Invited Review

Biologically Active Proteins from Natural Product Extracts[1]

Barry R. O'Keefe*

Molecular Targets Drug Discovery Program, Center for Cancer Research, National Cancer Institute−Frederick, Frederick, Maryland 21702-1201

Received July 9, 2001

The term "biologically active proteins" is almost redundant. All proteins produced by living creatures are, by their very nature, biologically active to some extent in their homologous species. In this review, a subset of these proteins will be discussed that are biologically active in heterologous systems. The isolation and characterization of novel proteins from natural product extracts including those derived from microorganisms, plants, insects, terrestrial vertebrates, and marine organisms will be reviewed and grouped into several distinct classes based on their biological activity and their structure.

Introduction

The history of pharmacognosy is, in part, defined by the ever-expanding catalog of naturally occurring, biologically active compounds that have been discovered and characterized. The chemical diversity and unique biological activities of these compounds have propelled further discoveries in both the chemical and biological sciences and provided therapeutic agents for many diseases. The continuing search for novel chemical entities has led to collection efforts in increasingly remote ecosystems such as rain forests, coral reefs, deep sea vents, hot springs, and severely polluted lakes. Similarly, chemical studies on the constituents of aqueous and organic solvent extracts have progressed from isolation of relatively simple lipophilic molecules to more complex organic structures, to small peptides, and to proteins. Recently, techniques for isolating and characterizing proteins have become both more automated and more affordable. These developments now make biologically active proteins from natural product extracts more accessible to the natural products chemist.

The proteinaceous constituents of natural product extracts represent a largely untapped source of potentially novel, biologically active molecules. This situation has arisen for several reasons. First, the isolation of an active protein to homogeneity is widely considered to be a difficult, time-consuming process utilizing buffer systems and chromatographic techniques not used in the separations familiar to most natural product chemists. Second, the elucidation of a protein's structure is usually not amenable to standard NMR techniques used for small molecules. In fact, until recently the tools for characterizing an isolated protein (i.e., *N*-terminal sequencing, LC/MS, amino acid analysis) were expensive and not widely available. In addition, proteins generally become less stable during the purification process and are often only isolated in microgram quantities. Perhaps the most significant reason that proteins from natural product extracts have not been studied more fully is the widely held belief that these bioactive molecules do not have the pharmacological characteristics necessary to become useful drugs.

Given these seemingly daunting challenges it is not surprising that very few researchers have specialized in the isolation and characterization of proteins from organisms other than humans. That several research groups are working in this nascent field despite these obstacles is testament to several other realities of working with proteins from natural product extracts: (1) there is an enormous amount of uncharted chemical diversity in the proteinaceous constituents of natural product extracts; (2) proteins, due to their size and structural complexity, occupy a large and varied subset of pharmacological space (i.e., the structural diversity of all pharmacologically active chemical classes combined), which often results in unique and specific interactions with other macromolecules in living systems; (3) advances over the past decade in the technologies for isolating and characterizing sub-microgram quantities of proteins have made the discovery of these compounds much more accessible; and (4) recombinant DNA technology provides a ready means of re-supply for bioactive proteins to be used in follow-up studies. Finally, the criticism that proteins may not be worth isolating because they are unlikely to be useful drugs has some inherent weaknesses. Rapid developments are being made in the targeted delivery of bioactive proteins into the human system, including mucosal delivery via both the intestine[2,3] and the lung,[4,5] while other studies have shown improved protein-based drug delivery using sustained-release, microencapsulated formulations for injection.[6−8] In addition, the recent discovery of specific peptides that can drive translocation across the plasma membrane of eukaryotic cells using an energy-independent pathway[9,10] provides a means for proteinaceous drug delivery and an example of why research into biologically active proteins can be useful in drug development.

This review will illustrate a variety of biologically active proteins that have been purified and characterized from nonhuman sources. Proteins from plants, microorganisms, marine organisms, insects, and terrestrial vertebrates will be discussed. Though many small, cyclic bioactive peptides have been isolated, especially from marine organisms (e.g.,

* To whom correspondence should be addressed. Tel: (301) 846-5332. Fax: (301) 846-6157. E-mail: okeefe@dtpax2.ncifcrf.gov.

10.1021/np0103362 CCC: $20.00
Published on Web 09/26/2001

depsipeptides), many of these compounds have been reviewed previously[11−13] and will not be covered here. Similarly, other small peptides that have highly unusual amino acids and appear to be biosynthesized through nonribosomal pathways (e.g., those produced by peptide synthase gene clusters) will not be included in this review. Finally, there is already a large body of literature devoted to characterization of the toxic constituents of poisonous species[14] such as snakes,[15] spiders,[16] cyanobacteria,[17] and eubacteria,[18] so these are not encompassed here.

Sources of Biologically Active Proteins

Microorganisms. Microorganisms have proven an excellent source of novel natural products including polyketide and peptide antibiotics as well as other classes of biologically active compounds.[19] One example of unusual proteinaceous compounds produced in bacteria (often lactic acid-producing bacteria) are the bacteriocins. The bacteriocins are ribosomally produced antibiotic peptides and proteins[20] that have been subdivided into several classes. These include the lantibiotics that are produced by Gram-positive bacteria and the microcins that are produced by Gram-negative bacteria.[21,22] The lantibiotics (e.g., nisin) have multiple postranslational modifications resulting in the presence of unusual thioether amino acids (i.e., lanthionine), while the microcins (e.g., J25) generally contain only common amino acids and are often heat stabile (up to 100 °C).[23] Both classes of antibiotic peptides are usually 20−40 amino acids in length (2−5 kDa), but there are several examples of the microcins ranging up to 8−10 kDa.[24] The biological activity of bacteriocins is mediated by their ability to form pores in cytoplasmic membranes of susceptible microorganisms.[22,25] This class of unique natural products is of significant interest in the food industry, as the bacteriocins are produced by many *Lactococcus* species used in the preservation of various foodstuffs (e.g., yogurt).

Recently, the general term "receptins" has been suggested for the spectrum of microbial proteins that specifically bind to mammmalian proteins (for a review see ref 26). An example of such a macromolecule is the elastin-binding protein from *Staphylococcus aureus* (epbS). This 25 kDa protein specifically binds to the extracellular matrix protein elastin and helps mediate attachment of *S. aureus* to tissues.[27] Many similar proteins have been isolated and characterized[26] and may be useful in elucidating the mechanisms of microbial infiltration and pathogenesis.

Bioactive proteins produced by fungi have also provided an interesting variety of structures and activities. The first was the discovery of the ion-gating polypeptide alamethicin from the fungus *Trichoderma viride* (for a review see ref 28). The fungal ribotoxins (including mitogillin, α-sarcin, and restrictocin) are small (17 kDa), basic proteins that act as specific ribonucleases by hydrolyzing a conserved domain in 23-28S rRNAs and are members of the protein superfamily of ribosome-inactivating proteins.[29] Another unusual fungal protein with a molecular weight of 10.4 kDa (RC-183) was isolated from the edible mushroom *Rozites caperata* and was found to inhibit herpes simplex virus 1 (HSV-1) and HSV-2 replication with an IC_{50} of $\leq 5\ \mu M$ and to inhibit HSV-1-induced keratitis in a murine model.[30] A 15 kDa protein isolated from a fungus in the genus *Helicosporium* was shown to inhibit neurite outgrowth from rat brain cortical neurons while promoting neurite outgrowth in PC12 cells, providing a useful biological probe for investigating the mechanisms of nerve growth.[31]

There are also several reports of biologically active lectins from various fungi. These include an 81 kDa protein from the fruiting body of *Pleurotus ostreatus* (the oyster mushroom) that displays potent antitumor activity in mice against the sarcoma cell line S-180 and the hepatoma cell line H-22.[32] Another lectin from the edible mushroom *Volvariella volvacea* (VVL), with a molecular weight of 32 kDa, has antiproliferative activity against cultured tumor cell lines. This activity was shown to be mediated by a concentration-dependent stimulation of the expression of cyclin kinase inhibitors (i.e., p21, p27, p53) resulting in cell-cycle arrest in the G2/M phase.[33] Many other fungal lectins have also been reported in the literature,[34,35] but the majority of these proteins have been isolated on the basis of either expected immunomodulatory effects or their carbohydrate-binding properties and, therefore, are not discussed further.

