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# ARTHROPOD ALKALOIDS IN POISON FROGS: A REVIEW OF THE 'DIETARY HYPOTHESIS'

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**Abstract** – Poison frogs are chemically defended from predators and/or microorganisms by the presence of alkaloids in dermal skin glands. Over the past 40 years, more than 800 alkaloids, which are generally organized into 28 structural classes, have been identified in several lineages of poison frogs worldwide. Originally, the presence of alkaloids in frogs was thought to be the result of biosynthesis, however research led largely by John W. Daly resulted in the discovery that most of these alkaloids are sequestered unchanged from dietary arthropods. In the present paper, we review the most significant findings and studies that led to the proposal of the 'dietary hypothesis'.

## INTRODUCTION

John W. Daly was an organic chemist and pharmacologist with a research program that encompassed several significant lines of investigation, as this issue of Heterocycles will illustrate. We had the opportunity to collaborate with him on one of his main research interests, the study of alkaloids found in poison frog skin and the discovery of their arthropod origin. The purpose of this paper is to provide a brief review of the ideas and research that ultimately led to the discovery that poison frogs sequester most of their lipophilic alkaloids from dietary arthropods, an idea that became known as the 'dietary hypothesis'.

Alkaloids in frog skin were originally discovered in members of the neotropical poison frog family Dendrobatidae, as a result of research carried out by Daly and his colleagues. Alkaloids have also been identified in members of three other anuran families (Mantellidae, *Mantella* – Madagascar; Bufonidae, *Melanophryniscus* - southern South America; and Myobatrachidae, *Pseudophryne* – Australia).[1] The term *poison frog* is used to collectively refer to alkaloid-containing frogs. Poison frogs from the four anuran families mentioned above have yielded more than 800 alkaloids, which are organized into approximately 28 structural classes. Table 1 is a listing of structural classes and the number of alkaloids within each class.

Table 1. The number of alkaloids arranged by structural class.

| | | |
|---|---|---|
| Steroidal alkaloids | Pumiliotoxins | Amides |
| batrachotoxins (6) | pumiliotoxins (30) | epiquinamide (1) |
| | allopumiliotoxins (20) | |
| Izidines | homopumiliotoxins (18) | Monocyclics |
| 3,5-pyrrolizidines (26) | dehydropumiliotoxins<br>desmethylpumiliotoxins (28)<br>deoxypumiliotoxins | 2,5-pyrrolidines (9) |
| 3,5-indolizidines (30) | | 2,6-piperidines (40) |
| 5,8-indolizidines (80) | | |
| dehydro-5,8-indolizidines (40) | | Tricyclics |
| 5,6,8-indolizidines (70) | Spiro-alkaloids | gephyrotoxins (2) |
| 4,6-quinolizidines (6) | histrionicotoxins (16) | coccinelline-like (5+55) |
| 1,4-quinolizidines (20) | spiropyrrolizidines (8) | cyclopentaquinolizines (10) |
| lehmizidines (10) | | pseudophrynamines (13) |
| hydroxyizidines (25) | Decahydroquinolines | |
| other dehydroizidines (16) | decahydroquinolines (50) | Indolic alkaloids (2) |
| | dimers (7) | |
| | | Other alkaloids (150) |
| | Pyridinic alkaloids | |
| | epibatidines (3) | |
| | other pyridinic alkaloids (4) | |

Batrachotoxins were the first frog alkaloids identified and were originally isolated from skins of three species of the genus *Phyllobates* (Dendrobatidae) found in Colombia.[2,3] The discovery of alkaloids in frogs marked the beginning of more than 40 years of research by J.W. Daly, and also led to an important collaboration between Daly and biologist Charles W. Myers of the American Museum of Natural History (AMNH; for review, see ref. 4). During their more than 30 years of research together, their collaboration led to the discovery of several frog species new to science, resulted in the isolation and structure elucidation of several hundred new alkaloids, provided key insights to our understanding of how chemistry and biology are related in poison frogs, and incidentally laid the foundation for the 'dietary hypothesis'.

## THE 'BIOSYNTHETIC HYPOTHESIS'

Daly was primarily interested in the pharmacological effects of poison frog alkaloids and their potential applications to human health, and from the initial discovery of frog alkaloids through the late 1980s, he logically assumed that the frogs synthesized these compounds. At the time, the only other amphibian suspected to produce alkaloids was the European Fire Salamander, *Salamandra salamandra*[5], which has been shown to synthesize samandarines.[6, 7] Support for the 'biosynthetic hypothesis' also came from the fact that particular alkaloids only occur in certain dendrobatid species. At the time, the alkaloids were thought to be chemical taxonomic markers.[3, 8-11] The most notable among these are the batrachotoxins (**1**) found only in frogs of the genus *Phyllobates*,[3] and epibatidine (**2**), the analgesic chloropyridylazabicycloheptane alkaloid, found only in frogs of the genus *Epipedobates*.[1, 10] Additional support for the 'biosynthetic hypothesis' came from observations that alkaloid profiles remained relatively constant in some dendrobatids after years of captivity.[3, 12-14] In most cases, it appeared that the quantity of alkaloids (and toxicity) in captive frogs decreased over time,[3, 12, 14, 15] but at that time, this was not considered evidence that frogs did not manufacture alkaloids. Referring to the observed declines in quantity of alkaloids in captive *Phyllobates terribilis*, Myers et al. 1978:336[3] state, "The reason for such declines in toxicity is not clear, although conceivably related to stress. But the fact that the frogs are still appreciably toxic after long periods of captivity does provide evidence that the toxins are not sequestered from some natural food item." The decrease in quantity of alkaloids observed in captive dendrobatids was largely attributed to stress, either the lack of natural stress (presumed from predation pressure) that might turn on the alkaloid biosynthetic machinery or the stress associated with being held in captivity; however, it should be noted that factors such as diet and seasonality were also considered possible.[3, 8, 12] Furthermore, Daly et al. 1980:1385[12] point out a specific example in which it appeared possible that the decrease in alkaloid amounts in captive frogs was directly related to stress. In that paper, it was noted that, "One observation was inconsistent with the tendency toward decreased production or accumulation of skin toxins in captive *P. terribilis*. A specimen caught at the type locality and maintained for 6 years and 4 months was killed 4 days after its body and limbs became grossly bloated because of water retention. Its skin contained 1150 $\mu$g of batrachotoxin-homobatrachotoxin, an amount equivalent to the original average value for the wild population and much higher than that of any other frog kept in captivity for more than a few weeks. The possibility should be considered that physiological stress stimulated toxin production in this individual. All other specimens tested for toxicity were apparently in good health when killed." All of these lines of evidence seemed to be compelling arguments against a possible 'dietary hypothesis', and thus, diet as a source of alkaloids was initially doubted.

