Chemical Studies of Marine Bacteria: Developing a New Resource

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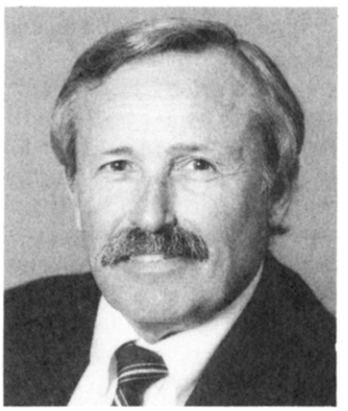

William Fenical was born in Chicago, IL, in 1941. He received his B.S. in Biochemistry from California State Polytechnic University in 1963 and his M.S. in Organic Chemistry from San Jose State University in 1965. In 1968, he completed his doctoral studies with Prof. P. Radlick at the University of California, Riverside. He remained at Riverside to work with Prof. D. R. Kearns on an American Cancer Society Postdoctoral Fellowship until 1969. He began as a lecturer at the Scripps Institution of Oceanography in 1973. He subsequently became a Professor of Oceanography in 1983 and the Director of the Marine Research Division in 1989. The author of over 200 papers, he has also been active as a consultant and an organizer of meetings and symposia, and he is currently the President-Elect of the International Society of Chemical Ecology.

I. Introduction

A. Bacteria and Their Chemistry

Microorganisms, and in particular the bacteria, have had a profound effect on the development of chemistry and upon medical science. Since the discovery of penicillin in 1929, intensive studies of mainly soil-derived bacteria and fungi have shown that microorganisms are a rich source of structurally-unique, bioactive substances. Over the past 60 years, between 30 000 and 50 000 natural products have been discovered from microorganisms. More than 10 000 of these compounds are biologically active and more than 8 000 are antibiotics and antitumor agents.[1,2] Today, over 100 microbial products continue to be used clinically as antibiotics, antitumor agents, and agrichemicals. The importance of these compounds in medicine has served to underscore their importance in chemistry. Throughout the years, extensive chemical programs developed worldwide to synthesize these compounds and to understand the structural foundations for their bioactivity. Still today, massive efforts are underway in the pharmaceutical industry to explore soil bacterial fermentation products (estimated at in excess of $9 billion per year!). These studies continue to show exciting results. The discoveries of bacterial metabolites in the FK-506 and esperamycin classes, for example, have generated new insight in basic mammalian immunology and provided new approaches in the treatment of cancer. It is therefore not surprising that chemists, on a worldwide basis, have dedicated tremendous efforts toward studying the conformations, reactivities, and structural variations of metabolites from bacterial fermentation.

Most metabolites of bacterial origin come from one group, Gram-positive soil bacteria of the order Actinomycetales. Although these bacteria continue to be studied extensively, it is clear that the rate of discovery for novel metabolites is decreasing. It can now be estimated that in excess of 90% of all bioactive cultures discovered produce previously known agents. This rate of duplication is discouraging and highly cost inefficient. It is also true that infectious diseases are rapidly developing resistance toward traditional antibiotics. Recently observed strains of *Mycobacterium*, the cause of tuberculosis, are multiply drug resistant, and antibiotics to control this specific bacterium are simply not available. For these reasons, and to assure the constant flow of novel new drug candidates, it is imperative that new sources of bioactive natural products be developed for the next decade.

The world's oceans compose over 70% of the earth's surface and over 90% of the volume of its crust. Microbiologically, the oceans are massively complex and consist of a diverse assemblage of life forms which occur in environments of extreme variations in pressure, salinity, and temperature. Marine microorganisms have developed unique metabolic and physiological capabilities that not only ensure survival in extreme habitats, but also offer the potential for the production of metabolites which would not be observed from terrestrial microorganisms.

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B. Early Studies of Marine Bacteria

In the late 1940s and 1950s, the pioneers of marine microbiology, such as Claude ZoBell, became active in delineating the vast numbers and diversity of true marine bacteria. Their potential in chemical synthesis was first recognized by Rosenfeld and ZoBell[3] and Grein and Meyers,[4] who showed that marine bacteria produce antimicrobial agents. Slightly later, the bactericidal property of seawater was recognized, and it was suspected that this was due to the production of antibiotics by planktonic algae[5] and bacteria.[6,7]

Despite these early observations, relatively little attention has been directed toward the study of natural products from marine bacteria. Apparently there has been the widespread perception that marine bacteria are extremely difficult to isolate and cultivate. It is true that only a small percentage of the viable bacterial cells in marine samples ultimately grow under standard culture conditions. However, the isolation and cultivation of marine bacteria can be readily undertaken, at least for the more common and well-known genera. Marine bacteria are uniquely adapted to saline environments, and for the most part, they require salt for growth. It is also true that few comprehensive investigations of the diversity and distribution of marine bacteria have been reported. The overall result is that few marine bacteria have been the subject of comprehensive chemical study.

In this chapter, I provide a brief overview of marine bacteria and a summary of most of the structurally-unique metabolites that have been discovered through fermentation. Most of the compounds discovered to date come from only a few groups of microorganisms, and as new and unusual marine microorganisms are studied, it is certain that new metabolites will be discovered from this developing resource.

II. Bacteria in the Marine Environment

A. Eubacteria

Most marine bacteria, including the cyanobacteria, are classified as eubacteria. These organisms are almost exclusively unicellular, and they are classified as Gram-negative or Gram-positive, depending upon the structures of their cell walls. The actinomyctes, or filamentous bacteria, which have been the single most important source of exciting metabolites from soil bacteria, are also members of the Gram-positive eubacteria.