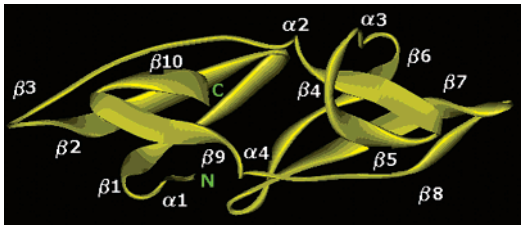

Figure 1. Ribbon diagram of the three-dimensional structure of cyanovirin-N as determined by NMR with β-sheets and α-helices marked.[38]

Another novel protein produced by a microorganism and named cyanovirin-N (CV-N) has generated significant interest in its potential utility as an anti-human immunodeficiency virus (HIV) prophylactic and/or therapeutic. The protein was originally isolated from the cyanobacterium *Nostoc ellipsosporum*.[36,37] CV-N is an 11 kDa protein with a novel primary amino acid sequence bearing no significant homology to any known protein. The three-dimensional structure of CV-N was elucidated by both NMR[38] and X-ray crystallography,[39] revealing that the tertiary structure represented a new superfamily of protein folds (Figure 1). CV-N displays potent virucidal activity against diverse primary isolates and laboratory strains of HIV-1 as well as HIV-2, with EC_{50} values generally in the 1−10 nM range.[36] The antiviral activity of CV-N is mediated through specific interactions with the HIV envelope glycoproteins gp120[36] and possibly also gp41.[40] Further investigation has indicated that CV-N binds to these proteins through unique interactions with the high-mannose oligosaccharides oligomannose-8 and -9 present in both glycoproteins.[41,42] CV-N therefore represents a new class of antiviral agent that specifically targets high-mannose glycans. CV-N is currently in preclinical development for use as a topical microbicide to prevent infection by HIV. As research increases into the relatively untapped proteome of the blue-green algae, it is likely that other novel biologically active proteins will be discovered.

Plants. Higher plants produce a variety of biologically active proteins with some classes (e.g., ribosome-inactivating proteins, defensins, cyclotides, lectins) that are also seen in other phylogenetic orders. Ribosome-inactivating proteins (RIPs) are a group of proteins with a wide variety of biological activities, including the ribonucleolytic activity for which the group is named. RIPs are often separated into single-chain (e.g., trichsanthin) and double-chain (e.g., ricin) classes and have been reviewed previously;[43,44] more recent reviews describe their uses as toxin conjugates[45] and their applications as biological probes of ribosome structure and function.[46] Examples of isolated single-chain RIPs include panaxagin and quinqueginsin, which were isolated from Chinese *Panax ginseng* and North American *P.*

quinquefolius, respectively, based on their antifungal and antiviral activity.[47,48] Other RIPs such as trichosanthin and TAP 29 (isolated from the tubers of *Trichosanthes kirilowii*),[49,50] MAP 30 (from *Momordica charantia*, a traditional Chinese medicinal herb),[51] and pokeweed antiviral protein (PAP, from *Phytolacca americana*)[52] have also been shown to be active against HIV and, in the case of MAP 30, also against HSV-1.[53] More recently, the RIP GAP 31 (from *Gelonium multiflorum*),[54] luffin (from *Luffa cylindrica*), and saporin (from *Saponaria officinalis*)[55] were reported to be potent inhibitors of HIV-1 integrase. Though RIPs are toxic, recent reports have noted that the RIP GAP31 and MAP30 are able to inhibit the growth of human breast tumor xenografts in mice at doses below the toxicity threshold.[56] The broad range of biological activities reported for the RIPs is indicative of their universal effect on mammalian ribosomes and interactions with other macromolecules. Whether or not any of these proteins have the specificity necessary for systemic therapeutics has yet to be conclusively demonstrated.

The plant antimicrobial peptides comprise another large group of bioactive products that have been further classified into several distinct families, including the thionins, defensins, and cyclotides. Recent reviews describe this group in general[57,58] as well as the thionins,[59] the defensins,[60] and the cyclotides[61] more specifically. The present survey covers each group only briefly; for a more exhaustive analysis of the individual families the aforementioned reviews are recommended.

The thionins are a group of small proteins generally from 45 to 47 amino acids in length that have four disulfide bonds and are basic or neutral in nature. The lead compound in this class is purothionin, originally isolated from wheat endosperm.[62] In addition to the Graminae, thionins have since been isolated from numerous other plant families, including the Loranthaceae (i.e., viscotoxins)[63] and the Leguminosae (i.e., the fabatins).[64] The biological activity of thionins has been associated with two unique characteristics of these peptides. First, they have been reported to selectively form disulfide bridges with other proteins,[65,66] and second, they have been shown to form ion channels in a variety of cell membranes[67] and in artificial lipid membranes.[68] This ability to permeabilize membranes is a common theme among many groups of antimicrobial peptides.[69]

The plant defensins are another group of cysteine-rich antimicrobial peptides that cause pore formation in cell membranes and which show structural homology to the insect and mammalian defensins.[70,71] The plant defensins were originally considered to be γ-thionins, but have since been reclassified based upon differences in their structure.[72] Plant defensins contain from 45 to 54 amino acids, have a net positive charge, and share sequence homology with respect to their eight cysteine residues and their three-dimensional structure.[60] The plant defensins are unusual in that, although they also permeabilize cell membranes, they appear to act specifically against fungal cell membranes with little activity against bacteria and none against plant or human cells.[60,73] This selectivity has been confirmed by reports of specific, high-affinity binding sites for the plant defensin Hs-AFP1 from *Heuchera sanguinea* on hyphae and microsomal membranes from *Neurospora crassa*[74] and has led to the successful genetic engineering of fungus-resistant potato with a similar plant defensin.[75]

The cyclotides are a family of proteins that has grown from the initial report of the circulins, a group of small, cyclic HIV-inhibitory proteins originally isolated from the tropical tree *Chassalia parvifolia*[76] which share a common disulfide linkage pattern, termed a "cysteine knot motif", with other members of the cyclotide family[61] (Figure 2). Many similar cyclic peptides have since been isolated from other plant species in the plant family Rubiaceae, including the kalata peptides from *Oldenlandia affinis*[77,78] and palicourein from *Palicourea condensata*.[79] Additional cyclotides such as the cycloviolacins from *Viola odorata*,[78] the varv peptides from *Viola arvensis*,[80] and the cycloviolins from *Leonia cymosa*[81] have also been found in the plant family Violaceae. The circulins were found to inhibit 10 different strains of HIV-1 with effective cytoprotective concentrations (EC_{50} values) from 40 to 275 nM.[76,82] The precise mechanism of action for the circulins and other plant cyclotides has not yet been fully elucidated, but initial studies suggest that these proteins, like the thionins, interact directly with cell membranes.[61]

The final group of bioactive proteins from plants to be discussed is also the largest. Plant lectins have been defined as proteins bearing a noncatalytic domain that binds reversibly to specific carbohydrates, normally via a monosaccharide-specific mechanism.[83] Lectins have been reported to have myriad biological activities, including antimicrobial activity,[57] immunostimulation/repression,[84,85] anti-HIV activity,[86,87] and antitumor activity.[88] Plant lectins such as *Helix pomatia* lectin[89,90] and jacalin from *Artocarpus heterophyllus*[91] have also been used as diagnostic agents to identify the presence of specific carbohydrates on proteins of interest in human cells. Lectins from *Urtica dioica*,[92] *Myrianthus holstii*,[93] and *Concanavalia* sp. (concanavalin A),[94] among many others,[95] have been reported to specifically target the HIV envelope protein gp120. The widespread use of plant lectins for glycobiology, as well as for their ability to specifically target glycoproteins associated with disease, will no doubt continue to expand as this vast group of proteins is further investigated.[96]

Insects. Several classes of antimicrobial peptides have been identified in insects, and recent reviews have been published on their structure and function[97] and their biological activity.[98] The insect peptides have been studied as components of the innate immune defense in insects and have generally not been investigated for additional biological activities in humans. The "defensins", from a variety of insects, include recent examples from the beetle *Oryctes rhinoceros*,[99] the termite *Pseudacanthotermes spiniger*,[100] the mosquito *Anopheles gambiae*,[101] and an unusual antifungal defensin from larvae of the moth *Heliothis virescens*.[102] Additional classes of antimicrobial peptides from insects include the antifungal drosomycin[103] and other antimicrobial peptides from *Drosophila melanogaster*,[104] several proline-rich peptides from species in the orders Hymenoptera (e.g., apidaecins) and the Hemiptera (e.g., pyrrhocoricin) (for a review see ref 105), and the cecropins, originally isolated from *Hyalophora cecropia*[106] and since found in many other species.[98]

Another novel biologically active protein has been reported from surface secretions of the caterpillar *Lonomia achelous*.[107] This protein causes a "bleeding syndrome" that is reported to be mediated by specific interactions with factor V in the blood coagulation cascade. Whether or not this interesting protein will also represent the first of another large class of insect bioactive proteins will require further study, but its unusual activity may open new areas of research into thrombosis.

Figure 2. Sequence alignment of cyclic proteins of the cyclotide family (displayed in linear form after cleavage of a single peptide bond with endoproteinase Glu-C). The disulfide bonding pattern shown ([___]) has been established for circulin A and kalata B1.[78] Cysteine residues are in red and underlined to indicate complete conservation; blue residues indicate homology to the consensus sequence (>50% conversion); green residues indicate residues that do not match the consensus sequence.

Interesting polypeptides have also been isolated from the venom of creatures that prey on insects, particularly arachnids and arthropods. Recent reviews on the ion-channel toxins from scorpions,[108] neurotoxins from spider venom,[109,110] and arthropod venom proteins in general[111] and their effects on the cardiovascular system[112] have been published.

