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Perspective

Ernest Guenther Award in Chemistry of Natural Products. Amphibian Skin: A Remarkable Source of Biologically Active Arthropod Alkaloids

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Chemistry in a biomedical institute has an imperative to have "the potential to have an impact on biomedical research". Thus, during my career of over 40 years at the National Institutes of Health, there was a need to select chemical problems with relevance to the biomedical mission. Natural products research was an ideal choice, since the field has had an incredible record for impact on biomedical research, leading to the introduction or development of the majority of all therapeutic agents. There have been two strategies for natural product chemists. The first, more classical approach has been to prepare extracts of a plant or other material and by fractionation and chromatography to isolate compounds for characterization and elucidation of structures. However, in many cases, the material to be extracted was chosen because of a known or reputed biological activity, often toxicity. The second approach, now widely practiced, is to isolate compounds, based on a biological activity or target, using sophisticated screening techniques and assays.

Our own natural products research has focused on amphibians and the biologically active substances found in their skin. Historically, amphibian skin has provided many biologically active compounds. Vasoactive, analgetic, and antibiotic peptides, such as physalaemin, caerulein, sauvagine, dermorphin, and magainin,[1,2] biogenic amines, including serotonins, tryptamines, histamines, and tyramines,[1] cardioactive steroidal bufadienolides,[3] tetrodotoxins,[4] and one novel structural class of steroidal alkaloids, the samandarines,[5] have been found in amphibian skin. Over the years, our research has produced a rich harvest of unique alkaloids, some of which have had a major impact on biomedical research (Figure 1). However, our initial foray into amphibian skin extracts came about because Indians on the Pacific coast of Colombia were using small, brightly colored frogs (*Phyllobates species*) to poison their blow-darts. It could hardly have been predicted that the toxic substances from such frogs would prove to be steroidal alkaloids and that such alkaloids, to be named batrachotoxins, would figure prominently in decades of research on sodium channel function and as a radioligand in defining local anesthetic, anticonvulsant, and antiarrhymic activity of various agents. At that time, the only alkaloids known from amphibians were the samandarines, highly toxic compounds from the European fire salamander. Fractionation of the skin extracts of the Colombian poison-dart frogs was guided at first by toxicity assays until the active principles were found to be alkaloids that could be detected by Ehrlich's pyrrole/indole reagent. Ultimately, three alkaloids were isolated and structures were determined through X-ray analysis of a *p*-bromobenzoate derivative of the least toxic one to define the steroid moiety, followed by NMR analyses to define the nature of a dialkylpyrrole carboxylate moiety and then by partial synthesis to confirm the structure.[6] Recently, the highly toxic batrachotoxins have been detected in skin and feathers from birds of Papua New Guinea.[7,8]

From the poison-dart frogs of Western Colombia, the research moved on to a variable species of brightly colored frogs (*Dendrobates pumilio*) of the same family Dendrobatidae that occurred on the Caribbean coast of

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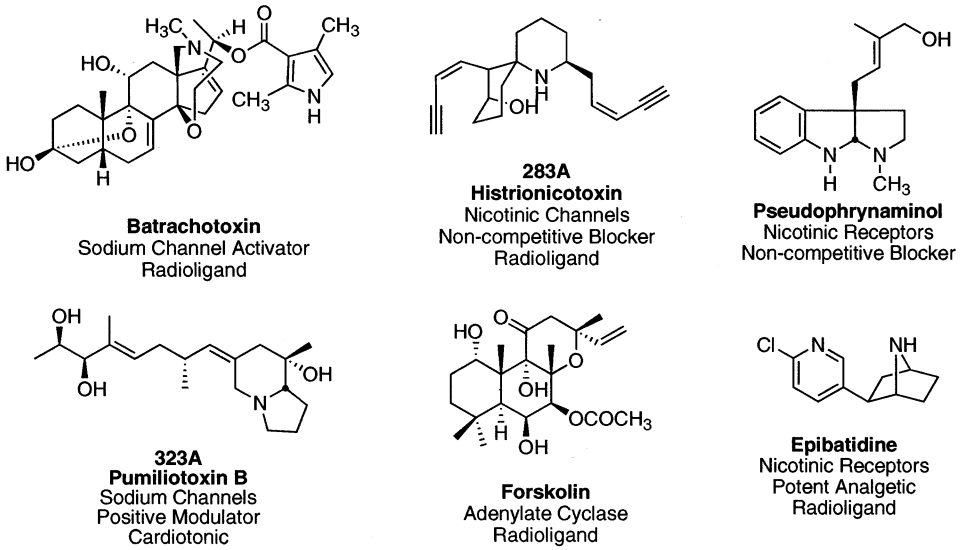


Figure 1. Natural products as pharmacodynamic probes: structures and targets. Development of radioligands is indicated.

