Drugs from the Sea

Nobuhiro Fusetani

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Introduction

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In 1967 the symposium entitled 'Drugs from the Sea' sponsored by the Marine Technology Society of the United States, was held at the University of Rhode Island, Kingston. Hugo D. Freudenthal, the editor, described 'a great future in sight' in development of drugs from marine organisms, in the proceedings published in 1968. The discovery of a large amount of prostaglandin A_2 methyl ester acetate in 1969 became an additional driving force to stimulate research activity of secondary metabolites in marine organisms.

Baslow's 'Marine Pharmacology' published in 1969 promised a great future for marine-derived drugs. Perhaps the term 'marine natural products' first appeared in Clifford Chan's review papers in the *Journal of Chemical Education* in 1973. This enthusiasm on marine metabolites resulted in the foundation of the 'Roche Institute of Marine Pharmacology' near Sydney. Since the Drugs from the Sea Symposium, more than 10,000 new compounds have been isolated from bacteria, fungi, microalgae, seaweeds, sponges, soft corals, opisthobranch mollusks, bryozoans, echinoderms, and ascidians (about 200 marine-derived compounds were listed in the review of E. Premuzic published in *Progress in Organic Chemistry of Natural Products* in 1971). Presumably, more than 0.1% of them are structurally unique and exhibit significant biological activity; naturally they are expected to be important drug leads or potential drugs. However, no drugs have been developed from these compounds. It should be noted that approximately 300 patents on marine natural products were issued between 1969 and 1999 [1].

There are two serious obstacles which prevent development of drugs from marine natural products. Obviously the most important is 'supply issue'. The majority of promising compounds have complex structures, which limit us to supply large amounts of samples by chemical synthesis. Usually their yields

from organisms are quite low as well, often less than 10^{-6}% based on wet weight. Many researchers believe that these compounds are actually produced by 'symbiotic' microorganisms. Yet bacteria or fungi which produce highly promising metabolites have not been isolated from parent organisms. However, Faulkner and co-workers have obtained fascinating results with sponge/bacteria association. If such symbiotic bacteria are culturable, we can expect a more promising future in 'Drugs from the Sea'. Another promising approach is 'production of potential drugs by aquaculture'. In fact, the culture of the gorgonian *Plexaura homomala* which contains large amounts of prostaglandins was performed by a pharmaceutical company 30 years ago. Recently, attempts at aquaculture of a sponge, a bryozoan, and an ascidian have been performed in several places, which show promising results (chapters by Mendola and by Hart et al.). It is remarkable if aquacutural production of drugs is succeeded. Most of the highly bioactive marine metabolites are also highly toxic, which is very serious. If enough materials are available, modification of parent compounds could be done to reduce their toxicity or to improve their activity. However, such attempts have not been accomplished because of limited amounts of materials and highly complex structures, except for readily synthesized substances such as didemnins and dolastatins. One of the solutions for this problem will be to create a 'tumor-specific' or other disease-targeted drug delivery system. Hart et al. also describe such an attempt.

Of course, the discovery of new structures is very important. Marine microbes are relatively new targets for such research. Bacteria and fungi isolated from marine environments, namely, seawater, sediments, and marine organisms, often produce metabolites identical or similar to those from terrestrial species. Unculturable microbes should be interesting to be explored. Jensen and Fenical summarize the present status and future prospect of research on marine microbial metabolites. In contrast, phytoplanktons produce a wide range of unusual metabolites with strong bioactivities, frequently unprecedented structural features. However, there are some problems; low growth rates, low productivity of metabolites, and complexity of structures would be negative factors. Shimizu points out these problems. Macroorganisms, especially sponges, opisthobranch mollusks, and ascidians, are an important source of pharmaceutically important metabolites. Contributions from Kobayashi and Yamada et al. deal with such metabolites found in sponges and opisthobranch mollusks, respectively. A predominant portion of bioactive marine metabolites is occupied by antitumor/cytotoxic compounds. Recently, metabolites possessing biological activities other than cytotoxicity have been reported. Kuramoto et al. report antiosteoporotic alkaloids isolated from a hexacoral.

At the first Drugs from the Sea Symposium, many participants might have dreamt that marine-derived drugs would be developed in a short time.

Table 1. Marine natural products and their analogues entered in clinical trials

Organisms	Compound	Disease area	Phase
Porifera			
Agelas mauritianus	KRN7000[1]	Cancer	I
Petrosia contignata	IPL-567[2]	Inflammation	I
Coelenterata			
Pseudopterogorgia elisabethae	Methopterosin[3]	Inflammation/wound	I
Nemertea			
Amphiponus lactifloreus	GST-21[4]	Alzheimer's/schizophrenia	I
Mollusca			
Dolabella auricularia	Dolastatin 10	Cancer	II
D. auricularia	LU-103793[5]	Cancer	I
Conus magnus	Ziconitide[6]	Pain	III
Bryozoa			
Bugula neritina	Bryostatin 1	Cancer	II
Urochordata			
Tridemnum solidum	Didemnin B	Cancer	II
Aplidium albicans	Dehydrodidemnin B	Cancer	II
Ecteinascidia turbinata	Ecteinascidin 743	Cancer	II
Chordata			
Squalus acanthias	Squalamine	Cancer	I

[1] Agelasphin analogue.
[2] Contignasterol analogue.
[3] Pseudopterosin analogue.
[4] Anabaseine analogue.
[5] Dolastatin 15 analogue.
[6] ω-Conotoxin MVIIA.

After more than 30 years, the dream seems to be coming true. More than ten marine-derived drugs were entered in the clinical trials summarized in table 1, while more substances are under preclinical examinations [2–4]. Shortly, ω-conotoxin MVIIA (SNX 111) will be approved as a painkilling drug in the United States. Olivera documents the discovery and development of ω-conotoxin MVIIA. A unique glycosphingolipid, KRN7000, developed from a sponge metabolite is under phase I trials. Its unique mode of action is described by Natori et al. Unfortunately, as a chapter on the present status of the development of ecteinascidin 743 and dehydrodidemnin B as anticancer drugs

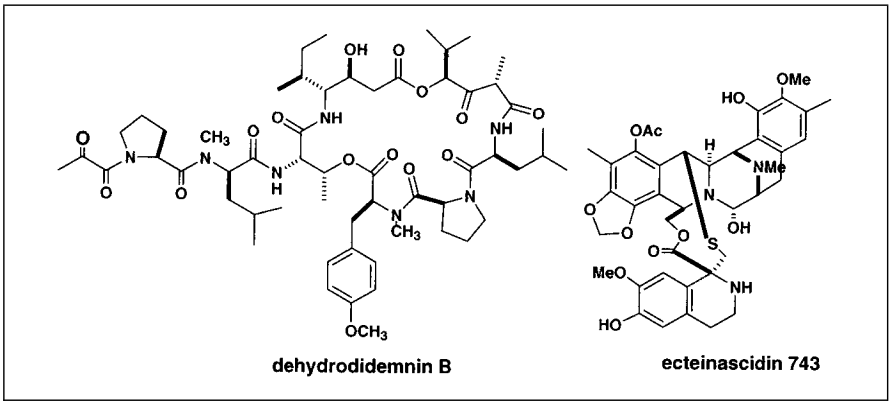

Fig. 1. Dehydrodidemnin B and ecteinascidin 743.

failed to arrive, I would briefly like to comment on these important anticancer drugs. Didemnin B, an antitumor depsipeptide discovered from the Caribbean tunicate *Tridemnum solidum*, had been a forerunner in the 'Drugs from the Sea' race; it proceeded to phase II clinical trials. However, its hepatotoxicity prevented the further development for an anticancer drug. Recently, dehydrodidemnin B (aplidine) isolated from the Mediterranean tunicate *Aplidium albicans* was found to be more active and less toxic; dehydrodidemnin B is generally 6–10 times as active as didemnin B [5, 6]. It showed a broad spectrum of activity, both in vitro and in vivo against leukemia, melanoma, breast, ovarian, colon, non-small cell lung cancers, and lymphomas. Didemnins show cytotoxic acitivity by interfering with protein synthesis through GTP-dependent inhibition of the elongation factor 1-α (fig. 1).

The Caribbean tunicate *Ecteinascidia turbinata* was first reported to be highly antitumorous in 1969 at the Food-Drugs from the Sea Symposium. However, the isolation and structure elucidation of the active constituents, ecteinascidins, were not completed until 1990. The ecteinascidins are tetrahydroisoquinoline alkaloids related to saframycins. Ecteincascidin 743 was found to be the most promising among them; it is particularly active agianst colon, melanoma, and renal tumor cell lines with IC_{50} values of < 1 pM. In vivo activity against melanoma, lung, breast, and ovarian cancers was quite remarkable [5, 7]. Ecteinascidins bind the N2 position of guanine in the minor groove of duplex DNA with the preference for GC-rich sequences, but its mode of action remains to be fully elucidated. Production of ecteinascidin 743 by aquaculture has been attempted in a few places as described by Mendola.

The majority of contributors to this book also participated in the Natio Conference on Chemical and Biological Basis for the Diversity of Marine Life

in October 1997. Their presentations deeply impressed the attendants of the conference, which prompted me to plan the publication of the present book entitled 'Drugs from the Sea'. Of course, the fruits of the marine natural products research have become ripe, and it therefore seems good timing to publish such a book.

Finally, I wish to thank all the contributors for their understanding, cooperation, and patience. Thanks are also due to Professors Isao Kitagawa, Takeshi Yasumoto, Nobutaka Takahashi and Akinori Suzuki for their encouragement and valuable discussions. This publication was partly supported by the Naito Foundation.

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Marine Microorganisms and Drug Discovery: Current Status and Future Potential

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Preface

In recent years, published reviews clearly indicate the tremendous potential of marine microorganisms as a source of new pharmaceuticals [1–9]. Rather than repeat a comprehensive review of microbial metabolites here, we have instead grouped compounds based on biomedical activities and discussed in more detail a few representative compounds that show promising activities in specific disease areas. In compiling this list and in an effort to emphasize the vast potential of this resource, an effort was made to choose biomedically relevant compounds produced by taxonomically diverse microorganisms isolated from a variety of marine sources. Although compounds are presented based upon activity, it should be noted that some of these compounds possess multiple activities and most have only been tested in a limited number of assays. With this in mind, the true drug potential of many of these compounds may not yet be fully realized. This review covers secondary metabolites from cultured, heterotrophic marine microorganisms, and does not include the many interesting compounds that have been isolated from cyanobacteria and photosynthetic microalgae.

Introduction

For more than two decades, there has been an ongoing quest to discover new drugs from the sea. This quest has been manifested in many forms but,

as is demonstrated by the other chapters in this book, most efforts have been directed towards chemical studies of marine invertebrates. Although these studies have indeed proven that marine invertebrates are an important source of new biomedical leads, a fact well demonstrated by the number of compounds currently in clinical trials, it has proven notoriously difficult to obtain adequate, reliable, and most importantly, renewable, supplies of these compounds from nature. Compounding the supply problem is the inherent structural complexity of many marine natural products which most often eliminates the possibility of commercially viable syntheses. Because of these problems, and the fact that more than 20 years of extensive research has made it increasingly difficult to isolate novel metabolites from marine invertebrates, a new avenue of study focusing on marine microorganisms has been garnering considerable attention. Although marine microorganisms are not well defined taxonomically, preliminary studies in this field unequivocally indicate that the wealth of microbial diversity in the world's oceans, coupled with its biochemical uniqueness, make this a promising frontier for the discovery of new medicines.

The foundation for chemical studies of marine microorganisms derives from the historical significance of terrestrial microbes as a source of pharmaceuticals. From the discovery of penicillin more than 60 years ago for the treatment of microbial infection, to the more recent anticancer chemotherapeutic agent Adriamycin and the important immunosuppressant drug cyclosporin A, microorganisms have yielded over 120 of today's most important medicines. The rate at which new compounds are being discovered from traditional microbial resources, however, has diminished significantly in recent decades as exhaustive studies of soil microorganisms repeatedly yield the same species which in turn produce an unacceptably large number of previously described compounds. In an effort to improve the rates at which new classes of secondary metabolites are discovered, new microbial resources are being sought. These efforts now include studies of marine microorganisms. Although special techniques are required for the isolation and cultivation of marine microorganisms, many of the principles developed for the commercial fermentation industry can be applied making this a controllable resource that does not face the same supply problems associated with the acquisition of compounds from marine invertebrates.

Unlike marine invertebrates, which can be clearly defined, there has been some debate as to what constitutes a marine microorganism. Marine bacteria are most generally defined by their requirement of seawater, or more specifically sodium, for growth [10]. In the case of marine fungi, which in general do not display specific ion requirements, obligate marine species are generally considered to be those that grow and sporulate exclusively in a marine habitat [11]. Although such definitions can prove useful, they tend to select for a

Table 1. Metabolites from marine bacteria

Producing strain	Source	Compound	Reference
Anticancer			
Actinomycete	Sediment	Lagunapyrones	12
Alteromonas haloplanktis	Sediment	Bisucaberin	13, 14
Alteromonas sp.	Sponge	Alteramide	15
Bacillus cereus	Mollusk	Homocereulide	16
Bacillus sp.	Sediment	Halobacillin	17
Bacillus sp.	Sediment	Isocoumarin	18
Chaina purpurogena	Sediment	SS-228Y	19, 20
LL-141352	Tunicate	LL-141352β	21
Micromonospora sp.	Soft coral	Thiocoraline	22, 23
Pelagiobacter sp.	Alga	Pelagiomicins	24
Streptomyces hygroscopicus	Fish gut	Halichomycin	25
Streptomyces sioyaensis	Sediment	Altemicidin	26, 27
Streptomyces sp.	Soft coral	Octalacins	28
Streptomyces sp.	Sediment	γ-indomycinone	29
Streptomyces sp.	Sediment	δ-indomycinone	30
Streptomycete	Mollusk	Aburatubolactam C	31
Vibrio sp.	Driftwood	Acyldepsipeptide	32
Antibacterial			
Actinomycete	Sediment	Marinone	33
Alcaligenes faecalis	Mollusk	B-1015	34
Alteromonas rava	Seawater	Thiomarinol	35–38
Bacillus sp.	Marine worm	Loloatins	39, 40
Chromobacterium sp.	Seawater	Bromopyrroles	41
Maduromycete	Sediment	Maduralide	42
Pseudoalteromonas	Alga	Korormicin	43
Pseudomonad	Seawater	Quinolinol	44
Pseudomonas aeruginosa	Sponge	Diketopiperazine	45
Pseudomonas bromoutilis	Seagrass	Pentabromopseudilin	46
Pseudomonas fluorescens	Ascidian	Andrimid, noiramides	47
Pseudomonas sp.	Alga	Massetolides	48
Streptomyces griseus	Sediment	Aplasmomycins	49–52
Streptomyces sp.	Sediment	Phenazines	53
Streptomyces sp.	Sponge	Urauchimycins	54
Streptomyces sp.	Sediment	Bioxalomycins	55
Streptomyces tenjimariensis	Sediment	Istamycins	56, 57
Streptomycete	Sediment	Wailupemycins	58
Vibrio gazogenes	Sediment	Magnesidins	59–61
Vibrio sp.	Sponge	Phenolic	62
Vibrio sp.	Sponge	Trisindoline	63

Table 1 (continued)

Antiviral			
Unidentified Gram-positive	Sediment	Macrolactins	64
Unidentified Gram-positive	Sediment	Caprolactins	65
Anti-inflammatory			
Actinomycete	Jellyfish	Salinamides	66, 67
Enzyme inhibition			
Agrobacterium aurantiacum	Not reported	Hydroxyakalone	68
Blastobacter sp.	Seawater	B-90063	69
Flavobacterium sp.	Bivalve	Flavocristamides	70
Streptomyces sp.	Sediment	Pyrostatins	71
Other targets			
Alteromonas rubra	Not reported	Aromatic acids	72
Streptomyces sp.	Mollusk	Aburatubolactam A	73

subset of the microorganisms that can be isolated from any one environment. This problem is compounded in the case of near-shore or estuarine samples where a large percentage of the resident microbes are adapted to varying degrees of marine exposure. For the purpose of microbial drug discovery, it seems only logical to study all microbes that can be isolated from the marine environment. Once new products are discovered, then questions about the in situ distributions and metabolic activities of the producing species can be asked.

Based on the species listed in tables 1 and 2, most of the new compounds reported from marine microorganisms were obtained from species that can, in principle, be isolated from both land and sea. Although these facultatively marine species are clearly a good source of novel metabolites, their ecological roles and degrees of adaptation to the marine environment remain largely unknown. What is clear about these isolates however, is that they are yielding unprecedented structures with potent biomedical activities. A finding that suggests that despite, in some cases, apparent taxonomic affiliations with terrestrial species, environmental differences are sufficient for novel compound production.

Biomedical Activities

The Discovery of New Antitumor Agents (fig. 1)

Despite aggressive research efforts, the age-adjusted mortality rate for cancer in the United States has not changed significantly in the last 30 years.

Table 2. Metabolites from marine fungi

Producing strain	Source	Compound	Reference
Anticancer			
Aspergillus fumigatus	Fish	Fumiquinazolines	74
Aspergillus fumigatus	Sediment	Tryprostatins	75
Aspergillus niger	Sponge	Asperazine	76
Aspergillus sp.	Sediment	Aspergillamides	77
Aspergillus versicolor	Alga	Sesquiterpene esters	78
Fusarium sp.	Wood	Neomangicols	79
Gymnasella dankaliensis	Sponge	Gymnastatins	80, 81
Gymnasella dankaliensis	Sponge	Gymnasterones	82
Leptosphaeria sp.	Alga	Leptosins	83–86
Penicillium fellutanum	Fish	Fellutamides	87
Penicillium sp.	Alga	Cummunesins	88
Penicillium sp.	Alga	Penochalasins	89
Penicillium sp.	Alga	Penostatins	90, 91
Penicillium waksmanii	Alga	Pyrenocines	92
Periconia sp.	Sea hare	Pericosines	93
Phomopsis sp.	Coral reef	Phomopsidin	94
Trichoderma harzianum	Sponge	Trichodenones, harzialactones	95
		Spiroxins	96
Antibacterial			
Coniothyrium sp.	Sponge	Hydroxyphenyl	97
Corollospora pulchella	Sand	Melinacidins, gancidin	98
Exophiala pisciphila	Sponge	Exophilin A	99
Microsphaeropsis sp.	Sponge	Microsphaeropsisin	97
Preussia aurantiaca	Mangrove	Auranticins	100
Unidentified fungus	Sponge	Secocurvularin	101
Unidentified fungus	Sponge	Hirsutanols	102
Antiviral			
Scytalidium sp.		Halovirs	103
Antifungal			
Aspergillus sp.	Alga	Mactanamide	104
Hypoxlon oceanicum	Wood	15G256γ	105
Enzyme inhibition			
Corollospora pulchella	Wood	Pulchellalactam	106
Microascus longirostris	Sponge	Epoxysuccinates	107
Other targets			
Penicillium sp.	Sediment	Epolactaene	108
Phoma sp.	Crab shell	Phomactins	109–111

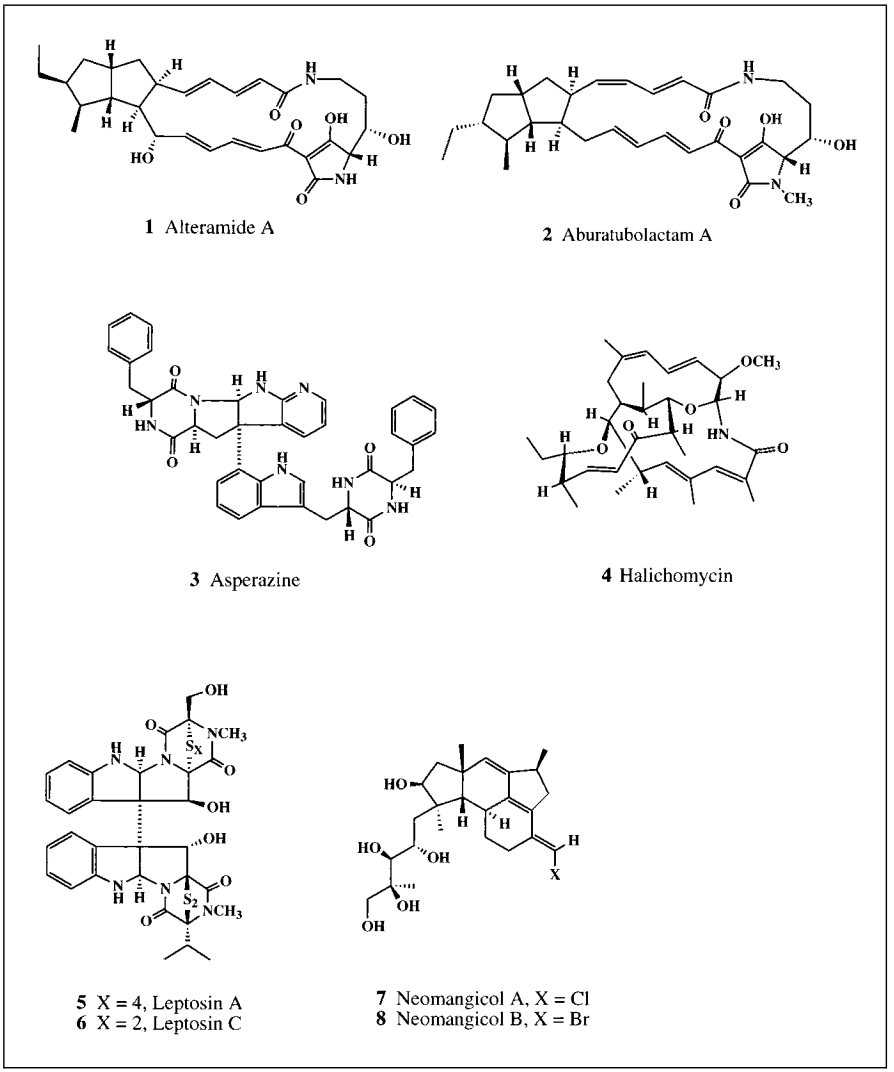


Fig. 1. Anticancer agents from marine microorganisms.

This disappointing statistic remains intact despite the fact that the US National Cancer Act of 1971 greatly expanded the level of funding for cancer research and the involvement of the National Cancer Institute in the research process. Part of this expansion included more aggressive efforts to evaluate natural products as a source of new cancer drugs. Although natural product screening has led to the discovery of important new drugs that are highly effective at

treating widely disseminated cancers such as leukemia, few of the approximately 40 cytotoxic or antiproliferative drugs in use today show clinical utility against the most common forms of slow-growing tumors [111]. In an effort to resolve this problem, the NCI phased out its P388 murine leukemia anticancer drug screening program during 1985–1990, and in its place established an in vitro primary screen consisting of 60 different human tumor cell lines. Compounds screened against this panel generate an activity 'fingerprint' that, when compared to a compound database, provide useful information about novelty, mechanism of action, and structure activity relationships [113]. The enormous efforts expended by the NCI to provide an effective mechanism to evaluate natural products for both in vitro and in vivo antitumor activities have proven invaluable in aiding the discovery and prioritization of new antitumor drug candidates.

In addition to the cell-based assays such as those performed at NCI, numerous academic and industrial laboratories have developed sophisticated molecular and biochemical screens that target specific cellular aspects of cancer growth and dissemination. These efforts, combined with new high throughput screening technologies, will undoubtedly lead to the discovery of new and less toxic, natural product-based chemotherapeutic agents. As is evident from tables 1 and 2, a significant fraction of the metabolites reported from marine microorganisms show anticancer activity. Five of these metabolites are highlighted below:

(1) The cytotoxic alkaloid alteramide A (**1**) was isolated from a 2-liter culture of a marine bacterium (*Alteromonas* sp.) obtained from the sponge *Halichondria okadai* [15]. Alteramide A is a new lactam that exhibited cytotoxicity against P388 leukemia, lymphoma L1210 and human epidermal carcinoma (KB cells) with IC_{50} values of 0.1, 1.7 and 5.0 μg/ml, respectively. A related macrocyclic lactam aburatubolactam A (**2**), which inhibited human neutrophil superoxide anion generation, was isolated from a 100-liter fermentation of a marine *Streptomyces* sp. [73]. These results clearly demonstrate how two taxonomically distinct bacteria, a Gram-negative *Alteromonas* sp. and a Gram-positive actinomycete can share similar biosynthetic capabilities. Of additional interest is the fact that the related compound cyrindramide was isolated from a marine sponge. This connection leads to the possibility that, as has been previously indicated [e.g. 114], microorganisms can in some instances be the true source of compounds isolated from marine invertebrates. It should be noted, however, that despite a great deal of speculation on this subject, there has been little experimental evidence that demonstrates the microbial production of invertebrate metabolites.

(2) Also studying microorganisms associated with marine sponges, Phil Crews and co-workers [67] at the University of California at Santa Cruz

recently reported the isolation of a selectively cytotoxic alkaloid, asperazine (**3**), from a strain of *Aspergillus niger* obtained from the sponge *Hyrtios* sp. Asperazine is an asymmetric diketopiperazine dimer containing highly modified tryptophan units. This compound, which is related to many similar amino acid dimers reported from terrestrial fungi, displayed significant leukemia-selective cytotoxicity at 50 μg/disk in a soft agar disk-diffusion assay. Asperazine showed no antibacterial (*B. subtilis*) or antifungal (*C. albicans*) activities.

(3) Atushi Numata's group [25] at the Osaka University of Pharmaceutical Sciences has made significant contributions to the study of marine microbial metabolites. Working with a streptomycete identified as *Streptomyces hygroscopicus*, isolated from the gastrointestinal tract of the marine fish *Halichoeres bleekeri*, this group reported the bioassay-guided isolation and structure elucidation of the cytotoxic macrolide halichomycin (**4**). Halichomycin, which represents a new macrolide class, was isolated from the ethyl acetate extract of the culture filtrate and exhibited potent in vitro cytotoxicity (IC_{50} 0.13 μg/ml) against P388 lymphocytic leukemia. Although fish gastrointestinal tracts are not recognized as a traditional actinomycete habitat, these types of habitats are not well studied and it remains possible that highly adapted actinomycete populations are associated with *H. bleekeri*.

(4) In addition to halicomycin, Numata and co-workers [84] reported a series of cytotoxic diketopiperazine dimers, leptosins A–F, from the obligate marine fungus *Leptosphaeria* sp. The producing strain was obtained from the marine alga *Sargassum tortile* and the six compounds displayed P388 in vitro cytotoxicities in the range of 1.75–8.6 μg/ml. More significantly, leptosins A (**5**) and C (**6**) displayed potent in vivo antitumor activity in a Sarcoma-180 ascites tumor model where they scored the exceptional T/C values (% survival time of treated versus control mice) of 260 and 293, respectively. Subsequent studies of this fungus led to the isolation of leptosins G–K [85, 86], all of which also demonstrated significant in vitro cytotoxicities.

(5) In our laboratory at the Scripps Institution of Oceanography, three novel sesterterpenes, neomangicols A–C, were isolated from the mycelial extract of a lignicolous (wood-inhabiting) marine fungus [80]. These compounds represent a new class of rearranged sesterterpenes that appear to be produced by atypical terpenoid biosynthesis. The producing strain was tentatively identified as the terrestrial fungus *Fusarium heterosporum* based on morpological characteristics, however compounds related to the neomangicols have not been previously reported from terrestrial isolates. That neomangicols A (**7**) and B (**8**) are chlorinated and brominated strongly suggests that the producing strain is adapted to marine conditions. We are currently performing 18s rRNA studies on this strain to determine its phylogenetic position. These studies should provide insight as to the degree to which marine adaptations

are characterized by genetic change. These compounds appear to be the first examples of halogenated sesterterpene natural products. Neomangicol A was the most cytotoxic of the three inhibiting MCF-7 (human breast carcinoma) and CACO-2 (human colon carcinoma) with IC_{50} values of 4.9 and 5.7 μM, respectively.

The Discovery of New Antibacterial Agents (fig. 2)

The stunning successes of the antibiotic era left society and the scientific community unprepared for the emergence of antibiotic resistance in bacteria. This resistance, which has come about largely from the indiscriminant use of antibiotics and the remarkable ability of bacteria to acquire resistance via genetic mutation or gene acquisition, has spread rapidly and left physicians with little recourse for the treatment of what were once routine infections. For example, enterococci, traditionally regarded as low-grade pathogens, are now the third most common cause of nosocomial infection in the United States [115]. Current threats posed by *Staphylococcus aureus* are even more frightening. In 1941, virtually all strains of *S. aureus* worldwide were susceptible to penicillin G. By 1992, 95% of these strains were antibiotic resistant [116]. Even the primary drug of last resort for methicillin-resistant *S. aureus*, the glycopeptide antibiotic vancomycin, has been rendered ineffective in at least three cases [117], spurring fears of a new generation of 'superbugs' that cannot be treated with any of the antibiotics currently available. Clearly the recent emergence and clinical significance of drug-resistant bacterial infection has created an urgent need for the rapid and continued development of new classes of antibiotics that can keep pace with the changing face of bacterial antibiotic susceptibility.

(1) One of the earliest reports of an antibiotic isolated from a cultured marine bacterium came from the pioneering work of Okami's group [19, 20] at the Institute of Microbial Chemistry in Tokyo. Here researchers isolated the hydroxyquinone SS-228Y (**9**) from a sediment-derived actinomycete identified as *Chainia* sp. Even in this early work, the importance of media composition was made clear by the finding that antibiotic activity was only produced when powdered seaweed was added to the medium, while traditional nutrients such as yeast extract and peptone resulted in a loss of activity. Compound SS-228Y inhibited multiple Gram-positive bacteria at MICs (minimum inhibitory concentrations) of 1–2 μg/ml. Compound SS-228Y also displayed activities against murine Ehrlich carcinoma and dopamine-β-hydroxylase.

(2) As part of our work, a new structure class of antibiotics was isolated from cultures of a marine sediment-derived actinomycete [33]. These compounds, marinone (**10**) and debromomarinone (**11**), are produced via mixed biosynthesis involving both acetate and terpene pathways. Both com-

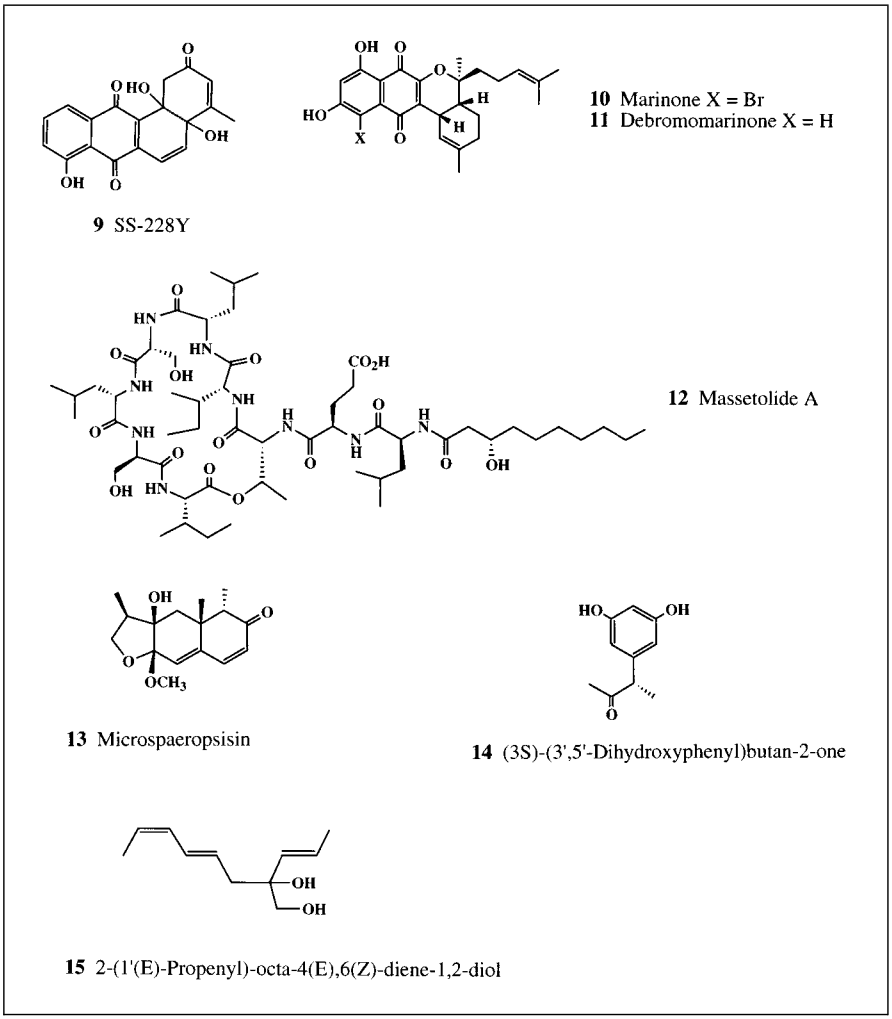


Fig. 2. Antibacterial agents from marine microorganisms.

pounds inhibited Gram-positive bacteria with MIC values ranging from 1 to 2 μg/ml.

(3) A variety of structurally unique, antibacterial cyclic peptides have been reported from taxonomically diverse marine microorganisms. In Raymond Andersen's laboratory [48] at the University of British Columbia, eight novel cyclic depsipeptides (massetolides A–H) were found to be produced by two *Pseudomonas* strains obtained from a marine alga and a tube worm. The

producing strains were grown on solid agar and, in the case of the algal-derived strain, an ethyl acetate extract of the cells yielded massetolides A–D. Massetolides E–H were obtained from the ethyl acetate extract of the agar medium following the growth of the tube worm-derived strain. Massetolide A (**12**) exhibited potent antimicrobial activity against *M. tuberculosis* and *M. avium-intracellulare* with MIC ranges of 5–10 and 2.5–5 μg/ml, respectively, and thus may be of potential use for the treatment of tuberculosis.

(4) In an effort to discover new antibiotics from sponge-associated fungi, Höller et al. [97] reported isolating more than 500 fungal strains from sponges collected in six different locations including the Caribbean Sea and the waters around Helgoland, Germany. Active fungal extracts led to the isolation of the new compound microsphaeropsisin (**13**) from *Microsphaeropsis* sp. and two new compounds (**14**, **15**) from a *Coniothyrium* sp. Compounds **13** and **14** possessed moderate antimicrobial activity while **15** decomposed prior to testing. Given the increasing number of new metabolites reported from sponge-associated fungi (table 2), it would be interesting to learn more about the occurrence and distributions of fungi in sponge tissues. Such information would be especially useful given that sponges feed by filtering seawater and it is possible that fungal spores accumulate in sponge tissues where they remain dormant until retrieved and induced to grow on a suitable microbiological medium. Answers to these questions are critical to our understanding of fungi in the ocean and the extent to which the compounds they produce represent specific marine adaptations.

The Discovery of New Antiviral Agents (fig. 3)

The emergence of drug-resistant viral pathogens and the continued spread of viruses such as HIV have created pressing needs for the development of new and more effective antiviral drugs. Adding to this urgency is the rapid increase of opportunistic viral infections in immunocompromised patients where, as in the case of AIDS, viruses such as herpes simplex are now a leading cause of viral morbidity and mortality. Even in the general population, the prevalence of herpes simplex type II infection has increased by 30% since the 1970s and is now detectable in roughly 1 of 5 persons 12 years or older in the United States [118]. The recent breakthroughs in developing HIV protease and reverse transcriptase inhibitors have increased acceptance that viral disease can be successfully treated with chemotherapy. Yet, despite these successes, there are few adequate treatments for many of today's most problematic viral infections and new drugs with novel modes of action are desperately needed to help overcome emerging drug resistance.

Although few marine microbial metabolites have been reported as possessing antiviral activity, this may well reflect a lack of focused screening in

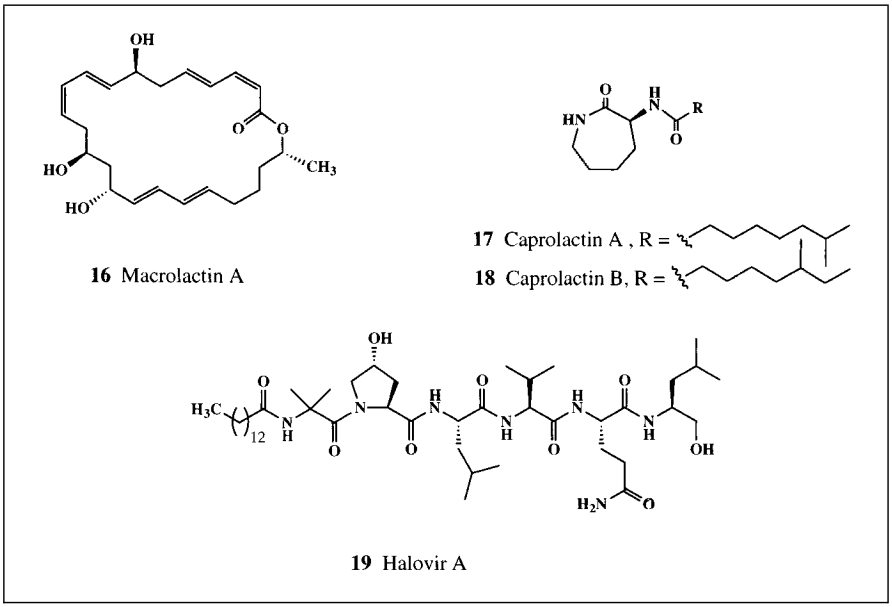


Fig. 3. Antiviral agents from marine microorganisms.

this disease area. Three antiviral compounds of marine microbial origin are discussed below:

(1) A Gram-positive deep-sea bacterium that had a requirement of salt for growth was isolated from a sediment sample obtained from −1,000 m along the California coast. Fermentation of this slow-growing bacterium yielded a series of novel cytotoxic and antiviral macrolides, macrolactins A–F [64]. The major metabolite, macrolactin A (**16**) was active against B16–F10 murine melanoma with an IC_{50} value of 3.5 μg/ml. More importantly, macrolactin A inhibited several viruses including herpes simplex ($IC_{50} = 5.0$ μg/ml) and human immunodeficiency virus, HIV ($IC_{50} = 10$ μg/ml).

(2) Caprolactins A (**17**) and B (**18**) were also isolated from a Gram-positive bacterium obtained from a deep-sea sediment sample [65]. These compounds showed moderate cytotoxicity and activity against herpes simplex type II. The caprolactins contain a rare cyclized lysine moiety that had previously been reported from marine sponges. This observation suggests that, as already discussed for cyrindramide, some sponge metabolites may be of bacterial origin.

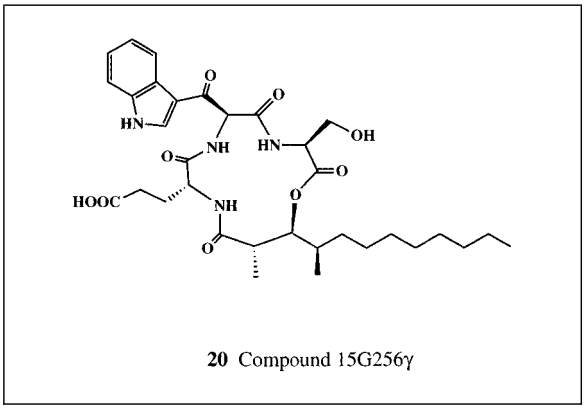
(3) We recently isolated a series of novel linear peptides from a marine fungus identified as a *Scytalidium* sp. [103]. These peptides, halovirs A–C,

are distantly related to a class of molecules known as peptaibols that are characterized by N-terminal acetylation, a reduced C-terminus, and a high proportion (20–50%) of amino isobutyric acid. Halovir A (**19**) is a modified peptaibol in that the N-terminus is substituted with a 14 carbon fatty acid side chain. This molecule shows potent antiviral activity against herpes simplex virus (type I) with an IC_{50} of 1.1 μg/ml. More importantly, a 30-min exposure to 0.6 μ*M* halovir A completely inhibits HSV-I-induced cell lysis, suggesting that the compound binds directly to the virus rendering it incapable of entering the cell. Thus, halovir A shows particular promise as a topical antiviral agent.