B. Archaebacteria

The more primitive archaebacteria are important bacterial inhabitants of extreme marine environments. The halophilic archaebacteria, for example, are commonly found only in saturated salt ponds. These organisms commonly require a minimum of 12–15% salt for growth, and some tolerate supersaturated solutions of up to 24% salt. Other examples are the thermoacidic archaebacteria, which grow in solutions of temperatures in excess of 90 °C and acidities in the range of pH 1, and the strictly anerobic methanogens, which reduce organic matter to methane gas. Although little is known of the metabolites of the archaebacteria, the potential for these primitive bacteria to produce unique metabolites has been recognized.[8]

C. Distributions in Marine Environments

In the context of developing a new chemical resource and effectively sampling marine bacteria, it will be essential to gain a solid understanding of the distributions of various bacterial groups in the complex marine environment. Overall, the distributions of marine bacteria are poorly known. Gram-negative bacteria, such as those of the common marine genus *Vibrio*, are found in abundance in seawater. Here the Gram-negative bacteria comprise approximately 90% of the bacterial flora. The remainder are Gram-positive forms of a variety of taxonomic affiliations including representatives of the genus *Bacillus*.[9] The other important microhabitats for marine bacteria are the sediments, animate and inanimate surfaces, and the internal spaces of invertebrate animals. Marine plants and animals are well known to have developed symbiotic relationships with numerous microorganisms. This is particularly true of the bacteria, which are widely distributed on the surfaces and within the tissues of marine plants and animals. The importance of bacterial symbiosis is growing in recognition that bacteria may be the true producers of many compounds isolated from sponges, ascidians, and other marine invertebrates.

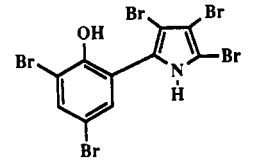
III. Chemistry of Marine Bacteria

A. Seawater-Derived Bacteria

The bacteria in seawater are mainly Gram-negative rods belonging to taxonomic groups which when isolated from the soil, have proven to be chemically unproductive. Nonetheless, some of the first metabolites from marine bacteria were those from bacteria isolated directly from seawater. To the best of my knowledge, the first marine bacterial metabolite to be reported was the highly brominated pyrrole antibiotic, 1 (Chart I), isolated by Burkholder and co-workers through fermentation of a bacterium obtained first from the surface of the Caribbean seagrass *Thalassia*.[10] Although this bacterium was isolated from the surface of a seagrass, it belongs to a well known group of Gram-negative seawater bacteria, and the bacterium has subsequently been repeatedly observed from seawater samples. The highly unique metabolite 1 was identified by X-ray crystallographic methods,[11] and is composed of more than 70% bromine by weight. The molecule showed impressive *in vitro* antibiotic properties against Gram-positive bacteria, with minimum inhibitory concentrations (MICs) ranging from 0.0063 to 0.2 μg/mL. The antibiotic was not active against Gram-negative bacteria however, and it proved inactive in whole animal assays. Although this bacterium was first assigned as *Pseudomonas bromoutilis*, the biochemical characteristics of this isolate were later found to indicate an affinity to the genus *Alteromonas*.[12] In more recent work, a synthesis of this compound (later named pentabromopseudiline) and its antitumor properties were reported.[13]

The discovery of this exceptional molecule suggests that marine microorganisms may be as unique as their macroscopic counterparts. Like the plants and inver-

Chart I. Compounds Isolated from Seawater-Derived Bacteria



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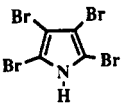

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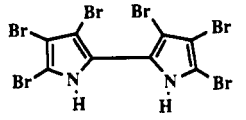

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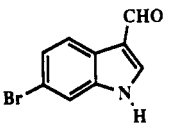

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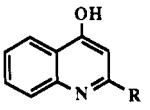
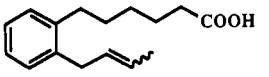


5, R=C_7H_{13}
6, R=C_5H_{11}



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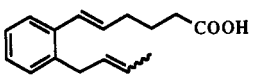

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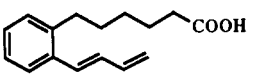

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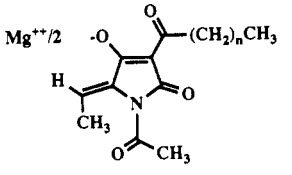
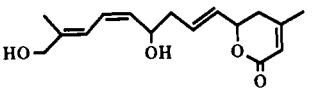


10, n = 4,6



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tebrates in marine habitats, some bacteria have the ability to incorporate bromine into organic compounds. This is a common mechanism in marine systems, resulting in bromination of metabolites which possess enhanced bioactivities. The bioactivities reported for these simple brominated metabolites, however, did not appear to provide useful leads in antibacterial or anticancer chemotherapy.

Several years later, the Faulkner group in Califronia isolated a purple-pigmented bacterium which also provided potent antibiotics.[14] The strain, originally defined as a *Chromobacterium* sp., (now revised by Baumann *et al.*[15] to the genus *Alteromonas*) was isolated from seawater samples collected in the North Pacific Ocean. Comprehensive chemical analysis showed that this organism produces, in culture, several antimicrobial compounds including the previously described pyrrole **1**, tetrabromopyrrole (**2**), hexabromo-2,2′-bipyrrole (**3**) and several simple phenolics including 4-hydroxybenzaldehyde and *n*-propyl 4-hydroxybenzoate. Tetrabromopyrrole (**2**) showed moderate antimicrobial activity *in vitro* against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*. It was even more active against a group of marine bacteria and showed autotoxicity against the producing *Chromobacterium* sp. itself.

In a continuing investigation, the same group later isolated an antibiotic producing bacterium from a La Jolla, CA, tide pool seawater sample.[16] The yellow strain was identified as a pseudomonad, and careful analysis of its metabolic products showed the production of 6-bromoindolecarboxaldehyde (**4**), its debromo analog, and a mixture of 2-*n*-pentyl- and 2-*n*-heptylquinolinol (**5** and **6**), the latter a known antibiotic produced by strains of *P. aeruginosa*. The most potent of these simple antibacterial agents was 2-*n*-pentylquinolinol (**6**), which showed its greatest activity against *Staphylococcus aureus*. The unique metabolite, 6-bromoindolecarboxaldehyde (**4**), lacked antibiotic properties.

Before its closure in the late 1970s, researchers at the Roche Research Institute in Australia had identified marine bacteria as a novel new target for the isolation of natural products. Although their program was only just beginning, they did initiate studies of a strain of *Alteromonas rubra*, a typical seawater Gram-negative bacterium. Under fermentation culture, the bacterium produced a series of C_{16} aromatic acids, **7**–**9**, which are acetogenins of obvious fatty acid synthetic origin.[17] The acids showed interesting pharmacological properties in bronchodilator assays and in neuromuscular assays designed to detect relaxant effects.