## THE 'DIETARY HYPOTHESIS'

The 'dietary hypothesis' was not something that immediately transpired from the study of poison frog alkaloids; rather it was the result of numerous years of collaborative research, carefully designed experiments, and in some cases, serendipity. Although J.W. Daly directed the majority of this research, there were many others involved in the formulation of this hypothesis.

For many years, the presence of alkaloids in poison frogs was entirely attributed to biosynthesis (see 'Biosynthetic Hypothesis' above). However, there were certain observations that were difficult to explain under the 'biosynthetic hypothesis', such as: (1) a 6-week study attempting to demonstrate alkaloid synthesis in dendrobatid frogs was unsuccessful, as "there was no detectable incorporation of injected radioactive acetate, mevalonate, or cholesterol into alkaloid fractions of *Oophaga* (= *Dendrobates*) *pumilio*[16] or *Phyllobates aurotaenia*, nor was there any detectable incorporation of radioactive serine into the batrachotoxin alkaloids of *P. aurotaenia*" (Daly et al. 1987:1064[10]),[17] (2) the apparent decrease in the amount of alkaloids of captive wild-caught dendrobatids,[3, 9, 10, 12, 14, 15] (3) alkaloids were absent in captive-bred dendrobatids,[12, 13] and (4) alkaloid profiles were variable among species, among populations within a species, and in some cases, over time.[3, 8-10, 18-20] These observations did not go unnoticed, and even in some of the earliest studies on dendrobatid alkaloids, it is clear that they were thought to be important. Referring to the observed decreases in alkaloid amounts of captive dendrobatids and variation in alkaloid profiles of *Oophaga* (= *Dendrobates*) *histrionica*,[16] Myers and Daly, 1976:217[8] stated, "Further studies are in progress on the possible influence of stress, diet, and seasonality."

The fact that dendrobatids appeared to slowly lose alkaloids while in captivity, and that captive-bred dendrobatids did not have alkaloids was puzzling. If the frogs were manufacturing alkaloids, then why were the alkaloids not produced when in captivity? At the time, Daly suggested that frogs in captivity did not produce alkaloids because they were predator-free, and lived a captive life free of stress. If this were the case, then one should be able to initiate alkaloid production through experimental stress. In experiments that began as early as 1980 (personal lab notebooks, J.W.D.), Daly tried to induce alkaloid biosynthesis in the face of stress. In a series of very imaginative experiments, such as frequent changes in terraria, supplementing terraria water with adrenaline, modification of diets, increased fluorescent lighting, changes in sound and temperature, swabbing, and a variety of visual threats such as placing a flopping dummy snake or real *Boiga* next to frog terraria, it proved impossible to induce alkaloid production in captive frogs.[13, 14] These experiments were done at the National Institutes of Health and at the National Aquarium in Baltimore (NAIB) in collaboration with Jack F. Cover, Jr.

Originally, differential genetic expression of the biosynthetic machinery responsible for alkaloid production was invoked to explain differing alkaloid profiles in dendrobatids, although unknown

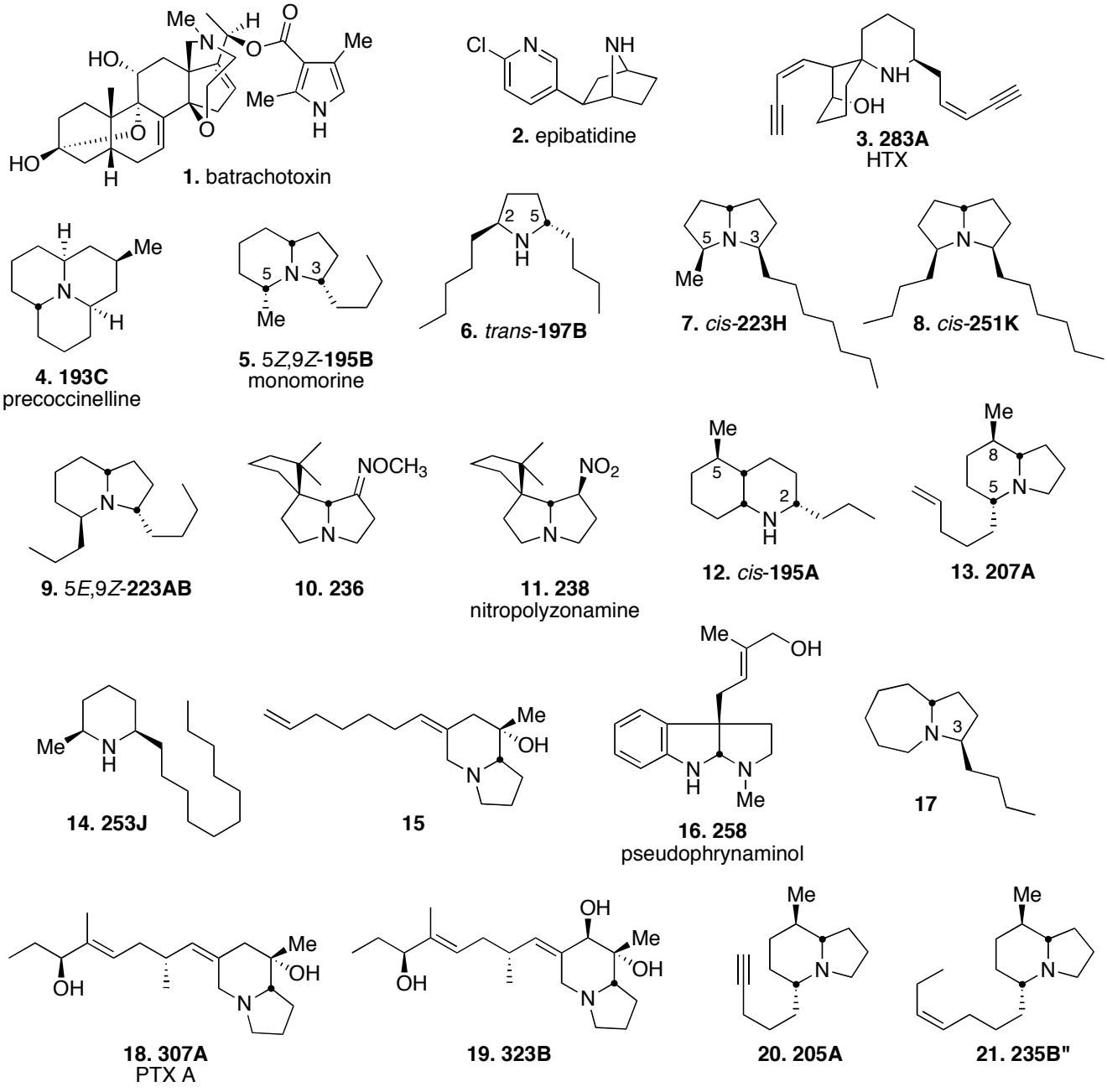
environmental factors were also considered as possible causes for the observed differences. Daly et al. 1987:1065,[10] claimed, "Genetic control of the expression of necessary biosynthetic pathways must be involved in the 200-odd dendrobatid alkaloids, since different species of frogs co-existing in neotropical forest usually exhibit quite different and fairly consistent alkaloid profiles. Nonetheless, the possibility cannot be excluded that symbiotic microorganisms or other environmental factors might play a necessary role in the initiation of alkaloid biosynthesis during ontogeny." At the time, only 200 alkaloids had been characterized from skins of dendrobatid frogs, however that number now exceeds 500 in dendrobatids and more than 800 in all poison frogs collectively.[1] Although the possibility of alkaloids originating from microorganisms has never been rigorously tested, Daly et al. 1994a:658[14] reported results suggesting it was unlikely, "Even after coexistence for one year and even breeding with wild-caught alkaloid-containing frogs (*Dendrobates auratus*), captive-raised frogs did not contain detectable levels of skin alkaloids, a result arguing against an essential symbiotic microorganism."

