# Evidence for an enantioselective pumiliotoxin 7-hydroxylase in dendrobatid poison frogs of the genus *Dendrobates*

**John W. Daly*†, H. Martin Garraffo*, Thomas F. Spande*, Valerie C. Clark*‡, Jingyuan Ma*§, Herman Ziffer*¶, and John F. Cover, Jr.‖**

*Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Department of Health and Human Services, Bethesda, MD 20892-0820; and ‖National Aquarium in Baltimore, 501 East Pratt Steet, Baltimore, MD 21202

Contributed by John W. Daly, July 15, 2003

**Dendrobatid poison frogs readily accumulate alkaloids from diet into skin, where such compounds serve as a chemical defense against predators. Arthropods seem to be the source of decahydroquinolines (DHQs), several izidines, coccinellines, spiropyrrolizidines, pumiliotoxins (PTXs), and allopumiliotoxins (aPTXs). A DHQ iso-223F, and PTX (+)-251D were fed to poison frogs of the dendrobatid genera *Dendrobates*, *Epipedobates*, and *Phyllobates*. The two alkaloids were accumulated in skin unchanged except for the three species of *Dendrobates*, where ≈80% of accumulated PTX (+)-251D was stereoselectively hydroxylated to aPTX (+)-267A. The unnatural enantiomer PTX (−)-251D was accumulated efficiently when fed to *Dendrobates auratus*, but was not hydroxylated. The enantiomers of PTX 251D and their desmethyl analogs were synthesized from *N*-Boc-protected (−)- and (+)-proline methyl esters. Both PTX (+)-251D and aPTX (+)-267A proved to be potent convulsants in mice, with (+)-267A being ≈5-fold more toxic than (+)-251D. Both alkaloids were hyperalgesic at the site of injection. The unnatural PTX (−)-251D caused no overt effect in mice. Thus, the evolutionary development of a pumiliotoxin 7-hydroxylase would have provided frogs of the genus *Dendrobates* with a means of enhancing the antipredator potency of ingested PTXs.**

Amphibian skin has over the past century been found to contain a variety of biologically active compounds (1). Such compounds seem to have evolved for chemical defense against infection and/or against predators. The many alkaloids, discovered in skin extracts of poison frogs of the neotropical family Dendrobatidae (2, 3), were at first thought to be elaborated by such frogs. Almost all of the ''dendrobatid alkaloids'' were unknown elsewhere in Nature. However, when dendrobatid frogs were raised in captivity they completely lacked alkaloids (4, 5). But when fed fruit flies dusted with alkaloid-containing powder or fed alkaloid-containing myrmicine ants, the frogs readily accumulated the alkaloids into skin (6, 7). It seemed that the frogs had no enzymes associated with alkaloid synthesis or metabolism but instead had developed or overexpressed an uptake system or systems designed to capture dietary alkaloids for use in skin secretions as a chemical defense. Alkaloids sequestered in the wild were retained during several years in captivity (4, 5). Many of the alkaloids, for example the batrachotoxins, the pumiliotoxins (PTXs) and allopumiliotoxins (aPTXs), and epibatidine, were highly toxic, whereas others, such as the decahydroquinolines (DHQs), the histrionicotoxins and the izidines, were of low toxicity, but would still serve as deterrents to predators because of bitter, unpleasant effects on buccal tissue (8, 9). ''Dendrobatid alkaloids'' are now known to occur in one genus from each of three other anuran families, namely *Mantella* from the Madagascan family Mantellidae (10, 11), the South American *Melanophryniscus* from the family Bufonidae (12) and the Australian *Pseudophryne* from the family Myobatrachidae (13). The *Mantella* frogs, like the dendrobatid frogs, seemed dependent on a dietary source (14). The *Pseudophryne* frogs seemed dependent on diet for their PTXs/aPTXs (15). However, raised in captivity, the *Pseudophryne* frogs still contained pseudophrynamines in skin and, thus, the frogs seem able to synthesize these unusual indolic alkaloids (15). The dietary source of the many classes of alkaloids found in skin of frog taxa has become a major challenge.

In the present alkaloid-feeding experiments, a synthetic DHQ iso-**223F**, an isomer of a natural DHQ, code name **223F** (3), was fed along with a simple PTX (+)-**251D**. DHQs were known to be readily accumulated unchanged into skin of dendrobatid frogs (6). The experiment was designed to determine for the first time the relative efficiency of uptake of two of the most common classes of alkaloids found in skin of dendrobatid frogs, namely DHQs of ant origin (16) and PTXs that originate from an arthropod source, as yet unidentified as to taxon (17). However, the results led to evidence for the evolutionary development in one lineage of dendrobatid frogs of an enantioselective pumiliotoxin 7-hydroxylase that converts a toxic PTX (+)-**251D** to a more toxic aPTX (+)-**267A** (Fig. 1). The nontoxic, unnatural (−)-**251D** enantiomer was not hydroxylated. The DHQ iso-**223F** was sequestered unchanged.

## Materials and Methods

**Alkaloids.** The synthetic DHQ iso-**223F** is the enantiomeric compound **18** of ref. 18 and was provided by A. G. Schultz (Rensselaer Polytechnic Institute, Troy, NY); the synthetic aPTX (+)-**267A** was provided by L. E. Overman (University of California at Irvine).