Two antibiotic pigments, the magnesidins (**10**), were isolated as a 1:1 mixture of methylene homologs (4- and 6-methylene groups) as minor metabolites of the marine bacterium *Pseudomonas magnesiorubra*.[18,19] While *P. magnesiorubra* was originally isolated from the surfaces of the tropical marine green alga *Caulerpa peltata*, it is closely related to the typical seawater bacteria and hence included here. These unique pigments are thought to be oxidation products of prodigiosin, a common tripyrrolic pigment produced by marine as well as terrestrial bacteria.

More recently, the Andersen group in British Columbia reported the isolation of a structurally-unprecedented lactone, oncorhyncolide (**11**), which is produced by a seawater-derived bacterium obtained from samples taken near a Chinook salmon (*Oncorhyncus tshawytscha*) net-pen farm.[20] Oncorhynoclide is a unique lactone of unknown biosynthetic origin. Current biosynthetic studies indicate that it could be of at least partial terpenoid origin, or derived by unique methylation reactions not yet observed in nature.

The seawater-derived bacteria studied so far have been taxonomically very limited and seemingly driven mostly by screening processes for new antibiotics. Because of this, the diversity of chemical products is yet to be fully defined. Programs which evaluate seawater bacteria using other criteria are quite likely to encounter the production of more diverse metabolites. New programs should keep in mind, however, that the Gram-negative bacteria are not generally prolific in their production of extracellular metabolites. Fermentation yields of less than 1 mg of total organics per liter from dense cultures are common, if not the rule. Anyone wishing to explore this potentially exciting new group of microorganisms should plan for scaleup

to the 100-liter range before anticipating success in this endeavor.

B. Sediment-Derived Bacteria

Although it is tempting to draw an analogy between marine sediments and terrestrial soil, they are generally not considered to be closely related microenvironments. Nonetheless, like the soil, marine sediments are a more nutrient-rich microhabitat which varies greatly in organic content from place to place. Sediments from estuaries and bays are obviously organic rich and likely to provide a diversity of bacteria not found in more nutrient-limited habitats. In shallow coastal waters, including temperate and tropical oceans, geologically diverse sediments are found with different chemical characteristics. It is not surprising that these sediments yield a diversity of bacterial flora. One consistent feature is the presence of surfaces which is known to stimulate and support enhanced bacterial colonization and growth. Because of the above, sediments and "marine muds" have provided the most prolific resource for the isolation of chemically-prolific marine bacteria.

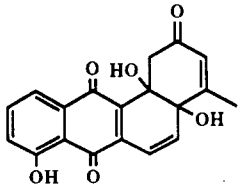
Our knowledge of the chemistry of marine sediment-derived bacteria, particularly the actinomycetes, is almost entirely the result of the pioneering work of researchers at the Institute of Microbial Chemistry in Tokyo. Beginning in the 1970s, Okami and co-workers were innovative in isolating bacteria from both the shallow- and deep-water sediments from the coasts of Japan. Their work, summarized in several recent reviews, provides the foundation for the development of this field.[21,22]

In 1975, the Institute of Microbial Chemistry group reported the isolation of an antibiotic-producing actinomycete, *Chainia purpurogena* SS-228, from mud samples obtained from Sagami Bay. The bacterium was found to produce the benzanthraquinone antibiotic **12** (Chart II), the structure of which is a modification of the well-known anthraquinone antibiotic class.[23,24] This compound showed selective inhibition of Gram-positive bacteria, with MIC values of between 1–2 μg/mL, and was an active antitumor agent *in vivo* against Ehrlich carcinoma in mice, showing significant life-span extension at low doses. In addition, quinone **12** deactivated dopamine-β-hydroxylase (producing 65% inhibition at 0.1 μg/mL). Most interestingly, the production of **12** was only observed in selected seawater media containing the unique Japanese seaweed product "Kobu Cha". Kobu Cha is a dried and pulverized powder produced in Japan from the brown seaweed *Laminaria*. This paper was among the first to show that bacteria of order Actinomycetales could be isolated from marine environments. It created some discussion, however, as actinomycetes had been considered rare or nonexistent in the oceans. Actinomycetes isolated from marine samples were considered to have entered the ocean via rivers and through runoff. What is clear is that actinomycetes are not as abundant in marine as in terrestrial samples. Recent observations, however, show that distinct populations of marine actinomycetes exist in tropical habitats and that these organisms require salt for growth.[25]

Another significant aspect of the *Chainia purpurogena* antibiotic study was the specificity of production of antibiotic only under selected, marine-specific nutrient conditions. This observation clearly shows that marine microorganisms have nutrient adaptations which relate to their natural nutrients in marine habitats. For future studies, there is obviously a clear need to develop selective marine isolation and mass culture media which utilize *natural* nutrients and growth factors derived directly from marine sources.

The istamycins A and B (**13** and **14**), are other examples of antibiotics isolated by the Tokyo group from the culture broths of marine actinomycetes.[26] The istamycins were produced by fermentation of the marine streptomycete, *Streptomyces tenjimariensis* SS-939, collected from a shallow-water mud sample from Sagami

Chart II. Compounds Isolated from Sediment-Derived Bacteria



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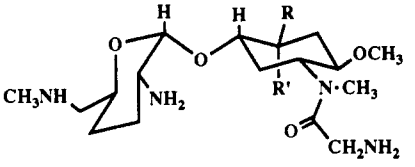
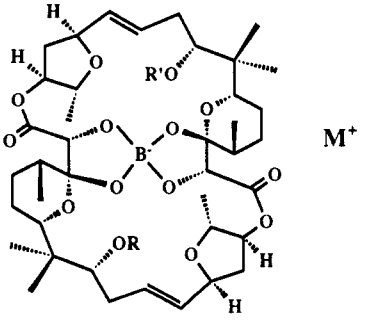
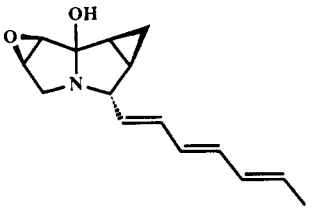


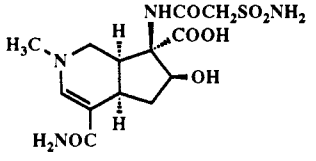
13, istamycin A, R=H, R'=NH_2
14, istamycin B, R=NH_2, R'=H



15, aplasmomycin A, R=R'=H
16, aplasmomycin B, R=H, R'=Ac
17, aplasmomycin C, R=R'=Ac



18



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Bay, Japan. The compounds show strong *in vitro* antibiotic activity against both Gram-negative and Gram-positive bacteria, including some strains which are known to be resistant to the aminoglycoside antibiotics. Istamycins A and B showed MIC values of between 0.10 and 3.0 μg/mL against various species and isolates of *Staphylococcus*, *Bacillus*, *Corynebacterium*, and *Escherichia*. The compounds were much less active against *Pseudomonas*, *Klebsiella*, and *Serratia* species. The istamycins are related to the fortimicins and sporaricins, aminoglycoside antibiotics produced by terrestrial actinomycetes. They appear more therapeutically important, however, because of their activity against typical aminoglycoside-resistant pathogens.

