ANTITUMOR-ANTIBIOTICS FROM MARINE MICROORGANISMS

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Résumé *: si les bactéries du sol, en particulier celles de l'ordre des Actinomycètes, sont une source bien connue de nouveaux composés antitumoraux et d'antibiotiques, aucun travail de recherche analogue n'a été entrepris dans le domaine des bactéries marines. Dans cet article, nous décrivons les études préliminaires menées dans le but de fournir un aperçu des ressources des microorganismes marins. L'accent est mis sur le développement de nouvelles méthodes d'isolement et de culture de souches de microorganismes exigeantes en sel, et sur le développement de l'isolement de produits nouveaux potentiellement utiles dans le traitement des maladies infectieuses et du cancer.*

Abstract *: although soil-derived bacteria, particularly those of the order Actinomycetales, are a well-studied resource for new antitumor-antibiotics, analogous comprehensive investigations of marine bacterial sources have not been undertaken. In this paper, we describe preliminary studies of marine bacteria aimed at providing an overall assessment of the scope of marine microbial resources. An emphasis is placed upon developing new methods for the isolation and culture of salt-requiring microorganisms, and on developing the isolation of new compounds with potential in the treatment of infectious diseases and cancer.*

As the field of marine natural products chemistry matures and becomes even more effectively interfaced with drug development, new biological resources will need to be explored. A frontier in this regard are the microorganisms of the oceans which represent an immense resource, the scope of which remains to be fully understood. The term "microorganism" is all encompassing. It includes all organisms which require magnification for clear evaluation, and includes both unicellular organisms, filamentous forms and multicellular organisms of small size. Further classifications are whether the organisms are phototrophic or chemotrophic (perform photosynthesis or chemical metabolism) or heterotrophic (require organic carbon sources) as a means of basic nutrition. Table I illustrates the diversity of microorganisms known from marine habitats. The variety is enormous, ranging from the primitive Archaebacteria to the Eucaryotes (algae, fungi, etc.). In the algae alone, there are at least 10 full plant Phyla known, with at least 100,000 species expected. In the other groups, the number of species can only be speculated.

The large numbers of "microorganisms" in the world's oceans indeed point to these organisms as a major resource for chemical and biomedical study. However, few marine microorganisms have been chemically-studied in detail. Problems in isolation and taxonomy, and in both small scale and mass scale cultivation have been formidable, thus far precluding comprehensive studies of these microbes.

Table I. Classifications of Marine Microorganisms

Archaebacteria

Chemoautotrophs

- -methanogens
- -thermoacidophiles(1)

Chemoheterotrophs

- -halophiles

Autotrophic Eubacteria

Photoautotrophs

- -anoxygenic photosynthesis
  - purple and green photosynthetic bacteria (Order Rhodospirillales)
- -oxygenic photosynthesis
  - cyanobacteria (Order Cyanobacteriales)
  - prochlorophytes (Order Prochlorales)

Chemoautotrophs

- -nitrifying bacteria (Family Nitrobacteraceae)
- -colorless sulfur-oxidizing bacteria(1)
- -methane-oxidizing bacteria (Family Methylococcaceae)

Chemoheterotrophic Eubacteria

Gram positive

- -endospore-forming rods and cocci
- -non spore-forming rods
- -non spore-forming cocci (Family Micrococceae)
- -actinomycetes (Order Actinomycetales) and related organisms

Gram negative

- -rods and cocci
  - aerobic (Family Pseudomonadaceae)
  - facultative (Family Vibrionaceae)
  - anaerobic (sulfur-reducing bacteria)
- -gliding bacteria (Orders Cytophagales and Beggiatoales)
- -spirochetes (Order Spirochaetales)
- -spiral and curved bacteria (Family Spirillaceae)
- -budding, and/or appendaged bacteria
- -mycoplasmas (Class Mollicutes)(2)

Eucaryotes

Photoautotrophs

- -microalgae

Chemoheterotrophs

- -protozoa
  - flagellates
  - amoebae
  - ciliates
- -fungi
  - higher fungi
    - Ascomycetes
    - Deuteromycetes
    - Basidiomycetes
  - lower fungi (Class Phycomycetes)