In 1988, Daly and M.A. Donnelly met at the AMNH when Donnelly was a postdoctoral fellow working with Myers on the evolution of feeding in poison frogs. When Daly discovered that Donnelly was interested in these frogs, he described to her the temporal variation in alkaloid profiles for dendrobatid frogs from Isla Bastimentos, Panama. Daly and Myers had observed differences in alkaloid profiles of certain populations of the dendrobatid frog, *Oophaga pumilio*, which had been sampled repeatedly since 1972.[10] Donnelly suggested that the variation in alkaloid profiles might be related to variation in diet. Donnelly (1991)[21] had described variation in patterns of consumption of ants, mites, and other arthropods by frogs in a population of the same species of poison frog in northeastern Costa Rica over a 15-month period. The composition of the diet varied through time, by age (adult vs. juvenile), and by sex. Donnelly described variation in small arthropod abundance through time and asked Daly if this variation in diet might be related to the differences observed in alkaloid profiles of frogs over time. While Daly was open-minded to this possibility, he had examined some stomach contents of wild-caught *Oophaga histrionica* and *Ameerega* (= *Dendrobates*) *trivittata*,[16] but found "No traces of toxins or potential alkaloid precursors…" (Myers et al. 1978:336[3]). Consequently, Daly considered the connection between diet and toxicity improbable as most of the evidence available at that time supported the 'biosynthetic hypothesis'. By 1992, however, it was clear that captive-raised and bred frogs did not contain skin alkaloids and that alkaloid profiles differed among populations of the same species, reducing support for the 'biosynthetic hypothesis'. In a pivotal 1992 paper,[13] Daly and co-authors described "markedly different" alkaloid profiles between populations of the dendrobatid frog, *Dendrobates auratus*, in Panama and Costa Rica. Furthermore, in a comparison of alkaloid profiles between populations of *D. auratus* that had been introduced to Hawaii in 1932 to that of the founding population of frogs from Panama, it was found that alkaloid profiles differed. Surprisingly, the Hawaiian populations contained no histrionicotoxin (**3**)

alkaloids (histrionicotoxins are alkaloids that were known from all but one population of *D. auratus* at the time[10]), but contained the tricyclic alkaloid, precoccinelline (**4**). Precoccinelline was known only from ladybug beetles in the family Coccinellidae.[22] In addition, the Hawaiian frogs also contained a diastereomer of the 3,5-disubstituted indolizidine **195B** (**5**),[23] monomorine, an alkaloid known to occur in the Pharaoh's ant, *Monomorium pharaonis*.[24] At around the same time, Cover was directing a captive breeding program for dendrobatids at NAIB and was very successful in rearing these brightly colored frogs, especially the Green and Black Poison frog (*D. auratus*). One of the *D. auratus* escaped the husbandry area and established itself in the tropical rain forest exhibit where it presumably fed on arthropods living inside the exhibit. The escaped frog was found dead, and the skin was sent to Daly. Daly had analyzed other captive-raised frogs in the past and found no alkaloids, but the escapee from NAIB contained monomorine ((5*Z*,9*Z*)-3-butyl-5-methylindolizidine) and trace amounts of 2-pentyl-5-butylpyrrolidine, **197B** (**6**), both of which were previously known from the ant *M. pharaonis*[24] (see above). These results were intriguing, because these alkaloids were known only from ants. Daly et al. 1992[13] also found that offspring of wild-caught Hawaiian frogs raised in indoor terrariums on a diet of crickets and fruit flies did not contain alkaloids, whereas offspring raised in outside terrariums and fed mainly wild-caught termites and fruit flies did contain alkaloids resembling those found in their wild-caught parents, albeit in smaller amounts. Crickets, fruitflies, and termites do not contain alkaloids, and therefore in retrospect, the most probable explanation for these results is that alkaloid-containing arthropods somehow entered the outside terrariums. Daly et al. 1992:890[13] concluded that the results of these studies "indicate that environmental factors have a remarkable role in either triggering or supporting alkaloid production in dendrobatid frogs."

