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Studied on the Antibacterial, Antifungal Components in Some Korean Marine Sponges

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Antimicrobial substances were screened by paper disk plate method in marine sponges, *Halichondria okadai, Halichondria* sp., *H. japonica and Haliclona permollis,* collected from the south coast of Korea. Antibacterial components were detected in two species, *H. okadai* and *Halichondria* sp.. Three components such as benzoic acid, okadaic acid(OA) and dinophysistoxin-1(DTX1) were identified from these sponges as the antimicrobial compounds by MS and NMR spectral data. OA(550~600μg/kg) and DTX1(400~490μg/kg) were determined from the wet *H. okadai* and *Halichondria* sp., respectively, by using fluorometric HPLC analysis with 9-anthryldiazomethane(ADAM) as fluorescent labelling reagent.

Introduction

Sponges, which constitute the phylum Porifera, are the most primative of the multicellula animals having no true tissues or organs. Except for hundreds of freshwaters species, several thousands species of sponges live in all seas, wherever there are rocks, shells, submerged timbers, coral or even on soft sand and mud bottoms to provide a suitable substratum(Barnes, 1982). They feed on microorganisms such as small planktons and bacteria through pumping mechanism and bear large amounts of symbionts with bacteria and blue-green algae (Wilkinson, 1978). Therefore, sponges have been considered as a valuable and interesting marine source both ecologically and biochemically. Many biological active substances have been successively isolated from sponges(Schmitz et al., 1984; Fusetani, 1987; Kitagawa, 1987). Recently, some species of genus *Halichondria* were known to produce cytotoxic or antitumor polyethers such as okadaic acid(OA, Tachibana et al., 1981, Fig. 1.) and halichondrins(Uemura, 1991).

Some sponges including *Halichondria* sp., widely distributed on the shore rocks of south coast of Korea, have been presumed to have any useful bioactive substances.

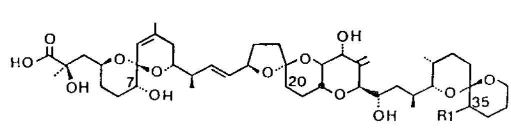

Fig. 1. Structures of Okadaic acid(OA) and Dinophysistoxin-1(DTX1).

In this paper, we screened antimicrobial compounds from the 3 species of genus *Halichondria* and one of genus *Haliclona* sp. collected at the Chungmu area by using paper disk plate method (Kondo et al., 1988) and firstly identified a large quantity of benzoic acid, OA and dinophysistoxin-1 (DTX1) as the antimicrobial components. And also, discussed the determination method of OA and DTX1 in the sponges by using fluorometric HPLC analysis.

Materials and Methods

Materials

H. okadai, *H. japonica*, *Haliclona permollis* and *Halichondria* sp.(Photo. 1) collected on the shore rocks at Sanyangmyeon, Tongyeonggun, Kyeongnam Prefecture, south coast of Korea during summer, 1990. A species of sponge(Photo. 1-B), living more deeper sea and fragile matrix than other species and getting greenish yellow color, was presumed genus *Halichondria* due to its structural type, having one rayed spicules, but not identified species name. Therefore, we used its name as *Halichondria* sp..

Screening of antimicrobial components

Acetone extracts of each homogenated sponge (1*kg*) were partitioned with hexane, methylene chloride, butanol(BuOH) and water layer, successively. An aliquot(50*g*) of each layer adsorbed into paper disk(thick, 0.8*cm* diameter, Tosoh Co.) and put on the microbial agar media. After incubated 24 hours, measured the diameter of clear zone formed around the colony. Fungi with potato dextrose agar media, yeasts with yeast malt agar media and bacteria with standard plate count agar media were used in screening, respectively. The microorganisms tested, were shown in Table 1.

Isolation and purification of active components

After washing with water and get rid of contaminants, *H. okadai*(161*kg*) and *Halichondria* sp.(23*kg*) were minced and extracted twice with two volumes of acetone, respectively. The acetone extracts further purified as shown in Fig. 2. Three active components, isolated from the two species, named ten-

Table 1. Distribution of antimicrobial activity of each fractions extracted from sponge(50*g*) screened by paper disk plate(0.8*cm*) method.

