nm, flow rate 1.5 ml/min) with a solvent system of MeOH-H_2O (7:3) to yield 40 mg of **1** as crystalline needles. Another active fraction, No. 1 (F-1, 2 g) (BST LC_{50} 0.4 μg/ml) was subjected to C_{18} reversed-phase vacuum flash cc with a gradient system of H_2O-MeOH (1:1→1:3→100% MeOH). The resulting fraction, No.1 (F′-1, 860 mg), was purified on a C_{18} reversed-phase hplc column (YMC-pack ODS-A, 10 μm, 10×250 mm, flow rate 1.8 ml/min) with a solvent system of H_2O-MeOH (7:3) to yield 2′,3′-didehydro-2′,3′-dideoxyuridine (3.3 mg) as an amorphous powder. Fraction No. 3 (P-3) from the flash column fraction of F-2 was purified on a C_{18} reversed-phase hplc column (YMC-pack, ODS-A, 10×250 mm, uv 254 nm, flow rate1.8 ml/min) with the solvent system H_2O-MeCN (3:7) to yield psammaplin A (25 mg).

Pectenotoxin II [**1**].—Crystalline needles; lrfabms *m/z* 882 $[M^+Na]^+$, 859 $[MH^+]$; ^{13}C- and ^{1}H-nmr signals were fully assigned utilizing COSY, TOCSY, HETCOR, long-range HETCOR, and homonuclear *J*-resolved 2D nmr experiments; ^{13}C nmr (125 MHz, Me_2CO-d_6) 172.6 (C-1), 48.7 (C-2), 76.6 (C-3), 30.2 (C-4), 22.3 (C-5), 34.7 (C-6), 107.8 (C-7), 33.1 (C-8), 22.5 (C-9), 81.3 (C-10), 75.7 (C-11), 81.9 (C-12), 44.4 (C-13), 213.5 (C-14), 79.8 (C-15), 71.6 (C-16), 36.6 (C-17), 80.4 (C-18), 28.8 (C-19), 34.2 (C-20), 109.7 (C-21), 79.7 (C-22), 29.9 (C-23), 37.9 (C-24), 84.5 (C-25), 50.9 (C-26), 30.9 (C-27), 140.8 (C-28), 130.9 (C-29), 135.3 (C-30), 122.2 (C-31), 83.2 (C-32), 74.9 (C-33), 33.9 (C-34), 82.6 (C-35), 97.8 (C-36), 71.4 (C-37), 30.3 (C-38), 27.9 (C-39), 60.9 (C-40) 15.5 (C-41), 23.3 (C-42), 26.3 (C-43), 26.8 (C-44), 23.7 (C-45), 12.6 (C-46), 17.9 (C-47); ^{1}H-nmr (500 MHz, Me_2CO-d_6) 2.24 (1H, dd, *J*=9.2 and 7.0 Hz, H-2), 3.45 (1H, ddd, *J*=11.0, 9.2, and 2.2 Hz, H-3), 1.12 (1H, dd, *J*=3.9 and 11.0 Hz, H-4a), 1.49 (1H, dd, *J*=10.9 and 2.2 Hz, H-4b), 1.79 (1H, dd, *J*=10.9 and 3.9 Hz, H-5a), 1.51 (1H, dd, *J*=13.0 and 9.7 Hz, H-5b), 1.61 (1H, m, H-6), 2.46 (1H, dd, 12.7 and 7.0 Hz, H-8a), 1.50 (1H, dd, *J*=12.7 and 5.1 Hz, H-8b), 2.05 (1H, m, H-9a), 1.66 (1H, m, H-9b), 4.27 (1H, ddd, *J*=8.3, 6.0, and 1.3 Hz, H-10), 3.96 (1H, d, *J*=1.3 Hz, H-11), 2.88 (1H, d, *J*=16.2 Hz, H-13a), 1.92 (1H, d, *J*=16.2 Hz, H-13b), 3.74 (1H, d, *J*=1.8 Hz, H-15), 4.19 (1H, ddd, *J*=12.5, 3.5, and 1.8 Hz, H-16), 2.01 (1H, dd, *J*=3.9 and 3.5 Hz, H-17a), 1.28 (1H, dd, *J*=12.5 and 3.9 Hz, H-17b), 2.21 (1H, dd, *J*=13.0 and 8.2 Hz, H-19a), 1.86 (1H, m, H-19b), 1.88 (1H, m, H-20a), 1.58 (1H, dd, *J*=10.0 and 1.4 Hz, H-20b), 3.81 (1H, dd, *J*=9.6 and 5.2 Hz, H-22), 2.02 (overlapped with solvent peak, H-23a), 1.61 (1H, t, *J*=11.0 Hz, H-23b), 1.39 (1H, dd, *J*=5.3 and 2.5 Hz, H-24a), 1.57 (1H, dd, *J*=11.0 and 5.3 Hz, H-24b), 1.64 (1H, dd, *J*=13.5 and 4.9 Hz, H-26a), 1.55 (1H, dd, *J*=12.3 and 11.0 Hz, H-26b), 2.60 (1H, m, H-27), 5.30 (1H, d, *J*=10.0 Hz, H-28), 6.38 (1H, dt, *J*=15.7 and 1.8 Hz, H-30), 5.38 (1H, dd, *J*=15.7 and 3.0 Hz, H-31), 4.69 (1H, q, *J*=3.0 Hz, H-32), 5.43 (1H, t, *J*=3.0 Hz, H-33), 2.23 (1H, ddd, *J*=13.0, 10.5, and 3.0 Hz, H-34a), 2.13 (1H, ddd, *J*=13.0, 6.0 and 1.5 Hz, H-34b), 4.46 (1H, dd, *J*=10.5 and 6.0 Hz, H-35), 3.30 (1H, dd, *J*=8.0 and 1.7 Hz, H-37), 2.07 (1H, m, H-38), 1.20 (1H, m, H-39a), 1.63 (1H, m, H-39b), 3.96 (1H, ddd, *J*=13.1, 10.9, and 2.5 Hz, H-40a), 3.56 (1H, dd, *J*=10.9 and 4.4 Hz, H-40b), 1.08 (3H, d, *J*=7.0 Hz, H-41), 1.17 (3H, s, H-42), 1.26 (3H, s, H-43), 1.16 (3H, s, H-44), 0.96 (3H, d, *J*=7.0 Hz, H-45), 1.70 (3H, s, H-46), 0.93 (3H, d, *J*=7.0 Hz, H-47), 3.32 (1H, s, OH-11), 4.33 (1H, br s, OH-36), 3.49 (1H, d, *J*=8.0 Hz, OH-37).

