MARINE PHARMACOLOGY:
Prospects for the 1990s

EDITORS
Robert S. Jacobs and Marianne de Carvalho

Summary of
a California
Sea Grant
Workshop

ROSEMARY AMIDEI
COMMUNICATIONS COORDINATOR

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May 7-9, 1990
University of California, Santa Barbara

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WELCOME

Gordon G. Hammes, Vice Chancellor, Academic Affairs,
University of California, Santa Barbara

On behalf of the University of California, Santa Barbara (UCSB), it is my pleasure to welcome you to this workshop on "Marine Pharmacology: Prospects for the 1990s."

It is very appropriate that this workshop be held at this campus. As many of you know, Robert S. Jacobs, a professor of pharmacology at UCSB, has been a key collaborator in California Sea Grant's pioneering Marine Pharmacology Program since 1977. This program was the first effort organized within the United States to discover and develop new pharmaceuticals from marine sources, and James Kennett will describe it more fully.

What you may not know is that UCSB is unique in having both an undergraduate and a graduate program in marine pharmacology. (The programs were initiated in 1974 and 1977, respectively). Since that time, in excess of 400 undergraduate students have received the bachelor's degree in pharmacology and 37 graduate students have received the master and Ph.D. degrees. In addition, we have recently completed a Marine Biotechnology Laboratory, the first of its kind in the nation.

The genetic diversity and uniqueness of marine organisms make them a prime resource for new medicinals and other products. The first steps in realizing the potential of these organisms have yet to be taken. We hope that this workshop will be instrumental in developing future research paths.

INTRODUCTION

James P. Kennett, Marine Science Institute,
University of California, Santa Barbara

On behalf of the Marine Science Institute, it is my pleasure to welcome you all to this national workshop on "Marine Pharmacology — Prospects for the 1990s." This meeting is sponsored by California Sea Grant and organized by Robert Jacobs, with the help of the staff of the Marine Science Institute.

The Marine Pharmacology Program within Sea Grant was the *first* organized effort within the United States to discover and develop new pharmaceuticals from marine organisms.

My first encounter with marine pharmacological research was through the work of my former colleague at the University of Rhode Island, Dr. Shimizu — it was also funded by Sea Grant — and related to his successful efforts to isolate the toxins of the red-tide dinoflagellate *Gonyaulax*. This work made apparent to me the existence of an enormous range of unknown compounds and substances used by marine organisms for a variety of purposes, such as biological defense. The taxonomic diversity of marine organisms is large, as is their biochemical and behavioral diversity, so it probably should have come as no surprise that this chemical diversity was even found to contain pesticide substances close to DDT and chlordane. It is clear that the study of these compounds has much potential benefit in the long-run for development of new agrichemical products, industrial chemicals, and materials for biomedical and pharmaceutical applications.

As a marine geologist, I work with foraminifera, a marine group of high diversity. At the last count, there were about 6,000 living species described. However, their taxonomy is still poorly known, let alone their biological characteristics. Most living foraminiferal species are known only on the basis of their shells; cytology, life cycles, behavior, and other factors are known for fewer than 50 species.

This is the case for most groups of marine microorganisms and also, as it happens, for many terrestrial groups associated with the tropical rain forests. Major concern has developed for retaining biodiversity in the rainforests. It is less well known, however, that certain coastal marine biotas (such as those in the Philippine Islands) are also under enormous pressure, and extinctions may be occurring. Given the expected growth in human populations and further stress on marine

environments, perhaps the marine pharmacological and biotechnological communities can argue for greater funding for the study of potentially useful compounds and substances from taxa in threatened marine communities. Who knows, for instance, what tropical molluscan species, on the verge of extinction through over-exploitation, may provide new products or drugs of anti-inflammatory or analgesic value or of use in counteracting viral diseases or in cancer chemotherapy.

One of the stated purposes of this workshop is to bring together leading scientists to discuss new research avenues that may open as a result of recent advances in molecular biology, chemistry, biochemistry, and biotechnology and that might impact marine pharmacology and natural product chemistry over the next decade. This is a timely meeting. It is also an appropriate location. The field was considerably strengthened beginning in the 1970s, by the initiation of collaborative efforts between the UC Santa Barbara group, led by Bob Jacobs, and workers at UC San Diego and UC Santa Cruz.

I hope that programs such as this, under the auspices of Sea Grant, will receive better funding attention in the future, especially given concerns regarding U.S. economic competitiveness.

INTRODUCTION

D. John Faulkner, Scripps Institution of Oceanography,
University of California, San Diego

On behalf of the participants in the Marine Chemistry and Pharmacology project of the California Sea Grant College, it is my pleasure to welcome you to this workshop and to describe the origins and history of the project.

This project was formally initiated in 1977, when Sea Grant persuaded three chemists, William Fenical, Philip Crews, and me, to collaborate with a pharmacologist, Robert Jacobs, to screen marine natural products in a wide range of assays with the hope that we might discover new medicinal agents. We were, in effect, a Sea Grant-sponsored "venture capital" or "start-up" company operating within the constraints of an educational program. This was not an easy marriage of interests. I was probably the most reluctant of the participants because I fiercely valued my independence and believed, at that time, that a university professor should be completely independent. The incentive to collaborate was an agreement that each investigator would attempt to maximize the original research in his own field and would minimize the "service" component of the program. Further, the graduate students (or Sea Grant trainees) would be required to perform original research at all times.

The need to balance academic research with the goals of drug development has been accomplished, but not without considerable soul-searching at times. The results of the project have been quite striking, both in terms of quantity and quality. In many instances, we have achieved our goal of discovering new marine natural products with unique pharmacological properties, the so-called "lead" compounds that form the basis of drug development programs in the pharmaceutical industry. Yet we are most proud of our record in educating chemists and pharmacologists, many of whom now hold positions of responsibility in industry or academia.

Why was this project so different from those that had preceded it? Prior to 1977, we chemists had isolated a large number of interesting marine natural products, but the screening of these compounds had usually been limited to <u>in vitro</u> antimicrobial assays. Only one compound from the pre-Sea Grant period was considered for patent protection and that was at the request of a pharmaceutical company. Our encounters with industry had been of the type that Bob Jacobs calls "mail-order pharmacology": the companies paid for samples of new compounds

and, in return, they eventually sent us a computer printout of relatively meaningless numbers.

The Sea Grant project was a refreshing change, for it developed into a true two-way collaboration. In addition to the raw screening data, Bob has provided the chemists with a rapid and realistic evaluation of pharmacological properties of each compound and has advised us of any potential medicinal value. It is that interpretation of the data, particularly when a compound was active in more than one assay, that has been most valuable to the chemists. Over the years, Bob has experienced firsthand the effort that goes into isolation and re-isolation of active compounds, and we chemists have witnessed the development of bioassays. This has led to an appreciation and understanding of each others' problems. In particular, our students have gained valuable experience in interdisciplinary research and are better prepared for research projects that require a team approach.

The success of the project can be evaluated in many ways. Some might restrict their criterion of success to those compounds that have been patented and/or evaluated by the pharmaceutical industry. Others, particularly in academia, will consider the publication record as the most critical factor. Our students will judge the project by how well they were prepared for their career goals. I am inclined to rate the project on the basis of what it has taught me, and I am well satisfied. For those who like numbers, I will cite some statistics.

During the first ten years, Bob Jacobs, his colleagues, graduate students, and undergraduate classes screened nearly 900 samples, predominantly pure compounds, in as many as 13 bioassays. Each activity was reconfirmed, and in many cases, a dose-response study was performed.

The productivity of the Sea Grant project, as measured by the usual academic yardsticks of publications, invited lectures, and degrees awarded has been very high. I believe that this is a direct outcome of our desire to maintain the highest academic standards for the project. The four project leaders, either separately or in collaboration, published about 150 research papers in the first 12 years and graduated 19 doctoral and 3 master's students who were either partially or completely supported as Sea Grant trainees. A closer look at the publications shows a trend toward joint, interdisciplinary papers. It also shows that the project has matured from simple isolation and screening to mechanistic and structure-activity studies. The group has also submitted 35 disclosures to the University of California Patent Office and, from these, 7 patents have been issued and 3 more are pending. The most important of these discoveries are in the anti-inflammatory area, with

compounds such as manoalide and pseudopterosin E as the lead compounds. We can even claim to have helped spawn a small business venture.

It is difficult to fully assess the results of the project because there are many compounds that may still provide new leads. We often find ourselves returning to investigate old friends, particularly when a new source of the compound is discovered. For example, when Stuart Abramson in Palmer Taylor's group at the University of California, San Diego, was looking for potential acetylcholinesterase inhibitors, we had just re-isolated onchidal, the toxic constituent of a marine pulmonate, for the first time in ten years. Bob Jacobs had demonstrated its activity in the guinea pig ilium assay in 1977 but had run out of material. Stuart was able to demonstrate the mechanism of action of this interesting compound using a very small quantity of compound. It is important to recognize the trend toward bioassays that require very small quantities of compounds, for this trend has allowed the screening of minor constituents of extracts of marine organisms and has allowed us to study smaller organisms. However, these compounds may never be available in large enough quantities for full evaluation, and the synthetic chemists can therefore be assured of a job if any of these compounds are required for clinical or preclinical testing.

During the course of this research project, we have continually modified the screening procedures as better assays became available. Our philosophy of screening pure compounds rather than crude extracts has always been controversial, but we tested the validity of our approach by contrasting the results from screening pure compounds with those from crude extracts. We were able to obtain reliable data by testing pure compounds in complex biological systems or complex mixtures in single component assays, but testing complex mixtures in complex biological systems gave poor results. Over the years, Bob has dropped some of the more expensive bioassays, especially those that yielded a low hit rate, and has increased the number of enzyme-based assays. These changes have led to a reduction in the quantity of material required for screening and has broadened the scope of chemical studies.

During the first ten years, the marine chemistry and pharmacology group worked with a wide range of collaborators. Our industrial collaborators have included Allergan Pharmaceuticals, Bristol-Myers, Eli Lilly Laboratories, Merck, Sharpe & Dohme, Shell Development, Smith Kline & French, Syntex, and Wyeth-Ayerst. Jon Clardy not only provided X-ray crystallographic studies, but also took an active role in several collection trips. Bob Jacobs has studied samples from Bruce

Howard, Valerie Paul, and Francis Schmitz, in addition to those from the California Sea Grant group. An international collaboration with Professor C.B. Rao of Andhra University in India has yielded a novel series of anti-inflammatory alkaloids. We thank all of our collaborators for their efforts in making this a successful project.

We have inevitably experienced some disappointments. There have been exciting new compounds that we felt sure would show great activity, but which failed miserably in the bioassays. I was particularly upset when our funding was reduced for budgetary reasons immediately after some of our best scientific achievements. We were disappointed that promising compounds had to be dropped because we lacked the funds to launch re-collection programs, and, in general, the program has been damaged by rapidly rising costs in some areas. Our interaction with industry has sometimes been less than satisfactory, although not at the scientist-to-scientist level. In retrospect, however, these disappointments have been more than outweighed by the success of the program.

The experience that we have gained in the first ten years of the project is an excellent start. We have learned to have the patience that is required for a project where all significant advances are long term. We have learned that we must continually modify our approaches to take advantage of new technology and instrumentation. We recognize that we have examined only a small fraction of the marine organisms that are easily available to us. This project could easily continue to be successful for many years into the future. The purpose of this workshop is to introduce some new research directions. I contend that we are well positioned to take advantage of these advances.

X-RAY CRYSTALLOGRAPHY AND MOLECULAR STRUCTURE OF MARINE NATURAL PRODUCTS

Jon Clardy, Department of Chemistry, Cornell University

The last twenty years have seen the creation and explosive growth of marine natural products research, and today we have a splendid opportunity to consider past successes and future directions.

What have we done? We've shown that marine organisms produce many of the most interesting compounds ever discovered. They are interesting to chemists because they have new carbon skeletons, unprecedented functionalities, and novel biosynthetic schemes. They are interesting to biologists because they illustrate novel ecological chemistry and because they have promising biological activities as anti-inflammatory, anticancer, antifungal, or antiviral agents. There are even indications that some marine natural products will serve as models for agricultural chemicals.

What can we expect from the future? Predictions of scientific progress invariably turn out to have been too conservative, so I'd like to ask a slightly different question. What specific research areas and tools are most likely to produce the spectacular results we all expect? The paradigms for marine natural products research are rapidly changing. When the field emerged, a wonderful result was the discovery of a novel, highly halogenated sesquiterpene from a red alga. While the discovery of new compounds still drives research, the mere cataloging of simple structures has little scientific cachet. Larger structures are increasingly interesting. A compound should have at least twenty chiral centers if you expect to impress your colleagues. Even more interesting than larger structures are compounds with biological activity. Assaying for biological activity has expanded greatly as a result of advances in biotechnology. Pharmacologists and molecular biologists can test for an enormous range of activities, often at the specific enzyme level, with small amounts of material.

The push to ever-more complex structures and detailed levels of testing has a simple consequence — structural information must be more definitive. If the quest for structure characterized the earlier phase of marine natural products research, the quest for shape and the relation of shape to function characterize the current phase. The shapes of molecules must be understood, not only the lowest energy shape, but all plausible shapes that a molecule can have under biologically relevant conditions.

Fortunately, we have the tools to obtain such information — X-ray diffraction to define molecular shapes and molecular mechanics to study the energetic consequences of altering molecular shapes. I shall give two short summaries of some recent work in our laboratory involving X-ray diffraction and molecular mechanics. Of course, many other projects are underway, but these two are representative of the key points I'd like to make.

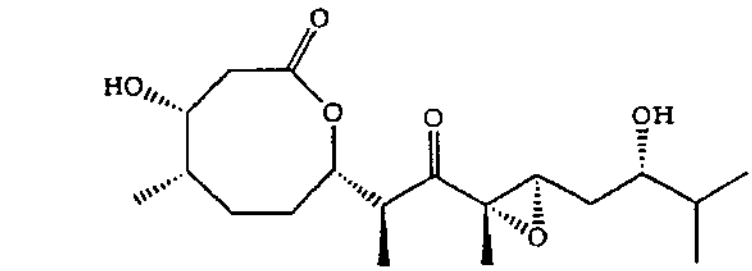
Octalactin A

Although marine plants and invertebrates have been the subject of extensive chemical investigations, studies of marine microorganisms are rare. The paucity of studies undoubtedly reflects difficulties in collecting, culturing, and identifying these organisms. Bill Fenical, at the Scripps Institution of Oceanography, has begun a program to remedy these problems, and, in conjunction with Bill's group, we've investigated the octalactins from the culture broth of *Streptomyces* sp. isolate PG-19. High level of <u>in vitro</u> cytotoxicity toward B-16-F10 murine melanoma (IC_{50} 7.2×10^{-3} μg/mL) and HCT-116 human colon tumor (IC_{50} 0.5μg/mL) cell lines provoked our interest in this particular organism. The structure of the most cytotoxic agent, octalactin A, is shown in Figure 1. The top drawing is the conventional structural representation used by chemists; the bottom, the actual structure in the crystal. These two representations are juxtaposed to make a point: the conventional way of representing molecular structures (top drawing) often does a miserable job of conveying the shape of a molecule (bottom drawing).

The octalactin A structure was obtained by X-ray diffraction studies, and, while it precisely defined the relative stereochemistry, the absolute stereochemistry remained unknown. In general, structural techniques can solve the problem of relative stereochemistry much better than they can address the issue of absolute stereochemistry. With the structure of octalactin A firmly in hand, Bill's group was able to determine the structure of octalactin B by conventional spectroscopic methods. X-ray diffraction typically defines the first structure in a family, and spectroscopically defines the remaining members.

Octalactin A was structurally novel in containing an eight-membered lactone ring. Small lactone rings, five- or six-membered, are common in chemistry, as are larger, fourteen- or sixteen-membered rings. But so-called medium-sized rings are rare. There is no reported natural product with an eight-membered lactone ring, so octalactin A emphasizes the ability of marine microorganisms to produce novel

Octalactin A



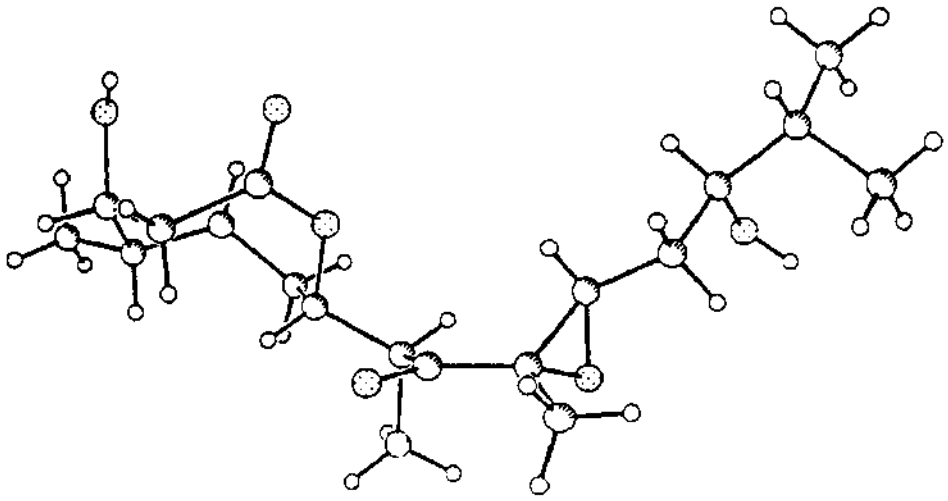


Figure 1. At top is a conventional structural drawing of octalactin A, showing the relative stereochemistry. The bottom is a computer-generated perspective drawing of the X-ray model.