Panama. Such frogs were known to have toxic secretions on handling and also to other frogs when put into the same collecting bag. The frog project would not have progressed so successfully had I not been contacted in 1965 about such Panamanian poison frogs by a herpetologist, Charles Myers, then doing his Ph.D. thesis on reptiles/amphibians of Panama. Again, it could not have been predicted that Myer's proposed project, namely, to determine whether the frog's bright warning coloration correlated with toxicity of skin extracts, would have a biomedical impact. There proved to be no correlation,[9] but the project led to years of collaboration with Myers and scores of joint expeditions to rain forests of the neotropics. The initial collaboration with Myers in Panama ultimately resulted in isolation and structure elucidation of unique new alkaloids,[10] which we designated as pumiliotoxins and allopumiliotoxins (7-hydroxypumiliotoxins) (Figure 1). Such alkaloids would prove to be found in almost all alkaloid-containing species and populations of frogs/toads from diverse habitats in Central and South America, Madagascar, and Australia.[11] The pumiliotoxins/allopumiliotoxins were shown to have myotonic and cardiotonic activity due to positive modulation of sodium channels and to have stimulatory effects, probably indirect, on the formation of inositol phosphates.[12−14] The cardiotonic activity of the pumiliotoxins deserves further study in an effort to develop an analogue that would have minimal cardiotoxic effects at higher dosages.

The field work with Myers then shifted to another brightly colored and variable poison frog species (*Dendrobates histrionicus*) of the family Dendrobatidae found on the Pacific coast of Colombia. From skin extracts of an abundant population collected in a Pacific river basin of Colombia near the Ecuadorean border, two members of a unique histrionicotoxin class of alkaloids (Figure 1) were isolated and structures were determined by X-ray analysis.[15] The histrionicotoxins were to become important "high-affinity" noncompetitive blockers for nicotinic channels.[16,17] By this point, it was clear not only that the investigation of alkaloids present in frog skin would yield structurally unique compounds but also that many would have remarkable biological activity.

The research of our group was not directed solely at discovery of new, biologically active natural products but also sought, through molecular pharmacology, to determine functional roles of enzymes and receptors and how second messengers and ion channels could be affected by "small molecules", including our frog alkaloids. Because of our reputation for defining sites of action of natural products and investigating second messenger and ion channel function, we were sent a sample of a diterpene, forskolin (Figure 1), that was known to stimulate cardiac function through an unknown mechanism. Our investigation of forskolin revealed that it directly stimulated adenylate cyclase and enhanced G-protein-mediated stimulation of this enzyme.[18] Forskolin rapidly became a powerful tool for investigation of adenylate cyclase and the role of cyclic AMP in physiological processes. For the batrachotoxins, histrionicotoxins, and forskolin, we developed radiolabeled probes to investigate sites of action.

Two of the other alkaloids, pseudophrynaminol and epibatidine, that we discovered (Figure 1) in frog skin extracts proved to be quite remarkable. Pseudophrynaminol represented one member of a novel indolic class of alkaloids, the pseudophrynamines, that had never been detected in nature until they were discovered in extracts from one genus (*Pseudophryne*) of Australian frogs of the family Myobatrachidae.[19] Structurally, such alkaloids resemble the physostigmine class but had no effect on acetylcholine esterase. Instead, like the histrionicotoxins, they were potent blockers of nicotinic channels.[20] Many of the other classes of frog skin alkaloids, including decahydroquinolines, various izidines, piperidines, pyrrolidines, and spiropyrrolizidines, also have proven to be blockers of nicotinic channels.[21,22] The pseudophrynamines, found in skin extracts from the Australian frogs, were accompanied by a limited range of pumiliotoxins and allopumiliotoxins.[23] Recent results indicate that these Australian frogs produce the pseudophrynamines while obtaining the pumiliotoxins from the diet.[24]