The Discovery of New Antifungal Agents (fig. 4)

During the last two decades, the frequency of life-threatening fungal infections has increased dramatically due in large part to the growing numbers of immunocompromised and severely ill patients. In one study, 84% of HIV-infected patients had oropharyngeal colonization by *Candida* sp. while 55% developed clinical thrush [119]. Although new antifungal drugs are aggressively being sought, the drug discovery process has been slow and, even for the newer azole drugs such as fluconazole, an alarming number of resistant strains are being isolated. A major reason why antifungal agents have proven so difficult to develop is that fungi, owing to their eukaryotic nature, share many characteristics in common with their human hosts. One rational approach to resolving this dilemma is to target fungal-specific biochemical or molecular features such as the cell wall. Although few marine microbial compounds have been reported specifically as antifungal agents, one cell-wall inhibitor is discussed below:

(1) The natural products research group at Wyeth-Ayerst has initiated a significant program to explore the biomedical potential of marine microorganisms. As a result of these efforts, the antifungal agent 15G256γ (**20**), which inhibits fungal cell wall synthesis and belongs to a new class of lipodepsipeptides, was recently reported from the marine fungus *Hypoxylon oceanicum* [105]. A number of interesting observations were made in this study that warrant discussion. First, two compounds previously described from terrestrial fungi (*Penicillium verruculosum* and *Scedosporium apiospermum*) were also isolated from *H. oceanicum*. This finding supports the concepts that secondary metabolite production is not species-specific and that marine and terrestrial species do not always produce distinct metabolites. The fact that diverse genera produce the same metabolites suggests that these compounds provide a selective survival advantage and that the biosynthetic pathways responsible for their production developed early in fungal evolution and were conserved as species diverged. It is also noteworthy in this case that compound yield increased over 100-fold by omitting seawater from the fermentation medium.



20 Compound 15G256γ

Fig. 4. Antifungal agents from marine microorganisms.

This observation suggests that a maximized compound titer is not required for any potential ecological effects that this antifungal metabolite may have in the marine environment. Given that *H. oceanicum* is an obligate marine species yet does not require seawater for growth suggests that some factor other then seawater limits its distribution.

The Discovery of New Anti-Inflammatory Agents (fig. 5)

The symptoms of many chronic diseases include inflammation and pain. The occurrence of some of these diseases, such as osteoarthritis, which afflicts 1 in 3 Americans over 60 years of age, continues to rise as life expectancies increase. Others, including the autoimmune diseases rheumatoid arthritis and inflammatory bowel syndrome, remain serious problems that are difficult to treat with current medicines. Because much of the discomfort associated with inflammatory diseases is the result of the inflammation process itself, there have been aggressive efforts to search for new and more effective anti-inflammatory drugs. Although anti-inflammatory activities have been reported for only a few marine microbial metabolites, the numbers could increase dramatically if this were to become a more common target. Two compounds with activities relevant to inflammation are discussed below.

(1) A series of unique depsipeptides, salinamides A and B, were reported from an actinomycete isolated from the surface of the jelly fish *Cassiopeia xamancha*. The structure of salinamide A (**21**) was determined by spectral methods while that of the crystalline, chlorinated isomer, salinamide B (**22**) was resolved by X-ray methods [66, 67]. These compounds were originally isolated based on activities against the Gram-positive bacterial pathogens *Strepto-*

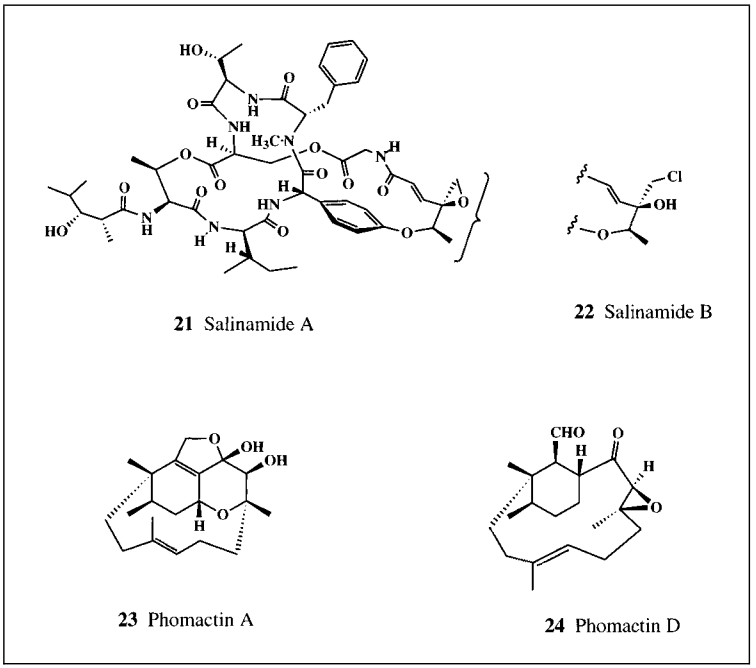
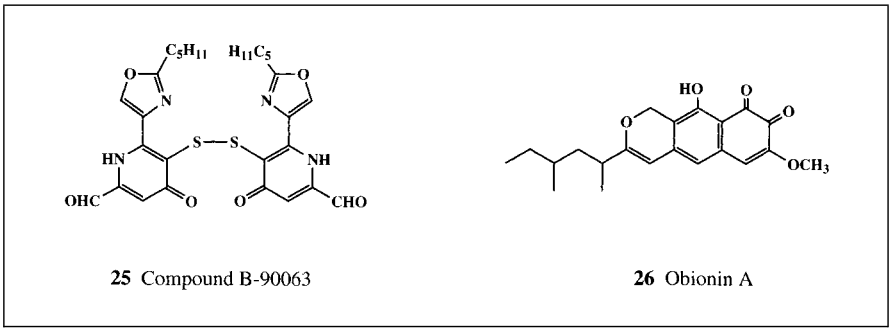


Fig. 5. Anti-inflammatory agents from marine microorganisms.

coccus pneumoniae and *Staphylococcus pyrogenes* (MIC values of 2–4 μg/ml). Subsequently, salinamides A and B were shown to have potent in vitro anti-inflammatory activities in a phorbol-ester-induced mouse ear edema assay, inhibiting inflammation by $>80\%$ at a dose of 50 μg/ear.

(2) Sugano et al. [109] screened lipophilic extracts of marine fungi for the inhibition of PAF (platelet-activating factor) receptor binding and PAF-induced platelet aggregation. These studies led to the isolation of phomactin A (**23**) from a *Phoma* sp. obtained from a crab shell. Subsequent large-scale fermentation (600 liters) followed by ethyl acetate extraction led to the isolation of the additional diterpenes phomactins B–G [110, 111]. Within this series, phomactin D (**24**) was the most active, inhibiting the binding of PAF to its receptors and PAF-induced platelet aggregation with IC_{50} values of 0.12 and 0.8 μ*M*, respectively. The structure-activity relationship of these compounds suggests that the conformation of the bicyclic ring system and the hydroxy group substitution patterns have significant effects on activity [110]. It is also interesting to note that sporulation of this *Phoma* sp. was only observed on a seawater-based medium although growth and metabolite production were observed in the absence of seawater.



25 Compound B-90063

26 Obionin A

Fig. 6. Enzyme inhibitors and agents targetting other therapeutic areas.

Other Therapeutic Targets (fig. 6)

Although the majority of biomedically relevant marine microbial metabolites reported to date show activities against cancer or infectious diseases, a growing number of molecules have been shown to act on more diverse biochemical targets including specific enzymes. Enzyme inhibitors have been reported from both marine fungi and bacteria (tables 1, 2) and one such compound is described below. In addition, a compound with activity in a central nervous system (CNS) screen is presented.

(1) From the research laboratories at Sankyo Co., Ltd in Japan, a novel endothelin-converting enzyme (ECE) inhibitor was isolated from a new marine species of the genus *Blastobacter* [69]. The producing strain was isolated from seawater and required seawater for growth, suggesting it is highly adapted to marine conditions. The compound (**25**) possessed the structurally unique 4-pyridone and oxazole skeletons and inhibited ECE with an IC_{50} of 1.0 μM, nearly equivalent to the naturally occurring ECE inhibitor phosphoramidon which has been shown to be effective in treating myocardial ischemia, hypertension, and renal failure in animal models.

(2) Jim Gloer's laboratory at the University of Iowa performed some of the earliest chemical studies of marine and estuarine fungi. This group reported the structure and biological activity of the new polyketide obionin A (**26**) from the obligate marine fungus *Leptosphaeria obiones* [100]. The producing strain, a halotolerant ascomycete, was isolated from the coastal march grass *Spartina alterniflora* where it is commonly found in the lower portion of the plant. In a screen for CNS activity, obionin A inhibited ligand binding to a dopamine-selective receptor with an IC_{50} of 2.5 μg/ml. It was reported that further studies of the biological activity of obionin A (**26**) and other *L. obionin* metabolites are under way.

Future Biomedical Potential

For the biomedical potential of marine microorganisms to be realized, it is critical that the full extent of marine microbial diversity be examined. It is clear that marine fungi and actinomycetes, as was the case with their terrestrial counterparts, represent a productive source of new metabolites. But what about other taxonomic groups, particularly those found exclusively in the sea? Certainly for the next millennium, unexplored, obligate marine taxa will become an important focus of study. For example, we now know that myxobacteria can be isolated from marine samples [120]. Given the historical significance of terrestrial myxobacteria as a source of novel secondary metabolites [121], marine representatives certainly warrant study. And what about the diverse microbes inhabiting the deep sea? This ecological group clearly has not yet been examined in sufficient detail. It is also evident that new cultivation methods must be developed as many marine microorganisms are not amenable to traditional yeast extract/peptone media. Marine microbial diversity represents one of the last great resources for natural product drug discovery and the development of new culture techniques and creative isolation methods will help ensure that the potential of this resource is realized.

It is worth spending a moment to again mention the fact that chemical studies of marine microorganisms will inevitably yield secondary metabolites that were previously described from terrestrial strains. This observation can, in part, be explained by the fact that diverse microbial taxa have been shown to produce the same or similar secondary metabolites. For example, in our laboratory, we recently isolated beauvericin from three different species of obligate marine fungi. Even though these species have never been reported from land, they share similar biosynthetic pathways with *Beauveria bassiana* the original, terrestrial source of this compound [122]. Although marine strains will continue to yield previously described compounds, it is our opinion that, relative to terrestrial strains, they also yield a significantly improved ratio of new to known compounds. And the greater the degree of adaptation to specific marine conditions, the more favorable this ratio becomes. For this reason, it seems logical to pursue chemical studies of highly adapted microorganisms such as symbionts or those inhabiting extreme environments such as the deep sea. Adaptations to these environments, even if found in species that are not obligately marine, will continue to yield strains with the capacity to produce secondary metabolites that are new to science.

It is also worth pointing out that traditional methods for microbial identification may not adequately delineate marine species. For example, in the case of fungi, the morphological characteristics used to assign species names may mask significant biochemical differences among strains. Marine strains may

produce unusual secondary metabolites as an environmental adaptation yet these adaptations may not affect the taxonomic markers, such as spore morphology, used for classification. Certainly, we have isolated strains with unique biosynthetic capabilities that when identified using traditional methods are classified as terrestrial species. Do these assignments adequately describe marine strains and their environmental adaptations? We believe they do not, however more research is needed before definitive answers to these questions can be obtained.

Although new, biomedically active compounds are being isolated from marine microorganisms, the potential for any one of these metabolites to reach the clinic is very much dependant upon the aggressiveness with which they are tested in diverse disease areas. In the academic setting, where the majority of marine microbial metabolites have been discovered, assays are generally selected based upon the source of the research funding or availability through collaborators. In industry, screen selection is obviously more focused but still may not encompass the full spectrum of assays available within different departments or disease programs. Regardless of selection criteria, many of the compounds being isolated from marine bacteria and fungi are not being screened effectively. This is disappointing given the effort required to obtain marine cultures and isolate new compounds, and the genuine need for new drugs. Encouraging greater collaboration between academic and industrial researchers will help ensure that the drug potential of marine microbial metabolites is better realized.

The biomedically relevant secondary metabolites reported to date from marine microorganisms represent diverse structure classes that include terpenes, peptides, polyketides, and compounds of mixed biosynthetic origin. The producing strains range from obligate marine bacteria such as *Flavobacterium ulginosum* to ubiquitous fungal genera such as *Penicillium*. The strains isolated have been obtained from diverse substrates and clearly represent only a small portion of the microbial diversity available for chemical study. How this field develops in the future will depend upon many factors including the number of academic research groups working in this area and the willingness of industry to invest in a new avenue of natural product research. The later is particularly important given the tremendous amounts of time and money required to bring a new drug to market. For many lead compounds, clinical utility is not readily apparent and, as exemplified by taxol, years of development can be required before medicinal utility is fully realized. A strong industrial push will be needed to bring marine microbial products to the clinic and this, by necessity, will require access to the most sophisticated in vivo models and the development of large-scale saline fermentation techniques and facilities. Hopefully, the clinical potential of marine microbial compounds will spark

sufficient industrial interest to enable new drug leads to be thoroughly evaluated. If this occurs, it will only be a matter of time before marine microbial-based pharmaceuticals join the current arsenal of useful medicines.

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Microalgae as a Drug Source

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Introduction

Recently, microalgal metabolites are attracting enormous attention, and the topic has been discussed by a number of authors. This author also reviewed the chemical and biochemical aspects of microalgal metabolites [1–3]. Therefore, emphasis in this chapter will be placed on some issues concerned with drug screening.

There are a couple of reasons behind the surge of interest in microagal metabolites. First, the traditional drug sources such as terrestrial higher plants and Actinomycetes have been examined very extensively for a length of time, and the screening works are suffering from a high rate of redundancy in both structural type and mechanism of pharmacological action. Here, the microalgal phyla have been recognized to provide chemical and pharmacological novelty and diversity. Another reason for the enthusiasm is the recognition that microalgae are the actual producers of some highly bioactive compounds found in marine invertebrates.

Marine organisms have been looked at as a very promising source of therapeutic drugs. In 1967, the first conference of drugs from the sea was convened at University of Rhode Island [4]. However, more than three decades later, no significant therapeutic drugs have been derived from marine organisms, while several thousand new chemical structures have been discovered from marine organisms [5]. This does not signify, however, that the marine compounds are barren with respect to pharmacological activity. On the contrary, there are strong indications that many of them possess novel bioactivity. The major obstacle to the drug development of marine natural products is the lack of sufficient material for comprehensive pharmacological evaluation. More often than not, compounds are isolated in milligram quantities, and

their structures are elucidated and published. This probably means that chances for the compounds to be developed to drugs will be lost for good. No serious enterprise will invest hundreds of millions of dollars for a compound without proper patent protection.

Invertebrates such as sponges, tunicates and soft corals, have been the richest sources of new compounds. With some exceptions, very limited numbers of organisms can be collected for chemical studies. Recollections of the organisms are often impossible or ecologically undesirable, and the procurement of enough material for wide-spectrum assays, not to mention the preclinical or clinical studies, is almost prohibitive. In the case of compounds such as bryostatins [6] or halichondrins [7], the source organisms are relatively abundant, but their low contents (~ppb) limit the advanced clinical studies. For those compounds having complex structures, total synthesis is not practical for a procurement purpose. Thus, without a reproducible resource to supply the compounds, it is impossible to move the compound forward for actual therapeutic use.

The true origins of compounds found in marine invertebrates have been a subject of constant discussion. They may vary case by case, but there are strong hints that dietary or symbiotic algae are one of the participants in the primary production of the metabolites. For example, as early as 1977, the blue-green alga, *Lyngbya majuscula* was recognized as the source of aplysiatoxin found in the sea slug *Aplysia* [8]. Similarly, a series of highly active antitumor compounds, dollastatins, isolated from sea slugs by Pettit's group are considered to be of blue-green algal origin. In fact, a close analogue of dolastatin 10 isolated from *Dollabella auricularia* was found to be a metabolite of a blue-green alga (fig. 1) [9]. While the aforementioned compounds are probably introduced into the invertebrates by the food chain, some others may have their origins in the symbiotic organisms. The antitumor, antiviral cyclic depsipeptides, didemnins [10], have been suspected to be produced by photosynthetic prokaryotes, prochlorons, which are symbionts in most *Didemnum* tunicates [11], although no proof for the production has been presented because of the unculturability of the organism. The cytotoxic sponge metabolites, swinholide and analogues, have close relatives such as tolytoxin in blue-green algae [12].

In the eukaryotic algae, various dinoflagellate metabolites are found in shellfish and other invertebrates as toxins. Well-known examples are paralytic shellfish toxins, brevetoxins, ciguatoxins, and dinophysistoxins [13]. From the sponge *Halichondria okadaii*, potent antitumor compounds, halichondrins, have been isolated [7]. The fact that the same sponge also contains okadaic acid, a known dinoflagellate metabolite, suggests that halichondrins with similar polyoxygenated alkyl structures are also of dinoflagellate origin [14, 15].

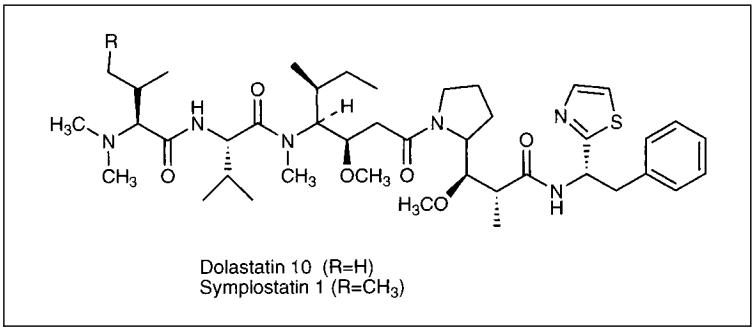

Fig. 1. Some of the highly active anticancer compounds found in seahares have their origins in the dietary blue-greens. Symplostatin, a close analogue of dolastatin 10, is found in a marine cyanobacterium *Symploca hydnoides*.

All these observations led to a high hope to produce the compounds by culturing microalgae.

Biodiversity and Chemical Diversity of Microalgae

Microalgae play the major role in the primary production in the oceans. They also play many important functions in the complex marine ecosystems such as coral reefs. Microalgae are usually found in seven divisions or phyla: Chlorophyta, Phaeophyta, Rhodophyta, Chrysophyta, Pyrrhophyta, Euglenophyta and Cryptophyta, which are further divided into classes and families. There are always disagreements among the taxonomists, and synonyms and assignments of different families and genera are quite common. However, there are two issues which need special explanations. One is the treatment of Cyanophyta or blue-green algae. They are prokaryotes and, in a strict sense, bacteria (cyanobacteria). However, physiologically and ecologically, these photosynthetic organisms share many similarities with the eukaryotic algae. Also, the isolation and culturing techniques for study are basically the same. Therefore, many researchers still treat blue-greens as microalgae, and this author also includes them in this chapter. Another aspect of microalgae which needs special attention is the ambiguity regarding the animal and plant distinction. Plants are characterized by their ability to do photosynthesis. However, in microalgae in the lower order of the phylogenic tree, this distinction of presence or absence of photosynthesis is not clear. Thus, a half of Pyrrhophyta (dinoflagellates) organisms are non-photosynthetic and heterotrophic. Even in the same genera or species, both photosynthetic and heterotrophic forms

exist. Phyla such as Pyrrhophyta and Euglenophyta are often classified as animals (protozoa). It is obvious at this point that the distinction between animals and plants is very artificial, and the term Proticista or Protoctista is more appropriate to cover these organisms [16].

There is no agreed number of existing microalgal species. Until recently, books cited an approximate number of 5,000. However, the number is grossly underestimated, and it is ever increasing. In addition, each species has many subspecies. There are a number of morphologically indistinguishable, but chemically different strains. If these chemical strains are brought into consideration, there are almost infinite possibilities with respect to metabolite production.

Some General Issues in Screening Microalgae

Screening of microalgae for bioactive compounds is basically the same as screening bacteria or fungi. However, the growth of algae is generally much, much slower than that of bacteria or fungi. The doubling time of most dinoflagellates in culture, for instance, is normally 3–4 days in comparison to 20 or 30 min in bacterial culture. In the physiological or ecological studies, cultures started from multiple cells from the same population are often used without problems. However, for chemical studies, the establishment of genetically uniform clonal cultures from a single cell (single cell isolates) is highly desirable, since the secondary metabolite profiles of individual cells vary even in the same population. After all, the production of secondary metabolites is not essential to the organisms, and it is a rather freak incident to them. For example, *Aphanizomenon flosaquae* is a ubiquitous blue-green alga, and most of them do not produce any secondary metabolites. However, a few strains are known to produce interesting metabolites, although they are morphologically identical with the rest of the strains. The possible genetic background of this chemical diversity was discussed before [3]. The same observation has been made with dinoflagellates. *Alexandrium* spp., for example, are known to produce paralytic shellfish toxins, but their toxin productivity differs tremendously from strain to strain even from a close proximity [17]. *Therefore, it is very dangerous to predetermine the potential of an organism solely based on the species name.*

Culturability of organisms is the most critical issue. For example, only a handful number of dinoflagellates are known to be cultured. *Dinophysis* spp., which are known to produce interesting compounds [18], have been resisting enormous efforts by many groups.

Obtaining enough biomass to obtain extracts for various assays is also a big problem with most of microalgae. Algae such as blue-greens and diatoms

are relatively easy to culture, but dinoflagellates are very difficult to grow en masse. A minimum amount of biomass needed for preliminary multiple assays using a 96-well plate is about 2 g of wet cells, which requires a minimum of about 10 liters of culture.

Maintenance of strains is also a problem with microalgal cultures. Preservation methods used for bacterial or fungal strains are not applicable to microalgae except blue-greens. Normally, reinoculation is required as often as once a month. A combination of these problems makes the screening of microalgae far more difficult and time-consuming than that of bacteria or fungi.

To date, Cyanophyta has shown the most chemical diversity. Dinoflagellates come next. Only limited information is available with the other phyla of microalgae. This may change in the future, because Cyanophyta is the most studied because of their relative easiness to isolate and culture. In the following, the individual phyla of organisms will be discussed.

Cyanophyta (Blue-Green Algae)

Drug screening of blue-green algae has been discussed by several authors including Moore et al. [19] and Gerwick et al. [20]. Blue-green algae are found widely in various environments. They appear in different morphologies: filamentous, unicellular, colonial, etc. The isolation of blue-green algae is very much like the isolation of fungi and bacteria; selection of colonies form on agar plates. Many of them are able to fix molecular nitrogen, and one of the standard isolation methods for these organisms is to use nitrogen-meager media (e.g. BG-11_0) [21] to discriminate other photosynthetic algae. Blue-greens are found not only in aquatic samples, but in soils, animals, macroalgae, higher plants, rock surface, etc. In particular, soil and mud samples have proved to be productive. For screening purposes, it is advisable to grow in more than one medium, because the productivity of secondary metabolites is greatly influenced by the nature of medium. The optimum growth does not mean the optimum condition for the biosynthesis of secondary metabolites.

Blue-greens have very rich chemistry [19, 20, 22]. The chemical diversity and novelty seen in blue-greens are comparable to those of Actinomycetes, which have turned out many important drugs. It is not unusual that a single species produces many different chemotypes. *L. majuscula* is a good example. The diversity of structures found in this ubiquitous filamentous alga is just incredible. The structures of representative compounds from individual chemotypes are shown below (fig. 2). Most of them possess characteristic biological

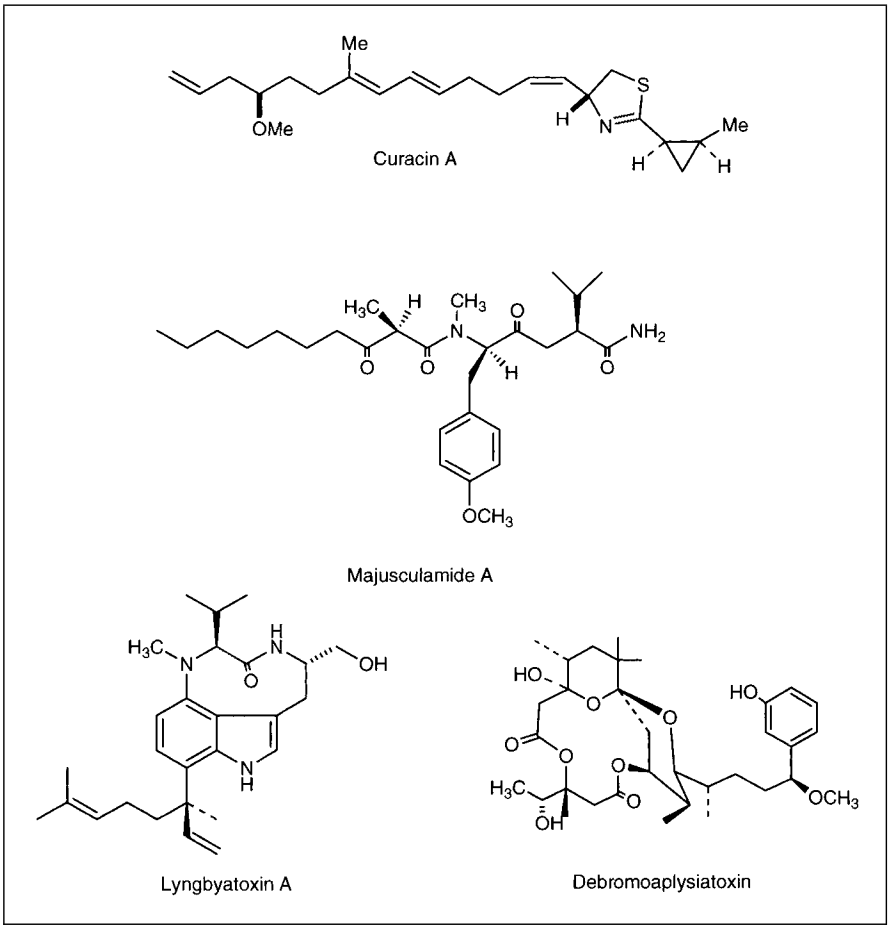

Fig. 2. Variant structural types found in an identical species of blue-green alga, *Lyngbya majuscula*.

activity. Curacin A isolated from a Curasao strain by Gerwick's group [23], for example, is recognized as a new pharmacophore to perturb microtubule assembly system. With a rather simple structure, it is an important lead compound for new types of cancer drugs.

Among many groups screening blue-green algae, Moore-Patterson's group [19, 20, 22] in Hawaii have done the most pioneering work and isolated many new compounds. In their work, species belonging to families such as Nostocaceae, Scytonemataceae and Oscillatoriaceae seem to be particularly rich in secondary metabolites. They include a variety of compounds: alkaloids

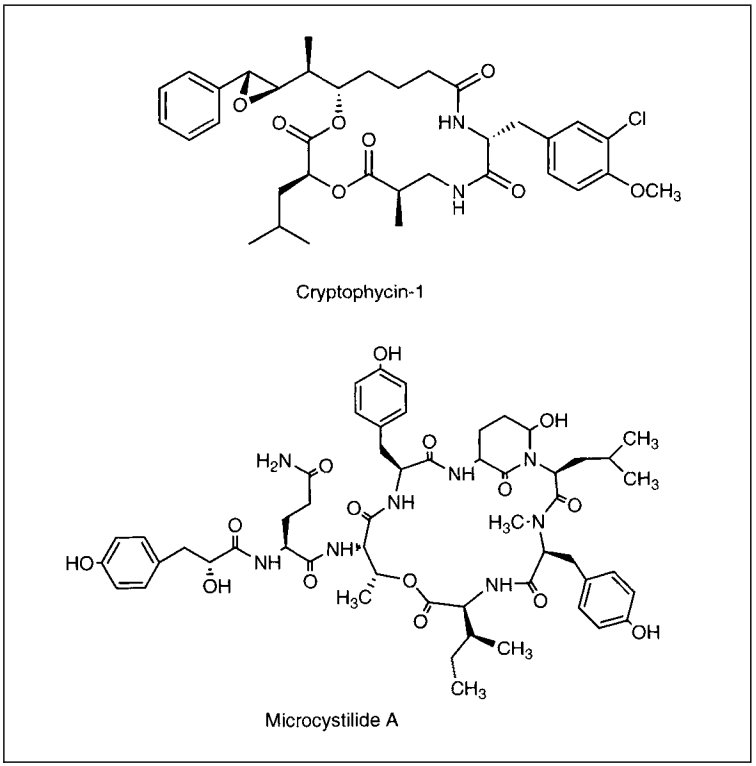

Fig. 3. Examples of interesting cyclic depsipeptides from blue-green algae. Cryptophycin is a microtubule depolymerizing compound, and microcystin A is a cell-differentiation promotor.

(e.g. lyngbyatoxin A), polyketides (e.g. tolytoxin), cyclic peptide (e.g. microcystin), depsipeptides (e.g. majusculamide), thiazole or oxazole derivatives (e.g. tantazole), etc. Again, many of these compounds demonstrated versatile biological activity. One of them, cryptophycin-1 from *Nostoc* sp., was a compound isolated by the Merck group as a fungicide. The compound was rediscovered by Moore's group [24] as a microtubule depolymerizing agent. The compound and its analogues are very effective against solid tumors (fig. 3).

Various enzyme inhibitors have been discovered in blue-green algae. For example, microcystins from *Microcystis aeruginosa* are potent protein phosphatase-1 and -2A inhibitors [25], but they also produce other types of peptides and depsipeptides. One of them, microcystilide A isolated at the author's laboratory, is a promotor of cell differentiation [26].

Pyrrhophyta (Dinoflagellates)

Dinoflagellates were first recognized as the causative organisms of paralytic shellfish poisons. Now it is known that dinoflagellates are the source organisms of several major marine toxins and produce very unique compounds not seen in organisms of other phyla. Most of them have also demonstrated very potent biological activity, which is sometimes associated with the toxicity.

Dinoflagellates are rather primitive eukaryotes. Their cells have large nuclei, which lack or have very little histone. They are placed between prokaryotes and eukaryotes, and sometimes called mesokaryotes [27]. This unique status to link between eukaryotes and prokaryotes makes the organisms very interesting with respect to secondary metabolite production.

Contrary to the general perception that dinoflagellates proliferate fast, they actually grow very slowly in culture. The normal doubling time in culture is usually 3–4 days and never exceeds 1 day. Many of them are difficult to put in sustaining culture. Many of them are heterotrophic or symbiotic, and only a handful of organisms found in nature have been cultured successfully. In the past 20 years, this author's laboratory has made about 1,000 new isolates of dinoflagellates for screening purposes, but the success rate of making cultures starting from a single wild cell has been about 20%. It takes a minimum of 6 months to scale up to a 10-liter culture from a single cell isolate. The metabolites seem to be both in the cells and culture media, but most past works have been done with the cells.

The structural types of dinoflagellate metabolites spread widely from heterocyclic compounds, polycyclic ethers, oxygenated polyketides, macrolides, etc. (fig. 4) [2, 28]. On the surface, these chemo-types seem to parallel those of blue-greens, but they are, in fact, very different except for saxitoxin derivatives. Polyketides of blue-greens, for example, are normal polyketides, but the dinoflagellate counterparts do not conform the polyketide rule of biosynthesis [1, 29]. The linear polycyclic ethers represented by brevetoxins are quite unique to dinoflagellates.

Many dinoflagellate metabolites show very conspicuous biological activity. The so-called PSP toxins such as saxitoxin, neosaxitoxin and gonyautoxins produced by *Alexandrium* and several other genera of dinoflagellates are highly selective sodium channel blockers [30]. On the other hand, brevetoxins produced by *Gymnodinium breve* are potent sodium channel activators. Another polycyclic ether, maitotoxin from *Gambierdiscus toxicus*, is a rare natural calcium channel activator [28].

A number of compounds are known to act on the signal transduction system in the cell. Okadaic acid and its derivatives found in *Prorocentrum* spp.

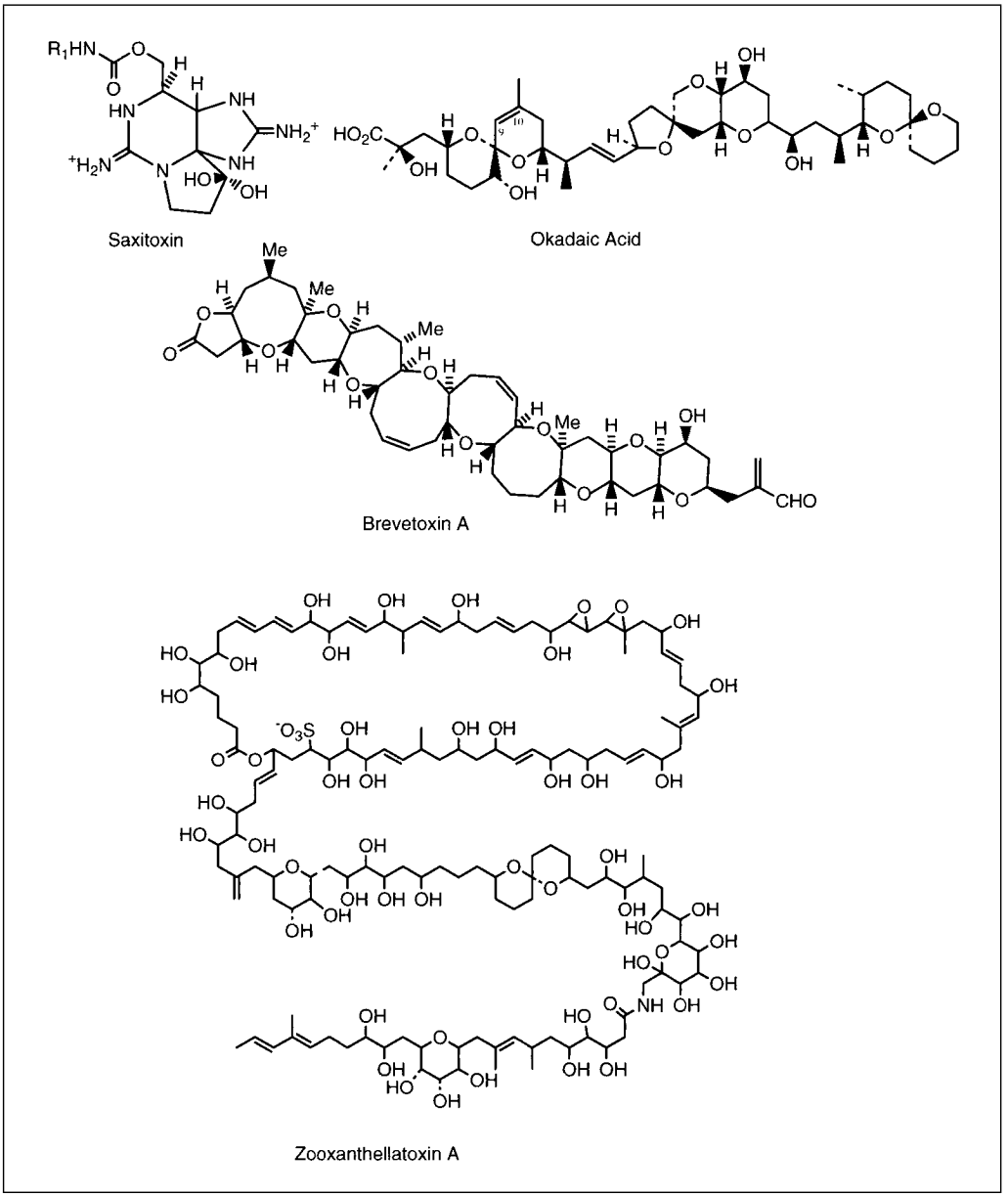

Fig. 4. Different structure types produced by dinoflagellates. Most of them are recognized as toxins and highly receptor-specific.

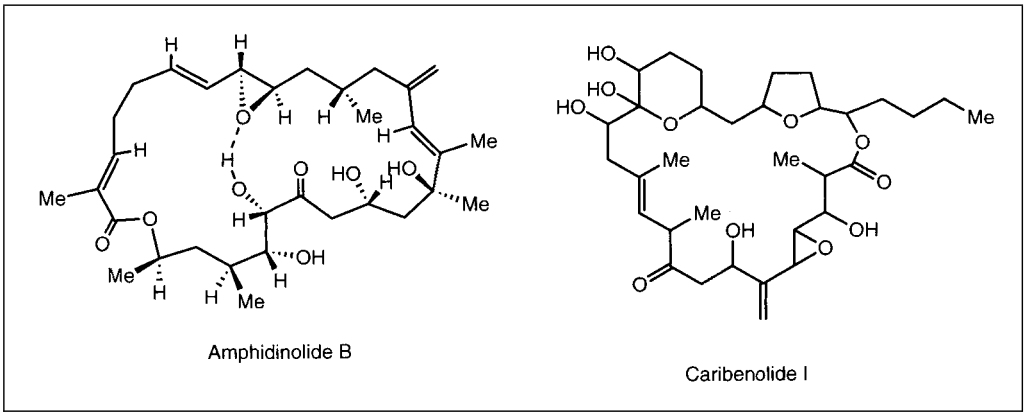

Fig. 5. Cytotoxic and antitumor macrolides represented by amhidinolide B and caribenolide I from the dinoflagellates *Amphidinium* spp.

and *Dinophysis* spp. are very potent inhibitors of serine/threonine-specific protein phosphatase 1 and 2A [31].

Kobayashi and Ishibashi [32] first reported that a symbiotic *Amphidinium* sp. from a flatworm contains a series of macrolides, amphidinolides. Most of them are very cytotoxic. This author's group, screening free-swimming *Amphidinium* spp. from Caribbeans for antitumor agents, also isolated a series of compounds including amphidinolide B reported by Kobayashi's group [33]. One of the compounds, caribenolide I, was extremely cytotoxic (IC_{50} 1.6 n*M*) against HCT 116) and active in vivo [34] (fig. 5).

A characteristic feature of dinoflagellate metabolites is that many of them have strong antifungal activity. Goniodomin A from *Goniodoma* (= *Alexandrium*) sp. is a strong antifungal agent [35]. Yasumoto's group [28] discovered potent antifungal agents, gambieric acids, in the culture medium of *Gambierdiscus toxicus* and subsequently polyhydroxylated alkyl compounds, amphidinols, in some strains of *Amphidinium* spp. (fig. 6).

Another noticeable structural feature seen in dinoflagellates is the occurrence of long-chain irregular polyketides with many oxygen functions and methyl groups. Biosynthetically, they may have the same origin as brevetoxin-type polycyclic ethers. They include the compounds with drastic biological activity like ostreopsins, close analogues of palytoxin [36]. The aforementioned amphidinols belong to the same category. Nakamura et al. [37] isolated vasoconstrictive compounds, zooxanthellatoxins from *Symbiodinium* sp. The compounds have a long alkyl chain and a lactone ring. The author's group found that related compounds are actually a cell wall constituent and widely distrib-

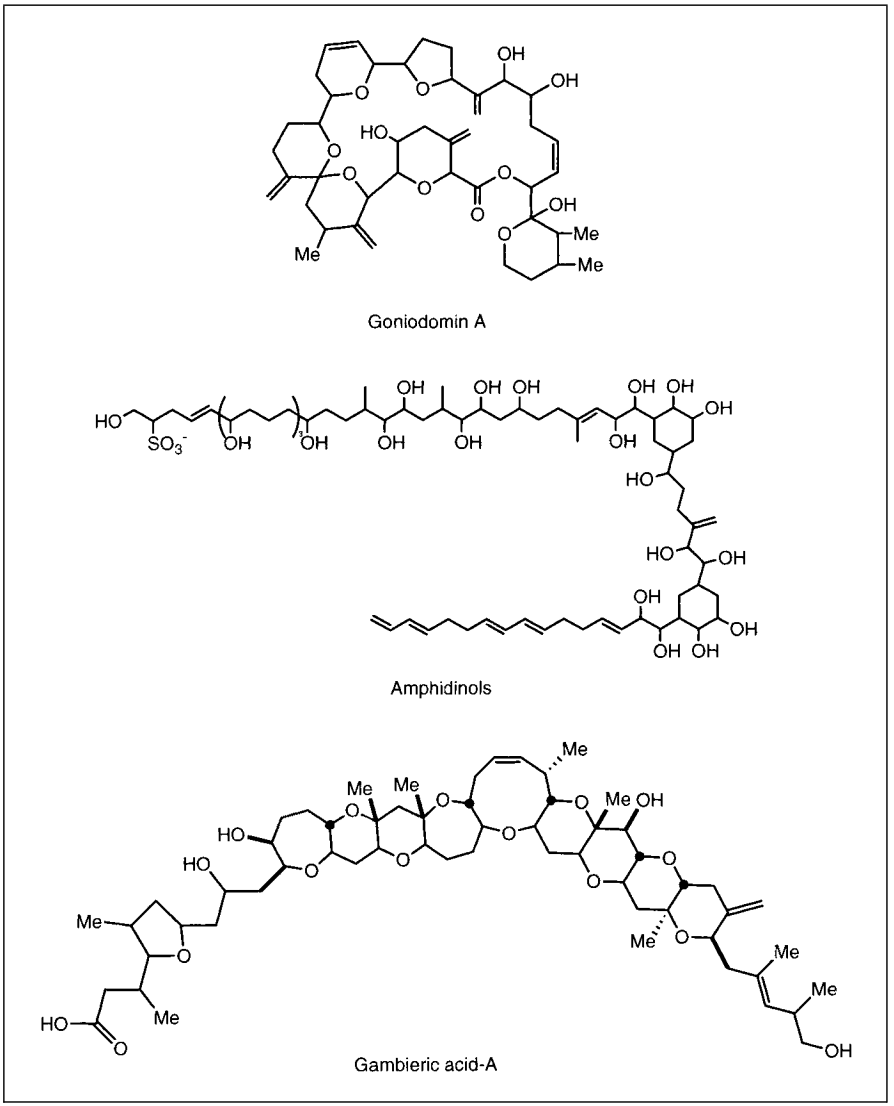

Fig. 6. Dinoflagellates produce potent antifungal agents.

uted in many strains of *Symbiodinium* spp. [Shimizu, unpubl. data]. The compounds are moderately cytotoxic, but not all of them have vasoconstrictive activity.