Vertebrates. Endogenous vertebrate antimicrobial peptides have become an increasingly active area of research over the past decade. Several classes of proteins and peptides have been identified from a variety of source organisms, including cathelicidin and related proteins, protegrins, defensins, and magainins. These peptides are generally considered to be a part of the innate immunity that has been widely conserved throughout evolution.[113−115]

The defensins were first isolated by Ganz et al. from granule-rich sediments of human neutrophils[116] and have since been found as a part of the functional immune system

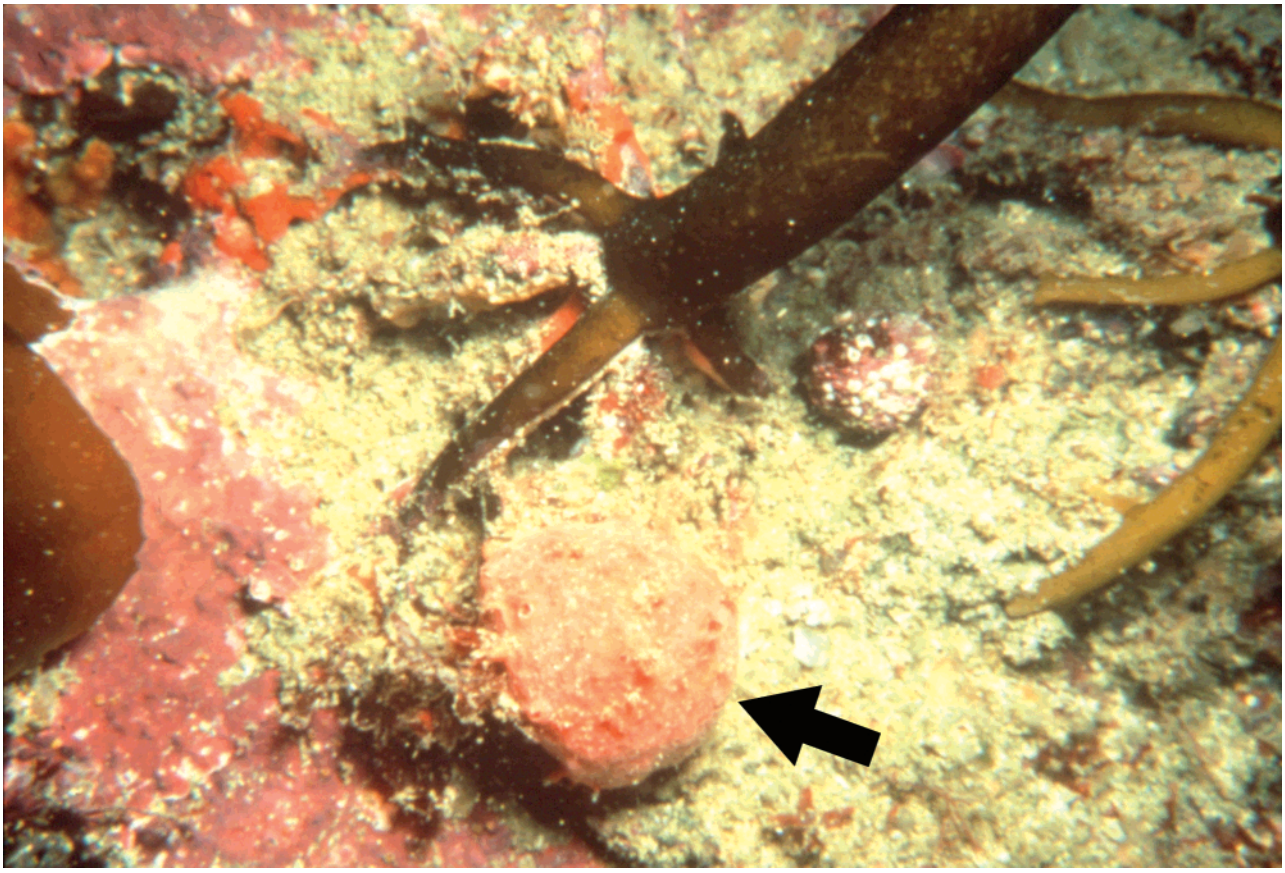

Figure 3. Photograph of the sponge *Tethya ingalli* (Hadromerida) collected at a depth of 20 m in Leigh Cove, Maori Island, New Zealand (collection #Q66D0064-Z), courtesy of the Natural Products Branch, DTP, NCI.

in both vertebrates and invertebrates.[117] Defensins are small cysteine-rich peptides approximately 30 amino acids in length. The defensins have been divided into alpha and beta subgroups on the basis of their disulfide linkage pattern consisting of three disulfide bonds between Cys-1/Cys-6, Cys-2/Cys-4, and Cys-3/Cys-5 for α-defensins and Cys-1/Cys-5, Cys-2/Cys-4, and Cys-3/Cys-6 for β-defensins.[118] These disulfide bonds provide stability to a unique predominantly β-sheet three-dimensional structure for defensins and separate this class of broad-spectrum antimicrobial peptides from others which typically form amphiphilic helices.[119] Like the plant and insect defensins, vertebrate defensins form voltage-gated pores in phospholipid bilayers[118] which destroy the membrane integrity of susceptible organisms and protect the host from infection.[120]

Amphibian skin has also been a source of many biologically active peptides, including bombesins and bombinins, bradykinins, dermorphins, and caruleins among others.[121,122] One particularly interesting group of these peptides with antimicrobial activity is the magainins, which were isolated from the skin of the frog *Xenopus laevis*.[123] Magainins are approximately 23 amino acid, pore-forming peptides[124] that form α-helices in lipid bilayers[125] and have bactericidal, fungicidal, and virucidal activity.[126] In addition, the magainins have been reported to selectively lyse tumor cells in vitro[127] and have in vivo efficacy against tumor xenografts in mice.[128] Magainin-based topical creams to treat impetigo and diabetic foot ulcers have been subjected to clinical trial in humans, but, at this time, none has yet been approved by the FDA for sale in the United States.

Protegrins, a class of antimicrobial peptides originally isolated from porcine leukocytes, are 16−18 amino acids in length and, though smaller, bear significant homology to defensins.[129] The protegrins, like the defensins, are pore-forming proteins and are largely β-sheet in structure.[130] These peptides were initially reported to have activity against *Escherichia coli, Listeria monocytogenes*, and *Candida albicans*[129] and have since also shown activity against the causative organism in several sexually transmitted diseases, including HSV-1 and 2,[131] *Chlamydia trachomatis*,[132] and *Neisseria gonorrheae*.[133] The protegrins have also shown good activity against a variety of periodontal pathogens[134] and are currently in clinical development for the prevention of chemotherapy- and radiation-induced oral mucositis.[135]

Cathelicidin-derived peptides are a class of broad-range antimicrobial proteins that were originally isolated from rabbits (CAP18).[136] Cathelicidins are made up of two domains, an *N*-terminal highly conserved "cathelin" domain and a *C*-terminal antimicrobial domain which can be structurally diverse (for a review see refs 137, 138). The bactericidal activity of the "cathelicidins" has been recently reviewed,[139] as have the structural features associated with this activity.[140] Cathelicidins become bactericidal after the cathelin domain has been cleaved from the antimicrobial domain. Members of this group have also been reported to bind to lipopolysaccharide and neutralize its activity.[140] Since the initial discovery of CAP18 in rabbits, cathelicidins have been isolated from mice,[141] sheep,[142] and humans.[143]

Marine Organisms. Terrestrial vertebrates produce many different biologically active proteins, with most falling into several well-defined classes. Marine organisms, on the other hand, produce a very wide array of bioactive proteins and peptides that do not share significant homology. Few recent general reviews of biologically active marine proteins are available (for the most recent see ref 144).