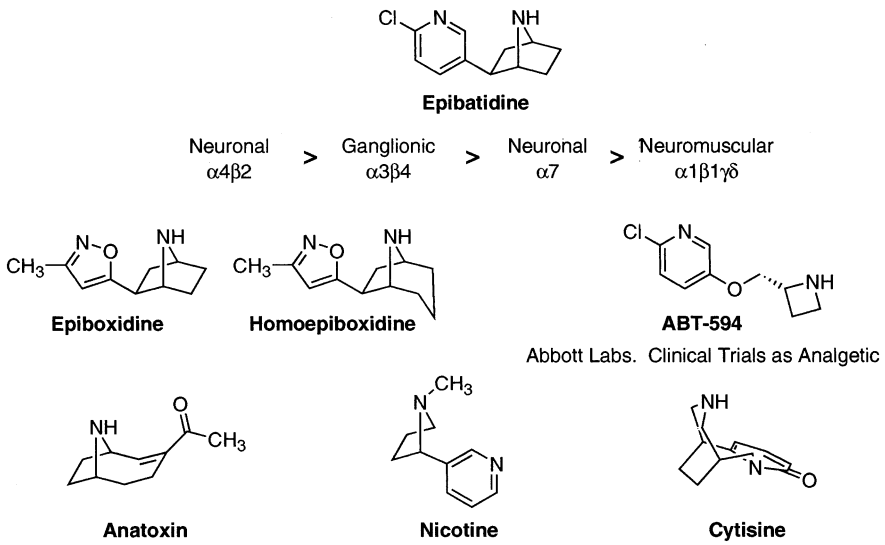


Figure 2. Nicotinic agents: epibatidine and analogues and other natural nicotinic agonists. Relative activity of epibatidine at four major subtypes of nicotinic receptors is indicated.

The discovery of epibatidine and subsequent definition of the basis for its potent analgetic activity has had a tremendous impact on research on nicotinic receptors and function. The presence of epibatidine might not have been suspected if an aliquot of the methanolic extract from an Ecuadorean frog collected in 1974 had not been injected into a mouse in a routine toxicity assay. The mouse was observed to arch its tail over its back, a drug reaction known as the Straub tail. Excited and suspecting an opioid alkaloid, a return with Myers to the collection site yielded several hundred frogs. The Straub tail provided an initial assay for isolation of an alkaloid that, after structure elucidation, we named epibatidine.[25] It was a trace alkaloid in the extracts at levels of 1 μg per frog. Structure elucidation by NMR spectral analysis of an *N*-acetyl derivative was finally accomplished in 1992. Epibatidine had proved to be 200-fold more potent than morphine as an analgetic. But to elucidate the basis of such activity required synthesis of sufficient material. With synthetic epibatidine from E. J. Corey's laboratory,[26] our group showed that the analgetic activity of epibatidine was due to potent agonist activity at nicotinic receptors.[27] The introduction of epibatidine as a nicotinic agonist and analgetic provided the impetus for major synthetic efforts to obtain an analogue that would retain analgetic activity while being much less toxic. One such analogue, ABT 594, underwent phase I and phase II clinical trials as an analgetic.[28] Undoubtably other analogues are still under investigation. Epibatidine, some analogues, and other natural nicotinic agonists are illustrated in Figure 2.

Other alkaloids from frog skin, including batrachotoxins, pumiliotoxins, allopumiliotoxins, homopumiliotoxins, decahydroquinolines, histrionicotoxins, and various izidines also have provided challenges for syntheses in laboratories throughout the world. Such synthetic work, prior to 1993, has been reviewed.[29] To our knowledge, there is no recent comprehensive review. In the case of pseudophrynaminol,[20] the spiropyrrolizidine oxime **236**,[30] lehmizidine **275A**,[31] and certain other alkaloids from frog skin, synthesis was required for structure elucidation and to supply sufficient material for biological evaluation because of the limited amounts available to us from natural sources. Our biological investigation of pumiliotoxins/allopumiliotoxins benefited greatly from synthetic efforts of L. E. Overman.[32]

Our research has focused on alkaloids, but certain skin extracts that we studied did not have alkaloids but had other classes of active substances. Research in our laboratory has led to the discovery of bufadienolides in genera of toads other than *Bufo* from Central and South America and Thailand (ref 33 and unpublished data from our laboratory). Some are structurally different from typical bufadienolides. The bufadienolides appear to be produced by toads of the genus *Atelopus*,[34] as had been previously demonstrated for toads of the genus *Bufo*.