Micro-	*H. okadai*			*Halichondria* sp.			*H. japonica*			*H. permollis*		
organisms	CH_2Cl_2	BuOH	H_2O	CH_2Cl_2	BuOH	H_2O	CH_2Cl_2	BuOH	H_2O	CH_2Cl_2	BuOH	H_2O
Staphylococcus sp.	++*	++	+	+	++	+	++	+	+	++	++	+
Bacillus subtilis	++	++	+	++	++++	++	+	++	+	+	−	−
Salmonella typhymurium	+	+	−	+	−	++	+	−	−	+	−	−
Pseudomonas aeruginosa	+	+	−	+	++	++	−	−	+	−	−	−
Escherichia coli	+	+	−	+	++	++	−	−	+	−	−	−
Candida albicans	+	−	−	+	−	−	−	−	−	−	−	−
Pichia pastoris	++	++	−	++	++	−	−	−	−	−	−	−
Saccharomyces diasticus	++	++	−	++	++	−	−	+	++	−	−	−
Aspergillus niger	+++	+	−	+++	+	−	−	−	−	−	−	−
Mucor sp.	+++	++	−	+++	+	−	−	−	−	−	−	−
Penicillium funiculosum	+++	++	−	+++	+	−	−	−	−	−	−	−

*Diameter of clear zone formed around the colony, −: not formed, +: below 1*cm*, ++: 1.1~2.0*cm*, +++: 2.1~3.0*cm*, ++++: 3.1~4.0*cm*.