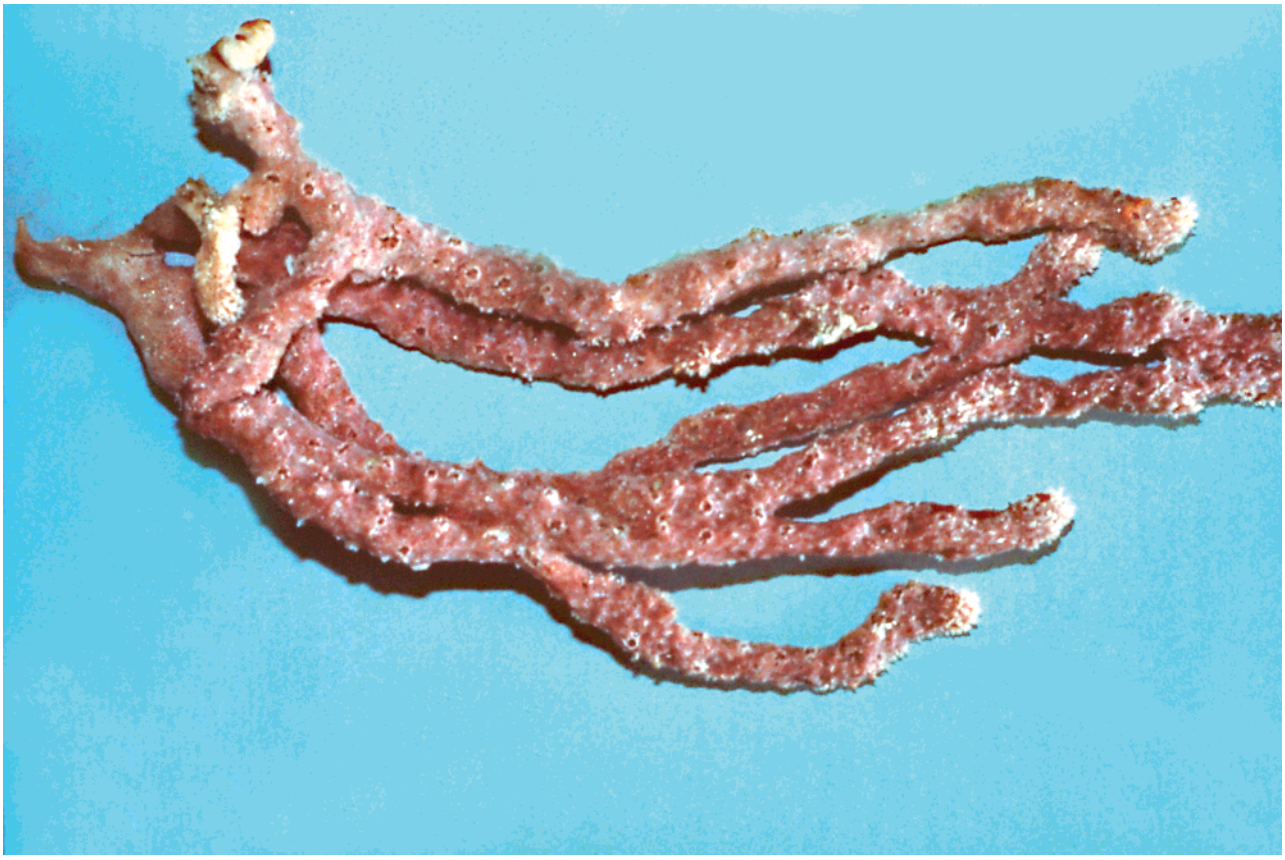

Figure 4. Photograph of the sponge *Niphates erecta* (Haplosclerida) collected at a depth of 36 m off Freeport-Lucaya, Grand Bahama Island, Bahamas (collection #Q66B0331-V), courtesy of the Natural Products Branch, DTP, NCI.

Highlighting the field of bioactive marine proteins are the conotoxin peptides derived from the venom of marine snails from the genus *Conus*. These peptides, originally discovered by Olivera, Cruz, and co-workers,[145] are initially biosynthesized as 70−120 amino acid prepropeptides that are then processed by both post-translational modification and proteolytic cleaveage to form a wide variety of novel small peptides, 10−50 amino acids in length.[146,147] Research on potential therapeutic applications of the conotoxins has been an extremely active area, after studies showed that these peptides interact uniquely with voltage-gated ion channels to induce a wide variety of pharmacological effects, including analgesia/anaesthesia and anticonvulsant activity.[148−150] General reviews of the conotoxins are available[146,151] as well as more detailed reviews of their structures[152] and potential therapeutic uses.[153−155] Currently, ziconotide (SNX-111), a conotoxin-derived peptide developed by Neurex Inc., is under regulatory review in the United States after successful Phase III clinical trials and an NDA filing by Elan Corp. in December 1999 for use of ziconotide in the treatment of chronic, opiate-resistant pain. It has been estimated that there are between 500 and 1000 different species of *Conus* snails, with each producing approximately 50−200 different venom peptides resulting in a potential "library" of approximately 100 000 different conotoxins.[153] It is clear that, with their wide array of biological activities and their impressive structural diversity, additional interesting developments from this group of marine proteins are likely in the future.

Cone snails are a predatory species, and their venom components are used to subdue and capture prey. In contrast, other shell-bearing marine organisms have also found a use for proteins and peptides as chemical defense agents. Several species of mussel including *Mytilus edulis* and *M. galloprovincialis* have been reported to produce defensin-like antibacterial peptides[156,157] as well as lectins that are cytotoxic to various marine *Vibrio* species.[158] In addition, horseshoe crabs from the genera *Tachypleus* have been reported to produce a variety of antibacterial peptides and proteins ranging from 2.3 to 42 kDa in size, including tachyplesins, tachystatins, and tachycitins as well as arthropod-like defensins.[159] Another group of antimicrobial peptides, the polyphemusins, was isolated from the hemolymph of the horseshoe crab *Limulus polyphemus*.[160] The polyphemusins are peptides consisting of 18 amino acids with two internal disulfide bonds that bear strong homology to the tachyplesins. Interestingly, a synthetic peptide based on the sequence of polyphemusin II with tyrosine residues inserted at amino acids 5 and 12 and a lysine residue inserted at amino acid 7, called T22, strongly inhibited the cytopathic effect of infection with HIV (EC_{50} = 0.008 μg/mL) on a MOLT-4 T-lymphoblastic cell line.[161] T22 has since been reported to interact with the HIV envelope protein gp120 and the cellular receptors CD4 and CXCR4 and to inhibit only T-tropic strains of HIV.[162,163]

Unlike mussels, snails, and horseshoe crabs, which all have some form of physical protection from attack, other less well-protected marine invertebrates rely on chemical defense molecules for their protection. As might be expected, some of these creatures produce interesting bioactive proteins for this purpose. The tunicate *Styela clava* has been reported to produce both 23-amino acid α-helical antimicrobial peptides called clavanins that have strong homology to the magainins and 32-amino acid phenylalanine-rich antibacterial peptides called styelins.[164,165] Another soft-bodied marine organism, the marine worm *Cerebratulus lacteus*, has been found to produce both polypeptide neurotoxins of approximately 6 kDa[166] and four

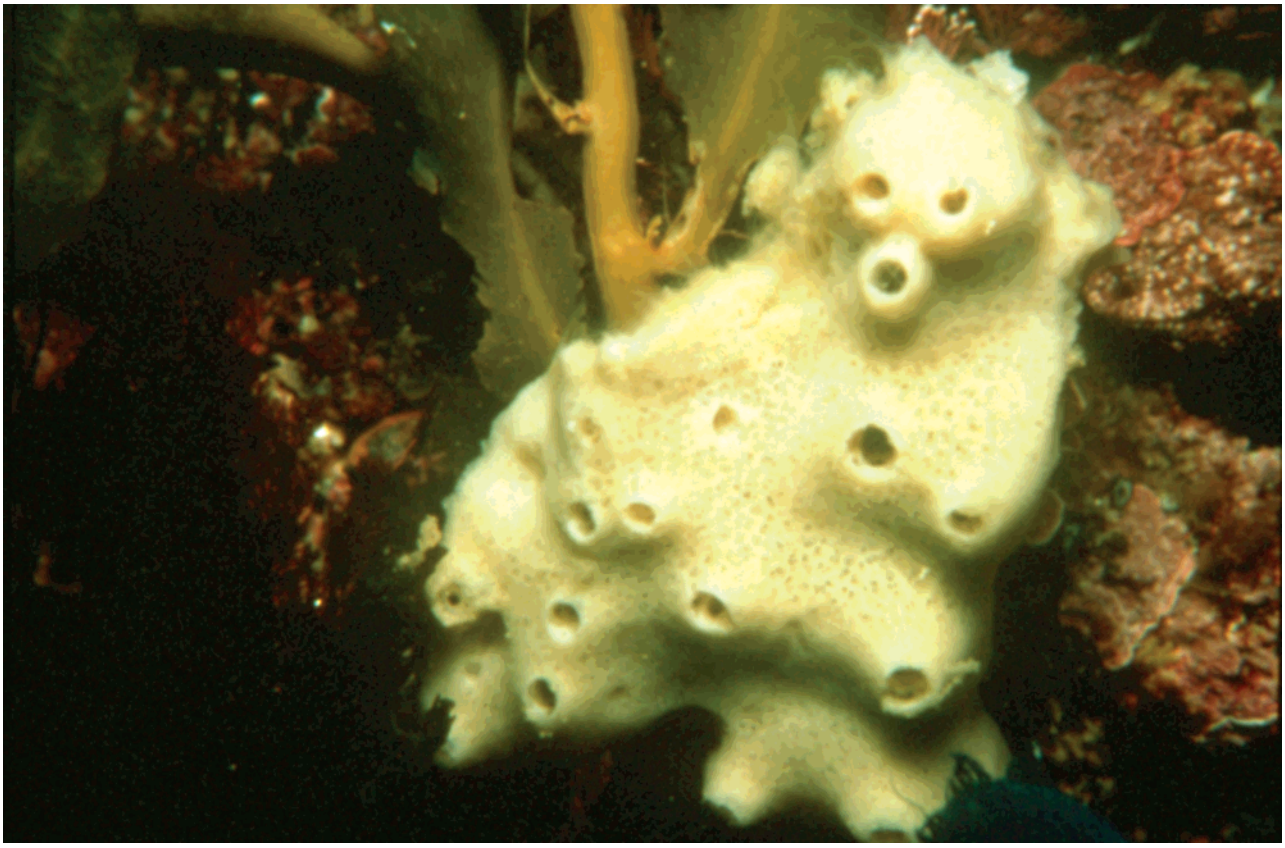

Figure 5. Photograph of the sponge *Adocia sp.* (Haplosclerida) collected at a depth of 14 m in Ocean Bay, Chatham Island, New Zealand (collection #Q66D293-Z), courtesy of the Natural Products Branch, DTP, NCI.