Psammaplin A.—Lreims (22 eV) *m/z* 668/667/666/664/663 (MH^+), 334/332 (5), 318/316 (2), 301/299 (1.5), 283/281 (2), 218 /216 (19), 213/211 (99), 201/199 (14), 138/136 (9), 133 (12), 132 (100); ^{1}H- and ^{13}C-nmr spectral data were identical with those published previously (8).

2′,3′-Didehydro-2′,3′-dideoxyuridine.—^{1}H- and ^{13}C-nmr spectral data were identical with those published previously (12).

BIOLOGICAL TESTING.—The extracts, fractions, and isolated compounds were evaluated routinely for lethality to brine shrimp larvae (BST) (1). The DNA cleavage assay (4) was performed employing ϕX174 RF I DNA (isolated from *E. coli* infected with ϕX174 *am* 3 *cs*

70, BioLabs, Beverly, MA) as a substrate. Bleomycin sulfate was employed as a positive control in the DNA cleavage assay. The brine shrimp and DNA cleavage assays were performed in-house. In vitro cytotoxicity assays of **1** against HT-29 (human colon adenocarcinoma), A-549 (human lung carcinoma), and MCF-7 (human breast carcinoma) were determined at the Cell Culture Laboratory, Purdue Cancer Center, Purdue University, following the protocols established by the National Cancer Institute. Adriamycin was employed as a positive control in this assay. In vitro cytotoxicity assays of psammaplin A against P-388 and A-549 were determined at the College of Pharmacy, Seoul National University, employing 5-fluorouracil as a positive control. Pectenotoxin II [**1**] was sent to the NCI, Bethesda, MD, for testing in human cancer cell line panels including leukemia, non-small cell lung cancer, colon cancer, CNS cancer, melanoma, ovarian cancer, renal cancer, prostate cancer, and breast cancer. In vitro antiviral activities against HSV-1 (*Herpes simplex* virus type 1) and HSV-2 (*Herpes simplex* virus type II) were evaluated at the Pharmaceutical Screening Center, Korea Research Institute of Chemical Technology. Antiviral activity was measured by a virus-induced CPE (cytopathic effect) inhibition assay emplying Vero cells (CCL 81) as the host cells. A rat plasma membrane assay (4) was performed at Dr. D.J. Morre's laboratory, Department of Medicinal Chemistry and Pharmacognosy, Purdue University. Rat liver plasma membrane was used as an reduction-oxidation enzyme fraction. Coenzyme Q, NADH, and the enzyme fraction were incubated with the test sample in Tris HCl buffer for 10 min. Enzyme inhibition was determined by measuring uv absorbance at 410 nm at 5 min, 10 min, and 15 min, respectively.

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