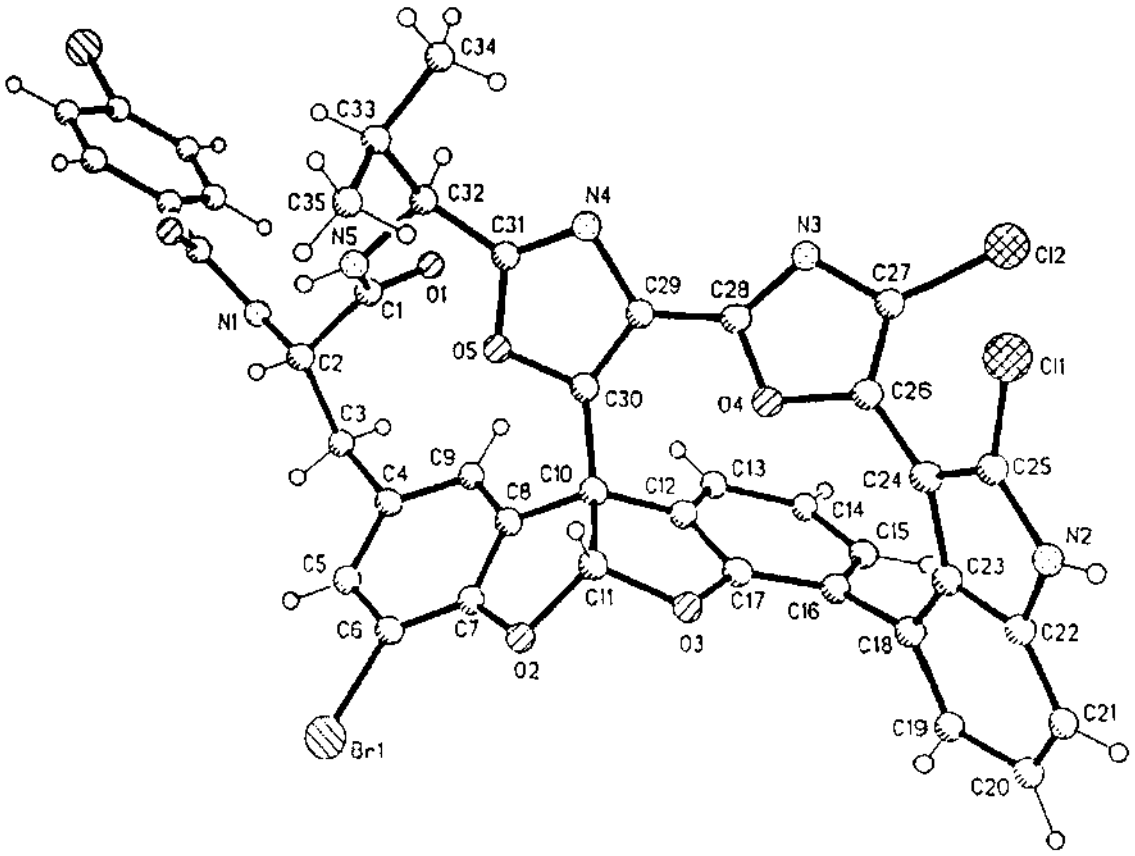


Figure 2. A computer-generated perspective drawing of the final X-ray structure of the diazonamide B derivative. The absolute configuration was set by anomalous scattering measurements.

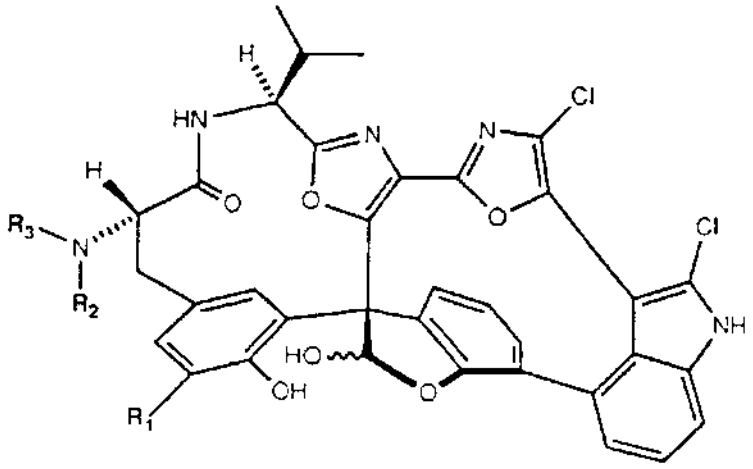


Figure 3. The structure of diazonamide B. The configuration of the hemiacetal carbon in the naturally occurring material has not been established.

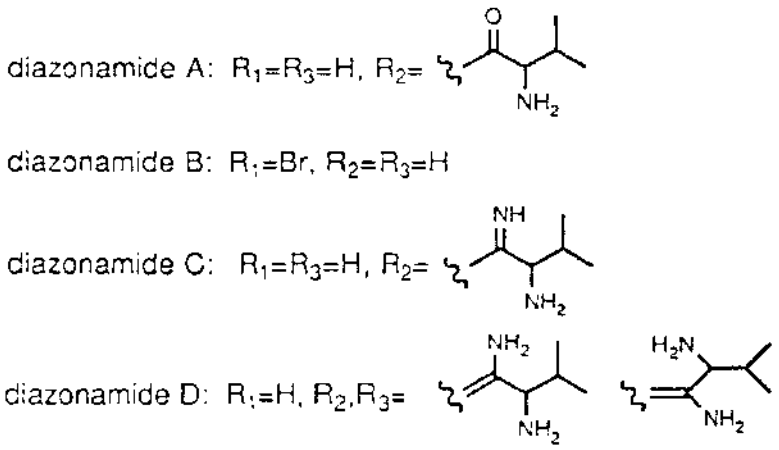


Figure 4. Drawings of all of the diazonamides characterized to date.

metabolites. Since eight-membered rings are rare, chemists know relatively little about their shape. Of course the shape of an eight-membered ring depends on its substituents, and the shape of a simple eight-membered ring may not represent the shape of octalactin. We used molecular mechanics (MACROMODEL) to explore the shapes of octalactin A and found six distinct conformations within 1.3 kcal/mole of the ground state. We are currently working on some of the consequences of these calculations, such as our ability to predict conformational biases during the course of a total synthesis and the possibility that octalactin A can translactonize to a fourteen-membered erythromycin-type lactone.

Diazonamide A and B

Ascidians have been a rich source of bioactive secondary metabolites with potent antimicrobial, cytotoxic, and antiviral activities. In collaboration with Bill Fenical's group, we've been working on the secondary metabolites of the ascidian *Diazona chinensis* from the Philippines. The most active compound was diazonamide A with in vitro activity against HCT-116 human colon carcinoma and B-16 murine melanoma (IC_{50}'s of less than 15 μg/mL). We were not able to crystallize diazonamide A or any of the diazonamides, but we were able to prepare a crystalline derivative of a degradation product of diazonamide B. Preparation of crystalline samples — an absolute necessity for diffraction studies — is a constant difficulty. One area that we are exploring is the systemization of strategies for crystallizing peptides and highly modified peptides from marine organisms.

In Figure 2, a drawing of the X-ray determined crystal structure of the diazonamide B derivative is presented. In this case, since the metabolite contained a number of heavy atoms, we were able to determine the absolute stereochemistry. With the diazonamide B derivative structure secured, we were able to elucidate the complete structure of diazonamide B, with one small point of ambiguity (Figure 3). Diazonamide B is a hemiacetal that was converted to an acetal during the process of making the derivative. We have been trying to establish the stereochemistry of the hemiacetal carbon via molecular mechanics, but, unfortunately, the energy differences are too small to allow a firm prediction. The question is too subtle to be settled by the current generation of molecular mechanics programs. Given the structure of diazonamide B, we have been able to establish the structure of diazonamide A, the most active component, and this is shown in Figure 4.

PROGRESS IN MARINE NATURAL PRODUCTS SYNTHESIS

Kim Albizati, Department of Chemistry, Wayne State University

One measure of the ongoing activity in a given field is the number of manuscripts published in that field as a function of time. Data on the isolation of marine natural products indicate that remarkable growth occurred in this area during the early to mid-1970s (see graph) [1]. A similar data analysis of the synthesis of marine natural products [2] indicates that synthesis in this field has only recently entered a similar growth phase. We have completed a near-comprehensive review of marine natural products total synthesis and observed trends similar to natural products synthesis as a whole. From the period 1960 to 1989, approximately 350 syntheses of about 200 different marine-derived metabolites were reported [3,4]. A breakdown of these data show the following.

- Approximately 50 percent of the syntheses involved terpenes, and nearly three-fourths of this number involved sesqui- or diterpenes;

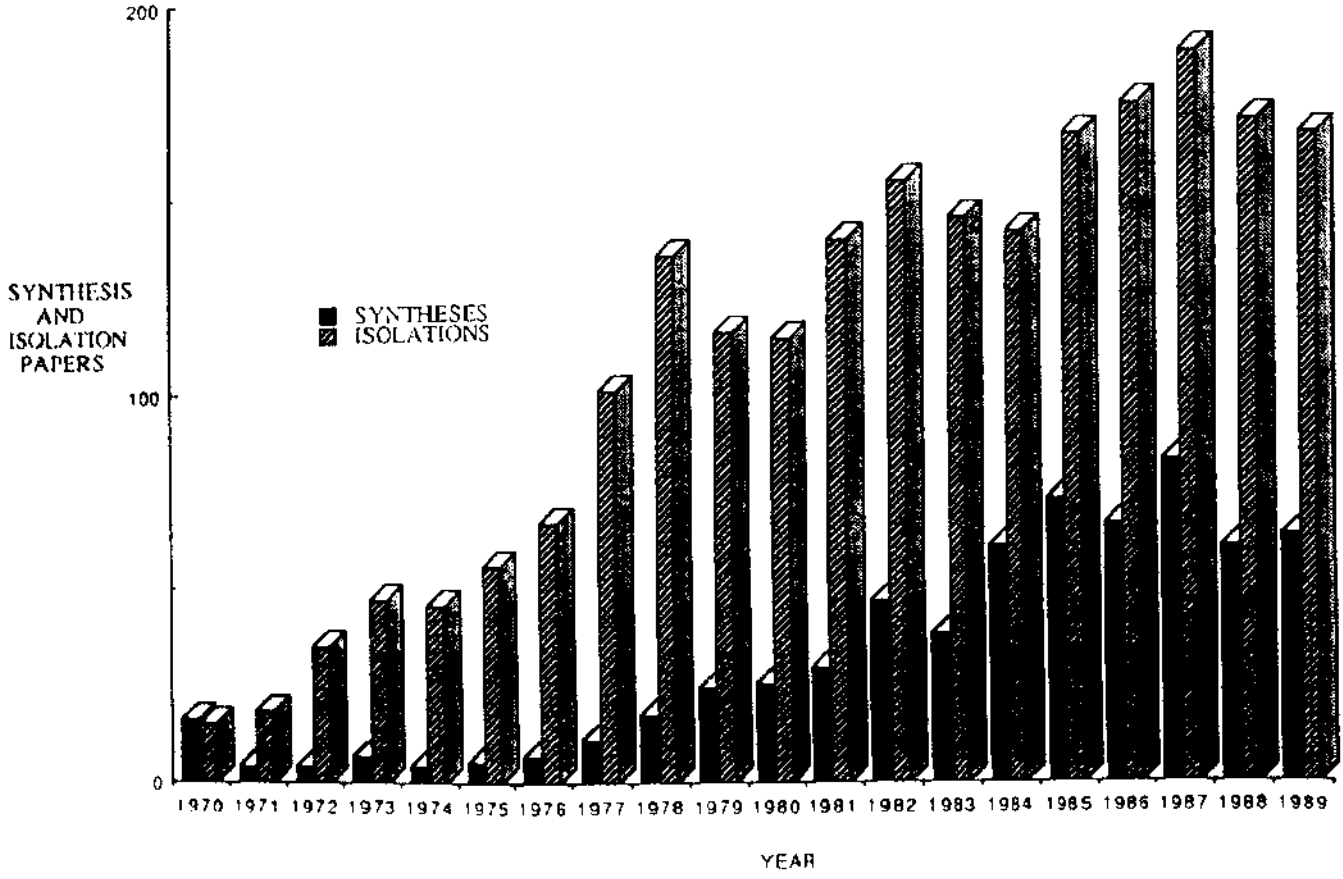
- Sponge, algal, and coral metabolites were the most frequent targets;

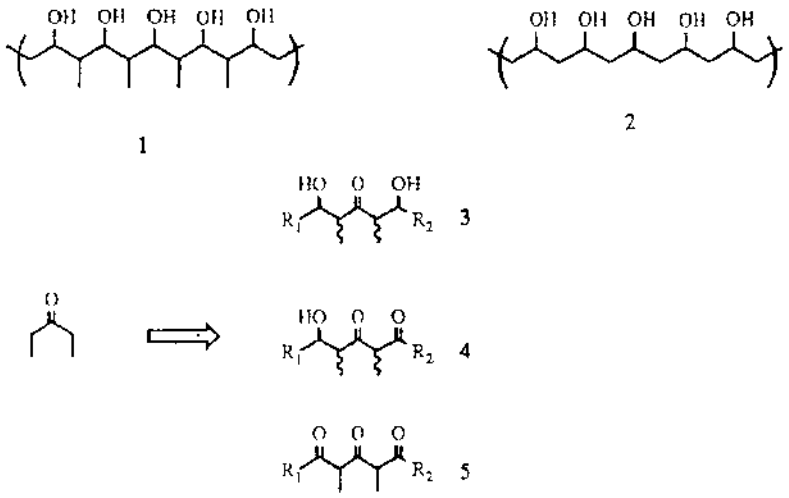
- Almost one-third of the syntheses proceeded to give final products in homochiral form.

As one might expect, the vast majority of the syntheses involved metabolites of relatively simple structure, although the largest nonpolymeric natural product synthesized to date is the marine-derived palytoxin. Thus, the synthesis of marine natural products in the past 30 years parallels natural products synthesis as a whole in every way. As long as marine organisms continue to be sources of compounds of biological importance or of interesting new structures, this area should experience continued growth.