At around the same time period, two of us (H.M. Garraffo & T.F. Spande) began to discover additional arthropod alkaloids in other poison frogs (Bufonidae, *Melanophryniscus*; Mantellidae, *Mantella*).[19, 20] In particular, certain alkaloids in the skin of the toad, *Melanophryniscus stelzneri*, from central Argentina and from several species of *Mantella* from Madagascar were likely of ant or beetle origin, which further suggested a possible dietary source for alkaloids in poison frogs.[19, 20] The alkaloids found in *M. stelzneri* included (1) **195B**, the 3-butyl-5-methylindolizidine (monomorine), a trail-marker component in the Pharaoh's ant, *Monomorium pharaonis*[24] (see above), (2) **223H** (**7**), the 3-heptyl-5-methylpyrrolizidine of the thief ant, *Solenopsis* (*Diplorhoptrum*) sp.,[25] (3) **251K** (**8**), the 3-butyl-5-hexylpyrrolizidine of the ant, *Megalomyrmex modestus*,[26] (4) **223AB** (**9**), the 3-butyl-5-propylindolizidine known from a fire ant, *Solenopsis* (*Diplorhoptrum*) sp. *molesta* group, from Puerto Rico (later published[27]), (5) **193C**, precoccinelline, of the ladybug family Coccinellidae[22] (see above), and (6) **236** (**10**), the pyrrolizidine oxime, which is closely related to nitropolyzonamine (**11**), an alkaloid found in the millipede, *Polyzonium rosalbum*[28] (now *Petaserpes cryptocephalus*[29]; for structures of alkaloids mentioned in the text of this

article, see Figure 1). Garraffo et al. 1993a:373[19] suggested that diet might somehow be associated with the presence of alkaloids in poison frogs, and stated, "The fact that ants and other insects represent the diet of bufonid toads and dendrobatid frogs, together with the wide variety of alkaloids found in such toads and frogs, raises the possibility of two processes, an alkaloid intake and *de novo* biosynthesis, as the source of amphibian skin alkaloids."



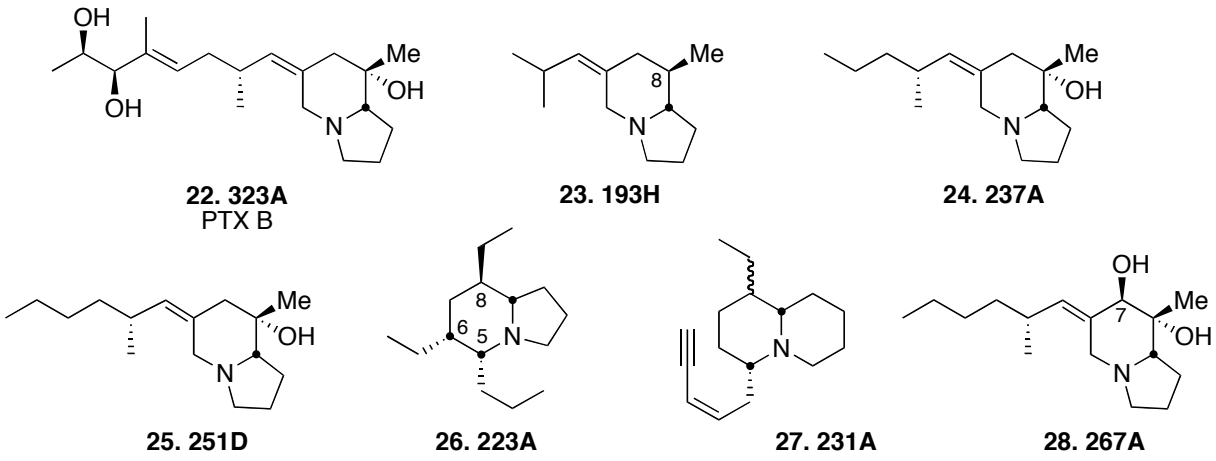



Figure 1. Alkaloid structures.

To test the hypothesis that poison frogs could sequester alkaloids, Daly began a series of now classic alkaloid feeding experiments – both lab and field based. Using captive-raised and wild-caught dendrobatid frogs, Daly fed frogs a diet of 'alkaloid-dusted' fruit flies (containing one or more different alkaloids) and experimentally demonstrated that certain alkaloids could be accumulated unmodified in skin.[4, 14, 30, 31] In addition, captive-raised frogs fed the alkaloid-containing ant, *Monomorium pharaonis*, being raised in his lab and which contained monomorine **195B** and the pyrrolidine **197B** as major alkaloids (see above), were shown to accumulate the indolizidine in incredibly high amounts but the pyrrolidine only in trace amounts.[14] In the fall of 1994, Daly began additional work on alkaloid uptake in dendrobatids (unpublished data, personal lab notebooks, J.W.D.). He orally administered a methanol-saline solution of the following alkaloids: decahydroquinoline **195A** (**12**), 5,8-disubstituted indolizidine **207A** (**13**), *cis*-2-methyl-6-undecylpiperidine **253J** (**14**)(an ant alkaloid),[32] and a non-natural pumiliotoxin analog of molecular weight 249 (**15**), to five captive-raised dendrobatids (*Dendrobates auratus*, *Hyloxalus* (= *Epipedobates*) *azureiventris*,[16, 33] *Oophaga histrionica*, *Epipedobates tricolor*, and *Phyllobates bicolor*). The alkaloid delivery occurred once a day for three days and then the frogs were sacrificed for alkaloid analysis. Of all of the species, *D. auratus* showed the best uptake and all of the delivered alkaloids appeared in skin extracts, although the pumiliotoxin was present in lower amounts and the piperidine was barely detectable. These and other experiments established the presence of an uptake system for pyrrolizidine, indolizidine, quinolizidine, decahydroquinoline, histrionicotoxin, pumiliotoxin, and batrachotoxin alkaloids.[14] The frogs did not significantly accumulate pyrrolidines and piperidines, moncyclic alkaloids that are secondary amines.[14] On the basis of these initial feeding experiments, the uptake system was considered to be present in alkaloid-containing dendrobatid frogs in the genera, *Dendrobates*, *Epipedobates*, and *Phyllobates*, but absent in the non alkaloid-containing frogs of the genus

*Colostethus*.[14] Future experimental feeding studies would reveal that the ability to sequester alkaloids is also present in members of the genus *Adelphobates*.[16, 34] In experiments that followed, Daly also established a similar dietary uptake system in the mantellid poison frogs of Madagascar,[35] whereas the myobatrachid poison frogs of Australia appeared to synthesize pseudophrynamine (**16**) alkaloids, yet were able to accumulate dietary pumiliotoxins.[36] Although not yet experimentally tested, it is likely that poison bufonids also possess an alkaloid uptake system.[19] These findings demonstrated that alkaloids "might derive from dietary sources" (Daly et al. 1994a:663[14]) and the presence of an uptake system in poison frogs "…strongly suggests that dietary alkaloids from insects or other small prey would accumulate in the skin and could account for some or even all of the alkaloids detected in skin of poison frogs" (Daly et al. 1994b:944[30]).