(1) includes heterotrophic genera
(2) only two reports documented

In our investigations of marine plants and invertebrates, we became aware that microorganisms, almost exclusively heterotrophic eubacteria, seemed to play important roles in survival, and, perhaps, in the synthesis of secondary metabolites. Marine bacteria include almost every known taxonomic group, and include many forms which are unique to the ocean environments (see Table I). Because of these concepts, four years ago, we began development of a program to isolate and culture marine bacteria. Of the vast diversity of microorganisms found in marine sources, we selected the heterotrophic eubacteria as the most likely candidates to yield biomedically interesting results. There were a number of reasons for this selection. First, there were preliminary studies which showed that some marine bacteria produce bioactive metabolites (1, 2). Second, similar bacteria from terrestrial sources are well-known to produce antibiotics and antitumor agents of very unique structures. The filamentous actinomycetes, for example, have been a major resource for the development of new fermentation antibiotics (there are approximately 115 antibiotic drugs sold today!).

Our investigations of marine bacteria have focussed upon those isolated from both animate surfaces and from sediments. Because many of the most chemically-prolific terrestrial bacteria are Gram positive organisms, we studied the distributions of these organisms in tropical marine habitats. Table II shows the results of this analysis from the coastline of Belize in the Caribbean Sea. To our surprize, and quite inconsistent with many reports, Gram positive bacteria were prevalent in marine habitats, especially on the surfaces of marine plants. Sediments, too, were a good source for these bacteria which included the actinomycetes.

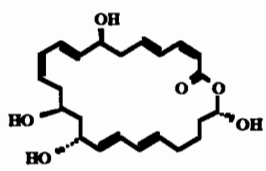
Our first successful examination of sediment-derived bacteria involved samples of marine sediments from deep ocean cores. Deep ocean drilling cores are available to us through the international "Deep Sea Drilling Program." From a sample taken at -1000 m off northern California, we isolated a Gram-negative bacterium showing a strong requirement for salt-based culture conditions. The bacterium was successfully cultured using rather standard techniques.

Sample	Colonies Tested	Gram-positive	% Gram-positive
Sediments	822	198	24.6
Seawater	559	73	15.9
Invertebrates	354	30	10.8
Algae	597	175	31.3
TOTAL	**2332**	**476**	

Table II: Distributions of Gram-positive bacteria (Belize Barrier Reef)

Extraction of the whole fermentation broth led to the isolation of several new metabolites, the macrolactins A-F (**1-6**) some of which possessed interesting cytotoxic and antiviral activities (3). The macrolactins are examples of a new structural class of macrolide antibiotics. Because of the pressures at -1000 m, the bacterium which produces these compounds is apparently a facultatively barophile. Although we attempted to identify the bacterium, classical biochemical methods failed to lead to a known genus. In a subsequent collaboration with Scott Rychnovsky, at the University of Minnesota, we were able to assign the absolute stereochemistry of macrolactin A (**1**) as shown in Figure 1 (4).

Figure 1. Bioactive Compounds Isolated from Marine Bacteria



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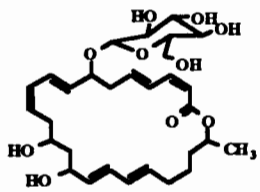

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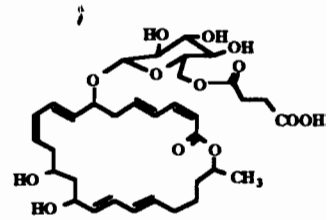

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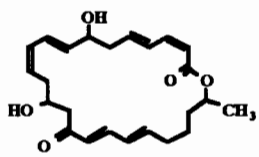

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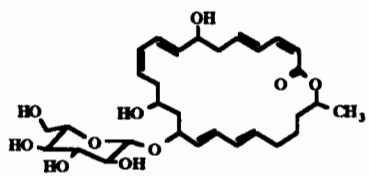

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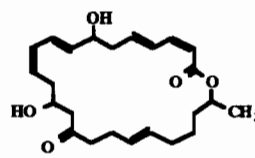

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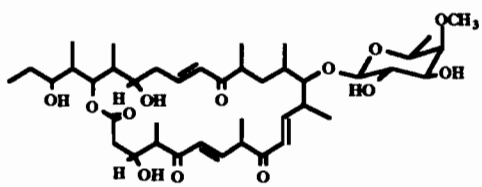

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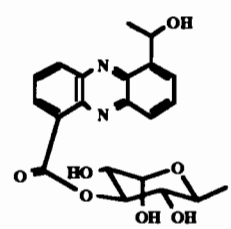