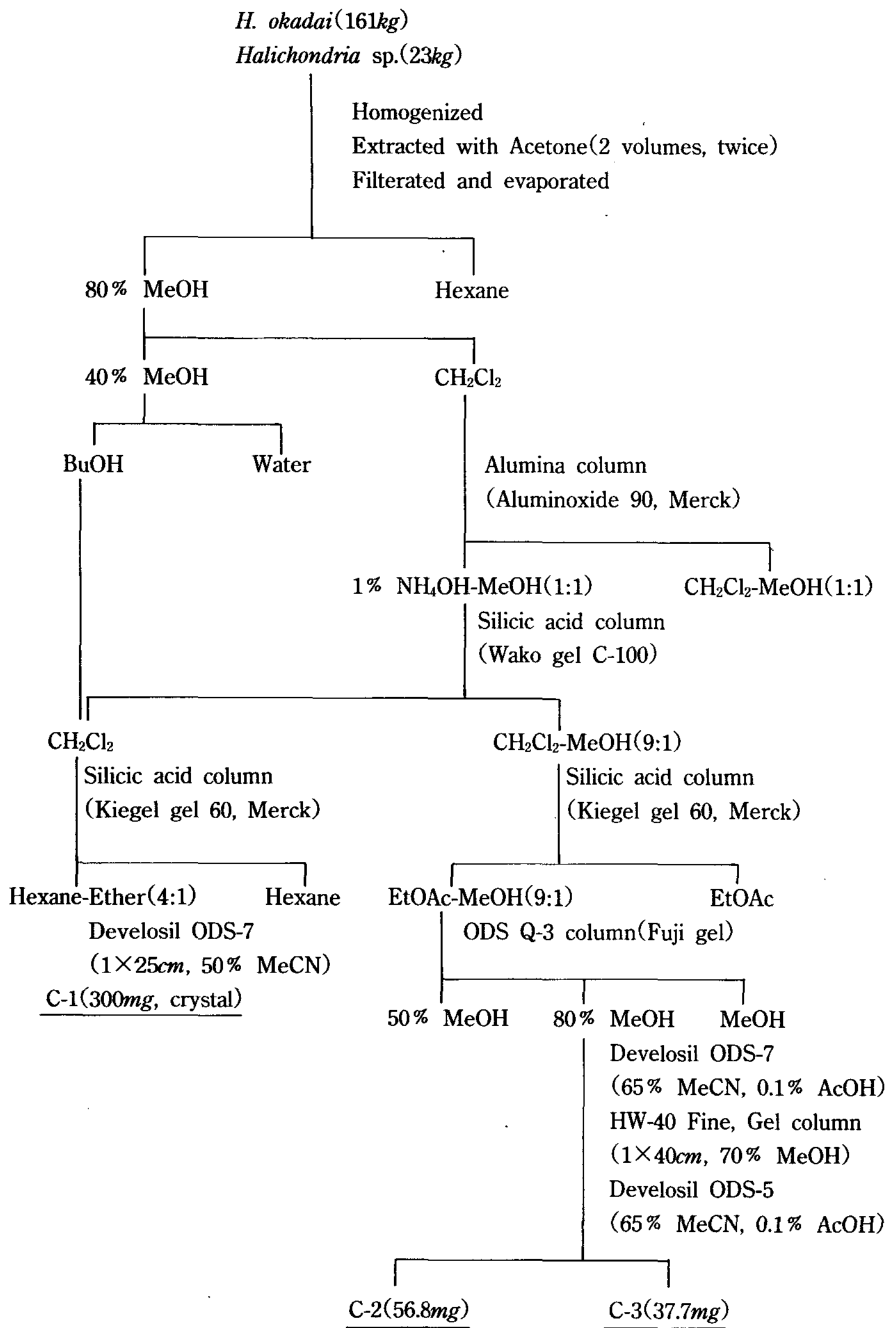

Fig. 2. Purification and isolation procedures of antimicrobial components from sponges.

tatively as C-1, C-2 and C-3.

Detection of active components

During the chromatographic purification, elutes of the active components was monitored with UV-flowmonitor, thin layer chromatography(TLC) and paper disk plate method using *Staphylococcus* sp. and *Aspergillus niger* as tracer. Wavelengths of UV-flowmonitor(JASCO UV III) were set at 254*nm* for detection of C-1 and 220*nm* for C-2 and C-3, respectively. TLC was carried out on the silica gel 60 plate(Merck, precoated) with hexane-ether(3:1) for C-1, and $CHCl_3$-MeOH-H_2O(100:15:1) for C-2 and C-3. Detection of active components was made by heating the plate with 50% methanolic sulfuric acid. For the C-2 and C-3, fluorometric HPLC analysis was also taken as follows. After 9-anthryl esterified C-2 and C-3 with 0.1% 9-anthryldiazomethane(ADAM), then analysed on the Develosil ODS-5 column(4.6×250*mm*, Nomura Chemical Co.). MeCN-MeOH-H_2O(8:1:1) was used as the mobile phase(flow rate, 1.1*ml/min.*) and maintained the column at 30℃ oven. The excitation and emission wavelengths were set at 365 and 412*nm*, respectively.

UV, IR, Mass and NMR spectrometry

UV spectra were measured on a U-best UV spectrometer(JASCO) in MeOH solution and IR spectra on a Hitachi IR spectrophotometer with KBr tablets. Electron impact(EI) mass spectrum of C-1, and the fast atom bombardment(FAB) negative ion mass spectra of C-2 and C-3 were measured on a JMX-DX303 HF mass spectrometer(JEOL) using glycerol as matrix. NMR spectra of C-1 in $CDCl_3$ and C-2, 3 in $CDCl_3$-CD_3OD(2:1) were measured on a GSX 400(400 MHz) FT NMR spectrometer (JEOL), respectively. All the spectral data of C-1 were compared with those of authentic benzoic acid (Wako Pure Chemical Co.) and C-2, 3 with OA and DTX1 isolated from Japanese scallop(Murata et al., 1982), *H. okadai* and *Prorocentrum lima*(Torigoe, 1991; Murakami et al., 1982).

Determination of OA and DTX1

Acetone extracts of sponge was evaporated and dissolved in 50% MeOH. After defatted with hexane, extracted with $CHCl_3$ and evaporated. The residue was dissolved in 50% MeOH, and 1*g* aliquot was charged into Sep-pak ODS column(Waters Co.). Then, washed with same solvent, and eluted with 80% MeOH and concentrated. The residue was dissolved in MeOH and 0.5*g* aliquot was taken into colored vial, then esterified with 50*μl* of 0.1% ADAM. Further clean up procedure carried out according to the determination method for diarrhetic shellfish toxins reported by Lee et al. (1987).

Examination of antimicrobial activities of OA and DTX1

Purified OA and DTX1 was dissolved in MeOH and each 10*μg* aliquot was adsorbed into paper disk and examined the antimicrobial activity by the paper disk plate method above mentioned. Species of the microorganisms were shown in Table 2.

Results

Distribution of antimicrobial activity

Table 2. Antimicrobial activity of Okadaic acid(OA)* and Dinophysistoxin-1(DTX1)* to various microorganisms.