Tetrodotoxins and bioactive peptides in frog/toad skin extracts have also been discovered during the course of our research. Tetrodotoxins, now known from several lineages of amphibians (see ref 35 and references therein), appear likely to be produced in *Atelopus* toads by a symbiotic microorganism.[34] A 33 amino acid peptide, adenoregulin, is produced by the hylid tree frog from which it was isolated[36] and has remarkable effects on the GTPase activity of G proteins involved in receptor signaling.[37] The frog was investigated because of the use of its skin secretions in a "hunting magic" ritual by Indians of the Amazon.

In the course of the 4 decades of analyzing for alkaloids, over 500 alkaloids of about 20 structural classes have been detected and characterized by our group.[38] Species of amphibians from over 80 genera and 11 families have been examined. There remain many further genera as yet not investigated. In our studies, alkaloids have been detected in only seven genera, four in the family Dendrobatidae and one genus in each of three other families, namely, South American *Melanophryniscus* of the family Bufonidae, *Mantella* of the Madagascan family Mantellidae, and *Pseudophryne* of the Australian family Myobatrachidae. In the family Dendrobatidae, the batrachotoxins occur only in the genus *Phyllobates*, the pumiliotoxins in all four genera (*Phyllobates*, *Dendrobates*, *Epipedobates*, *Minyobates*), the histrionicotoxins in all but *Minyobates*, and epiba-

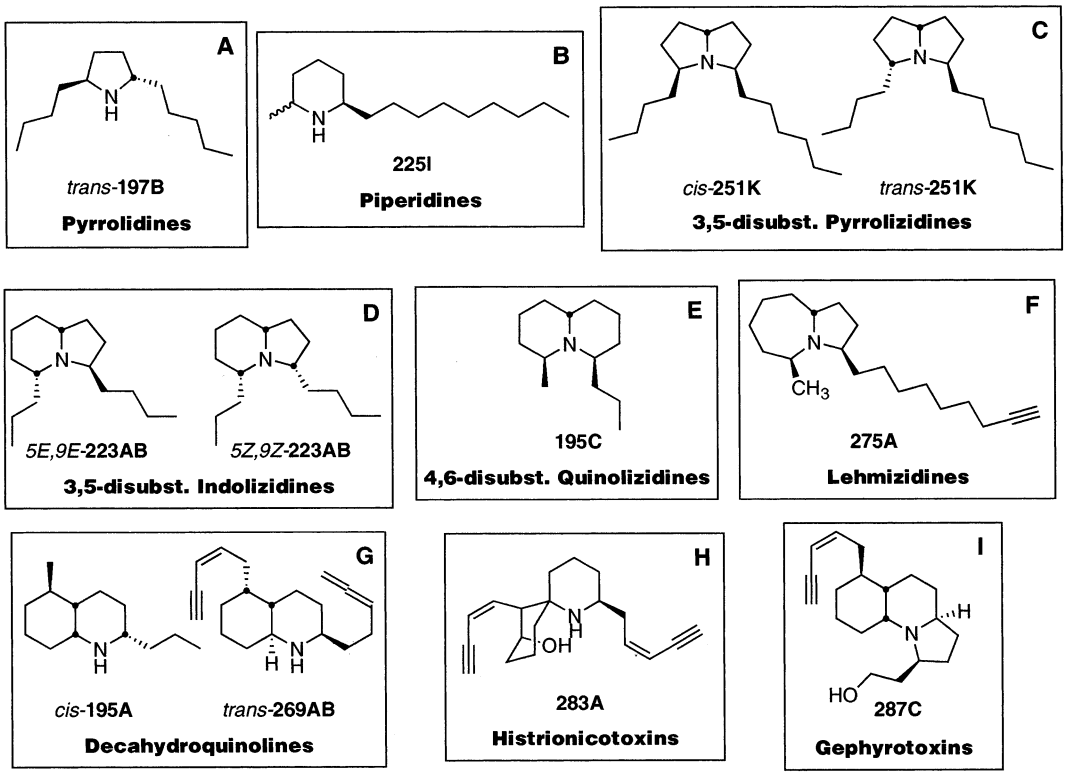


Figure 3. Frog skin alkaloids either demonstrated (A−E, G) or expected (F, H, I) to be present in myrmicine ants. Such ants are a major component in the diet of many alkaloid-containing poison frogs.