Probability to find bioactive compounds in dinoflagellates seems to be very high. In our program of screening for anticancer compounds, about 3%

Table 1. Approximate culture volumes estimated to procure 100 mg of representative bioactive metabolites of dinoflagellates

Organism	Metabolite	Liters of culture needed to obtain 100 mg
Alexandrium tamarense	Gonyautoxin-2, 3	700
Gymnodinium breve	Brevetoxin A	3,300
	Brevetoxin B	600
Gambierdiscus toxicus	Gambieric acid	893,000
	Maitotoxin	20,000
Amphidnium sp.	Amphidinolide B	25,000
	Caribenolide I	50,000

of the extracts passed the primary test using several human cancer cell lines. However, when it comes to actual drug screening or development, dinoflagellates have serious shortfalls. As mentioned earlier, the organisms are slow growing, and generally the biomass productivity is very low. Typically, a 10-liter culture, which requires an about 10% volume of inoculum, reaches the harvesting stage after more than a month. The biomass yield is about a few grams, which is equivalent to a few leaves of a higher plant. The contents of active metabolites are also not so encouraging. Table 1 shows approximate amounts of culture needed to obtain 100 mg of the compounds [38]. It is quite evident from the data that, unless the structures of the compounds are simple enough to be synthesized in reasonable yields, chances of the compounds becoming drugs are almost nil. Development of a new mass culturing method or dramatic increase in productivity by genetic manipulation will be required for real drug development.

In this respect, the author's group has recently tried a 15,000-liter tank for the culture of *Amphidinium* and other organisms rather successfully, but the productivity of the desired metabolites was not par with the small batch culture [39].

Bacillariophyceae (Diatoms)

Bacillariophyceae is a versatile and abundant family, which is probably the most important in the primary production in the oceans. Diatoms are

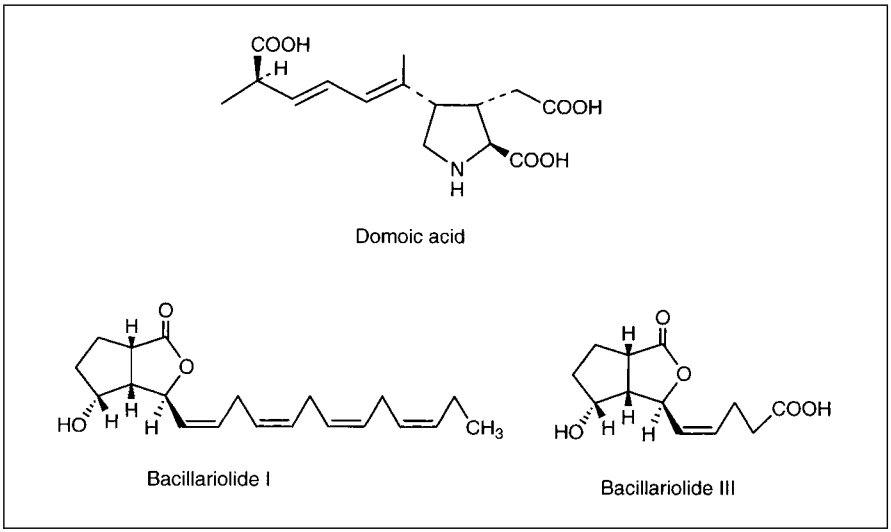

Fig. 7. Examples of bioactive compounds isolated from diatoms.

fast growing and relatively easy to culture on a large scale. However, unlike dinoflagellates, very few secondary metabolites have been reported from the diatoms. One of them, an excitatory amino acid, domoic acid, from *Pseudo-nitzschia multiseries* and some other species, is a harmful glutamate agonist, which causes amnesic shellfish poisoning (ASP) [40]. The same organism also produces a new type of cyclic eicosanoid bacillariolides [41, 42]. Bacillariolide I has inhibitory activity against phospholipase A_2 [3]. The compounds are formed from eicosapentaenoic acid through a mechanism different from prostaglandin biosynthesis. Bacillariolide III is an extracellular metabolite [43]. It was suggested that many of diatom metabolites might not be retained in the cells and excreted into the media [3]. If so, the past works which focused on the harvested cells may need reexamination (fig. 7).

Other Algal Families

A vast number of organisms which belong to such phyla as Chrysophyta (Chrysophyceae, Xanthophyceae, Prymnesiophyceae), Euglenophyta and Cryptophyta remain virtually unexplored. In many respects, they are close to the very productive dinoflagellates, and there is a good possibility to find new types of compounds and bioactivity in these algae. In fact, we can have a

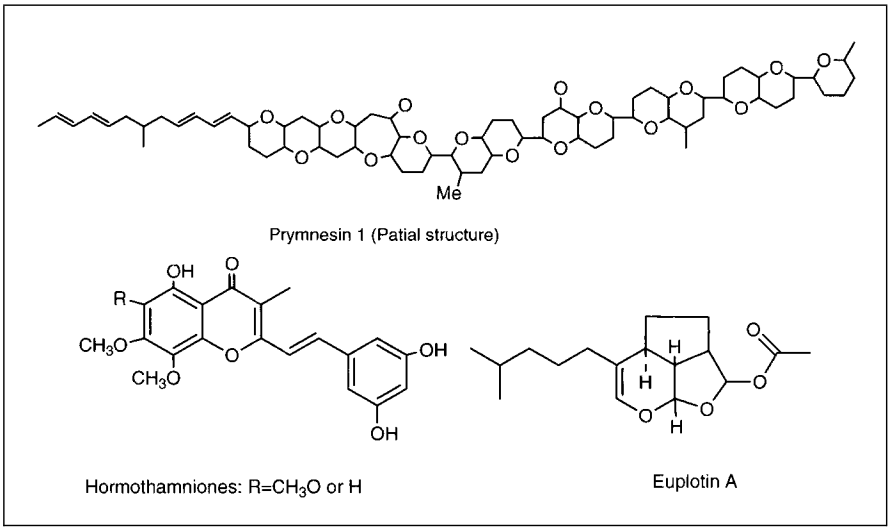

Fig. 8. Structure types found in Premnesiophyceae (Prymnesin), Cryptophyta (hormothamnione) and Ciliophora (euplotin).

glimpse of it in a few reported examples. Prymnesins, toxins produced by *Prymnesium* sp., have been shown to have polycyclic ether structures resembling those found in dinoflagellates by Yasumoto's group [44]. Gerwick's group [45] isolated highly cytotoxic styrylchromones called hormothamniones from the marine cryptophyte *Chrysophaeum taylosi*.

The ciliate *Euplotes crassus* produces euplotin, a sesquiterpene. Ciliates (Phylum, Ciliophora) are heterotrophic, but they are very close to Pyrrhophyta or Eugenophyta in the phylogenic tree [46] (fig. 8).

Conclusion

The data presented in the past 10 or 20 years clearly indicate that microalgae are a promising source of new secondary metabolites. Most isolated compounds have unprecedented structures and demonstrated highly selective pharmacological activity. While most of the past efforts have been made in the blue-greens, which are relatively easy to grow, most of other phyla have been scarcely touched. The unculturability, slow growth and poor productivity of secondary metabolites are discouraging factors. In order for these organisms to be qualified as a real drug resource, the development of new culture techniques and/or enhanced production by gene manipulation seems to be required.

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Search for Biologically Active Substances from Marine Sponges

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Introduction

Marine sponges (Porifera) are sessile invertebrates, which often lack of physical defense. Instead, they have evolved to develop chemical defense against predators and larval settlement of other sessile organisms. In fact, marine sponges are one of the richest sources of interesting chemicals among marine organisms.

In 1955, Bergmann and Burk [1] isolated unusual nucleosides named spongothymidine (arabinosyl thymidine) and spongouridine (arabinosyl uridine) from the Caribbean sponge *Cryptotethya crypta*. Further biological and synthetic studies led to the development of Ara-C (Cytarabin®, arabinosyl cytosine) as an anticancer drug for leukemia and Ara-A (Vidarabine®, arabinosyl adenine) as an antiviral drug. At present, Ara-A is supplied by culture of the terrestrial bacterium *Streptomyces antibioticus*.

In the 1970s, natural product chemists embarked on exploitation of bioactive compounds from marine invertebrates by use of antimicrobial or cytotoxic assays. Later, several pharmaceutical companies joined these races; they used more sophisticated assay systems including enzyme inhibition assays. Hitherto, several promising candidates for new pharmaceuticals have been discovered from marine sponges (fig. 1).

Manoalide [2], an antibacterial sesterterpene originally isolated from the Palauan *Luffariella variabilis*, was later found to be a potent inhibitor of phospholipase A_2 (PLA_2 from bee venom: IC_{50} 0.05 μM) and development of an anti-inflammatory drug of a new type was anticipated. A large number of analogues has been synthesized and tested for anti-inflammatory activity. However, they were dropped in preclinical stages. Manoalide is widely used

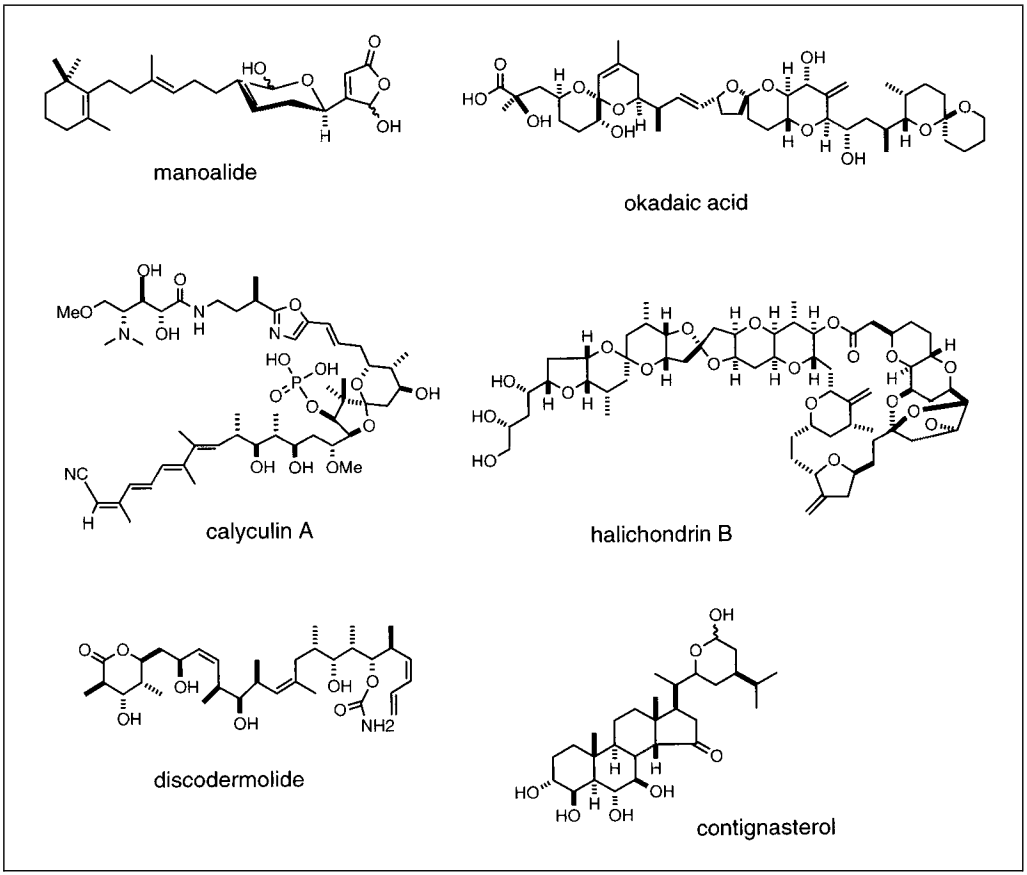


Fig. 1. Promising candidates for new pharmaceuticals from marine sponge.

as a pharmacological tool. Contignasterol [3], a highly oxygenated sterol isolated from *Petrosia contignata* collected in Papua New Guinea, exhibited anti-inflammatory activity. Again, numbers of its analogues have been tested, and a candidate is scheduled to enter clinical trials shortly.

Okadaic acid [4] and calyculin A [5], both cytotoxic polyketides from *Halichondria okadai* and *Discodermia calyx*, respectively, are selective inhibitors of protein phosphatase 1 and 2A and widely used as research tools in life science fields.

Halichondrin B [6], a metabolite of *H. okadai*, is a promising anticancer drug which has entered clinical trials in Canada (for details, see pp 134–153). Agelasphin isolated from *Agelas mauritiana* is a unique antitumor glyco-

sphingolipid [7], from which a new anticancer drug (KRN7000) has been developed and entered phase 1 trials in Europe (see pp 86–97).

Discodermolide [8] was first isolated as an immunosuppressive agent from deep water specimens of *Discodermia dissoluta*. Later, it was found to be more promising as an anticancer drug. Discodermolide stabilizes microtubules in a fashion similar to taxol [9]. It is in preclinical trials for taxol-resistant cancers.

In search of new drug leads from marine organisms, we have been studying bioactive metabolites of marine sponges [10]. Bioassay-guided separation using L1210 and KB cell lines led us to isolate several potent cytotoxins from marine sponges which were collected from Okinawan coral reefs and other Japanese waters.

Altohyrtins, Potent Cytotoxic Macrolides from *Hyrtios altum*

The acetone extract of *H. altum* (collected at Aragusuku Island, Okinawa, Japan) showed strong cytotoxic activity (IC_{50} 0.56 μg/ml) against KB cells. Bioassay-guided separation (cytotoxicity against KB and L1210 cells) of the ethyl acetate soluble portion provided an active fraction (5.8% based on the ethyl acetate soluble portion) [IC_{50} 0.002 μg/ml (KB)] which exhibited potent antitumor activity against P388 murine leukemia (T/C 155% at 10 mg/kg i.p. injection in mice on days 1 and 5). Repeated SiO_2 column chromatography and HPLC of this fraction furnished significantly cytotoxic macrolides named altohyrtins A (**1**), B (**2**), and C (**3**), and 5-desacetylaltohyrtin A (**4**) in yields of 3.4×10^{-3}, 2.2×10^{-4}, 2.2×10^{-4}, and 2.1×10^{-3}%, respectively on the basis of the AcOEt-soluble portion. **1**, **2**, **3** and **4** exhibited extremely potent cytotoxicity against KB (IC_{50} 0.01, 0.02, 0.4 and 0.3 ng/ml) and L1210 (IC_{50} 0.1, 0.03, 1.3 and 2.3 ng/ml) cell lines, respectively [11] (fig. 2).

On the other hand, Fusetani's group [12] isolated cinachyrolide A (**5**) from a marine sponge *Cinachyra* sp., while Pettit's [13] group reported spongistatins 1 (**6**) to 9 from two marine sponges *Spongia* sp. and *Spirastrella spinispirulifera*. These macrolides appeared to have the same carbon framework installed with two spiroketal moieties and one halogen atom. They were also reported to exhibit extremely potent cytotoxicity against cultured tumor cells. As for the mechanism of cytotoxicity of these macrolides, spongistatin 1 inhibited microtubule assembly by binding to the vinca alkaloid site of tubulin, which inhibits displacement of GDP bound in exchangeable site of tubulin.

Recently, Evans et al. [14] and Kishi et al. [15] succeeded in the total synthesis of altohyrtin C and altohyrtin A, respectively. These synthetic compounds were identical with natural altohyrtins, thereby confirming validity of the absolute stereostructure of altohyrtins proposed by us. We also isolated

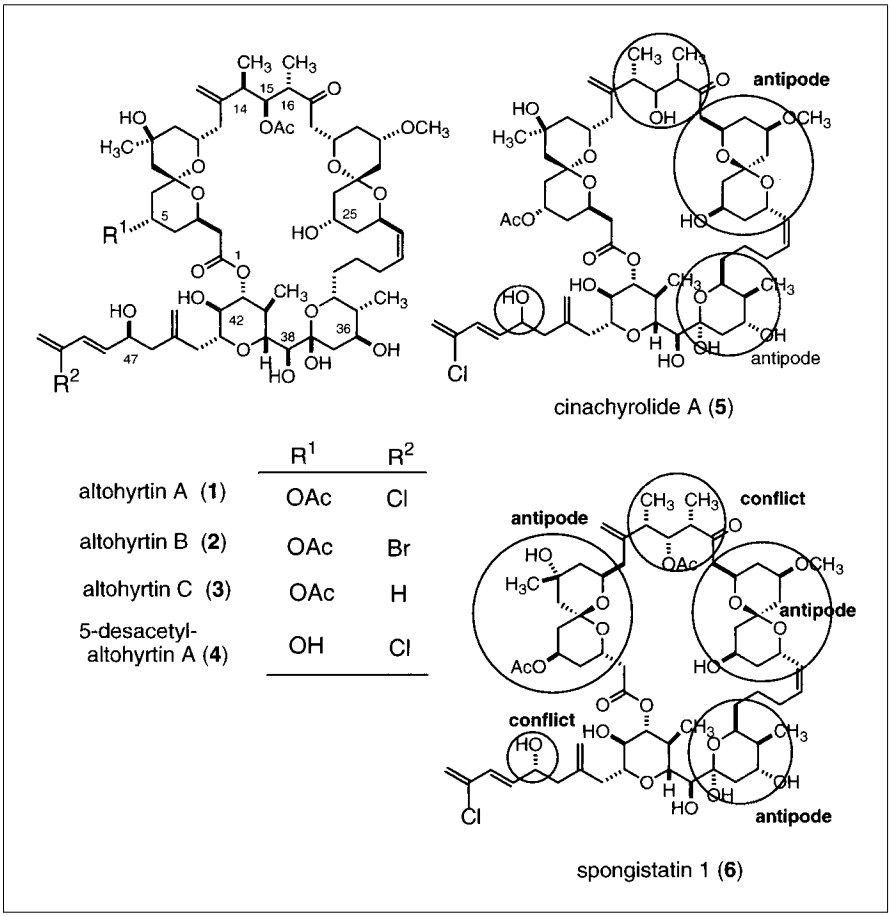


Fig. 2. Chemical structures of altohyrtins and the related macrolides.

altohyrtins A (**1**) and C (**3**) from *Haliclona* sp. which was collected off Amami Island, southwestern Japan. Thus, we presume that symbiotic microorganism(s) may be responsible for production of these macrolides.

Arenastatin A, an Extremely Potent Cytotoxic Depsipeptide from the *Dysidea arenaria*

The acetone extract of *D. arenaria* (collected off Iriomote Island of the Ryukyu Archipelago) exhibited cytotoxicity against KB cells with an IC_{50} value

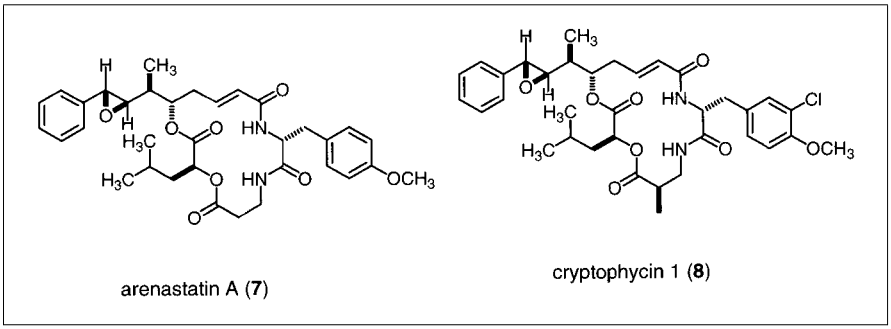


Fig. 3. Cytotoxic depsipeptides isolated from marine sponge and cyanobacteria.

of 3.7 μg/ml. Bioassay-guided separation of the ethyl acetate-soluble portion afforded a cytotoxic depsipeptide named arenastatin A (**7**) (1.4×10^{-3}% based on the AcOEt-soluble portion) which showed extremely potent cytotoxic activity against KB cell line with an IC50 value of 5 pg/ml (fig. 3) [16].

In 1990, the Merck group isolated an antifungal depsipeptide named cryptophycin from culture of a terrestrial cyanobacterium *Nostoc* sp. and proposed the plane structure [17]. Later, Moore's group [18] isolated cryptophycin 1 (**8**) and its analogues as cytotoxic constituents from *Nostoc* sp. The chemical structure of arenastatin A was very similar to that of cryptophycin 1 including absolute stereostructure; arenastatin A (**7**) was in fact identical with cryptophycin-24.

Since arenastatin A (**7**) was obtained in a very small amount, we attempted at the asymmetric total synthesis of **7** as well as of several stereoisomers as shown figure 4. Surprisingly, only arenastatin A showed extremely potent cytotoxic activity, while other stereoisomers [7,8-epimer (epoxy), 6-epimer, and *L*-tyrosines] were inactive at concentrations < 0.1 μg/ml except for the 15-epimer which was cytotoxic at 20 ng/ml. Thus, the natural-type stereochemistry at each asymmetric carbon in arenastatin A is essential to express the potent cytotoxic activity. Arenastatin A inhibits polymerization of tubulin at concentrations < 5 μ*M* [19], while the stereoisomers **9**, **10** and **11** were inactive at 10 μ*M*. Furthermore, rhizoxin was found to be a competitive inhibitor of arenastatin A, while vinblastine inhibited in a partial competitive manner. From Scatchard analysis, arenastatin A was found to bind tubulin at 1:1 ratio [19].

Arenastatin A showed antitumor activity against P388 murine leukemia only when administered intraperitoneally; intravenous injection of arenastatin A was not promising. On the contrary, cryptophycin-1 which possesses a methyl group at β-alanine part showed strong antitumor activity. Since arenastatin A has two ester linkages in the ring structure, we presumed that these two esters were easily metabolized into inactive compounds by enzymatic hydrolysis.

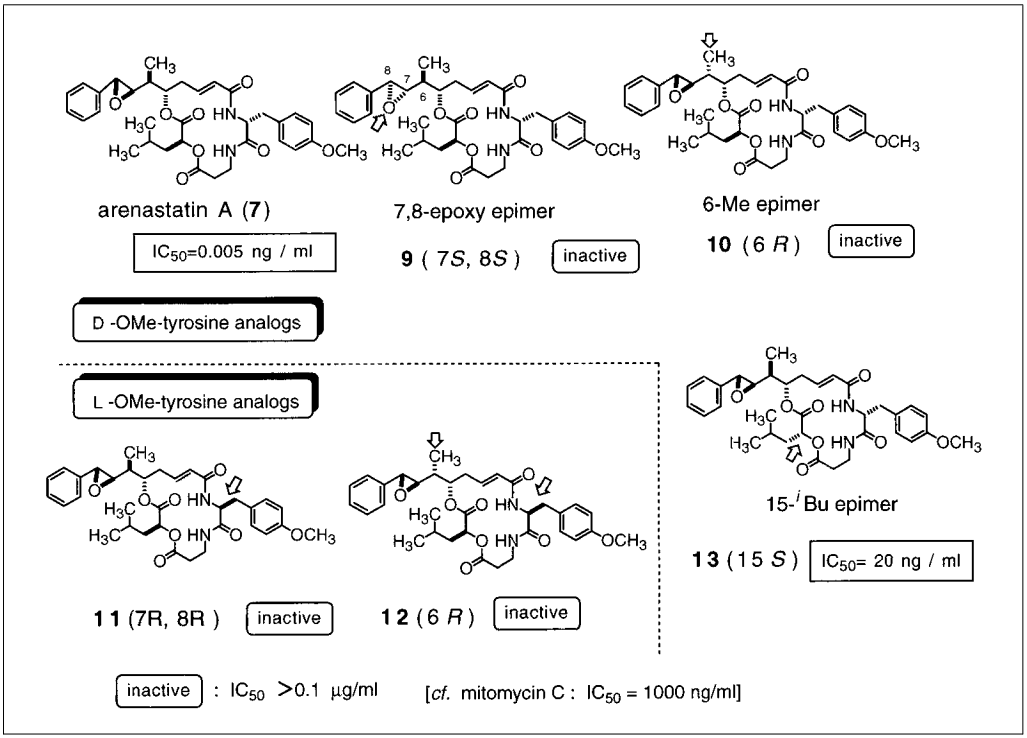


Fig. 4. Cytotoxic activities of arenastatin A and analogues against KB cells.

Thus, arenastatin A was incubated with mouse serum at 37 °C, which resulted in the disappearance of arenastatin A in 5 h. In order to obtain more evidence, we further synthesized three amide analogs; two esters were replaced by either one amide or two (fig. 5). The triamide analog-II (**15**) and tetraamide (**16**) were stable in the serum; only triamide-II (**15**) showed strong cytotoxic activity, even though 1,000 times weaker than arenastatin A. However, these three analogs were hardly soluble in water and other solvents.

Recently, the synthetic cryptophycin 1 (**8**) has been placed under phase 1 trials in the United States.

Callystatin A, an Extremely Potent Cytotoxic Polyketide from *Callyspongia truncata*

The acetone extract of *C. truncata* (collected off the Goto Islands, south-western Japan) exhibited cytotoxicity [98% inhibition at 10 µg/ml (KB cells)].

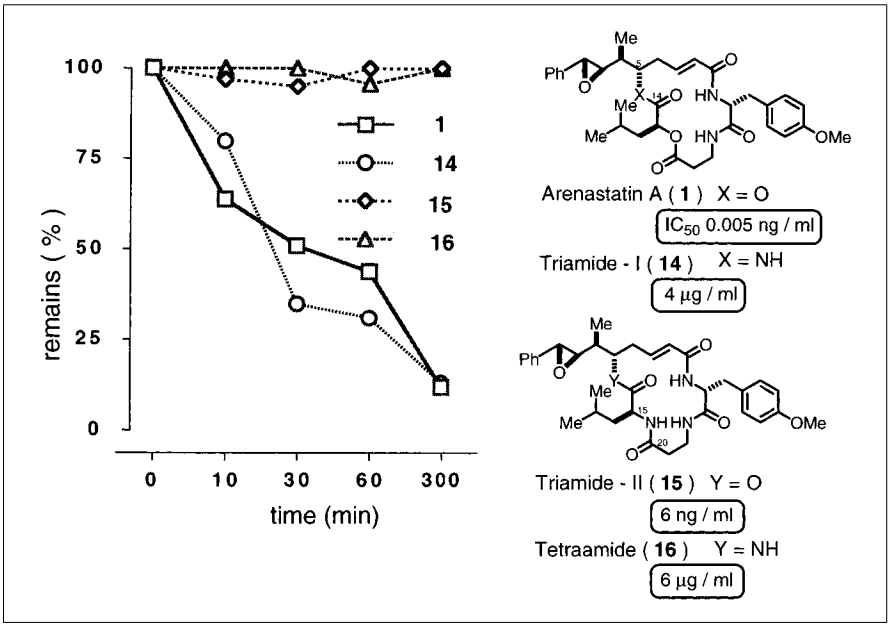


Fig. 5. Stability of arenastatin A and its analogues in serum.

Repeated SiO_2 column chromatography of the ethyl acetate-soluble portion furnished an active fraction [86% inhibition at 0.01 μg/ml (KB)] which was further purified by reversed-phase HPLC to provide a new cytotoxic polyketide named callystatin A (**17**) (1.0×10^{-2}% from the AcOEt-soluble portion) [20]. Callystatin A exhibited extremely potent cytotoxicity against KB cells (IC_{50} 10 pg/ml) (fig. 6).

The limited supply of callystatin A (**17**) prompted us to engage in a total synthesis, especially for further biological evaluation, and we succeeded in total synthesis of callystatin A [21]. To obtain information of the structure requirement for extremely potent cytotoxic activity, we synthesized model compounds IIa-c (**18–20**), which showed moderate cytotoxic activity with an IC_{50} value of 0.01 μg/ml, while the synthetic C-5 epimer **21** was 350 times weaker than the parent compound. Interestingly, 10-*epi*-callystatin A (**22**) was highly active.

The plane structure elucidated for callystatin A (**17**) was very similar to those of several antitumor antibiotics (e.g. leptomycin [22], kazusamycin [23], anguinomycin [24], and leptofuranin [25]) isolated from several strains of *Streptomyces* (fig. 7). We also isolated callystatin A from a marine sponge *Stelletta* sp. as well as from an unidentified tunicate, which were collected at the same collection site as *C. truncata*. These facts may indicate the possible participation of a symbiotic microorganism(s) in the biosynthesis of cally-

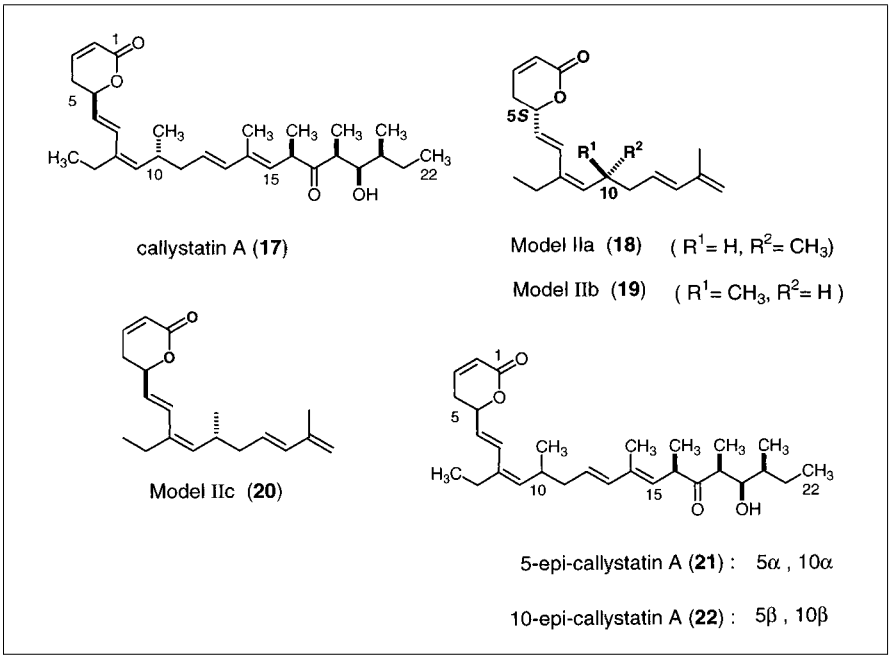
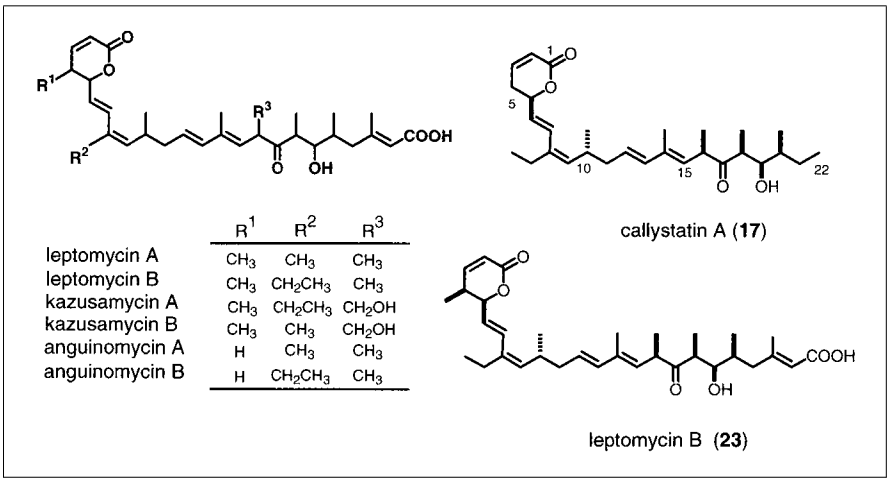


Fig. 6. Callystatin A and its synthetic analogs.



	R^1	R^2	R^3
leptomycin A	CH_3	CH_3	CH_3
leptomycin B	CH_3	CH_2CH_3	CH_3
kazusamycin A	CH_3	CH_2CH_3	CH_2OH
kazusamycin B	CH_3	CH_3	CH_2OH
anguinomycin A	H	CH_3	CH_3
anguinomycin B	H	CH_2CH_3	CH_3

Fig. 7. Callystatin A and the related antitumor antibiotics.

statin A. Recently, leptomycin B was found to inhibit an essential step for the initiation of DNA synthesis, which occurs at the end of G1 and G2 phases. Furthermore, leptomycin B specifically binds chromosome maintenance region 1 (CRM1) protein and inhibits nuclear export signal (NES)-mediated transport of Rev and U snRNA protein [26]. Callystatin A also binds CRM1 protein and inhibits nuclear export of NES-mediated protein.

Agosterol, an MDR-Reversing Polyhydroxylated Sterol Acetate from *Spongia* sp.

The development of multidrug resistance in tumor cells has been recognized as one of the major clinical problems in successful cancer chemotherapy. A major mechanism underlying this multidrug resistance is the overexpression of membrane protein. One of these membrane proteins is well known as P-glycoprotein [27]. P-glycoprotein is believed to function as an energy-dependent pump for efflux of anticancer agents from the tumor cells. To obtain the substances inhibiting the function of this membrane protein, we use two tumor cell lines named KB-C2 and KB-CV60, which were raised by Prof. S. Akiyama of Kagoshima University. KB-C2 cell line was selected and maintained in the selection medium containing colchicine from parental KB 3-1 cells. This tumor cell line overexpresses P-glycoprotein. KB-C2 cell line is resistant not only to colchicine but also to vincristine and Adriamycin. KB-CV60 cell line overexpresses multidrug resistance-associated protein (MRP) [28] but not P-glycoprotein, and exhibits drug resistance against vincristine, etoposide, and Adriamycin. Both cell lines are resistant to antitumor agents by two-digit concentration compared with parental KB 3-1 cell line. As for the procedure of this bioassay, to MDR tumor cells preincubated for 24 h were added test sample and antitumor agents, and the mixture incubated for 48 h and growth inhibition was measured by MTT assay. We selected 0.1 μg/ml dose of antitumor agent because a large difference in growth inhibition was observed between KB-C2 or KB-CV60 and parental KB 3-1 cell line at this concentration. To exclude a false reversing effect by cytotoxicity of test samples, we also check their cytotoxic activity against parental KB 3-1 cell line by MTT assay.

The ethyl acetate-soluble portion of the acetone extract of *Spongia* sp. collected off the Kii Peninsula showed the growth inhibition against KB-C2 cell in the presence of 0.1 μg/ml of colchicine. At the same dose, the ethyl acetate-soluble portion showed no cytotoxicity against parental KB 3-1 cell. The bioassay-guided separation afforded the major active substance named agosterol A (**24**) [29] which reversed resistance to colchicine in KB-C2 cell at a concentration of 1 μg/ml (fig. 8).

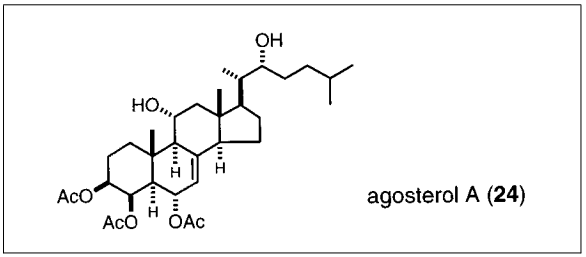


Fig. 8. MDR-reversing polyhydroxylated sterol from marine sponge.

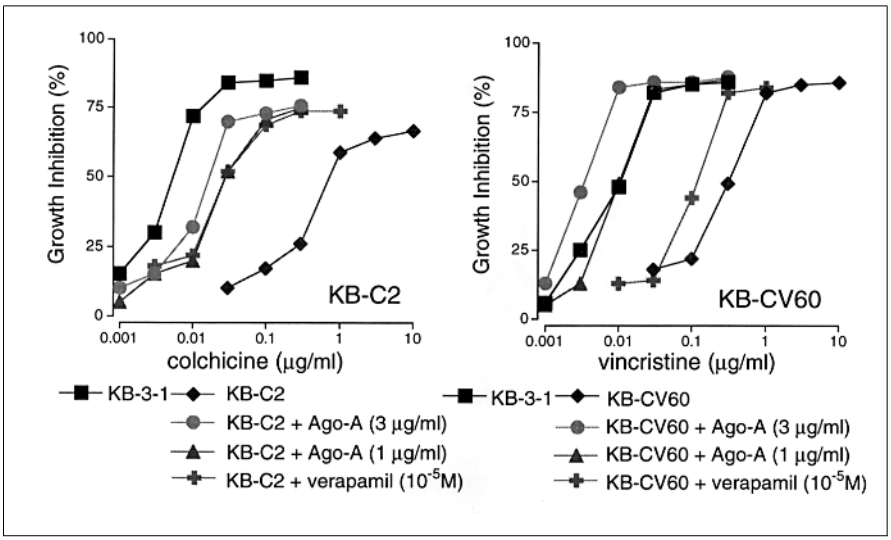


Fig. 9. Reversal of MDR in tumor cells by agosterol A (Ago-A).

Agosterol A completely reversed the resistance to colchicine in KB-C2 cells at 3 μg/ml. It also completely reversed the resistance to vincristine in KB-CV60 cells at 1 μg/ml as shown in figure 9. Verapamil showed weak MDR reversing activity against KB-CV60 at 10^{-5} *M*. These data show that agosterol A reverses MDR not only against KB-C2 cell overexpressing P-glycoprotein but also against KB-CV60 cell overexpressing MRP membrane protein.

Furthermore, agosterol A reversed the accumulation of vincristine in both of KB-C2 and KB-CV60 cell dose-dependently. In the case of KB-CV60 cells, agosterol A showed much stronger MDR reversing activity compared with that of verapamil. So far, there are few agents which reverse MDR caused by

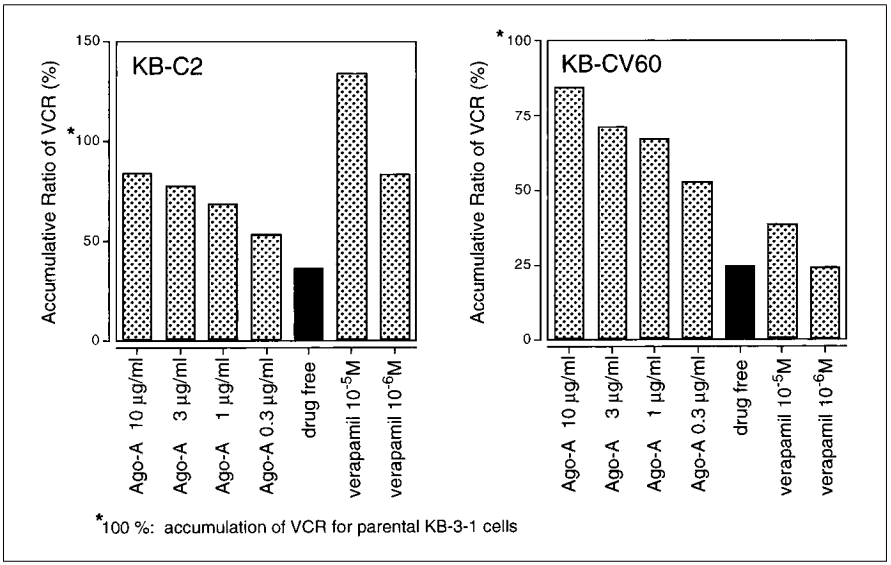


Fig. 10. Accumulation of vincristine (VCR) in KB-C2 and KB-CV60 cells in the presence of agosterol A (Ago-A) and verapamil.

overexpression of MRP. Agosterol A may be a useful lead for reversing MDR and also useful for detailed explication of the molecular mechanism of MRP (fig. 10).