Microorganisms	Activity OA	Activity DTX1	Media
Staphylococcus aureus	tr**	tr	Nutrient Agar
Escherichia coli	tr	tr	〃
Pseudomonas aeruginosa	tr	tr	〃
Proteus vulgaris	tr	tr	〃
Vibrio vulnificus	tr	tr	〃 (3% NaCl)
Candida albicans	1.1	1.5	Yeast Malt Agar
Pichia pastoris	1.5	2.0	〃
Saccharomyces diasticus	2.4	3.0	〃
Saccharomyces acidifaciens	1.5	2.0	〃
Aspergillus versicolor	3.5	4.0	Potato Dextrose Agar
Helminthosporium sp.	3.5	4.0	〃
Mucor sp.	2.5	3.0	〃
Penicillium funiculosum	2.5	3.0	〃

* Each 10*ug* of OA and DTX1 was adsorbed into paper disk(thin, 0.8*cm*, diameter) and confirmed the diameter(*cm*) of clear zone around the colony ** below 0.9*cm*.

All the hexane fractions of sponges extracts showed no activity to various bacteria, yeasts and molds tested. Antibacterial activity were shown in all species except for *H. permollis,* showed no activity to *E. coli.* The growth inhibition activity to yeasts were contained in the 3 species of genus *Halichondria.* On the other hands, antifungal activity was only shown in methylene chloride and BuOH fractions of *H. okadai and Halichondria* sp.(Table 1).

Identification of active components

C-1(300*mg*) was obtained as white crystalline with unpleasantable odor from the BuOH and methylene chloride fraction, respectively. The EIMS showed prominent ion peaks at *m/z* 122, 105 and 77(Fig. 3), which were assignable to $[M]^+$, $[M-OH]^+$, $[M-COOH]^+$ of authentic benzoic acid, respectively. UV, IR and ^1H NMR spectra(Fig. 3) were agreed well with those of benzoic acid. The carbon signal of carboxyl group was further assigned on the ^{13}C NMR spectrum(δ: 172.58*ppm*). The C-1 was thus identified as benzoic acid.

C-2(56.8*mg*), eluted from the alumina column with 1% NH_4OH-H_2O(1:1), was obtained as a colorless niddle-like crystal in MeOH solution after eluted on the ODS column. On the TLC(*Rf*:0.5) and fluorometric HPLC(Fig. 4), it was indistinguishable with those of OA. All the proton signals on the ^1H NMR specturm(Fig. 5), including 5 methyl protons at δ:0.90, 1.01, 1.03, 1.34, 1.73*ppm*, were assigned well with those of OA. The FABMS exhibited $[M-H]^-$ peak at *m/z* 803(Fig. 6) corresponding

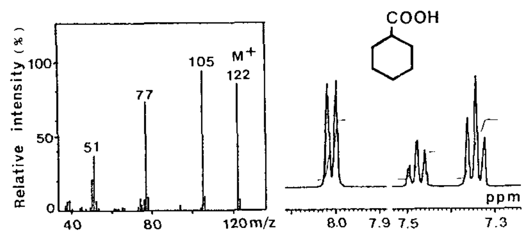

Fig. 3. EI mass and ^1H NMR spectra of component C-1($CDCl_3$, 400MHz).

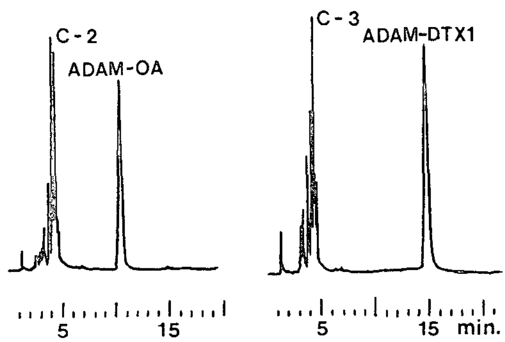

Fig. 4. Fluorometric HPLC chromatograms of component C-2 and C-3 esterified with 9-anthryldiazomethane(ADAM).