Daly turned his attention to field-based experimentation in an attempt to link naturally occurring alkaloid-containing arthropods with alkaloids in the frogs skin. In collaboration with A. Stanley Rand, a biologist from the Smithsonian Tropical Research Institute, captive-born *Dendrobates auratus* "were raised in Panama in inside terraria either on wingless fruit flies or on leaf-litter arthropods collected from a site where a population of this dendrobatid frog occurs" (Daly et al. 1994b:944[30]). It was discovered that the frogs fed the wingless fruit fly diet lacked the alkaloid profiles of wild frogs, whereas the frogs fed the leaf-litter arthropod diet had alkaloid profiles that matched wild-type frogs from that locality, albeit at reduced levels. In an initial attempt to identify some of the potential arthropod sources, Daly et al. 1994b[30] also examined combined leaf-litter collections of arthropods for alkaloids in an approach later referred to as "combinatorial bioprospecting" (see Identifying Dietary Sources below). They found the pyrrolizidine oxime **236** (suspected to be of millipede origin; see above), the millipede alkaloid nitropolyzonamine, **238**,[28] and the beetle alkaloid precoccinelline, **193C**[37] (see above), all of which were also found in wild-caught *D. auratus* from the same location. In a follow-up study, conducted shortly after the previous study, but published years later, additional *D. auratus* raised in outdoor terraria and also provided with leaf-litter from the frog's habitat were shown to accumulate a variety of alkaloids into skin.[38] This more detailed study also indicated that queens but not workers from a nest of *Solenopis* (*Diplorhoptrum*) contained **195A**, *cis*-2-propyl-5-methyldecahydroquinoline, with the same absolute configuration as **195A** found in wild-caught *D. auratus* from the same area. Furthermore, some collections of another myrmicine ant, *Megalomyrmex sylvestri*, from Isla Taboga, Panama had two diastereomers of 3-butyl-5-hexylpyrrolizidine **251K** also occurring in sympatric *D. auratus* (previously, *trans*-**251K** had been identified in *Megalomyrmex foreli* of Costa Rica, but at a site at which dendrobatid frogs did not occur; published later[27]). It was now clear that poison frogs could take up alkaloids from dietary sources and that these dietary sources were alkaloid-containing arthropods available in the local environment of the frogs. The findings from these two studies "strongly suggest a contribution from

alkaloids of leaf-litter prey to the profile of alkaloids in dendrobatid frogs" (Daly et al. 1994b:948[30]), however many questions still remained concerning the 'dietary hypothesis', most notably with regard to the source of the several other major classes of alkaloids found in poison frogs. Daly et al. 1994b:954[30] suggest, "an extensive study on the complete set of arthropods, including flying insects and other small creatures that could serve as food for dendrobatid frogs, and on the alkaloids present in such food sources needs to be conducted." Thus began a new chapter in the 'dietary hypothesis' – the search for alkaloid-containing arthropod sources.

## IDENTIFYING DIETARY SOURCES

Experimental evidence now indicated support for a 'dietary hypothesis' of alkaloid presence in poison frogs, however one of the major challenges that remained was the identification of the supposed dietary sources for most of the more than 800 alkaloids from poison frogs worldwide.[1] Although arthropod sources had been identified for some of the alkaloid classes, "the vast majority of over 500 alkaloids (the current number now exceeds 800 alkaloids[1]) detected in frog skin extracts have not yet been identified from a possible dietary source" (Daly et al. 2000:76[38]). In this section, we will briefly describe some of the recent studies that have led to the identification of many of the arthropod sources for alkaloids in poison frogs.

By the year 2000, it was well known that the diet of some dendrobatids, as well as other poison frogs, were composed largely of ants and mites,[21, 39-45] and indeed, six of the 28 structural classes of frog alkaloids had been identified in myrmicine ants.[27, 38, 46-48] These alkaloid classes included 2,5-dialkylpyrrolidines, 2,6-dialkylpiperidines, 3,5-diakylpyrrolizidines, 3,5-dialkylindolizidines, 4,6-dialkylquinolizidines, and 2,5-dialkyldecahydroquinolines.[38] The histrionicotoxins, gephyrotoxins, and 3,5-disubstituted lehmizidines share certain structural features (like the previous six structural classes, they could derive biosynthetically from a precursor with a linear carbon chain) with those of known ant alkaloids, and it was expected that they would be of myrmicine ant origin (this expectation remains today). Recently, a monosubstituted lehmizidine, 3-butyllehmizidine (**17**) was identified in the venom of an Indonesian myrmicine ant, *Myrmicaria melanogaster*.[49] On the basis of dietary and chemical data, it appeared that ants were a large source of alkaloids in poison frogs (see Table 2 for a listing of all alkaloids common to ants and poison frogs). Two other alkaloid classes found in frog skin were known to also occur in arthropods, namely the tricyclic coccinelline class, know from coccinellid beetles, and the spiropyrrolizidine class, known from a millipede (see above). At the time, even though alkaloids had been identified in ants, beetles, and millipedes, "very few of the alkaloids had been identified in arthropods from a region where the frogs occur." (Daly et al. 2000:76[38]). It was clear that a truly detailed study of alkaloid-containing arthropods from regions where poison frogs occurred was necessary.