**Synthesis of Pumiliotoxins.** PTXs (−)-**251D** and (+)-**251D** and their desmethyl analogs were synthesized as shown in Fig. 2. The synthesis of (+)-**251D** (**9**), (−)-**251D** (**12**) and the two enantiomeric desmethyl analogs **13** and **14** followed methodology developed by L. E. Overman (19–21) and by Barger *et al.* (22). In the case of (+)-**251D** (**9**), the synthesis began with the *N*-Boc derivative of L-proline methyl ester (**1**), which was reacted with methyl magnesium iodide, followed by dehydration of the resultant crude alcohol with $SOCl_2$/pyridine to provide the alkene **2**. Epoxidation with *m*-chloroperbenzoic acid gave a mixture of diastereomeric epoxides **3**. These procedures follow those of Overman and Bell (19) as described for *N*-Cbz-L-proline methyl ester. *R*-2-Methylhexanol (**4**) was converted to the dibromoalkene **6** in two steps by Swern oxidation, followed by a Wittig

Abbreviations: DHQ, decahydroquinoline; PTX, pumiliotoxin; aPTX, allopumiliotoxin.

†To whom correspondence should be addressed. E-mail: jdaly@nih.gov.

‡Present address: Department of Chemistry, Columbia University, Havenmeyer Hall, New York, NY 10027.

§Present address: Metabolex Inc., Hayward, CA 94545.

¶Retired, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health.





www.pnas.org/cgi/doi/10.1073/pnas.1834430100

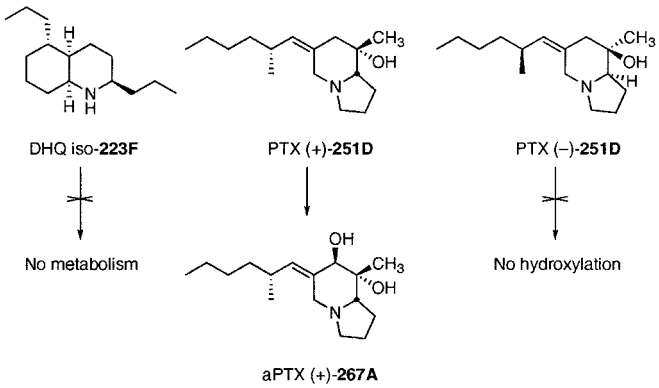


**Fig. 1.** Sequestration and hydroxylation of alkaloids of the DHQ class and the PTX class fed to dendrobatid poison frogs of the genus *Dendrobates*. Enantioselective conversion of (+)-PTX **251D** to aPTX (+)-**267A**.

reaction of the crude aldehyde **5** with triphenylphosphine and $CBr_4$. These steps follow those of Caderas *et al.* (21). The dibromoalkene **6** was converted to the lithium salt of the corresponding alkyne with *n*-butyl lithium, followed by reaction with the diastereomeric epoxides (**3**) in the presence of $Et_2AlCl$ to afford **7**. Deprotection of **7** by treatment with camphorsulfonic acid was followed by cyclization/iodination with NaI and paraformaldehyde. The resultant crude vinyl iodide **8** was treated with *s*-butyl lithium to yield after purification the hydrochloride of (+)-**251D** (**9**). This sequence of reactions was based on that of Barger *et al.* (22). The synthesis of (−)-**251D** (**12**) followed the same overall procedure, starting with the *N*-Boc derivative of D-proline methyl ester (**10**) and by using *S*-2-methylhexanol (**11**) as the source of the dibromoalkene. The desmethyl analog **13** of (+)-**251D** was synthesized from the diastereomeric epoxides (**3**) by reaction with the lithium salt of 1-heptyne to form the desmethyl analog of **7**, followed as above with acid, NaI, and paraformaldehyde to yield a desmethyl analog of **8**, which was deiodinated to yield after purification the hydrochloride of the desmethyl analog **13**. The desmethyl analog **14** of (−)-**251D** was synthesized from the diastereomeric epoxides obtained from **10** in the same manner. Physical properties of **2**, **3**, **6**, **7**, **9**, and **12**-**14** are provided in *Supporting Text*, which is published as supporting information on the PNAS web site, www.pnas.org. *R*- and *S*-2-Methylhexanol and 1-heptyne were from commercial sources, and **5** and **8** were used without purification.

**Frog Experiments.** The following species were captive-raised to adulthood at the National Aquarium in Baltimore: *Dendrobates auratus* (Girard, 1855); *Dendrobates galactonotus* (Steindachner, 1864); *Dendrobates castaneoticus* (Caldwell and Myers, 1990); *Epipedobates tricolor* (Boulenger, 1899), and *Phyllobates bicolor* (Bibron, 1841). Before and during alkaloid-feeding experiments they were housed in glass terraria and fed termites and/or wingless fruit flies. Immediately before feeding the frogs, the termites or fruit flies were dusted with a vitamin-mineral powder (Nekton-Rep, Nekton, Clearwater, FL) containing ≈1% by weight of a 1:1 mixture of DHQ iso-**223F** and either PTX (+)-**251D** or the unnatural enantiomer PTX (−)-**251D**. After 1–6 wk of alkaloid-feeding, as indicated in the legend to Fig. 3, the frogs were killed, and skin extracts were prepared and partitioned as described (15) to yield alkaloid fractions. Alkaloids were detected with a flame-ionization detector after gas chromatography on a six-feet 1.5% OV-1 packed column (2 mm, i.d.), programmed from 150 to 280°C at 10°/min with helium as the carrier gas at ≈30 ml/min. Injection volume was 2 μl, corresponding to 2 mg wet weight frog skin. Gas chromatograms are shown in Fig. 3. The alkaloids were then analyzed by gas chromatography-mass spectrometry as described (15).