Concluding Remark

More than 2,700 research papers concerning sponge metabolites have been published. However, the lack of pharmacological evaluation of these compounds and limited sample supply have hampered the development of drugs. Pharmaceutical companies have started the high-throughput screenings for sponge extracts, and it is likely that sponge-derived drugs will be developed in the future.

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Cytotoxic Substances from Opisthobranch Mollusks

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Introduction

The phylum Mollusca includes bivalves, cephalopods and gastropods, the last of which contains prosobranchs, opisthobranchs and plumonates. Sea hares of the opisthobranch mollusks are shell-less and slow-moving benthic marine animals that feed on a variety of marine algae. These mollusks are postulated to have chemical defense substances, which appear to deter predators. The poisonous properties of the sea hare secretions were already recorded in the Roman times. Sea hares have been known to be a rich source of unique bioactive compounds, which have generally been isolated as very minute constituents of sea hares and are considered to be of dietary origin and/or to be produced by symbiotic microbes. Since the notable report on the isolation of aplysin (**1**) (fig. 1), a bromine-containing sesquiterpene, by Hirata and Yamamura [1] in 1963, sea hares of the genus *Aplysia* were a target for studies of secondary metabolites, most of which were halogenated. Intensive investigations on bioactive constituents of sea hares of the genus *Dolabella* collected in the Indian Ocean were executed by Pettit and co-workers [2], culminating in the isolation of the cytostatic and antineoplastic compounds named dolastatins 1–15. We intensively examined the bioactive constituents of two species of the sea hares, *Aplysia kurodai* and *Dolabella auricularia*, collected off the coast of the Shima Peninsula, Mie Prefecture, Japan, and isolated a number of bioactive compounds, most of which were cytotoxic.

In this article we describe isolation of cytotoxic and antitumor compounds from two species of the sea hares, *A. kurodai* and *D. auricularia*, as well as

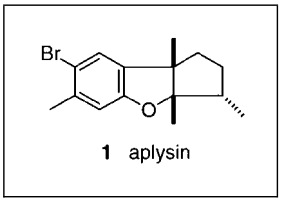


Fig. 1. Structure of aplysin (**1**).

their chemistry and bioactivities with the emphasis on the compounds obtained by our group.

Cytotoxic and Antitumor Compounds from the Sea Hare *A. kurodai*

Aplyronines

The ethyl acetate extract of *A. kurodai* that was collected at a specific area of the Shima Peninsula, Mie Prefecture, Japan, showed potent cytotoxicity against tumor (HeLa S_3) cells. Originally aplyronine A (**2**) [3] was isolated by eight-step chromatographic separation of this ethyl acetate extract guided by cytotoxicity test utilizing HeLa S_3 cells. A more efficient method for the isolation of **2** was developed using about 300 kg of the animal to afford seven minor congeners, aplyronines B (**3**), C (**4**) [3], and D–H (**5–9**) [unpubl. result], in addition to **2** (fig. 2). The aplyronines showed potent cytotoxicity against HeLa S_3 cells (fig. 3). It should be noted that aplyronine A (**2**) exhibited exceedingly potent antitumor activity in vivo (table 1). The gross structure of **2** was determined on the basis of the spectral data. Although the NMR spectra of **2** were complicated by the doubled NMR signals for some protons and carbons arising from the restricted rotation about the *N*-methyl-*N*-vinylformamide terminus (2:1 ratio) and the presence of two scalemic amino acid portions (1.1:1 and 3:1 ratios for *N,N,O*-trimethylserine and *N,N*-dimethylalanine parts, respectively), detailed analysis of 2D NMR spectra of **2** and its diacetate enabled us to establish the gross structure of **2**.

The stereostructure of aplyronine A (**2**) was determined by a combination of NMR spectroscopy and organic synthetic method [4–6]. The enantioselective synthesis of aplyronine A (**2**) was accomplished in a convergent manner for the purposes of confirming the stereostructure and the potent cytotoxicity [7–9]. The overall yield of the synthesis, based on the longest linear sequence (47 steps), was 0.35%. So far, the amount of **2** supplied from the natural source and by synthesis has not been sufficient for further evaluation of the antitumor properties of **2**. Thus, the scarcity of the sample of **2** has hampered the detailed studies on **2** as an antitumor agent in the advanced stages. The syntheses

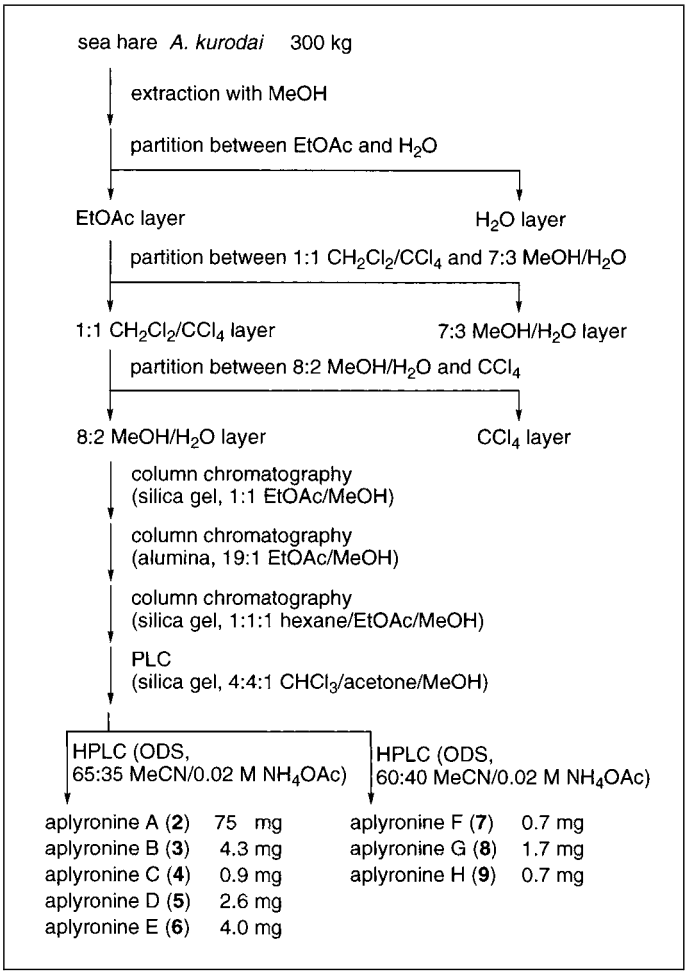
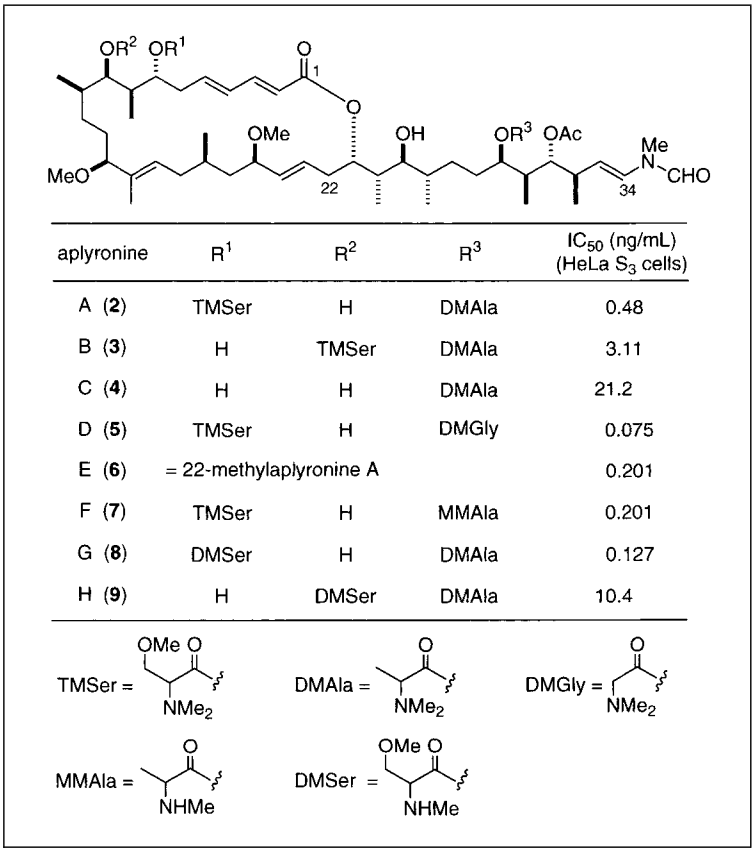


Fig. 2. Isolation procedure for aplyronines A–H (**2**–**9**).

of aplyronines B (**3**) and C (**4**) were also achieved, which established their stereostructures unambiguously [9, 10].

Generally, the target biomolecules for antitumor agents are considered to be the following three kinds of molecules: (i) DNA, (ii) tubulin and (iii) cell cycle regulating enzymes. Aplyronine A (**2**) did not react with any of these molecules, but interacted with actin, the major protein in cytoskeleton. Aplyronine A (**2**) not only binds to G-actin in a 1:1 molecular ratio to inhibit polymerization of G-actin to F-actin but also depolymerizes F-actin to G-actin by severing [11]. Although a number of proteins that interact with actin have



aplyronine	R^1	R^2	R^3	IC_{50} (ng/mL) (HeLa S_3 cells)
A (**2**)	TMSer	H	DMAla	0.48
B (**3**)	H	TMSer	DMAla	3.11
C (**4**)	H	H	DMAla	21.2
D (**5**)	TMSer	H	DMGly	0.075
E (**6**)	= 22-methylaplyronine A			0.201
F (**7**)	TMSer	H	MMAla	0.201
G (**8**)	DMSer	H	DMAla	0.127
H (**9**)	H	DMSer	DMAla	10.4

Fig. 3. Structures and cytotoxicity of aplyronines A–H (**2**–**9**).

been known, there are only a few compounds of low molecular weights that interact with actin: the examples are cytochalasins, phalloidin, and recently discovered marine macrolides such as latrunculins, mycalolide B [12, 13], bistheonellides and swinholides. Interesting is the fact that the mode of action of aplyronine A (**2**) toward actin is different from those of cytochalasins and phalloidin. To investigate the structure-bioactivity relationships of **2**, 14 artificial analogs were synthesized and their bioactivities were evaluated [14]. The presence and the length of the side chain portion of **2** proved to be crucial for both cytotoxicity and actin depolymerizing activity: for example, whereas the synthetic analog **10** that corresponds to the macrolide ring of **2** was shown to be about 4,400-fold less cytotoxic than **2** and to have no actin depolymerizing activity [14], the dimethylalanine-containing linear compound **11** that mimics

Table 1. Antitumor activity of aplyronine A (**2**)

Tumor	Dose mg/kg/day[1]	Median survival time, days	Relative life span, %	Number of survivors on day 60
P388 leukemia	0.08	59.9	545	4/6
	0 (controls)	11.0	100	0/7
Lewis lung carcinoma	0.04	60.1	556	6/6
	0 (controls)	10.8	100	0/8
Ehrlich carcinoma	0.04	59.7	398	2/6
	0 (controls)	15.0	100	0/8
Colon 26 carcinoma	0.08	40.0	255	0/6
	0 (controls)	15.7	100	0/10
B16 melanoma	0.04	46.8	201	0/6
	0 (controls)	23.3	100	1/9

[1] Administered by i.p. injection on days 1, 2, 3, 4 and 5.

the side chain of **2** was found to retain approximately 10% of the actin depolymerizing activity [unpubl. result] (fig. 4).

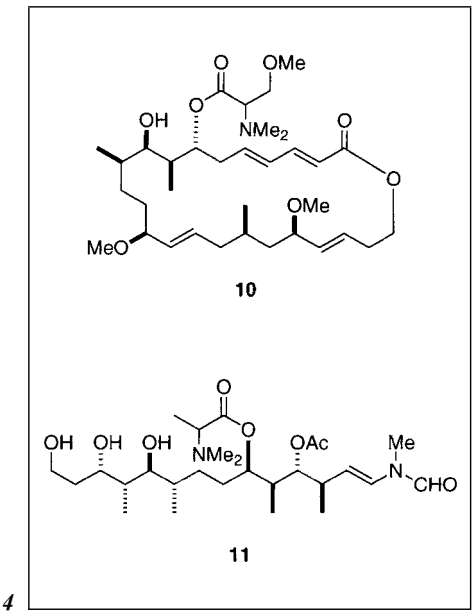
Aplaminone and the Related Metabolites

Three cytotoxic alkaloids, aplaminone (**12**), neoaplaminone (**13**) and neoaplaminone sulfate (**14**) were isolated from *A. kurodai*, among which **13** was shown to be highly cytotoxic (IC_{50} 1.6×10^{-7} μg/ml) against HeLa S_3 cells (fig. 5) [15]. The structures were elucidated by spectral analysis and organic synthesis [16].

Cytotoxic and Antitumor Compounds from the Sea Hare *D. auricularia*

Dolastatin H, Isodolastatin H and Doliculide (fig. 6)

Since 1965, Pettit and co-workers [2] have intensively examined the cytostatic and antineoplastic constituents of the Indian Ocean sea hare *D. auricularia*, resulting in the isolation of 15 structurally novel peptide- and depsipeptide-type compounds termed dolastatins 1–15. Most of them are extraordinarily strong cell growth inhibitors: for example, the ED_{50} value of dolastatin 10 (**15**), a well-known antitumor agent, was 0.046 ng/ml against P388 cells. A large number of sea hares was required to obtain dolastatins 1–15,



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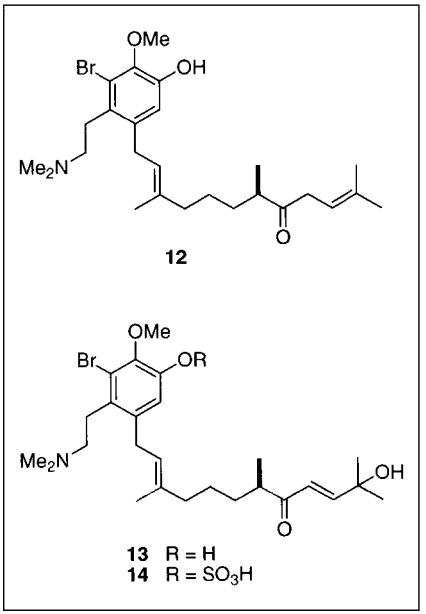

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Fig. 4. Structures of analogs of aplyronine A (**2**).
Fig. 5. Structures of aplaminone (**12**) and the related metabolites.

since they were trace components of the sea hare. Among them, dolastatin 10 (**15**) is especially important, which displayed unprecedented potency in experimental antineoplastic and tubulin assembly systems [2]. Both dolastatin 10 (**15**) and a derivative of dolastatin 15 (**17**) were reported to be in phase 1 clinical trials as anticancer agents.

Symplostatin 1 (**16**), a closely related analog of dolastatin 10 (**15**), was isolated from the marine cyanobacterium (blue-green alga) *Symploca hydnoides* by Moore and co-workers [17] and this finding supports the proposal that the minute constituents such as dolastatin 10 (**15**) of *D. auricularia* are not metabolites of the sea hare but are derived from its diet.

We have performed the bioassay-directed investigation of the cytotoxic constituents of the Japanese specimens of *D. auricularia* and isolated cytotoxic peptides and depsipeptides such as dolastatins C [18], D [19], E [20, 21], G [22, 23], and H (**18**) [24], isodolastatin H (**19**) [24], dolastatin I [25], doliculide (**20**) [26–28], aurilide (**24**) [29], and dolabellin [30] together with a cytotoxic terpene aurilol [31] and cytotoxic polyketides such as aurisides A and B [32, 33], auripyrones A and B [34], and dolabelides A, B, C and D [35, 36]. All of them were minute constituents of the sea hare *D. auricularia* and some of them were obtained in

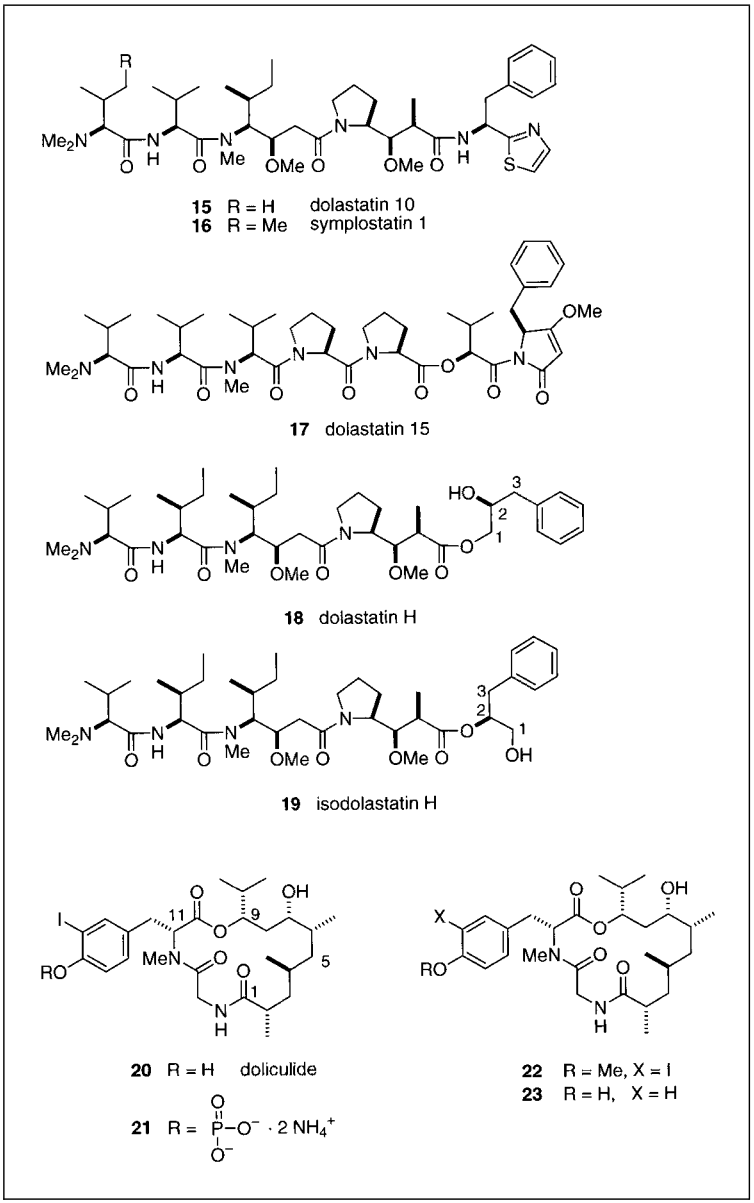

Fig. 6. Structures of dolastatins (**15–19**), doliculide (**20**) and its analogs.

Table 2. Cytotoxicity and yields of metabolites of the sea hare *D. auricularia*

Compound	Cytotoxicity against HeLa S_3 cells IC_{50}, μg/ml	Yield mg from 100 kg of the sea hare
Dolastatin C	17.0	5.1
Dolastatin D	2.2	5.2
Dolastatin E	22.0	3.0
Dolastatin G	1.0	88
Dolastatin H (**18**)	0.0022	0.9
Isodolastatin H (**19**)	0.0016	0.9
Dolastatin I	12.0	0.7
Doliculide (**20**)	0.005	24.6
Aurilide (**24**)	0.011	0.2
Dolabellin	6.1	18
Aurilol	4.3	4.5
Auriside A	0.17	0.3
Auriside B	1.2	0.3
Auripyrone A	0.26	0.2
Auripyrone B	0.48	0.4
Dolabelide A	6.3	88
Dolabelide B	1.3	24
Dolabelide C	1.9	71
Dolabelide D	1.5	1.5

sub-milligram quantities (cf. table 2). Cytotoxicities of these compounds against HeLa S_3 cells were evaluated and are summarized in table 2. Among the isolated compounds, dolastatin H (**18**), isodolastatin H (**19**) and doliculide (**20**) were strongly cytotoxic compounds (table 2), the studies of which were performed in detail.

Dolastatin H (**18**) and isodolastatin H (**19**) were isolated in trace amounts (fig. 7), and on the basis of the spectroscopic analysis they proved to be peptides that were closely related to dolastatin 10 (**15**). The enantioselective synthesis of dolastatin H (**18**) and isodolastatin H (**19**) was achieved, which not only determined the absolute stereostructures of **18** and **19** unambiguously, but also enabled us to supply the amounts sufficient for the evaluation of cytotoxicity and antitumor activity for **18** and **19** [24]. It is noteworthy that the C2-epimers of **18** and **19** are much less cytotoxic (IC_{50} 0.020 and 0.029 μg/ml, respectively) than **18** and **19** themselves. Thus, the stereochemistry of the 3-phenylpropane-1,2-diol moiety at the C-terminus in dolastatin H (**18**) and isodolastatin H (**19**) turned out to play an important role in the cytotoxicity. Dolastatin H (**18**) showed acute toxicity against mice at doses >10 mg/kg by i.v. injection. On the basis of

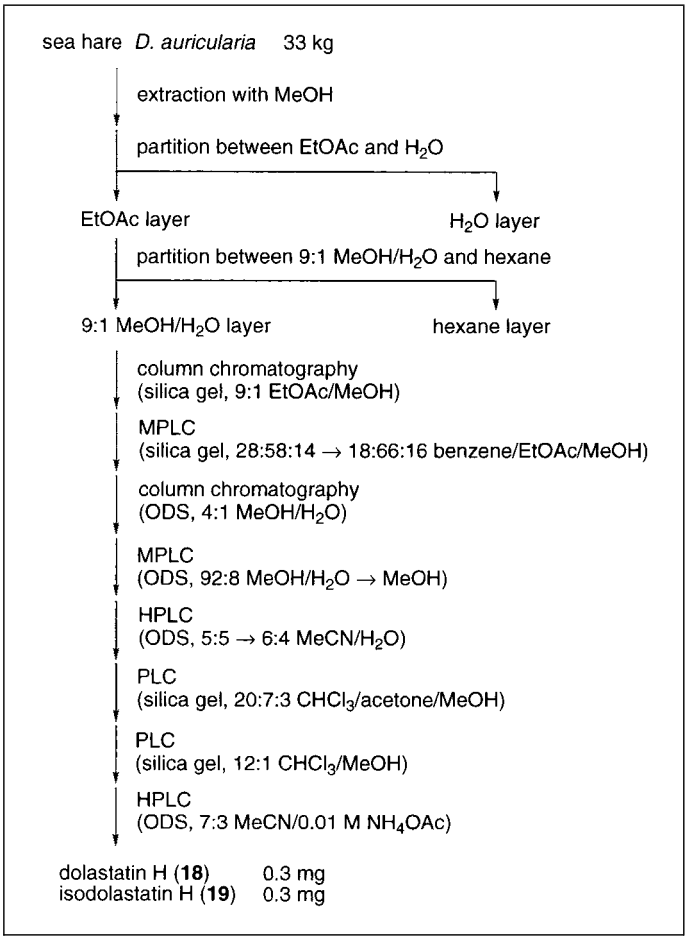

Fig. 7. Isolation procedure for dolastatin H (**18**) and isodolastatin H (**19**).

this toxicity test, in vivo antitumor activities of **18** and **19** were examined. Whereas no significant activity was shown for dolastatin H (**18**), isodolastatin H (**19**) exhibited antitumor activity with a T/C of 141% at a dose of 6 mg/kg/day against P388 leukemia (i.p. tumor inoculation-i.v. drug administration). This antitumor activity for **19** is a little weaker than that for dolastatin 10 (**15**) (T/C 155% at 6.5 μg/kg/day against P388 leukemia), although a much higher dose of **19** is required than that of **15**.

Doliculide (**20**) was also isolated as a minute constituent of the sea hare *D. auricularia*, and its structure was elucidated on the basis of spectral and chemical methods to be a cyclodepsipeptide of mixed peptide-polyketide biogenesis [26].

Table 3. Cytotoxicity of doliculide (**20**) and its synthetic analogs against HeLa S_3 cells

Compound	Doliculide (**20**)	11-*epi*-Doliculide	**22**	**23**
IC_{50}, μg/ml	0.005	5.0	1.7	0.83

Doliculide (**20**) is structurally related to cyclodepsipeptides such as geodiamolides [37, 38] and jaspamide (jasplakinolide) [39, 40]. Enantioselective synthesis of doliculide (**20**) and its analogs was carried out, which made it possible to examine the structure-activity relationships in detail [27, 28]. Some selected data of the structure-activity relationships of **20** are shown in table 3. Comparison of cytotoxicity of **20** (0.005 μg/ml) with those of doliculide methyl ether (**22**) (1.7 μg/ml) and deiododoliculide (**23**) (0.83 μg/ml) suggested that both phenolic hydroxyl group and iodine atom in **20** are responsible for the strong cytotoxicity of **20**. It should be noted that 11-*epi*-doliculide (5.0 μg/ml) is 1,000-fold less cytotoxic than doliculide (**20**); this finding suggested that the conformation of the 16-membered dilactam-lactone ring may be important for cytotoxicity, because the conformation of doliculide (**20**) is significantly different from that of 11-*epi*-doliculide. With synthetic doliculide (**20**) in hand, preliminary experiments on antitumor activity of **20** were executed using doliculide phosphate (**21**): doliculide phosphate (**21**) showed antitumor effect to a considerable extent against Lu-61 xenograft in nude mice. Owing to the scarcity of the sample no further studies on the antitumor activity of **20** have been made.

Aurilide

Aurilide (**24**) was isolated as a trace constituent of the sea hare *D. auricularia* (0.5 mg from 262 kg of the sea hare) [29]. Figure 8 illustrates the isolation procedure for aurilide (**24**) that involves two-step solvent partition and subsequent ten-step chromatographic separation. The gross structure of aurilide (**24**) was determined by the spectroscopic method and the absolute stereostructure of the peptide moiety was determined by chiral HPLC analysis of the component amino acids and hydroxy acid obtained by acid hydrolysis of **24**. The absolute stereostructure of a new dihydroxylated fatty acid part in aurilide (**24**) was determined by the enantioselective synthesis of four possible diastereomers of the fragment **25** derived from **24** and comparison of their ^{1}H-NMR and CD data with those of the natural fragment **25** (fig. 9). Thus, the absolute stereostructure of aurilide was determined to be a 26-membered cyclodepsipeptide as depicted in **24**. The efficient enantioselective synthesis of aurilide (**24**) was achieved in 12% overall yield based on the longest linear sequence (16

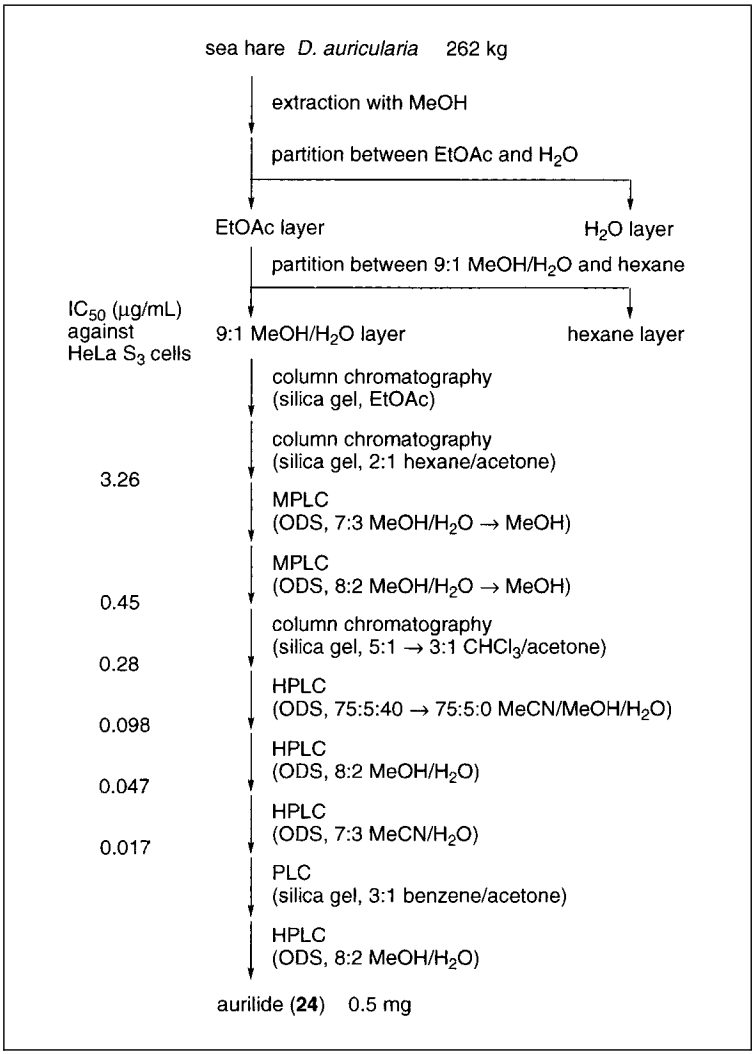

Fig. 8. Isolation procedure for aurilide (**24**).

steps) [41] and so far about 1.5 g of aurilide (**24**) has been synthesized. Although aurilide (**24**) was isolated from a strongly cytotoxic fraction of the extracts of the sea hare (fig. 8), the scarcity of the natural sample prevented the evaluation of its cytotoxicity. Thus, the cytotoxicity of aurilide (**24**) was evaluated by employing the synthetic sample and it was found that **24** exhibited strong cytotoxicity against HeLa S_3 cells with an IC_{50} value of 0.011 μg/ml. It is

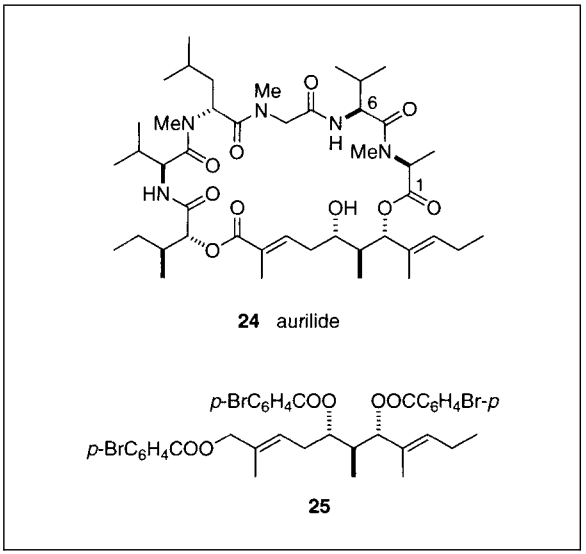

Fig. 9. Structures of aurilide (**24**) and its fragment.

interesting to note that the cytotoxicity of 6-*epi*-aurilide obtained by synthesis ($IC_{50} > 4$ μg/ml) is much weaker than that of aurilide (**24**). The NCI's human cancer cell panel indicated that aurilide (**24**) exhibited a high level of cytotoxicity (the mean panel GI_{50} concentration was 0.12 μg/ml), and that **24** is selectively active against lung, ovarian, renal and prostate cancer cell lines. Interestingly, aurilide (**24**) was not cytocidal but cytostatic against human leukemia cell lines. Aurilide (**24**) met the criteria of the NCI's hollow-fiber assay in high scores, which is a preliminary in vivo test for assessing the potential anticancer activity of compounds identified by the in vitro human cancer cell panel. Based on this promising result concerning the hollow-fiber assay, a standard in vivo human tumor xenograft test is now under way.

Concluding Remarks

From two species of sea hares of the opisthobranch mollusks, *A. kurodai* and *D. auricularia*, a variety of new cytotoxic compounds have been isolated and structurally characterized. Some of them have been synthesized enantioselectively. These compounds were contained in very small quantities in the animals, many of which are presumably of dietary origin and/or originate from symbiotic microorganisms such as cyanobacteria. Among the isolated

compounds, detailed chemical and biological studies as well as the antitumoral evaluation have been executed on the highly cytotoxic compounds: aplyronine A (**2**), dolastatin H (**18**), isodolastatin H (**19**), doliculide (**20**), and aurilide (**24**), some of which exhibited promising antitumor activities and thus will serve as leads for the antitumor drugs.

A serious problem in the present studies is unavailability of the large quantities of cytotoxic compounds from the sea hares. In particular cases, one of the solutions to this problem is provided by the organic synthesis, which enables us to secure the amount necessary for further biological and medicinal evaluation of a cytotoxic compound: for example, whereas the natural sample of aurilide (**24**) was obtained from the sea hare *D. auricularia* in sub-milligram quantities, the synthetic sample was available on a gram scale.

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ω-Conotoxin MVIIA: From Marine Snail Venom to Analgesic Drug

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Introduction

A 25 amino acid peptide, ω-conotoxin MVIIA, from the venom of a predatory cone snail, *Conus magus*, is a marine natural product in advanced development as a therapeutic agent. The current status (as of March 2000) is that clinical trials by a biotechnology company, Neurex, are complete for two therapeutic indications, and a new drug application has been submitted to the US Federal Drug Administration. Although originally designated ω-conotoxin MVIIA, as a pharmaceutical the peptide will be known by the generic name ziconitide.

This potential peptide drug is noteworthy in several ways; first, the commercial product ziconitide is identical to the natural peptide from the marine snail; no chemical modifications have been introduced. The natural peptide is directly translated from the genome of *C. magus*, and would be the first example of a gene-encoded invertebrate polypeptide to be used clinically. Finally, the compound was not discovered by a medicinal chemist at a pharmaceutical company, but by a young undergraduate, barely out of high school.

This review is written as scientific history, with a bit of personal memoir thrown in. The somewhat convoluted pathway of drug development of ω-conotoxin MVIIA may well prove to be paradigmatic for many other marine natural products. A noteworthy aspect of the ω-conotoxin MVIIA story is that the exploration of potential therapeutic applications were closely intertwined with advances in basic research.

Background Biology: Early Biochemical and Pharmacological Characterization of *Conus* Venoms

Ziconitide (ω-conotoxin MVIIA) was originally found in the venom of the Indo-Pacific marine snail *Conus magus*, the 'magician's cone' (fig. 1). All 500 species of the genus *Conus* are venomous predators [1, 2]. Several centuries ago, the great Dutch naturalist Rumphius noted that cone snails were venomous enough to cause human fatality – however, it took Alan Kohn's work as a graduate student more than 250 years later to provide a rationale for why stings of some of these snails were potentially lethal to man. Kohn [3] showed that the natural prey of the most dangerous cone snails were vertebrates, i.e. fish [3].

Lourdes J. Cruz, at the University of the Philippines, and I began to systematically collect *Conus* venoms and purify the biologically active components of the venoms from several fish-hunting cone snails in the early 1970s. The very first venom component characterized was α-conotoxin GI, a small peptide (13 amino acids) that proved to be a competitive nicotinic antagonist [4]. Several other α-conotoxins, as well as three μ-conotoxins, which were channel blockers of the muscle subtype of voltage-gated sodium channels, were characterized soon afterwards [for an overview, see 5]. These peptides cause paralysis in mice. The characterization of α- and μ-conotoxins explained the high frequency of fatality from stings of cone snail species such as *Conus geographus*: such a sting is equivalent to a combination of a cobra snakebite (which exposes the victim to a physiologically-similar competitive nicotinic antagonist) and eating a lethal dose of badly-prepared 'fugu', the Japanese puffer fish (which contains the well-known and deadly sodium channel blocker, tetrodotoxin). This early biochemical work established that active components in cone snail venoms are small, highly cross-linked peptides. However, the enormous pharmacological diversity present in these venoms was not yet apparent.

Purification and Characterization of ω-Conotoxin MVIIA

The characterization of ω-conotoxin MVIIA at the University of Utah became possible because of two advances: first, the introduction of high-performance liquid chromatography for purification and second, a new assay for following biological activities of venom peptides.

All the previous purification of α- and μ-conotoxins had been done by screening for paralytic activities following mice i.p. injection, which detects fractions that interfere with neuromuscular transmission. A 19–year-old under-

Fig. 1. *C. magus*, the magician's cone snail. This is the *Conus* species whose venom was the original source of ω-conotoxin MVIIA. ***a*** Various shells of *C. magus* from different localities in the Philippines. *C. magus* has perhaps the most variable shell pattern in the entire genus. ***b*** A living specimen of *C. magus*, which has just captured a fish; the fish can still be seen within the mouth of the snail. Note that the living *C. magus* has a thick organic layer which covers the shell pattern.

graduate student in our laboratory, Craig Clark, got what was, in retrospect, the brilliant idea of injecting components of the venom directly into the central nervous system of a mouse. Craig learned how to do intracranial injection simply and effectively so that control injections gave no obvious symptoms. Using his assay, we quickly discovered that *Conus* venoms were exceedingly

complex pharmacologically. Different fractions from the venom of a fish-hunting cone snail elicit a wide range of different symptoms. Craig Clark himself went on to purify several peptides including one that induced a sleep-like state in mice [6]. These peptides, now known as conantokins, were later shown to be NMDA receptor antagonists, and are presently being explored for their therapeutic potential (by Cognetix, Inc., Salt Lake City, Utah, USA).

Michael McIntosh (fig. 2), who started doing research at the University of Utah even before he graduated from high school, examined the venom of *C. magus* using Craig Clark's newly developed assay. One venom fraction caused mice to shake continuously (see fig. 3); in our laboratories at the time, this extremely distinctive symptomatology was referred to as the 'shaker' syndrome.

Michael identified and purified the first shaker activity from *C. magus* venom (see fig. 3). On the basis of McIntosh's work, a post-doctoral fellow, Antonio Luque, detected and purified another shaker activity from *C. geographus* [7]. Amino acid sequences were determined in the laboratory of William Gray, and both peptides were synthesized by Jean Rivier's laboratory at the Salk Institute [8]. The shaker peptides from *C. magus* and *C. geographus* venoms are known today as ω-conotoxins MVIIA and GVIA; the structure of ω-conotoxin MVIIA is shown in figure 4.

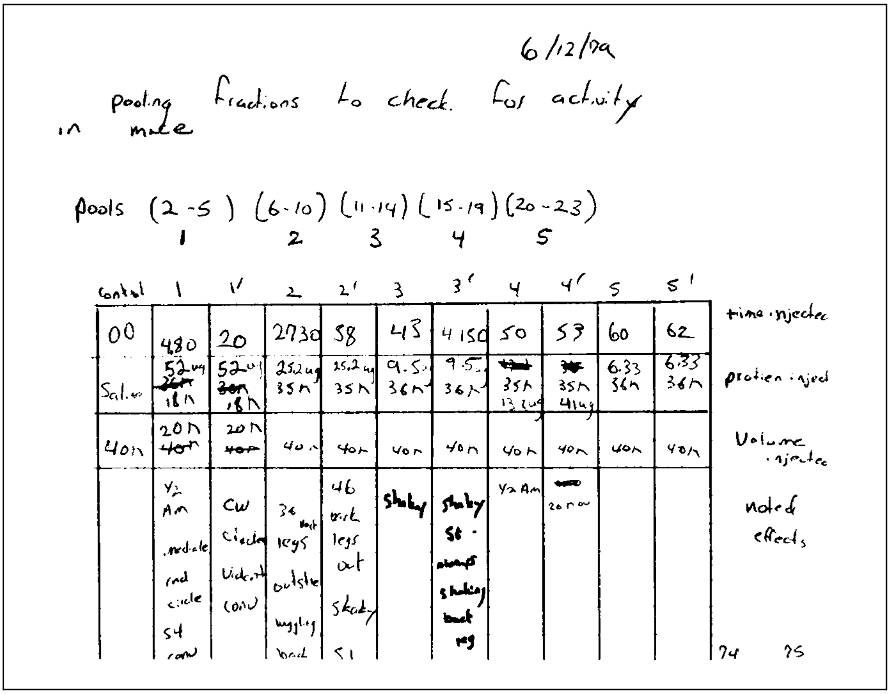
Both ω-conotoxins MVIIA and GVIA were paralytic to fish, suggesting that they might interfere with neuromuscular transmission. The effects of the peptides at the frog neuromuscular junction were analyzed using electrophysiological techniques by Lynn Kerr and Doju Yoshikami [9] at Utah.