homologous ~10 kDa cytolytic proteins[167] which insert into lipid membranes and permeabilize a variety of cells.[168]

Sponges, the source of so many interesting secondary metabolites, have also been found to contain many different biologically active proteins. For example, lectins from the sponges *Chondrilla nucula* and *Geodia cydonium* have been used for histochemical labeling of both melanoma and breast and thyroid carcinomas.[169] This class of protein is common in sponges with lectins found in 12 different sponge families in a recent survey off the coast of Venezuela.[170] Other proteins such as iotrochotin, an 18 kDa protein isolated from the sponge *Iotrchota birotulata*, and polytheonamide B, a linear 48-amino acid peptide from *Theonella swinhoei*, were reported to mediate their toxicity by selectively permeabilizing membranes causing them to release small molecular weight components.[171,172] In contrast, the protein mapacalcine, a dimeric 19 kDa protein isolated from the sponge *Cliona vastifica*, specifically blocked non-L-type calcium channels in mouse duodenal myocytes but did not affect T-type calcium flux or potassium and chloride currents.[173]

Proteins from sponges have also been reported to selectively kill human tumor cells. In the case of the cytolytic protein from the sponge *Tethya ingalli* (Figure 3), the active 21 kDa protein, similar to a previously isolated hemolysin from *T. lycinurium*,[174] selectively lysed sensitive human ovarian cancer cells with an EC_{50} value of 0.16 μg/mL.[175] Interestingly, this protein was co-purified with a novel Kunitz family protease inhibitor. This inhibitor could act by inhibiting the proteolysis of the hemolysin and thus allow it time to affect potential predators. Another antitumor glycoprotein was isolated from the sponge *Pachymatisma johnstonii.* This 46 kDa protein (30% sugar), named pachymatismin, was found to inhibit the proliferation of human tumor cells with an IC_{50} value in the range 0.8−2 μg/mL.[176] In the same study, this protein also exhibited a unique mechanism of action whereby cell growth in a human non-small-cell-bronchopulmonary carcinoma line (NSCLC-N6) was inhibited at the G_0/G_1 phase of the cell cycle. This glycoprotein was also later shown to have anti-leishmanial activity (IC_{50} = 1 μg/mL) and to be cytotoxic to the parasite at the promastigote and amastigote stages of the life cycle.[177]

Members of the final group of sponge proteins to be discussed have all been shown to protect cells from the cytopathic effects of HIV infection. The protein niphatevirin, a 19 kDa glycoprotein isolated from the sponge *Niphates erecta* (Figure 4), inhibited HIV-induced cytopathic effects, cell−cell fusion, and sycytium formation with an EC_{50} of 10 nM.[178] Niphatevirin was shown to specifically interact with the cellular receptor CD4 in a manner that prevented the subsequent association of the viral envelope protein gp120 but did not cause either hemagglutination or hemolysis.[178] Another anti-HIV protein was isolated from the Haplosclerid sponge *Adocia* sp. (Figure 5) that bound to both CD4 and gp120. The protein, adociavirin, was found to be a disulfide-linked homodimer weighing 37 kDa that inhibited diverse strains of HIV with EC_{50} values ranging from 0.4 to >400 nM, displaying greater potency in macrophage cell lines than T-lymphocytic cell lines.[179] More recently, a non-self-binding domain of the aggregation factor of the sponge *Microciona prolifera* (MAF) was reported to bind to gp120 and protect T-lymphoblastoid cells from infection with HIV with an EC_{50} value of ≤0.12 μg/mL.[180]

Conclusions

As research into the bioactive constituents of natural product extracts continues, it is certain that more new and

unusual proteins, not only from sponges and other marine organisms, but also from plants, microbes, insects, and vertebrates, will be discovered. The structural, biochemical, and functional diversity of the proteins found in nature provides an exceptional opportunity for future research. Though increasingly extensive efforts are being devoted to understanding the proteome of humans and certain model organisms, an enormous amount of proteinaceous diversity yet remains to be investigated in the nonmammalian natural world.

Acknowledgment. I give special thanks to Dr. Michael R. Boyd, MTDDP, CCR, NCI, for his continued support of research into natural products and his encouragement of my interest in bioactive proteins. I also thank Drs. Shilpa S. Kurian (MTDDP), David J. Newman (NPB, DTP, NCI), and Raymond C. Sowder II (SAIC-Frederick) for assistance with the figures. Finally I thank my colleagues at the NCI both at the MTDDP and at the Natural Products Branch for their help in pursuing this area of research and for their thoughtful reviews of the manuscript.