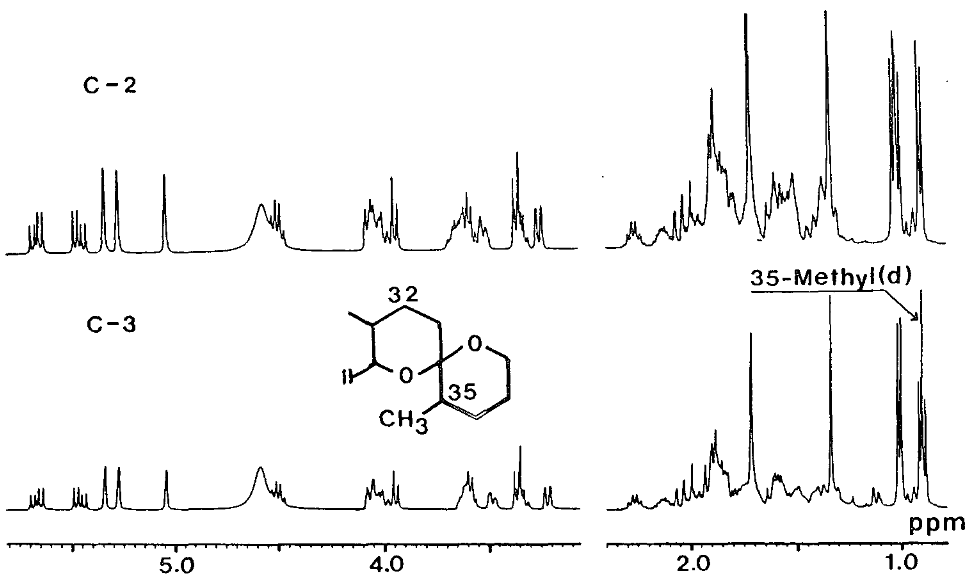

Fig. 5. ^1H NMR spectra of component C-2 and C-3($CDCl_3$-CD_3OD, 2:1, 400MHz).

to that of OA. From these data, C-2 was identified unambiguously as OA(Fig. 1).

C-3(37.7*mg*), having similar behavors with C-2 on the alumina and silicic acid colomn, separated from C-2 on the ODS column as colorless solid. It showed no absorption maximum in UV region and eluted after than C-2 on the fluorometric HPLC (Fig. 4), which had same retention time with DTX1-anthryl ester(ADAM-DTX1). Comparing ^1H NMR spectra of C-2(OA) and C-3, the feature of proton signals of C-3 were resumbled to those of C-2 except appeared an additional methyl proton at δ:0.93*ppm*(doublet, Fig. 5). It was further confirmed on the FABMS which showed $[M-H]^-$ of C-3 at *m/z* 817(Fig. 6) meaning 14 mass units higher than that of OA(*m/z* 803). Including newly observed methyl, all the other spectral data were well coincided with those of DTX1 which substituted a proton of 35 position with a methyl in OA. Therefore, C-3 was elucidated unanimously as DTX1, 35-methyl OA(Fig. 1).

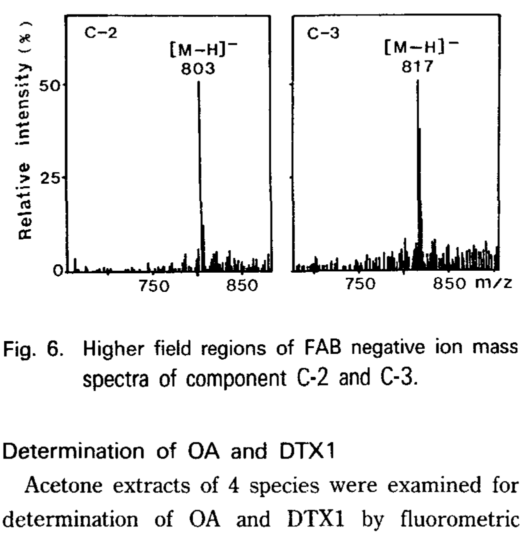

Fig. 6. Higher field regions of FAB negative ion mass spectra of component C-2 and C-3.

Determination of OA and DTX1

Acetone extracts of 4 species were examined for determination of OA and DTX1 by fluorometric HPLC analysis. OA and DTX1 were only detected from two species, *H. okadai* and *Halichondria* sp. (Fig. 7). The content of OA in *H. okadai* and *Halichondria* sp. was between 550~600*μg/kg* wet base. On the other hands, DTX1 was detected between 400~490*μg/kg* in these two species.

Antimicrobial activity of OA and DTX1

Table 2 was shown antimicrobial activity of OA and DTX1 to various microorganisms. Growth of bacteria was not so inhibited in the agar media contained 10*μg* of OA or DTX1 disk plate but, large clear zones were formed around the fungi. The activity was not so much difference between OA and DTX1.

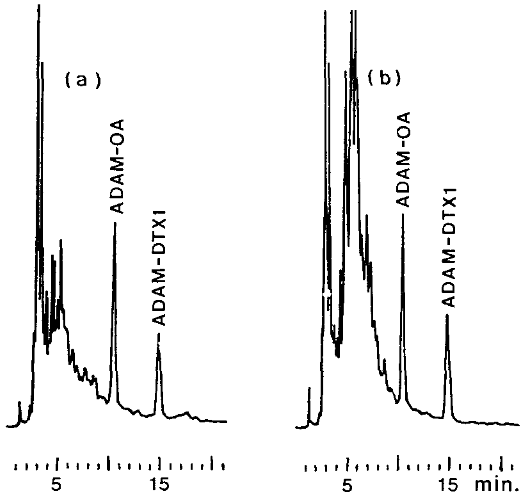

Fig. 7. Fluorometric HPLC chromatograms of sponge extracts, (a) *Halichondria okadai* and (b) *Halichondria* sp..