Table 2. Alkaloids common to ants and poison frogs.

| | Structural Classes | | | | | | |
|---|---|---|---|---|---|---|---|
| | DHQ | 3,5-I | 3,5-P | 4,6-Q | Pyr | Pip | PTX |
| 1 | ***cis*-195A** | **167E** | **195F** | **195C** | **197B** | **223K** | **307A** [a] |
| 2 | **195J** | **195B** | **223H** | | **225C** | **225I** | **323A** |
| 3 | ***cis*-275B** | **223AB** [a] | **251K** | | **223N** | **253J** | |
| 4 | | **223R** | **251O** | | **225H** | | |
| 5 | | | | | **253I** | | |

All data are from ref. 19, 20, 27, 38, 47, 49, 54, 55, and 57. For alkaloid structures, see Figure 1 and ref. 1. Abbreviations for alkaloid classes are as follows: DHQ, 2,5-disubstituted decahydroquinoline; 3,5-I, 3,5-disubstituted indolizidine (up to 4 isomers can be present); 3,5-P, 3,5-disubstituted pyrrolizidine (1 or 2 isomers are generally present); 4,6-Q, 4,6-disubstituted quinolizidine; Pyr, 2,5-disubstituted pyrrolidine; Pip, 2,6-disubstituted piperidine; PTX, pumiliotoxin. [a] also identified in an oribatid mite.[56] An isomer of the frog alkaloid **217B** was reported in an ant from Madagascar,[57] however in the absence of structural data (particularly the lack of infrared data ), this presumably new alkaloid is not included in our table. Underlined alkaloids are discussed further within this paper.

Daly and Myers had studied alkaloid profiles of *Oophaga pumilio* from Isla Bastimentos, Panama for almost 30 years, and this seemed like a logical place to begin a detailed search for arthropod alkaloid sources. In 2000, Daly and Donnelly traveled to Isla Bastimentos to begin the search for the sources of dendrobatid alkaloids. Donnelly had field experience with small leaf-litter arthropods and with frog diet in *O. pumilio*, and therefore she was an appropriate collaborator on this project. In addition, Daly and Donnelly had spent time exploring tepuis in Venezuela during the late 1980s through the mid-1990s, as part of AMNH field expeditions headed by Myers, and knew they could manage the fieldwork.[50-53] On Isla Bastimentos, Daly, Donnelly, and Alex Espinosa collected arthropods and frogs from multiple locations that had been sampled in the past[10], and they discovered several alkaloids from their mixed samples of arthropods (referred to as "combinatorial bioprospecting").[54] These alkaloids included pumiliotoxin **307A** (**18**), allopumiliotoxin **323B** (**19**), 5,8-disubstituted indolizidines **205A** (**20**) and **235B** (**21**), decahydroquinoline **195A**, and the spiropyrrolizidine **236**.[54] These findings demonstrated that these alkaloids had an arthropod source, however, when Donnelly tried to identify which arthropods were the sources of these frog alkaloids, she discovered that identification was not possible. Some samples shared alkaloids but did not share the same organisms, and she suggested that the field methods needed to be refined to, (1) eliminate cross-contamination in the handling and sample preparation and (2) determine unambiguously which arthropod contained which alkaloid. To accomplish this they recruited one of Donnelly's graduate students, Ralph A. Saporito, to work with them as they searched for the sources of

dendrobatid alkaloids. The laboratory work led to collaboration with Daly, the authors of this paper, and a variety of arthropod specialists.[29, 55, 56]

Daly, Donnelly, and Saporito went to Panama together for the first time in 2003 to sample individual leaf-litter arthropods to identify the likely sources of alkaloids in *Oophaga pumilio*. Using forceps (cleaned with methanol between sampling events to reduce contamination), arthropod samples were collected directly from leaf-litter, from living plants (mostly *Heliconia* species), and from coarse woody debris. Using these methods, Saporito et al. (2003)[29] identified the siphonotid millipede, *Rhinotus purpureus*, as a likely source of the spiropyrrolizidine oxime **236** and nitropolyzonamine **238**, and Saporito et al. (2004)[55] identified formicine ants in the genera *Paratrichina* and *Brachymyrmex* as a source of the pumiliotoxins **307A** and **323A** (**22**). It was expected to find a millipede source for the spiropyrrolizidines, as a result of earlier work by Meinwald et al. (1975)[28] and the discovery of **236** and **238** in a mixed sample of leaf-litter arthropods from another region of Panama (see 'The Dietary Hypothesis'). Spiropyrrolizidines (including **236** and **238**) were later found in *R. purpureus* from Madagascar, which then established this species as a probable source of these alkaloids in mantellid poison frogs.[57] Recently, spiropyrrolizidine **236** was identified in a Japanese millipede, *Kiusiozonium okai*.[57] However, it was a surprise to discover the pumiliotoxins (**307A** and **323A**) in formicine ants, which are well known for their use of formic acid as a defensive compound. While some alkaloids such as pyrazines are known from formicine ants, it was unexpected to find pumiliotoxin alkaloids, with their apparently isoprene-derived side-chains, in this family of ants.[55] Up to this point, ant alkaloids common to frogs were only known from myrmicine ants and were all derived from straight-chain precursors.[27, 38, 46-48] This discovery further stressed the importance of dietary ants to the chemical defenses of dendrobatids (and other poison frogs).[55] Ultimately, the discovery of spiropyrrolizidines in millipedes and pumiliotoxins in ants demonstrated that individual sampling of arthropods from locations in which poison frogs occurred, could, indeed result in the identification of potential alkaloid sources.

By the year 2005, several alkaloids common to poison frogs had been identified in arthropods, many of which were beginning to be found in locations where frogs occurred.[29, 38, 47, 55, 57] However, even after multiple detailed field collections of arthropods, only a fraction of the more than 800 alkaloids known from poison frogs had been identified (26 alkaloids from ants, five from beetles, and six from millipedes[56]), and furthermore some of the more common alkaloid classes had yet to be identified in an arthropod. If the majority of alkaloids were in fact being sequestered from arthropod sources, then why was it so difficult to identify these arthropods – which based on the number and amount of alkaloids present in frogs, should have been fairly abundant? At the time, ants were considered the major source of alkaloids and most of our efforts were aimed at collecting ants, however, in 2005, Takada et al.[58`] published a seminal paper on the presence of alkaloids in oribatid mites from Japan that would begin to