tidine only in *Epipedobates*. At one time, it was felt that the skin alkaloids were produced by the frogs. However, frogs raised in captivity had no skin alkaloids and it eventually became clear that the skin alkaloids were sequestered unchanged from alkaloid-containing arthropods.[39] Alkaloids fed to captive-raised dendrobatid and mantellid frogs were sequestered unchanged into skin glands.[40,41] Representatives of many of the frog skin alkaloid classes have now been detected in arthropods.[42] The pyrrolidines, piperidines, pyrrolizidines, certain indolizidines, certain quinolizidines, and decahydroquinolines have been detected in ants, while it is suspected that the lehmizidines, histrionicotoxins, and gephyrotoxins ultimately will be found in ants, based on structural similarities to known ant alkaloids, namely, an unbranched carbon skeleton and unsaturation (enes, ynes, and allenes) in the side chains (Figure 3). Frog skin tricyclic alkaloids, including or related to the coccinellines, undoubtedly come from beetles, while the spiropyrrolizidine oximes undoubtedly come from millipedes (Figure 4). However, the putative arthropod source of the batrachotoxins, pumiliotoxins, allopumiliotoxins, homopumiliotoxins, certain izidines, and epibatidine remained a mystery.

It was obvious that in order to identify the dietary source, one would have to collect and analyze leaf-litter arthropods from a locale where the frog had high levels of the particular alkaloid of interest. For the batrachotoxins, this would mean the remote Rió Saija drainage on the Pacific coast of Colombia, where the frog *Phyllobates terribilis* contains about 1 mg of batrachotoxins per skin. However, permits for such field work in Colombia are now impossible to obtain. For the histrionicotoxins, there are several possible sites of which the ones in Costa Rica are most accessible. For

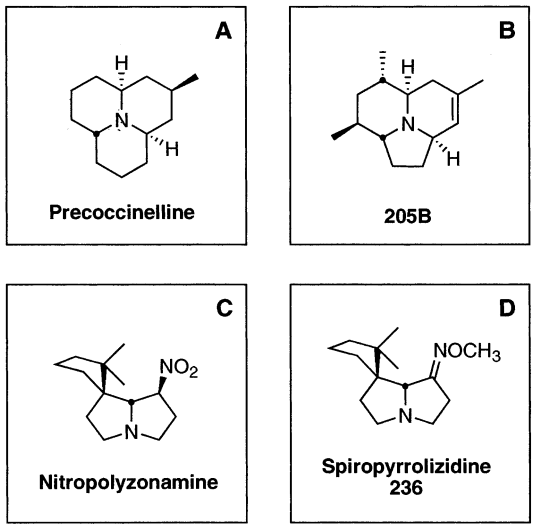


Figure 4. Frog skin alkaloids either demonstrated (A, C, D) or expected to be present in beetles (A, B) or millipedes (C, D).

epibatidine, sites in Ecuador, where epibatidine was found in the frog *Epipedobates tricolor*, would most likely provide the dietary source. At one site, epibatidine was present at about 1 μg per skin, while at a nearby riparian site it was present at only 100 ng per skin. It appears likely that a food chain from a plant may pertain. Thus, a plant may produce the nicotine-like epibatidine, which is taken up by an arthropod and then passes to the frog. For the pumiliotoxins, sites on a small island, Isla Bastimentos in Panama, where the frog *Dendrobates pumilio* had high levels of pumiliotoxins in skin, were easily accessible for collecting leaf-litter arthropods. But it was realized that hundreds of dif-

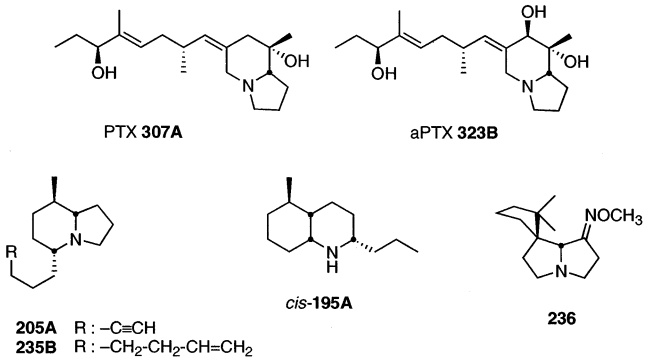


Figure 5. Combinatorial bioprospecting: alkaloids detected in extracts of combined collections of leaf-litter arthropods from eight sites on Isla Bastimentos, Panama.[42] All were present in skin extracts of dendrobatid frogs from the same sites.