Discussions

Antibacterial components, as shown in Table 1, were presumed to be contained in all species tested, much or less. The antibacterial activity may be affected by commonly distributed components in sponges such as fatty acids or organic acids, having antimicrobial activity(Branen et al., 1983) and inorganic salts in water layers. In this study, therefore, we focused firstly to the active components in the methylene chloride and BuOH fractions of two species which having both antibacterial and antifungal activities.

The occurrence of a large amount of benzoic acid in sponges was firstly confirmed. As well known, benzoic acid and its derivatives have been permitted to use in cosmetics, drugs and some foods as preservatives(Chipley, 1983). It was thought that need to clear the biochemical roles and formation mechanisms of benzoic acid in sponges.

After the OA was isolated first by Tachibana et al.(1981) from sponges as cytotoxic complex polyether derivative of C_{38} fatty acid, many OA analogues were found from various organisms. It have been proved OA and DTX1 are produced by the dinoflagellates, *Dinophysis* spp.(Lee et al., 1989) and *P. lima*(Murakami et al., 1982). On the other hands, OA and its derivatives had been also isolated from intoxicated bivalves as toxic principles causing diarrhetic shellfish poisoning(Yasumoto et al., 1985; Kumagai et al., 1986).

Including cytotoxicity and acute toxicity of OA and its derivatives, recently, biochemically interesting activities such as non-TPA(12-o-tetradecanoylphorbol-13-acetate) type tumor promotor(Fujuki et al., 1988), anticachexia activity(Uemura et al., 1991), calcium independent phosphorylation of smooth muscle(Ozaki et al., 1987), inhibition of interleukin-1 synthesis(Hokama et al., 1989) and stimulation of mouse bone mellow cells(Oka et al., 1989) were reported.

For the detection of OA analogues in sponges, fungi were more specific and sensitive than other bacteria and fluorometric HPLC analysis method, using ADAM as fluorescent labelling reagent, was also very useful. Interferences can be able to remove, effectively, during subsequent biphasial partition and Sep-pak treatment. Content and composition of OA and DTX1 in *H. okadai* and *Halichondria* sp. were not so much differences. However, they were presumed large regional and seasonal variation(Yasumoto, 1991).

In spite of wide and large distribution of sponges in the mediolittoral zone on the south coast of Korea, it have been treated as no useful marine animal. This is our first attempt to isolate biological active substances from the Korean sponges and firstly found OA analogues as antimicrobial components. If we use several kinds of screening systems for bioactive substances, it will doubtless to find various active compounds from the marine sponges. And also, these compounds will be used as not only chemicals of potential importance but also tools for investigating biochemical functions.

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References

Barnes, R. D., 1982, Invertebrate Zoology(4th), Holt-Saunders, Japan, p. 89.

Branen, A. L., P. M. Davidson, 1983, Antimicrobials in Food, Marcel Dekker Inc., New York and Basel, pp. 1~140.

Chipley, J. R. 1983, Sodium Benzoate and Benzoic Acid, in *ibid.*, pp. 11~35.

Fujiki, H., M. Suganuma, H. Suguri, S. Yoshizawa, K. Takagi, N. Uda, T. Yasumoto, and T. Sugimura, 1988, Diarrhetic Shellfish Toxin, Dinophysistoxin-1, Is a Potent Tumor Promotor on Mouse Skin, Jpn. J. Cancer Res(Gann)., 79, pp. 1089~1093.

Fusetani, N., 1987, Antimicrobial and Antitumor Substances from Marine Invertebrates, in Bioactive Marine Natural Products(Eds. by Yasumoto T., and Kamiya, H., Suisangaku series 65), Koseisha Koseikaku, Japan, pp. 32~45.

Hokama, Y., P. J. Scheur, and T. Yasumoto, 1989, Effect of a Marie Toxin on Human Peripheral Blood Monocytes, J. of Clinical Laboratory analysis, 3, pp. 215~221.