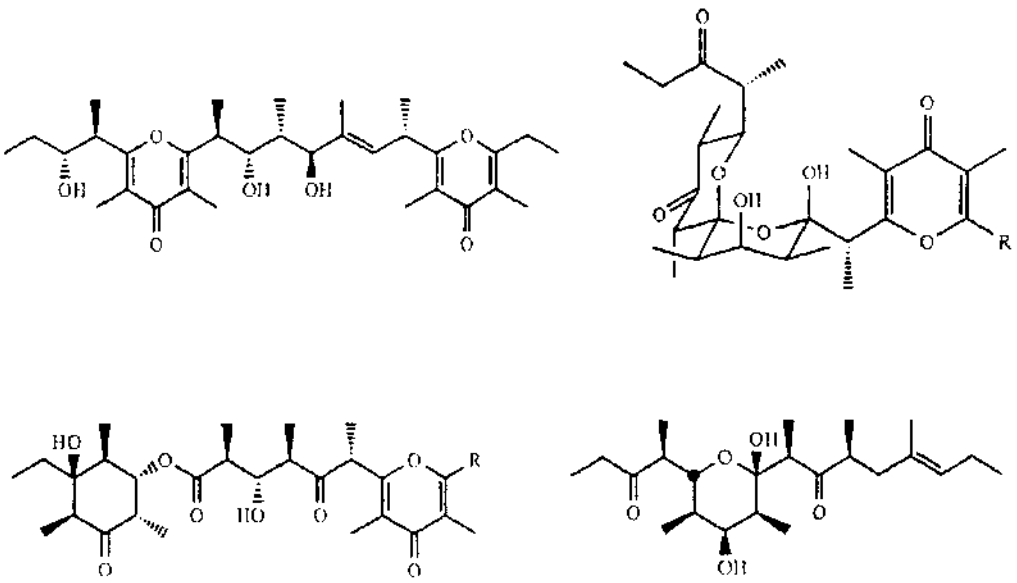
Our own work in marine natural products synthesis has recently involved a new approach to polypropionate synthesis. The target molecules that have stimulated this chemistry are the metabolites of *Siphonaria*, air-breathing marine molluscs which are the source of about two dozen (to date) truly polypropionate-derived substances [5]. Using the aldolate dianion technology recently developed in our laboratory [6], we have developed a number of rapid entries into structural

Growth of Marine Natural Products Chemistry

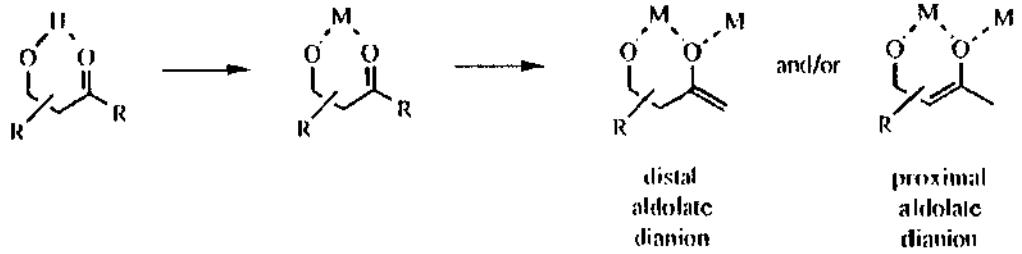




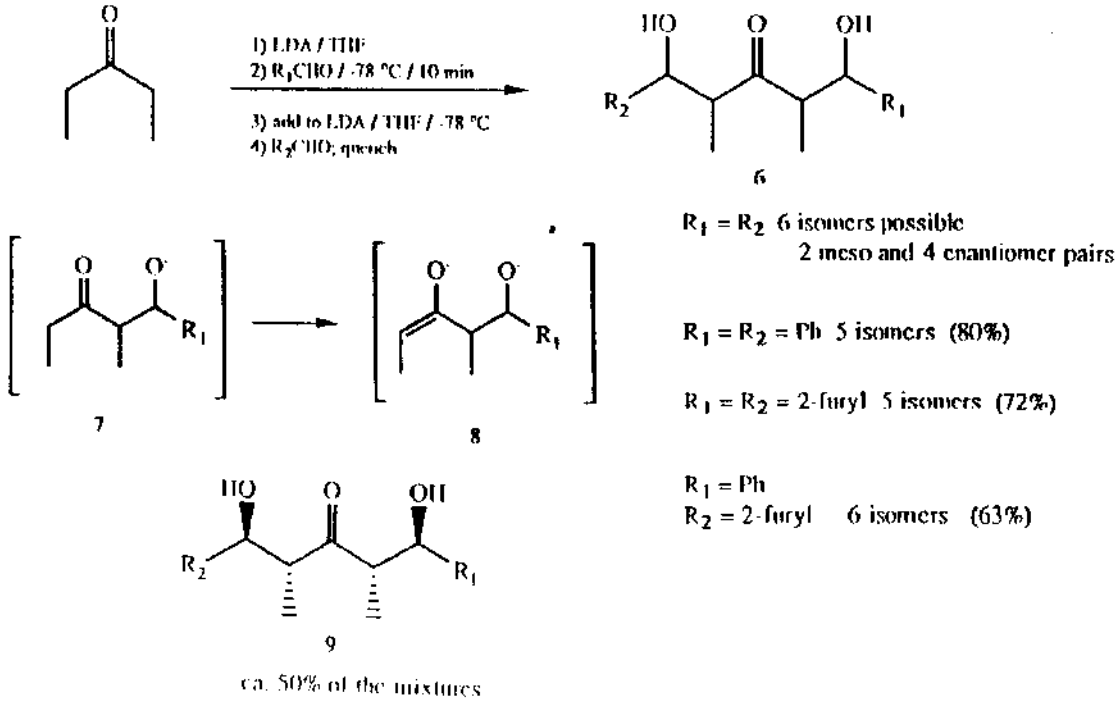
Scheme 1. Some Metabolites of *Siphonaria sp.*



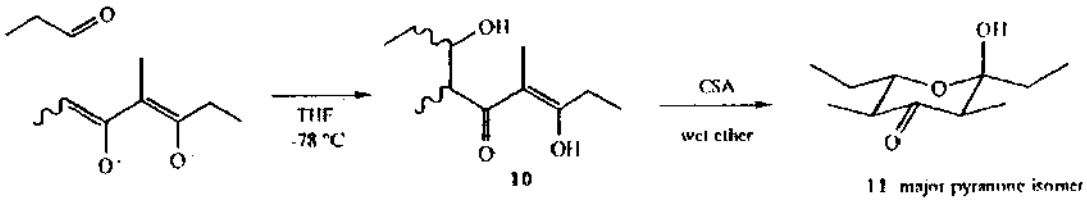
Scheme 2

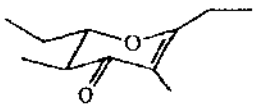


Scheme 3



Scheme 4





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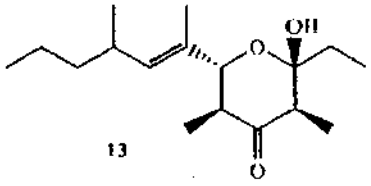
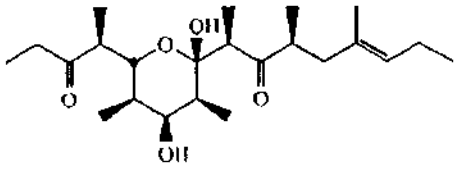


13

Siphonaria australis



denticulatin B

chains containing alternating methyl-bearing and oxygenated carbons. To briefly summarize, we have found that β–hydroxyketones can be doubly deprotonated under standard conditions to provide distal aldolate dianions in which the presence of the β-oxido group enables regiospecific formation of the enolate anion. Such enolates react with a variety of electrophiles including silyl halides, aldehydes, and acyl cyanides in good to excellent yield. Extensive study has led to a simple working model for the deprotonation process in which the hydrogen-bonded substrate is first deprotonated at the heteroatom to form a cyclic metal chelate, which then undergoes subsequent enolization leading to the observed aldolate dianion species.

To implement our strategy as applied to the metabolites of *Siphonaria*, rapid and nonstereoselective access to the three substructures (3-5) was desired. We have developed a number of protocols, some of which are based on aldolate dianion chemistry. A one-step method for the production of β, β'-dihydroxyketones is the tandem aldol process; it consists of two aldol reactions run consecutively in the same reaction vessel, taking advantage of the regiospecific production of aldolate dianions (8) from the aldol reaction intermediate 7. Although a large number of isomers can be isolated, it appears that the *anti-syn anti*-isomer 9 is the major (about 30 percent) product of this process.

Along with the tandem aldol process, alternative and complementary processes have also been utilized to assemble the desired substrate chains. These have made a variety of polyoxygenated chains available for study.

In the simplest test case, we have found that the δ-hydroxy-β-diketone 10 can be cyclized with equilibration of all alkyl substituents to equatorial positions to give the saturated 2-hydroxy-4-pyranone 11 as the major saturates isomer (Scheme 4). The major side product is the equilibrated 2,3-dihydro-4-pyrone 12. It is noteworthy that 10 was used as a mixture predominating (80:20) in the stereochemistry opposite to that at C5 and C6 in 11 or 12. The structural analogy of 11 to the *Siphonaria* metabolite 13 and denticulatins A and B is straightforward.

Notes

1. These data were provided by Professor John Faulkner.
2. These data take into account total syntheses. Partial and formal syntheses have not been included.

3. Albizati, K.F., V.A. Martin, M. Agharahimi, et al. 1991. "Synthesis of Marine Natural Products." In *Bioorganic Marine Chemistry*, P.J. Scheuer, ed. Verlag Chemie, Heidelberg.
4. We did not review membrane synthesis or synthesis of fresh water metabolites.
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NEW PERSPECTIVES IN MARINE NATURAL PRODUCT CHEMISTRY

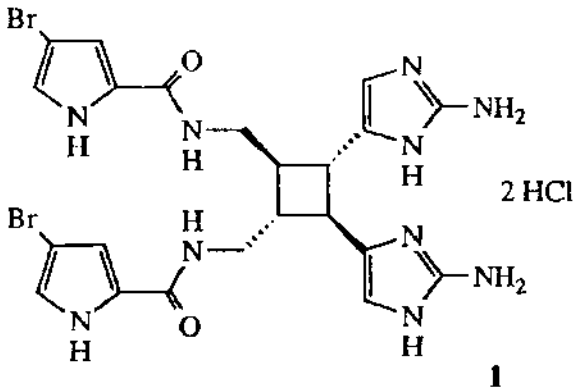
D. John Faulkner, Scripps Institution of Oceanography,
University of California, San Diego

Before speaking about new directions in marine natural product chemistry, I would like to emphasize the novelty of those compounds already isolated and identified by my research group and screened by Professor Jacobs. Many of the compounds belong to structural classes that are unique to marine sources or have chemical functionality that has not been described elsewhere. These include small cyclic polysulfides, carbonimidic dichlorides, cyclic peroxides, polychlorinated amino-acid derivatives, sesquiterpene thiocyanates, 9-methyladenine derivatives of diterpenes, and individual compounds like sceptrin (1), jaspamide (2), and the renieramycins (3,4).

Most of the marine organisms that have been studied were chosen because it was strongly suspected, on the basis of field observations, that the organisms employed chemical defenses against predation. It is therefore not surprising that Dr. Jacobs has recorded positive bioassay results in one pharmacological screen or another for more than half of the compounds tested. This is a much higher "hit" rate than is generally experienced with compounds from terrestrial or fermentation sources. Although it is now more difficult to find truly novel compounds, they still appear with regular frequency. There is no doubt in my mind that the study of marine organisms will continue to provide new pharmaceutical leads for at least another decade.

The research being performed by my Sea Grant trainee is typical of Ph.D. research at Scripps in that it involves the testing of a general hypothesis. Steve Bobzin is examining the hypothesis that nudibranchs select the most biologically active compounds from the sponges (or other invertebrates) that comprise their diet. If true, the application of this hypothesis will enable us to track down the more biologically active compounds in each environment by examining the metabolites concentrated by nudibranchs.

I anticipate that my next Sea Grant trainee will be Barbara Potts, who is studying the chemical mechanism of the irreversible reaction of manoalide (5) with phospholipase A_2 (PLA_2). She has recently demonstrated that primary amines, which can be considered as models for the lysine residues of PLA_2 that were known to be involved in the interaction, react at the gamma hydroxybutenolide ring of manoalide analogues to form Schiff bases rather than the Michael addition products that were



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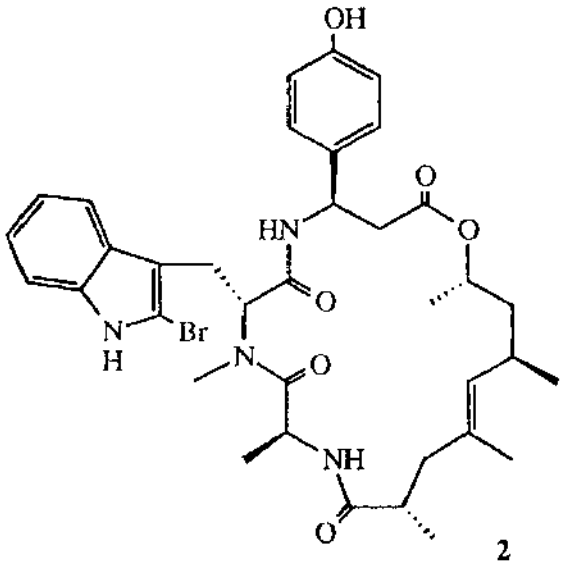

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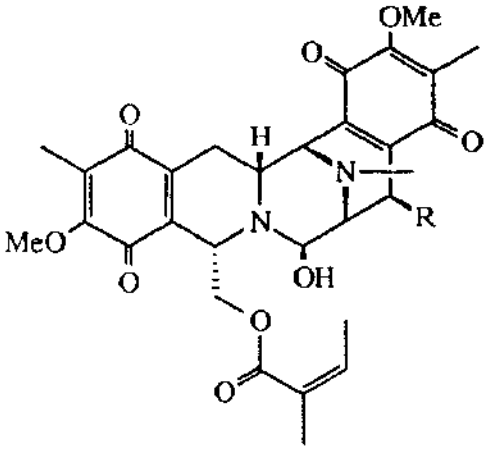
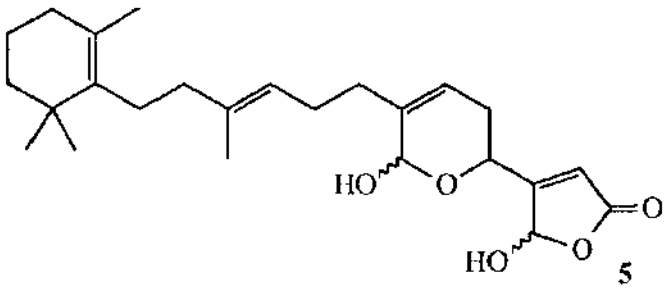


3 R = H
4 R = OMe



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proposed previously. The advantage of the NMR method that we employ is that we can identify transient intermediates and measure approximate rates for the different reactions that occur. We have proposed that manoalide reacts with two lysine residues of PLA_2 to form two Schiff bases, a reaction that is essentially irreversible at physiological pH.

Our future research program is really dependent on current and future advances, particularly in what I call the miniaturization of bioassays. As new screens are developed that require smaller quantities of material for each bioassay, we will be able to study a broader range of pharmacological activities with the same amount of compound or examine new organisms that are very small and difficult to collect.

The collections are the key to successful discovery programs. Although many areas of the world seem to have been heavily collected, we have only looked at a very small area of the available shallow water environments. Collections from new locations and particularly the study of micro-environments will yield new organisms or concentrations of organisms that are rare in adjacent locations. Collaboration with researchers in third-world countries, similar to our very productive joint research program with Professor Rao at Andhra University, may be the key to future collection and screening programs. Ultimately, however, our success in discovering new drugs will depend on the ingenuity of those developing new bioassays and their skill in interpreting the <u>in vitro</u> bioassay data to provide potential treatments for human diseases.

SYNTHESIS OF MANOALIDE

Michael E. Garst (with Gary Lee and Elizabeth Syage),
Department of Chemical Sciences, Allergan Pharmaceuticals, Inc.

Manoalide (1), a naturally occurring sesterpene isolated from *Luffariella variabilis* [1], has been shown to inhibit phospholipase A_2 [2], phospholipase C [3], 5-lipoxagenase [4], and calcium flux [5]. This spectrum of activity makes manoalide an attractive lead structure for the development of novel anti-inflammatives. This presentation summarizes some of our synthetic work using manoalide.

Using the general reaction sequence that we developed for the synthesis of manoalide [6], four analogs of 1 have been prepared containing different side chains. The activities of these analogs were comparable to manoalide [7].

To help determine the key structural elements of manoalide, we prepared simple fragments of 1. One of these analogs, 3, possesses biological activity like 1 [8]. The efficient synthesis of 3 required a novel synthesis of furan 2. Furthermore, to facilitate the large-scale synthesis of 3, the method for oxidation of furans to hydroxy-butenolides was dramatically improved by the inclusion of water as co-solvent [9].

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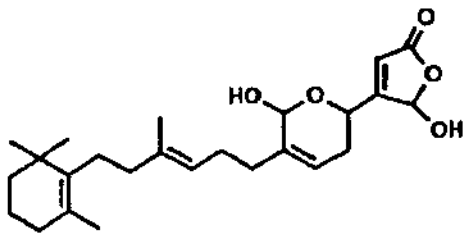

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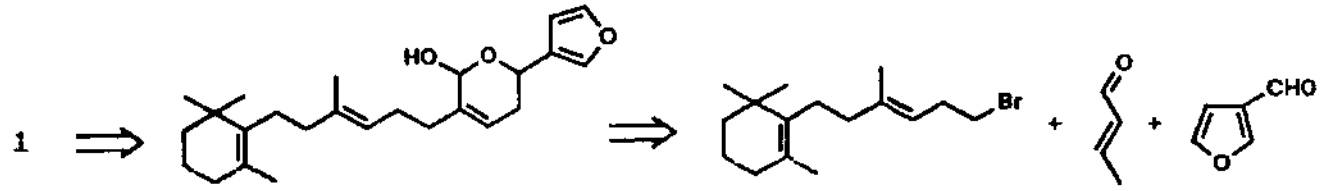

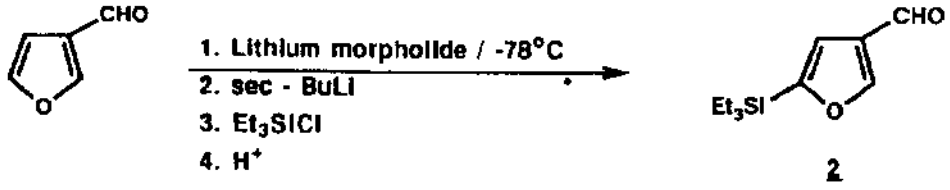

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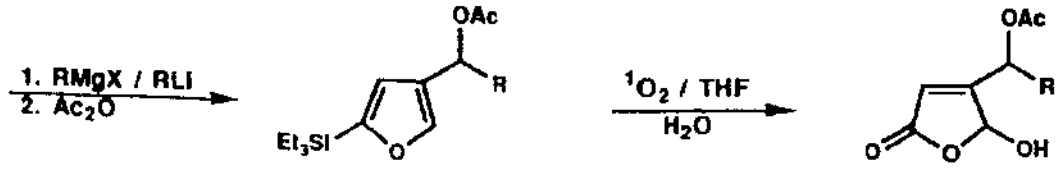

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APPROACHES TO THE DISCOVERY OF DRUGS TO TREAT INFECTIOUS DISEASE

Elizabeth B. Fraser-Smith, Syntex Corporation

About 640 extracts have been tested at Syntex in the past year for activity against helminths, viruses, fungi, and bacteria. These natural products were derived mainly from marine sponges and tunicates and provided by Phil Crews' laboratory at the University of California, Santa Cruz.