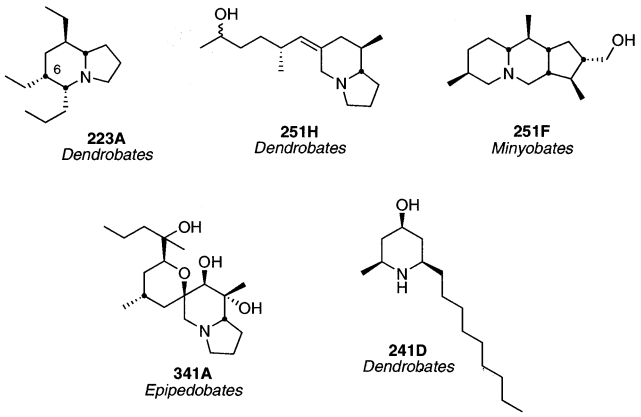


Figure 6. Further unique alkaloids from neotropical dendrobatid frogs. The genus from which each was isolated is indicated. The original proposed relative configuration at C-6 of alkaloid **223A** proved to be incorrect and is as shown.[51]

ferent species of small arthropods would occur at such sites and that an exhaustive collection of each species still might fail because of seasonal hatches or annual population fluctuations of the sought-after alkaloid-containing arthropods. Thus, for an initial effort "combinatorial bioprospecting" was attempted, wherein all the arthropod species were collected together at several sites on Isla Bastimentos in Panama, where the resident dendrobatid frog had high levels of pumiliotoxins. With three collectors and several collection sites, a total of 22 mixed arthropod collections were obtained. There appeared to be over 200 species of arthropods in these mixed collections. Analysis revealed the presence of one pumiliotoxin, one allopumiliotoxin, two 5,8-disubstituted indolizidines, and one decahydroquinoline.[43] The structures are shown in Figure 5. Thus, further collection at these sites of separate arthropod species and analysis undoubtably will reveal the arthropod source of the widely distributed frog skin pumiliotoxins and 5,8-disubstituted indolizidines. Such studies are likely to reveal new arthropod genera/families for investigation of novel biologically active alkaloids.

Our initial research had led to isolation and pharmacological characterization of major alkaloids, namely, the batrachotoxins, pumiliotoxins, and histrionicotoxins, found in skin extracts from often large numbers of frogs. However, for the past 2 decades, the challenge has been to characterize and elucidate the structure of minor and trace alkaloids. Permits to collect large numbers of often abundant frogs are no longer possible to obtain. Synthesis now usually is needed to confirm structures and to provide sufficient material for biological investigation. Thus, the characterization of further new alkaloids is truly a major challenge; only limited numbers of frogs can be collected, and an extract from a single population of frogs can contain upward of 80 alkaloids. Spectroscopic techniques, including gas chromatography, high-performance liquid chromatography−mass spectrometry, vapor-phase Fourier transform infrared spectroscopy (FTIR), and after isolation, nuclear magnetic resonance (NMR) spectral analysis on alkaloids, frequently available only in amounts of a few hundred micrograms or less, now play a major role in structure elucidation. Chemical ionization tandem mass spectrometry with collision-activated fragmentation has proven to be complementary to the usual electron-impact mass spectral analysis,[31,44,45] while vapor-phase FTIR spectra have provided important stereochemical data.[42,46,47] In addition, microchemical manipulations have been developed to provide further structural insights.