References and Notes

(1) Matt Suffness Award lecture, presented at the 41st Annual Meeting of the American Society of Pharmacognosy, Seattle, WA, July 25, 2000.
(2) Lee, V. H. L.; Chu, C.; Mahlin, E. D.; Basu, S. K.; Ann, D. K.; Bolger, M. B.; Haworth, I. S.; Yeung, A. K.; Wu, S. K.; Hamm-Alvarez, S.; Okamoto, C. T. *J. Controlled Release* **1999**, *62*, 129−140.
(3) Russell-Jones, G. J. *Crit. Rev. Ther. Drug Carrier Syst.* **1998**, *15*, 557−586.
(4) Patton, J. *Nature (Biotechnol.)* **1998**, *16*, 141−143.
(5) Gonda, I. *J. Pharm. Sci.* **2000**, *89*, 940−945.
(6) Putney, S. D.; Burke, P. A. *Nature (Biotechnol.)* **1998**, *16*, 153−157.
(7) Chang, P. L.; Van Raamsdonk, J. M.; Hortelano, G.; Barsoum, S. C.; MacDonald, N. C.; Stockley, T. L. *Trends Biotechnol.* **1999**, *17*, 78−83.
(8) Peppas, N. A.; Bures, P.; Leobandung, W.; Ichikawa, H. *Eur. J. Pharm. Biopharm.* **1999**, *50*, 27−46.
(9) Wender, P. A.; Mitchell, D. J.; Pattabiraman, K.; Pelkey, E. T.; Steinman, L.; Rothbard, J. B. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13003−13008.
(10) Lindgren, M.; Hällbrink, M.; Prochiantz, A.; Langel, Ü. *Trends Pharm. Sci.* **2000**, *21*, 99−103.
(11) Rinehart, K. L. *Med. Res. Rev.* **2000**, *20*, 1−27.
(12) Pettit, G. R. *Fortschr. Chem. Org. Naturst.* **1997**, *70*, 1−79.
(13) Moore, R. E. *J. Ind. Microbiol.* **1996**, *16*, 134−143.
(14) Kourie, J. I.; Shorthouse, A. A. *Am. J. Physiol. Cell Physiol.* **2000**, *278*, C1063−C1087.
(15) Harvey A. L. *Toxicon* **2001**, *39*, 15−26.
(16) Escoubas, P.; Diochot, S.; Corzo, G. *Biochimie* **2000**, *82*, 893−907.
(17) Dawson, R. M. *Toxicon* **1998**, *36*, 953−962.
(18) Richard, J.-F.; Petit, L.; Gibert, M.; Marvaud, J. C.; Popoff, M. R. *Int. Microbiol.* **1999**, *2*, 185−194.
(19) Demain, A. L. *Appl. Microbiol. Biotechnol.* **1999**, *52*, 455−463.
(20) Kolter, R.; Moreno, F. *Annu. Rev. Microbiol.* **1992**, *46*, 141−163.
(21) Jack, R. W.; Jung, G. *Curr. Opin. Chem. Biol.* **2000**, *4*, 310−317.
(22) Ennahar, S.; Sashihara, T.; Sonomoto, K.; Ishizaki, A. *FEMS Microbiol. Rev.* **2000**, *24*, 85−106.
(23) Guder, A.; Wiedmann, I.; Sahl, H. G. *Biopolymers* **2000**, *55*, 62−73.
(24) Gaillard-Gendron, S.; Vignon, D.; Cottenceau, G.; Graber, M.; Zorn, N.; van Dorsselaer, A.; Pons, A.-M. *FEMS Microbiol. Lett.* **2000**, *193*, 95−98.
(25) Sahl, H.-G.; Bierbaum, G. *Annu. Rev. Microbiol.* **1998**, *52*, 41−79.
(26) Kronvall, G.; Jonsson, K. *J. Mol. Recognit.* **1999**, *12*, 38−44.
(27) Park, P. W.; Rosenbloom, J.; Abrams, W. R.; Rosenbloom, J.; Mecham, R. P. *J. Biol. Chem.* **1996**, *271*, 15803−15809.
(28) Cafiso, D. S. *Annu. Rev. Biophys. Biomol. Struct.* **1994**, *23*, 141−165.
(29) Kao, R.; Davies, J. *Biochem. Cell Biol.* **1995**, *73*, 1151−1159.
(30) Piraino, F.; Brandt, C. R. *Antiviral Res.* **1999**, *43*, 67−78.
(31) Hanada, T.; Sato, T.; Arioka, M., Uramoto, M.; Yamasaki, M. *Biochem. Biophys. Res. Commun.* **1996**, *228*, 209−215.
(32) Wang, H.; Gao, J.; Ng, T. B. *Biochem. Biophys. Res. Commun.* **2000**, *275*, 810−816.
(33) Liu, W.-K.; Ho, J. C. K.; Ng, T. B. *Biochem. Pharmacol.* **2001**, *61*, 33−37.
(34) Mo, H.; Winter, H. C.; Goldstein, I. J. *J. Biol. Chem.* **2000**, *275*, 10623−10629.
(35) Wasser, S. P.; Weis, A. L. *Crit. Rev. Immunol.* **1999**, *19*, 65−96.
(36) Boyd, M. R.; Gustafson, K. R.; McMahon, J. B.; Shoemaker, R. H.; O'Keefe, B. R.; Mori, T.; Gulakowski, R. J.; Wu, L.; Rivera, M. I.; Laurencot, C. M.; Currens, M. J.; Cardellina, J. H., II; Buckheit, R. W., Jr.; Nara, P. R.; Pannell, L. K.; Sowder, R. C., II; Henderson, L. E. *Antimicrob. Agents Chemother.* **1997**, *41*, 1521−1530.
(37) Gustafson, K. R.; Sowder, R. C., II.; Henderson, L. E.; Cardellina, J. H., II; McMahon, J. B.; Rajamani, U.; Pannell, L. K.; Boyd, M. R. *Biochem. Biophys. Res. Commun.* **1997**, *238*, 223−228.
(38) Bewley, C. A.; Gustafson, K. R.; Boyd, M. R.; Covell, D. G.; Bax, A.; Clore, G. M.; Gronenborn, A. M. *Nature (Struct. Biol.)* **1998**, *5*, 571−578.
(39) Yang, F.; Bewley, C. A.; Bax, A.; Louis, J. M.; Clore, G. M.; Gronenborn, A. M.; Gustafson, K. R.; Boyd, M. R.; Wlowdower, A. *J. Mol. Biol.* **1999**, *288*, 403−412.
(40) O'Keefe, B. R.; Shenoy, S. R.; Xie, D.; Zhang, W.; Muschik, J. M.; Currens, M. J.; Chaiken, I.; Boyd, M. R. *Mol. Pharm.* **2000**, *58*, 982−992.
(41) Bolmstedt A. J.; O'Keefe, B. R.; Shenoy, S. R.; McMahon, J. B.; Boyd, M. R. *Mol. Pharm.* **2001**, *59*, 949−954.
(42) Shenoy S. R.; O'Keefe, B. R.; Bolmstedt A. J.; Cartner, L. K.; Boyd, M. R *J. Pharmacol. Exp. Ther.* **2001**, *297*, 704−710.
(43) Stirpe, F.; Barbieri, L.; Batelli, M. G.; Soria, M.; Lappi, D. A. *Bio/Tecnology* **1992**, *10*, 405−412.
(44) Girbés, T.; Ferreras, J. M.; Iglesias, R., Citores, L.; DeTorre, C.; Carbajales, M. L.; Jiménez, P.; De Benito, F. M.; Munoz, R. *Cell. Mol. Biol.* **1996**, *42*, 461−471.
(45) Singh R. C.; Singh V. *Indian J. Biochem. Biophys.* **2000**, *37*, 1−5.
(46) Liu, W. Y.; Pu, Z. *J. Nat. Toxins* **1999**, *8*, 385−394.
(47) Wang, H. X.; Ng, T. B. *Life Sci.* **2001**, *68*, 739−749.
(48) Wang, H. X.; Ng, T. B. *Biochem. Biophys. Res. Commun.* **2000**, *269*, 203−208.
(49) Zarling, J. M.; Moiran, P. A.; Haffer, O.; Sins, J.; Richman, D. J.; Spina, C. A.; Myers, D.; Keubelbeck, V.; Ledbetter, J. A.; Uckun, F. M. *Nature* **1990**, *347*, 92−95.
(50) Lee-Huang, S.; Huang, P. L.; Kung, H.-F.; Li, B.-Q.; Huang, P. L.; Huang, P.; Huang, H. I.; Chen, H.-C. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6570−6574.
(51) Lee-Huang, S.; Huang, P. L.; Nara, P. L.; Chen, H.-C.; Kung, H.-F.; Huang, P.; Huang, H. I.; Huang, P. L. *FEBS Lett.* **1990**, *272*, 12−18.
(52) McGrath, M. S.; Huang, K. M.; Caldwell, S. E.; Garion, L.; Luc, K. C.; Wu, P.; Ng, V. C.; Crow, S.; Danich, J.; Manh, J.; Dienhart, T.; Lekas, P. V.; Vennari, J. C.; Yeng, H. W.; Lipon, J. D. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 2844−2848.
(53) Bourinbaiar, A. S.; Lee-Huang, S. *Biochem. Biophys. Res. Commun.* **1996**, *219*, 923−929.
(54) Lee Huang, S.; Huang, P. L.; Huang, P. L.; Bourinbaiar, A. S.; Chen, H.-C.; Kung, H.-F. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 8818−8822.
(55) Au, T. K.; Collins, R. A.; Lam, T. L.; Ng, T. B.; Fong, W. P.; Wan, D. C. C. *FEBS Lett.* **2000**, *471*, 169−172.
(56) Lee-Huang, S.; Huang, P. L.; Sun, Y.; Chen, H.-C.; Kung, H.-F.; Huang, P. L.; Murphy, W. J. *Cancer Res.* **2000**, *20*, 653−660.
(57) Cowan, M. M. *Clin. Microbiol. Rev.* **1999**, *12*, 564−582.
(58) Garcia-Olmedo, F.; Molina, F.; Alamillo, J. M.; Rodríguez-Palenzuéla, P. *Biopolymers* **1998**, *47*, 479−491.
(59) Florack, D. E.; Stiekema, W. J. *Plant. Mol. Biol.* **1994**, *26*, 25−37.
(60) Broekaert, W. F.: Terras, F. R. G.; Cammue, B. P. A.; Osborn, R. W. *Plant Physiol.* **1995**, *108*, 1353−1358.
(61) Craik, D. J.; Daly, N. L.; Waine, C. *Toxicon* **2001**, *39*, 43−60.
(62) Balls, A. K.; Hale, W. S.; Harris, T. H. *Cereal Chem.* **1942**, *19*, 279−288.
(63) Olson, T.; Samuelsson, G. *Acta Chem. Scand.* **1970**, *24*, 720−721.
(64) Zhang, Y.; Lewis, K. FEMS *Microbiol. Lett.* **1997**, *149*, 59−64.
(65) Diaz, I.; Carmona, M. J.; Garcia-Olmedo, F. *FEBS Lett.* **1992**, *296*, 279−282.
(66) Pineiro, M.; Diaz, I.; Rodríguez-Palenzuéla, P.; Titarenko, E.; Garcia-Omelda, F. *FEBS Lett.* **1995**, *369*, 239−242.
(67) Evans, J.; Wang, Y. D.; Shaw, K. P.; Vernon, L. P. *Proc. Natl. Acad. Sci. U.S.A.* **1989**, *86*, 5849−5853.
(68) Hughes, P.; Dennis, E.; Whitecross, M.; Llewellyn, D.; Gage, P. *J. Biol. Chem.* **2000**, *275*, 823−827.
(69) Froy, O.; Gurevitz, M. *FASEB J.* **1998**, *12*, 1793−1796.
(70) Hoffman, J. A.; Hetru, C. *Immunol. Today* **1992**, *13*, 411−415.
(71) Ganz T.; Lehrer, R. I. *Curr. Opin. Immunol.* **1994**, *6*, 584−589.
(72) Bruix, M.; Jimenez, M. A.; Santoro, J.; Gonzalez, C.; Colilla, F. J.; Mendez, E.; Rico, M. *Biochemistry* **1993**, *32*, 715−724.
(73) Terras, F. R. G.; Schoofs, H. M. E.; De Bolle, M. F. C.; Van Leuven, F.; Rees, S. B.; Vanderleyden, J.; Cammue, B. P. A.; Broekaert, W. F. *J. Biol. Chem.* **1992**, *267*, 15301−15309.
(74) Thevissen K.; Osborn, R. W.; Acland, D. P.; Broekaert, W. F. *J. Biol. Chem.* **1997**, *272*, 32176−32181.
(75) Gao, A.-G.; Hakimi, S. M.; Mittanck, C. A.; Wu, Y.; Woerner, B. M.; Stark, D. M.; Shah, D. M.; Liang, J.; Rommens, C. M. T. *Nature (Biotechnol.)* **2000**, *18*, 1307−1310.
(76) Gustafson, K. R.; Sowder, R. C., II; Henderson, L. E.; Parsons, I. C.; Kashman, Y.; Cardellina, J. H., II; Buckheit, R. W., Jr.; Pannell, L. K.; Boyd, M. R. *J. Am. Chem. Soc.* **1994**, *116*, 9337−9338.
(77) Saether, O.; Craik, D. J.; Campbell, I. D.; Sletten, K.; Juul, J.; Norman, D. G. *Biochemistry* **1995**, *34*, 4147−4158.
(78) Craik, D. J.; Daly, N. L.; Bond, T.; Waine, C. *J. Mol. Biol.* **1999**, *294*, 1327−1336.
(79) Bokesch, H. R.; Pannell, L. K.; Cochran, P. K.; Sowder, R. C., II; McKee, T. C.; Boyd, M. R. *J. Nat. Prod.* **2001**, *64*, 249−250.
(80) Göransson, U.; Luijendijk, T.; Johansson, S.; Bohlin, L.; Claeson, P. *J. Nat. Prod.* **1999**, *62*, 283−286.
(81) Hallock, Y. F.; Sowder, R. C., II; Pannell, L. K.; Hughes, C. B.; Johnson, D. G.; Gulakowski, R.; Cardellina, J. H., II; Boyd, M. R. *J. Org. Chem.* **2000**, *65*, 124−128.
(82) Gustafson, K. R.; Walton, L. K.; Sowder, R. C., II; Johnson, D. G.; Pannell, L. K.; Cardellina, J. H., II; Boyd, M. R. *J. Nat. Prod.* **2000**, *63*, 176−178.
(83) Peumans, W. J.; Van Damme, E. J. M. *Plant. Physiol.* **1995**, *109*, 347−352.

