Evidence for Biosynthesis of Pseudophrynamine Alkaloids by an Australian Myobatrachid Frog (*Pseudophryne*) and for Sequestration of Dietary Pumiliotoxins

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Australian myobatrachid frogs of the genus *Pseudophryne* have only two classes of alkaloids in skin extracts, pseudophrynamines (PSs) and pumiliotoxins (PTXs). The former are unique to such Australian frogs, while the PTXs occur worldwide in all other genera of frogs/toads that contain lipophilic alkaloids. The major alkaloid of wild-caught frogs from one population of *Pseudophryne semimarmorata* was PTX **267C**, while PSs were only minor or trace alkaloids. Captive-raised frogs from the same parental stock had no PTXs, but had larger amounts of PSs. A PTX fed to captive-raised frogs accumulated into skin along with dihydro and hydroxy metabolites. Thus, *Pseudophryne* frogs appear to biosynthesize PSs, but to sequester into skin dietary PTXs. In addition, biosynthesis of PSs appears reduced when high levels of dietary PTXs have accumulated into skin. This is the first evidence indicating that certain frogs are capable of synthesizing rather than merely sequestering alkaloids. A wide range of PSs, including many with molecular weights >500, were detected using both GC−mass spectral and LC−mass spectral analysis.

Biologically active peptides, amines, steroidal bufadienolides, and samandarine alkaloids represent some of the classes of compounds that amphibians elaborate and store in granular skin glands, apparently for chemical defense against microbial skin infections and/or predators.[1] However, the wide variety of lipophilic alkaloids, discovered in skin of dendrobatid frogs,[2] do not appear to be synthesized by such frogs, since captive-raised dendrobatid frogs completely lacked such alkaloids, but could sequester alkaloids unchanged into skin.[2] Ants, beetles, and millipedes were found to represent the dietary source for many of the "dendrobatid alkaloids".[3,4]

The pseudophrynamines (PSs), a unique class of indolic alkaloids with isoprenoid side chains, were discovered to be present in extracts of skin of Australian myobatrachid frogs of the genus *Pseudophryne*.[5,6] Such alkaloids had not been detected in skin extracts of dendrobatid frogs. Structures for three of these alkaloids, namely, pseudophrynaminol (code designation **258A**), pseudophrynamine A (**512**), and pseudophrynamine **286A**, were established[5] and later confirmed by synthesis.[7−9] For a review of frog skin alkaloids, including code designations (based on nominal molecular weights), see ref 2. A variety of PSs were present in skins of several species and populations of *Pseudophryne* frogs of Queensland, New South Wales, Victoria, and Western Australia[2,6] (see Figure 1 for structures and tentative structures). PSs are unknown in nature except from *Pseudophryne* frogs, but do contain the same ring system as the eserine-class alkaloids from leguminosae plants and the flustramines from marine fungi. In addition to the unique PSs, frogs of the genus *Pseudophryne* also contain PTXs,[5,6] sometimes as major alkaloids in the case of PTXs **267C** and **323A** and alloPTX **323B** (see Figure 1 for structures). PTXs occur in all other frogs/toads that are known to contain lipophilic alkaloids in skin extracts, namely, four genera of neotropical dendrobatid frogs, one genus of Madagascan mantellid frogs, and one genus of South American bufonid toads.[2] PTXs and most of the simpler mono- and bicyclic alkaloids that have been found in such frogs are now known to have a dietary origin (refs 2−4 and unpublished results). It was, therefore, natural to assume that the PSs also were derived from dietary arthropods. The absence of such PSs from skin of all of the other alkaloid-containing lineages suggested the existence of either an endemic Australian arthropod or a nocturnal arthropod, which would be available to nocturnal *Pseudophryne*, but not to the other lineages of alkaloid-containing frogs/toads, all of which are diurnal. However, the present results indicate that the *Pseudophryne* frogs synthesize the PSs, while obtaining the PTXs from an unknown dietary source.