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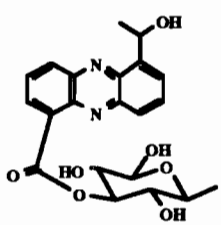

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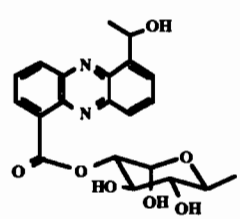

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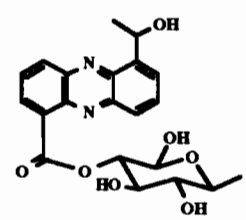

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Subsequent to this investigation, sediment samples from shallow bays have been studied. From a sediment sample from the Bodega Bay, in northern California, we isolated an interesting actinomycete which was identified only as a maduramycete. Fermentation led to the isolation of a new macrolide glycoside, maduralide (7) possessing a rare 24-membered lactone ring (5). Maduralide is also unique in that it possesses a rare 3O-methyl-6-deoxy-talose sugar. This observation suggested that shallow marine sediments were a rich source for actinomycetes, perhaps even those of less common taxonomic groups. However, although shallow sediments have been found to yield actinomycetes, the classes observed are generally those of the common streptomycete group. Table III shows the distributions of streptomycetes verses total actinomycetes and their percentages of bioactivity in *in vitro* cytotoxicity and antibiotic assays. In addition, the 11 active streptomycetes were tested *in vivo* in a murine P-388 lymphocytic leukemia assay. Three of the extracts showed *in vivo* activity in excess of the minimum level of T/C = 125.

	# Tested	# Cytotoxic*	Antimicrobial Biotesting (# active)		
			B. subtilis	*E. coli*	*S. cerevisiae*
Actinoplanetes	33	9	2	0	2
Streptomycetes	86	11	10	9	4
Total Actinomycetes	119	20	12	9	6

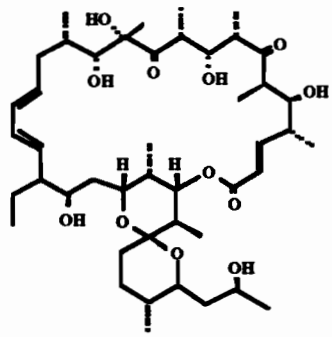
* MIC activity of less than 2 µg/ml against human colon tumor cells.

Table III : Actinomycete *in vitro* testing data

Another sediment-derived bacterium from Bodega Bay, isolate # CNB-253; also an unidentified *Streptomyces sp.*, was found to produce metabolites with significant antibacterial activity. Fermentation in mass scale, followed by isolation and extraction, gave crude mixtures of new phenazine alkaloids as the active molecules. Purification by HPLC resulted in the isolation of four phenazinesugar esters assigned as **8-11** (6). The new compounds are members of the rare phenazine class, but more importantly they are **L**-quinovose esters at the C2' and C3' positions. This appears to be the first case in which **L**-quinovose has been observed in nature. The phenazine esters show minimum inhibitory activity (MIC) in the 1-4 µg/ml range against Gram-positive bacteria. Although the MIC values for these compounds are modest at best, their selective activity against Gram-positive bacteria is of interest.

Animate surfaces are unique in the marine environment. Because bacteria are freely dispersed in aquatic habitats, pathogens which are free to colonize surfaces are a potentially devastating problem. We have found that some non-pathogenic bacteria have adapted to live on the surfaces of marine plants and invertebrates (7, 8). Further, we feel that some marine bacteria protect their hosts through the formation of true symbiotic relationships. In some cases, the symbiotic bacteria produce antibiotic substances which protect the host by reducing the likelihood of pathogen encroachment. In return, the associated bacteria are guaranteed a surface rich in nutrients. Thus, we feel that surfaces are a unique microhabitat from which to sample for bioactive bacterial strains.