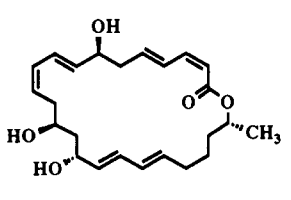
Among the more unusual of the compounds isolated from marine actinomycetes are the aplasmomycins A–C (15–17), also reported by the Tokyo group. The compounds are produced by a marine actinomycete identified as *Streptomyces griseus* SS-20, isolated from shallow mud samples also from Sagami Bay.[27–29] Here too, this organism only produced the aplasmomycins when cultured with Kobu Cha-containing media or when grown under conditions (27 °C and very low nutrients) which relate to the natural environment of Sagami Bay. The aplasmomycins are antibiotics which inhibit Gram-positive bacteria *in vitro* with MIC values of between 0.8 and 3.0 μg/mL against *Staphylococcus aureus*, *Bacillus subtilis*, *Bacillus anthracis*, and *Corynebacterium smegmatis*. More importantly, aplasmomycin is an effective antimalarial agent *in vivo*, resulting in cures of *Plasmodium berghei*-infected mice. It was on the basis of this potent antiplasmodium activity that aplasmomycin received its name. The structure elucidation of aplasmomycin A was performed by X-ray crystallographic methods. To the surprise of the Tokyo group, the molecule contained an unexpected boron atom in the center of the complex.[30] Although quite remarkable structures, these compounds are precedented by the boromycins, similar boron-containing ionophores produced by a terrestrial actinomycete. Recently, an aplasmomycin C-producing actinomycete was isolated from sandy sediment sample in California. Unexpectedly, the structure of aplasmomycin C was found to be even more complex than originally observed. By NMR and X-ray methods, aplasmomycin C (17) has been recently found to exist in several chromatographically-stable ring conformers.[31]

The Tokyo group has also reported manipulations of the expression of antibiotic synthesis in some marine actinomycetes. When protoplasts of the istamycin-producing strain *S. tenjimariensis* SS-939 were fused with the aplasmomycin-producing *S. griseus* SS-20, a new clone, SK2-52, was obtained which produced a new indolizine antibiotic, indolizomycin (18).[22,32,33] While similar studies of antibiotic gene regulation have been reported from terrestrial actinomycetes, this innovative study pointed to the power of modern molecular methods as applied to the developing field of marine microbiology.

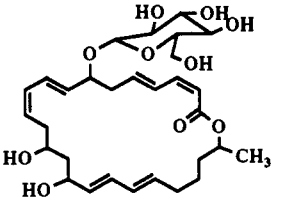
The most recent work from the Tokyo group is a paper reporting the isolation of a structurally-novel alkaloid, altemicidin (19), which is produced by a marine strain of *Streptomyces sioyaensis* SA-1758.[34,35] This exceptional alkaloid was detected by screening cultures for toxicity against the common brine shrimp *Artemia salina*. Earlier reports have shown that *Artemia* toxicity translates amazingly well to antitumor activity.[36] Indeed, altemicidin showed potent antitumor activity *in vitro* against L1210 murine leukemia and IMC carcinoma cell lines with IC_{50} values of 0.84 and 0.82 μg/mL, respectively. The new compound showed weak antibacterial activity, but was relatively toxic in mice (LD_{50} = 0.3 mg/kg iv), probably limiting its chemotherapeutic uses. Altemicidin is an exceptionally novel sulfur- and nitrogen-containing microbial metabolite. While its biosynthesis has not been defined, altemicidin possesses a monoterpene carbon skeleton, indicating it may be produced via the mevalonate pathway.

Our efforts in this field have focused upon explorations of bacteria found in tropical and subtropical marine habitats and upon developing cultures of bacteria from the deep seas. Screening for antitumor effects, a deep-sea bacterium was isolated from a sediment sample obtained from −1000 m along the California coast. Fermentation of the slow-growing bacterium in a salt-based medium yielded a series of novel cytotoxic and antiviral macrolides, the macrolactins A–F (20–25) (Chart III).[37] The bacterium, isolate C-237, was Gram-positive, had a requirement for salt, and could not be identified by standard biochemical

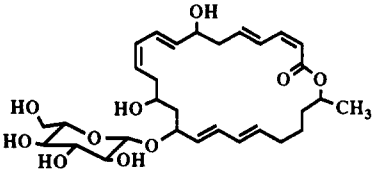
Chart III. Compounds Isolated from Sediment-Derived Bacteria



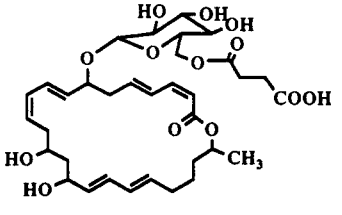
20, macrolactin A



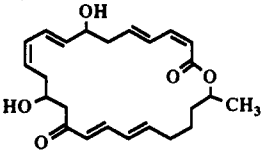
21, macrolactin B



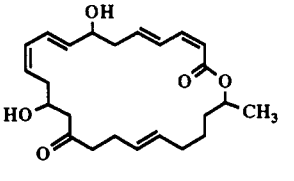
22, macrolactin C



23, macrolactin D

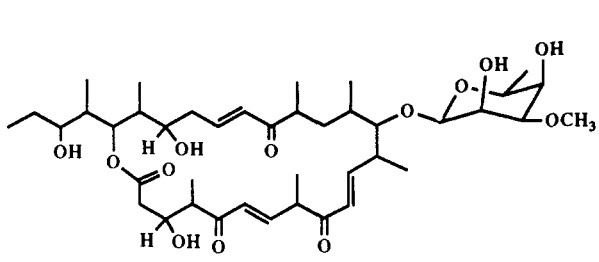


24, macrolactin E



25, macrolactin F

Chart IV. Compounds Isolated from Sediment-Derived Bacteria



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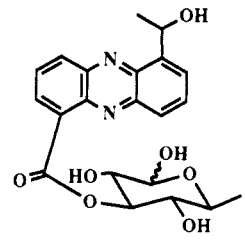