To test for anthelmintic activity, an in vitro assay with the nematode *Nippostrongylus brasiliensis* was used to determine reductions in the number of larvae which molt to the adult. From 23 active extracts that were found (3.5 percent hit rate), 16 purified compounds in 11 chemical families were identified. A mixed helminth infection in mice was then used to test these compounds for in vivo activity. Reductions in intestinal-worm burden of the nematode *Nematospiroides dubius* and the cestode *Hymenolepis nana* were measured following treatment. Three compounds had moderate activity against either the nematode or the cestode, but not both. Another compound was toxic to the mice with no efficacy at subtoxic doses. The rest were not active in vivo or had no in vitro activity when prepared synthetically. When compared to known anthelmintics, none of the compounds had sufficient efficacy to be selected for development.

To test for antiviral activity, in vitro cell assays with human immunodeficiency virus (HIV) and herpes simplex virus-1 and -2 (HSV-1, HSV-2) were used to determine the inhibitory concentration of drug which reduced either pathogen by 50 percent (IC_{50}). Five of 490 extracts were found to be active against HIV (1 percent hit rate). Three pure compounds were subsequently identified from these extracts; two of these molecules did not have a favorable therapeutic ratio. Testing of the third compound is ongoing. Three extracts were found active against HSV (0.6 percent hit rate); no pure compounds have been identified from these extracts.

To test for antifungal or antibacterial activity, in vitro disk assays to determine the zone of growth inhibition of either *Candida* and *Trichophyton* or of *Pseudomonas, Escherichia, Staphylococcus,* and *Streptococcus,* respectively, were used. When tested against the fungi, 7 of 540 extracts were active against *Trichophyton* (1.3 percent hit rate) and none was active against *Candida*. Antibacterial testing revealed four extracts active against both *Staphylococcus* and

Streptococcus (0.7 percent hit rate). Although these efficacies were comparable to the antimicrobial activities of known antifungal and antibacterial agents, the identification of compounds from these extracts has not been pursued to date.

In an effort to speed up the process of identifying useful antimicrobial agents from natural products, new techniques are being designed. The development of spectrophotometric assays, correlating optical density with the number of viable cells or other parameters, will allow data to be read electronically. These methods, coupled with automatic plate harvesters and other robotic techniques, will allow testing of thousands of extracts per year instead of hundreds. These techniques will soon be available for both HIV and HSV assays.

Molecular biology techniques will also contribute substantially to the development of rapid assays. By taking advantage of rapid bacterial doubling times, essential targets of various pathogens can be cloned into bacteria and the effects of drug treatment on growth determined in one day rather than one to two weeks. For example, with cloning techniques, a mutant bacterium which does not grow on its own theoretically could be made dependent on an HIV enzyme for growth. If growth occurs when a drug is added, the drug is inactive. With no growth, the compound is a potential anti-HIV agent. Controls utilizing the normal bacterium are run to ensure that the effect is specific for the cloned HIV target enzyme. Innumerable other molecular biology techniques are possible using bacteria, yeast, tissue cells, bacterial reporter genes, and target genes in various combinations. In addition to speeding up assays, the new techniques often avoid the risk of working directly with deadly pathogens so that the handling and disposal of hazardous wastes is minimized.

Many hurdles await a compound after it has been shown to be active in vitro and before it can be developed commercially. If the compound is active in an enzyme assay, the molecule may be inactive when tested in vitro against the pathogen itself. In this case, the compound often is not capable of getting inside the cell to exert its effect. More frequently, a compound that is active against the pathogen in vitro fails to have in vivo efficacy when run in a subsequent animal-infection model. The drug is either not absorbed or not bioavailable, often being serum-bound or metabolized too rapidly. Then, too, the inherent toxicity of the drug may first become apparent in an animal-infection model. For example, body-weight loss, atrophy of organs or tissues, hematological toxicity or immunosuppression may occur. Finally, in long-term animal testing, the

compound may be eliminated because it has proved to be carcinogenic or teratogenic.

Thus, the road to drug development is a long and arduous one with many steps at which the drug may fail along the way. However, the possibility that unique molecules with good antimicrobial efficacy will continue to be discovered makes this effort well worthwhile.

MECHANISM-BASED APPROACHES TO ANTI-TUMOR DRUG DISCOVERY WITH NATURAL PRODUCTS

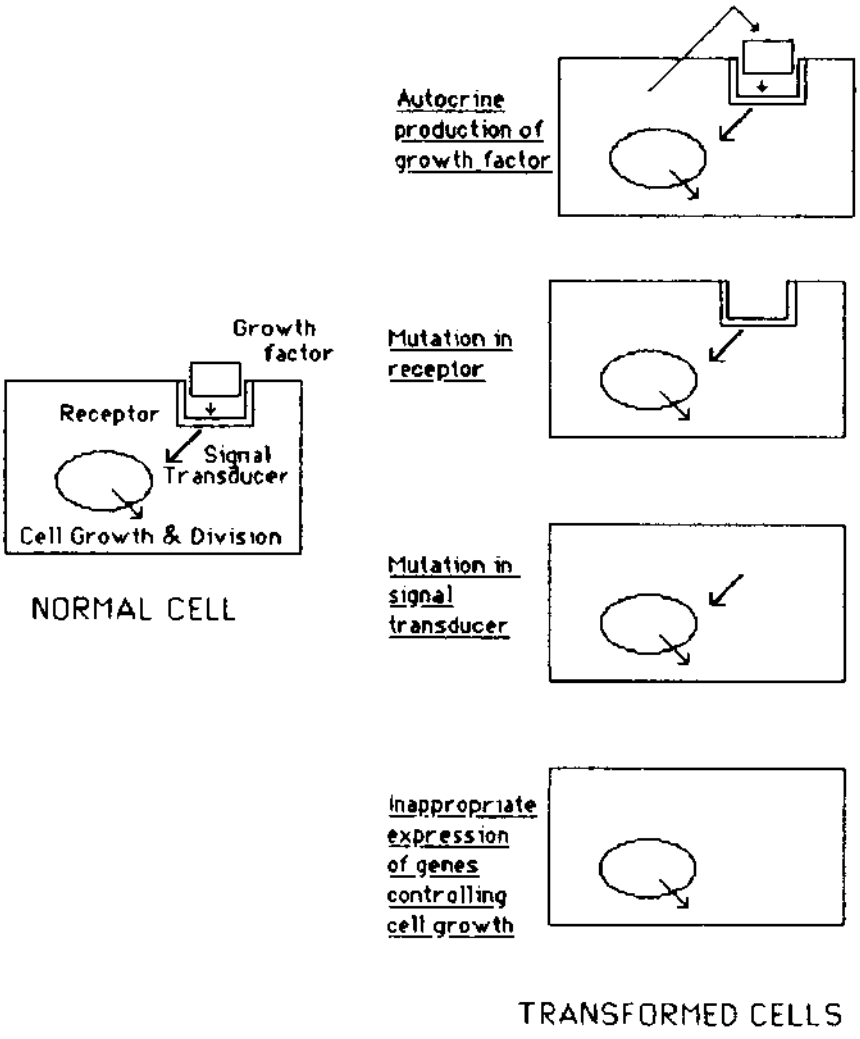
Doris L. Slate, Syntex Corporation

Current chemotherapy for cancer has proven successful in only a limited number of human neoplasms, and such major solid tumors as lung and colon carcinoma remain refractory to available treatment. Even if a primary tumor can be adequately managed, the patient often succumbs to regional or distant metastases. In addition, the host toxicity of current drugs is often severe, leading to debilitating side effects and in some cases to iatrogenic mortality. These observations highlight the major problems with existing chemotherapy: drug resistance, ineffectiveness toward metastatic disease, and lack of tumor specificity.

Potential approaches for improving cancer chemotherapy are as varied as the types of human neoplasia. As the mechanisms of action for current drugs become better understood, second and third generation agents (often closely related analogs of known compounds) can be synthesized and developed. Approaches taking advantage of natural host defense mechanisms, such as the use of lymphokines and cytokines, are also being tested clinically. While new therapies are likely to emerge from these strategies, our group in the Syntex Institute of Cancer and Developmental Biology has decided to focus on the biology of the tumor cell itself, particularly those facets which distinguish it from its normal, nontransformed counterparts.

Recent advances in cell and molecular biology have provided new insights into the alterations in growth regulation which underlie the aberrant proliferation observed in neoplastic disease. The "rediscovery" of cellular oncogenes has revealed distinct differences between normal and tumor cells in the expression or form of certain proteins regulating cell growth and differentiation. The products of cellular oncogenes can be grouped into several discrete functional classes, e.g., growth factors, receptors, tyrosine and serine/threonine protein kinases, other known or presumed signal transducers, and nuclear DNA binding and transcription factors. This categorization suggests that cancer, presenting clinically as a collection of many distinct diseases, may be due to alterations in a conceptually limited number of regulatory molecules (see Figure). The recent identification of a so-called "anti-oncogene" product, the rb (retinoblastoma) protein, which can be found associated with a variety of oncogene products, also strengthens this hypothesis.

Growth and Regulation and Possible Mechanisms for Oncogenesis



As the biochemical functions of oncogene products have been elucidated, assays for identifying inhibitors of these functions have been developed. For example, over the last few years, small molecule inhibitors of protein tyrosine kinases have been identified from natural sources and by synthetic pursuit of these microbial products. Recently, several of these were shown to inhibit cell proliferation triggered by epidermal growth factor. As the evidence for the involvement of individual oncogenes in particular human tumors mounts, and as cell lines are engineered to overexpress normal and mutant oncogene products, it should be possible to test the following simple hypothesis: If a particular oncogene product is necessary for tumor development and progression, then an inhibitor of its biochemical function should be useful in blocking tumor growth and/or metastasis. A corollary of this hypothesis would suggest that since tumor cells have altered forms or elevated levels of oncogene products compared with normal cells, some useful degree of tumor specificity may be obtained.

Transfection of certain cell lines, especially NIH 3T3, with oncogenes of the ras and tyrosine kinase families has been shown to confer full tumorigenic and metastatic ability. These two families have been chosen as our initial oncogene targets for drug screening. Many retroviral oncogenes encode protein tyrosine kinases, and these enzymes are known to be crucial for cell growth and differentiation. Cell lines which overexpress these oncogene products have been obtained, and characterization of spontaneous tumors for the expression of these proteins is being performed. Consistent elevation of pp60-src specific activity has been noted in human colon tumors, and other carcinomas have been shown to contain high levels of phosphotyrosine in immunohistochemical studies of tissue sections. Activation of ras oncogenes and overexpression of p21 proteins have been shown to occur frequently in human tumors, with virtually all pancreatic carcinomas and 40 to 50 percent of colon carcinomas possessing activated Kras genes.

The possible involvement of enzymes of the protein kinase C family in tumor formation also makes them potential screening targets. Various oncogene products and growth factors have been shown to stimulate the protein kinase C activation pathway by enhancing phosphoinositide turnover, and protein kinase C activation can trigger oncogene expression. Phorbol esters, which bind and activate protein kinase C, have long been known as potent tumor promoters. Some transformed cells have been reported to overexpress protein kinase C, and its overexpression in transfected NIH 3T3 cells alters cell growth regulation and enhances tumorigenicity.

Patients may present with inherently drug resistant tumors, such as colon or pancreatic carcinoma, or may develop resistant tumors following initial, apparently successful, courses of chemotherapy. One particularly interesting type of drug resistance is classical multidrug resistance, which encompasses the major classes of natural product antitumor agents (the antracyclines, vinca alkaloids, and epipodophyllotoxins). Classical multidrug resistance arises following the overexpression of p170 (P-glycoprotein), a membrane-spanning glycoprotein which functions as an energy-dependent drug efflux pump. A number of different agents, such as calcium entry blockers and cyclosporin A, have been shown to reverse drug resistance in animal models employing multidrug resistant tumor cells. These compounds bind to p170 and compete with antitumor agents for outward drug transport. Unfortunately, since these compounds were optimized for other pharmacological activities, they are not well suited for reversing multidrug resistance in cancer patients, although preliminary reports suggest some promise for the concept.

Metastasis continues to produce morbidity and mortality even when a patient's primary tumor can be managed by surgery or radiation therapy. Since most cancer patients probably have occult metastases at the time of presentation, approaches to inhibit the outgrowth or further spread of these secondary foci should provide new therapies. A variety of proteolytic enzymes have been shown to be required for extracellular matrix and basement membrane degradation, among them plasminogen activators, type IV collagenase, and transin/stromelysins; these enzymes can also be targeted in drug discovery efforts.

Models at the secondary and tertiary stages of screening must emphasize cells that possess the alterations in target protein level/activity for the mechanisms that have been identified for primary targeting. For example, cells overexpressing p170 should show renewed sensitivity to drugs involved in the multidrug resistance phenotype when a resistance reversal agent is co-administered.

Historically, natural products and their derivatives (e.g., the anthracyclines, vinca alkaloids, and epipodophyllotoxins) have been a mainstay in cancer therapy. Our program hopes to identify novel compounds from marine organisms which will address the current failings of chemotherapy outlined above: specificity, resistance, and metastatic progression. Targeting proteins whose altered activity or overproduction is characteristic of specific tumors when compared with normal tissue should permit the discovery of drugs with better therapeutic indices. Targeting proteins involved in mediating different types of drug resistance should

permit broader or repeated use of currently available (or related) agents. Targeting proteases involved in invasion, metastasis, migration, and growth control should result in the identification of novel compounds to interfere with these processes. The screening of extracts of marine organisms will complement ongoing rational medicinal chemistry approaches to drug discovery.

INTRACELLULAR RECEPTORS: NEW APPROACHES TO DRUG DISCOVERY

Tina S. Berger, New Leads Discovery, Ligand Pharmaceuticals, Inc.

Recently, important discoveries in the molecular biology of intracellular receptors have been made by Ronald M. Evans, Ph.D., at the Salk Institute in San Diego. As a result of these discoveries, fundamental insights have been gained concerning the molecular structure of these important proteins and the manner in which they interact with hormones to regulate physiological processes.

A novel co-transfection assay used to measure intracellular receptor activity was developed in Dr. Evans' laboratory. This assay is unique compared to conventional "grind and bind" assays in that it enables scientists to measure the degree to which ligands bind to receptors and initiate gene activation. The assay is functional, highly sensitive, and quantitative. Cultured cells are transfected with the receptor cDNA in an expression vector. This vector provides for the efficient production of the receptor in cells that do not normally express the receptor gene. A second transfected vector contains a luciferase gene coupled to a hormone-responsive promoter. Addition of hormone or experimental drug will activate the luciferase gene, causing light to be emitted from cell extracts. The level of light emitted is directly proportional to the effectiveness of the hormone receptor complex in activating gene expression. Ligand Pharmaceuticals has exclusively licensed the resulting technology and is using it as the foundation of its program to discover new drugs for treating hormonal dysfunction and related diseases.

Ligand Pharmaceuticals has spent the last year optimizing the co-transfection assay. Initially, only 80 data points were collected per week, but with the same number of personnel, the data output has increased to over 5000 data points per week. This was made possible by the extensive optimization of the assay and the introduction of automated robotic systems. In addition, experienced molecular biologists at Ligand have developed proprietary methods of receptor modification, which have resulted in an increase in assay response and sensitivity by tenfold for all receptors. Using this methodology, Ligand is the first research group to successfully design a functional assay for both the human progesterone receptor and the human androgen receptor. The company believes that, armed with its knowledge in the intracellular receptor area, it will be a leader in the development of new products for both human and nonhuman applications.

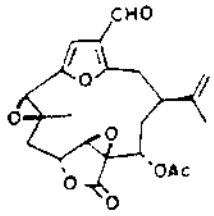
In conjunction with William Fenical of the Scripps Institution of Oceanography, Ligand has initiated screening of organic extracts of oceanographic bacterial soil samples. To date, a total of 150 samples have been screened in the co-transfection assay with all of the known and orphan human intracellular receptors. Two compounds were positive in the initial screen of crude extracts, and these compounds have been highly purified and identified. These include an agonist of the vitamin D receptor and an antagonist of the mineralocorticoid receptor. These compounds are now being tested in animal models for in vivo activity as well. In a relatively short period of time, we have been able to demonstrate the utility of our system and the role of intracellular receptors in the identification of potential pharmaceuticals from oceanographic samples.