change our thinking on arthropod sources for alkaloids. Although poison frogs do not occur in Japan, Takada et al.[58] identified several alkaloids in oribatid mites that are common to frogs, namely deoxy-pumiliotoxin **193H** (**23**), pumiliotoxins **237A** (**24**) and **251D** (**25**), the tricyclic **193C**, the 5,6,8-trisubstituted indolizidine **223A** (**26**), and the 1,4-disubstituted quinolizidine **231A** (**27**). To collect oribatid mites from regions in which poison frogs occurred, Saporito, Donnelly, and Daly began collecting arthropods from leaf-litter samples throughout Panama and Costa Rica using Berlese funnels[59] (a method adopted from previous studies[30, 38]). These collections led to the identification of more than 80 alkaloids from extracts of oribatid mites, representing 11 of the 28 structural classes of poison frog alkaloids, including 5,8-disubstituted and 5,6,8-trisubstituted indolizidines (indolizidines are the most abundant and common class of alkaloids found in frog skin worldwide), pumiliotoxins, a 1,4-disubstituted quinolizidine, a 4,6-disubstituted quinolizidine, a 3,5-disubstituted indolizidine, two pyrrolidines, a spiropyrrolizidine, a tricyclic, and several unclassified alkaloids (i.e., alkaloids that are awaiting structural determination and have not yet been assigned to any structural class).[56] Many of these alkaloids were common to the dendrobatid frog *Oophaga pumilio* and other poison frogs (Table 3), however an equal number were new alkaloids not previously known from poison frogs.[56] It now appears that ants are the likely dietary source of unbranched alkaloids (meaning that they appear to be derived from a precursor with a linear carbon chain), whereas mites are the likely dietary source for branched alkaloids. Figure 2 illustrates the difference between unbranched (e.g., 3,5-disubstituted indolizidines) and branched alkaloids (e.g., 5,8-disubstituted indolizidines); for a complete list of unbranched and branched poison frog alkaloids, see ref. 1. On the basis of these studies, oribatid mites are a significant repository for a large number of alkaloids of diverse structures, and represent a major dietary source for alkaloids in poison frogs, perhaps *the* major source. Although there still remain a large number of individual frog skin alkaloids with no known arthropod source, the majority of the structural classes of alkaloids have now been identified in putative arthropod sources. Of the 28 structural classes of alkaloids found in poison frogs, 17 have also been identified in a potential dietary source, including mites, ants, beetles, and millipedes.

The discovery of alkaloids in oribatid mites begins yet another chapter of the 'dietary hypothesis'. The large number and diversity of alkaloids found in mites appears to explain some of the initial difficulties in identifying arthropod sources for alkaloids, but also opens the door to a variety of new research questions. Many of the alkaloids that have been identified in mites are also found in other arthropod taxa (e.g., pumiliotoxins, which are also known from ants; tricyclics, which are also known from beetles, and spiropyrrolizidines, which are also known from millipedes), suggesting that there may be multiple dietary sources for alkaloids in poison frogs or a common mite-ant precursor, such as a symbiont. In addition,

Table 3. Alkaloids common to oribatid mites and poison frogs.

| | Structural Classes | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 5,8-I | d-5,8-I | 5,6,8-I | PTX | hPTX | deoxy-PTX | 1,4-Q | 4,6-Q | 3,5-I | Pyr | Spiro | Tri | Unclassified |
| 1 | **195I** | **205L** | **195G** | **251D** | **251R** | **193H** | **233A** | **231A** | **223AB** [c] | **183B** | **236** [d] | **193C** [e] | **181C** |
| 2 | **203A** | **243F** | **223A** | **237A** | | | | **237I** | | **253I** | | | **209G** |
| 3 | **205A** | **269D** | **235E** | **307A** [b] | | | | | | | | | **227** |
| 4 | **207A** | | **237C** | **307F** | | | | | | | | | **265K** |
| 5 | **209S** | | **237L** | | | | | | | | | | **279I** |
| 6 | **219F/L** [a] | | **251T** | | | | | | | | | | **323I** |
| 7 | **223D** | | **253H** | | | | | | | | | | |
| 8 | **223V** | | **259C** | | | | | | | | | | |
| 9 | **225D** | | | | | | | | | | | | |
| 10 | **231C** | | | | | | | | | | | | |
| 11 | **235B"** | | | | | | | | | | | | |
| 12 | **237D** | | | | | | | | | | | | |
| 13 | **261D** | | | | | | | | | | | | |

All data are from ref. 56 and 58. For alkaloid structures, see Figure 1 and ref. 1. Abbreviations for alkaloid classes are as follows: 5,8-I, 5,8-disubstituted indolizidine; d-5,8-I, dehydro-5,8-disubstituted indolizidine; 5,6,8-I, 5,6,8-trisubstituted indolizidine; PTX, pumiliotoxin; hPTX, homopumiliotoxin; deoxy-PTX, deoxypumiliotoxin; 1,4-Q, 1,4-disubstituted quinolizidine; 3,5-I, 3,5-disubstituted indolizidine; Pyr, 2,5-disubstituted pyrrolidine; Spiro, spiropyrrolizidine; Tri, Tricyclic. [a] The identity of the 5,8-Is **219F** and **219L** could not be determined from the GC-MS data[56]; [b] **307A** has also been identified in formicine ants[55]; [c] **223AB** has also been identified in a myrmicine ant[47]; [d] **236** has also been identified in a siphonotid millipede[29]; [e] **193C** has also been identified in a coccinellid beetle[37]. Underlined alkaloids are discussed further within this paper.

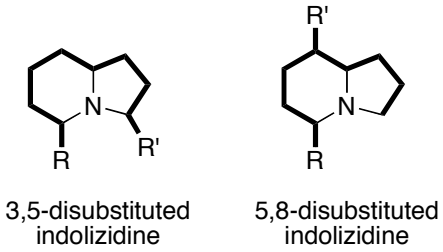



Figure 2. Unbranched vs. branched alkaloids.

oribatid mites contain a large number of alkaloids previously unreported and new from any natural source, which has resulted in a new interest for studying arthropods as sources for novel compounds. Clearly, the identification of alkaloids in mites is in its early stages, and as Saporito et al. 2007:8890[56] suggest, "the investigation of the presence, distribution, chemical nature, and function of mite alkaloids has just begun."