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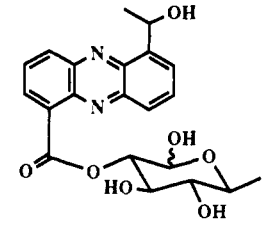

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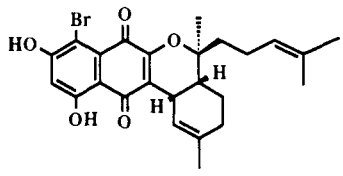

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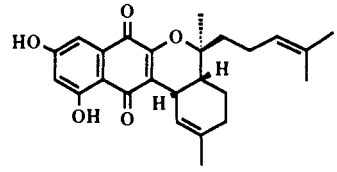

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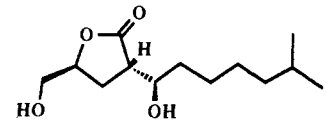


31

methods. Under standard fermentation at atmospheric pressure, bacterium C-237 produced the six macrolides and two open-chain hydroxy acids in varying amounts. Macrolactin A (**20**) was produced as the major metabolite (ca. 4–8 mg/L) in most of the fermentations. The majority of the biological properties were due to macrolactin A, which showed modest antibacterial activity, but was active against B16-F10 murine melanoma *in vitro* with IC_{50} values 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). The structures of these compounds were determined by combined spectral methods and did not include the absolute stereochemistries at the four chiral centers. In a collaborative effort with Rychnovsky and co-workers at the University of Minnesota, we have recently established the complete stereostructure for macrolactin A as shown.[38]

Of the marine actinomycetes studies, those of the genus *Streptomyces* have clearly predominated. It must be noted, however, that non-streptomycetes are common marine actinomycetes[25] and that their chemical behaviors remain virtually unknown. One example of studies of a marine non-streptomycete is our recent investigation of a sediment-derived actinomycete (CNB-032) found in the shallow sediments of Bodega Bay, CA. On the basis of its cell wall constituents and whole cell sugar composition, this organism keys to a member of the suprageneric group Maduramycetes. Fermentation of this organism resulted in the production of maduralide (**26**) (Chart IV), a new member of a rare class of macrolide. Although the biological properties of this macrolide are not yet known, the compound is a member of a rare 24-membered ring lactone group represented previously only by rectilavendomycin.[39] The compound was not crystalline, hence X-ray methods could not be applied to the structure determination. The structure assignment, yielding the molecule without stereochemical detals, was determined by combined spectral methods emphasizing 2-D NMR.[40]

In related studies of the sediment bacteria from Bodega Bay, CA, we isolated a *Streptomyces* sp., isolate no. CNB-253, which produced some possibly useful antibacterial compounds.[41] Purification of the extract of the whole fermentation broth yielded four new compounds (α- and β-**27** and α- and β-**28**) of the phenazine class. These compounds are phenazine esters of the rare sugar, L-quinovose, at the sugar 2′ and 3′ positions. Although the α and β anomers in each series were readily isolated by silica HPLC, they were found to interconvert. The new metabolites show antibacterial activities against a range of Gram-negative and Gram-positive bacteria, with MIC values in the 1–4 μg/mL. They were not appreciably cytotoxic against human tumor cells *in vitro*.

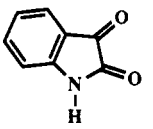
In a similar study from California, an unidentified estuarine actinomycete, isolate CNB-632, (probably a *Streptomyces* sp.) was found to produce two metabolites of a unique mixed biosynthetic origin. Marinone and debromomarinone (**29** and **30**) are antibacterial metabolites possessing common naphthoquinone, but rare sesquiterpenoid structural components. In addition, marinone (**29**) possesses a bromine substituent in the dihydroxybenzene ring, a position typical for bromination in marine metabolites. Both molecules show antibacterial activities, in the range of 1–2 μg/mL, against Gram-positive bacteria of the genera *Bacillus* and *Staphylococcus*.[42]

Lastly, we reported the structure of a simple C_{13} butanolide, **31**, which was isolated from a sediment bacterium, actinomycete no. CNB-228, isolated from coral reef carbonate sediments (~80 ft) from the Bahamas.[43] The simple butanolide was closely related to a series of signal compounds previously known to regulate secondary metabolite synthesis in these microorganisms. As part of the structure elucidation process for **31**, an enantioselective synthesis was performed.[43]

C. Surface-Associated and Symbiotic Bacteria

As more evidence is obtained, it is becoming abundantly clear that bacteria form highly specific, symbiotic relationships with marine plants and animals. Given this situation, one begins to wonder about the true origins of marine metabolites previously isolated from sponges, ascidians, alcyonarians, and other filter-feeding

Chart V. Compounds Isolated from Surface-Associated and Symbiotic Bacteria



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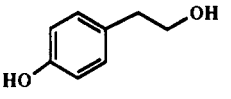

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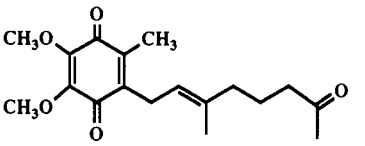

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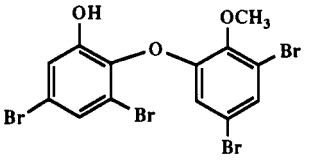

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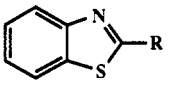
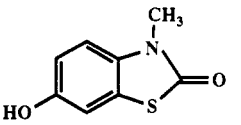


36, R= SH
37, R= CH_3
38, R= OH



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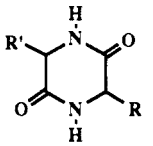
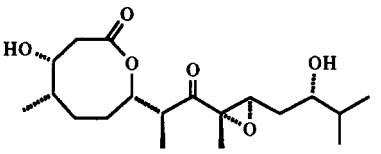
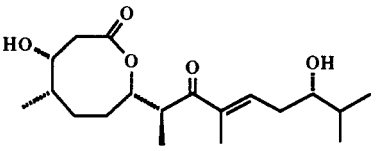