Kerr and Yoshikami established that at the amphibian neuromuscular junction, ω-conotoxin GVIA potently blocked neuromuscular transmission; in contrast, ω-conotoxin MVIIA was far less potent (this appears to be a phylogenetic quirk; ω-conotoxins from *C. magus* venom are, for unknown reasons, less potent in amphibian systems than in other vertebrates). Because of its greater affinity, the more detailed characterization was carried out on ω-conotoxin GVIA. Kerr and Yoshikami [9] provided strong evidence that ω-conotoxin GVIA blocked presynaptic voltage-gated calcium channels. However, at this time, a molecular characterization of Ca channels had not yet been carried out.

The Role of ω-Conotoxins in Defining Voltage-Gated Calcium Channel Subtypes

At the time ω-conotoxins were discovered, most electrophysiologists recognized two classes of voltage-gated calcium channels, high- and low-voltageactivated channels (based on the depolarization required to open the channel).

a



b

Fig. 2. a Michael McIntosh, photographed at around the time he first purified and characterized ω-conotoxin MVIIA. ***b*** The original entry from McIntosh's notebook, showing the first time he detected the 'shaker' symptomatology elicited by ω-conotoxin MVIIA. The entry is dated on June 12, 1979; McIntosh had graduated from high school a few days earlier. He entered college at the University of Utah in the fall of 1979.

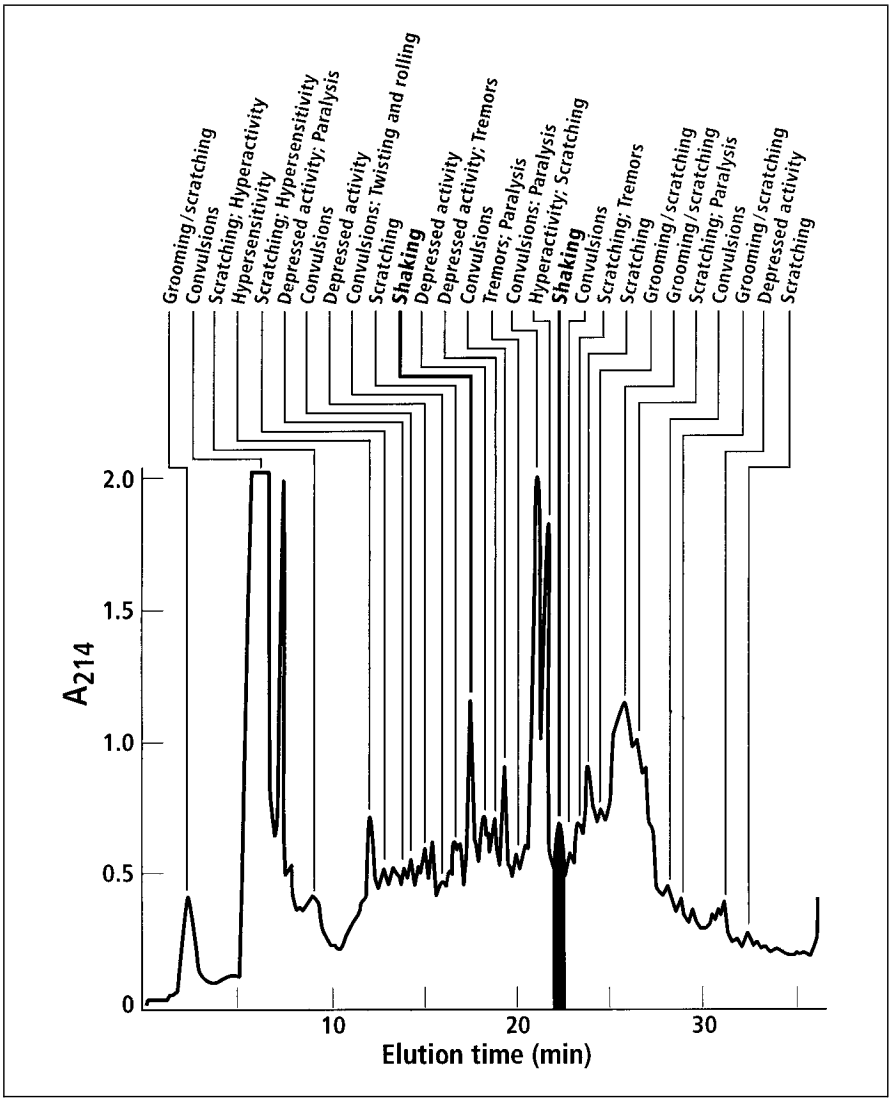


Fig. 3. An HPLC chromatographic trace of *C. magus* venom, showing the biological activity elicited by fractions. The fraction that eventually yielded ω-conotoxin MVIIA caused a shaking syndrome and is highlighted in black. Most fractions are not pure, and comprise a mixture of peptides. The other 'shaker' fraction eluting earlier contains ω-conotoxin MVIIB.

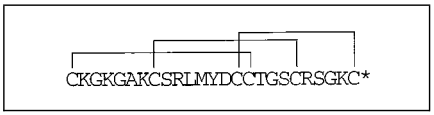


Fig. 4. The primary structure of ω-conotoxin MVIIA (ziconitide), showing the disulfide bonds. The asterisk represents an amidated C-terminus.

The most widely used pharmacological tools for calcium channels were the dihydropyridines, known to inhibit high-voltage-activated calcium channels in many systems [for a more detailed general review, see 10].

However, several investigators had found high-voltage-activated calcium channels that were not inhibited by dihydropyridine drugs. A further subdivision of calcium channels based both on pharmacological and electrophysiological criteria was proposed by R. Tsien and collaborators; single-channel studies were consistent with at least two types of high-voltage-activated calcium channels. In their nomenclature, the largely noninactivating, dihydropyridine-sensitive channels were called L-type, and low-voltage-activated channels, responsible for transient currents, were referred to as T-type Ca channels. The dihydropyridine-insensitive high-voltage-activated Ca channels suggested by the single channel recording studies were postulated to be neither L- nor T- and were called N-type calcium channels. However, on the basis of electrophysiology alone, it was not easy to distinguish between voltage-gated calcium channel subtypes.

The discovery of ω-conotoxins occurred at an opportune time to play a key role in defining calcium channel subtypes. A collaboration between the Utah groups and the Tsien laboratory established that ω-conotoxins GVIA and MVIIA were specific N-type Ca channel inhibitors [11]. Thus, high-voltage-activated Ca channels could now be divided into at least two classes based on clear-cut pharmacological criteria: L-type channels that were dihydropyridine-sensitive but resistant to ω-conotoxins GVIA and MVIIA, and N-type calcium channels which were dihydropyridine-insensitive, but blocked by ω-conotoxins GVIA and MVIIA. ω-Conotoxins have become standard tools for identifying N-type calcium channels and for inhibiting synaptic transmission. Subsequently, two additional subtypes of high-voltage-activated Ca channels, resistant to both dihydropyridines and ω-conotoxin MVIIA, were identified [10].

Biochemistry and Molecular Biology of ω-Conotoxin MVIIA Target

The rapid progress made in understanding Ca channel subtypes has been reviewed [10], and a brief historical chronology of the major highlights is

outlined here. Benson and Caterall purified the skeletal muscle L-type calcium channel and showed that it was a multiple subunit complex. The α_1 subunit of this channel was successfully cloned and expressed by the Numa lab shortly thereafter. T. Snutch and collaborators provided the first molecular evidence for multiple calcium channel subtypes, and they proposed the nomenclature for Ca channels which is now widely used. The different Ca channel classes are based primarily on the α_1 subunits; at present, nine α_1 genes have been identified by cloning.

With the availability of ω-conotoxins and cloned sequences of other calcium channels, the characterization of the N-type calcium channel progressed rapidly. The mammalian N-type calcium channel was purified in the laboratory of Kevin Campbell using ω-conotoxins as affinity reagents, and then first cloned and expressed by Harpold and collaborators at SIBIA and designated α_{1B}. When expressed in oocytes (or other heterologous expression systems), the α_{1B} Ca channel is inhibited by ω-conotoxins GVIA and MVIIA, and had other properties consistent with N-type Ca channels. Thus within a few years of the characterization of the shaker peptides, a large body of data firmly established that they were highly specific blockers of one specific molecular class of voltage-gated Ca channels in mammalian systems, the subset which had an α_{1B} subunit.

G. Miljanich, and the Introduction of ω-Conotoxins to Neurex

Since all synaptic transmission requires Ca entry for neurotransmitter release to occur, several laboratories, including that of George Miljanich (then at the University of Southern California), began using ω-conotoxins to evaluate their effects on synaptic transmission in diverse systems. Miljanich's laboratory used synaptosomes derived from the electric organ of the ray, *Ommata discopyge*, for studying neurotransmitter release. They assessed the effects of both ω-conotoxins MVIIA and GVIA on neurotransmitter release by the synaptosomes. In this system, ω-conotoxin MVIIA was the more potent inhibitor of neurotransmitter release. Miljanich joined Neurex in 1988; at this point, the commercial development of ω-conotoxin MVIIA began. George Miljanich introduced ω-conotoxin MVIIA to Neurex and would play a major role in all aspects of its subsequent development as a therapeutic [12].

The decision to focus Neurex's resources on conotoxins was made by J. Ramachandran and Jim Wilson, who were the Vice President for research and CEO, respectively. Scott Bitner, a behavioral pharmacologist then at Neurex, together with Miljanich and Ramachandran, initiated a major effort to explore the potential of ω-conotoxin MVIIA as a neuroprotective agent. Neuronal

cell death after hypoxic-ischemic episodes was known to be initiated by excess intracellular calcium, which then triggered a complex cascade culminating in the destruction of the cell's DNA. ω-Conotoxin MVIIA might prevent excessive intracellular Ca levels in neurons after a heart attack or stroke by two possible mechanisms. First, Ca entry through N-type calcium channels on the cell body or dendrites would be blocked. In addition, glutamate release from presynaptic termini would be inhibited by blocking presynaptic Ca entry. When excessive glutamate is released by cells, glutamate-gated ion channels open, and a subclass of these (the NMDA receptors) are known to allow large amounts of calcium to enter neurons. Thus, inhibiting glutamate release should prevent excessive Ca entry and be neuroprotective. Because of Miljanich's earlier work on the inhibition of neurotransmitter release, he was well sensitized to the potential utility of ω-conotoxin MVIIA in this respect.

The neuroprotection tests using animal models proved encouraging enough for Neurex to begin clinical trials on ω-conotoxin MVIIA for brain ischemia in 1993. These investigations were made possible by the large-scale synthesis of ω-conotoxin MVIIA; the original chemical synthesis developed by Jean Rivier was further refined by D. Yamashiro, at Neurex, to make large batches of peptide available for preclinical and phase I clinical studies. Phase II clinical trials for neuroprotection after head trauma and coronary artery graft surgery have recently been completed.

Development of ω-Conotoxin MVIIA as an Analgesic

Scott Bitner also pressed for testing of ω-conotoxin MVIIA in animal models of pain. One proposed mechanism for the analgesic action of morphine was attenuation of N-type calcium channel activity in the spinal cord; ω-conotoxin MVIIA was a potent blocker of those very Ca channels. When ω-conotoxin MVIIA was tested on the rat tail-flick test, a simple animal model for analgesia, encouraging initial results were obtained.

Autoradiography was carried out to determine the distribution of radiolabeled ω-conotoxin MVIIA binding sites in the spinal cord. These sites proved to be almost exclusively limited to the outer laminae of the dorsal horn, precisely where synapses between sensory neurons and higher sensory pathways are located. This distribution of ω-conotoxin MVIIA sites was similar to that seen with morphine, providing further support for the potential of ω-conotoxin MVIIA as an analgesic. Importantly, these sites are present essentially nowhere else in the spinal cord, a favorable harbinger of few side effects (fig. 5).

These results led Neurex to collaborate with Tony Yaksh at the University of California, San Diego, who had developed more sophisticated analgesic

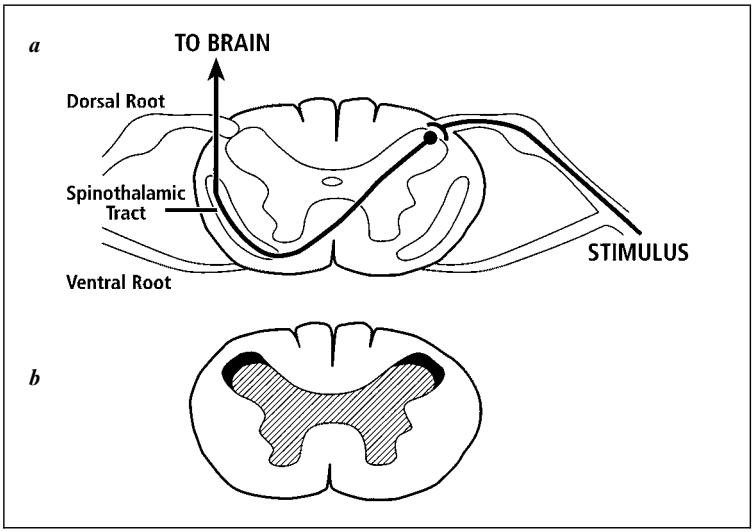


Fig. 5. How ω-conotoxin MVIIA suppresses pain. The peptide is therapeutically applied to the spinal cord. The figure shows the relevant circuitry in the spinal cord which is affected by the conus peptide. ***a*** A cross section of the cord illustrating how a pain stimulus enters the spinal cord through the dorsal horn region, providing synaptic input to a neuron (black dot) that carries the signal to the brain, leading to the perception of pain. The analgesic activity of ω-conotoxin MVIIA is believed to be due to an interruption in communication between an incoming pain fiber and the spinal cord neuron which transmits the signal to the brain. ***b*** Diagram of the results of an autoradiographic analysis of specific ω-conotoxin MVIIA binding sites (black), presumably N-type channels, which appear to be restricted to the dorsal horn with no labeling of the ventral horn. In contrast, the hatched area shows labeling by ω-conotoxin MVIIC, which binds to a broader set of voltage-gated channels besides the N-type calcium channel. Comparing ***a*** and ***b*** demonstrates that the highly labeled ω-conotoxin MVIIA target region is precisely the region where synaptic connections occur between a peripheral axon carrying the initiating stimulus and a spinal cord neuron which relays the information to the brain. These results clearly suggested ω-conotoxin MVIIA might inhibit neurotransmitter release from incoming sensory fibers, which should suppress the perception of pain (based on information provided by G. Miljanich, Neurex Corp.).

tests. Using an animal model of nociceptic pain (which results from a noxious external stimulus), Yaksh and colleagues showed that ω-conotoxin MVIIA was indeed a potent analgesic, approximately 1,000 times more potent than morphine. However, the peptide also proved effective in an animal model of neuropathic pain, which results from abnormally hyperactive responses of the nervous system to what are normally benign stimuli. Clinically, the latter type of pain is generally unresponsive to opiates. Additional animal models for pain were tested by Scott Bowersox and collaborators at Neurex.

The Neurex/UC San Diego collaboration demonstrated an additional promising feature of ω-conotoxin MVIIA. A major problem with opiates is development of tolerance; when morphine is taken chronically, progressively higher doses are required to maintain the same level of relief. It was found that no tolerance to ω-conotoxin MVIIA administration developed during chronic treatment.

The original designation for the peptide 'ω-conotoxin MVIIA' was regarded as unfortunate for a potential therapeutic, since 'toxin' would have negative connotations for a commercial drug. In the publications from Neurex of this period, the terminology SNX111 was used in referring to the peptide. It should be also remarked parenthetically that at Neurex, peptide chemists (led by L. Nasdasdi) synthesized numerous analogs of ω-conotoxin MVIIA and other ω-conotoxins to determine whether any might have superior qualities as an analgesic. In the end, the decision was made to proceed with unmodified peptide for human clinical trials for analgesia. Thus, the Neurex synthetic material is indistinguishable from the natural product synthesized in the venom of the *Conus* snail.

The animal studies provided evidence that ω-conotoxin MVIIA might solve an unmet medical need, alleviating intractable forms of severe pain. These promising preclinical results led to the initiation of clinical trials with human patients. Initially, permission was given to Neurex by the FDA to carry out clinical trials on the peptide only on terminal cancer patients and AIDS patients, whose pain was no longer relieved by morphine. The initial trial which involved a small patient pool proved sufficiently promising so that permission was extended to other classes of patient with intractable pain. An account of these clinical trials, as well as the preclinical work, has been given by Miljanich and other Neurex investigators [12, 13]; a less technical account, including individual patient histories, has also been reported in an article in *Worth Magazine* [14]. Successful phase III clinical trials for two therapeutic applications of ω-conotoxin MVIIA as an analgesic have been completed: to alleviate pain associated with malignant disease (i.e. cancer and AIDS) and as an analgesic for nonmalignant neuropathic pain. The peptide is still undergoing clinical trials for long-term toxicity. A new drug application has been submitted.

Postscript – An Update (Dec. 1998)

Scott Bitner, L. Nasderdi, J. Ramachandran, J. Wilson, and D. Yamashiro have all left Neurex, the biotechnology company which developed ziconitide. Neurex ceased to exist as an independent entity on August 15, 1998. It was

purchased by Elan, a multinational pharmaceutical company, for USD 700 million. Tragically, Craig Clark, who became a neurosurgeon, passed away suddenly in 1994. Michael McIntosh, the young undergraduate who purified ω-conotoxin MVIIA, trained as a psychiatrist, and is a faculty member of the Department of Psychiatry and Biology at the University of Utah. His research interests are to use *Conus* peptides to study receptors and ion channels relevant to mental illness. He has pioneered in developing specific ligands for neuronal nicotine receptor subtypes, and has been particularly active in directing the research of University of Utah undergraduates, most of whom are purifying new peptides from *Conus* venoms.

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KRN7000 as a New Type of Antitumor and Immunostimulatory Drug

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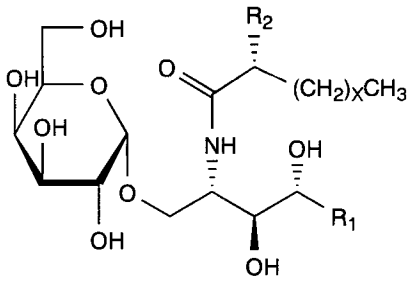
Introduction

The search of new, effective anticancer drugs continues to be an important activity in the cancer research. In particular, the search of anticancer leads has been a mainstream of the marine natural product research. Several marine-derived compounds have recently entered in clinical trials as anticancer drugs, while many marine compounds are under preclinical trials. All of these compounds were discovered in the screening of cytotoxic effects, and hence they are strongly cytotoxic.

In our search for antitumor compounds from marine organisms, we adopted the in vivo assay using mice bearing tumor without pointing to cytotoxic compounds. In this screening, we found from the Okinawan sponge *Agelas mauritianus* a series of potent antitumor compounds, agelasphins, which also showed immunostimulatory activity in the mixed lymphocyte reaction (MLR) assay, but no in vitro cytotoxicity. This finding led us to develop a synthetic analogue, KRN7000, as a candidate drug for cancer chemotherapy. This is an entirely new type of antitumor agent as it is not cytotoxic. In this article we describe the isolation of agelasphins, the development of KRN7000, its antitumor activity and mode of action.

Discovery of Agelasphins

In our in vivo screening using B16 melanoma-bearing mice, a lipophilic extract from the sponge *A. mauritianus* significantly prolonged the survival



	X	R_1	R_2
Agelasphin-7a	21	$-(CH_2)_{11}-CH_3$	-OH
Agelasphin-9a	21	$-(CH_2)_{12}-CH_3$	-OH
Agelasphin-9b	21	$-(CH_2)_{11}-CH(CH_3)_2$	-OH
Agelasphin-11	21	$-(CH_2)_{11}-CH(CH_3)-C_2H_5$	-OH
Agelasphin-13	22	$-(CH_2)_{11}-CH(CH_3)-C_2H_5$	-OH
KRN7000	23	$-(CH_2)_{13}-CH_3$	-H

Fig. 1. Chemical structures of some agelasphins and synthetic analogue KRN7000.

period of the mice. The extract also exhibited marked activity in the lymphocytic proliferation in allogeneic MLR in vitro. These results led us to undertake bioassay-guided fractionation of the extract. We first obtained several known series of terpenoids that showed weak cytotoxicity, but none of them exhibited in vivo antitumor activity. This finding prompted us to search in vivo active substances present in the crude extract. We finally isolated novel active substances named agelasphins (AGLs; fig. 1). The structures were determined by spectroscopic and chemical methods. The AGLs are glycosphingolipids with a characteristic α-galactosylceramide (α-GalCer) structure, that is, the linkage between a galactose and a ceramide is in an α-configuration [1]. The stereochemistry of AGLs was elucidated by a total synthesis [2, 3]. Administration of 100 μg/kg of AGLs significantly prolonged the survival period of tumor-bearing mice and showed extremely weak acute toxicity ($LD_{50} > 10$ mg/kg). In addition, AGLs markedly stimulated the proliferation of spleen cells in allogeneic MLR.

It is interesting to note that β-GalCer has been reported to exhibit no antitumor activity against tumor-bearing mice at a dose of 100 mg/kg, while AGLs with an α-GalCer structure showed strong antitumor and immunostimulatory effects.

Structure-Activity Relationship Studies

In order to clarify the difference in the biological effects of α-GalCers and β-GalCers, we first synthesized α-GalCers and β-GalCers, which have the same ceramide moiety [4], and compared their antitumor and immunostimulatory activities. A comparative study demonstrated that α-GalCers possessed much stronger antitumor and immunostimulatory activities than β-GalCers [5–7] and suggested that α-GalCers should be selected as lead compounds to be used as an antitumor and/or immunostimulatory agents. We next synthesized a number of α-GalCers having various types of ceramide moieties for the structure-activity relationship studies using B16-bearing mice. It was found that the length of the fatty acid chain, the length of the long-chain base, and the existence of 3-hydroxyl group greatly affected the antitumor activity of α-GalCers [8]. We also examined the effects of the sugar moiety using the glycosphingolipids having various α-sugars, including galactose, glucose, 2-deoxygalactose, 6-deoxygalactose, and several oligosaccharides. It was found that α-GalCer was the best, exhibiting the strongest activity [9–11]. Taking these findings on biological potency and ease of chemical synthesis for a large-scale preparation together, we finally selected a compound coded KRN7000, (2*S*,3*S*,4*R*)-1-*O*-(α-*D*-galactopyranosyl)-2-(*N*-hexacosanoylamino)-1,3,4-octadecanetriol, as a candidate for clinical application (fig. 1).

For a small-scale preparation, KRN7000 was synthesized in 18 steps starting from *D*-galactose, but it was necessary to develop a shorter and simpler process that would be suitable for a large-scale synthesis in order to supply a substantial amount of KRN7000 for preclinical and clinical application. Then we used *D*-lyxose as a chiral starting material to construct the long-chain base moiety. It allowed simple operations for the protection of the hydroxyl groups and direct alkylation [12]. Having established a large-scale synthesis, we next studied detailed biological effects of KRN7000 and its mode of action.

Detailed Biological Activity of KRN7000

Antitumor Activity

KRN7000 (100 μg/kg, i.v.) markedly suppressed tumor growth in mice subcutaneously (s.c.) inoculated with B16 melanoma cells [5, 8]. KRN7000 (0.1–100 μg/kg, i.v.) prolonged the survival period of mice with B16 pulmonary metastasis in a dose-dependent manner [13]. Its potency at 100 μg/kg was stronger than those of mitomycin C (MMC), OK432 and lentinan at their optimal doses [13], the last two of which are biological response modifiers (BRMs).

In addition to the profound antitumor activities in s.c.-implanted and pulmonary metastasis model, most remarkable effects of KRN7000 were evident in hepatic metastasis models. Treatment with KRN7000 (10 ng/kg to 100 μg/kg, i.v.), beginning 1 day after tumor inoculation, significantly inhibited the growth of murine adenocarcinoma Colon26 cells in the liver [14]. The potency of KRN7000 at the dose of 100 μg/kg (i.v.) was nearly equal to that of intraperitoneally (i.p.) administered interleukin (IL)-12 at the dose of about 50 μg/kg (fig. 2), and that was stronger than those of chemotherapeutic agents such as MMC, Adriamycin (ADR) and 5-fluorouracil (5-FU) used at their optimal doses and administration schedules. Moreover, treatment with KRN7000, beginning 3 days after tumor inoculation, could eradicate established Colon26 tumor nodules in the liver, resulting in a high cure rate. Such remarkable effects were observed in other hepatic metastasis models with B16 or murine T lymphoma EL-4 [15, and unpubl. data].

Liver is a major site of metastasis for various types of tumors. In humans, colorectal liver metastasis is especially frequent and lethal. Liver metastasis is seen in 20–25% of patients at the time of colon resection and will eventually develop up to 75% of patients. Most of these patients die within 2 years of diagnosis despite various therapies. Thus, improved treatment of hepatic metastasis is urgently needed. Our results provide the possible application of KRN7000 for the treatment of various types of liver metastasis.

Since immunotherapy is combined, in some cases, with immunosuppressive radiotherapy or chemotherapy to expect the restoration of impaired host immunity and augmentation of therapeutic efficacy, we attempted the combined treatment with KRN7000 and radiation in mice implanted with Colon26 or Meth A tumor cells in their right flank or hind limb. KRN7000 (100 μg/kg, i.v.) in combination with high-dose (20–30 Gy) local irradiation or low-dose (3 Gy) fractionated whole-body irradiation exerted synergistic antitumor effects in Meth A- or Colon26-bearing mice compared with radiotherapy or KRN7000 treatment alone. Besides, 60% of the mice were cured by the combination therapy [16]. These results strongly suggested that the combination of KRN7000 with radiotherapy would be very hopeful for the treatment of cancer patients resistant to radiotherapy or chemotherapy alone.

Immunostimulatory Activity

KRN7000 (1–100 ng/ml) significantly stimulated both allogeneic and syngeneic immune response of murine spleen cells in a concentration-dependent fashion (fig. 3a). Furthermore, it stimulated the proliferation of mononuclear cells (MNC) prepared from human umbilical cord blood and peripheral blood at similar concentrations, thus demonstrating that KRN7000 stimulates the proliferation of murine and human lymphocytic cells [8, 11, 17].

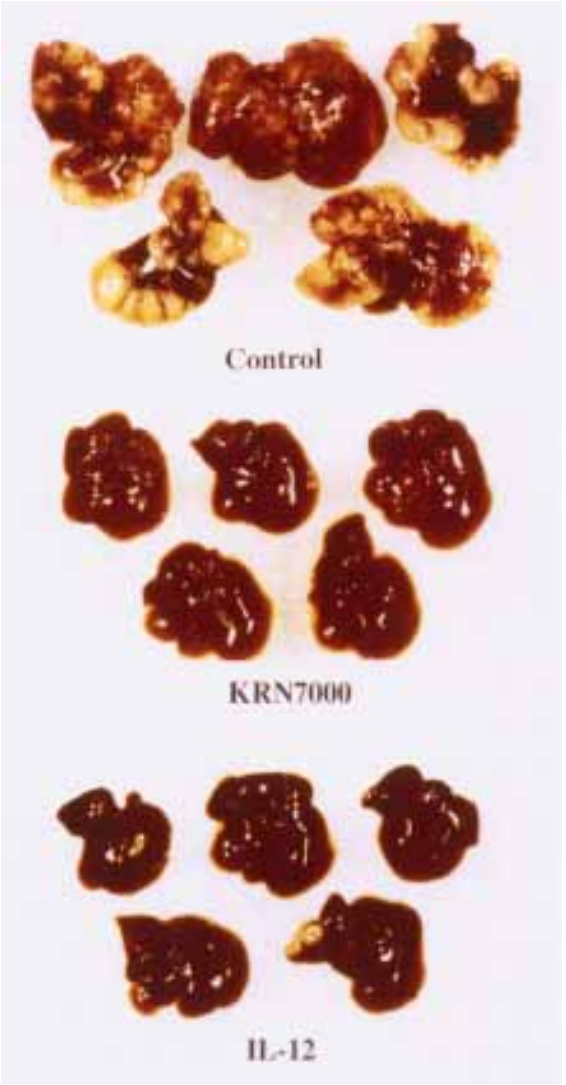


Fig. 2. Antitumor activities of KRN7000 and IL-12 against liver metastasis of Colon26 cells in mice. Antitumor activity of KRN7000 was compared with IL-12 in Colon26 liver metastasis model. Colon26 adenocarcinoma cells (2×10^6 cells/mouse) were intrasplenically injected into female BALB/c on day 0. KRN7000 (100 μg/kg) or IL-12 (1 μg/mouse) was administered i.v. on days 1, 5 and 9, or i.p. on days 1, 3, 5, 7 and 9, respectively. Control indicates no treatment. Macroscopic appearances of each liver on day 18 are shown in photographs.

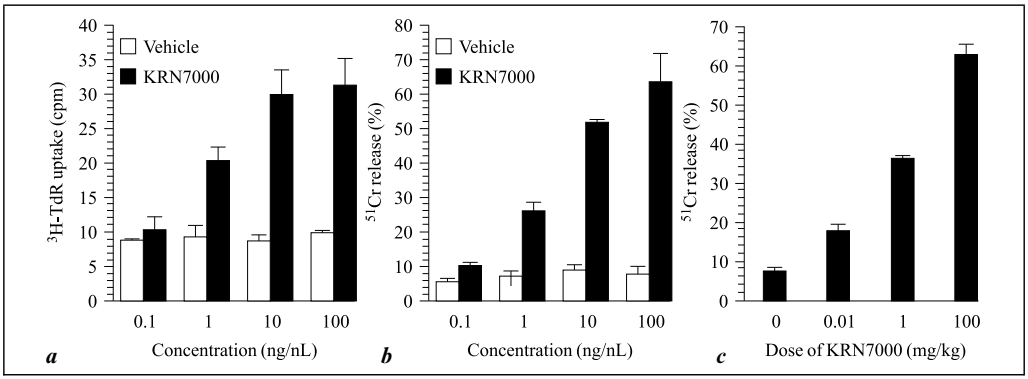


Fig. 3. Effects of KRN7000 on proliferative response and lytic activity of spleen cells. ***a, b*** Proliferative response and lytic activity of spleen cells cultured in vitro with KRN7000 were assessed. Spleen cells obtained from intact BALB/c mice were plated at 2.5×10^5 cells/well and cultured in the presence of various concentrations of KRN7000 or control vehicle solutions. Proliferative responses of spleen cells were assessed after 3 days of culture by pulsing with ^{3}H-TdR for an additional 6 h. Lytic activities of spleen cells against YAC-1 cells were evaluated after 4 days culture by 4 h ^{51}Cr-release method. ***c*** Lytic activities of spleen cells from KRN7000-treated BDF_1 mice were assessed. Two days after in vivo treatment with KRN7000 (0.01, 1 and 100 μg/kg, i.v.), spleen cells from intact mice or KRN7000-treated mice were prepared and used as effector cells. Lytic activity against ^{51}Cr-labeled YAC-1 cells at a E/T ratio of 100:1 were evaluated by 4 h ^{51}Cr-release method.

KRN7000 significantly augmented the lytic activity of murine spleen cells against natural killer (NK)-sensitive YAC-1 cells in a dose-dependent manner at the concentrations of 1, 10 and 100 ng/ml (fig. 3b). This response was parallel to the stimulatory effects of KRN7000 on the spleen cell proliferation (fig. 3a). In addition, when KRN7000 was intravenously (i.v.) administered into mice at the doses ranging from 0.01 to 100 μg/kg, a dose-dependent augmentation of the lytic activity of spleen cells against YAC-1 cells was observed (fig. 3c) [13]. These results demonstrated that KRN7000-triggered immune response led to the induction of killer cells both in vitro and in vivo, and that the antitumor activity of KRN7000 was mediated by host immunity.

Mode of Action of KRN7000

Involvement of Dendritic Cells and NKT Cells in Triggering Immune Response

It is well known that antigen-presenting cells (APCs) such as dendritic cells (DC), macrophages (Mϕ) and B cells are responsible for the initiation

of immune response. To elucidate whether APCs were involved in KRN7000-induced immune response, we examined the effect of KRN7000 on fractionated murine DC, Mϕ and B cells. When we performed syngeneic MLR using splenic DC, splenic or peritoneal Mϕ and splenic B cells as stimulator cells, KRN7000-pulsed DC substantially enhanced proliferative response of naive spleen cells [18]. KRN7000-pulsed splenic Mϕ also stimulated the proliferation of spleen cells, but its stimulatory effect was somewhat lower than that of KRN7000-pulsed splenic DC. In contrast, KRN7000-pulsed peritoneal Mϕ and splenic B cells exhibited quite slight, but apparently detectable, stimulatory effects. These results demonstrated that immunostimulatory activity of KRN7000 was related to its effect on the APCs, preferentially DC.

In our syngeneic MLR assay, splenic responder cells contained various lymphoid cells including T cells, B cells, NK cells and NKT cells, and therefore responder cell types remained to be unclear. In order to identify which type(s) of cells would respond to KRN7000-pulsed DC, we assessed proliferative responses of spleen cells from wild-type littermates having all lymphocyte lineages, NKT-transgenic (NKT^{tg}) mice having only NKT cells, NKT-deficient mice lacking only NKT cells, and $RAG^{-/-}$ (NK) mice having only NK cells. KRN7000-pulsed DC stimulated the proliferation of spleen cells including NKT cells from NKT^{tg} mouse and wild-type, but not those of spleen cells lacking NKT cells from NK mouse and NKT-deficient mouse (fig. 4). Intravenous treatment with KRN7000 at a dose of 100 μg/kg strikingly enhanced splenic killer activity of wild-type and NKT^{tg} mice against YAC-1 cells, whereas no augmentations were observed in NK mouse and NKT-deficient mouse [19]. In addition, KRN7000 almost completely inhibited the growth of B16 tumor cells in the liver of wild-type and NKT^{tg} mice at a dose of 100 μg/kg (i.v.), but failed to inhibit that of NKT-deficient mouse [20]. These results demonstrated that NKT cells, a novel lymphoid lineage, were the major responder to KRN7000-pulsed DC and contributed to tumor rejection.

Involvement of CD1d Molecule in NKT Cells Activation

It appeared that KRN7000-mediated NKT cells activation was restricted by CD1d molecule, a nonclassical antigen-presenting molecule, because preincubation of DC with anti-CD1d monoclonal antibody concentration-dependently inhibited the proliferation of NKT cells induced by KRN7000-pulsed DC [19]. To confirm this finding, we examined whether KRN7000-pulsed murine CD1d-transfectant could activate NKT cell hybridomas. KRN7000-pulsed CD1d-transfectant stimulated IL-2 production from NKT hybridomas, while KRN7000-pulsed parental cells did not. Moreover,

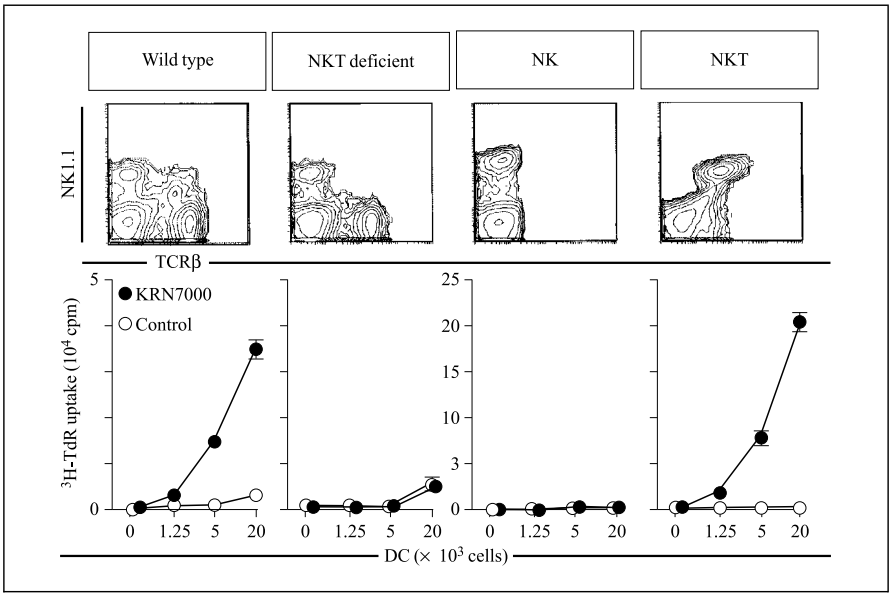


Fig. 4. Selective stimulation of Vα14$^+$ NKT cells by KRN7000. Fluorescence-activated cell sorter (FACS) profiles of spleen cells from wild-type littermates, NKT-deficient mice, RAG$^{-/-}$ NK mice, and NKT^{tg} mice are shown in the upper panel. It is apparent that wild-type littermates all have lymphocyte lineage, NK cells (NK1.1$^+$TCRβ$^-$ cells), T cells (NK1.1$^-$ TCRβ$^+$ cells) and NKT cells (NK1.1$^+$TCRβ$^+$ cells). In contrast, other mice lack or have a limited lymphoid population. The proliferative responses of their spleen cells to KRN7000-pulsed splenic DCs prepared from NK mice are shown in the lower panel.

KRN7000 showed no immunostimulatory activity in CD1 knockout mice in contrast to the marked effects in its control littermate [21]. These results demonstrated that KRN7000 was presented to NKT cells through the CD1d molecule on APCs.

It is of interest to evaluate that KRN7000-mediated and CD1d-restricted NKT activation is applicable to human immune system. Human CD1d molecule is likely to present KRN7000, because it is highly homologous to murine CD1d. In fact, our results clearly revealed that KRN7000 was presented to human NKT cell clones in a CD1d-restricted fashion [22, 23]. More interestingly, both murine and human CD1d-transfectant could activate human NKT cells as well as murine NKT cells by the pulsing with KRN7000 [24]. These results demonstrated that CD1d-mediated recognition of KRN7000 by NKT cells was highly conserved between mouse and humans, and suggested the potential application for clinical use.

Involvement of Cytokines in the Augmentation of Immune Response

It is known that several cytokines such as IL-2, IFN-γ and IL-12 are released from DC, NKT cells or other lymphocytes and activate killer cells to eliminate tumor cells. We therefore examined whether KRN7000 has an ability to induce both cytokines in vivo and in vitro. It was found that circulating IL-2 and IFN-γ levels were extremely elevated by KRN7000 treatment (100 μg/kg i.v.) [15], and that concentrations of both cytokines in the supernatant of whole spleen cells culture and mixed cultures consisting of KRN7000-pulsed DC and naive T cells fraction markedly increased upon stimulation with KRN7000 [18]. In syngeneic MLR using KRN7000-pulsed DC and spleen cells from NKT mice, large amounts of IFN-γ and IL-4 could be secreted from NKT cells in the presence of KRN7000-pulsed DC [19]. Furthermore, it was clearly demonstrated that the interaction between NKT cells and DC led to IL-12 secretion from DC through CD40-mediated signal pathway [25, 26]. Thus, these cytokines seemed to contribute, at least to a substantial extent, to augment antitumor immune response and to induce other killer cells than NKT cells.

Involvement of Antitumor Effector Cells

In order to clarify the effector mechanism, we assessed which phenotypes of killer cells were activated by KRN7000. Since KRN7000 proved to be most effective in the liver metastasis model, our aim was focused on liver-resident killer cells. Hepatic MNC prepared from mice treated with KRN7000 (100 μg/kg, i.v.) showed a strong lytic activity against YAC-1 and Colon26 cells, although hepatic MNC of the control mice showed little or no lytic activity. Based on the results that KRN7000 enhanced the lytic activity against NK-sensitive tumors, we sorted hepatic NK ($NK1.1^+CD3^-$) cells from hepatic MNC activated by KRN7000 and evaluated their lytic activity against Colon26 cells. The sorted NK cells showed marked lytic activity, and their potency was similar to the parental MNC [14]. In addition, lytic activity of liver-associated Mϕ (Kupffer cells) was significantly augmented by in vivo treatment with KRN7000 (100 μg/kg, i.v.) [27]. Moreover, cured mice by the treatment with KRN7000 in the liver metastasis model rejected secondly s.c. challenged same tumor, but not another syngeneic tumor, thereby demonstrating that cured mice systemically acquired tumor-specific immunity. All these results implied that tumor cells metastasizing into the liver were completely eliminated by nonspecific killer cells (NK cells, Kupffer cells and presumably NKT cells) as well as by tumor-specific killer cells induced by KRN7000.