**Toxicity.** The toxicities of (+)-**251D**, (−)-**251D**, the two desmethyl analogs **13** and **14**, and (+)-**267A** were compared on s.c. injection

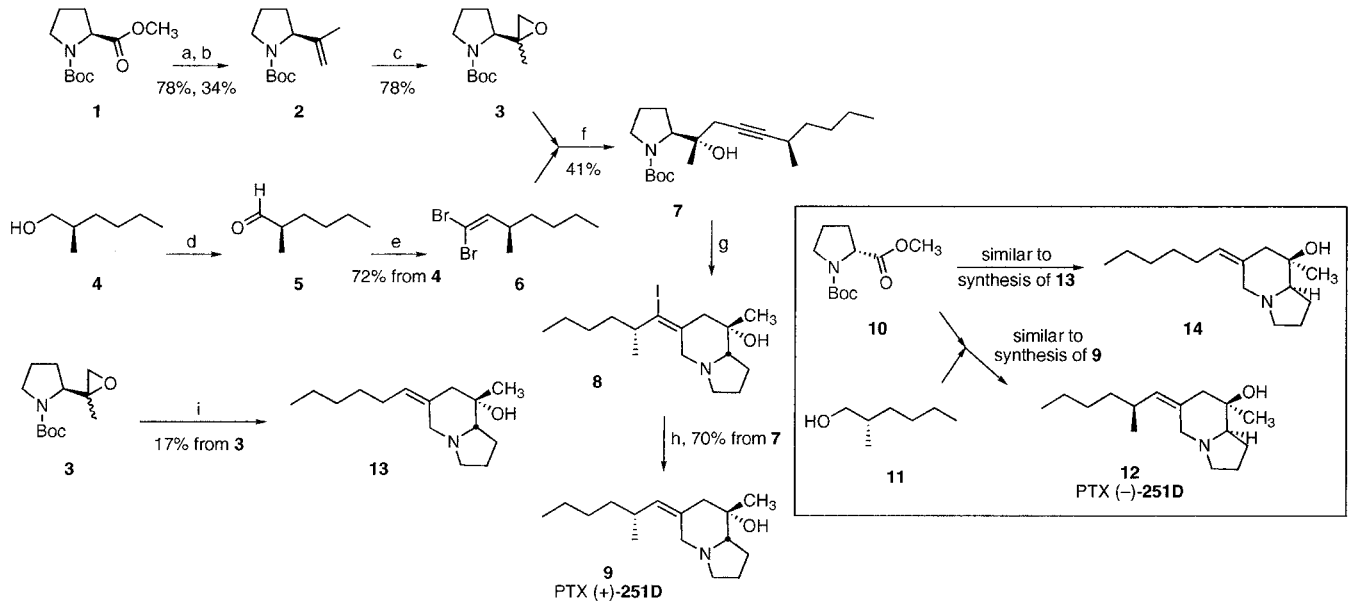


**Fig. 2.** Synthetic routes to (+)-**251D** (**9**), (−)-**251D** (**12**) and the desmethyl analogs **13** and **14**. a, MeMgI, $Et_2O$, reflux 1 h; b, $SOCl_2$, $NEt_3$, THF, −45°C, 1 h; c, *m*-chloroperbenzoic acid, $NaHCO_3$, $CH_2Cl_2$, at 0°C, then room temperature overnight; d, Swern oxidation with oxalyl chloride and DMSO, $CH_2Cl_2$, at −78°C, 30 min, add $NEt_3$, after 1 h warm to 0°C, and quench with aqueous HCl; e, $Ph_3P$ in $CH_2Cl_2$, at −15°C, add $CBr_4$, cool to −78°C, add **5**, after 30 min warm to −20°C, 1 h; f, **6** in toluene at −78°C, add *s*-BuLi, 2 h, warm to −10°C, add $Et_2AlCl$, after 1 h add **3**, warm to 0°C, 2 h, quench with aqueous $NH_4Cl$; g, camphorsulfonic acid, $H_2O$/MeCN, heat to 80°C, after 3 h add NaI, $(CH_2O)_n$, heat to 100°C, 5 h; a $CH_2Cl_2$ extract yields the crude product **8**; h, crude **8** in anhydrous $Et_2O$, add *s*-BuLi at −78°, slowly warm to −20°C, cool to −78°C, add $CH_3OH$, then room temperature overnight; i, as in f, with 1-heptyne in place of **6** and then continue as in g and h.