MARINE TOXINS THAT AFFECT CHOLINERGIC NEUROTRANSMISSION

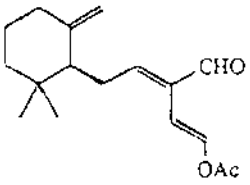
Palmer Taylor and Stewart Abramson,
Department of Pharmacology, University of California, San Diego
William Fenical, and D. John Faulkner,
Scripps Institution of Oceangraphy, University of California, San Diego

We have characterized the molecular mode of action of two non-nitrogenous toxins which exhibit distinct modes of action in blocking neuromuscular transmission.

Lophotoxin, a cyclic diterpene isolated from several soft corals of the genus *Lophogorgia*, was initially characterized as a long-acting neuromuscular blocking agent (see Figure). Our studies on its action in cell culture and on isolated nicotinic acetylcholine receptors from *Torpedo californica* reveal that lophotoxin reacts irreversibly with the α-subunit of the receptor. Its irreversible action can be blocked with agonists and antagonists that bind to the acetylcholine-recognition site but not with noncompetitive allosteric inhibitors that bind within the ion channel. Labeling studies using a tritiated lophotoxin analog reveal that the irreversible action is a consequence of covalent conjugation with tyrosine 190. The lophotoxin reaction is not blocked by prior reduction of cysteine 192-193 or alkylation of the reduced disulfide with iodoacetate or N-ethylmaleimide. On the other hand, alkylation with the bulkier site-directed agent malemidobenzyltrimethyl-ammonium results in block of the lophotoxin reaction. These results suggest that Tyr^{190} interacts with the quaternary ammonium group present in most agonists and antagonists. Structure and activity analyses of lophotoxin action have indicated that the reactive portion of the molecule includes a lactone and a reactive epoxide at the position analogous to the quaternary ammonium group of acetylcholine. Because of the similarities between the structures of lophotoxin and acetylcholine, it appears that the acetylcholine ester binds in an antiparallel fashion to the amide bonds of the peptide chain in the receptor. Hence the region of the acetylcholine binding site involved in binding the acetyl moiety is proximal to cysteine 192-193, whereas the quaternary ammonium moiety on acetylcholine is positioned near Tyr^{190} in the receptor. Lophotoxin shows a broad range of reaction with acetylcholine receptor subtypes in nerve and muscle and may prove useful in the treatment in some types of muscle spasms.



Lophotoxin



Onchidal

A second compound, onchidal, shows some structural similarity to lophotoxin in that both toxins contain an acetate ester and an α, β unsaturated aldehyde. However, onchidal does not inhibit the nicotinic acetylcholine receptor but rather it irreversibly blocks acetylcholinesterase. Several characteristics of the kinetics of inhibition of acetylcholinesterase suggest that onchidal is acting as a suicide substrate, where its irreversible action occurs at an intermediate step in catalysis. Our kinetic analyses suggest that approximately 3250 catalytic steps occur for every inhibitory step. Reversible inhibitors of the active center protect against onchidal inhibition, while peripheral site inhibitors only provide partial protection. Of particular interest is that onchidal inhibition, in contrast to the inhibition seen by organophosphates, is not reversed by oximes. This would argue against acylation of the active-site serine as a mechanism of inhibition. Because catalysis gives rise to a structure which could tautomerize to form a 1,4 dialdehyde, conjugation with an amino or a guanidinum group in the acetylcholinesterase structure is a likely possibility for its mechanism of action.

NEW PHARMACEUTICALS FROM MARINE MICROORGANISMS

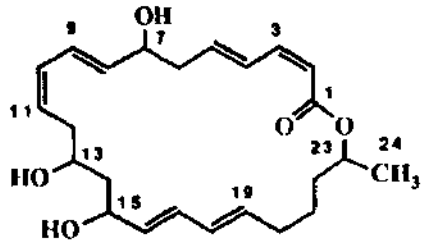
William Fenical, Scripps Institution of Oceanography,
University of California, San Diego

Although marine plants and invertebrates have been the subject of extensive biomedical study, investigations of marine microorganisms are rare and apparently limited by difficulties in culture and identification. Considering the immense importance soil bacteria have played in the development of clinically important antibiotics and antitumor compounds, it is surprising that so little attention has been placed on the massive diversity of bacteria found in marine environments.

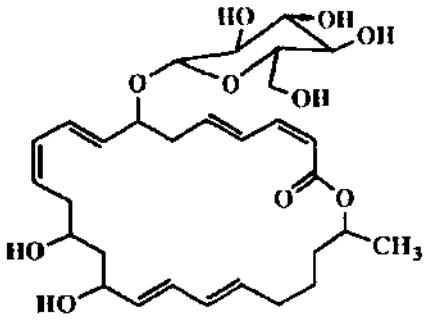
Marine sediments resemble soils in that there is an immense diversity of bacteria present. In addition, marine plants and invertebrates provide suitable host surfaces, which are adapted niches for specific bacteria that appear to play symbiotic roles crucial for survival. As part of our Sea Grant biomedical research program, we have been exploring new biomedical resources including marine microorganisms. Our approach has been to focus attention on the development of techniques to isolate and mass culture obligate marine halophiles and other bacteria from unique marine environments. Although this is, at present, a very minor component of our Sea Grant program, we expect to expand into this area in the future.

Current research in my group with microorganisms includes preliminary studies of deep-sea bacteria and surface-dwelling microorganisms from gorgonians and other soft-bodied invertebrates. While it is early in the development of this field, the initial results are encouraging. An unidentified deep-sea bacterium isolated from marine muds collected at -1000 feet in Northern California has been found to produce a unique group of macrocyclic lactones, the macrolactins (1,2). These compounds are antiviral and cytotoxic agents of a new chemical class. Macrolactin A (1) inhibits the replication of human immunodeficiency virus (HIV), the causative agent in AIDS. This compound is of current interest, since it may indicate that new drug therapies for AIDS may be discovered in marine sources.

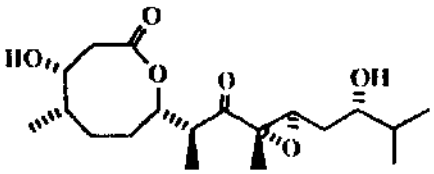
In other work, we have identified two new 8-member ring lactones, the octalactins A and B (3,4) produced by a bacterium found on the surface of a Pacific gorgonian of the genus *Pacifigorgia*. These novel compounds are cytotoxic agents active against human colon carcinoma cancer cells, and thus indicate the potential for the isolation of new anticancer agents from these sources.



1, Macrolactin A



2, Macrolactin B



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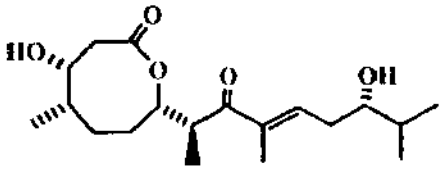

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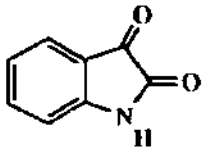

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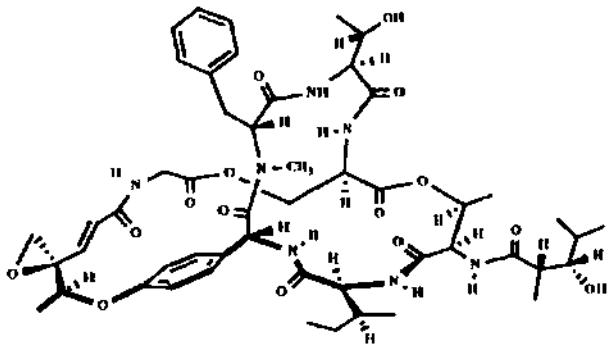

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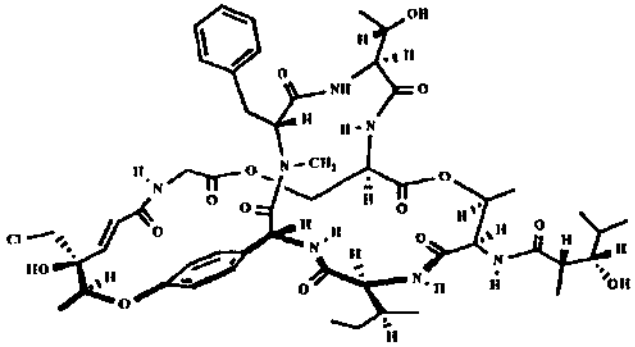

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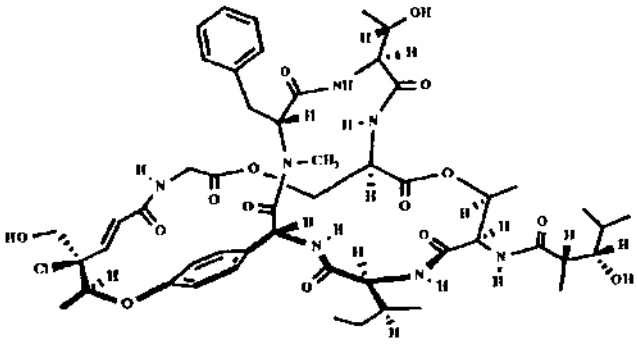

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Many soft-bodied invertebrates indeed possess surface bacteria that appear essential for their survival. Eggs of the estuarian shrimp *Palemon macrodactylus* have been found to produce a unique metabolite, 2,3-indolinedione (5), which shows potent antifungal properties. The potency of this compound, particularly against the marine pathogenic fungus *Lagenidium callinectes*, has suggested that it may be an effective agent useful in the control of diseases in aquaculture.

In another investigation, we have isolated a unique actinomycete of the genus *Streptomyces* from the surface of a Caribbean jellyfish. This bacterium produces, in culture, a group of complex bicyclic depsipeptides of a radically new type. Three of these compounds, (6-8), are unique antibiotics with selective inhibitory properties against gram-positive bacteria.

Part of our studies of marine microorganisms have focused on the concept of the marine or terrestrial origins of marine actinomycetes. Prior proposals have been that marine actinomycetes are of terrestrial origin and that their presence in the ocean is a result of rainwater runoff. Although this explanation has some validity, we have found that actinomycetes have very specific distribution in marine habitats. In studies of bacteria associated with coral reefs, we have illustrated that some actinomycetes, particularly streptomycetes, are found in abundance in very shallow marine waters (ca. 1-2 ft.). These are abundant only in near-shore environments. However, our work has also shown that actinomycetes of group actinoplanetes are unequally distributed in deeper marine sediments. Sampling in tropical reefs at >90 ft. in depth has illustrated a large diversity of unique actinoplanetes which are of obligate marine origin. Culture studies have shown that these bacteria absolutely require seawater for cell division to occur.

Forecasting to the future, we believe that marine microorganisms represent a unique source for the development of bioactive agents. Studies of this type may lead not only to new drug development, but also to a more accurate understanding of the roles of microorganisms in the ecology of the sea.

WIDENING THE SEARCH FOR MARINE BIOACTIVE PRODUCTS

Francis J. Schmitz, Department of Chemistry, University of Oklahoma

In considering the issue of widening the search for marine bioactive products, it seems appropriate to review how our past efforts have been directed and to examine what we have done with our most significant discoveries.

We have given top priority to discovery of potential antitumor agents, second priority to central nervous system active agents and anti-inflammatory agents, and some attention to cardiovascular active compounds. Thus, in terms of types of bioactivities, our search has not been very wide. In terms of source organisms, our search has been reasonably broad; but it has been limited by the practical factors of accessibility and availability of adequate quantities of the organism to provide sufficient amounts of pure active compounds for in-depth pharmacological evaluation. Two ways we can broaden our search are by expanding the types of bioactivity we screen for and by direct attention to types of organisms we have not yet examined.

The most potent and structurally novel cytotoxins we have isolated are acanthifolicin [1], okadaic acid [2], and tedanolide [3] (see the Figure). Although all three were toxic to murine leukemia cells in vitro at 10^{-4} to 10^{-5} micrograms/mL, none showed sufficient activity in vivo to warrant further development. Because of the scarcity of these materials and their high levels of toxicity to whole animals, we did not pursue the mechanism of action of these compounds. This, in retrospect, was a mistake.

Mechanism of action studies were carried out on one of our early isolates, dactylyne, which had been found to increase pentobarbitol-induced sleep time in mice [4]. Follow-up studies revealed that dactylyne was a potent inhibitor of the metabolism of pentobarbitol, but had no hypnotic effect of its own.

Okadaic acid was found by Suganuma et al. to be a nonphorbol-ester-type of tumor promoter [5]. Additional studies revealed that it is a very selective and powerful inhibitor of protein phosphatase 1 and 2A [6]. Okadaic acid is now much in demand as a biological and pharmacological tool. This case illustrates again that natural products are important as tools for research as well as for clinical application.

At present, considerable emphasis is being given to mechanism-based bioassays rather than to overall cell toxicity for identifying anticancer drug

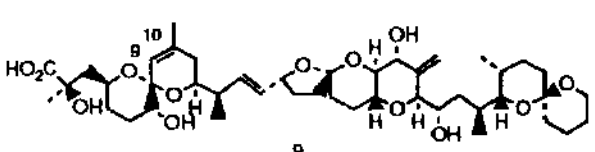
candidates. Emphasis is also being given to use of slow-growing tumor cell lines and drug resistant cell lines. It is anticipated that these approaches will be more effective in identifying promising drug candidates for the more recalcitrant solid tumors. However, individual investigators may not be able to conduct many of these assays or a broad spectrum of them, and collaboration with industry, medical centers, or pharmacology departments will be needed.

One obvious way to broaden the search for drugs from marine sources is to examine each extract with many more bioassays than we have in the past. This may become more possible with the emergence of enzyme- and receptor-based bioassays that are conducive to miniaturization and automation with direct connection to computer bases. These techniques should make it possible to evaluate a small amount of extract for a broad spectrum of activities and hence lead us to identify more of the types of bioactive compounds that are present in extracts of marine organisms.

Another approach to broadening the search for drugs from marine organisms is to pay more attention to organisms that have been underemphasized in the past. These include small or encrusting species in the already commonly studied phyla or more representatives from phyla that have not yet been studied extensively, e.g., hydroids. A major area for future studies is that of microorganisms, e.g., bacteria, fungi, dinoflagellates, and blue-green algae. Considerably more work is involved in culturing and investigating microorganisms, but the work completed to date suggests that this will be a fertile area, and it has the obvious advantage that cultured organisms constitute a renewable, manageable source of many good drug candidates.

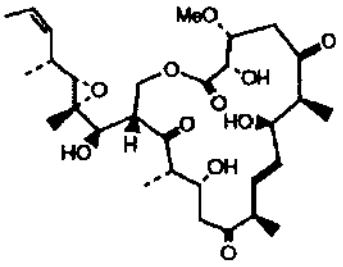
We are initiating studies of anaerobic marine bacteria. Limited studies on methanogens have revealed some significant biochemical differences between aerobic and anaerobic bacteria [7]. For example, the latter contain isopranyl glyceryl ethers as membrane lipids and utilize some novel coenzymes, e.g., methanopterin instead of folic acid. These fundamental biochemical distinctions suggest that anaerobes may also produce novel secondary metabolites. Indeed, a few unusual lipopeptides have already been isolated from anaerobic bacteria.

In summary, utilizing new bioassays; much broader screening of extracts using new microscale bioassays; devoting attention to organisms in unusual ecological niches and to small macroscopic organisms; and, in particular,

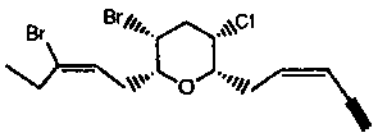


Okadaic acid: Δ^9

Acanthifolicin: 9,10 



Tedanolide



Dactylyne

intensifying our efforts in the area of cultured marine microorganisms should all contribute to the discovery of many new bioactive marine natural products.

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EICOSANOID BIOSYNTHESIS IN MARINE ORGANISMS

William H. Gerwick, College of Pharmacy, Oregon State University

During the past 20 years of marine natural products studies, the theme has emerged that many classes of primitive marine organisms produce eicosanoid-type natural products in much larger abundance than do their mammalian counterparts. While some of these marine eicosanoids have structures identical to those produced by mammals (for example $PGF_{2\alpha}$ and 12(S)-HETE), others have novel structures, without direct parallel in higher animals. These novel eicosanoids are of great interest because of their potential to have selective pharmacological action at eicosanoid receptors in mammals; and hence, they represent potential treatments for a variety of human diseases in which eicosanoids play a role in the underlying etiology. Furthermore, the mechanisms for the biosynthesis of marine eicosanoids, both those with standard structures as well as the novel ones, are entirely different from those produced by terrestrial mammals. For these reasons, our group at Oregon State has made the study of eicosanoids from marine plants, from discovery and structural description to biosynthesis and pharmacological characterization, our central focus over the past several years.

Our discovery that marine algae of the division Rhodophyta are a rich source of eicosanoid-type natural products was initiated through work with the intertidal Oregon alga *Ptilota filicina*. Originally studied for its moderately antibiotic crude lipid extract, this alga yielded a series of eicosapentaenoic acid derivatives of novel structure [eicosa-(5(Z),7(E),9(E),14(Z),17(Z)-pentaenoic acid and 11-hydroxy-16-keto-eicosa-5(Z),8(Z),12(E),14(E),17(E)-pentaenoic acid = ptilodene]. Through this work, we became aware that many red algal crude lipid extracts contained tlc and nmr characteristics comparable to the novel *Ptilota* compounds and, hence, deserved examination in greater detail with this chemical theme in mind.