The research has been hindered by difficulties in obtaining permits to collect any amphibians for scientific investigation, especially in neotropical countries of Central and South America, where the alkaloid-containing dendrobatid frogs are found. For this reason, in the past decade our research has shifted to bufonid frogs of Argentina[48] and to mantellid frogs of Madagascar.[49] The mantellid frogs of the genus *Mantella* have afforded a variety of further structural classes of alkaloids.[11,50] Some are still under investigation, including the following: (1) alkaloid **235C**, formerly proposed to be a dehydrohomopumiliotoxin,[49] but now known to represent a unique new class; (2) a tricyclic alkaloid **261C**, related to the coccinellines, but with a 6,5,5 ring system rather than 6,6,6 and with ethyl, propyl, and allyl substituents; (3) a highly oxygenated alkaloid **392** with an empirical formula of $C_{22}H_{36}N_2O_4$; (4) a series of alkaloids, including **384A/B**, that appear likely to be adducts of the Diels−Alder type of octa- and hexahydroquinoline analogues of decahydroquinoline **195A**.[38]

Further new structural classes of alkaloids (see Figures 6 and 7) continue to be defined from both "old" and newly obtained extracts of frogs/toads[51−55] as the power and sensitivity of spectroscopic techniques continue to increase. The challenge of returning to an activity-driven investigation of extracts is now being met with multiwell microfluorometric or radiometric techniques that require very little material. The first frog skin extract whose HPLC fractions were analyzed for nicotinic activity has yielded a novel new structural class of nicotinic agonists (unpublished results of R. Fitch, T. Spande, H. M. Garraffo, H. J. C. Yeh, and J. W. Daly). There remain over 100 alkaloids detected in frog skin extracts for which we cannot as yet assign a structure.

From a biodiversity standpoint, the marked variation in alkaloid profiles in skin extracts from different populations of the same species of frogs[50,56] suggests that certain alkaloid-containing arthropods have a very limited distribution. Thus, the profiles can differ remarkably even for populations close together on a single island.[43] In addition, the profile and presence or absence

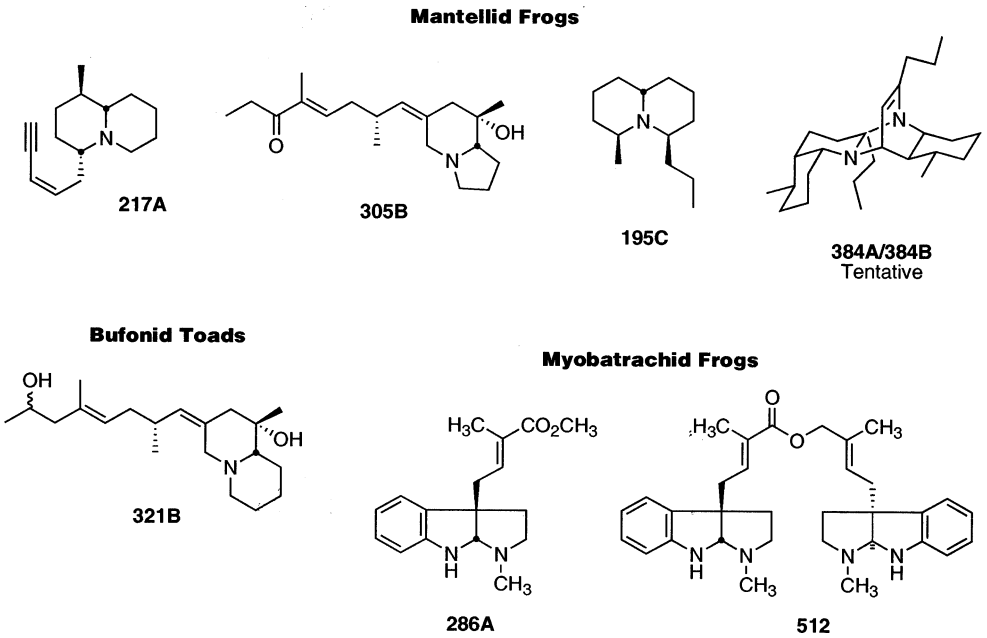


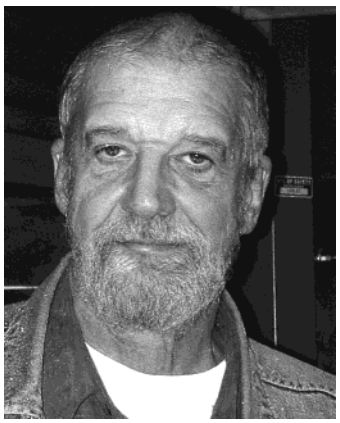
Figure 7. Further unique alkaloids from Madagascan mantellid frogs (*Mantella*), South American bufonid toads (*Melanophryniscus*), and Australian myobatrachid frogs (*Pseudophryne*). Additional novel alkaloids (**235C**, **261C**, **392**, and **434**) from mantellid frogs are still under investigation (see text).

of alkaloids can change markedly over the years probably because of alteration of ecosystems by human inroads. Thus, destruction or alteration at a single site can likely cause extinction of an arthropod species that was the source of a unique alkaloid whose structure and possible activity will never be known.