40, various amino acids



41, octalactin A



42, octalactin B

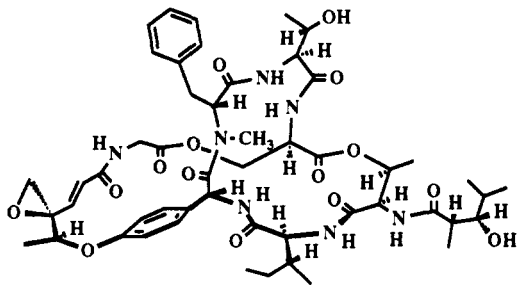
marine invertebrates. Are bacterial and cyanobacterial symbionts responsible for this production? At this point we have insufficient information to make an informed decision. A major problem encountered in working with symbiotic bacteria (symbiotic defined here as simply living together on a species-specific basis) is the need to rigorously establish that a true symbiosis exists. Frequently, bacterial isolates have been reported as being symbionts without any information relating to the actual presence of the bacteria on or within the tissues of the animals in question. A major problem, and one perhaps solved by the utilization of taxon-specific molecular probes, is to establish that a bacterium is indeed specific to the host. A bacterium found throughout the marine environment, or found only sporadically in association with invertebrates, cannot be considered a true symbiont, but only a nonobligate associate.

Despite the difficulty in defining true symbiotic bacteria, the surfaces and internal spaces of marine organisms are a unique microhabitat in which bacteria are regularly observed. These environments are more nutrient rich than seawater and most sediments, thus they would likely be a unique niche for the isolation of diverse bacteria, perhaps even the Gram-positive bacteria which are more commonly observed in organic-rich habitats.

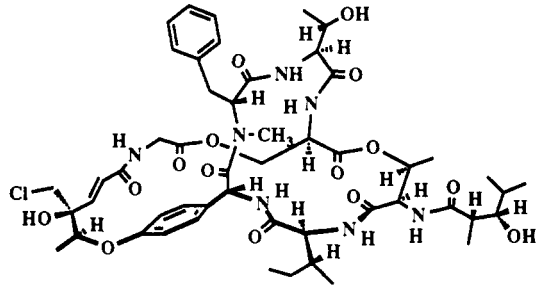
Our experiences in this area began with a study of the pathogen resistance of the estuarine shrimp *Palaemon macrodactylus*. We observed that the eggs of this animal possess significant surface bacteria, which when removed by treatment with antibiotics, led to the rapid infestation of the eggs of pathogenic fungi, especially the known crustacean pathogen *Lagenidium callinectes*. Although there are many plausible mechanisms to explain this protective phenomenon, with *Palaemon* the answer appears to lie in the bacterial production of defensive antifungal agents. Fermentation of the isolated bacterium (an *Alteromonas* sp.) led to the isolation of an unexpectedly potent antifungal agent, 2,3-indolinedione, also known as isatin (**32**) (Chart V).[44] This compound had been known for years as a synthetic compound produced by the nitric acid oxidation of tryptophan. Isatin was not recognized as an antifungal agent, although related indolinediones have shown various therapeutically relevant pharmacological properties. In this case, subsequent studies by scanning electron microscopy (SEM) gave strong confirmatory evidence that *Alteromonas* sp. is a true surface symbiont. In a similar study, the American lobster, *Homarus americanus*, was investigated leading to similar results. SEM analysis showed the eggs of *Homarus* were totally covered with an unidentified unicellular bacterium. In a similar fashion, the cultured bacterium produced very large amounts of tyrosol, 2-(*p*-hydroxyphenyl)ethanol (**33**). At the concentration observed, it appears that this simple phenolic would be effective in controlling the encroachment of pathogenic microorganisms.[45] Similarly, the tropical filamentous cyanobacterium *Microcoleus lyngbyaceus* from Puerto Rico appears to possess highly specific eubacteria on its surface.[46] Samples of *Microcoleus lyngbyaceus* were collected from 65 sites around the island, and the bacteria were isolated and compared. To our surprise, four specific strains of highly-colored bacteria (one purple, one red, and two yellow) were consistently obtained only from the surface of the cyanobacterium. These strains were not isolated from adjacent seawater samples nor from proximate, morphologically-similar filamentous green algae. Fermentation of the bacterium under high-nutrient conditions led to unique results. All four strains produced the same antibiotic material, identified as the quinone **34**. This molecule, produced at levels of 20 mg/L, possesses significant antifungal and antibacterial activities, and it had never been observed as a natural product. Biosynthetically, this compound appears to be produced by oxidative cleavage of a ubiquinone-type precursor.

Several investigations of the bacteria associated with sponges have been reported. Most important is a recent

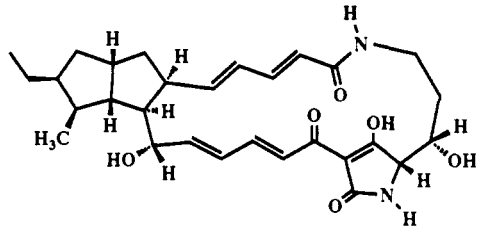
Chart VI. Compounds Isolated from Surface-Associated and Symbiotic Bacteria



43, salinamide A



44, salinamide B



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report, by the Vladivostok group, of the successful isolation of a bacterium, a *Vibrio* sp., from an Indian Ocean sponge, *Dysidea* sp. In culture, the bacterium appears to produce small amounts of the bis(dibromophenyl) ether **35**.[47,48] The bis(bromophenyl) ethers are typical products isolated from some *Dysidea* sponges, and it has been speculated that they may be bacterial in origin. This is the first report which provides experimental evidence to support the hypothesis of microbial production. In several other cases, bacteria have been isolated from sponges and their metabolites identified. As part of a study to assess the true origin of metabolites from the Caribbean sponge *Tedania ignis*, studies of bacteria isolated from the ground tissues were reported. From a cultured *Micrococcus* sp., the benzothiazoles **36–39** were observed. The same isolate also produced a series of diketopiperazines **40** composed of various amino acids.[49] Since the diketopiperazines were also isolated from the sponge, this was presented as evidence of symbiont synthesis.[50] This evidence of bacterial production is not strong, however, as in excess of 90% of all Gram-negative bacteria produce these same diketopiperazines when grown in nutrient-rich media.