Kitagawa, K., 1987, Biological Active Substances in Marine Sponges and Soft Coral, in Marine Natural Products Chemistry(Ed. Kitagawa K., Kagaku zoukan 111), Japan, pp. 133~144.

Kondo, M., T. Fujino, and T. Hata, 1988, Hygienic Microbiology, Kodansha Scientific, Tokyo, p. 312.

Kumagai, M. T. Yanagi, Y. Oshima, T. Yasumoto, M. Kat, P. Lassus, and J. A. Rodrigez-Vazquez, 1986, Okadaic acid as the Causative Toxin of Diarrhetic Shellfish Poisoning in Europe, Agr.

Biol. Chem., 50, pp. 2853～2857.

Lee, J. S., T. Yanagi, R. Kenma, and T. Yasumoto, 1987, Fluorometric Determination of Diarrhetic Shellfish Toxins by High Performance Liquid Chromatography, Agr. Biol. Chem., 51, 877～881.

Lee, J. S., T. Igarashi, S. Fraga, E. Dahl, P. Hovgaard, and T. Yasumoto, 1989, Determination of Diarrhetic Shellfish Toxins in Various Dinoflagellates species, Applied Phycology, 2, 147～152.

Murakami, Y., Y. Oshima, and T. Yasumoto, 1982, Identification of Okadaic aicd as a Toxic Component of a Marine Dinoflagellate, *Prorocentrum lima,* Nippon Suisan Gakkaishi, 48, pp. 69～72.

Murata, M., M. Shimatani, H. Sugitani, Y. Oshima, and T. Yasumoto, 1982, Isolation and Structural Elucidation of the Causative Toxins of the Diarrhetic Shellfish Poisoning, Nippon Suisan Gakkaishi, 48, pp. 549～552.

Oka, S., K. Nakagomi, H. Tanaka, and T. Yasumoto, 1989, Effect of Okadaic acid on Mouse Hemopoietic Cells, Biochemical and Biophysical Res. Comm., 165, pp. 539～546.

Ozaki, H., H. Ishihara, K. Kohama, Y. Yonomura, Y. Nonomura, S. Shibata and H. Karaki, 1987, Calcium-independent Phosphorylation of Smooth Muscle Myosin Light Chain by Okadaic acid Isolated from Black Sponge J. Pharmacol. Exp. Ther., 243, pp. 1167～1173.

Schmitz, F. J., S. P. Gunasekera, G. Yalamanchili, M. B. Hossain, and D. var der Helm, 1984, Tedanolide, A potent Cytotoxic Macrolide from the Carribean Sponge, *Tedania ignis,* J. Am. Chem. Soc., 106, pp. 7251～7252.

Tachibana, K., P. J. Scheur, Y. Tsukitani, H. Kikuchi, D. V. Engen, J. Clardy, Y. Gopichand, and F. J. Schmitz, 1981, Okadaic acid, a Cytotoxic Polyether from Two Marine Spnges of the Genus *Halichondria,* J. Am. Chem. Soc., 103, pp. 2469～2471.

Torigoe, K. 1991, Studies on the Structures and Biosynthesis of Bioactive Components Produced by Dinoflagellate, *Prorocentrum lima,* Doctral Thesis of Tohoku University.

Uemura, D., 1991, Antitumor Polyethers from Marine Animals, in Bioorganic Marine Chemistry, (vol. 4, Ed, by P. J. Scheur), Springer-Verlag, New York, "in press"

Wilkinson, C. R., 1978, Microbial Associates in Sponges, I, II, III, Marine Biology, 49, pp. 169～186.

Yasumoto, T., M. Murata, Y. Oshima, M. Sano, G. K. Matsumoto, and J. Clardy, 1985, Diarrhetic Shellfish Toxins, Tetrahedron, 41, pp. 1019～1025.

Yasumoto, T., 1991, "personal communication"

