Pharming Frogs

Chemist finds precious alkaloids in poisonous amphibians

A colorful dendrobatid frog (below) and Epipedobates tricolor *(right), which contains a chemical that may be a better analgesic than morphine.*

George Grall/National Aquarium

By ELIZABETH PENNISI

John W. Daly mines the skins of tropical frogs — not for mineral treasures, but for new medicines. A molecular pharmacologist at the National Institutes of Health (NIH) in Bethesda, Md., he has staked his claim early in what may become the 21st century's gold rush.

As part of that rush, dozens of chemical prospectors have begun taking biopsies of the world's flora and fauna. Concern about lost species fuels this rush, as does a growing appreciation of the untapped pharmacological and chemical resources in many of these organisms.

For almost 30 years, Daly has panned for alkaloids — complex, often bitter compounds most often produced by plants (see p.42). During the 1960s, he teamed up with Charles W. Myers, a herpetologist now with the American Museum of Natural History in New York City, to collect frogs in Panama. Myers hoped Daly's chemical analysis of the content of frog skins would help resolve the very fuzzy evolutionary history of these amphibians.

The two have collaborated ever since. They have traveled to tropical rain forests throughout South and Central America, identified dozens of new species of frogs, and collected many other hitherto unnamed organisms.

Like prospecting for gold, the search for useful alkaloids has demanded perseverance — and luck. Altogether, Daly has surveyed 11 amphibian families, sampling species from about 75 genera.

Any strikes? Seven.

One group of frogs, a family called Dendrobatidae, contains four genera with alkaloid skins. The other strikes came from unexpected species: a yellow-and-black Australian frog, a Madagascan genus supplied to Daly by a pet dealer, and a red-bellied toad Daly happened to collect while in Brazil.

Over the years, Daly and his NIH group have characterized more than 225 different compounds. Daly and Myers found that they could use the presence of one alkaloid, batrachotoxin, to show that several species had a common ancestor. But other frogs, only distantly related and living in very different habitats, produced very similar alkaloids. In some cases, frogs of a single species varied considerably in their alkaloid chemistries. Myers and Daly still don't understand why.

Some alkaloids, such as batrachotoxin, have helped neurobiologists study sodium channels — molecular pores in nerve cell membranes that help control the excitation of nerve cells. Also thanks to batrachotoxin, neurobiologists now understand better how local anesthetics work, Daly says. One alkaloid, called epibatidine, numbs more effectively than morphine, and several others show promise as heart attack medicines.

Chemical prospecting comes with its share of unexpected obstacles. Some alkaloids proved easy to identify; others took many years. And while Daly need not worry unduly about claim jumpers, he does have to deal with international conventions to safeguard these colorful amphibians from extinction. These regulations have largely cut him off from his sources. Finally, some frogs will breed in the laboratory but grow up barren of alkaloids, adding to Daly's frustration and confusion about the biological origins and significance of these chemicals. So even with years of prospecting behind him, many more years lie ahead before his strikes yield those sought-after medicines.

In 1977, while collecting in Ecuador, Daly and Myers caught seven specimens of an unfamiliar frog, *Epipedobates tricolor.* As always, Daly extracted the chemical contents of their skins, then injected a small amount of the extract into a mouse. Up went the mouse's tail, arching over its back, a response usually triggered by opioid compounds such as morphine. But when Daly gave the mouse a compound that blocks morphine's analgesic effects, the mouse still arched its tail. Daly was surprised and excited.

He knew then that he had discovered a new kind of anesthetic. "It works differently from morphine and it's manyfold more active," says Daly. But he lacked a large enough sample to decipher the compound's identity.

So the next year, he returned to Ecuador and collected enough frogs to make 60 milligrams of alkaloid extract. He then began to isolate the active ingredient. It took a decade, but this spring, he and his colleagues described the structure of epibatidine in the April 22 JOURNAL OF THE AMERICAN CHEMICAL SOCIETY.

The analysis took a lot of work.