Although marine actinomycetes are significant inhabitants of shallow marine environments, their distributions on living surfaces in the ocean remains unclear. Very few actinomycetes exist on the surfaces of invertebrates, but those that do may be chemically interesting. We isolated an unidentified streptomycete from the surface of a gorgonian coral (*Pacifigorgia* sp.) from the Gulf of California, Mexico. This isolate (strain PG-19), when grown in marine media, produced a series of undescribed metabolites possessing cytotoxic and antibiotic properties. Two of the derivatives isolated are the 20-hydroxy derivative of oligomycin-A and the 5-deoxy derivative of enterocin, two previously described classes of antibiotics. These compounds possess antibiotic properties similar to their parent molecules. In addition, this organism yielded two totally new cytotoxic agents, octalactins A and B (**41** and **42**), belonging to a new structure class. The compounds are C_{19} ketones possessing rare 8-membered ring lactone functionalities. Octalactin A (**41**) possesses potent *in vitro* cytotoxicity against B16-F10 murine melanoma (IC_{50} = 7.2 × 10^{-3} μg/mL) and HCT-116 human carcinoma (IC_{50} = 0.5 μg/mL) cell lines. Octalactin B is devoid of cytotoxic activity, leading to the conclusion that the epoxy ketone functionality is essential for cytotoxicity. The structures of these compounds were finalized by X-ray studies on octalactin A, but the absolute stereochemistry was not defined.[51]

The surface of an undescribed jellyfish from the Florida Keys has also recently yielded an actinomycete which produces unique peptide metabolites. This organism was identified as a *Streptomyces* sp. (CNB-091). Extracts of this culture were found to possess significant antibiotic activity. Investigation of the source of this activity led to the isolation of two new bicyclic peptides, salinamides A and B (**43** and **44**, Chart VI), which possess novel depsipeptide backbones. The structure of salinamide A was derived first by spectral methods and by hydrolysis to yield several chiral amino acids. Subsequently, the crystalline isomer, salinamide B (**44**), was isolated and its structure determined by X-ray methods.[52] Conversion of **43** to **44** with HCl in methanol further linked these molecules confirming both their relative and absolute stereochemistries. The salinamides are antibiotics of moderate potency with selective activity against Gram-positive bacteria. The phenyl ether ring illustrates restricted rotation with all four aromatic protons shown at distinct NMR chemical shifts. This property, and its selective antibiotic activity, have opened the question of its relatedness to the vancomycin antibiotics which act by binding peptide cell wall precursors. Studies are now in progress to determine any mechanistic similarities between vancomycin and the salinamides.

Finally, there is a very recent report which further underscores the potential of associated marine bacteria to produce unique metabolites. As part of a study to evaluate the bacteria associated with the pacific sponge

Halichondria okadai, the Kobayashi group in Hakkaido isolated an *Alteromonas* strain which produced the unique tetracyclic alkaloid alteramide (**45**).[53] Alteramide represents a new structure class of bioactive metabolites possessing significant anticancer activity *in vitro* against P-388 lymphocytic leukemia, L1210 murine lymphoma, and human epidermoid carcinoma. Although this bacterium was described as a symbiont of the sponge, no evidence was presented to make this conclusion. Indeed, if significant cell densities of *Alteromonas* were present in *Halichondria okadai*, one would expect the compound to be present in extracts of the sponge. No such observations have been made despite the fact that comprehensive chemical studies of the sponge have been undertaken.

D. Bacterial Production of Marine Toxins

In the context of symbiotic bacteria, one must refer to recent information which delineates the true origins of many marine toxins. Evidence of bacterial toxin production was first obtained for the novel, but not widespread toxin, neosurugatoxin (**46**, Chart VII). First found along with prosurugatoxin as an unexpected toxicant in the edible Japanese mollusc, *Babylonia japonica*,[54] this unique toxin was later traced to an unexpected source, a Gram-positive, coryneform bacterium isolated directly from the digestive gland of the animal.[55] It appears that environmental conditions were generated which allowed this bacterium to attain large numbers in *Babylonia*. It does not appear that the bacterium is a true symbiont of the mollusk, since animals from other regions are known to be nontoxic.

The potent marine neurotoxin, tetrodotoxin (TTX, **47**), has been known for many years. Until recently, its origin has been considered to be pufferfish of the family Tetraodontidae. In Japan, the pufferfish, known as "Fugu", are considered a delicacy and great care is taken in preparing and consuming the flesh of these toxic fish. Despite great care and training, a few deaths are reported each year from pufferfish intoxication. The origin of TTX has been a subject of vigorous debate. Although mainly recognized from pufferfish, TTX has been isolated from crabs, an octopus, a goby, mollusks, flatworms, and even a terrestrial amphibian, all suggesting a microbial source. Recent data have shown conclusively that TTX is produced by numerous unicellular marine bacteria.[56-58] These organisms span a diverse range of bacterial groups, with toxin being detected in at least 15 genera of shallow- and deep-sea isolates.[59,60] It is indeed unusual to find the same molecule being produced so widely and by numerous bacterial genera. Perhaps toxin production is linked to a plasmid which can be transferred within marine environments. Continued study of this possible phenomenon could clarify aspects of the bacterial production of TTX.