This led to our efforts with the tropical red alga *Murrayella periclados*, which has subsequently turned out to be one of the most prolific producers of interesting eicosanoid natural products, including 12(S)-HETE, hepoxilin B_3 and B_4, and 6(E)-leukotriene B_4. Additional studies with other tropical and temperate water red algae have yielded many of these same compounds which were initially discovered in *Murrayella*. They point out an important generalization in marine plant eicosanoid metabolism: most marine plants utilize a 12-lipoxygenase manifold in the metabolism of arachidonic acid and its congeners.

One seaweed-derived compound that most likely emanates from the 12-lipoxygenase pathway, 12(R),13(S)-diHETE, has occupied a great deal of interest and attention in our laboratory. It was initially discovered in the Oregon red alga *Farlowia mollis*, and subsequently described from *Gracilariopsis lemaneiformis*, also from the Pacific Northwest. At the time of its initial isolation, we also characterized several pharmacological properties of the molecule, including its ability to inhibit mammalian Na^+/K^+ ATPase, 5-lipoxygenase, and generation of O_2^- by fMLP-stimulated human neutrophils. Continued study of the natural products of *G. lemaneiformis* led to the isolation and structural description of several related 12(R),13(S)-diHETE-type natural products, including 18-keto-12(R),13(S)-diHEPE and several monogalactosyldiglycerides and digalactosyldiglycerides in which some of the acyl substituents are this same 12(R),13(S)-diHETE unit. Recently, we have demonstrated that acetone powder preparations of *G. lemaneiformis* are extremely efficient in their metabolism of arachidonic acid to 12(R),13(S)-diHETE as well as a number of other 12-lipoxygenase metabolites. Use of this cell-free system will enhance our ability to do biosynthetic experiments aimed at understanding the mechanistic chemistry involved in the production of this novel arachidonic acid metabolite.

Another recent project with an Oregon coastal seaweed has yielded an exciting class of novel carbocyclized eicosanoids. The mushroom-shaped alga *Constantinea simplex* seasonally produces a number of cyclopropyl- and lactone-containing eicosanoids of extremely interesting structure. A biogenesis deriving from initial metabolism by a 12-lipoxygenase seems likely for these *C. simplex* metabolites, and cell-free studies with this alga are underway as well.

Because mammalian eicosanoids play such a fundamental role in regulating normal physiological events in mammals, the study of unique marine eicosanoids promises to yield many new insights into the chemistry and potential pharmacological properties of this structure class.

ARACHIDONIC ACID METABOLISM IN CALCIFYING RED ALGAE: A MODEL FOR STUDYING THE ROLE OF EICOSANOIDS IN THE BIOMINERALIZATION PROCESS IN MAMMALS

Jay Burgess, Department of Biology, University of California, Santa Barbara

Arachidonic acid (AA), when released from the cell membrane, gives rise to various biologically active oxidation products, collectively termed eicosanoids. Three major enzymatic pathways have been defined in mammals: cytochrome P450 catalyzes the formation of (R)-HETEs, epoxy-FAs, and 19,20-hydroxy eicosanoids; lipoxygenase catalyzes the formation of HPETEs, HETEs, leukotrienes, lipoxins, and hepoxilins; and cyclo-oxygenase catalyzes the formation of prostaglandins, thromboxane, and prostacyclin. Although many unique eicosanoid-like compounds have been identified in marine systems, very little is known about the enzymes involved. The purpose of this research is to develop a model system for studying eicosanoid biosynthesis activity in red algae. These results may provide a new insight into pharmacological approaches to drug therapy in humans.

The presence of an eicosanoid biosynthetic pathway was analyzed in red algae by measuring samples for phospholipase A_2 (PLA_2) and lipoxygenase (LO) activities. PLA_2 activity was measured using a fluorescent substrate (NBD-caproyl-PC), and the amount of activity was quantified by measuring the fluorescence of the free caproic acid product. LO-activity was measured after incubation of AA with the algae homogenates both polarographically and by scanning the UV spectrum (200-400 nm) of the organic extracts as a measure of conjugated olefin formation. A survey of lipoxygenase and PLA_2 activities in homogenates of selected species of Rhodophyta clearly shows that both enzymes are present in most species studied.

Structural determination of this product was undertaken using a local (Santa Barbara) calcified red algae species, *Bossiella orbigniana*. The enzymatic activity responsible for the formation of the conjugated tetraene formed from AA by this species was associated with a non-uniform cell membrane protein. Purification of the product was accomplished by HPLC (Supelco 449:449:1:1). Proton, ^{13}C, and 2D NMR analysis indicated that the major product was 5,8,10,12,14-eicosapentaenoic acid, and it was named "bosseopentaene" (BP)[1].

The enzymatic activity was pH and substrate dependent. The pH optimum was 7. The double reciprocal plot of substrate saturation curve gave K_m = 125 μM and V_{max} = 112.4 nmoles.60min^{-1} mL^{-1}. Furthermore, the enzyme could utilize

5,8,11,14,17-eicosapentanoic acid (EPA) as a substrate as well as AA, but the methyl ester of AA was not an efficient substrate. The associated formation of BP from AA coralline red algae suggests that this unique fatty acid may have a role to play in biomineralization. This possibility is being investigated.

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IRON AND BACTERIAL GROWTH: SOME OBSERVATIONS ON THE ROLES OF IRON IN METABOLISM, PROTECTION FROM OXIDATIVE STRESS, AND GENE REGULATION

James A. Fee, Los Alamos National Laboratory

It has been known for some time that iron is essential to the major metabolic processes of primary production: photosynthesis, respiration, and nitrogen fixation. Recent results suggest that iron may play an even more profound role in biology, and some speculations relevant to iron and marine organisms are given at the end of this abstract.

Fe^{3+} is extremely insoluble in water, having $K_{sol} \sim 10^{-38}$ [1], whereas Fe^{2+} is much more soluble but is sensitive to oxidation by O_2 to Fe^{3+}. A variety of microorganisms excrete iron-chelating materials (siderphores) which, once having bound Fe^{3+}, are specifically recognized by surface proteins and transported back into the cell where iron is apparently released as Fe^{2+}. By this mechanism, some organisms are able to grow at extremely low iron availability.

There may be a fundamental connection between free iron and oxygen through the formal process:

$$Fe^{2+} + O_2 = Fe^{3+} + O_2^-$$

This reaction has been studied over a wide range of iron coordination environments and is known to proceed in either direction depending on redox potential. In this regard, we have found [2] that expression of the superoxide dismutase genes of *Escherichia coli* is substantially regulated by the ferric uptake regulation (*fur*) locus. Here, Fur, an iron/DNA binding regulatory protein, appears to act as a classical iron-dependent repressor of the *sodA* gene (MnSOD), and as an iron-dependent activator of *sodB* (FeSOD).

Evidence from other laboratories [3,4] strongly suggests that the regulatory process by which *E. coli* switches from aerobic respiration to anaerobic respiration is also dependent on iron. Since the Fnr (fumarate nitrate reduction) protein plays a central role in this process, there is some suggestion that Fnr is an iron/DNA binding protein which senses Fe^{2+} concentrations much as does the Fur protein. It is possible that a process analogous to the reaction given above, may be responsible for affecting Fe^{2+} concentration, which is sensed by the cell.

Together these observations portend a major role for iron in the global regulation of prokaryotic gene expression and are the basis for some of the following speculations and commentary related to iron in the marine environment:

- The hypothesis that low iron concentration may limit primary production in certain parts of the oceans (cf. Refs. 5 and 6 and references therein) is an extremely important one. We extend this somewhat here and suggest that a gradation of effects will occur as iron availability proceeds from an absolute limitation to complete sufficiency. In this fashion, one might, for example, expect iron availability to affect speciation. Those organisms best able to adapt to iron deficiency will dominate under that condition; as iron availability increases, organisms less well-suited to iron deficiency may persist, and so on. Examples of this are available among terrestrial organisms [7].
- The well-studied siderophore-based mechanisms for extracting iron from the terrestrial environment may not be appropriate for free-living marine microorganisms. It is simply counter-intuitive that siderophores could be efficiently recovered in the extremely dilute environment of the open ocean. One circumvention of this entropic effect is the use of siderophores attached to the surface of the cell [8].
- Organisms able to grow at a particular iron availability will adapt their metabolism to that level of iron. This is of particular importance to marine pharmacologists working with cultured organisms because the secondary metabolic processes, those responsible for synthesis of physiologically active substances, may be strongly affected by iron availability [7].
- Finally, any serious effort to "seed" the oceans with iron [9] should be preceded by small scale studies coupled with investigations into the fundamental biochemistry of iron in marine organisms.

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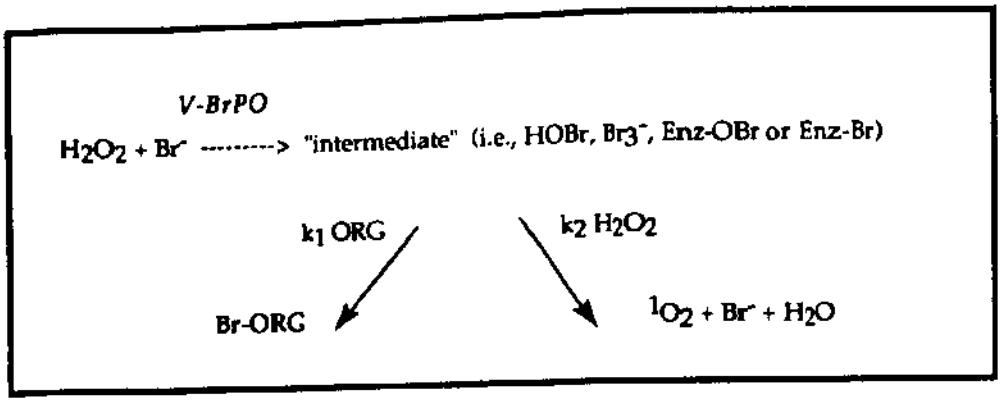
VANADIUM BROMOPEROXIDASES FROM MARINE ALGAE: BROMINATION AND OXIDASE REACTIVITY

Alison Butler, Department of Chemistry, University of California, Santa Barbara

The marine environment is unique and extreme in its metal ion composition. After molybdenum, vanadium is the second most abundant transition-metal ion in seawater, and both are far more concentrated than iron. Thus, it is not surprising that the first vanadium enzyme, vanadium bromoperoxidase (V-BrPO), was discovered in the marine environment [1] and has been found to be abundant in Rhodophyta (red algae) and Phaeophyta (brown algae). Halogenated compounds and certain selectively oxidized compounds are also abundant in marine organisms; many have important biological activities, including the chiral halogenated sesquiterpenes (antineoplastic agents) and bi-indoles (antifungal agents; specialty dyes). We are interested in the general mechanism of V-BrPO and the enzyme-catalyzed oxidation of indoles forming bi-indoles.

Vanadium bromoperoxidase catalyzes the oxidation of bromide by hydrogen peroxide, which results in the bromination of a suitable substrate. In the absence of an appropriate organic substrate, V-BrPO catalyzes the production of dioxygen, as summarized in Scheme 1. We have shown that: a) $k_2[H_2O_2]$ is competitive with $k_1[Org]$ when the organic compound, Org, is monochlorodimedone (2-chloro-5,5-dimethyl-1,3-dimedone, MCD; i.e., the usual substrate used to characterize bromoperoxidase activity) [2]; b) both dioxygen formation and MCD bromination occur via the formation of a common intermediate, [2,3]; c) singlet oxygen is produced quantitatively [3]; and, d) bromamines are readily produced and detected when peracetic acid is used as the peroxide source [4]. The striking feature of V-BrPO is its exceptional stability; V-BrPO is not inactivated by singlet oxygen or oxidized bromine intermediates, contrary to the Fe-Heme haloperoxidases, lactoperoxidase, and chloroperoxidase [3]. The steady state kinetics of dioxygen formation is consistent with a substrate-inhibited bi bi ping-pong mechanism in which bromide [5] and hydrogen peroxide [Soedjak and Butler, unpublished results] inhibit at certain pH values. MCD bromination also fits a bromide-inhibited bi bi ping pong mechanism [5,6] with nearly identical parameters as determined from the bromide-assisted catalase reaction.



Scheme 1

The identity of the intermediate in Scheme 1 has not been established unambiguously. Reaction of hydrogen peroxide with any of the proposed intermediates is consistent with singlet oxygen production, since it is a well-established product of the reduction of HOBr/Br_2/Br_3^-, or bromamines by hydrogen peroxide [3,7].

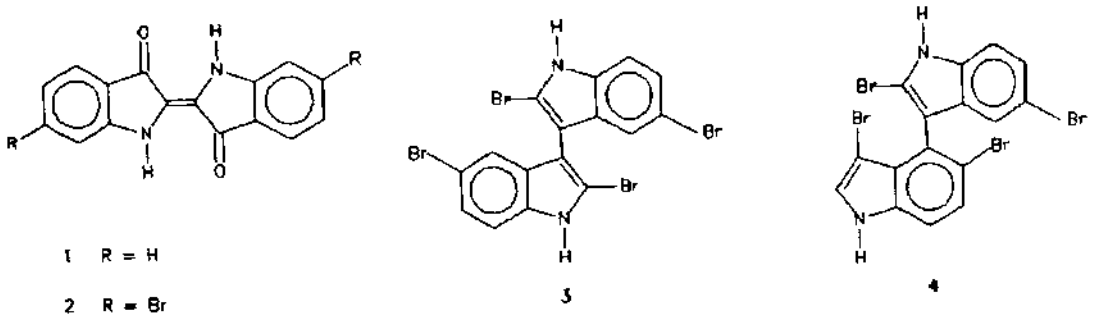
The quantitative conversion of hydrogen peroxide to singlet oxygen by V-BrPO and the unusual stability of V-BrPO in the presence of high concentrations of singlet oxygen or oxidized bromide species raises questions about the primary biological function of B-BrPO. In particular, does V-BrPO produce singlet oxygen to carry out specific oxidation reactions? V-BrPO can carry out oxidation reactions relevant to the biosynthesis of the halogenated and nonhalogenated bi-indole natural products (see compounds 1-4 (Fig. 1); 1 is indigo, 2 is the ancient dye, Tyrian purple). Such oxidations could arise from initial bromination followed by bromide displacement, resulting in an oxidation reaction. In this case the *primary* function of V-BrPO would be considered a bromoperoxidase. On the other hand, V-BrPO could also effect direct oxidation by singlet oxygen, in which case the *primary* function might be considered an oxidase.

V-BrPO catalyzes the oxidative coupling of indole to indigo in the presence of hydrogen peroxide and bromide, in a reaction that requires the presence of bromide, but does not produce brominated indigo (Fig. 2). V-BrPO also catalyzes the bromination of 1-methylindole, 2-methylindole, and 1,2-dimethylindole, forming the 3-bromo-derivatives; and the oxidation of 3-methylindole, forming 3-methyl-2-oxindole. Only one equivalent of hydrogen peroxide is required for complete

reaction of indole and the indole derivatives. This stoichiometry indicates that the oxidized-bromine species is the reactive oxidizing or brominating species as opposed to oxidation or oxidative coupling by singlet oxygen. A singlet oxygen-mediated oxidation would require consumption of two equivalents of hydrogen peroxide per indole oxidized. The oxidative coupling of indole to indigo is unique to V-BrPO. The FeHeme haloperoxidases, chloroperoxidase, and lactoperoxidase produce 2-oxindole, exclusively (and not indigo) which must reflect the oxidative reactivity of the Compound I, [FeO^{2+}-$Heme^{+}$], state. The mechanism of the brominated indigo compounds (e.g., Tyrian purple, 2, above) is under investigation.

Many of the halogenated marine natural products have important industrial applications as drugs in the pharmaceutical industry and as specialty chemicals. Their industrial development has been hampered by the lack of sufficient quantities to meet needs in basic and applied research. This problem can be alleviated by developing synthetic methods based on the endogenous enzyme-catalyzed processes of the marine organism. As established, V-BrPO is a robust enzyme that is not inactivated by strong oxidants, even in high concentrations. V-BrPO also catalyzes the novel oxidative coupling of idole to indigo, a process important to the specialty chemical dye industry, and to the pharmaceutical industry for antifungal applications.

Figure 1.