(84) Wimer, B. M. *Cancer Biother. Radiopharm.* **1997**, *12*, 195−212.
(85) Wimer, B. M. *Cancer Biother. Radiopharm.* **1998**, *13*, 99−107.
(86) De Clercq, E. *Med. Res. Rev.* **2000**, *20*, 323−349.
(87) Vleitinck, A. J.; De Bruyne, T.; Apers, S.; Pieters, L. A. *Planta Med.* **1998**, *64*, 97−109.
(88) Abdullaev, F. I.; de Mejia, E. G. *Nat. Toxins* **1997**, *5*, 157−163.
(89) Brooks, S. A. *Histol. Histopathol.* **2000**, *15*, 143−158.
(90) Mitchell, B. S.; Schumacher, U. *Histol. Histopathol.* **1999**, *14*, 217−226.
(91) Kabir, S. *J. Immunol. Methods* **1998**, *212*, 193-211.
(92) Balzarini, J.; Neyts, J.; Schols, D.; Hosoya, M.; Van Damme, E.; Peumans, W. J.; De Clercq, E. *Antiviral. Res.* **1992**, *18*, 191−207.
(93) Charan, R. D.; Munro, M. H. G.; O'Keefe, B. R.; Sowder, R. C., II.; McKee, T. C.; Currens, M. J.; Pannell, L. K.; Boyd, M. R. *J. Nat. Prod.* **2000**, *63*, 1170−1174.
(94) Robinson, W. E., Jr.; Montefiori, D. C.; Mitchell, W. M. *AIDS Res. Hum. Retroviruses* **1987**, *3*, 265−282.
(95) Astoul, C. H.; Peumans, W. J.; Van Damme, E. J. M. Rougé *Biochem. Biophys. Res. Commun.* **2000**, *274*, 455−460.
(96) Rüdiger, H. *Acta Anat.* **1998**, *161*, 130−152.
(97) Bulet, P.; Hetru, C.; Dimarcq, J.-L.; Hoffman, D. *Dev. Comput. Immunol.* **1999**, *23*, 329−344.
(98) Otvos, L., Jr. *J. Pept. Sci.* **2000**, *6*, 497−511.
(99) Ishibashi, J.; Saido-Sakanaka, H.; Yang, J.; Sagisaka, A.; Yamakawa, M. *Eur. J. Biochem.* **1999**, *266*, 616−632.
(100) Lamberty, M.; Ades, S.; Uttenweiler-Joseph, S.; Brookhart, G.; Bushey, D.; Hoffmann, J. A.; Bulet, P. *J. Biol. Chem.* **2001**, *276*, 4085−4092.
(101) Eggleston, P.; Lu, W.; Zhao, Y. *Insect Mol. Biol.* **2000**, *9*, 481−490.
(102) Lamberty, M.; Ades, S.; Uttenweiler-Joseph, S.; Brookhart, G.; Bushey, D.; Hoffmann, J. A.; Bulet, P. *J. Biol. Chem.* **1999**, *274*, 9320−9326.
(103) Fehlbaum, P.; Bulet, P.; Michaut, L.; Lagueux, M.; Broekaert, W. F.; Hetru, C.; Hoffmann, J. A. *J. Biol. Chem.* **1994**, *269*, 33159−33163.
(104) Meister, M.; Hetru, C.; Hoffman, J. A. *Curr. Top. Microbiol. Immunol.* **2000**, *248*, 17−36.
(105) Hetru, C.; Hoffmann, D.; Bulet, P. In *Molecular Mechanisms of Immune Responses in Insects*; Brey, P. T., Hultmark, D., Eds.; Chapman & Hall Publishers: New York, 1998; pp 40−66.
(106) Steiner, H.; Hultmark, D.; Engstrom, A.; Bennich, H.; Boman, H. G. *Nature* **1981**, *292*, 246−248.
(107) Lopez, M.; Gil, A.; Arocha-Pinango, C. L. *Thrombosis Res.* **2000**, *98*, 103−110.
(108) Possani, L. D.; Merino, E.; Corona, M.; Bolivar, F.; Becerril, B. *Biochimie* **2000**, *82*, 861−868.
(109) Grishin, E. *Eur. J. Biochem.* **1999**, *264*, 276−280.
(110) Escoubas, P.; Diochot, S.; Corzo, G. *Biochimie* **2000**, *82*, 893−907.
(111) Jones, D. *Adv. Exp. Med. Biol.* **1996**, *391*, 379−386.
(112) Gueron, M.; Ilia, R.; Margulis, G. *Am. J. Emerg. Med.* **2000**, *18*, 708−14.
(113) Risso, A. *J. Leukoc. Biol.* **2000**, *68*, 785−792.
(114) Mitta, G.; Vandenbulcke, F.; Roch, P. *FEBS Lett.* **2000**, *486*, 185−190.
(115) Zasloff, M. *Curr. Opin. Immunol.* **1992**, *4*, 3−7.
(116) Ganz, T.; Selsted, M. E.; Szklarek, D.; Harwig, S. S.; Daher, K.; Bainton, D. F.; Lehrer, R. I. *J. Clin. Invest.* **1985**, *76*, 1427−1435.
(117) Hughes, A. L. *Cell Mol. Life Sci.* **1999**, *56*, 94−103.
(118) Lehrer, R. I.; Ganz, T. *Ann. NY Acad. Sci.* **1996**, *797*, 228−239.
(119) White, S. H.; Wimley, W. C.; Selsted M. E. *Curr. Opin. Struct. Biol.* **1995**, *5*, 521−527.
(120) Gudmundsson, G. H.; Agerberth, B. *J. Immunol. Methods* **1999**, *232*, 45−54.
(121) Erspamer, V.; Falconieri-Erspamer, G.; Cei, J. M. *Comp. Biochem. Physiol. C* **1986**, *85*, 125−137.
(122) Roseghini, M.; Falconieri-Erspamer, G.; Severini, C.; Simmaco, M. *Comput. Biochem. Physiol. C* **1989**, *94*, 455−460.
(123) Zasloff, M. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 5449−5453.
(124) Matsuzaki, K. *Biochim. Biophys. Acta* **1998**, *1376*, 391−400.
(125) Ramamoorthy, A.; Marassi, F. M.; Zasloff, M.; Opella, S. J. *J. Biomol. NMR* **1995**, *6*, 329−334.
(126) Bechinger, B. *J. Membr. Biol.* **1997**, *156*, 197−211.
(127) Cruciani, R. A.; Barker, J. L.; Zasloff, M.; Chen, H. C.; Colomonici, O. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 3792−3796.
(128) Baker, M. A.; Maloy, W. L.; Zasloff, M.; Jacob, L. S. *Cancer Res.* **1993**, *53*, 3052−3057.
(129) Kokryakov, V. N.; Harwig, S. S.; Panyutich, E. A.; Shevchenko, A. A.; Aleshina, G. M.; Shamova, O. V.; Korneva, H. A.; Lehrer, R. I. *FEBS Lett.* **1993**, *327*, 231−236.
(130) Sokolov, Y.; Mirzabekov, T.; Martin, D. W.; Lehrer, R. I.; Kagan, B. L. *Biochim. Biophys. Acta* **1999**, *1420*, 23−29.
(131) Yasin, B.; Lehrer, R. I.; Harwig, S. S.; Wagar, E. A. *Eur. J. Clin. Microbiol. Infect. Dis.* **2000**, *19*, 187−194.
(132) Yasin, B.; Lehrer, R. I.; Harwig, S. S.; Wagar, E. A. *Infect. Immunol.* **1996**, *64*, 4863−4866.
(133) Qu, X.-D.; Harwig, S. S.; Oren, A.; Shafer, W. M.; Lehrer, R. I. *Infect. Immunol.* **1996**, *64*, 1240−1245.
(134) Miyasaki, K. T.; Iofel, R.; Lehrer, R. I. *J. Dent. Res.* **1997**, *76*, 1453−1459.
(135) Bellm, L.; Lehrer, R. I.; Ganz, T. *Expert Opin. Investig. Drugs* **2000**, *9*, 1731−1742.
(136) Larrick, J. W.; Morgan, J. G.; Palings, I.; Hirata, M.; Yen, M. H. *Biochem. Biophys. Res. Commun.* **1991**, *179*, 170−175.