Some examples of alkaloids with limited geographic occurrence in frogs are the following: (1) batrachotoxins, which are present in high levels only in the three *Phyllobates species* of Western Colombia and, indeed, are absent in most populations of a related Panamanian species, *Phyllobates lugubris*; (2) epibatidine, which was detected in "significant" amounts only in two populations of *Epipedobates tricolor* but was absent in several other populations in Ecuador; (3) the histrionicotoxins, which have been detected only in neotropical frogs of the family Dendrobatidae and then only in certain populations; (4) the tricyclic alkaloid **205B**, which was abundant only in certain Panamanian populations of the dendrobatid frog *Dendrobates pumilio*; (5) the unusual tricyclic **251F**, which was present in significant amounts only in a tiny dendrobatid frog, *Minyobates bombetes*, found in a remnant forest some 50 km from Cali, Colombia; (6) the lehmizidines, which were present in significant amounts only in the dendrobatid frog *Dendrobates lehmanni*, found in cloud forests near Cali, Colombia (remarkably, the *D. lehmanni* frogs lack the histrionicotoxins that are characteristic of a closely related frog, *Dendrobates histrionicus*, found at lower elevations throughout the Pacific Coast of Colombia); (7) the tricyclic alkaloid **261C**, which was present only in frogs of the genus *Mantella* that are found in arid habitats of western Madagascar; (8) certain alkaloids, such as **235C** and **392**, the structures of which are still under investigation, which were present only in *Mantella* species from swamp habitats in eastern Madagascar. Thus, the ongoing efforts of natural product chemists to discover by chance new substances with unsuspected and perhaps important biological activities depend on both the preservation of such ecosystems and the permission for scientists to sample and study the components of such systems. There can be no doubt that further bioprospecting, driven both by the presence of unique compounds and by microassays for biological activities, will provide agents that will have a major impact not only on biomedical research but also on the role of such compounds in the biology and ecology of our biosphere.

Acknowledgment. Our natural products program at NIH has succeeded because of the contributions of many individuals. I most gratefully acknowledge the contributions of my mentor and companion for over 3 decades in field work, biologist Charles W. Myers, now retired, of the American Museum of Natural History in New York; chemist Takashi Tokuyama, now retired, who began investigating batrachotoxin with me in the 1960s and continued for over 3 decades to collaborate with our group; X-ray crystallographer Isabella L. Karle; NMR spectroscopist H. J. C. Yeh; pharmacologist Edson X Albuquerque for his insightful studies on the pharmacology of the batrachotoxins and histrionicotoxins; chemist Tappey H. Jones for his contributions to the frog−arthropod connection; and many outstanding students and pharmacologists. Many others have contributed, but special homage goes to molecular pharmacologist Fabian Gusovsky, formerly of my group, and to two chemist colleagues of my group, H. Martin Garraffo and Thomas F. Spande. The last two have for the past 2 decades contributed in so many ways to our natural products program. Many chemists throughout the world have taken the synthetic challenge posed by the frog skin alkaloids and generously provided the synthetic products to us for biological evaluation. Finally, I thank Dr. Bernhard Witkop for starting me on this most rewarding sojourn.

Biography



John W. Daly was born in Portland, Oregon, and received his B.S. and M.A. degrees in biochemistry and organic chemistry, respectively, at Oregon State College and subsequently a Ph.D. in organic chemistry from Stanford University in 1958. After postdoctoral work at NIH, he became a permanent staff member in 1960 and was the founding chief of the Laboratory of Bioorganic Chemistry in 1981. He has pioneered the isolation of natural products, many of which have unusual chemical structures and biological activities. A principal effort has been in the isolation of alkaloids from frog skin, where he has discovered over 500 members from some 20 structural classes. He has received many honors during his career, including membership in the National Academy of Sciences in 1997. Outside of science, John W. Daly is an avid fisherman.

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