Postulated Immunostimulatory and Antitumor Mechanisms

Taking all these findings together, the following hypotheses for the antitumor activity of KRN7000 have been proposed: (1) NKT cells recognize

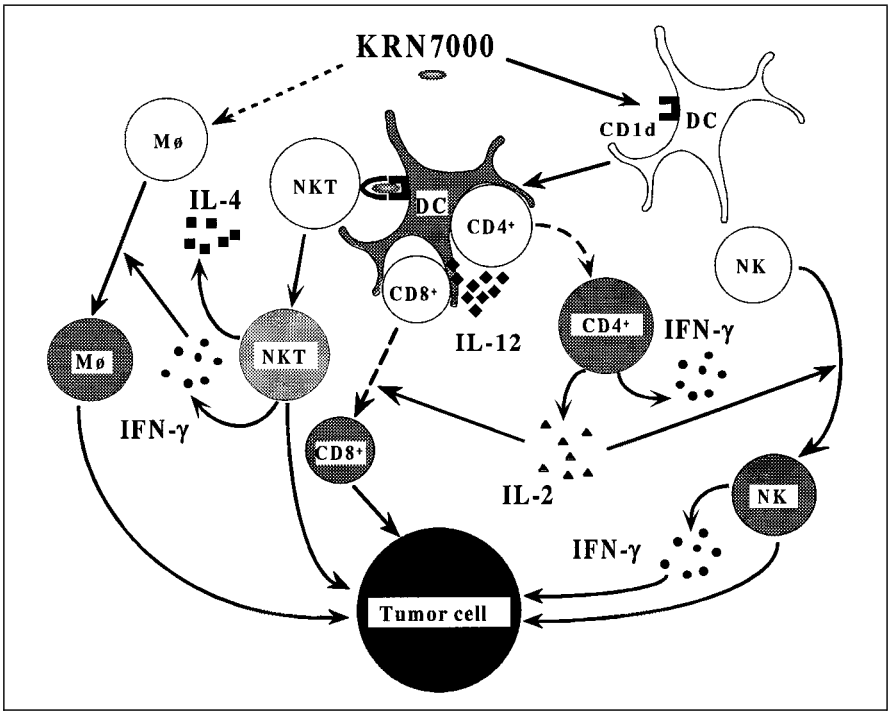


Fig. 5. Proposed mechanism for the immunostimulatory activity of KRN7000 (unshaded cells: naive cells/shaded cells: activated cells).

KRN7000 in the context of CD1d molecule and the interaction between NKT and APCs results in functional activation of both cells. (2) IL-12 and IFN-γ are secreted from APCs and NKT cells upon the stimulation with KRN7000 and secondly activate NK cells, Mϕ and T cells. (3) Activate NKT cells, NK cells and Mϕ, nonspecifically killed tumor cells. (4) Tumor-specific $CD8^+$ CTLs, which are presumably primed by tumor antigen-presenting DC and some cytokines, are involved in the late phase of tumor rejection and persistent tumor-specific memory (fig. 5).

Toxicity of KRN7000

Preclinical studies using mice, rats and monkeys have demonstrated no vital or treatment-related changes in the mortality, general vital signs, or hematological and biochemical parameters by the administration of KRN7000, even at the relatively high dose of 2,200 μg/kg for 28 days [unpubl. data].

Clinical Trial of KRN7000

Based on all the data mentioned above, a clinical trial of KRN7000 is underway in patients with various types of solid tumors.

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Zoanthamines, Antiosteoporotic Alkaloids

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Introduction

Zoanthamine (**1**) (fig. 1), a new class of alkaloid, was first isolated by Rao et al. [1] from an Indian zoanthid *Zoanthus* sp. which also contained zoanthenamine (**2**) and zoanthamide (**3**) [2]. Interestingly, zoanthamines inhibited the phorbol myristate acetate (PMA)-induced inflammation in the mouse ear. Zoanthaminone (**4**), another zoanthamine-type alkaloid, was later obtained from an Arabian Sea species of the genus *Zoanthus* [3].

More recently, the authors' group isolated five new zoanthamine alkaloids, norzoanthamine (**5**), oxyzoanthamine (**6**), norzoanthaminone (**7**), cyclozoanthamine (**8**), and epinorzoanthamine (**9**) from a Japanese *Zoanthus* sp. [4] They inhibited the growth of P388 murine leukemia cells with IC_{50} values of 24, 7.0, 1.0, 24 and 2.6 μg/ml, respectively. More significantly, norzoanthamine inhibited production of interleukin-6 (IL-6) in preosteoblastic MC3T3-E1 cells [5].

Although zoanthamines have been regarded as terpenoids based on their structures, they are most likely to be synthesized through a polyketide biosynthetic pathway [6]. In fact, zooxathellamine (**10**) isolated from a symbiotic dinoflagellate *Symbiodinium* sp. has been suggested to be a polyketide from a feeding experiment [7].

Recently, we have discovered that norzoanthamine shows antiosteoporotic activity as described in this chapter.

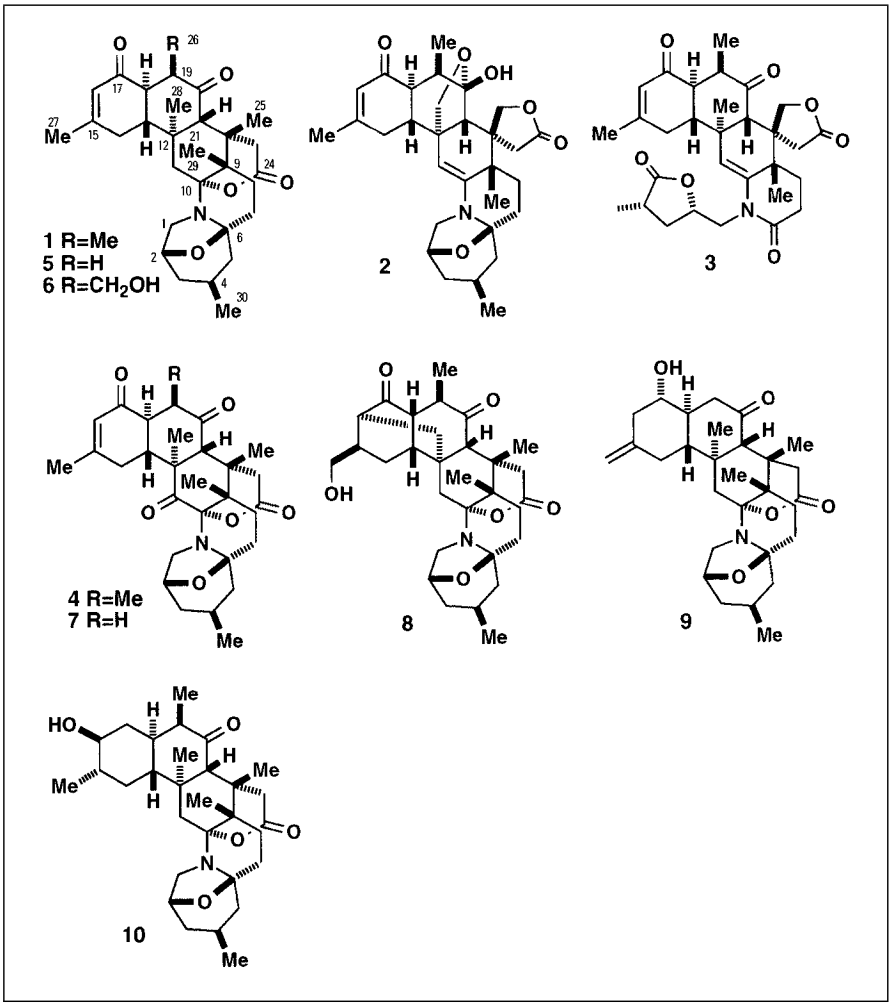

Fig. 1. Structures of zoanthamine alkaloids.

Bioassay System

Norzoanthamine and norzoanthamine hydrochloride inhibit IL-6 induction in preosteoblastic MC3T3-E1 cells stimulated by parathyroid hormone at concentrations of 13 and 4.7 μg/ml, respectively [5]. IL-6 is known to stimulate osteoclast formation, and therefore suppression of IL-6 secretion would prevent osteoporosis. Osteoporosis is caused by an imbalance between bone resorption and bone formation, which results in bone loss and fractures after

mineral flux. The frequency of fracture is significantly increased in osteoporosis; hip fracture in senile patients is a very serious problem, because it often limits their quality of life. Therefore, in addition to prevention of losing bone mass, the maintenance of bone mechanical strength is very important [8].

To discover candidates for new antiosteoporotic drugs, simple and sensitive in vitro assay systems are essential. Although a variety of in vitro screening systems for antiosteoporotic drugs have been developed, the results of in vitro experiments do not always match those of in vivo tests. Furthermore, there is no in vitro assay system to evaluate the mechanical strength of bone and its morphology. Thus, we have developed an in vivo assay system using an osteoporosis model in mice [9]. This postmenopausal osteoporosis model using ovariectomized mice is useful for evaluating antiosteoporotic drugs, since several parameters are clearly decreased within 4 or 5 weeks after the ovariectomy. The effects of a drug can be evaluated in terms of bone mass, biomechanical properties, and bone morphology in this model.

During the in vivo screening of antiosteoporotic drugs, we found that norzoanthamine hydrochloride shows a potent suppressive effect on osteoporosis in ovariectomized mice.

Ovariectomized Mice

Four-week-old female ddY mice were maintained at 25 ± 0.5 ºC with commercial diet. Ovariectomies and sham operations were carried out under ketamine hydrochloride anesthesia. The mice were divided into 5 groups: sham-operated, ovariectomized control, and norzoanthamine hydrochloride-treated ovariectomized groups (0.08, 0.4, 2 mg/kg/day, p.o.). Each group consisted of 5 mice. The vehicle or norzoanthamine hydrochloride dissolved in sterilized water was administered 5 days a week for 4 weeks, beginning the day after the operations. After 4 weeks, all mice were weighed and sacrificed to retrieve femurs, humeri and uterus. The right femurs were used to measure length and dry weight (dried at 60 ºC for 24 h), while the left femurs were used to measure failure load and yield energy. The right humeri were used for the observation of bone morphology.

Effect on Body Weight and Uterine Weight

The ovariectomy, norzoanthamine hydrochloride, and 17β-estradiol did not affect the body weight in our experiments. Although 17β-estradiol significantly and dose-dependently increased the uterine weight, which was significantly reduced by ovariectomy, norzoanthamine hydrochloride did not affect the uterine weight (fig. 2). This uterine atrophy was caused by the depletion of the estrogen supplied by the ovary, thus indicating that the ovary had been completely removed by the operation. The uterine hypertrophy caused by 17β-

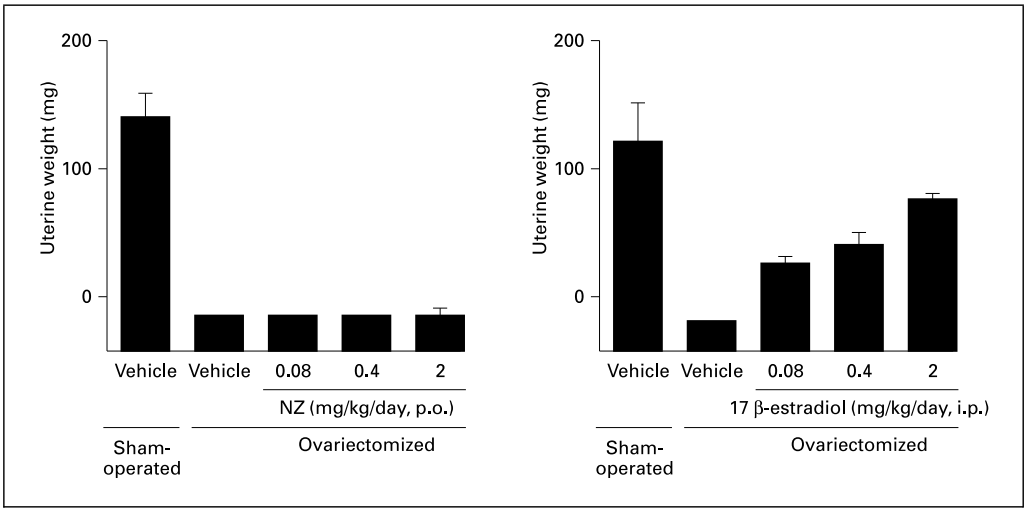

Fig. 2. Effects of norzoanthamine hydrochloride and 17β-estradiol on uterine weights.

estradiol was a side effect on reproductive organs, while norzoanthamine hydrochloride did not have an estrogen-like side effect on the reproductive organs. 17β-Estradiol is known to regulate bone metabolism by binding to estrogen receptors. Since norzoanthamine hydrochloride did not have an estrogen-like effect on reproductive organs, it should regulate bone metabolism like estrogen through a process different from that of 17β-estradiol. It is possible that norzoanthamine hydrochloride is a selective agonist of estrogen receptor β [10].

Effect on Femoral Length and Femoral Weight

Although norzoanthamine hydrochloride did not affect femoral length, it significantly affected femoral weight. The administration of norzoanthamine hydrochloride significantly suppressed the loss of bone weight caused by ovariectomy at doses of 0.08–2 mg/kg/day (p.o.) (fig. 3). Similarly, 17β-estradiol did not affect femoral length but did suppress the loss of bone weight caused by ovariectomy at a dose of 0.08 mg/kg/day (i.p.) (fig. 3). It did not suppress bone loss at a dose of 0.4 mg/kg/day. This could be explained by downregulation of the estrogen receptor.

Furthermore, failure load and yield energy of the femoral were maintained by the administration of norzoanthamine hydrochloride at a dose of 0.4 mg/kg/day (p.o.), respectively (fig. 4). 17β-Estradiol exhibited effects similar to those of norzoanthamine hydrochloride on the failure load and the yield energy at doses of 0.016–0.4 mg/kg/day (i.p.).

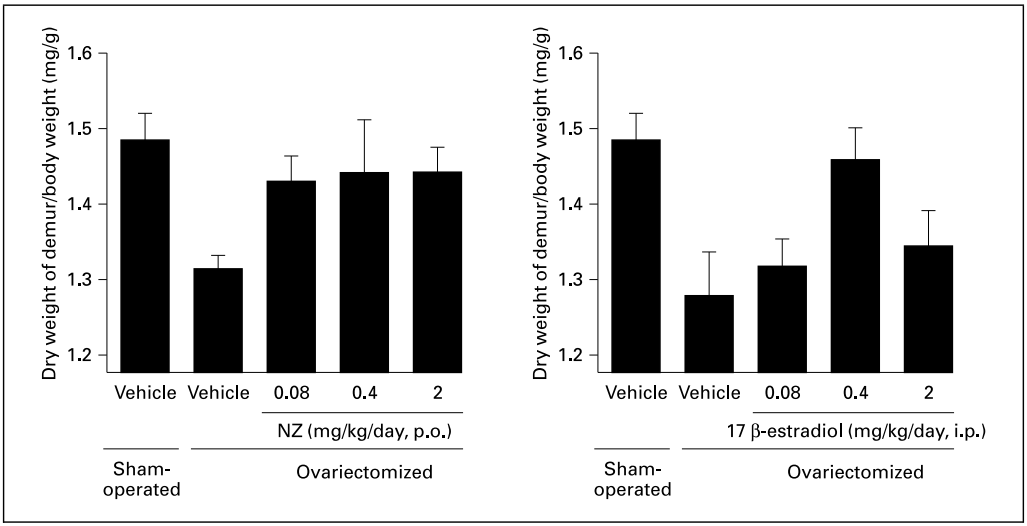

Fig. 3. Effects of norzoanthamine hydrochloride and 17β-estradiol on femoral weights.

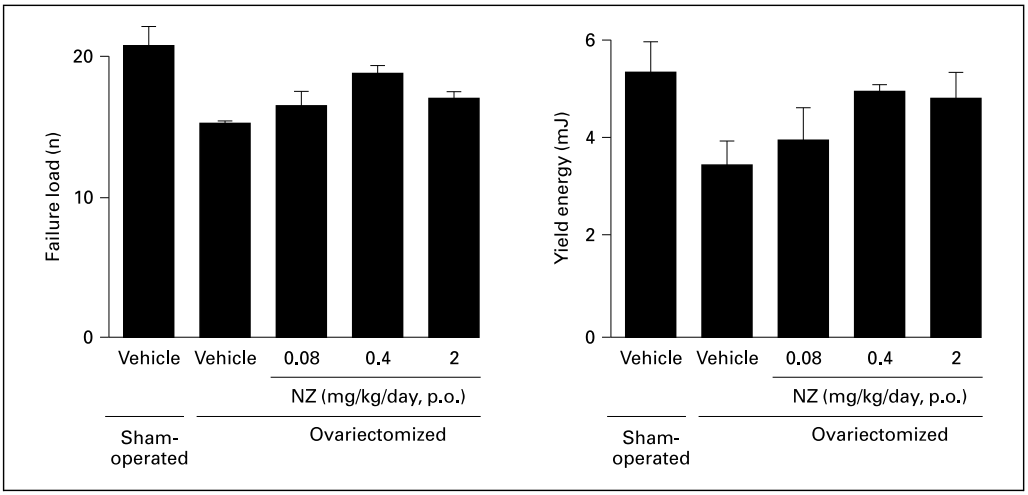

Fig. 4. Effect of norzoanthamine hydrochloride on failure load and yield energy.

Effect of Norzoanthamine Hydrochloride on Bone Morphology

Dried humeri were ground from the back using a fine grindstone. The ground humeri were then washed with water, acetone, and bleaching solution containing 0.5% sodium hypochlorite. The humeri were dried again and observed under a microscope. The thickness of the cortical bone was measured from a photograph of the ground bone (fig. 5).

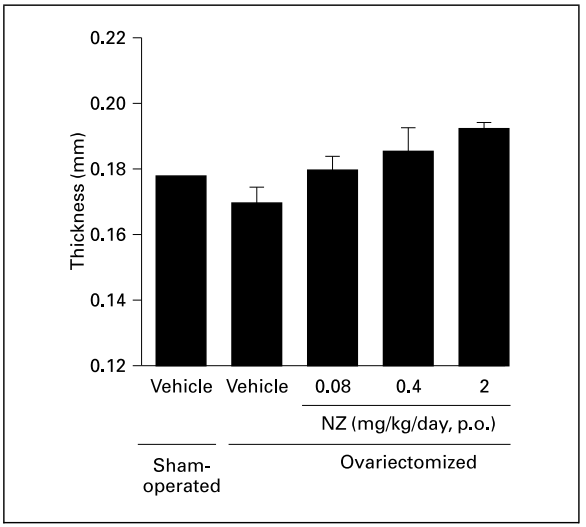

Fig. 5. Effect of norzoanthamine hydrochloride on thickness of cortical bone.

Ovariectomy caused a decrease in the humeralis trabeculae. Norzoanthamine hydrochloride significantly suppressed this decrease in a dose-dependent manner (fig. 6C–E). In ovariectomized mice treated with norzoanthamine hydrochloride, the primary spongiosa was not significantly increased and the morphology of the metaphysis was nearly normal.

Furthermore, norzoanthamine hydrochloride increased the thickness of the cortical bone in the humeralis diaphysis (fig. 5). Thus, norzoanthamine hydrochloride had protective effects on both the trabecular and cortical bone in the humerus.

Activity of Norzoanthamine against Osteoporosis and Structure-Activity Relationships

Studies on structure-activity relationships of norzoanthamine have been done to obtain more active derivatives. The presence of the double bond (C15–16) and lactone moiety has attracted our attention. Therefore, norzoanthamine was transformed to several derivatives (**11, 12, 13, 14, 15, 16** and **17**) (fig. 7). Norzoanthamine (**5**) was reduced with $NaBH_4$ to furnish deoxynorzoanthamine (**11**) and deoxydihydronorzoanthamine (**12**), the latter of which was treated with acetic anhydride and pyridine to afford monoacetate **13**. Norzoanthamine (**5**) was reduced with H_2 on Pd/C to obtain 15,16-dihydronorzoanthamine (**14**). Oxidation of **5** with OsO_4 followed by treatment with $NaHSO_3$ gave the desired 15,16-dihydroxy-15,16-dihydronorzoan-

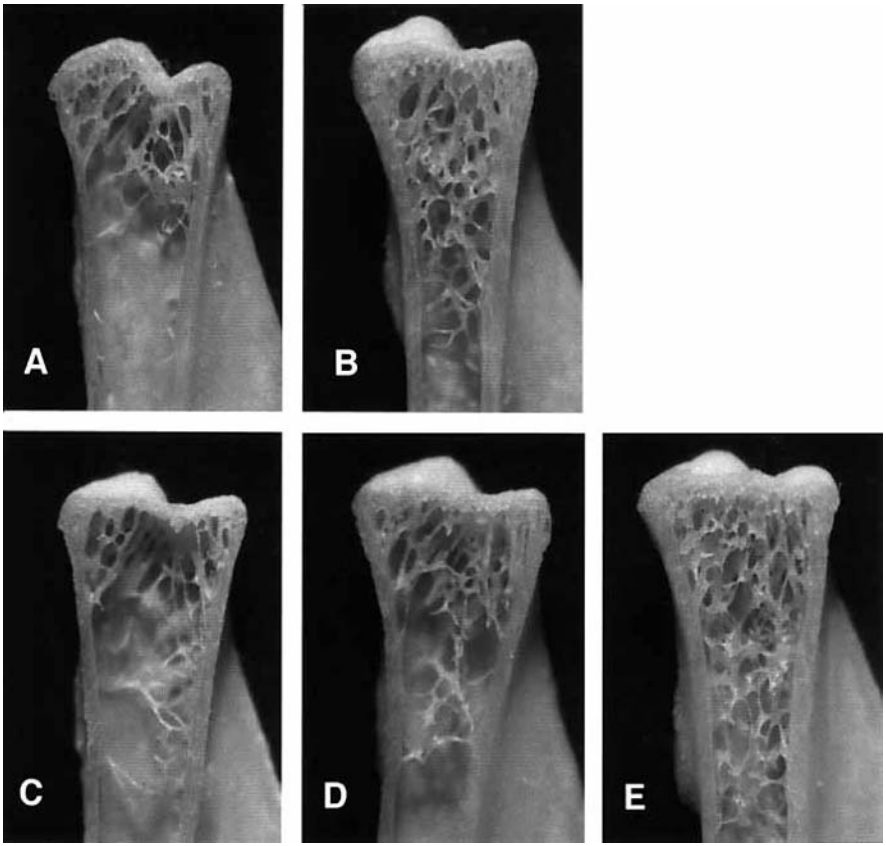

Fig. 6. Effect of norzoanthamine hydrochloride on humeralis morphology in ovariectomized mice. ***A*** Ovariectomized mouse treated with vehicle. ***B*** Sham-operated mouse treated with vehicle. ***C–E*** Ovariectomized mouse treated with norzoanthamine hydrochloride at doses of 0.08, 0.4 and 2.0 mg/kg/day, respectively. Each mouse was treated for 4 weeks after the operation.

thamine (**15**). When norzoanthamine was treated with $NaBH_3CN$, the carboxylic acid (**16**) was obtained. Methyl ester **17** was obtained by treatment of **5** with CH_3I-Ag_2O. Inhibition of IL-6 production was tested for these derivatives; the activity of norzoanthamine was reduced by transformation as shown in table 1. These data indicated the importance of the double bond (C15–C16) and lactone moiety for the activity. Elucidation of the target molecule will provide new ideas for the discovery of novel antiosteoporotic agents. Further detailed studies with norzoanthamine hydrochloride will be needed.

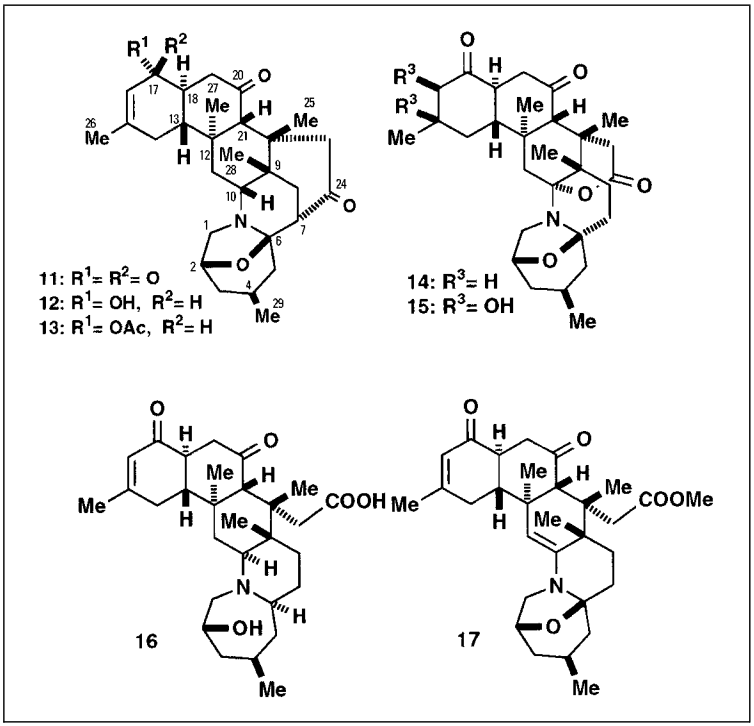

Fig. 7. Structures of norzoanthamine derivatives.

Table 1. Inhibition of IL-6 induction in MC3T3-E1 cells with norzoanthamine and its derivatives

Compound	IC_{50}, μg/ml
Norzoanthamine (**5**)	13
11	~25
12	30
13	23
14	45
15	35
16	42
17	>100

Conclusion

Norzoanthamine hydrochloride significantly suppressed the decrease in bone weight, failure load and yield energy caused by ovariectomy through an increase in trabecular and cortical bone. Unlike 17β-estradiol, norzoanthamine hydrochloride did not affect the uterine weight. However, it exhibited the effect

on bone morphology similar to that of 17β-estradiol in ovariectomized mice. Norzoanthamine hydrochloride is expected to act through a mechanism different from those of known antiosteoporotic drugs.

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Symbiotic Bacteria in Sponges: Sources of Bioactive Substances

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Introduction

Sponges are considered to be the most primitive metazoans (multicellular animals) because they do not possess true tissues or organs. When they appeared in the fossil record more than 500 million years ago, prior to the rise of scleractinian corals, they were often found in association with bacterial mats in reef-like formations [1]. Although the early association of sponges with bacteria may in part explain their apparent tolerance of bacterial symbionts, it does little to explain the observed selectivity of sponge-bacterial symbioses. Chemistry may well hold the key to understanding this interesting phenomenon.

Traditionally, the role of symbiotic microorganisms in sponges was considered to be related to their ability to recycle nutrients or, in the case of cyanobacteria and other chemoautotrophic bacteria, to supplement the diet of the sponge by fixing carbon and nitrogen [2]. Our current interest in bacteria associated with sponges stems from the possibility that the bacteria may produce some of the potential pharmaceuticals that have been isolated from sponges. Assuming that some of these potential pharmaceuticals are the same bioactive compounds that enhance the ability of sponges to compete with other organisms, symbiotic bacteria may also have played an important role in the successful evolution of the host sponge. If a bacterium from a sponge can be shown to produce a bioactive compound, there is an implication of a symbiotic relationship in which the sponge tolerates or encourages bacteria to grow within its tissues in return for some degree of chemical protection. It

is the concept of mutual benefit and the consistency of the relationship that separates the symbiotic bacteria from all other bacteria that may be more loosely associated with the sponge.

In recent years, it has been fashionable for marine natural products chemists to propose that certain compounds and classes of chemicals are produced by symbiotic bacteria. While they may indeed be correct in some cases, the arguments used to support their proposals are often simplistic and require closer examination. Is it prudent to assume that a sponge metabolite was produced by a bacterial symbiont just because the structure of the sponge metabolite resembles that of a metabolite from a cultured bacterium, which has usually been isolated from a terrestrial source? While these observations might be helpful in designing an experimental program, they are by no means sufficient to define a bacterial source for a sponge metabolite. Other tests must be applied before assigning the source of a natural product to a symbiont. The most obvious is the requirement that the host contains symbiotic microorganisms and that they be present in sufficient quantity to account for the amount of natural product isolated. The abundance of bacteria is usually estimated using transmission electron microscopy, which is not always available to natural products chemists. However, the abundance of photosynthetic microorganisms such as cyanobacteria can be inferred from the chlorophyll content of the crude extract of a sponge. Since most sponges contain some microorganisms, the most difficult task is to distinguish symbiotic microbes from those being consumed by the sponge and those that are surface contaminants. From a practical viewpoint, when any microorganism is found in high abundance and in the same relative location within the tissues of several specimens of the same sponge species, one may assume a stable, probably symbiotic, association between the microorganism and the sponge. However, the mutual adaptation of the sponge/microbe association that defines a symbiotic relationship also suggests that symbionts will be difficult, if not impossible, to culture in the absence of the host.

Sponge Metabolites Produced by Cultured Microorganisms

There is no doubt that the most powerful proof that symbionts can produce sponge metabolites would be to isolate and culture the symbiont responsible for the production of a unique bioactive natural product and then demonstrate metabolite production in culture. A critical examination of the literature suggests that this has not yet been accomplished, although there is reason to believe that some examples will soon appear [3]. The isolation of diketopiperazines, especially those containing proline, from both sponges and

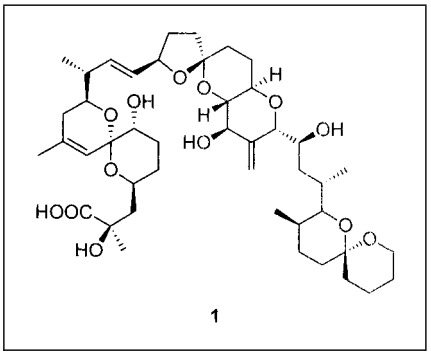

Fig. 1. Okadaic acid (**1**).

marine bacterial cultures suggested, perhaps correctly, that the sponge metabolites might be produced by bacteria [4]. However, no evidence was presented to support a specific association between the bacteria and the sponge. As further studies have been performed, the lack of specificity has become very apparent since the same diketopiperazines have been found, although seldom reported, in many other bacterial and fungal cultures and in other sponges [5]. We now believe that the diketopiperazines are common metabolites that arise during the catabolism of proteins and cannot be considered specific marker compounds.

The sponges *Halichondria okadai* from Japan and *Halichondria melanodocia* from the Caribbean both contain okadaic acid (**1**) (fig. 1) [6], which is produced by dinoflagellates of the genus *Prorocentrum* [7, 8]. It is extremely unlikely that the sponges also produce okadaic acid. Instead, it is proposed that the sponges concentrate okadaic acid produced by dinoflagellates with which they coexist in shallow habitats. Since there is currently no evidence of a symbiotic relationship between the sponge and the dinoflagellate, okadaic acid is best regarded as a dietary-derived metabolite until a definitive study has been performed.

A Chemistry-Based Strategy for the Study of Symbiotic Associations

In order to increase the probability of culturing symbiotic organisms that produce bioactive metabolites, we argued that it would first be necessary to identify the symbiont so that an appropriate culture medium could be selected. This led us to propose a logical series of experiments to reach that goal. We reasoned that the first task was to choose a bioactive metabolite that could readily be identified and that was suspected to be of microbial origin. We

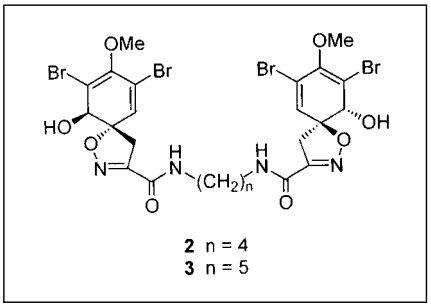

Fig. 2. Aerothionin (**2**) and homoaerothionin (**3**).

could then use a variety of methods to determine which type of cell contained the metabolite. If the metabolite was contained within a specific microbial cell type, the next step would be to isolate the symbiont and perform a 16S rRNA study. An analysis of the 16S rRNA sequence would then allow the symbiont to be classified and the microbes with the closest sequences identified. Application of the culture methods used to grow related organisms would offer the best hope for culturing the symbiont. Although this is a lengthy process, it may be preferable to the shotgun approach of isolating bacteria in a random manner, particularly if the symbiont has highly specialized growth requirements.

Chemical Studies of Symbiosis in Sponges

Our first study of the localization of sponge metabolites was undertaken during an interdisciplinary study of the chemical ecology of *Aplysina fistularis*. Thompson and Walker [9] measured the exudation rates of aerothionin (**2**) and homoaerothionin (**3**) both under natural conditions and when stressed by a simulated attack (fig. 2). They found a 100-fold increase in the rate of exudation of brominated metabolites during the simulated attack. This was in part explained when it was shown that the spherulous cells, found predominantly near the exhalent canals, contained much higher concentrations of bromine, presumably due to the brominated metabolites, than did other sponge cell types or bacterial cells [10]. We proposed that external pressure applied during a real or simulated attack caused the spherulous cells to rupture and release the brominated metabolites into the exhalent canals. In hindsight, we now recognize that *A. fistularis* contained remarkably few bacterial cells and that it would not be logical to suggest that the brominated metabolites, which are major metabolites of the sponge, could be produced by bacteria.

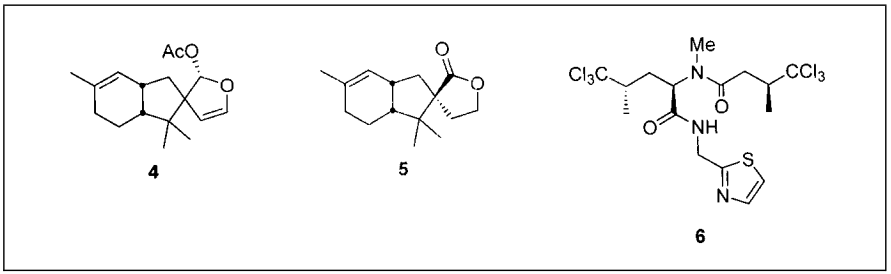

Fig. 3. Spirodysin (**4**), herbadysidolide (**5**), and 13-demethylisodysidolide (**6**).

When we first started our studies to determine if sponge metabolites were produced by symbiotic microorganisms, we realized that it would be important to choose a chemically rich sponge that contained large populations of a symbiont that had already been studied and defined by marine biologists. *Dysidea herbacea* provided us with a nearly perfect model system since it contains the cyanobacterial symbiont *Oscillatoria spongelliae* to the extent of 50% of the cellular volume [11]. Furthermore, specimens of *D. herbacea* could be subdivided into two major chemotypes, one of which contained mixtures of terpenes and polychlorinated amino acid derivatives while the other contained large quantities of polybrominated biphenyl ethers and no terpenes. We first examined specimens of *D. herbacea* from the Great Barrier Reef that contained the sesquiterpenes spirodysin (**4**) and herbadysidolide (**5**) and the chlorinated amino acid derivative 13-demethylisodysidenin (**6**) (fig. 3). Attempts to separate the various freshly dissociated cell types by centrifugation on a Ficoll density gradient did not provide clean separations. However, a significant breakthrough in cellular localization studies came when we found that metabolites could be recovered in good yield from cells that had been fixed with formaldehyde or glutaraldehyde. After disruption of the sponge and fixation of the dissociated cells, the cyanobacterial cells were separated from all other cell types using a cell sorter to detect and separate those cells containing fluorescent photosynthetic pigments. 13-Demethylisodysidenin (**6**) was identified as a major metabolite of the fraction containing the fluorescent cyanobacterial cells. The nonfluorescent fraction, which consisted of predominantly sponge cells together with some bacterial cells, contained the sesquiterpenes spirodysin (**4**) and herbadysidolide (**5**) [12].

We next examined a specimen of *D. herbacea* from Palau that contained the brominated biphenyl ether **7** in high yields of 5–12% dry weight (fig. 4). Using the same cell separation methods, we found that **7** was located in the cyanobacterial cells and not in the sponge cells. However, examination of

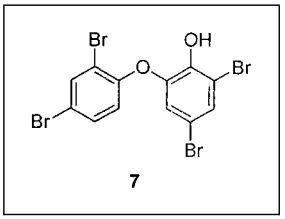

Fig. 4. 2′,4,4′,6-Tetrabromo-2-phenoxyphenol (**7**).

sponge sections by polarized light microscopy revealed crystals of the brominated biphenyl ether **7** situated just below the surface of the sponge. This suggested that the cyanobacterium had been actively excreting the brominated metabolite, which crystallized on exposure to seawater [13].

This research raised almost as many questions as it answered. In particular, why do the two specimens of *D. herbacea*, both of which appear to contain the same symbiotic cyanobacterium *O. spongelliae*, contain such different metabolites? The collecting site is irrelevant since specimens of both chemotypes are found at both locations. A more likely explanation is that the cyanobacteria are either different strains of *O. spongelliae* or even different species. The most convenient method to distinguish between these possibilities will be comparison of the 16S rRNA sequences for the two cyanobacteria.

Garson's group [14] has recently confirmed the major conclusions of our study but their studies have added two important new observations. They found that spirodysin (**4**) was concentrated in sponge cell preparations containing archaeocytes and choanocytes and that chlorinated diketopiperazines were located in cyanobacterial cell fractions. However, after separation on a second Percoll gradient, they isolated one cyanobacterial fraction that lacked chlorinated metabolites, which may add further support to the concept that *D. herbacea* might contain multiple strains or species of cyanobacteria.

We next studied the role of symbiotic microorganisms in the production of the antifungal cyclic peptides and cytotoxic macrolides ascribed to lithistid sponges. We selected *Theonella swinhoei* as our study organism because the chemistry appeared to be consistent and well defined and also because filamentous microorganisms, tentatively identified as cyanobacteria, had been observed within the sponge. Although it was later determined that the chemistry was not as consistent as we first imagined and that the filaments were not cyanobacteria, our choice of study organisms was a fortunate one.

The cyclic peptides found in two specimens of *T. swinhoei* from the Philippines and from Palau differed slightly from each other and from the cyclic peptide described previously by Matsunaga and Fusetani's group [15]. Theonegramide (**8**) (fig. 5) from Negros Island, the Philippines, was the first example

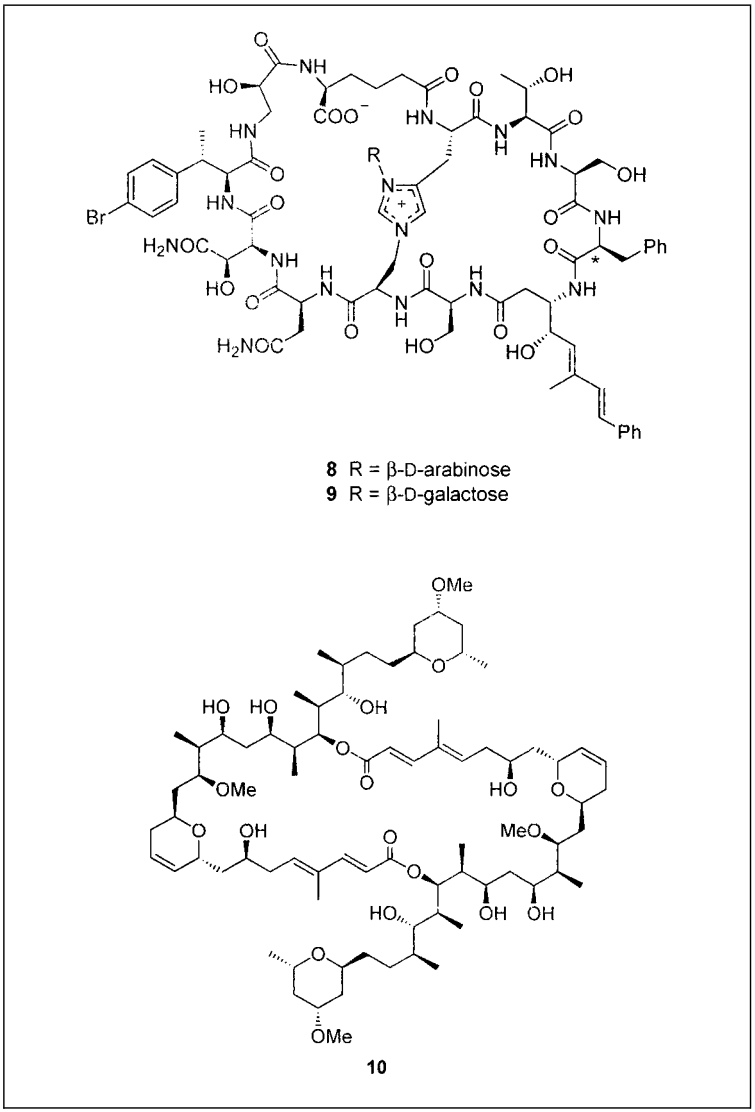

Fig. 5. Theonegramide (**8**), theopalauamide (**9**), and swinholide A (**10**).

of a glycopeptide from a lithistid sponge [16]. The major difference between theopalauamide (**9**) from a Palauan specimen of *T. swinhoei* and theonegramide (**8**) was in the identity of the sugar unit but in addition, theopalauamide underwent an annoying but interesting isomerization about one of the Cα-CO bonds (*) [17]. As it turned out, the isomerization of theopalauamide did not

present a serious impediment to our studies of the cellular localization of metabolites from the Palauan *T. swinhoei*.