First, Daly separated the alkaline components from one another using high-pressure liquid chromatography. Then he used mass spectrometry to determine the size of the molecules in the component that made the mouse arch its tail. Mass spectral data indicate the size of whole molecules — the key to determining the number of hydrogen, nitrogen, car-

bon, and other atoms in each component. He found that this new compound's molecular weight was either 208 or 210.

Next, Daly chopped up the alkaloid's molecules and analyzed the sizes and atomic constituents of the fragments. "Each class of alkaloids gives a characteristic breakdown," he explains. He learned that two major fragments made up epibatidine.

David Barker/National Aquarium

Daly focused first on the larger fragment. He considered the possible combination of atoms that could add up to its molecular weight and decided it must consist of six carbon atoms, 10 hydrogen atoms, and one nitrogen atom. He exposed the compound to deuterium and monitored the increase in molecular weight, which helped him determine that nitrogen bonded to only one hydrogen atom.

Five carbon, one nitrogen, three hydrogen, and a chlorine atom made up the second fragment. In some molecules, a heavy chlorine isotope resulted in a molecular weight of 210, not 208.

But that was as far as Daly got in his analysis. By this point, his stock of the compound was down to 500 micrograms. "Because of the tiny quantity, I was hesitant to try to do more," he says.

So he stored the compound and went on to other projects.

Nine years later, Daly decided to try again, hoping that technological advances in analytical equipment would make up for the small sample. "We knew if we were lucky, we could do the NMR [nuclear magnetic resonance] analysis," he recalls. Also, he had obtained a new, highly sensitive infrared spectrometer, which would aid identification.

Daly exposed the alkaloid molecule to infrared light of different wavelengths. Each type of bond between atoms absorbed a characteristic wavelength. So by measuring the amount of absorption of each wavelength and comparing those data with absorption data from known compounds, Daly concluded that in one part of the epibatidine molecule, a ring with three double bonds connected five carbon atoms and one nitrogen atom. The chlorine atom stuck out from one of the ring's carbon atoms.

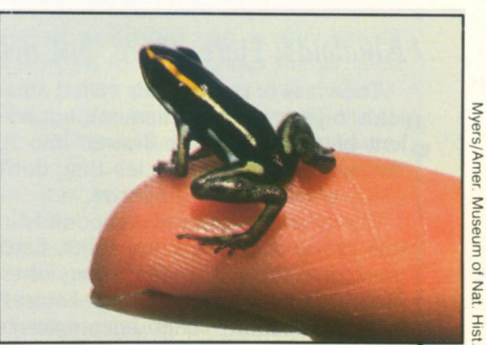
But the other fragment still stumped him. He knew by the ratio of carbon atoms to hydrogen atoms that this fragment contained two rings. Infrared spectroscopy told him the rings lacked double bonds. But he could not determine the arrangement of atoms in this fragment or how the fragments linked up. Also, the sample at that time contained a few impurities that confounded the data.

Then Daly's group came up with a way to purify the sample. They exposed it to a compound called acetic anhydride, which reacted only with the nitrogen in epibatidine, changing it slightly so it could be separated from the sample's impurities. The group then analyzed the resulting N-acetylepibatidine with NMR.

The plan worked—too well. "It gave us the structure, which was nice, but we couldn't get the acetyl group back off without destroying the molecule," says Daly. It turns out that the other nitrogen atom, which exists in the ring of the second fragment, made it difficult for chemists to get plain epibatidine. Pharmacologists need this plain form so they can study the compound's therapeutic potential.

Those two nitrogen atoms have also bedeviled Daly's synthetic chemists. For a year now they have sought to make epibatidine in the lab. Typically, chemists try to build complex molecules piece by piece. But to do so, they need to control where each new atom attaches. Also, new additions must not change the rest of a half-built molecule.

"But the nitrogens dominate the reactivity," Daly says. "So it's hard to do

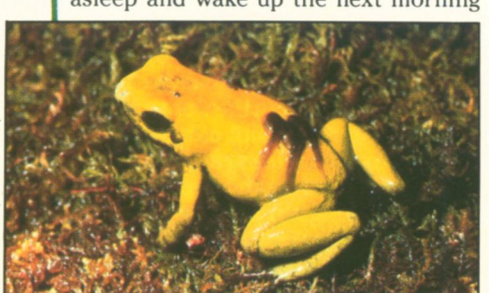


Myers/Amer. Museum of Nat. Hist.