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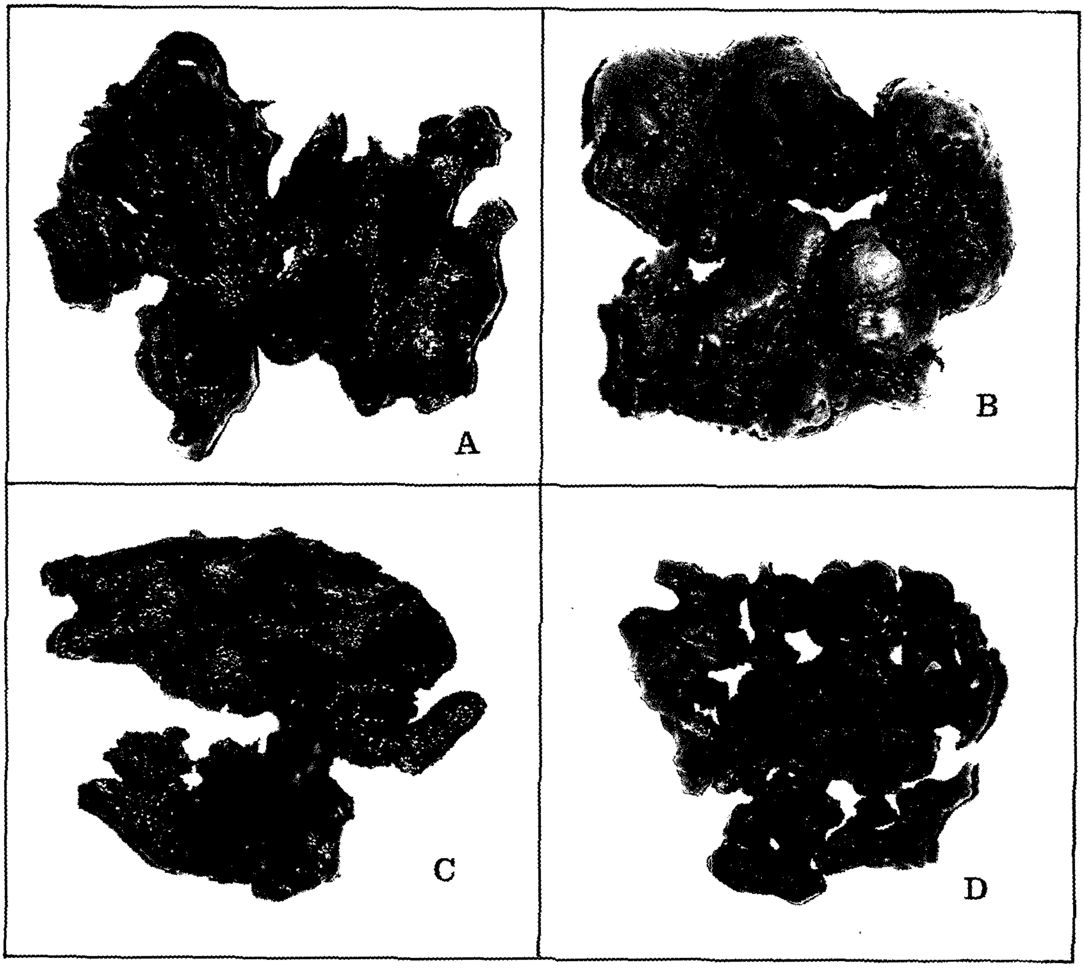

Photo 1. Photos of Marine Sponges, A: *Halichondria okadai*, B: *Halichondria* sp., C: *Halichondria japonica*, D: *Haliclona permollis*.

韓國產 海綿類中의 抗菌, 抗곰팡이 物質에 관한 研究

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4種類의 南海岸 海綿 *Galichondria* sp., *H. okadai, H. japonica* 및 *Haliclona permollis* 中의 抗菌, 抗곰팡이 物質을 paper disk plate法으로 檢索하였다. 抗菌物質은 4種類의 海綿에 모두 存在하였으나 抗곰팡이 物質은 *Halichondria* sp.와 *H. okadai*의 CH_2Cl_2區와 BuOH區에만 나타났다. 이들 두 種類의 海綿(184*kg*)을 아세톤으로 抽出하여 각종 column을 利用하여 精製 후 核磁氣共鳴 및 質量分析 등 각종 機器分析을 통하여 benzoic acid, okadaic acid(OA), dinophysistoxin-1(DTX1)의 3成分을 抗菌, 抗곰팡이 物質로 同定하였다. 한천培地상에서의 OA 및 DTX1의 微生物 生育沮止能力은 비슷하였으며 특히 곰팡이에 대하여 沮止効果가 크게 나타났다. 9-Anthryldiazomethane(ADAM)을 利用한 螢光 HPLC法을 開發하여 海綿中의 OA 및 DTX1을 分析한 結果 이들 成分은 *H. okadai* 및 *Halichondria* sp. 에만 存在하였다. 또한, 이들 두 種類의 海綿中의 含量은 濕中量 1*kg*당 OA가 550~600*μg*, DTX1이 400~490*μg*이었다.