## ALTERNATIVES TO THE 'DIETARY HYPOTHESIS'

The majority of poison frog alkaloids appear to be sequestered directly from a natural diet of alkaloid-containing mites, ants, beetles, and millipedes. Although this dietary hypothesis has received ample and widespread support from lab and field-based experiments/observations, and is generally accepted as the major route for alkaloid presence in poison frogs, it should be mentioned that this source of alkaloids is not exclusive of alternative routes, such as biosynthesis and alkaloid modification (i.e., metabolism). Biosynthesis *de novo* has been demonstrated for the pseudophrynamine class of alkaloids (cyclized and isoprenylated *N*-methyltryptamines) found in myobatrachid poison frogs of the genus *Pseudophryne* from Australia.[36] Currently, this is the only alkaloid class that is known to be synthesized by poison frogs, and is restricted to frogs of the genus *Pseudophryne*. Interestingly, although these frogs synthesize pseudophrynamines, the accompanying pumiliotoxins appear to be sequestered directly from dietary arthropods.[36] Modification of alkaloids (i.e., metabolism) that have been obtained from diet has also been demonstrated in certain poison frogs. Dendrobatid poison frogs in the genera *Dendrobates* and *Adelphobates* have been shown to efficiently and stereoselectively hydroxylate dietary pumiliotoxin (+)-**251D** to a more toxic allopumiliotoxin (+)-**267A** (**28**)[34], and one frog in the genus *Pseudophryne* has been shown to reduce/hydroxylate dietary pumiliotoxin **307A**.[36] To date, these are the only two known examples of alkaloid modification in poison frogs. Although these additional pathways only account for a small number of alkaloids present in poison frogs, they do suggest that not all alkaloids are products *per se* explainable only by the 'dietary hypothesis'.

## CONCLUSIONS AND FUTURE DIRECTIONS

This brief review would seem to indicate that (1) alkaloid-containing frogs are very common, and the survey and discovery of these frogs is complete, (2) the 'dietary hypothesis' is fully understood, and (3) the search for dietary arthropods is complete. However, it should be stressed that this field is still in its infancy and there is much more work to be done. In the summer of 2005, Daly presented the following figure (Figure 3)[60] as part of a presentation on poison frog alkaloids and the 'dietary hypothesis'.

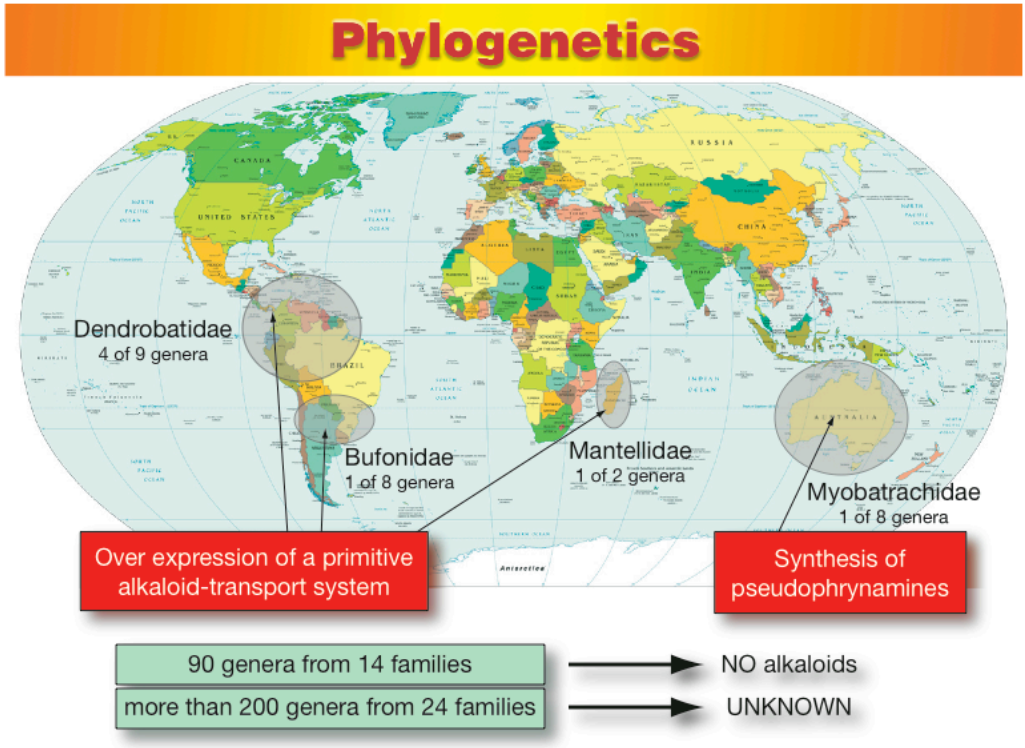



Figure 3

Daly used this photo to illustrate that although poison frogs are found worldwide, they are not very common, as they have only been identified in several genera from four families[61]. The photo also illustrates that although a large amount of work has been done in search of alkaloid-containing frogs, only a small fraction of anuran diversity[61, 62] has been examined and therefore much more work remains to be done.

In this paper, we have reviewed the research and ideas that have led to the formulation of the 'dietary hypothesis', whereby it has been demonstrated that poison frogs sequester alkaloids directly from a diet of alkaloid-containing arthropods. This research was led largely by the efforts of Daly and colleagues, and represents the culmination of more than 40 years of research on alkaloids in poison frogs. Although

our understanding of the 'dietary hypothesis' has improved greatly due to the contributions of Daly, there still remain many unanswered questions, such as, (1) is the ability to sequester alkaloids an overexpression of a primitive alkaloid-transport system?, (2) what is the mechanism by which frogs take up alkaloids for storage in skin glands and is there perhaps a biomedical relevance?, (3) are there other frog species that are able to sequester alkaloids from diet?, (4) how widespread is biosynthesis and metabolism of alkaloids by poison frogs?, and (5) what are the arthropod sources for the hundreds of alkaloids that have not yet been identified in an arthropod? As we continue to address some of these questions as well as discover and describe new alkaloids and their sources, we will be carrying forward John W. Daly's legacy as a pioneer in the discovery, isolation, and chemical and pharmacological characterization of these amazing bioactive compounds.

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62. Note: As a result of the taxonomic revision of amphibians,[61] the number and diversity of frogs listed in Figure 3 has changed, and in general, has increased. However, the general pattern remains the same, with a large number of frogs not having been examined for the presence of alkaloids.
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