Bright, Bitter, Baffling Frogs

More than unusual chemistries draw researchers to these poison frogs. Anthropologists are intrigued by rituals and stories involving these animals. And biologists find their behavior atypical of amphibians.

A supposed source of "hunting magic," one type of frog provides an ointment used in prehunt ceremonies. During the all-night preparations, hunters in the rain forest burn themselves, then rub a stick coated with this frog chemical on their wounds. They fall asleep and wake up the next morning with much keener senses, so the story goes.



David Barker/National Aquarium

Another frog's skin is so potent that natives just rub their arrows and darts against the frogs' backs. From these few species comes the common name dart-poison or poison-arrow frog. One specimen contains enough toxin to kill about a hundred people, says John W. Daly, an NIH scientist. "You don't want to handle those with your bare hands."

Secretions from another frog supposedly will change the color of parrots. Pluck a plume, rub a little frog secretion on the spot, and the new feather will grow back in a different hue. South American natives often rub frogs on wounds and cuts: The amphibian's skin contains potent antiseptic peptides.

In addition, poison frogs befuddle scientists by their variety and by breaking many of the rules by which other frogs live.

Many care for their young, says Daly. Either the male or female of a species will stay with the egg mass, which was laid on land. Newly hatched tadpoles then climb onto a parent's back for transport—not to a pond but to an aerial plant called a bromeliad. The adult frog deposits the tadpoles in the water-filled centers of these plants, one per bromeliad. One kind of frog goes a step further. Each day, the mother returns to this floral nursery to feed her young an unfertilized egg.

Some poison frogs grow no bigger than a fingernail. Others vary so much in color that even experts mistake cousins as different species. Most live on land, away from water. Despite their family name—Dendrobatidae—and feet trimmed with suction cups, these frogs prefer to hang out among the leaves that litter the rain forest floor, not the trees.

—E. Pennisi

something to one side [of the molecule] without affecting the other side."

Molecules aren't the only slippery things about Daly's business. Consider the frogs. Daly finds them ever harder to obtain.

During the early years of his research, he needed just a few specimens because the alkaloids he studied existed in large amounts in each specimen. But during the 1970s, he began focusing on alkaloids that, like epibatidine, occur in trace amounts. So he needed hundreds of frogs for his analyses.

In those days, frogs were plentiful. Some locales packed as many as half a dozen frogs per square meter of habitat, notes Myers. Once, Daly recalls, he had to flee town because, in a single morning, its residents collected about a thousand specimens and he had run out of money to pay for more.

But now, because development is rapidly destroying their habitat, even abundant species fall under "threatened" status according to the Convention on the International Trade of Endangered Species, which controls the import and export of animals. "Now I'd be lucky to get permits for 10 frogs," Daly says.

Raising the frogs in captivity would solve the supply problem. About six years ago, less than 50 miles from Daly's lab, herpetologist Jack Cover at Baltimore's National Aquarium did just that (SN: 4/16/88, p.247). But when Daly tried to sample the alkaloids in these aquarium-grown frogs, he came up empty-handed.

This came as a surprise. Other species of amphibians raised in captivity still produce toxins, and for years everyone assumed that captive frogs would produce toxins as well. Except for a few frog alkaloids that look like those found in millipedes and ladybugs or other insects, "All the rest have never been found in nature, so it's logical to assume the frog was making them, not getting them from plants or insects," Daly says.

"We're not sure what is going on with these frogs," Cover adds.

Captivity may deprive the frogs of the variety and amount of sensory input provided by real forests. Or natural diets may differ in key ways from the captive frogs' diets of crickets and fruitflies. In their natural habitat, the frogs may ingest a microbe that helps them produce the alkaloids or that provides chemical precursors the frogs then convert to alkaloids. Or because the lab-grown frogs lack some dietary or environmental cue, the genes that drive the production of the enzymes needed to make the alkaloids may never turn on.