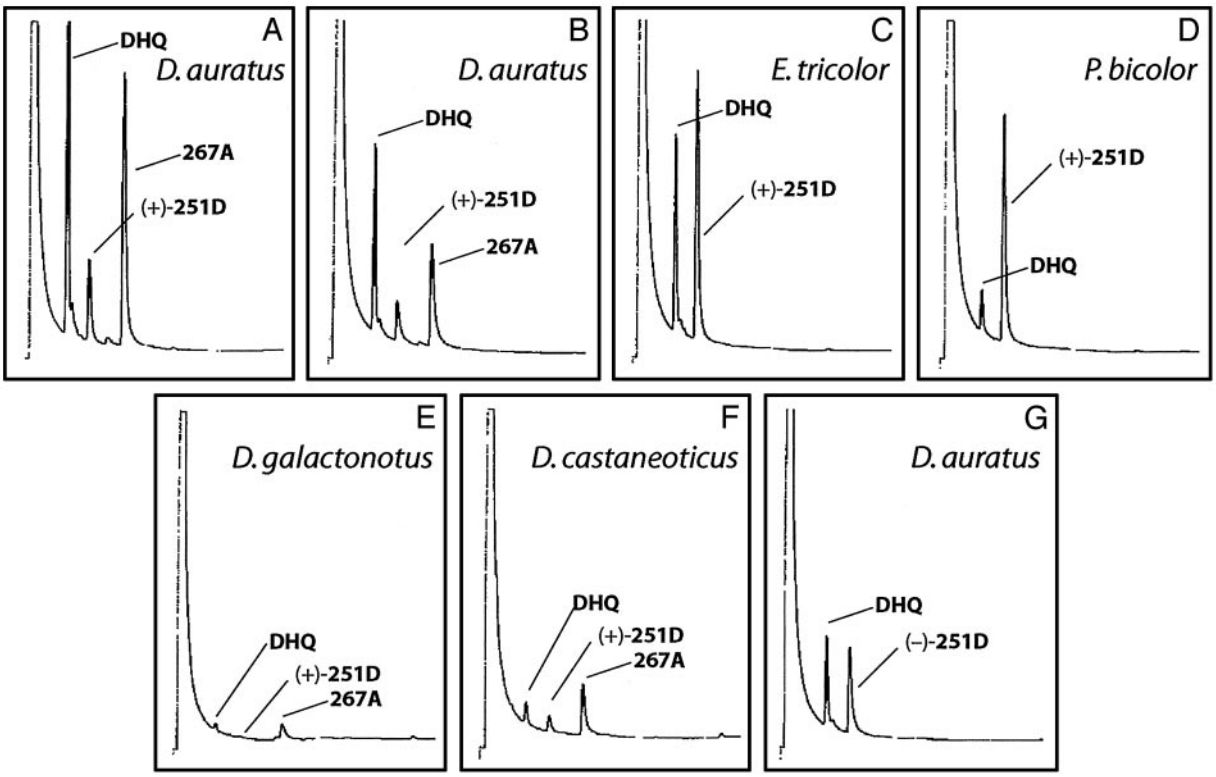


**Fig. 3.** Gas chromatographic profiles for alkaloids in skin of dendrobatid frogs fed alkaloid-dusted termites and/or fruit flies. Standard 1:1 mixtures of DHQ iso-**223F** and PTX (+)-**251D** were fed to frogs on dusted fruit flies unless otherwise noted. (*A*) *Dendrobates auratus*; DHQ and (+)-**251D**, primarily on termites, 6 wk. (*B*) *D. auratus*; DHQ and (+)-**251D**, 4 wk. (*C*) *Epipedobates tricolor*; DHQ and (+)-**251D**, 4 wk. (*D*) *Phyllobates bicolor*; DHQ and (+)-**251D**, 4 wk. (*E*) *D. galactonotus*; DHQ and (+)-**251D**, 1 wk. Frogs ate poorly and both died in ≈1 wk. (*F*) *D. castaneoticus*; DHQ and (+)-**251D**, 2 wk. Frogs ate poorly. (*G*) *D. auratus*; DHQ and unnatural (−)-**251D**, 5 wk. In each experiment, nearly identical results were obtained with 1 or 2 additional frogs.

in male 25- to 30-g NIH Swiss mice in a 1:4 mixture of methanol and isotonic saline. The injection volume was 0.2 ml. In further experiments, anticonvulsants (phenobarbital, carbamazepine, diazepam, and dizocilpine) were injected s.c. 10 min before 10 mg/kg (+)-**251D**. Two or three mice were used for each treatment.

## Results

**Feeding Experiments.** The initial feeding experiments, using mainly alkaloid-dusted termites, with *D. auratus*, a Central American species, provided a completely unexpected result. The major portion of the dietary PTX (+)-**251D** was present in skin as the 7$\beta$-hydroxy derivative aPTX (+)-**267A**. The vapor phase infrared spectrum (not shown) demonstrated clearly that (+)-**267A** had been formed, not the 7-epimer. Thus, the hydroxylation was stereospecific. The DHQ iso-**223F** accumulated into skin unchanged, as expected. The second experiment used alkaloid-dusted fruit flies with *D. auratus* and gave nearly the same result; no metabolism of the DHQ and extensive metabolism of (+)-**251D** to (+)-**267A** (compare Fig. 3 *A* and *B*). The 7-hydroxylation of PTX (+)-**251D** did not occur with an *Epipedobates* species (Fig. 3*C*) or a *Phyllobates* species (Fig. 3*D*). The third feeding experiment compared results with *D. auratus* (Fig. 3*B*) with those for two other species of the genus *Dendrobates*, namely *D. galactonotus* (Fig. 3*E*) and *D. castaneoticus* (Fig. 3*F*). Both also metabolized PTX (+)-**251D** to (+)-**267A**, but these Amazonian species ate very little, probably due to suboptimal ambient temperatures of only 20–21°C. Finally, the DHQ iso-**223F** and the unnatural enantiomer PTX (−)-**251D** were fed to *D. auratus*. Both alkaloids were sequestered into skin, but there was no detectable conversion of (−)-**251D** to a **267A** (Fig. 3*G*).

**Toxicity.** At a dose of 2 mg/kg, PTX (+)-**251D** caused only some initial hyperactivity in the mice, but no further effects. However, at 10 mg/kg, there was apparent marked pain at the injection site and hyperactivity. In ≈1 min, running convulsions commenced, leading to death after several minutes. Remarkably, the unnatural (−)-**251D** had virtually no effect on mice at 10 mg/kg. The aPTX (+)-**267A** was ≈5-fold more toxic than PTX (+)-**251D**. At 2 mg/kg aPTX (+)-**267A**, there were symptoms similar to those evoked by 10 mg/kg of PTX (+)-**251D,** with death in 6 min. Even at 0.5 mg/kg (+)-**267A**, there was apparent pain at the site of injection and pronounced long-lasting hyperactivity, with recovery in ≈1 h. The toxic effects (hyperactivity, convulsions, and death) evoked by 10 mg/kg of PTX (+)-**251D** were reduced by prior administration of the anticonvulsants phenobarbital (50 mg/kg) and carbamazepine (30 mg/kg), while being little affected by diazepam (5 mg/kg) or dizocilpine (0.5 mg/kg). The desmethyl analog **13** of PTX (+)-**251D** at 10 mg/kg seemed to cause only slight pain at the site of injection, followed by initial hyperactivity with complete recovery in 20 min. At 20 mg/kg, marked pain at the injection site and initial hyperactivity was followed in ≈10 min by prostration and finally loss of righting reflex and minor convulsions after ≈40–60 min. There was little recovery even after 3 h. The desmethyl analog **14** at 20 mg/kg caused no apparent pain at the injection site and had minimal effects on activity.