Figure 1. (continued)



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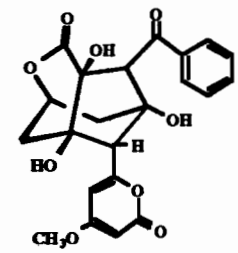

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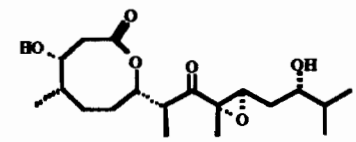

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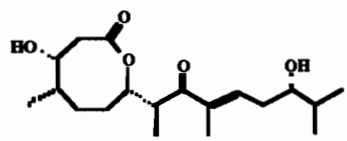

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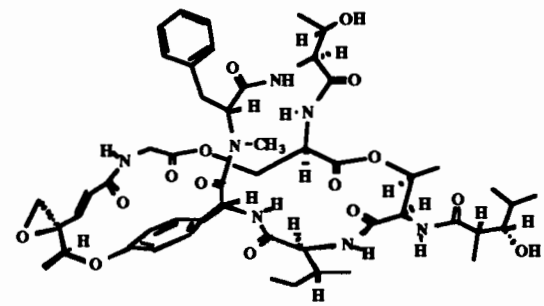

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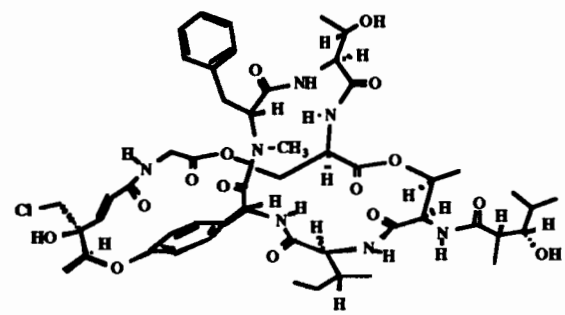

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In connection with surface derived bacteria, we investigated the chemistry of several bacteria isolated from the surface of an unidentified species of the pacific sea fan, *Pacifigorgia*, collected in the Gulf of California. *Pacifigorgia* species are true sea "fans", in that they possess broad flattened branchlets interconnected with coenenchyme tissues to construct small mesh openings. Because of the structures of these cnidarians, they are highly effective filters of seawater and should accumulate bacteria on their surfaces. One *Pacifigorgia* species studied yielded an interesting actinomycete, a *Streptomyces* sp. (strain PG-19), which when grown in marine media produced extracts with significant antibacterial activity and cytotoxicity toward human cancer cells. The bacterium was cultured in mass scale to yield complex mixtures of bioactive metabolites. Because of the complexity of the mixture obtained, it was difficult to sort out the various bioactivities observed. After comprehensive chromatography of the extract, four new compounds were ultimately obtained. The first of these compounds was a crystalline macrolide antibiotic related to the oligomycin group. Comprehensive spectral analysis led to the assignment of this molecule as **12** (Figure 1), however without assignment of its stereochemical features. X-ray analysis by the Clardy group at Cornell University, however, showed the assignment was correct and provided the relative stereochemical structure shown. Oligomycin derivative **12** shows *in vitro* cytotoxicity and antibiotic properties which are, in potency, well below those of existing products. Another metabolite, assigned as the enterocin derivative **13** was also isolated and identified by spectral methods. This molecule is also unremarkable in its potency and was not followed further. The most interesting metabolites produced by strain PG-19 were two related 8-membered ring lactones, the octalactins A and B (**14, 15**), which were obtained in small yield. The structures of these compounds were also determined initially by spectral and chemical methods and later by X-ray crystallography (9). Octalactin A, (**14**), is an epoxyketone with potent antitumor properties. In *in vitro* testing, **14** showed IC_{50} values of 7.2 x 10^{-3} µg/ml and 0.5 µg/ml against B16-F10 murine melanoma and HCT-116 human colon carcinoma cell lines, respectively. Based upon the metabolites isolated, it appears that the bioactivities of the crude extract were a rough combination of the activities of the four metabolites isolated. The most promising compound for antitumor research is octalactin A, since it possesses a novel structure not yet reported. Unfortunately, octalactin A was a very minor metabolite of the mixture and it was not obtained in sufficient yield to perform *in vivo* studies. Attempts to re-ferment this bacterium led to complex mixtures with only minor amounts of octalactin A. We are currently exploring modifying the conditions of culture to maximize the yield of this compound.