Daly and his collaborators have tried many experiments to find the right answer. In one series, a graduate student "stressed" the frogs by chasing them around their terrariums, moving them around, altering the light or temperature of their habitat, even putting a caged snake next to them. Still the frogs produced no alkaloids.

In other experiments, NIH scientists fed the frogs ants collected from the lawn outside their lab. Then they tried ants known to contain alkaloids, then crickets and fruitflies dusted with alkaloids. The frogs stored some of the ingested alkaloids in their skins, but not others. Those alkaloids that the frogs did sequester were not those typically found in large amounts in their bodies.

"So my feeling is that they are not accumulating alkaloids from their diet," Daly says. "I'm convinced they have the enzymes to make these things."

Yet diet and environment must play some role. At the National Aquarium, Cover raised a black-and-green poison frog in the "wilds" of the backstage area of the aquarium's rain forest exhibit. It produced small amounts of alkaloids. A Panamanian poison frog introduced into Hawaii 25 years ago now makes a somewhat different repertoire of alkaloids. "An alkaloid that wasn't detectable [in the Panamanian populations] is now a major alkaloid [in the Hawaiian frog]," says Daly.

Next Daly hopes that colleagues in Hawaii and in Panama will raise frogs outdoors in cages large enough to simulate the amphibians' native habitat. Some frogs will eat a cricket-fruitfly diet, but others will have a more natural diet of insects collected by the researchers from the forest floor.

Those experiments could take years.

Meanwhile, Daly's group waits. Chemists suspect the frog alkaloids are a byproduct of metabolism. But they cannot really test that idea until they can raise enough of the alkaloid-producing frogs to study the animals' metabolic pathways in detail. Many alkaloids not yet characterized exist only in minute amounts. So without more frogs, Daly's group must hope that analytical techniques improve sufficiently to enable scientists to glean chemical structures from microgram quantities.

Daly must even put the work on the new analgesic on hold until either the chemists make epibatidine synthetically or the biologists figure out how to get the captive-raised frogs to produce it.

"We're stymied," says Thomas F. Spande, a chemist who works with Daly. "Until we crack it, some of the frog work is going to be limited." □

George Grall/National Aquarium

Alkaloids: Here, there, but not everywhere

The class of compounds called alkaloids represents the chemist's equivalent of a kitchen junk drawer. Into it, researchers toss molecules they don't really know how to categorize.

The several thousand compounds in this group do share some traits. Each must contain a nitrogen atom, often incorporated as part of a ring of atoms; each is alkaline. That encompasses quite a range. "They run from very simple to very complex compounds for which the biosynthetic pathways are all different," says John W. Daly, a molecular pharmacologist with the National Institutes of Health.

However, no one alkaloid can be too widespread. By tradition, each must occur in a limited number of plants or animals. So alkaline amino acids such as arginine and proline don't qualify as alkaloids, but nicotine, caffeine, and cocaine fit neatly into the drawer. So do the antimalarial medication quinine, the cancer drug vincaleukoblastine, and the painkiller morphine. These, like most alkaloids, come from plants. Ladybugs, millipedes, and ants also produce a few alkaloids. And Daly's work has shown that a few bright frogs and a toad have come up with alkaloids of their own.

Sometimes scientists stumble across these alkaloids. Recently, John Dumbacher, a graduate student at the University of Chicago, and his colleagues caught a yellow-and-black bird while doing field work in New Guinea. The bird bit Dumbacher, who instinctively licked his nicked finger. His mouth numbed. So did the fingers of anyone who handled the bird.

Dumbacher sent samples of feathers, the beak, muscles, and skin to Daly, who studied them in typical fashion. He divided the chemical contents of these samples into neutral, alkaline, and water-soluble components. As Daly suspected, the alkaline part proved the source of the numbing effect. But to his surprise, that part contained batrachotoxin, the same chemical found in some frogs.

"There's no example of a bird having a chemical toxin as a defense," says Daly. "But no one has ever looked." In the past, ornithologists had assumed that a bird's bright feathers helped attract a mate. But for this New Guinea bird, they may also warn potential predators, Daly speculates. — *E. Pennisi.*