## Discussion

An alkaloid-sequestering system has been proposed to be the common evolutionary event allowing certain lineages of frogs from four different families to accumulate dietary alkaloids into secretory granular glands of skin for use in chemical defense

against predators (23). It seemed likely that this sequestering system was a primitive one and had been merely overexpressed in such frogs, who then were dependent on sequestered dietary alkaloids for chemical defense. The lineages proposed as having overexpressed this system were neotropical dendrobatid frogs of genera *Dendrobates*, *Epipedobates*, *Minyobates*, and *Phyllobates*, South American bufonid toads of the genus *Melanophryniscus*, Madagascan mantellid frogs of the genus *Mantella*, and myobatrachid frogs of the Australian genus *Pseudophryne*. Further experiments have now shown that frogs of the genus *Pseudophryne* do not have the same sequestering system, but instead seem likely to have an uptake system designed to retain the indolic pseudophrynamines that are synthesized by such frogs (15). The uptake system of the *Pseudophryne* did accumulate a PTX, albeit poorly, but not a simple DHQ or izidines. The selectivity of the uptake system probably accounts for the lack of the ant alkaloids of the DHQ and izidine classes in frogs of this genus, frogs known to be "ant specialists" (24).

Based on alkaloid-feeding experiments with frogs of the genera *Dendrobates*, *Epipedobates*, *Phyllobates*, and *Mantella* (5–7, 14), it had been proposed that the sequestering systems in such frogs were the same or very similar. Because no significant metabolism of any fed alkaloid had been observed with these frogs, it had also been assumed that all alkaloids would be sequestered unchanged. The alkaloids that have been fed and sequestered without metabolic change into skin of dendrobatid frogs (refs. 6 and 7, and unpublished results) include batrachotoxinin-A, a steroidal alkaloid of unknown dietary origin, and the following classes of alkaloids, demonstrated or presumed to be of ant origin (refs. 25 and 26, and unpublished results): pyrrolizidines, indolizidines, decahydroquinolines, and histrionicotoxins. A decahydroquinoline, two indolizidines, a histrionicotoxin, and aPTX **267A**, fed to frogs of the genus *Mantella*, were sequestered unchanged into skin (14). A piperidine and a pyrrolidine did not seem to be effectively sequestered when fed to either dendrobatid or mantellid frogs (6, 14). Dietary alkaloids were not accumulated into skin of dendrobatid frogs of the genus *Colostethus* (6); wild-caught *Colostethus* frogs do not have detectable lipophilic skin alkaloids.

In view of the results of earlier feeding experiments, it was entirely unexpected to find that PTX (+)-**251D** was metabolized extensively when fed to a dendrobatid frog, *D. auratus* (Fig. 3 *A* and *B*). Further experiments revealed that another frog, *D. galactonotus*, which, like *D. auratus*, is a member of the *Dendrobates tinctorius* group (27), also extensively metabolized PTX (+)-**251D** to aPTX (+)-**267A** (Fig. 3*E*). In addition, *D. castaneoticus*, a member of the *Dendrobates quinquevittatus* group (28), also hydroxylated PTX (+)-**251D** (Fig. 3*F*). Thus, it would seem that the expression of a pumiliotoxin 7-hydroxylase may prove to be common to all poison frogs of the genus *Dendrobates*. However, no frog of the third group of *Dendrobates*, namely the *Dendrobates histrionicus* group (29), has been tested, nor have frogs of a proposed dendrobatid genus *Minyobates* (30). No metabolism of PTX (+)-**251D** occurred in frogs of two other dendrobatid genera, namely *Phyllobates* and *Epipedobates* (Fig. 3 *C* and *D*).

Examination of our data on occurrence of PTX **251D** and aPTX **267A** in extracts from dendrobatid, mantellid, and bufonid anurans supports the conclusion that the presence of a pumiliotoxin 7-hydroxylase is probably associated as a synaptomorphy with frogs of the dendrobatid genus *Dendrobates*. In frogs of the genus *Dendrobates*, including members of the *D. histrionicus* group (29), the presence of significant amounts of PTX **251D** always was accompanied by significant amounts of the putative hydroxylation product aPTX **267A**. Representative examples are presented in Table 1. In some cases, aPTX **267A** was present, whereas PTX **251D** was not detected. In such cases, the aPTX **267A** may have been of dietary origin or dietary PTX **251D** may have been completely converted to aPTX **267A**. Certainly, the question remains unanswered as to how much of the frog skin aPTX **267A** is formed by 7-hydroxylation of ingested PTX **251D** and how much is obtained directly from a dietary source. The aPTXs probably do originate in some cases from dietary sources, a possibility indicated by the recent discovery of aPTX **323B** in mixed collections of Panamanian leaf-litter arthropods that were

**Table 1. Occurrence of pumiliotoxin 251D and allopumiliotoxin 267A in skin extracts of frogs of the dendrobatid genera *Dendrobates, Epipedobates, Minyobates,* and *Phyllobates****