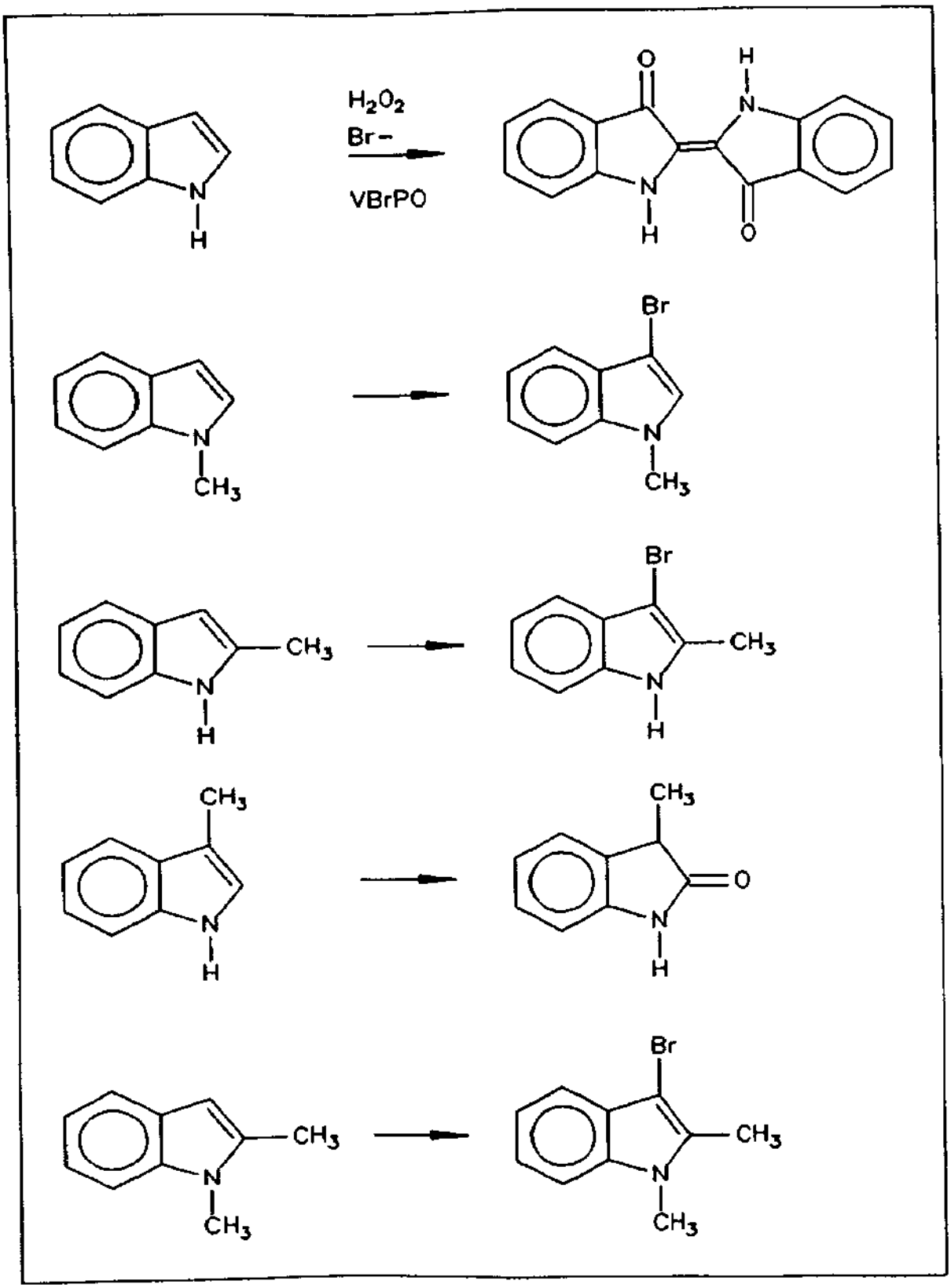

H2O2
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Figure 2.

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THE PARADOX OF PHOSPHOLIPASE A_2

Keith B. Glaser, Division of Immunopharmacology, Wyeth-Ayerst Research

The paradox of phospholipase A_2 is in reference not to the enzyme itself but to the development of inhibitors of this enzyme. Inhibition of phospholipase A_2 (PLA_2) as a potential therapeutic target for the treatment of inflammatory disorders arose from studies on the mechanism of the classical nonsteroidal anti-inflammatory agents and anti-inflammatory steroids. These types of anti-inflammatory drugs prevent the generation of proinflammatory eicosanoids, common mediators derived from arachidonic acid, which is found predominantly in the sn-2 position of membrane phospholipids. Therefore, the most direct mechanism that would release arachidonic acid is the hydrolysis of membrane phospholipids by a phospholipase A_2.

There are, however, other mechanisms which would result in the release of arachidonic acid. Activation of a phospholipase A_1 would generate cytolytic lysophospholipids, which are rapidly hydrolyzed by lysophospholipases, releasing arachidonic acid. A phospholipase C acting upon phosphatidylinositol would release a diacylglycerol, which would be acted upon by nonspecific lipase to release arachidonic acid. It is evident that in the intact cell there are multiple mechanisms that could release arachidonic acid from membrane phospholipids. To date, however, the experimental evidence implicates the phospholipase A_2 mechanism as the source of the bulk of arachidonic acid made available for eicosanoid biosynthesis.

The use of the $P388D_1$ macrophage-like cell line as a model to evaluate the role of phospholipase A_2 in arachidonic acid release was made possible by the use of a marine natural product that is a direct inhibitor of phospholipase A_2. Manoalide and a synthetic analog, manoalogue, are potent irreversible inhibitors of extracellular-type phospholipase A_2 [1,2]. However, inhibition of the $P388D_1$ phospholipase A_2 by these compounds appears to be reversible [3]. Inhibition of PGE_2 production and [^{3}H] arachidonic acid release by manoalide and manoalogue occurs in parallel, suggesting that inhibition of phospholipase A_2 is their mechanism of action in the intact cell [3]. These results implicate phospholipase A_2 as the enzyme that regulates the availability of arachidonic acid for eicosanoid biosynthesis in the $P388D_1$ cell line. However, despite the amount of information

available on the enzyme phospholipase A_2, the mechanisms which activate or regulate phospholipase A_2 activity in the intact cell are poorly understood.

To investigate the regulation of phospholipase A_2 in the intact $P388D_1$ cell, it was necessary to use a receptor-mediated activation that results in the generation of eicosanoids. $P388D_1$ cells are stimulated by platelet-activating factor (PAF) to release arachidonic acid metabolites through a receptor-mediated event [4]. While the release of PGE_2 in response to PAF is only two to three times the constitutive PGE_2 production, bacterial lipopolysaccharides (LPS) are able to prime $P388D_1$ cells for enhanced arachidonic acid metabolism, increasing PAF-stimulated PGE_2 production to 9-12 times the constitutive PGE_2 production [4]. Primed PAF-stimulation of PGE_2 production was dose-dependent with an ED_{50} for PGE_2 production in a similar concentration range as the K_d for PAF binding in $P388D_1$ cells and was blocked by the specific PAF antagonist L-659,989 [5]. The extent and rate of [^{3}H] arachidonic acid release from prelabeled $P388D_1$ cells are also increased in primed cells relative to unprimed cells in response to PAF-stimulation [6]. The increased PGE_2 production and [^{3}H] arachidonic acid release are not due to increased cyclo-oxygenase or phospholipase A_2 activity in the primed $P388D_1$ cells. LPS from either *Salmonella* Re595 or *Escherichia coli* 0111:B4 prime $P388D_1$ cells in a concentration-dependent manner but have themselves no ability to stimulate arachidonic acid metabolism. The priming of $P388D_1$ cells by LPS is time-dependent and results in a hyporesponsive state upon prolonged LPS exposure. LPS priming is sensitive to inhibition by actinomycin D, while primed PAF-stimulation of PGE_2 production is blocked by cyclohexamide, which implicates a protein that is rapidly turning over. Primed PAF-stimulation of PGE_2 is also inhibited by manoalogue, with a similar IC_{50} value to calcium ionophore A23187 stimulated PGE_2 production. Primed PAF-stimulated PGE_2 production is inhibited by the tyrosine-specific protein kinase inhibitor genistein, but not by the kinase inhibitor H-7.

The results suggest that LPS priming of macrophages amplifies the signal-transduction pathways for PAF-mediated arachidonic acid release, which results in increased arachidonic acid availability for eicosanoid biosynthesis. LPS priming is regulated primarily through transcriptional mechanisms, and the primed PAF-stimulated PGE_2 production is regulated by translational mechanisms as well as a phosphorylation mechanism, possibly a tyrosine-specific phosphorylation. The relationship between these events and arachidonic acid release and the cellular phospholipase A_2 are currently under investigation at a molecular level in the $P388D_1$ macrophage-like cell.

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USE OF MANOALIDE AND ITS ANALOGS IN UNDERSTANDING CALCIUM SIGNALING PATHWAYS

Larry A. Wheeler, Allergan Pharmaceuticals, Inc.

Manoalide was originally discovered to be a potent phospholipase A_2 (PLA_2) inhibitor [1,2]. Subsequent studies have shown that manoalide can also block phospholipase C (PLC) in vitro [3,4] but not inhibit the production of inositol triphosphate (IP_3) in intact whole cell assays [5]. The release of Ca^{2+} from intracellular stores is mediated by the activity of PLC, the release of IP_3, and subsequent binding to IP_3 receptors on the rough endoplasmic reticulum. Manoalide blocks the mobilization of Ca^{2+} after TRH in GH_3 cells but does not block IP_3 release [5]. These studies suggest that manoalide has a unique action on cytosolic Ca^{2+} ($[Ca^{2+}]_i$) regulation. Studies on whether manoalide blocked IP_3 induced Ca^{2+} release are in progress. In addition, manoalide can block Ca^{2+} entry through the plasma membrane channels [5]. Mechanism(s) of entry across the plasma membrane of non-excitable cells are not well understood. One current hypothesis for receptor activated Ca^{2+} entry is the activation of PLA_2 and release of arachidonic acid (AA) that then opens a Ca^{2+} entry pathway [6,7]. We have explored the AA hypothesis in the human ciliary NPE cell line ODM-2, using the muscarinic agonist carbachol. Cloning techniques have shown the existence of five subtypes of muscarinic receptors, M1 through M5. M2 and M4 are negatively coupled to adenylate cyclase, whereas M1, 3, and 5 regulate intracellular Ca^{2+} [8]. Pharmacological dissection of the muscarinic receptors depends on the use of selective antagonists: pirenzepine, an M1 antagonist; AF-DX 116, an M2 antagonist; and 4-DAMP, an M3 antagonist. In the case of the ciliary body, ODM2 cells respond to carbachol by a release of intracellular Ca^{2+} and by Ca^{2+} entry across the plasma membrane, giving the characteristic Ca^{2+} transient followed by an elevated $[Ca^{2+}]_i$ (Fig. 1). The Ca^{2+} signal was blocked selectively by 4-DAMP, showing that this was an M3 receptor, characteristic of muscarinic receptors in other secretory epithelia. A dose response curve to 4-DAMP, however, showed that the initial Ca^{2+} transient was blocked by 4-DAMP with little effect on the steady state Ca^{2+} signal (Fig. 2). AA at 1, 5, or 10 μM resulted in Ca^{2+} entry, but no Ca^{2+} transient (Fig. 3). Indomethacin and NDGA at 10 μM did not inhibit the arachidonic acid response, showing that conversion to eicosanoids was not involved. On the other hand, a selective phospholipase A2 inhibitor, AGN190576, blocked the steady state Ca^{2+} signal, with

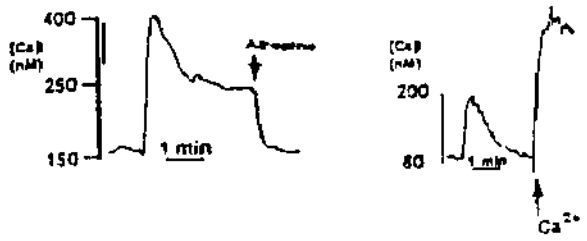

Figure 1. Carbachol produces biphasic response in $[CA^{2+}]$ in.

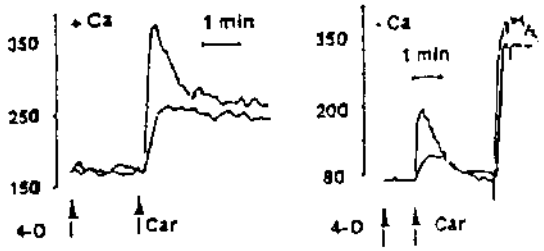

Figure 2. Peak calcium response.

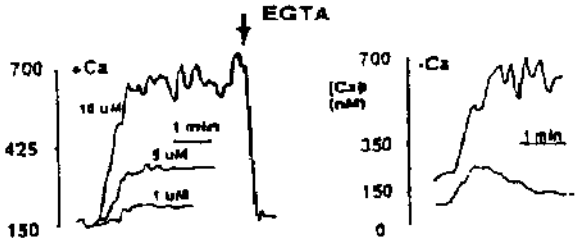

Figure 3. Arachidonic acid increased intracellular calcium.

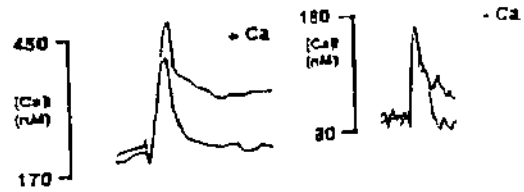

Figure 4. Effect of AGN 190576 on carbachol response.

little effect on the Ca^{2+} spike (Fig. 4). It is suggested that the M3 receptor of the NPE cell is coupled to phospholipase C for the Ca^{2+} transient, and probably to phospholipase A2 for Ca^{2+} entry. In this cell, the receptor-operated Ca^{2+} channel (ROC) can be activated by arachidonic acid.

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PROGRESS IN INFLAMMATION RESEARCH

Phil Davies, Inflammation Research, Merck Sharp & Dohme Research Laboratories

The last decade has seen broad-based advances in our understanding of the fundamental processes underlying host defense mechanisms and their disorders in immune-based inflammatory diseases. Significant discoveries have been made regarding the structure of molecules that determine the nature and the specificity of the afferent limb of the immune response, notably at the level of the MHC complexes of antigen-presenting cells and the antigen receptor of the T lymphocyte.

Significant discoveries have also been made regarding the mechanisms by which cells of the host defense system recognize one another and the cells of various tissues and organs. Superfamilies of molecules serving complementary functions as receptors and ligands on leukocytes (e.g., CD 18/CD 11a,b,c) and endothelial cells (ICAM 1 and ICAM 2), respectively, have been identified. Deficiencies in some of these molecules, such as CD18, result in serious defects in host defense mechanisms, while inhibition of receptor function can result in effective anti-inflammatory activity in a wide range of animal models of acute inflammation. These molecules effectively complement the activity of chemotactic stimuli generated at sites of inflammation which serve both to provide directional movement of phagocytes and to up-regulate appropriate adhesion molecules.

Our knowledge of the complexity and the apparent redundancy of the mediators of the effector arm of the host defense system has expanded greatly with the identification of a large number of new molecules, both low and high molecular weight, which contribute to acute and chronic inflammation. The establishment of their structure and the identification of specific receptors have allowed intensive synthetic chemical efforts to discover specific and potent antagonists. The lipid mediator field has been particularly fruitful in this respect with the discovery of PAF and leukotriene antagonists which have reached early clinical development and promise to provide definitive information on the significance of these mediators in a wide range of inflammatory and allergic diseases.

A large number of peptide mediators of inflammation have been characterized. The cytokine family has emerged as a highly integrated set of molecules with many complementary functions. Although specific receptors for these molecules have been identified, the development of specific antagonists has

not been successfully achieved, but alternate approaches to their pharmacological modulation using specific antibodies has been successful in some instances.

Tissue degradation is a prominent feature of inflammatory diseases. Proteolytic enzymes mediating this destruction are derived from both leukocytes and connective tissue cells. The matrix metalloproteinases have emerged as a family of enzymes with a wide variety of connective tissue substrates and the potential to mediate much of the cartilage destruction seen in rheumatoid- and osteoarthritis. A family of serine proteinases has been characterized in polymorphonuclear leukocytes, and one of these, elastase, has been a target for the development of specific inhibitors. This has been accomplished with a class of synthetic cephalosporin-based inhibitors, which are highly selective for elastase and are active against the enzyme in a number of in vivo models of tissue destruction.

The availability of a large number of well defined, specific targets in the host defense system as potential points of intervention in inflammatory diseases holds much promise for discovery of novel anti-inflammatory agents. The evaluation of new structural entities from marine sources should receive intensive attention in this context.

COMMENTARY ON MARINE BIOTECHNOLOGY

David H. Attaway, National Sea Grant College Program

I am grateful to have participated in an extremely stimulating short-course in marine chemistry and pharmacology by experts of the highest calibre. I am particularly gratified to see the strong participation of industry in this conference and the many examples of interdisciplinary and interinstitutional collaboration, including very productive collaboration between academe and industry. I know that making these interactions work even reasonably well is not easy because of institutional impediments on both sides which are beyond the control of the principals.

I will make brief remarks about Sea Grant and mention some general ideas for future research in support of marine biotechnology. Most of these ideas have been reinforced by what I have heard here. Still, there are troubling issues in regard to how we should proceed in the future. It is important that they be resolved, or at least recognized, so that we can be prepared to seize the opportunities for advancing science, training students, and promoting the development of new technologies in a very competitive world.