T. swinhoei from Palau is a good source of both theopalauamide (**9**) and swinholide A (**10**). Within the lithistid sponges, Bewley [18] had noted a strong correlation between the isolation of antifungal cyclic peptides containing aromatic β-amino acids and the presence of filaments within the sponge. Since the same class of cyclic peptides had also been isolated from many cyanobacteria, it was not surprising that some chemists would assume that the filaments were in fact filamentous cyanobacteria. However, this made no sense when one considered that the architecture of lithistid sponges, which often grow in caves and other shaded locations, prevents light from entering the interior of the sponge, thus depriving photosynthetic cyanobacteria of their energy source. We therefore argued that the filaments were unlikely to be cyanobacteria and since they contained no photosynthetic pigments, they could not be separated with a cell sorter using fluorescence detection.

Bewley et al. [19] were able to separate *T. swinhoei* into four fractions containing different cell types by using differential centrifugation. The first step was to peel off the outer layer of the sponge (the ectosome) and isolate the true cyanobacteria (*Aphanocapsa feldmanni*). The cells from internal tissues (the endosome) were dissociated, fixed and then separated by differential centrifugation into fractions which contained, in order of decreasing density, sponge cells, the filaments and unicellular bacteria. Each fraction was subjected to repeated centrifugation in order to obtain products having satisfactory enrichment. The fractions were thoroughly washed to remove excess fixative and then extracted with appropriate solvents to yield extracts that were analyzed by HPLC coupled with UV spectrometry and by ^{1}H-NMR spectroscopy. Both methods of analysis showed unambiguously that theopalauamide (**9**) was localized in the filaments and that swinholide A (**10**) was associated with the fraction that contained many types of unicellular bacteria. The sponge cells and unicellular cyanobacteria were devoid of these metabolites.

The next step along our strategic pathway was to identify the filaments. Examination of the filaments using transmission electron microscopy clearly showed that the filaments were not normal cyanobacteria because they lack the characteristic thylakoid structures. This observation is in good agreement with the absence of photosynthetic pigments in extracts of the filaments. Comparison of electron micrographs of the filaments with the ultrastructure of other filamentous nonphotosynthetic prokaryotes suggested a strong resemblance to filamentous gliding bacteria of the family Beggiatoaceae. There was, however, no real evidence to support this assignment. We then turned to analysis of the 16S rRNA gene sequence. After experiencing some initial difficulty in performing PCR amplification of the bacterial DNA from the

filaments, we devised a method using denaturing gradient gel electrophoresis and specific PCR primers to amplify and sequence the 16S rRNA gene and obtained almost the entire sequence (1,350 bp). This sequence did not show a close relationship to 16S rRNA sequences in the Genbank and Ribosomal Database Project databases. When 1,126 alignable characters were used for bootstrapping, the symbiont was grouped among the δ-proteobacteria as a sister taxon to Myxococcales with a bootstrap value (100 replicates) of 88%. The theopalauamide-containing filamentous symbiont has been assigned the taxonomic status '*Candidatus* Entotheonella palauensis' [20]. Starting from culture conditions appropriate for δ-proteobacteria we developed a culture medium that allowed the filaments to survive but not reproduce, which is an improvement over the majority of culture conditions that caused the filaments to rapidly lyse. Additional experiments are in progress.

Marine Natural Products Localized in Sponge Cells

It would be wrong to create the impression that biologically active metabolites from sponges are commonly produced by symbiotic microorganisms. Although chemists frequently speculate that marine natural products may be produced by associated microorganisms, it is probable that a minority of sponge metabolites will be shown to be produced by symbionts. An increasing number of marine natural products have been shown to be localized in sponge cells [9, 12, 21–26]. We investigated one such example in detail.

Pyridoacridine alkaloids, many of which are cytotoxic, have been reported from sponges, ascidians, certain molluscs, and one coelenterate [27]. While the pyridoacridine alkaloids found in the mollusc *Chelynotus semperi* were undoubtedly obtained from an ascidian dietary source [28], their distribution across three other phyla led to the suggestion that the alkaloids might be produced by the same or similar microorganisms associated with the different hosts. In order to test this hypothesis, we investigated the localization of dercitamide (**11**) (fig. 6) in the sponge *Oceanapia sagittaria* using the natural fluorescence of dercitamide to detect its presence in cells. Dercitamide (**11**), a representative pyridoacridine alkaloid that has been found in both sponges and an ascidian (under the name kuanoniamine C) [28], exhibits a pH-dependent UV-visible spectrum and changes color from yellow at pH 7.0 to red at pH 6.0. The color change was readily observed using a light microscope when a thick section of the sponge was acidified by exposure to trifluoroacetic acid (TFA) vapor. Unlike several other pyridoacridines that we have screened, dercitamide also has a pH-dependent fluorescence spectrum that allowed its detection in thick sections using fluorescence microscopy. Although the color

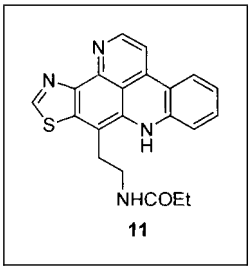

Fig. 6. Dercitamide (**11**).

change associated with dercitamide (**11**) could be readily observed in the tissue, the cells containing the compound could not be identified because of the excess fluorescence arising from cells that were out of the focal plane. Since the compound is soluble in alcohol, it was not possible to dehydrate and embed the tissue to make thin sections suitable for identification of specific cells. Attempts to separate the cells using a cell sorter were unsuccessful due to the relatively weak fluorescence of dercitamide (**11**) at the wavelength range of the cell sorter. We therefore used confocal laser scanning microscopy and transmission electron microscopy (TEM) to localize and identify the cells that contained dercitamide. The confocal microscope was necessary to provide a sharp fluorescence emission image of a single plane of tissue, allowing a more precise method of localizing dercitamide in situ. We examined both thick sections of fixed sponge tissue as well as fixed dissociated cells that had been separated and semipurified by Percoll density centrifugation. Examination of the sections and the enriched cell fractions under the confocal microscope at both neutral and acidic pHs led to the conclusion that dercitamide (**11**) was localized in sponge cells containing 10–20 spherical inclusions. Although we were confident that dercitamide was only found in specific sponge cells and not in associated intercellular symbionts, we also used TEM to determine that there were no intracellular bacteria that could be responsible for the chemistry. The TEM analysis also allowed characterization but not classification of the dercitamide-containing sponge cells. This appears to be the first time that confocal microscopy has been employed to locate marine natural products on the basis of their autofluorescence.

Conclusions

Although we have presented a few examples in which bioactive sponge metabolites are produced by symbiotic microorganisms, we believe that these are probably the exception rather than the rule. We initially chose to study only

those sponges that contained large populations of symbiotic cyanobacteria and filamentous bacteria, thereby enhancing our chances of discovering biosynthetically-active symbionts. The majority of sponges do not appear to contain such large populations of symbiotic microorganisms and the metabolites of these sponges, particularly those found in $>0.01\%$ dry weight, are unlikely to be produced by symbionts. This leaves the most interesting group of sponges, namely, those that contain small concentrations ($<0.0001\%$ dry weight) of highly bioactive compounds. There is a chance that some of these compounds could be produced by symbionts but, because of the low concentrations, they will be much more difficult to identify. To determine the cellular location of these minor metabolites will require far more sensitive methods than have been employed to date. Methods such as secondary ion mass spectrometry and fluorescent staining, using either the more traditional polyclonal antibodies that react with individual compounds or DNA probes for specific sequences involved in the biosynthesis of the target compounds, all appear to hold promise and will probably be reported before long, perhaps before this book is published.

One of the major problems that marine natural products chemists cannot ignore is that of supplying sufficient quantities of a sponge metabolite to allow for pharmaceutical development. The harvesting of wild populations of sponges seems inconsistent with modern goals of biodiversity conservation. The aquaculture of sponges is still in its infancy and little success has been reported in growing sponge cells in culture media. Accordingly, one of the major goals of sponge symbiont research is to find appropriate culture conditions for symbiotic microorganisms that produce pharmacologically active metabolites. Cultured symbionts can provide a renewable resource and thus preserve natural populations of the sponges. To date, the culture of obligate symbionts has been an elusive goal. We know very little about the chemical and physical environment provided by the host, which may hold the key to the formulation of suitable culture media. Rather than use a shotgun approach, we propose to start by identifying the symbiont's closest neighbours to provide a starting point for designing the required culture media. Another possible approach to alleviate the supply problem is the cloning of entire biosynthetic gene clusters. Advances in genetic engineering have provided the possibility of circumventing the symbiont altogether by identifying DNA that encodes for key steps in the biosynthesis. Provided that all of the DNA required for the biosynthesis of a bioactive metabolite is clustered, it is now possible to transfer relatively large segments of DNA to *Escherichia coli* or other bacteria and engineer a bacterium capable of producing the bioactive compound. Although these experiments involving genetic engineering remain to be accomplished, they will of necessity be based on today's studies of the role of

symbiotic microorganisms in the production of bioactive sponge metabolites, which provide the tools to create enriched fractions for DNA cloning and to select the correct expression vectors.

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Aquacultural Production of Bryostatin 1 and Ecteinascidin 743

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Introduction

Is it possible to grow drugs from the sea? This question, posed in 1986 by a Sterling Pharmaceuticals (Kodak) executive, led the author and coworkers to an investigation of using aquaculture techniques to grow marine organisms for their bioactive chemical constituents. In addition to the questions of technical feasibility, what would the costs be? And was there potential for profit from such endeavors?

In the mid-1980s, bryostatin 1, a complex polyketide, was entering the preclinical phase of its development, having shown potent antineoplastic activity during in vitro testing at the National Cancer Institute (NCI), and in the laboratory of its discoverers at the Cancer Research Institute at Arizona State University. The only known source of the compound was the sparsely distributed, and somewhat ephemeral, sessile marine bryozoan *Bugula neritina*. This arborescent bryozoan is a ubiquitous fouling organism found throughout the temperate world ocean. However, only a few populations from California and the Sea of Cortez were known to contain an octa-2,4-dienoate ester at the C-20 position of the molecule, which differentiates bryostatin 1 from the 17 chemical congeners found in other *Bugula* populations.

The NCI, which was gearing up to sponsor human clinical testing of bryostatin 1, projected a need for a reliable, renewable source of supply. Perhaps extant aquaculture techniques could be applied to produce crops of *B. neritina*, either in tanks or in the ocean to deliver commercial quantities of the compound.

During the same time period, a second marine invertebrate-derived natural product, ecteinascidin 743, entered preclinical testing as a potent antitumor

agent. Based on the sparse distribution and ecologically sensitive natural environment of the source organism (the Caribbean 'Mangrove tunicate' *Ecteinascidia turbinata*), aquaculture was proposed.

Two case histories will be presented which chronicle the development of these similar aquaculture technologies. Both technologies are now in the prototype testing phases of their development, funded by grants and contracts from the US government's SBIR program for small businesses.

Case History 1: *B. neritina* Aquaculture System Development

The Early Days

The initial feasibility project sponsored by the NCI was conducted in a small laboratory located on the shore of the Agua Hedionda Lagoon, 35 miles north of San Diego, California. For the initial experiments, 20 kg of gravid parent or 'broodstock' colonies of *B. neritina* were collected from a population which was known to contain bryostatin 1, and transported to the laboratory. The next morning, and in response to bright sunlight, literally thousands of 100-μm-sized larvae were seen scurrying in the direction of the incoming light at the water surface. The larvae were siphoned off and transferred into a 'settling tank' containing plates of different types of plastic, ceramic, and other materials, onto which the larvae could settle based upon their particular 'preferences'. Some hours after settlement the larvae metamorphosed into 'ancestrula' or starter zooids from which the next generation of *Bugula* colonies was to bud asexually – one zooid at a time – to form second-generation colonies (fig. 1).

The first experiments revealed that a majority of the larvae sought out each other's 'company', rather than settling more randomly over the surfaces of the settlement plates. Great balls of larvae could be seen settled on top of one another in just a few places and on only some of the plates. This larval clumping behavior had been described in the literature by *Bugula* researcher Michael Keough as a form of 'kin recognition', and was most likely mediated by a pheromone [1]. Keough reasoned that clumping of larger numbers of larvae in one place would have adaptive significance for survival (i.e. strength in numbers). However, for aquaculture, this clumping of valuable larvae was seen as undesirable, when compared to an even spreading of larvae over all of the plates. Solving this larval dispersion problem became the first technical challenge in pursuit of a viable aquaculture method. Air dispersion techniques borrowed from larval shrimp aquaculture were employed and fine-tuned as a partial solution for the problem. Use of heavily perforated PVC plastic for the settlement plates finished the job, in that the tiny larvae each sought out

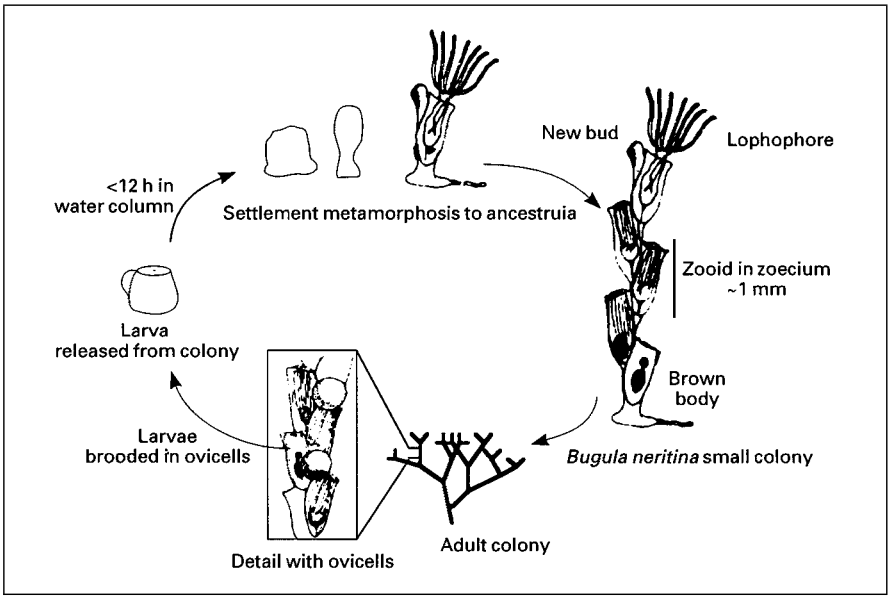

Fig. 1. Life cycle of *B. neritina* (figure provided by Seana Davidson, UCSD/SIO).

their own individual small hole as sanctuary, thereby distributing themselves more or less evenly over the plate surfaces.

Next came the food system. Nature provides each *Bugula* larva with sufficient energy stores to allow it to live for 1–24 h before its biological imperative compels it to settle onto a hard substratum and metamorphose into an ancestrula zooid. Whereas the ancestrula does not develop feeding appendages, the second and subsequent zooids of the colony do. The second zooid buds from the first, the third from it, and so forth, with each developing a ringlet of tentacles know as a lophophore which serves as a relatively efficient plankton capture mechanism. The new colony takes shape, and now has apparatus to capture phyto- and zooplankton from the current-stream passing by. Thus with each new, budding zooid there is a new mouth to feed – and given exponential growth of zooids and the large number of colonies which would be required for commercial-scale aquaculture, they add up fast!

Ingested phytoplankton cells pass through the short gut in about 20 min, and although there were diurnal patterns of greater and lesser feeding activity (they were more active at night), it was very apparent that large quantities of phytoplankton would have to be cultured in order to keep all the 'mouths' well fed. For the aquaculture trials, large cylindrical 'Sun-Tubes' of thin, clear fiberglass were employed for culture of 5–8 species of small-sized phytoplank-

Fig. 2. One of two prototype 5,000-liter aquaculture growout tanks for *B. neritina.*

ters. And as was first feared, food culture to serve the ever-increasing mass of aquacultured colonies became the major occupation of the aquaculturists. Even though artificial food components were added to the diet mix in an attempt to reduce the labor requirement for plankton culture, it was obvious after a couple of 6–8 month trials that no matter how hard we worked at food culture, no matter what novel artificial component we employed, we were not able to provide the requisites for healthy, normal growth of the cultured *Bugula* colonies (fig. 2). At harvest, the tank-cultured colonies were comparatively weak in appearance, lacked the healthy color of natural colonies, and contained only relatively small amounts of bryostatin 1 (2–14 μg/g d.w. vs. an average of 13–15 μg/g for natural colonies).

'To the Sea – To the Sea!'

What we were not able to accomplish using tank culture, we reasoned Mother Nature might provide in the sea. So, an in-sea aquaculture system was conceived and designed. To test the in-sea concept, a 7-m prototype structure was built for the company by the marine sciences development shop of the Scripps Institution of Oceanography (SIO) in La Jolla, California. With Scripps technician/divers assisting, the structure was launched and anchored 1 km offshore at a mean depth of 12 m in 40 m of water (fig. 3). The structure was designed to hold 20 0.5 m^2 perforated PVC plastic panels, each of which would eventually be settled with tens of thousands of bryozoan larvae in a shore-based hatchery. Following a pregrowth period of 3–6 weeks in hatchery tanks, the settled panels were transferred by divers to the undersea structure for eventual growout into harvestable sized colonies.

Fig. 3. The 7-m steel prototype in-sea aquaculture structure for *B. neritina*, showing culture panels, flotation sphere and orientation propeller, being launched from the pier of Scripps Institution of Oceanography, La Jolla, Calif.

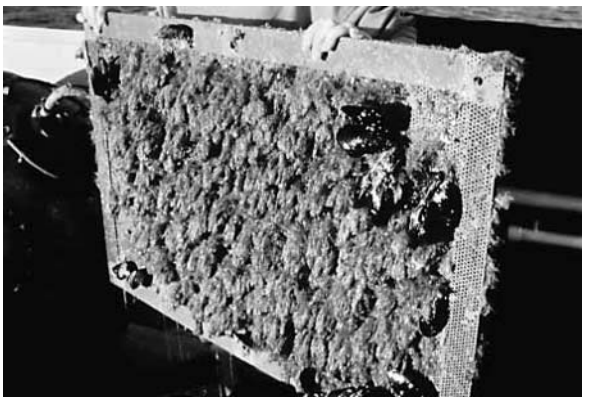

Fig. 4. A 0.5-m^2 aquaculture plate after 4.5 months growout in the ocean. Note the dense *B. neritina* colonies and the wild mussels settled only around the periphery.

For the first in-sea test, 15 well-settled panels were used. Four-and-a-half months later, 6 of the most mature panels were harvested, and to the delight of all team members, biomass densities surpassed expectations and project goals (average density was 2.88 kg/m^2 of panel area on a wet weight basis; fig. 4). Bryostatin 1 levels were found to be within the normal range for ocean

populations (3–14 μg/g d.w., average 7.5 μg/g). Analyses were performed by the NCI using HPLC and standards isolated and purified from the NCI's original large-scale collection from Palos Verdes, California in 1990.

This first in-sea growth trial showed that Mother Nature could in fact provide what we aquaculturists could not using the land-based tank system and cultured foodstuffs. There was room to improve bryostain 1 yields from the harvests, and longer growout periods might produce even higher amounts of bryostatin 1. Calculations of potential production showed that approximately 0.2 g of pure bryostatin 1 could be produced from just one 20-panel structure. Given the exceptional potency of the compound, the potential yield from one undersea structure could treat over 100 leukemia patients each year at the therapeutic weekly dose of 25 μg/m body surface, established in the phase I trials [2].

Economic analysis of the process produced yearly amortized costs for deployment and maintenance of a single 20-panel structure at approximately USD 4,300. Amortized annual costs for a larger, 72-panel, commercial-sized structure were calculated at USD 3,500, showing a clear economy of scale for the process. Given a projected yearly need for bryostatin 1 in the range of 100–200 g, approximately 200, 72-panel structures producing bryostatin 1 at a slightly better rate (13 μg/g d.w.) than did the prototype structure would be required to supply this level of market demand. This calculation does not take into consideration the use of semisynthetic chemical conversion of bryostatin 2 to 1, which could, if applied, boost the final yields considerably and reduce the final number of structures needed to serve market demand [3]. And although formidable, this level of ocean engineering and deployment is well within the state-of-the-art for modern oceanography.

In a commercial setting, structures would be distributed in 'pods' of varying numbers strategically anchored along the California coast. Structures would be located in the lee of channel islands or in other sheltered locations to protect them from storms, and at sufficient depth to avoid thermal assault from El Niño-mediated surface currents (see below). Contract growers would receive *Bugula*-settled panels from a central hatchery for growout in the ocean, and return the harvested biomass for processing, extraction, and purification of bryostatin 1. Incremental 'clip-harvesting' by divers, and vegetative regrowth of the clipped colonies, would obviate the need to return to the hatchery for each new crop – a tremendous savings for the process.

Trials and Tribulations

In the sea aquaculture for bryostatin 1, production has proven itself to be commercially viable. However, the process is not without its inherent problems. Primarily, any farming operation is vulnerable to the vagaries of

Fig. 5. Nudibranch *H. crassicornis* feeding on 1-month-old *B. neritina* colonies in situ on the prototype in-sea aquaculture structure (photo provided by Deeanne Edwards, UCSD/SIO).

nature and the potential for economic devastation as a result of weather-related phenomena.

In particular, El Niño-mediated weather ravaged the Southern California coast during the first half of the 1990s, and raised particular havoc with *Bugula* populations – both cultured and natural. The storms produced deluges of rain and huge quantities of silt runoff into the nearshore waters, devastating the *Bugula* beds. During 1994–95 El Niño, the sea surface water temperature in La Jolla Bay was unseasonably elevated by as much as 5 ºC, down to a depth of 15 m or more. This thermal 'capping' of the nutrient-rich bottom waters all but eliminated the usual turnover of nutrients into the euphotic zone, and either directly or indirectly (through reduced food availability) the *Bugula* populations on the in-sea structure all perished.

Bugula for Lunch?

Most likely due to the noxious and toxic effects of bryostatin itself, few predators or diseases are known to appreciably damage adult colonies. There is, however, reason to be concerned for newly settled colonies used early in the aquaculture process. In California, two predatory nudibranchs (*Polycera atra* and *Hermissenda crassicornis*) both have a particular appetite for the 'tender shoots' of young *Bugula* colonies (fig. 5). In the development of hatchery culture protocols, particular attention was paid to methods for separating

coexisting nudibranch larvae from *Bugula* larvae prior to settlement. After much trial and error, a method was discovered that proved to be 99% effective at eliminating the nudibranch larvae. Once in the ocean, the *Bugula* colonies grow quickly, outstripping the appetites of the relatively few adult nudibranchs which happen onto the aquaculture panels.

Bugula *Aquaculture – Yea or Nay?*

The success of the aquaculture technology project herein described cannot be disputed, and the projected costs for aquacultural production of *B. neritina* biomass in California waters are not out of hand when compared to those for natural collections. However, the question remains: Would a pharmaceutical company bank on aquacultural production for underpinning their worldwide supply needs? To date no pharmaceutical company has stepped to the fore to relicense the drug from its university discoverers. The reliability factor for large-scale ocean-based aquaculture using CalBioMarine's technology is yet to be determined, and aquaculture is not a familiar technology of 'Big Pharma'. The risks seem high compared to the perceived value, especially since bryostatin 1 is still being tested for efficacy in phase I and phase II clinical trials in the USA, Canada, and in Great Britian. Its initial testing as a single-agent anticancer compound is being augmented with a growing number of dual-agent studies to see if bryostatin can be administered as an adjuvant therapy to boost the effects of other known cancer drugs. The jury is still out, and in the meantime the patent life of the compound is fast-dwindling, so in this perspective the supply issue has taken a somewhat bizarre back-seat. In the interim since the aquaculture technology's development, more elegant biotechnologies which hope to produce bryostatins through fermentation of a naturally occurring microbial symbiont, or through a transgenic system of expression are under investigation – as are elegant chemical syntheses. In this scenario, aquaculture will have to wait in the background to see if it becomes the technology of choice for supply of commercial quantities of biomass and drug for the future drug market.

Case History 2: *E. turbinata* Aquaculture System

The Organism

E. turbinata (ET) is a colonial tunicate (ascidian) which inhabits mangrove roots, turtle grass blades, gorgonians, sponges and limestone substrates on coral reefs, as well as man-made materials such as ropes, docks, and pilings (fig. 6). It is broadly distributed in the Florida Keys, the Bahamas, Gulf of Mexico, and the Caribbean Sea, but is only abundant at a few sites in the

Fig. 6. Wild *E. turbinata* colonies, Lower Matecumbe Key, Florida (photo by Erich Bartels, CBMT).

Bahamas and Florida Keys. It occurs in water ranging from depths of 0.3–12 m, most typically at 1–3 m.

The tunicate grows with a compound colonial organization, and individuals (zooids) are united by short stolons to form tuft-like colonies. A shared circulatory system is present. Individual zooids are typically 0.5–2 cm long by 0.25–0.75 cm in diameter and generally occur in numbers ranging from 50 to 200 or more per colony. Individual zooids are covered by a transparent mantle (tunic) with two openings (siphons) at the free end through which water flows in and out of the branchial cavity. The body and internal organs are translucent to transparent, and the tunic possesses a characteristic bright orange pigment. However, at some times only an orange or pink ring is seen at the apical margin [4, 5].

ET is a filter feeder and removes plankton as small as a few microns in diameter from the current of water that passes through the pharynx. Root-like extensions (also covered with tunic) called stolons ramify from the base forming a permanent attachment to the substratum. It is known from previous work that the residual stolon masses of ET from mangrove root collections in the Florida Keys contain the same concentration levels of ecteinascidins as do the adult zooids. This implies a chemical protection function of the compound for the organism [Amy Wright, pers. commun., Harbor Branch Oceanographic Institution, Ft. Pierce, Fla.].

The ascidian is hermaphroditic and reproduces primarily from late March to April or May in most populations [A. Wright, op. cit.], although reproductive zooids may be found all year long. Fertilization takes place within the atrium, and embryos are brooded to a fully competent appendicularia (tadpole) larva within the atrium. The tadpole larvae are bright orange in color, 4.5 mm long, and easily visible to the naked eye through the tunic [6–8]. Each zooid may produce dozens of larvae, with two or three mature at any time. After a free-swimming period of a few seconds to some hours (settlement is typically within minutes), the larva settles and attaches at the anterior end. Metamorphosis to the adult form occurs within 48 h, and asexual budding from the stolons forms the new zooids of the second-generation colony.

Healthy ET colonies do not have epibionts (attached macroscopic organisms) and a clean surface is maintained by sloughing of tunic material. The animal is often found associated with other encrusting and innervating macroorganisms (primarily hydroids, sponges and other ascidians). No photosynthetic symbionts are present within the animal. Microorganisms are found on and within the ascidian, although whether or not a symbiotic relationship exists with any microorganism associates is not known.

In all species of ascidians so far examined, including *E. turbinata* [6, 8], light has been found to control the timing of larval release, usually following a period of dark adaptation [7, 9]. The rate of light-induced larval release appears to be positively correlated with the extent of the previous dark adaptation period [8]. These observations suggest that larval release might be accelerated (e.g. for aquacultural use) by extending the period of dark adaptation and by increasing the photon flux density of subsequently applied illumination. Natural metamorphosis inducers, which have been identified for other tunicates, have not been found for ET [10].

The Drug

Extracts of colonies sampled from locales throughout the Caribbean show the presence of a family of tetrahydroisoquinoline alkaloids collectively known as the ecteinascidins. One member of this group, ET-743, has entered human clinical testing as an antitumoral agent [11]. Supplies for preclinical and clinical testing have come from widespread natural collections, but it is generally agreed that the scale of collections which would be necessary to support commercial drug sales on a year-to-year basis would be both environmentally damaging and difficult to manage.

ET Aquaculture

Aquaculture research conducted in the Florida Keys has shown that it is indeed possible to aquaculture ET both in controlled-environment tanks,

Fig. 7. PVC pipe and poly-rope 'ET' growout units in situ in waters off of Long Key, Fla. (photo by Erich Bartels, CBMT).

and in the sea. The phase I project involved collecting gravid colonies from nature, settling the expelled larvae onto various types of materials in tanks, and conducting growout trials in both tanks and the ocean. The project successfully proved the concept that larvae could be settled onto various materials, and thereafter multiply in number to form sexually mature, ecteinascidin-containing harvestable colonies in both tanks and in the sea.

Interim results of scaled-up culture trials in phase II of the project have also been most encouraging. As of this writing, greater than 50,000 larvae have been successfully settled onto artifical substrates held in hatchery tanks. Following the concept for ocean growout which was proven on a smaller scale during the phase I project, 10-m rope grids with lab-settled colonies attached were established in the waters surrounding Long Key. The in-sea results have shown production in the range of 50–60 kg of biomass produced in 60–90 days from the larvae of just 1 kg of wild-collected parentstock. Average production from a single 1.5-m poly-rope approximates 500 g of tunicate biomasss per harvested crop – with crop periods varying from 2–3 weeks to a more usual 4–6 weeks. The ocean growouts have proven to be very reliable and highly successful, paving the way for commercial scale-up of the technology. Presently, modest scale-up tests with newly configured growout units are being tested (fig. 7) and allied investigations designed to explore production rates in relation to varying habitat types in the ocean are underway.

The in-tank growouts have been remarkably successful as well. And although the tank-cultured colonies have taken a bit longer to reach maturity, production rates are good and the method is still being considered a viable

Fig. 8. Sixty-day tank-cultured colonies of *E. turbinata* settled onto 0.8-cm diameter poly-rope (photo by Erich Bartels, CBMT).

option for scale-up (fig. 8). ET-743 analyses of both tank-reared and ocean-reared colonies have shown similar content and within-sample variability as have previously analyzed wild-collected samples (i.e. $0.015 \pm 0.010\%$ of biomass analyzed on a dry weight basis).

A commercial-scale ET aquaculture enterprise is envisioned as a 'distributed system' wherein a number of production units are set up in appropriate growing locales throughout the Caribbean basin. Such a distributed system would minimize inevitable losses from seasonal hurricanes, and in general is considered to be the best strategy for attaining the relatively large amount of biomass required.

Summary and Conclusions

The two case histories presented have illustrated that it is indeed technically possible to provide biomass and drug through application of modern aquaculture techniques. And as of this writing, economical chemical syntheses and/or other biotechnical sources such as fermentation, are not available for either bryostatin 1 or ET-743, leaving natural collections or aquaculture as the only possible options for supply.

No matter which source is chosen for each drug, it must be capable of delivering the needed quantities, reliable (i.e. year-to-year) and cost-effective. Both in-sea aquaculture and natural collections are subject to the uncertainties of nature, which bear on their reliability. However, with aquaculture, more

control can be exercised by placing in-sea units out of the most probable pathways of storms, El Niño currents and the like, giving aquaculture a significant edge over natural collection.

In the case of bryostatin 1, the quantity of drug which might ultimately be needed is still unknown. For treating the various forms of leukemia alone, estimates note that as little as 50–100 g/year might be sufficient. For supplying the growing possibilities for using bryostatin as an adjuvant therapy drug administered in combination with any one of a number of other anticancer therapeutics, needed quantities would have to be dramatically increased, possibly to as high as several hundred or a thousand grams per year. Could natural collections possibly deliver these amounts? We take as an example from the 1988 large-scale collection contracted by the NCI from bryostatin 1-containing populations of Southern California. This collection delivered 13,000 kg of biomass to the NCI, which took nearly 2 years to gather. After solvent extraction and chemical isolation, the yield was 18 g of drug [12]. These results imply that new bryostatin 1-containing beds would first have to be discovered before even 50 g of drug could be reaped from nature. Aquaculture or another yet-to-be-completed biotechnical method will have to fill in the difference.

In the case of ET, aquaculture (possibly a combination of both in-sea and tank culture) is the only logical choice for delivering commercial quantities of biomass and drug, provided an economic chemical synthesis is not developed. Given the sparse distribution of the organism in nature, and the ecologically sensitive habitats in which it is found, it is doubtful that large-scale collecting permits would be issued by source nations to provide the quantities of drug projected for yearly commercial sales (1,000 g of drug, or more, derived from upwards of 1,000 MT of biomass).

Costs estimations for commercial-scale aquaculture production have been extrapolated from the results of the prototype projects. They show production costs to be competitive with large-scale natural collections for both species. The costs are comparable. The environmental consequences are not. Therefore, the drug license holders must decide whether or not aquaculture becomes the method of choice for these two highly promising anticancer agents.

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The Halichondrins: Chemistry, Biology, Supply and Delivery

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Discovery

Norhalichondrin A was the first in a series of potent antitumour compounds reported by Uemura et al. [1] in 1985. The isolation of a further seven halichondrins from the same Japanese sponge (*Halichondria okadai* Kadota) was subsequently reported in 1986 [2]. These halichondrins, norhalichondrins A, B and C, halichondrins B and C, and homohalichondrins A, B and C, represent three classes of halichondrin (fig. 1). The A, B and C families are distinguished by the degree of oxidation at C12 and C13. Within each family, variation occurs beyond the C45 position.

The structures are characterized by a novel 2,6,9-trioxatricyclo[3.3.2.$0^{3,7}$]-decane system (rings C–E), a 22-membered lactone ring (C1–C30), two exocyclic olefinic groups and several pyranose and furanose rings.

Comparison of the cytotoxicity of all of the halichondrins isolated against the in vitro B-16 melanoma cell line showed that halichondrin B (**1**) (fig. 2) was the most cytotoxic with an IC_{50} in the 0.1 ng/ml range, establishing that members of this series were of very high potency. The in vitro testing also established that halichondrins with B-type C–E rings and homo-, or hali-type terminal moieties gave the best results [2].

The antitumour activities of halichondrin B (**1**) and homohalichondrin B (**2**) were also investigated by in vivo testing on mice with B-16 melanoma, P388 leukaemia and L-1210 leukaemia tumours. A 300% increase in the life expectancy of the P388 leukaemia afflicted mice was reported relative to the control group (no halichondrin administered) for halichondrin B [2]. Similar results were observed for the other two cell lines and for homohalichondrin B (**2**) [2].

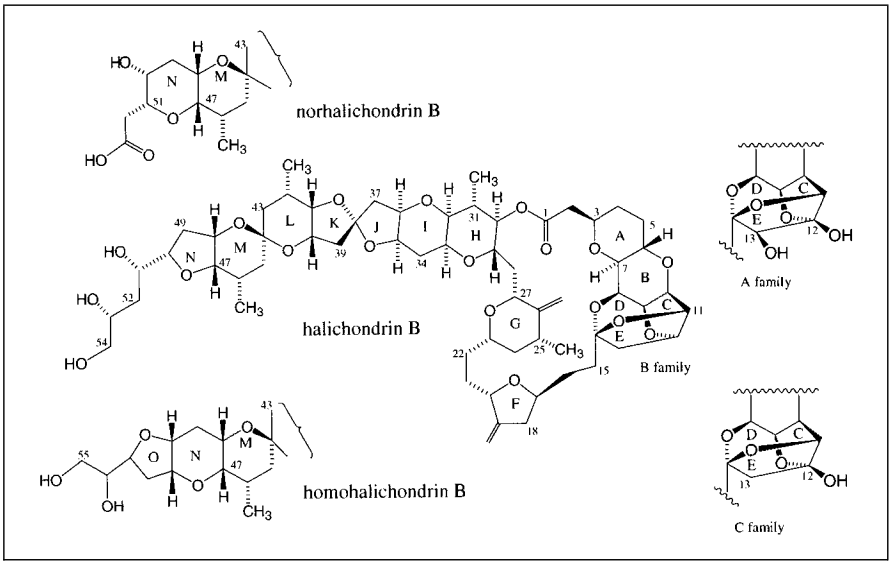

Fig. 1. The original halichondrins.

The isolation of halichondrin B (**1**) and homohalichondrin B (**2**) from an unrelated sponge *Axinella* sp., collected in Palau, was reported in 1991 [3]. Two new halichondrin compounds, halistatins 1 and 2, were reported by Pettit et al. [4] in 1993. Halistatin 1 (**3**) is 10α-hydroxyhalichondrin B and halistatin 2 (**4**) is 10α-hydroxyhomohalichondrin B. Halistatin 1 (**3**) was isolated from an east Indian Ocean sponge *Phakellia carteri* along with halichondrin B (**1**) and homohalichondrin B (**2**). These three halichondrins were subsequently isolated from an extract of the western Indian Ocean sponge *Axinella* cf. *carteri* Dendy together with halistatin 2 (**4**) [5]. Halistatin 1, halistatin 2 and halichondrin B were tested in the NCI's human tumour primary in vitro screen [5]. The overall mean GI_{50} values were 7.1×10^{-10}, 6.8×10^{-10} and 2.3×10^{-10} *M*, respectively.

In 1987, halichondrin-like compounds were identified in two unrelated New Zealand species of sponge [6]. Trace quantities of halichondrins were detected in an extract of *Raspalia agminata* (order Axinellida, family Raspailiidae), a black shallow-water sponge from the Leigh area of the North Island. However, a more assured source was a slimy yellow sponge, obtained by benthic dredging (ca. –100 m) off the Kaikoura Coast, identified as a new species of the genus *Lissodendoryx* Topsent (class Demospongiae, order Poecilosclerida, family Myxillidae). The Kaikoura sponge is higher yielding (~1 mg total halichondrins/kg wet weight of sponge) than any of the other halichondrin-producing sponges (typically 0.1 mg/kg or less).

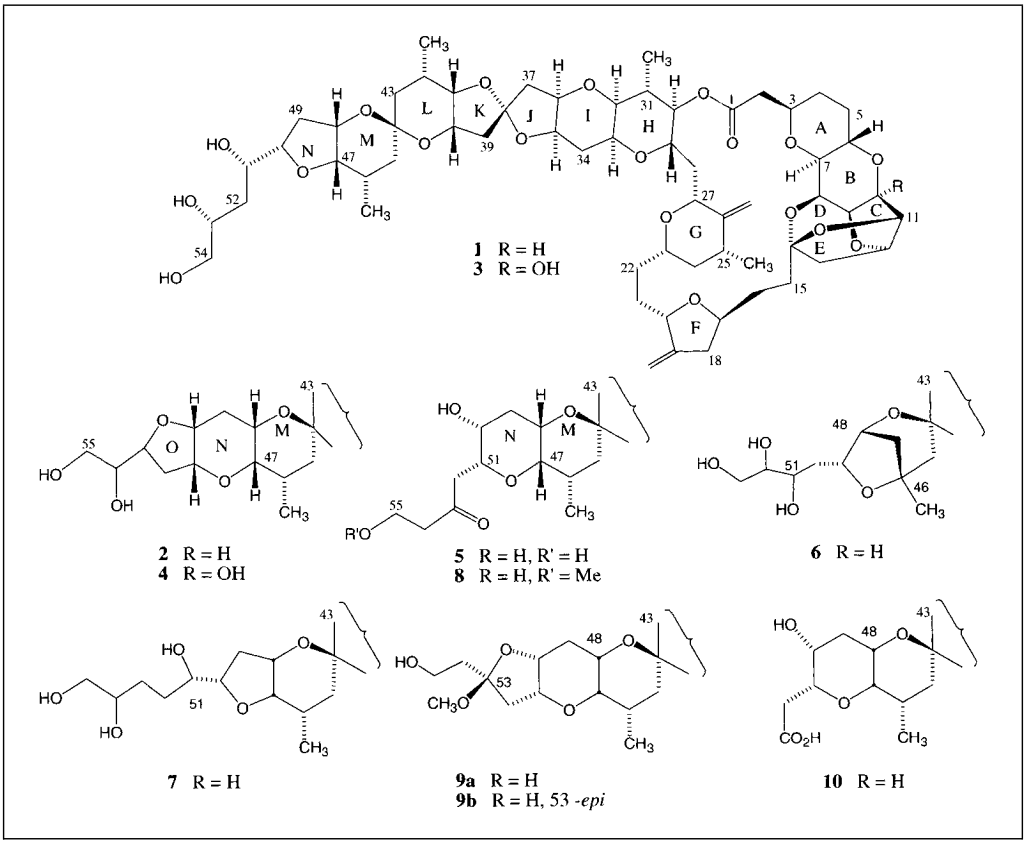

Fig. 2. New halichondrins.