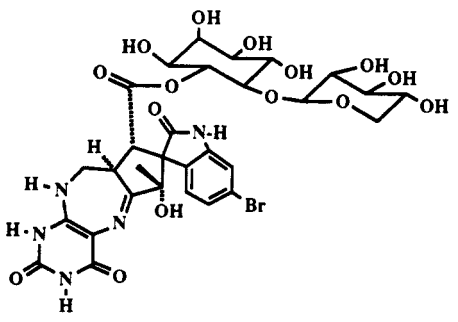
The causative toxin involved in "paralytic shellfish poisoning" (PSP) has, for many years, been known to be saxitoxin (STX, **48**) and structurally-related toxins (e.g., the gonyautoxins), metabolites known to be produced mainly by marine dinoflagellates of the genus *Alexandrium* from cold-water marine habitats. While this source has been generally accepted, there have been inconsistencies which have caused this concept to be questioned. Toxic shellfish have been observed in areas devoid of *Alexandrium*, for example.[61] Recently, Kodama and co-workers have provided startling evidence that a marine bacterium, identified as a *Moraxella* sp., isolated directly from *P. tamarensis*, is the true producer of STX toxins.[62]

E. Marine Bacterial Siderophores

Screening with a novel macrophage effector bioassay, the Tokyo group has also isolated an interesting new 22-membered ring siderophore, bisucaberin (**49**, Chart VIII), from the unicellular marine bacterium *Alteromonas haloplanktis* SB-1123.[63,64] Strain SB-1123 was isolated from a deep-sea mud sample collected at −3 300 m off the Aomori Prefecture coast. The bacterium required seawater for growth and produced large quantities of bisucaberin (ca. 700 mg/L) using sardine and cuttlefish powders and maltose as the major carbon sources. Although bacteria derived from deep sediments often require up to 700 atm pressure to allow growth, this organism appears to grow well at 1 atm. The fermentation was conducted for 2 days at 27 °C, a temperature well above the normally low temperatures recorded for the deep seas. Bisucaberin has the unique biological property of rendering tumor cells susceptible to the cytolytic action of murine peritoneal macrophages. This property has been proposed as a biorational approach to utilize natural immunological methods as alternatives to the use of potent cytotoxins in cancer chemotherapy. No data have been subsequently reported on the whole animal testing of this compound.

A very novel siderophore, anguibactin (**50**), has also been isolated from a cultured strain the marine fish pathogen *Vibrio anguillarum*.[65] Anguibactin is a novel

Chart VII. Marine Toxins of Bacterial Origin



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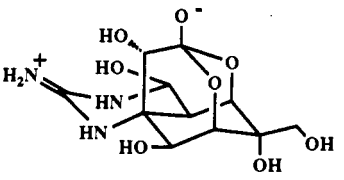

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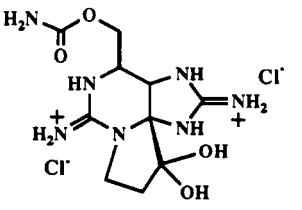

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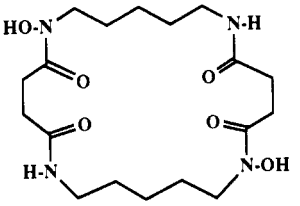
Chart VIII. Marine Bacterial Siderophores



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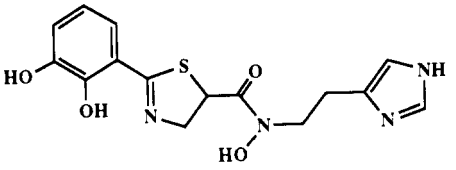

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catechol with thiazoline and imidazole functionalities. The structure of this siderophore was determined by X-ray methods on a crystalline synthetic analog, anhydroanguibactin, the fully aromatized thiazole and *N*-deoxy derivative. Because of this, the stereochemistry of the side chain on the thiazoline ring could not be assigned. Interestingly, the production of anguibactin was linked to a 65-kb plasmid, designed pJM1. Anguibactin, and its required plasmid, were demonstrated to be important factors in the virulence of this pathogen by the demonstration of the ability to cross-feed a nonproducing strain thus allowing the new strain to become established in the host.

Significantly, iron is one of the limiting nutrients in marine environments. Because of this, it seems reasonable to predict that many marine bacteria will have evolved the ability to produce extracellular iron siderophores. Thus, this area of research is likely to expand greatly and to potentially provide exciting new siderophore structures.

IV. Conclusions, Prospects, and Concerns

From the studies summarized in this chapter, it seems clear that marine bacteria are emerging as a significant chemical resource. But, with the relatively few studies conducted, two main questions remain to be answered. Are marine bacteria generally chemically prolific? Are the metabolites from marine bacteria as novel as those from marine plants and invertebrates? We now know that some marine bacteria produce structurally-novel, bioactive metabolites. But, the cultivation of bacteria for a new chemical discovery program is a tedious and costly process built on a statistical approach. It is still difficult to compare marine bacteria with marine invertebrates as a source for novel compounds, since less than 1% of the bacteria isolated will be worthy of study. While I would like to claim that marine bacterial metabolites are unique, and there is indeed some evidence to suggest this, I am also aware of the numerous "terrestrial metabolites" isolated by workers in this field, especially in studies of near-shore actinomycetes. One wonders just how distinctly the line between marine and terrestrial bacteria has been drawn. However, if one considers the deep seas and geothermal marine environments, and the anoxic habitats of the world's oceans, it seems clear that many marine bacteria must be environmentally distinct from their terrestrial counterparts. Given this concept, there is no question that the possibility exists for unusual biosynthetic processes to be found in the bacteria isolated from these extreme environments.

The lack of in-depth knowledge of the nutritional requirements of marine microorganisms, however, poses the question of how to effectively develop this resource. In my opinion, it will not be productive to exploit marine bacteria without major programs for developing an understanding of their the basic biology. Unfortunately, we know little about the specific, natural nutrients and growth factors required for cultivation. Common media constituents, such as peptone, simple sugars, etc., are unrealistic marine nutrients and, in the marine environment, are apparently replaced with complex carbon sources such as chitin, sulfated polysaccharides, and marine proteins. In addition, we have little understanding of the effects of uncommon inorganic elements, such as lithium, silicon, etc., which are also abundant in marine sediments.

It is estimated that less than 5% of the bacteria observed in marine samples by microscopic methods are found cultivable under "standard" conditions. This fact will greatly limit our ability to isolate and culture the majority of the interesting and new bacterial forms present. It is clear that more emphasis must be placed upon the basic biology of marine bacteria before this resource can be effectively developed.

V. Acknowledgments

Exploratory studies of marine bacteria have required a significant investment with small rewards. I want to thank some insightful colleagues, Dr. Ken Nealson, Dr. Terry Doyle, and Dr. Sal Forenza, for their enthusiastic financial and intellectual support for my program. The majority of the scientific studies in my group have been supported by the National Institutes of Health, National Cancer Institute, under grants CA44848 and CA50750. I also wish to thank the California Sea Grant Program for providing partial support for some of this work. Paul Jensen, the leader of our microbiological effort, deserves special thanks for his perseverance and innovation in developing this field.

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