| Dendrobatidae | PTX **251D** | aPTX **267A** |
|---|---|---|
| *Dendrobates* | | |
| *D. arboreus* | Minor | Major |
| *D. auratus* (5A)†‡ | Minor | Major |
| *D. azureus* | ND§ | Major |
| *D. granuliferus* | ND | Major |
| *D. histrionicus* (14A)†‡ | Minor | Major |
| *D. histrionicus* (14C)† | ND | Major |
| *D. lehmanni* | Trace | Major |
| *D. leucomelas* (16A)† | ND | Major |
| *D. occultator* | ND | ND |
| *D. pumilio* (25A)†‡ | ND | Trace |
| *D. pumilio* (25F)† | Minor | Major |
| *D. quinquevittatus* | ND | Minor |
| *D. reticulates* | ND | Minor |
| *D. speciosus* (29A)† | Minor | Minor |
| *D. tinctorius* | ND | Major |
| *D. truncates* | ND | ND |
| *D. ventrimaculatus* (35A)† | ND | Minor |
| *D. vicentei*¶ (8A)† | Major | Major |
| *Epipedobates*‡ | | |
| *E. erythromos* | ND | Major |
| *E. espinosai* | Minor | Minor |
| *E. parvulus* (22A)† | ND | ND |
| *E. petersi* | ND | ND |
| *E. pictus* (24 A)† | ND | ND |
| *E. silverstonei* | Major | ND |
| *E. tricolor* | Major | Trace |
| *E. trivittatus* | ND | ND |
| *Minyobates* | | |
| *M. abditus* | ND | ND |
| *M. altobueyensis* | ND | Major |
| *M. bombetes* (7A)† | Major | ND |
| *M. claudiae*‖ (17A)† | Minor | Minor |
| *M. fulguritus* | ND | Major |
| *M. viridis* (36A)† | ND | ND |
| *M. minutus (18A)*† | Trace | Major |
| *M. opisthomelas* | ND | ND |
| *M. steyermarki* | Trace | Major |
| *Phyllobates*‡ | | |
| *P. aurotaenia* | Trace | ND |

Data primarily from table 5 of ref. 1.

*The generic classifications are those of Myers (30). It has been suggested, based on very limited molecular data, that the frogs of the proposed genus *Minyobates* should be returned to the genus *Dendrobates* (31, 32). The present results suggest that further molecular data are required.

†Representative populations of this species are given. Code designations are from table 5 of ref. 1. For other populations see that table.

‡In certain populations of *D. auratus, D. histrionicus,* and *D. pumilio* and in *Epipedobates anthonyi, E. femoralis, E. myersi, Phyllobates bicolor, Phyllobates lugubris, Phyllobates terribilis* and *Phyllobates vittatus* neither PTX **251D** nor aPTX **267A** were detected (see tables 3 and 5 of ref. 1).

§ND, not detected.

¶Described in ref. 33, previously cited as *D. species* (8A, 8B) in table 5 of ref. 1.

‖Described as *D. claudiae* of the *minutus* group in ref. 34, previously cited as *D. species* (17A, 17B) in table 5 of ref. 1.

microsympatric with *Dendrobates pumilio* frogs containing aPTX **323B** (17).

Both PTX **251D** and aPTX **267A** have been found to occur in skin from dendrobatid frogs of the polyphyletic genus *Epipedobates* (Table 1). In two species of *Epipedobates*, aPTX **267A** was absent or a trace, whereas PTX **251D** was a major alkaloid (Table 1). In the most striking example, the major alkaloid in skin extracts from one population of the Ecuadoran *E. tricolor* was PTX (+)-**251D** (≈300 μg per frog; ref. 35) whereas only trace amounts of aPTX **267A** were detected. In the present study, *E. tricolor* did not convert PTX **251D** to aPTX **267A** (Fig. 3*C*). In *E. erythromosus*, aPTX **267A** was a major alkaloid whereas PTX **251D** was not detected. In *E. espinosai*, both PTX **251D** and aPTX **267A** were present as minor alkaloids (Table 1). The aPTX **267A** presumably must have a dietary origin, if these species, like *E. tricolor*, do not have a pumiliotoxin 7-hydroxylase.

In species of the proposed dendrobatid genus *Minyobates* (ref. 30; see footnote *, Table 1), for which feeding experiments were not conducted, the data from extracts are difficult to interpret for this probably polyphyletic genus. Two populations of *Minyobates bombetes* had PTX **251D** as a major alkaloid whereas aPTX **267A** was not detected, indicating lack of the 7-hydroxylase (Table 1). In different populations of *Minyobates minutus*, a species suggested on the basis of molecular data to group with *Dendrobates* (32), aPTX **267A** was always a major alkaloid whereas PTX **251D** varied from not detectable to being a major alkaloid. In *Minyobates claudiae* and *Minyobates viridis*, PTX **251D** was a minor alkaloid and was accompanied by aPTX **267A** as a minor or major alkaloid, respectively. In *Minyobates fulguritus* extracts, a species suggested on the basis of mitochondrial and ribosomal RNA analyses to group with *Dendrobates* (31), aPTX **267A** was a major alkaloid, whereas PTX **251D** was not detected. The data from *Minyobates bombetes* with major amounts of PTX **251D** and no aPTX **267A** in skin extracts indicate the lack of a pumiliotoxin 7-hydroxylase in this species, but conclusions regarding other *Minyobates* are not yet warranted. Such tiny frogs are "mite specialists" (36).

In poison-dart frogs of the genus *Phyllobates*, PTX **251D** was absent except for a trace amount in one species (*Phyllobates aurotaenia*), and aPTX **267A** was not detected (Table 1). PTX **251D** was readily taken up by *Phyllobates bicolor*, but was not hydroxylated (Fig. 3*D*).

Madagascan mantellid frogs of the genus *Mantella* contain a variety of PTXs, some aPTXs, and some homopumiliotoxins (10, 11). PTX **251D** was detected as a major alkaloid in populations of *Mantella cowani* and *Mantella expectata*, and as a minor or trace alkaloid in populations of *Mantella betsileo* and *Mantella baroni*. However, aPTX (+)-**267A** was not detected in *Mantella* frogs, although the 7-epimer was present as a trace alkaloid in extracts of one population of *Mantella cowani*, where PTX **251D** was a major alkaloid. Thus, it seems that *Mantella* frogs do not have a pumiliotoxin 7-hydroxylase.

In bufonid toads of the South American genus *Melanophryniscus*, PTX **251D** has been detected as a minor or trace alkaloid, but aPTX **267A** has never been detected in the six species from Argentina, Uruguay, and Brazil that have been studied (ref. 12, and unpublished results). Thus, such toads apparently do not have a pumiliotoxin 7-hydroxylase.