A new halichondrin-type compound was isolated by bioassay-directed fractionation from the extract of *Lissodendoryx* n.sp.1 in addition to halichondrin B (**1**) and homohalichondrin B (**2**). This new halichondrin was characterized using 2D NMR and MS techniques and named isohomohalichondrin (**5**), as it was isobaric with homohalichondrin B (**2**) [7]. The difference between the two compounds exists beyond C48, where isohomohalichondrin (**5**) has the ring opened form of the terminal furanose unit of **2**. Interestingly, this new halichondrin is the major component in the extracts from *Lissodendoryx* n. sp. 1.

A large-scale extraction of *Lissodendoryx* sp. (ca. 200 kg) yielded four minor compounds in addition to halichondrin B (**1**), homohalichondrin B (**2**) and isohomohalichondrin B (**5**), together with the known norhalichondrin B

(**10**) [8]. The structures of the minor halichondrin compounds were established as neonorhalichondrin B (**6**), neohomohalichondrin B (**7**), 55-methoxyisohomohalichondrin B (**8**) and 53-methoxyneoisohomohalichondrin B (**9a**). 53-Methoxyneoisohomohalichondrin B (**9a**) was observed to transform to isohomohalichondrin B (**5**) in solution in an NMR tube, presumably via acetal hydrolysis. The reverse reaction, although much slower, was realized on a sample of isohomohalichondrin B (**5**) in CD_3OD [9]. It is assumed that this compound was most probably an artefact of the extraction and purification procedure. A small amount of the C53 ketal diastereoisomer (**9b**) was also isolated. 55-Methoxyisohomohalichondrin B (**8**) is the C55 methoxyl derivative of isohomohalichondrin B (**5**). Neohomohalichondrin B (**7**) has a structure similar to halichondrin B (**1**) except for an additional methylene group inserted between the two secondary alcohol groups in the terminal moiety. Finally, neonorhalichondrin B (**6**) displays a unique terminal M and N ring fusion.

Mode of Action of the Halichondrins

A feature of the NCI's in vitro panel of 60 human tumor cell lines is the ability to generate additional, important biological information about a compound [10, 11]. As well as providing data on differential responses, the screen provides insights into the biological mode of action of test compounds [11]. For example, compounds with similar mechanisms of action will display similar, characteristic patterns of differential cytotoxicity across the 60 cell lines. The degree of similarity between compounds is assessed by the COMPARE pattern recognition program [11] utilizing Pearson correlation coefficients, which are extracted from the primary screening data. The calculation of the COMPARE correlation coefficient involves comparison of a selected mean graph profile, such as a 'seed' compound with a known mechanism of action, to the mean graph profile of the test compound. Use of COMPARE has enabled the ready identification of novel structural classes of compounds operating by a known biochemical mechanism of action [11, 12]. Indeed, halichondrin B (**1**) was one of the first published examples of this use of COMPARE to predict mechanism of action [13]. An evaluation of the pattern of differential activity (mean graph profile) of halichondrin B (**1**) against the NCI 60 human tumour cell line panel using the COMPARE algorithm found that the halichondrin pattern most closely resembled those of tubulin-binding agents such as vincristine [13].

Drugs which bind to tubulin can be subdivided into separate classes depending on the effect the drug has on the binding of five well-characterized tubulin-binding agents. The drugs are categorized depending on whether they

prevent the binding of colchicine, the binding of the vinca alkaloids, vinblastine and vincristine, or the binding of rhizoxin and maytansine to tubulin [14]. Some drugs do not interfere with the binding of these agents, having affinity for a separate distinct region of tubulin. The vinca alkaloids induce the destabilization of polymerized tubulin by binding to a site recently localized on β-tubulin [14]. The 'vinca site' represents the region of tubulin where the vinca alkaloids and their competitive inhibitors (e.g. maytansine) bind. The peptide antimitotics dolastatin 10 and phomopsin A are noncompetitive inhibitors of vinca alkaloid binding to tubulin. This is interpreted as binding in a region physically near to, but distinct from, the vinca site. This alternative site is termed the 'vinca domain'.

Investigations by Bai et al. [13] noted the accumulation of cells arrested in mitosis at cytotoxic concentrations of either halichondrin B (**1**), or homohalichondrin B (**2**) for L-1210 murine leukaemia cells in vitro [13]. These compounds were also found to inhibit the polymerization of purified tubulin and microtubule assembly dependent on microtubule-associated proteins. Despite its potent cytotoxicity, halichondrin B (**1**) was found to be a relatively weak inhibitor of tubulin polymerization, did not interfere with colchicine binding to tubulin, inhibited net GTP hydrolysis and nucleotide exchange on tubulin, and was a noncompetitive inhibitor of the vinca alkaloid vinblastine. This suggested that halichondrin B was binding in the 'vinca domain' of tubulin.

Isohomohalichondrin B (**5**) has also been shown to break up microtubule networks by direct interaction with tubulin, resulting in the decreased binding of GTP to the protein [15]. In a recent paper, **5** was shown to cause a delayed progression through S-phase, a mitotic block, tetraploidy and apoptosis in a range of human cancer cell lines [16] confirming the earlier results and hypotheses put forward for the mode of action of the halichondrins. Isohomohalichondrin B (**5**) is under development as an anticancer drug by the Spanish pharmaceutical company Pharma Mar SA.

Structure-Activity Relationships in the Halichondrin Series

Our initial evaluation of the bioactivities of the naturally occurring halichondrins was made using the murine leukaemia cell line P388 assay (IC_{50} data). Subsequently, the NCI's in vitro panel of 60 human tumor cell lines (GI_{50} data) was utilized. The variations in bioactivity (IC_{50} or GI_{50}) between the major natural halichondrins **1, 2, 5** and **10** observed in these assays prompted the production and investigation of a wider range of halichondrin derivatives to establish the structural (and conformational) features essential for high levels of biological activity. The first stage of this investigation was

Table 1. Activities of halichondrin derivatives in the NCI 60 cell line panel and against the murine leukaemia cell line P388

Compound	COMPARE[a]	Mean $GI_{50} \times 10^{-10} M$	CEM $GI_{50} \times 10^{-10} M$	P388 $IC_{50} \times 10^{-10} M$
1	1.00	1.4	1.1	7
2	0.95	3.2	2	2
5	0.74	1.2	2	1.6
6	0.79	12	6.6	3.7
7	0.9	3.4	3	7.1
8	0.72	2	2	88
9a	0.9	1.2	2.1	0.88
10		18		
11	0.87	360	280	110
12	0.69	480	510	75
13	0.86	480	370	130
14	0.91	120	63	37
15	0.81	850	33	47
16	0.71	320	320	5,500
17	0.73	490	400	500
18	0.82	96	46	190
19	0.85	1.6	2.4	1.9
20	0.87	1.8	1.9	5.3
21	0.8	2	1.4	4.5

[a] Correlation coefficients from the COMPARE pattern recognition algorithm were calculated using the GI_{50}-centred mean graph profiles of differential cellular sensitivities to each of the compounds. The GI_{50}-centred mean graph profile of halichondrin B (**5**) was used as the 'seed' for all of the comparisons. Ideally, TGI-centred profiles should be used, but all the necessary data was not available due to the wide concentration range over which bioactivity was observed for the various derivatives (**1, 2, 2–21**).

a study of the acid rearrangement products, followed by alterations at the terminal region of the molecule, alterations of the lactone ring (C1–C30) and modification of the olefinic groups at positions C19 and C26 of the more readily available homohalichondrin B (**2**) and isohomohalichondrin B (**5**). The structures of the analogues were assigned through the use of a range of MS and NMR spectroscopic techniques. These derivatives, along with the range of naturally occurring halichondrins (19 compounds in all), were analysed against the NCI's panel (GI_{50}) and in-house P388 assay (IC_{50}). The results are summarized in table 1. In general, it is apparent that significant decreases in cytotoxicity are observed in both the P388 assay and the NCI screen for the acid products (**12–14**), the lactone-opened compounds (**15–17**), and the olefin-

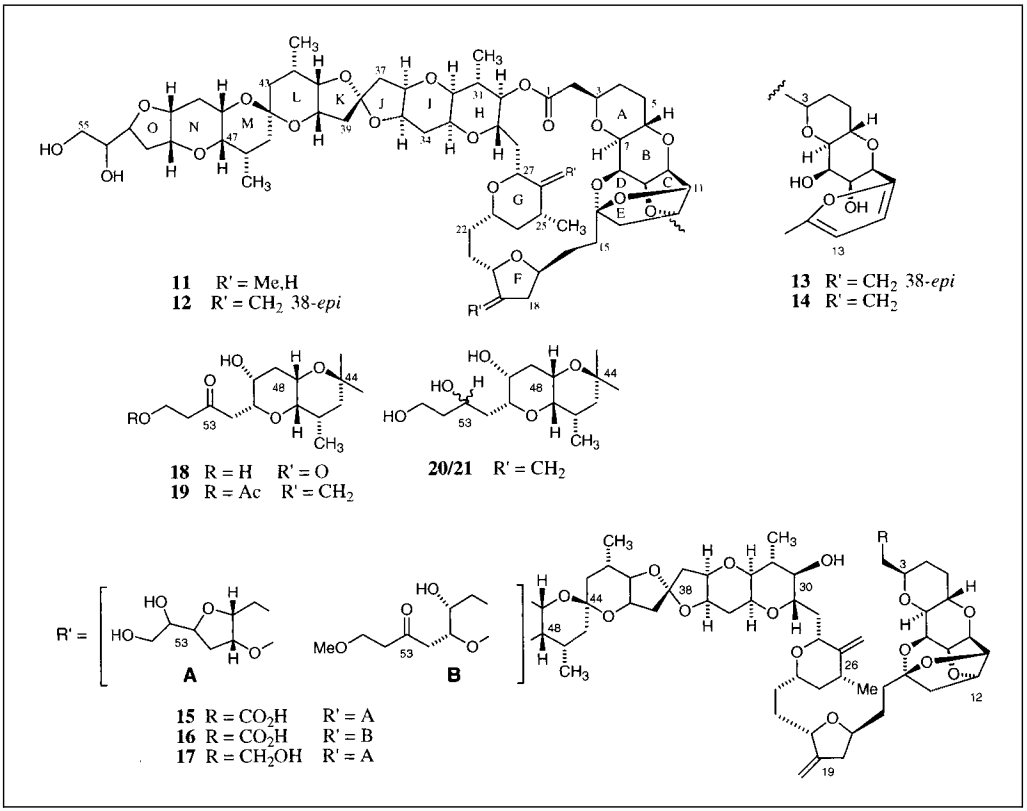

Fig. 3. Chemically modified halichondrins.

modified compounds (**11, 18**) relative to the naturally occurring halichondrins (**1, 2, 5, 10**). Interestingly, modifications to the terminal regions of the halichondrins (**6–9, 19–21**) did not result in significant changes in the biological activity of the resulting halichondrins (fig. 3).

The mechanism of cellular entry by the halichondrins is undetermined, but a unique combination of structural and conformational features will most likely be necessary for receptor-mediated cell entry, and the characteristic effects that are then observed. Once inside the cell, it is the interaction with tubulin that will then determine the ability of the halichondrin analogue to display a halichondrin-like mechanistic profile (a high COMPARE correlation coefficient) *and* display significant cytotoxicity. To examine the relationships between antimitotic ability and cytotoxicity, the GI_{50} data relative to the COMPARE correlation coefficients were plotted (fig. 4) for the naturally occurring halichondrins (**1, 2, 5–10**) and the hemisynthetic analogues (**11–21**).

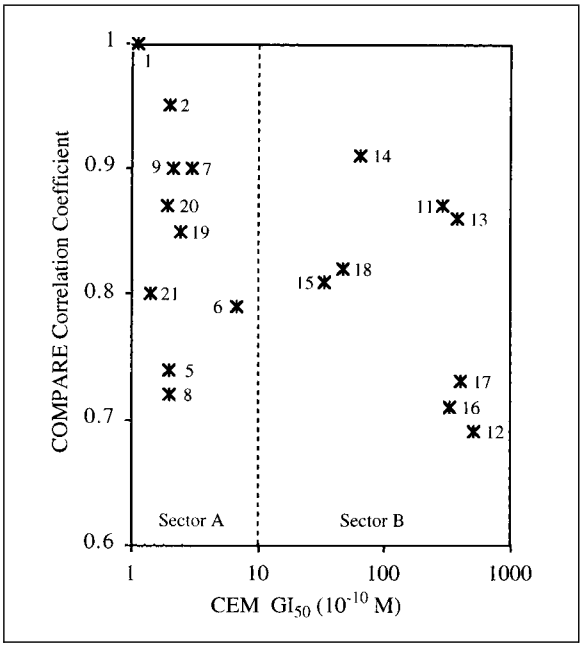

Fig. 4. Plot of COMPARE coefficients vs. GI_{50} from CEM cell line for a range of halichondrin derivatives.

Halichondrin B (**1**) was used as the 'seed' compound for the COMPARE correlation calculations with a COMPARE value of 1.0 by definition [11, 12]. The GI_{50} data derived from the CCRF-CEM cell line are used as the measure of cytotoxicity, as this cell line is most similar to the murine P388 cell line used in the other assay [17]. The GI_{50} data are plotted in log format because of the wide range of activities observed. Figure 4 has been arbitrarily divided into two sectors, A and B, to facilitate analysis of the data. The division at $GI_{50} = 10 \times 10^{-10}$ *M* separates those compounds which were relatively cytotoxic (appearing to the left of the line) from those displaying a significantly reduced level of cytotoxicity (appearing to the right of the line). The cytotoxicity, or GI_{50} value, can essentially be considered to be the biological outcome (i.e. the resultant) of all of the biological effects, and as such is a quantitative assessment of biological potency. The COMPARE correlation coefficient, in contrast, gives a qualitative assessment of the ability of the compound to act in a mechanistically predefined manner. If there were a simple loss of potency with concomitant fall-off in antimitotic ability then a negative correlation would be observed. Given that this was not the case, and that compounds with

reduced cytotoxicity but high COMPARE values (>0.85) (e.g. **11, 13, 14**) were identified as well as the reverse situation (e.g. **5, 8, 21, 6**), a more complex, underlying relationship between structure and the expression of biological activity in the halichondrin series is implicated.

As noted above, it is most likely that a unique combination of structural and conformational features will be necessary for receptor-mediated cell entry. As it is structural features that primarily define the conformation of large, flexible molecules such as the halichondrins, even small changes in structure are likely to have an influence on the solution conformation(s) available to the halichondrin skeleton. To examine the possibility of a link between structure-activity relationships in the halichondrin family and conformational features, an assessment of the average solution conformations of selected halichondrins was undertaken by various NMR spectroscopic techniques for $CDCl_3$ solutions. The average solution conformations of halichondrin B (**1**) and homohalichondrin B (**2**) (in $CDCl_3$) were first established as being similar to that found in the X-ray crystal structure of the 12,13-di-*p*-bromophenacyl derivative of norhalichondrin A [1]. In the NOESY NMR spectra of **1** and **2**, key correlations were observed between the following pairs of protons: H2′ and H20, H2′ and 19=CH, and between H29 and 19=CH, indicating that these pairs of protons were situated close in space (<3 Å) (fig. 5).[1] The interatomic distances for these pairs of protons in the X-ray crystal structure of the norhalichondrin A derivative were within the range over which NOE observations may be observed (2.8 Å (19=CH-H29), 2.6 Å (19=CH-H2′) and 2.9 Å (H20-H2′)). Using selective 1D and 2D-TOCSY experiments, vicinal coupling constants were next measured for **1**, and using the Karplus equation [18], these vicinal couplings were converted to dihedral angles and correlated, where applicable (H2-H43), to the dihedral angles found in the X-ray crystal structure. In general, the calculated dihedral angles correlated well with those of the X-ray crystal structure conformation, although there was a suggestion of some minor twisting in the ring systems from C29 to C44, relative to the X-ray crystal structure.

Having established that the average solution conformations of the parent halichondrins **1** and **2** were comparable to those found in the solid state, the relationships between the antimitotic ability (COMPARE correlation coefficient) and cytotoxicity (GI_{50}) could now be examined from the perspective of induced changes in conformation with alteration of structural features.

Sector A: The nine compounds falling into sector A are those halichondrins displaying high levels of cytotoxicity, while retaining the distinctive anti-

[1] In conventionally drawn diagrams of the halichondrin skeleton (e.g. **1** and **5**), these pairs of protons appear to be located on opposite sides of the lactone ring.

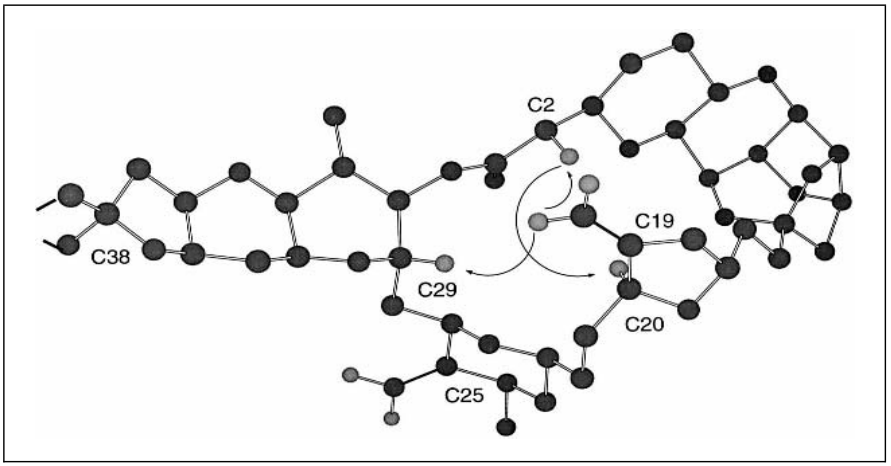

Fig. 5. Stereochemical diagram based on norhalichondrin A X-ray crystallographic data, showing the relative disposition of the C2, C19 = CH_2, C20, C25 = CH_2 and C29 hydrogens and the NOESY correlations observed.

mitotic mean graph profile of halichondrin B (**1**) (COMPARE values >0.7). Six of these compounds are naturally-occurring halichondrins (**2, 5–9**) and three are terminal-modified derivatives (**19–21**) which exhibit similar mechanistic properties (as seen by the strong COMPARE correlation coefficient values) and similar cytotoxicity levels (low GI_{50} values) to those of halichondrin B (**1**). Changing functional groups or oxidation level in the terminal region of the molecule (C50–C55), relative to **1**, did not result in significant changes in biological activity. Furthermore, the average solution conformations of these halichondrins (in $CDCl_3$) appeared to be similar to that of halichondrin B (**1**), as determined by NOE and NOESY NMR spectroscopy.

Sector B: All the compounds in this sector (**11–18**) are modified halichondrins and are significantly less cytotoxic relative to halichondrin B (**1**). The average $CDCl_3$ solution conformations displayed by these derivatives were unlike those of the parent halichondrins (**1** and **2**). For example, the NOESY spectrum of the furan acid product (**14**) lacked the H2′-H20, H2′-19 = CH and H29 to 19 = CH correlations described for halichondrins **1** and **2**. Computer modelling of the furan region of the molecule that included these implied inter-proton distances from the NOE measurements produced a lactone ring conformation very different from that determined for halichondrin B (**1**) and homohalichondrin B (**2**). Similarly, the *epi*-furan acid product (**13**), the lactone-opened **15–17** and oxidised derivative **18** all lacked the NOESY correlations observed across the lactone ring of halichondrins **1, 2** and **5**.

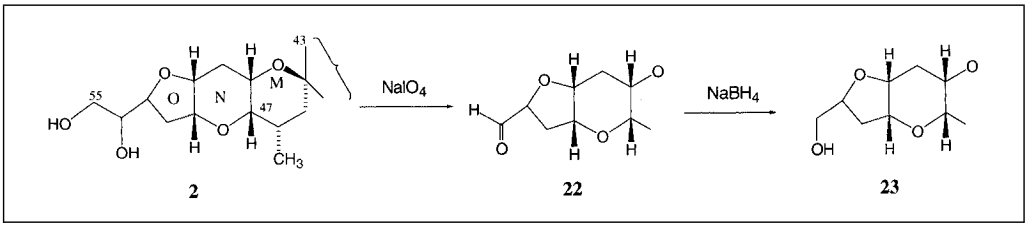

Fig. 6. Oxidative cleavage of homohalichondrin B.

While the NOESY spectrum of the *epi*-acid product (**12**) showed the distinctive H2′ to 19 = CH correlation across the lactone ring, computer modelling indicated significant conformational changes had occurred around the C38 centre to accommodate the change in stereochemistry, resulting in a 'kink' in the molecule around the C38 centre.

The analysis of the natural and modified halichondrins established that conformation is an important factor in determining overall cytotoxicity in the halichondrin series, and that only those derivatives possessing a 'halichondrin B-like' conformation retained significant cytotoxicity. Any change in the structure of the halichondrin skeleton that brought about a conformational change relative to halichondrin B (**1**) produced a significant decrease in the measured cytotoxicity. This is seen particularly with those derivatives where the sp^2 character at C19 or C26 is lost (e.g. **11**), or the lactone ring is opened (**15–17**). However, the conformational change did not necessarily impinge on the ability of the compound to display the characteristic antimitotic profile observed for the more cytotoxic halichondrins (**1** and **2**). This suggested that there are competing mechanisms associated with the conformation(s) observed in the halichondrin series which determine the ability of the halichondrin to access tubulin in the cell, or alternatively, to effectively interact with tubulin to produce a high level of cytotoxicity.

The implication that can be drawn from these structure-activity correlations is that the essential biological activity of the skeleton is captured by the right half of the molecule up to at least C38. Structural variations beyond this point have relatively little impact on the antimitotic properties and the cytotoxicity. This is exemplified by recent synthetic work by Kishi's group [19] and recent hemisynthetic work on the halichondrin skeleton. For example, homohalichondrin B (**2**) was cleaved with periodate to the formyl derivative and after reaction with borohydride to the norhomohalichondrin B (**2** to **22** to **23**) (fig. 6). The biological data for this series (table 2) have the expected high cytotoxicities and high COMPARE correlation coefficients.

Table 2. In vitro cytotoxicities of hemisynthetic homohalichondrins

Compound	COMPARE	Mean $GI_{50} \times 10^{-10}$ *M*	P388 $IC_{50} \times 10^{-10}$ *M*
Halichondrin B (**1**)	1.00	1.4	7
Homohalichondrin B (**2**)	0.95	3.2	2
Aldehyde (**22**)	0.98	3.0	3
Nor-alcohol (**23**)	0.99	1.2	6

In vivo Activity of the Halichondrins

The antitumour activities of halichondrin B (**1**) and homohalichondrin B (**2**) were initially investigated by Uemura and Hirata [2] using murine B-16 melanoma, P388 leukaemia and L-1210 models. A variety of regimens were used via intraperitoneal (i.p.) and intravenous (i.v.) routes, and excellent results were obtained against all three tumours at dosages as low as 5 μg/kg, with increases in life expectancy (T/C) of up to 300% recorded. The NCI involvement in the detailed biological evaluation of the halichondrins commenced in 1988–89, as the halichondrins represented a novel antitumour chemotype in a mechanistic class of considerable interest that were potent in vitro and in vivo and had demonstrable in vivo activity. By 1992 the NCI had demonstrated that halichondrin B (**1**) was active in vivo against selected, slow-growing, chemoresistant human solid tumour xenografts [subcutaneous (s.c.) implanted LOX melanoma, KM20L colon with i.p. drug administration, i.p. implanted FEMX melanoma, and OVCAR-3 ovarian also with i.p. administration]. In March 1992, the NCI Decision Network Committee selected halichondrin B (**1**) for anticancer drug development (DNIIA status). These initial evaluations [20], as well as subsequent work, established that halichondrin B (**1**) effectively increased life-span and/or decreased tumour growth rates in human tumour xenograft models which have included LOX IM IV (melanoma), NCI H522 (lung), OVCAR-3 (ovarian) and MDA-MB-435 (breast). In a recent study [17] the optimum route and scheduling was examined using s.c. tumour implants. All of the five regimens tested (qDx5, i.p. and i.v.; q4Dx3, i.p. and i.v. and qDx9 i.p.) in this study resulted in tumour growth suppression, but it was the intermittent q4Dx3 i.v. regimen against MDA-MB-435 that resulted in the highest frequency of regressions (8/10) and the longest duration of remissions (18.9 days) with minimal toxicity. In contrast, daily dosage regimens resulted in fewer regressions and greater toxicity.

These data confirm earlier results that halichondrin B (**1**) is in vivo active by an i.v. route at a remote (s.c.) location against a range of tumour types.

Other factors that are favourable for further development of halichondrin B (**1**) relate to the stability of the compound at physiological pHs, and the solubility of this general class of compound in a wide range of solvents, including some water solubility at concentrations appropriate to dose regimens. Finally, there is the potency of halichondrin B (**1**). The compound is active in vitro in the picomolar range, and typical dose regimens in vivo in the range of 50 μg/kg/dose reflect this potency. However, two factors in particular have mitigated against the further development of halichondrin B at the NCI in the 7 years since the compound first became a candidate for preclinical development. Firstly, and most importantly, there is the supply problem, and until this is unequivocally solved it is unlikely that the Decision Network Committee at the NCI will advance halichondrin B (**1**) from DN-IIA status to DN-IIB, for the next phase of development. The second factor relates to the potency of the halichondrin series of compounds and the magnitude of the therapeutic index. This can be summarized as problems associated with delivery of the drug. The balance of this paper is focused on the 'Supply Problem' and 'Delivery' and an examination of the potential of the halichondrin family of compounds, not just halichondrin B, as potential anticancer compounds.

The Supply Problem

In examining the problem of supplying halichondrins in bulk it is illustrative to make the assumption that this class of compounds will succeed as anticancer drugs and proceed from there. Taking into account the potency of halichondrin B (**1**) in the in vivo trials and projecting dose regimes for human use, the likely requirement for clinical trials would be about 10 g, and something in the order of 1–5 kg/annum as a commercial drug. Currently, the world availability of halichondrins is just 300 mg each of halichondrin B (**1**) and isohomohalichondrin B (**5**). This material originated from a 1-tonne collection of *Lissodendoryx* n. sp. 1 sponge off Kaikoura. The options available for supply of the halichondrins are:

Collection from the Wild: Lissodendoryx n. sp. 1 is a rare, deep water species (–80 to –100 m) found exclusively off the Kaikoura Peninsula on the East Coast of the South Island of New Zealand [21]. An extensive environmental survey using an ROV and a benthic camera was carried out in 1993, and established that the sponge was restricted to an area of only ~ 5 km^2. The mean biomass and abundance of sponge was estimated to be 69 ± 21 g/m^2, with 1.1 ± 0.1 individuals/m^2 over the sponge field. This survey gave an estimated total biomass of the *Lissodendoryx* sponge of just 289 ± 90 tonnes [21, 22] establishing quite unambiguously that it would not be possible to supply the necessary quantities

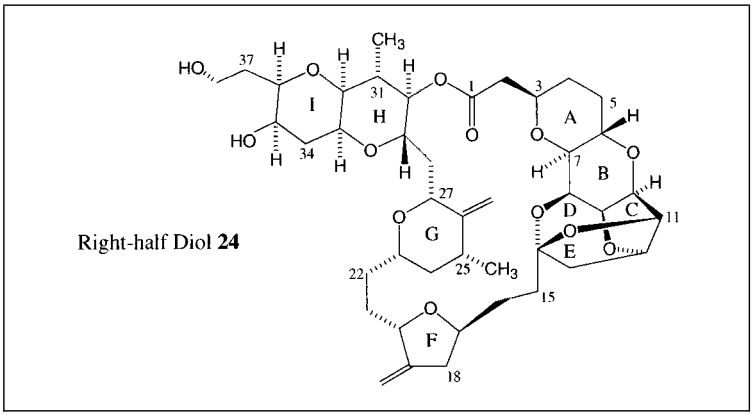

Fig. 7. Right-half diol from halichondrin B.

of halichondrins on a commercial scale by collection from the wild. It was only after the environmental work had been concluded that permission was obtained from Government authorities to allow the collection of just 1 tonne of sponge. To obtain sufficient halichondrins to carry out trials up to the clinical level, at least 15 tonnes would be required. It would be unlikely that permission to undertake such a collection would be obtained, considering the relatively small biomass established for this sponge [21, 22].

Synthesis: The total synthesis of the halichondrins provides another option for the supply of a large amount of halichondrin material. This is by no means a trivial task. Kishi's group [23] first reported the total synthesis of halichondrin B (**1**) and norhalichondrin B (**10**) in 1992. Although the starting points for the synthesis relied upon inexpensive compounds of known absolute stereochemistry, the synthesis was over 100 reaction steps. For the bulk total synthesis of halichondrins to be cost competitive, relative to the bulk extraction methods, a great deal of synthetic refinement will be required. Significant contributions to the synthesis of halichondrin B have also been made by the groups of Salomon, Horita and Yonemitsu, and Burke [see 24].

The commercial supply of halichondrin B (**1**) by total synthesis is unlikely. This is purely an accident of fate and not a comment on the synthetic routes. Not all compounds are amenable to synthesis on a large scale due to the complexity of the skeleton, the number of stereocentres that have to be controlled, or a combination of both of these factors. The halichondrin skeleton certainly falls into this category on both counts. However, the structure-activity analysis of the halichondrin skeleton with its requirement for the right half of the molecule up to C38 offers other potential options. Synthesis of just the right-half diol structure (**24**) (fig. 7) may offer the possibility of a commercially

viable synthesis. It was recently reported that this diol (**24**) exhibits the same activity pattern as the parent halichondrin B (**1**), with a reported cytotoxicity within one order of magnitude of the parent compound [20]. This discovery has concentrated synthetic efforts on the C1–C38 segment of halichondrin B, with a new approach to the C14–C38 segment reported in 1997 [20]. Although this strategy actually contains additional steps, it is an improvement in terms of a practical scalable synthesis. So, bulk supply by synthesis (of a truncated halichondrin with properties comparable to the parent system) should not be discounted.

Aquaculture: Over the past 7 years the National Institute of Water and Atmospheric Research (NIWA), in collaboration with the University of Canterbury and the NCI, have carried out aquaculture feasibility trials on *Lissodendoryx* n. sp. 1. Early work established that small explants were capable of extremely rapid growth, and trial aquaculture experiments over an 18-month period examined the variability of growth and halichondrin production with respect to season, location, site (within location) and depth. It was quickly established that summer transplants were not successful, but winter explants (April) at all sites were generally successful, with high growth rates observed through to December. The mortality rate of the sponges was high in summer, especially at shallower depths, and a critical temperature of 18 ºC has been identified above which the sponge will not survive [22]. This work also established that the halichondrins were being produced under these conditions, but the overall halichondrin content of the cultured sponge was not as high as that of the wild sponge. The observed rate of production was, however, still significant, with yields of halichondrins/kg of sponge ranging from 30 to 60% of that found in the wild sponge.

Bearing in mind that the necessary production of halichondrins should be measured in g or kg and not mg, it was necessary to examine the potential for the large-scale aquaculture production of the halichondrins. The initial experiments showed that the sponge could be grown successfully in small-scale trials, and that the halichondrins continued to be produced at acceptable levels. The move to simulate commercial production conditions was carried out in late 1997. This approach used a variety of culture methods: lantern, tray, bag or disc, all at 10 m depth [25]. It was found that sponges cultured in trays or on discs did not grow well, with fouling becoming a significant problem. The best initial growth rate was observed in lantern cultures, but ultimately bag-type culture systems proved to be the best, both in terms of promoting as well as maintaining fast growth. It was this method of culture that was also the most readily adaptable to a mechanized approach and could be developed to a large-scale stocking-type culture system, not unlike traditional mussel-seeding aquaculture [25].

These initial results demonstrated for the first time the commercial potential for the production of sponge metabolites, and confirmed that aquaculture of sponges is a viable and reliable option for creating extractable biomass. An earlier assessment of sponge biomass production by aquaculture suggested that the annual production of 5 tonnes of sponge/100 m of longline was achievable [22]. However, these estimates were based on use of scallop lanterns. The use of stocking-type systems should influence the likely yields as well as enhancing the economics of production, allowing the production of sufficient sponge mass to take the halichondrins through clinical trials (15 tonnes?). The big question that then needs answering is whether or not aquaculture of *Lissodendoryx* n. sp. 1 can be scaled up to say 1,000–5,000 tonnes/annum.

Tissue Culture: Sponge cell culture has been investigated as a possible source of halichondrins [26]. Attempts to develop long-term, continuous cell lines from marine sponges are still very much in their preliminary stages. Indeed, it has recently been suggested that even the so-called long-term cultures from several marine sponges are likely to be artefactual. The 'sponge cells' that have been maintained in culture for an extended period of time have been identified as thraustochytrids [27]. At this point there should be no expectation that the supply problem could be solved by this approach.

Genome Transfer: Genome transfer to a vector appropriate for fermentation requires the identification of the producing organism. This is not clear cut in the case of the halichondrins, with some thought that a symbiont could be responsible for their production. However, preliminary cell separation studies on *Lissodendoryx* sp. indicate that the halichondrins are associated with the sponge cells [26, 28]. It is hoped that a cell separation study of *Lissodendoryx* n. sp. 1 currently being undertaken by NIWA, in collaboration with the University of Canterbury Marine Chemistry Group, will confirm this result.

Drug Delivery

A poor therapeutic index is a problem frequently encountered in drug development. The high potency of many of the current finds from marine organisms (spongistatins, bryostatins, didemnins, etc.) only serves to exacerbate this problem. The development of a successful pharmaceutical requires that attention also be paid to delivery of the drug. This is because the potent antiproliferative properties of these anticancer compounds alone will never overcome solid tumours because of the sensitivity of the surrounding tissue to the fatal effects of exposure to such drugs. This lack of selectivity for the tumour and the sensitivity of surrounding tissues is observed in current

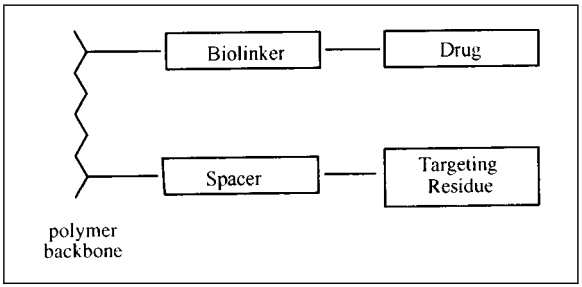

Fig. 8. Schematic of a polymer drug conjugate.

chemotherapy as adverse side effects (hair loss, bleeding gums, nausea, etc.). Alternative approaches are required that specifically target tumours. One approach, known as polymer therapeutics, is a rapidly growing multidisciplinary field requiring the combined talents of organic chemists, polymer chemists, pharmacologists and oncologists [29]. In polymer therapeutics the drug is attached via a biodegradable linker to a water-soluble polymer, and, if necessary, specific targeting residues can also be added (see fig. 8).

It is the physical properties of the polymer therapeutics that result in better targeting of tumour tissue, which in turn leads to higher selectivity. Macromolecules, such as polymer drug conjugates, cannot enter cells by diffusion in the manner of unbound low-molecular-weight drugs. The normal mechanism whereby a polymer passes through the cell membrane is by endocytosis. This restriction of cellular uptake to endocytosis enhances the residence time of the polymer drug in the bloodstream. In addition, tumour vasculature often displays a discontinuous endothelium (i.e. is leaky) which allows macromolecular extravasation of the polymer therapeutic which, when coupled with a lack of effective lymphatic drainage within the tumour, leads to accumulation of the polymer therapeutic at the desired site of action. This phenomenon is known as the enhanced permeability and retention (EPR) effect [30]. Figure 9 illustrates the two main factors giving rise to the EPR effect.

The success of this approach has been confirmed with drugs such as PK1 which are currently in phase I/II clinical trials. In the PK1 construct, the anticancer drug doxorubicin has been attached via a tetrapeptide linker to a water-soluble hydroxypropyl-methacrylamide (HPMA) polymeric backbone. The tetrapeptide linker was designed to resist peptidase activity in the bloodstream, but be susceptible to lysosomal enzymatic hydrolysis following the transfer by endocytosis to the interior of the target tumour cells [31].

At the University of Canterbury we are currently exploring the possibilities of polymer therapeutics as a means of enhancing the value of marine toxins.

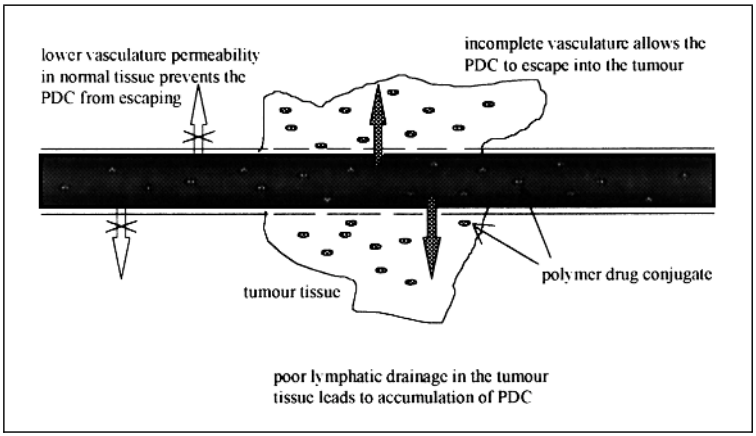

Fig. 9. Enhanced permeability and petention (EPR) effect.

While the potential of compounds such as the halichondrins as anticancer compounds has already been established, we consider that modifications that might enhance pharmacokinetic properties, reduce required plasma concentrations and exhibit enhanced selectivity can only be considered advantageous. To this end, we are working in collaboration with the NCI and the London School of Pharmacy on the development of polymeric therapeutics based on marine toxins. Initially, halichondrin-based polymer therapeutics will be prepared. Already, an amino derivative of the halichondrin skeleton has been synthesized and converted into a polymeric form comparable to PK1. Other marine-based polymer therapeutic drugs will follow, utilizing other extreme toxins such as the mycalamides [32], the discorhabdins [33], pateamine [34], calyculinamides and swinholides [35] that have been isolated from New Zealand marine organisms.

Conclusions

The halichondrin story is that of a group of interesting, but stalled, anticancer compounds and is illustrative of the problems associated with attempts to develop a pharmaceutical from a marine macroorganism. No matter how effective a compound might be as a drug candidate, the ongoing supply will always be a critical question. In the case of the halichondrins the biology has been established, the pharmacophore defined, alternative and enhanced means of delivery examined and, most importantly, production by

aquaculture (or synthesis) addressed. Unfortunately, the aquaculture has only been evaluated on a small scale, so the economics of production remain unknown. It is a classic Catch 22 situation. Halichondrin B will not advance at the NCI until supplies of the drug are assured. The supply of the drug cannot be assured until money is invested in the scale-up of the aquaculture operation.

For any pharmaceutical lead from a marine macroorganism, supply issues will always be a problem, and unless supply can be addressed in an economically feasible fashion, the dream of new effective drugs from the sea will falter.

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