(137) Zanetti, M.; Gennaro, R.; Romeo, D. *Ann. N.Y. Acad. Sci.* **1997**, *832*, 147−162.
(138) Zanetti, M.; Gennaro, R.; Romeo, D. *FEBS Lett.* **1995**, *374*, 1−5.
(139) Travis, S. M.; Anderson, N. N.; Forsyth, W. R.; Espiritu, C.; Conway, B. D.; Greenberg, E. P.; McCray, P. B., Jr.; Lehrer, R. I.; Welsh, M. J.; Tack, B. F. *Infect. Immunol.* **2000**, *68*, 2748−2755.
(140) Gennaro, R.; Zanetti, M. *Biopolymers* **2000**, *55*, 31−49.
(141) Popsueva, A. E.; Zinovjeva, M. V.; Visser, J. W.; Zijlmans, J. M.; Fibbe, W. E.; Belyavsky, A. V. *FEBS Lett.* **1996**, *391*, 5−8.
(142) Mahoney, M. M.; Lee, A. Y.; Brezinski-Caliguri, D. J.; Huttner, K. M. *FEBS Lett.* **1995**, *377*, 519−522.
(143) Larrick, J. W.; Hirata, M.; Balint, R. F.; Lee, J.; Zhong, J.; Wright, S. C. *Infect. Immunol.* **1995**, *63*, 1291−1297.
(144) Sharma, G. M.; Sahni, M. K. In *Marine Proteins in Clinical Chemistry in Marine Biotechnology, Volume 1: Pharmaceutical and Bioactive Natural Products*; Attaway D. H., Zaborsky, O. R., Eds.; Plenum Press: New York, 1993; pp 153−180.
(145) Gray, W. R.; Luque, A.; Olivera, B. M.; Barrett, J.; Cruz, L. J. *J. Biol. Chem.* **1981**, *256*, 4734−4740.
(146) Arias, H. R.; Blanton, M. P. *Int. J. Biochem. Cell Biol.* **2000**, *32*, 1017−1028.
(147) Craig, A. G.; Bandyopadhyay, P.; Olivera, B. M., *Eur. J. Biochem.* **1999**, *264*, 271−275.
(148) Nielson, K. J.; Schroeder, T.; Lewis, R. *J. Mol. Recognit.* **2000**, *13*, 55−70.
(149) McIntosh, J. M.; Santos, A. D.; Olivera, B. M *Annu. Rev. Biochem.* **1999**, *68*, 59−88.
(150) Favreau, P.; Le Gall, F.; Benoit, E.; Molgo, J. *Acta Physiol. Pharmacol. Ther. Latinoam.* **1999**, *49*, 257−267.
(151) Olivera, B. M.; Cruz, L. J. *Toxicon* **2001**, *39*, 7−14.
(152) Cruz, L. J. In *Natural Toxins II*; Singh, B. R., Tu, A. T., Eds.; Plenum Press: New York, 1996; pp 155−167.
(153) Jones, R. M.; Bulaj, G. *Curr. Pharm. Des.* **2000**, *6*, 1249−1285.
(154) Chiang, J. S.-T. *Acta Anaesthiol. Sin* **2000**, *38*, 31−36.
(155) Dutton, J. L.; Craik, D. J. *Curr. Med. Opin.* **2001**, *8*, 327−344.
(156) Charlet, M.; Chernysh, S.; Philippe, H.; Hetru, C.; Hoffman, J.; Bulet, P. *J. Biol. Chem.* **1996**, *271*, 21808−21813.
(157) Hubert, F.; Noel, T.; Roch, P. *Eur. J. Biochem.* **1996**, *240*, 302−306.
(158) Tunkijjanukij, S.; Olafsen, J. A. *Dev. Comput. Immunol.* **1998**, *22*, 139−150.
(159) Iwanga, S.; Kawabata, S.-I. *Front. Biosci.* **1998**, *3*, 973−984.
(160) Miyata, T.; Tokunaga, F.; Yoneya, T.; Yoshikawa, K.; Iwanaga, S.; Niwa, M.; Takao, T.; Shimonishi, Y. *J. Biochem. (Tokyo)* **1989**, *106*, 663−668.
(161) Nakashima, H.; Masuda, M.; Murakami, T.; Koyanagi, Y.; Matsumoto, A.; Fujii, N.; Yamamoto, N. *Antimicrob. Agents Chemother.* **1992**, *36*, 1249−1255.
(162) Tamamura, H.; Otaka, A.; Murakami, T.; Ibuka, T.; Sakano, K.; Waki, M.; Matsumoto, A.; Yamamoto, N.; Fujii, N. *Biochem. Biophys. Res. Commun.* **1996**, *229*, 648−652.
(163) Murakami, T.; Nakajima, T.; Koyanagi, Y.; Tachibana, K.; Fujii, N.; Tamamura, H.; Yoshida, N.; Waki, M.; Matsumoto, A.; Yoshie, O.; Kishimoto, T.; Yamamoto, N.; Nagasawa, T. *J. Exp. Med.* **1997**, *186*, 1389−1393.
(164) Lee, I. H.; Zhao, C.; Cho, Y.; Harwig, S. S.; Cooper, E. L.; Lehrer, R. I. *FEBS Lett.* **1997**, *400*, 158−162.
(165) Lee, I. H.; Cho, Y.; Lehrer, R. I. *Comp. Biochem. Physiol.* **1997**, *118B*, 515−521.
(166) Kem, W. R. *J. Biol. Chem.* **1976**, *251*, 4184−4189.
(167) Kem, W. R.; Blumenthal, K. M. *J. Biol. Chem.* **1978**, *253*, 5257−5262.
(168) Kem, W. R. *Toxicology* **1994**, *87*, 189−203.
(169) Opric, M. M.; Poznanovic, S.; Kljajic, Z.; Sladic, D.; Pupic, G.; Perunovic, B.; Gasic, M. J. *Eur. J. Histochem.* **1996**, *40*, 211−218.
(170) Miarons, P. B.; Fresno, M. *J. Biol. Chem.* **2000**, *275*, 29283−29289.
(171) Martin, J. V.; Koenig, M. L.; McClure, W. O. *Toxicon* **1992**, *30*, 1001−1010.
(172) Oiki, S.; Muramatsu, I.; Matsunaga, S.; Fusetani, N. *Nippon Yakurigaku Zasshi* **1997**, *110* (Suppl. 1), 195−198.
(173) Morel, J.-L.; Drobecq, H.; Sautiere, P.; Tatar, A.; Mironneau, J.; Qar, J.; Lavie J.-L.; Hughes, M. *Mol. Pharmacol.* **1997**, *51*, 1042−1052.
(174) Mangel, A.; Leitao, J. M.; Batel, R.; Zimmermann, H.; Müller, W. E. G.; Schröder, H. C. *Eur. J. Biochem.* **1992**, *210*, 499−507.
(175) O′Keefe, B. R.; Beutler, J. A.; Cardellina, J. C., II; Prather, T. R.; Shoemaker, R. H.; Sowder, R. C., II; Henderson, L. E.; Pannell, L. K.; Boyd, M. R. *J. Nat. Prod.* **1997**, *60*, 1094−1099.
(176) Zidane, M.; Pondaven, P.; Roussakis, C.; Quéméner, B.; Moré, M. T. *Comp. Biochem. Physiol.* **1996**, *115C*, 47−53.
(177) Le Pape, P.; Zidane, M.; Abdala, H.; Moré, M. T. *Cell Biol. Int.* **2000**, *24*, 51−56.
(178) O'Keefe, B. R.; Beutler, J. A.; Cardellina, J. H., II; Gulakowski, R. J.; Krepps, B. L.; McMahon, J. B.; Sowder, R. C., II; Henderson, L. E.; Pannell, L. K.; Pomponi, S. A.; Boyd, M. R. *Eur. J. Biochem.* **1997**, *245*, 47−53.
(179) O′Keefe, B. R.; Erim, T.; Beutler, J. A.; Cardellina, J. H., II; Gulakowski, R. J.; Krepps, B. L.; McMahon, J. B.; Sowder, R. C., II; Johnson, D. G.; Buckheit, R. W., Jr.; Halliday, S.; Boyd, M. R. *FEBS Lett.* **1998**, *431*, 85−90.
(180) MacKenzie, R.; Newman, D.; Burger, M. M.; Roy, R.; Kuhns, W. J. *Biol. Bull.* **2000**, *199*, 209−211.

NP0103362