Neither PTX **251D** nor aPTX **267A** have been detected in skin extracts of myobatrachid frogs of the Australian genus *Pseudophryne*. However a side-chain hydroxylated analog of **251D**, namely PTX **267C**, does occur in *Pseudophryne*, often as a major alkaloid (13). An aPTX that would result from 7-hydroxylation of PTX **267C** has not been detected in *Pseudophryne*. PTX **307A**, when fed to *Pseudophryne semimarmorata*, was accumulated to a limited extent, accompanied by apparent conversion to reduced and/or side-chain hydroxylated congeners (15). Thus, it is unlikely that *Pseudophryne* frogs have a pumiliotoxin 7-hydroxylase.

Until the structural requirements for 7-hydroxylation of PTXs are defined, the importance of this pathway for conversion of other PTXs, such as PTX **307A** to aPTX **323B**, and of PTX **323A** to aPTX **339A**, remains uncertain. Both PTX **307A** and aPTX **323B** have been reported from mixed extracts of leaf-litter arthropods from Panamanian sites, where the dendrobatid frog *D. pumilio* has both alkaloids in its skin (17). As yet, neither PTX **251D** nor aPTX **267A** has been detected in arthropod extracts.

In addition to defining the remarkable evolutionary development of a pumiliotoxin 7-hydroxylase in frogs of the genus *Dendrobates*, the present results provide evidence suggesting that the alkaloid-sequestering systems of frogs is not rigorously conserved across genera. Thus, the DHQ iso-**223F** was accumulated quite efficiently relative to **251D**/**267A** in *D. auratus* (Fig. 3 *A* and *B*) and *E. tricolor* (Fig. 3*C*), compared with *Phyllobates bicolor* (Fig. 3*D*). Frogs of the genus *Dendrobates* have been characterized as "ant specialists" (37–39). Thus, development of a sequestering system with enhanced efficiency toward putative ant alkaloids, such as the DHQ class, might have been advantageous to the "ant specialists." However, DHQ iso-**223F** was not efficiently accumulated in the two Amazonian *Dendrobates spp.* (Figs. 3 *E* and *F*).

The mechanism and target underlying the unexpectedly high toxicity and hyperalgesic effects of PTX (+)-**251D** and aPTX (+)-**267A** are of great pharmacological import. Such high toxicity does not seem to correlate with the only known mechanism of action of PTXs, namely as positive modulators of sodium channels (40, 41), where PTX **251D** and aPTX **267A** seemed ineffective and only weakly active, respectively (42–44). It is noteworthy that, in initial experiments with anticonvulsants (45), the toxic effects of PTX (+)-**251D** were reduced by carbamazepine, an anticonvulsant that does target sodium channels, whereas diazepam, which targets γ-aminobutyric acid type A ($GABA_A$) receptors, and dizocilpine, which targets *N*-methyl-D-aspartate (NMDA) receptors, were relatively ineffective. Phenobarbital, which targets both $GABA_A$ receptors and calcium channels, did reduce the toxic effects of PTX (+)-**251D**. Whatever the target is, it must be highly selective because the unnatural enantiomer PTX (−)-**251D**, administered at a comparable dose to PTX (+)-**251D**, had no observable toxic effects in mice. In addition, the side-chain methyl substituent of (+)-**251D** contributes to toxicity, in particular to the running convulsions, because the desmethyl analog **13** was less toxic and elicited prostration rather than marked running convulsions. The enantiomeric desmethyl analog **14**, like unnatural PTX (−)-**251D**, was relatively inactive. The effects of PTXs/aPTXs on sodium channels and inositol trisphosphate formation have been proposed to account for the cardiotonic effects of such alkaloids (43), but other sites of action with marked stereoselectivity now must be considered to account for the convulsant activity of PTX **251D** and aPTX **267A**.

Further investigation needs to focus on the following: (*i*) characterization of the alkaloid-sequestering systems and their possible derivation from primitive transporters of unknown functional significance; (*ii*) characterization of the unique enantiospecific pumiliotoxin 7-hydroxylase and its relationship to other hydroxylases; (*iii*) the presence or absence of the hydroxylase in other species of dendrobatid frogs as a defining taxonomic feature indicative of synaptomorphy; (*iv*) the advantage to frogs of the conversion of PTXs to aPTXs (the increase in toxicity to predators may be the sole advantage to frogs); (*v*) the biological role of PTXs and aPTXs in arthropods, where both occur; and (*vi*) the molecular target(s) underlying the toxic effects of PTX **251D** and aPTX **267A**.