In 1966, the United States government established the National Sea Grant College Program. The program's purpose is to conduct academic research, education, and advisory services that will contribute to the development and wise use of marine resources. This program, which is a component of the National Oceanic and Atmospheric Administration, has developed a network of 29 state or regional programs. Hundreds of researchers, educators, and advisory specialists in most of the major universities in the U.S. coastal and Great Lakes' states participate in this program, which allies itself closely with interests of the marine industry and of governmental agencies that manage natural resources. Sea Grant funds a spectrum of fundamental and applied research in the physical, biological, social, economic, and engineering sciences. The program annually directs over $2 million of its approximately $40 million in federal funds to research in support of marine biotechnology. An additional $1.5 million in matching funds help support research in this broadly defined program in marine biotechnology.

Marine biotechnology can be defined as the use of marine organisms or their components to provide goods and services—goods such as polymers and other chemicals for use in medicine, agriculture, and the chemical industry; services such as biological detoxification of wastes, control of biofouling and associated corrosion, and

sensitive measurement with biosensors. This broad definition encompasses more than products and processes resulting from DNA technology.

Although the production of certain marine products, such as agar and other polysaccharides from marine algae, accounts for significant commercial trade, marine biotechnology and the fundamental research to support its development have had relatively little attention in the academic and commercial sectors. However, results of research so far indicate that marine biotechnology has the potential to be increasingly important to science and technology and, just as importantly, to education. We have heard quite remarkable examples of this at this conference. I will mention other examples along different lines of research that also show this potential.

Plant pathologist L.A. Hadwiger and his students at Washington State University have shown that the polymer chitosan, derived from crab shells, is effective in protecting certain agricultural crops against pathogenic fungi and other diseases. More importantly, their studies show that inexpensive treatment of wheat seeds with chitosan increases yields by about 10 percent. The research indicates that the treatment, which costs less than two cents per harvested bushel, also increases the protein content of wheat seeds by up to one percent. This is an important finding for highly managed agriculture because high protein content raises the value of wheat by about 3.5 cents per bushel for each 0.1 percent increase in protein.

Part of chitosan's effect is thought to result from its ability to selectively activate genes whose function is an essential part of the plant's ability to resist disease. If this is true, that is, if genes of higher organisms can be controlled with foreign substances, then development of drugs and agrichemicals of a type different from those presently marketed might be possible. Defining chitosan's mechanisms of action is the focus of Professor Hadwiger's current research.

I should point out that Sea Grant's pilot-scale production of chitosan in the early 1970s, its distribution of samples to researchers around the world, and its support of research on this unique material now form a primary basis for a rapidly developing industry. One trade journal recently predicted annual sales of chitosan products within five years of $2 billion in the food, agricultural, cosmetics, and health-care industries.

Genetic engineering and other types of molecular biology have the potential to enhance the use of living marine resources. Fast-growing, disease-resistant aquaculture strains would be one important application of this technology. There are others, such as the diagnosis and control of fish diseases. For example, Joanne Leong and her students at Oregon State University developed vaccines for viral diseases that

can devastate production in aquacultural facilities—infectious pancreatic necrosis and infectious hematopoietic necrosis. They cloned and expressed in *Escherichia coli* portions of genes encoding for the surface proteins of the viruses. Crude extracts of the bacteria expressing these proteins have proven to be effective vaccines against lethal doses of the viruses. Thus, recombinant DNA technology can be used to meet industrial requirements for safe and effective, yet inexpensive, vaccines for fish.

Thomas Chen of the University of Maryland and Dennis Powers, previously with The Johns Hopkins University and now with Stanford University, applied DNA technology to production of transgenic fish, with growth up to 40 percent faster than their parents. They and their associates showed that the rainbow trout has two genes for growth hormone (GH). They also showed that biosynthetic preparations of these hormones in *E. coli* enhance growth of yearling trout through weekly intramuscular injection. Using carp and loach as test animals, these researchers have shown that introduction of additional copies of the GH gene results in transgenic fish that produce elevated levels of growth hormone and grow faster than controls. A small proportion of the first generation of fast-growing fish can pass this trait to their offspring. Thus, it appears that true-breeding fish strains of altered character can be produced.

Along quite a different line of research, A. P. Wheeler and C. S. Sikes of Clemson University and the University of South Alabama, working collaboratively, made the observation that proteinaceous components of the matrix in oyster shell inhibit rather than promote precipitation of calcium carbonate. They showed that certain synthetic substances modeled after the matrix and subunits of it, a variety of polyaminoacids, also are potent inhibitors of calcium carbonate precipitation, and thus of mineral scaling and biomineral fouling of marine surfaces. These findings have been reported in the scientific and patent literature and applied to technological developments. They are leading to new commercial products and new methods for preventing or controlling inorganic scaling in industrial processing. Their research is also leading to a fundamental understanding of calcification in marine molluscs.

These advancements in science and technology and those reported at this conference are just some examples of results of only limited investment in research. They suggest that marine biotechnological research of broader scope and larger scale could play an important role in the development of products and technology for the future. What are some of the opportunities?

For most marine products, natural function in marine systems, mechanism of action, and behavior in a wide array of biological and pharmacological assays are

unknown. These gaps in knowledge represent opportunities for research. By and large, research on marine natural products has focused on lipid extracts of macroalgae and invertebrates. Few scientists have tuned their attention to aqueous extracts, enzymes, other bioactive macromolecules, or to marine bacteria, fungi, and microalgae as subjects of study. These neglected topics and classes of organism also represent areas of opportunity for research as Bill Fenical has discussed here earlier.

Few marine invertebrates or macroalgae have been subjected to cell or tissue culture. Successful research to this end with organisms that produce useful metabolites could have several benefits. For example, it could help set the stage for using cells in bioreactors to economically produce pharmaceuticals, enzymes, growth regulators, pigments, and other chemicals. It could provide techniques useful in studying the basic physiology and nutrition of organisms and for determining natural regulation of secondary metabolism. It could enhance the study of biochemical relationships between invertebrates and the symbiotic organisms associated with many of them.

Biosensors promise to meet some important measurement needs for drugs, metabolites, and other biomolecules. Biosensors use an immobilized biological material, even a living material, in contact with a transducer to convert biochemical signals into quantifiable electrical signals. For example, the antennules of the blue crab have been used as a source of chemoreceptive nerve fibers for use in a biosensor. Other chemoreceptors, which are biomolecular assemblies involved in numerous physiologic functions, such as olfaction, are candidates for molecular recognition and application in biosensors.

Here are examples of several broad research activities that I believe offer special opportunities for advancing basic science and laying a foundation for new products and processes

- Determining the natural function of bioactive compounds;
- Defining the properties of secondary metabolites in a broad spectrum of biological assays and determining their mechanisms of action;
- Identifying secondary metabolites and defining the biochemistry of marine microorganisms and other little known marine organisms;
- Elucidating biochemical pathways and identifying associated enzymes;
- Studying the bioactivity of aqueous extracts and macromolecules;

- Exploring the biochemical and physical properties of enzymes and biopolymers;
- Defining nutritional and environmental controls on secondary metabolism in marine organisms;
- Developing the technology for cell culture of invertebrates and microorganisms and defining associated physiology and nutrition;
- Defining biochemical engineering parameters for bioreaction, photobioreaction, fermentation, and immobilized biocatalyst reaction with marine species;
- Exploiting microalgae for energy-rich substances, such as molecular hydrogen;
- Studying organelle functioning and associated biochemistry for application in biosensors;
- Developing techniques for genetic engineering of marine plants, animals, and microorganisms; and
- Cloning and expressing gene clusters encoding for enzymes responsible for biosynthesis of secondary metabolites.

These topics and others offer opportunities for studying basic issues in biological systems. Their study would benefit from interdisciplinary approaches to the fundamental science on which new technologies can be based. Certainly this group does not need convincing on this point. Some of you may recall the conclusion of Weinberg in his landmark paper of 1963 on criteria for scientific choice, "That field has the most scientific merit which contributes most heavily to and illuminates most brightly its neighboring scientific disciplines."

The interdisciplinary nature and requirements of research in support of marine biotechnology make this field particularly fertile ground for illuminating scientific phenomena and advancing socially responsible technology. The Japanese Ministry of International Trade and Industry (MITI), working with major industrial firms and academic collaborators, has recognized its importance by establishing the Marine Biotechnology Institute. It is founded on the premise that "marine biotechnology is the greatest remaining technological and industrial frontier." MITI expects marine biotechnology to provide technology for the 21st century.

Under MITI's management the National Research and Development Program, known more popularly as the Large-Scale Project, was started in 1966 to foster large-scale and high-risk research and development aimed at technology development in areas of exceptional promise to Japan. Its primary objective has been to unite national

laboratories, academic organizations, and private firms in order to raise the standard of technological know-how. One of the ten projects currently in the Large-Scale Project, and entitled "Fine Chemicals from Marine Organisms," is the basis for the new Marine Biotechnology Institute Company (MBI).

MBI will carry out its activities under the auspices of MITI's Research Center for the Industrial Utilization of Marine Organisms at two locations in Japan that are sites for two new experimental centers of marine biotechnology. One is located in Kamaishi City in the north of Honshu to take advantage of organisms found in the cold current on Japan's east coast. The other is located in Shimizu, Shizuoka Prefecture, where the currents are warm. The Kamaishi institute will focus more on biological studies, and the Shimizu institute on chemical studies. Construction of each facility is projected to cost about $25 million. A further approximate $190 million will be invested in operations over the next 10 years. Each facility of the MBI will have approximately 30 full-time professional scientists supported by a staff of about 20.

MBI also will operate a research vessel, the *Sohgen-Maru*, equipped with wet laboratories, sophisticated deep-sea exploration capability, and peripheral data systems, including a satellite data link. In all, ten multifunctional laboratories will be available, along with an examination room and sample preservation and freezer units. The vessel also has a total of 17 winches to conduct underwater-exploration activities to a depth of 11,000 m with exploration probes. A personnel complement of 87 is envisioned: a crew of 55 and a scientific staff of 32.

The Japanese Ministry of Agriculture, Forestry and Fisheries (MAFF) also recently completed plans to build a new facility for marine biotechnology at Kamaishi. It will cost the same amount as the MBI facility and also will have a professional scientific staff of 30. MAFF will expend six billion yen to build a new oceanic research ship, the *Kaiyomaru*, equipped with advanced facilities.

MAFF's five-year plan for research in marine biotechnology, which had a budget of 1.5 billion yen in fiscal year 1989, is focusing on methods to use marine organisms in Japanese waters, and development of technology to use normally wasted parts of edible species—internal organs, bones, etc. Its scientists and administrators expect these materials to provide a wide variety of products, both fine and industrial chemicals. The work, which will be conducted in collaboration with academic scientists and industry, will encompass the following:

- Screening of substances for bioactivity, including pheromonal activity, and useful color, odor, and taste properties;
- Developing effective and efficient separation and extraction technologies; and
- Evaluating applications of potentially useful substances in the food and aquacultural industries.

Now back to efforts in the United States. While the value of interdisciplinary efforts in marine chemistry and pharmacology is now well appreciated, and various kinds of collaboration between industrial and academic researchers have evolved, there still is not an ideal model for broad collaboration in the future. This may become a major issue if marine resources are to play a significant function in development of new processes and materials in the United States. I would like to leave you with several other issues that may merit further discussion.

- How do we maintain and communicate the intellectual content of this field to broad audiences?
- How should we address the lack of a universal model for effective collaboration between academe and industry?
- What is the appropriate role of academic science in drug development?
- How is a lead compound recognized? How can we most efficiently transfer promising compounds to industry?
- How can we ensure the feedback of industrial information to academic scientists in regard to compounds meriting detailed study?
- With regard to the resupply of bioactive substances for industrial evaluation — who should do it, who should pay for it?
- How can the patent process be made more efficient? Are there regulations that hinder collaboration?
- How can we stimulate interdisciplinary efforts in universities where such efforts are still not highly regarded?
- What can be done about the lack of good taxonomic information on many classes of marine organisms?
- Should there be centralized management of data on bioactive marine natural products?
- How can we stimulate research in neglected research areas?

PARTICIPANTS

Stewart N. Abramson
Department of Pharmacology M-036
University of California, San Diego
La Jolla, CA 92093

Kim Albizati
Department of Chemistry
Wayne State University
Detroit, MI 46202

David Attaway
National Sea Grant College Program
Office of Oceanic Research Programs
NOAA, R/OR
SSMB-1, Fifth Floor
1335 East-West Highway
Silver Spring, MD 20910

Tina S. Berger
Director, New Leads Discovery
Ligand Pharmaceuticals, Inc.
9393 Towne Centre Drive, Suite 100
San Diego, CA 92121

Jay Burgess
Department of Biological Sciences
University of California
Santa Barbara, CA 93106

Alison Butler
Department of Chemistry
University of California
Santa Barbara, CA 93106

Jon Clardy
Department of Chemistry
Cornell University
Ithaca, NY 14853-1301

Phil Crews
Department of Chemistry
University of California
Santa Cruz, CA 95064

Philip Davies
Executive Director, Inflammation Research
Merck Sharp & Dohme Research Laboratories
Rahway, NJ 07065-0900

Marianne de Carvalho [Kavanaugh]
University of California, Santa Barbara
Presently:
Department of Pediatrics
D402
National Jewish Center
1400 Jackson Street
Denver, CO 80206

Gerald W. DeVries
Allergan, Inc.
2525 Dupont Drive
Irvine, CA 92715

D. John Faulkner
Scripps Institution of Oceanography
University of California, San Diego
La Jolla, CA 92093

James A. Fee
Section Leader, Isotope and Structural
Chemistry Group
Los Alamos National Laboratory
Los Alamos, NM 87545

William Fenical
Scripps Institution of Oceanography
University of California, San Diego
La Jolla, CA 92093

Elizabeth Fraser-Smith
Staff Researcher
Syntex Corporation
3401 Hillview Avenue
Palo Alto, CA 94304

Taichiro Fujimura
Marine Science Institute
University of California
Santa Barbara, CA 93106

Michael E. Garst
Vice President, Chemical Sciences
Allergan Pharmaceuticals, Inc.
2525 Dupont Avenue
Irvine, CA 92713

John. T. Gerig
Department of Chemistry
University of California
Santa Barbara, CA 93106

William H. Gerwick
College of Pharmacy
Oregon State University
Corvallis, OR 97331

Keith B. Glaser
Division of Immunopharmacology
Wyeth-Ayerst Research
CN-8000
Princeton, NJ 08543

Gordon G. Hammes,
Vice Chancellor, Academic Affairs
University of California
Santa Barbara, CA 93106

Charles Hart
Affymax Research Institute
3180 Porter Drive
Palo Alto, CA 94304

Robert W. Holmes
Department of Biological Sciences
University of California
Santa Barbara, CA 93106

Yong-Ki Hong
Marine Science Institute
University of California
Santa Barbara, CA 93106

Chris M. Ireland
Department of Medicinal Chemistry
University of Utah
Salt Lake City, UT 84112

Robert S. Jacobs
Department of Biological Sciences
University of California
Santa Barbara, CA 93106

Alec Karma
Science and Engineering
950 Cañon Road
Santa Barbara, CA 93110

Beatrice Karma
Science and Engineering
950 Cañon Road
Santa Barbara, CA 93110

Deborah D. Kaska
Marine Science Institute
University of California
Santa Barbara, CA 93106

James P. Kennett
Director, Marine Science Institute
University of California
Santa Barbara, CA 93106

Tom Matthews
Syntex Corporation
3401 Hillview Avenue
Palo Alto, CA 94304

Dominick Mendola
California Bio-Marine Products Company
1001 Capri Road
Leucadia, CA 92024

Richard Moore
University of Hawaii
2545 The Mall
Honolulu, HI 96822

Aileen Morse
Marine Science Institute
University of California
Santa Barbara, CA 93106

Daniel E. Morse
Marine Science Institute
University of California
Santa Barbara, CA 93106

Michael Neushul
Department of Biological Sciences
University of California
Santa Barbara, CA 93106

Miriam Polne-Fuller
Marine Science Institute
University of California
Santa Barbara, CA 93106

Barbara Potts
Scripps Institution of Oceanography
University of California, San Diego
La Jolla, CA 92093

Laure Reynolds
Department of Chemistry
University of California, San Diego
La Jolla, CA 92093

Bruce Rickborn
Department of Chemistry
University of California
Santa Barbara, CA 93106

David Salisbury
Public Information Office
University of California
Santa Barbara, CA 93106

Francis J. Schmitz
Department of Chemistry
University of Oklahoma
620 Parrington Oval
Norman, OK 73069

Doris L. Slate
Syntex Corporation
3401 Hillview Avenue
Palo Alto, CA 94304

Palmer Taylor
Chair, Department of Pharmacology
University of California, San Diego
La Jolla, CA 92093

Carol Vandenberg
Department of Biological Sciences
University of California
Santa Barbara, CA 93106

Larry A. Wheeler
Director of Biological Science
Allergan Pharmaceuticals, Inc.
2525 Dupont Avenue
Irvine, CA 92713

Linda Wudl
Amgen, Inc.
Amgen Center
Thousand Oaks, CA 91320