In a similar study, we isolated bacteria from the surface of an unidentified jellyfish collected in the Florida Keys in 1988. After several months of subculturing, we isolated an actinomycete which produces a series of antibiotic depsipeptides. Through considerable spectral work, and hydrolysis to yield several of the respective amino acids, we were able to assign a gross structure to the salinamides A and B (**16, 17**) (10). Salinamide A was a noncrystalline epoxide with several non-peptide regions within the molecule. Using modern NMR methods, particularly HMBC and HMQC pulse sequence methods, we were able to construct the overall structure of **16**. Chiral HPLC analyses of the derivatized amino acids from hydrolysis of **16**, led to the absolute stereochemistry of part of the molecule. Ultimately, we were able to produce a crystal of the chlorohydrin **17** which proved suitable for X-ray analysis. Interconversion of salinamide A to B was accomplished with hydrochloric acid in aqueous acetone, thus allowing the complete structures of these molecules to be described. Much like the glycopeptide antibiotics, exemplified by vancomycin, the salinamides are selective antibiotics against Gram-positive bacteria. In an attempt to determine if salinamide B mimics vancomycin in its mechanism of antibiotic action, we collaborated with Tom Stout and Jon Clardy at Cornell University to repeat the classical proton NMR experiments showing solution binding to D-alanine dipeptides. The results to date have been inconclusive, but there is evidence that salinamide B is a weak binding agent for peptidoglycan cell wall precursors.

In the form of concluding remarks, I must pass along some of our findings and frustrations which tend to illustrate the early status in the development of this field of study. Marine microorganisms are very poorly known and we are sure that our studies are not representative of the the diversity of bacterial groups found in the marine environment. New methods must be developed to isolate and culture marine bacteria. New methods must be developed to provide bacteria with unique nutrients which are those from the natural environment. To rely upon peptone or similar "non-marine" protein sources simply does not represent a viable approach to sample complex marine-adapted microorganisms. Media must be developed with natural ingredients such as polysaccharides, i.e. chitin, etc., which are present in nature. New methods of isolation must be explored that select for diverse strains of bacteria with unique requirements. Some bacteria which grow extremely slowly, or which require very low nutrient levels, or which have specific needs as yet unperceived, are simply not being isolated in our sampling schemes.

Some of the very preliminary work described here is severely hampered by a lack of reproducibility we have encountered overall with marine isolates. Although it is not uncommon for terrestrial bacteria to loose the ability to produce unique molecules, we have found this problem to possibly be greatly accentuated with marine isolates. Cultures found "active" in early screening are, in over half of the regrows, found inactive. Compounds isolated from the first culture broths are, in perhaps > 50% of the time, never seen again, or they are found in such low yield that working with the culture is deemed fruitless. Clearly, if we are to develop this resource, studies must focus upon the *biology* of marine microorganisms, rather that a screening approach with bacteria that will simply grow on trivial media. We need to comprehend the nutrient and growth factor requirements that these unique bacteria demand. Further, our studies suggest that highly controlled fermentation conditions will be required to reproduce initial findings. With appropriate attention to the concept that marine microorganisms are a unique ***new*** resource requiring new methods and procedures, this may very well be developed into a major source for new antitumor-antibiotics.

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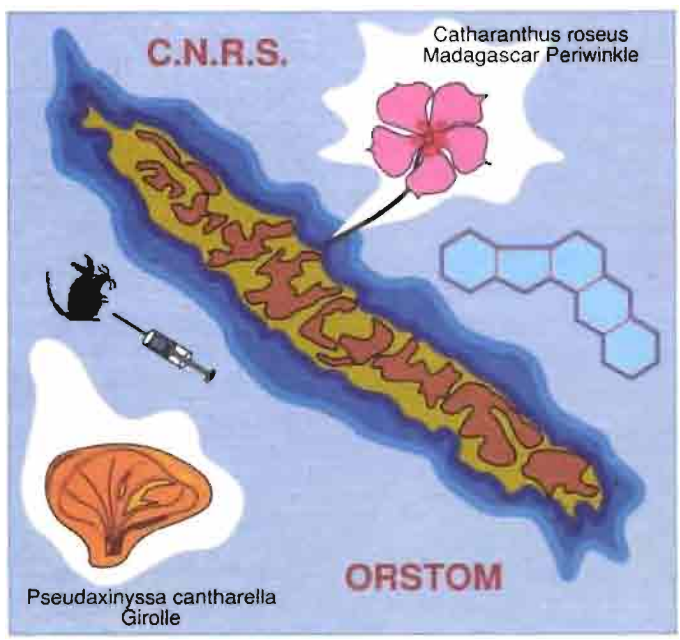

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