1. Daly, J. W., Myers, C. W. & Whittaker, N. (1987) *Toxicon* **25,** 1023–1095.
2. Daly, J. W., Brown, C. B., Mensah-Dwumah, M. & Myers, C. W. (1978) *Toxicon* **16,** 163–188.
3. Daly, J. W., Garraffo, H. M. & Spande, T. F. (1999) in *Alkaloids: Chemical and Biological Perspectives*, ed. Pelletier, S. W. (Pergamon, New York), pp. 1–161.
4. Daly, J. W., Myers, C. W., Warnick, J. E. & Albuquerque, E. X. (1980) *Science* **208,** 1383–1385.
5. Daly, J. W., Secunda, S. I., Garraffo, H. M., Spande, T. F., Wisnieski, A., Nishihara, C. & Cover, J. F., Jr. (1992) *Toxicon* **30,** 887–898.
6. Daly, J. W., Secunda, S. I., Garraffo, H. M., Spande, T. F., Wisnieski, A. & Cover, J. F., Jr. (1994) *Toxicon* **32,** 657–663.
7. Daly, J. W., Garraffo, H. M., Spande, T. F., Jaramillo, C. & Rand, S. (1994) *J. Chem. Ecol.* **20,** 943–955.
8. Myers, C. W. & Daly, J. W. (1976) *Bull. Am. Mus. Nat. Hist.* **157,** 173–262.
9. Myers, C. W., Daly, J. W. & Malkin, B. (1978) *Bull. Amer. Mus. Nat. Hist.* **161,** 307–366.
10. Garraffo, H. M., Caceres, J., Daly, J. W., Spande, T. F., Andriamaharavo, N. R. & Andriantsiferana, M. (1993) *J. Nat. Prod.* **56,** 1016–1038.
11. Daly, J. W., Andriamaharavo, N. R., Andriantsiferana, M. & Myers, C. W. (1996) *Am. Mus. Novitates* **3177,** 1–34.
12. Garraffo, H. M., Spande, T. F., Daly, J. W., Baldessari, A. & Gros, E. G. (1993) *J. Nat. Prod.* **56,** 357–373.
13. Daly, J. W., Garraffo, H. M., Pannell, L. K. & Spande, T. F. (1990) *J. Nat. Prod.* **53,** 407–421.
14. Daly, J. W., Garraffo, H. M., Hall, G. S. E. & Cover, J. F., Jr. (1997) *Toxicon* **35,** 1131–1135.
15. Smith, B. P., Tyler, M. J., Kaneko, T., Garraffo, H. M., Spande, T. F. & Daly, J. W. (2002) *J. Nat. Prod.* **65,** 439–447.
16. Daly, J. W., Garraffo, H. M., Jain, P., Spande, T. F., Snelling, R. R., Jaramillo, C. & Rand, A. S. (2000) *J. Chem. Ecol.* **26,** 73–85.
17. Daly, J. W., Kaneko, T., Wilham, J., Garraffo, H. M., Spande, T. F., Espinosa, A. & Donnelly, M. A. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 13996–14001.
18. McCloskey, P. J. & Schultz, A. G. (1988) *J. Org. Chem.* **53,** 1380–1383.
19. Overman, L. E. & Bell, K. L. (1981) *J. Am. Chem. Soc.* **103,** 1851–1853.
20. Overman, L. E., Bell, K. L. & Ito, F. (1984) *J. Am. Chem. Soc.* **106,** 4192–4201.
21. Caderas, C., Lett, R., Overman, L. E., Rabinowitz, M. H., Robinson, L. A., Sharp, M. J. & Zablocki, J. (1996) *J. Am. Chem. Soc.* **118,** 9073–9083.
22. Barger, T. M., Lett, R. M., Johnson, P. L., Hunter, J. E., Chang, C. P., Pernich, D. J., Sabol, M. R. & Dick, M. R. (1995) *J. Agric. Food Chem.* **43,** 1044–1051.
23. Daly, J. W. (1998) *J. Nat. Prod.* **61,** 162–172.
24. Webb, G. A. (1995) *Herpetofauna* **25,** 36–44.
25. Jones, T. H., Gorman, J. S. T., Snelling, R. R., DeLabie, H. C., Blum, M. S., Garraffo, H. M., Jain, P., Daly, J. W. & Spande, T. F. (1999) *J. Chem. Ecol.* **25,** 1179–1193.
26. Garraffo, H. M., Jain, P., Spande, T. F., Daly, J. W., Jones, T. H., Smith, L. J. & Zottig, V. E. (2001) *J. Nat. Prod.* **64,** 421–427.
27. Silverstone, P. A. (1975) *Nat. Hist. Mus. Los Angeles County Sci. Bull.* **27,** 1–55.
28. Caldwell, J. P. & Myers, C. W. (1990) *Am. Mus. Novitates* **2988,** 1–21.
29. Myers, C. W., Daly, J. W. & Martinez, V. (1984) *Am. Mus. Novitates* **2783,** 1–20.
30. Myers, C. W. (1987) *Papéis Avulsos Zool. S. Paulo* **36,** 301–306.
31. Vences, M., Kosuch, J., Lötters, S., Widmer, A., Jungfor, K.-H., Köhler, J. & Veith, M. (2000) *Mol. Phylogenet. Evol.* **15,** 34–40.
32. Clough, M. & Summers, K. (2000) *Biol. J. Linnean Soc.* **70,** 515–540.
33. Jungfer, K.-H., Weygoldt, P. & Jurasko, N. (1996) *Herpetofauna* **18,** 17–26.
34. Jungfer, K.-H., Lötters, S. & Jörgens, D. (2000) *Herpetofauna* **22,** 11–18.
35. Daly, J. W., Tokuyama, T., Fujiwara, T., Highet, R. J. & Karle, I. L. (1980) *J. Am. Chem. Soc.* **102,** 830–836.
36. Simon, M. P. & Toft, C. A. (1991) *Oikos* **61,** 263–278.
37. Caldwell, J. P. (1996) *J. Zool.* **240,** 75–101.
38. Donnelly, M. A. (1991) *Copeia* **3,** 723–730.
39. Toft, C. A. (1995) *Herpetologica* **51,** 202–216.
40. Gusovsky, F., Rossignol, D. P., McNeal, E. T. & Daly, J. W. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 1272–1276.
41. Gusovsky, F., Padgett, W. L., Creveling, C. R. & Daly, J. W. (1992) *Mol. Pharmacol.* **42,** 1104–1108.
42. Daly, J. W., McNeal, E. T., Overman, L. E. & Ellison, D. H. (1985) *J. Med. Chem.* **28,** 482–486.
43. Daly, J. W., Gusovsky, F., McNeal, E. T., Secunda, S. S., Bell, M., Creveling, C. R., Nishizawa, Y., Overman, L. E., Sharp, M. J. & Rossignol, D. P. (1990) *Biochem. Pharmacol.* **40,** 315–326.
44. Daly, J. W., McNeal, E., Gusovsky, F., Ito, F. & Overman, L. E. (1988) *J. Med. Chem.* **31,** 477–480.
45. McNamara, J. O. (1995) in *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, eds. Hardman, J. G., Gilman, A. G. & Limbird, L. E. (McGraw–Hill, New York), pp. 461–486.