Results

GC-FID Analysis. Alkaloid extracts from skin of eight wild-caught *Pseudophryne semimarmorata* (Table 1) from Holey Plains State Park were analyzed by GC-FID to provide relative amounts of alkaloids (Figure 2). However, higher molecular weight PSs (mol wt >500) do not gas chromatograph under these conditions or those of GC−MS analysis. In addition, PS **300** and PS **330** apparently do not gas chromatograph well (compare data of Figures 4 and 5). The major alkaloid in each extract was found on GC−MS analysis to be PTX **267C**, present at about 150 μg or more per frog, the amount not differing greatly between individual frogs (Figure 2A−D, and traces not shown) regardless of sex, size, or exact origin in the State Park. Minor amounts of other alkaloids were present in two of the eight wild-caught frogs (see Figure 2A,C) as detected by GC-FID and were identified by GC−MS to be PSs **302**

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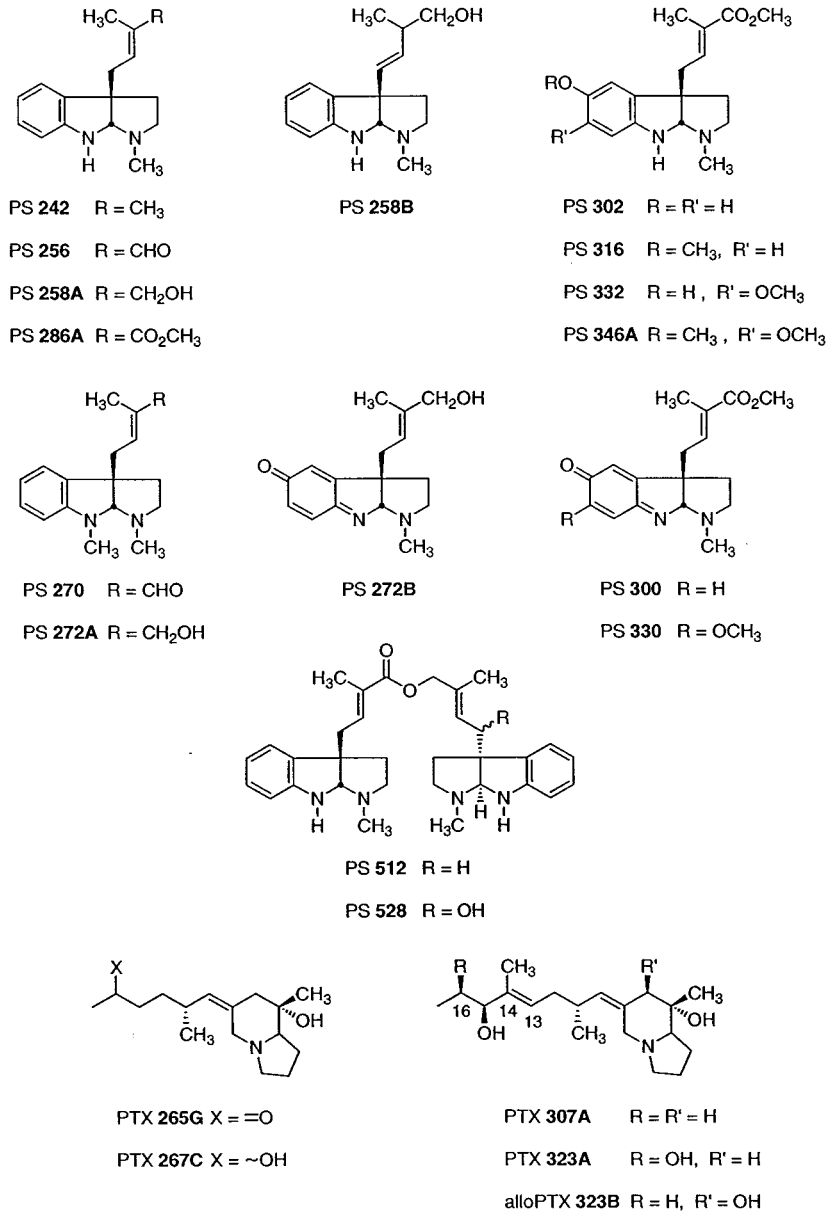

Figure 1. Structures of pseudophrynamines (PSs) and pumiliotoxins (PTXs). Absolute stereochemistry shown for PSs is based on that of (−)-pseudophrynaminol (**258A**). Structures for PSs **258B**, **270**, **272A**, and **272B** are tentative, as is the position of the methoxy group in PSs **316**, **332**, and **346A**. Structures are based primarily on mass spectral data including deuterium exchange (refs 2, 6 and unpublished data). No structures are proposed for trace PSs **240**, **274**, **278**, **362**, **524**, **526**, **530**, **538**, **540**, **542**, **556**, **572**, **574**, and **600** (see Tables 1, 2). More than one isomer was sometimes detected for PSs **300**, **302**, **330**, **332**, and **542**. Typical PSs, such as PSs **258A** and **286A**, under LC-MS APCI conditions showed a significant loss of a neutral 31 amu fragment proposed to be methylamine. PSs **242**, **274**, **316**, **526**, **528**, **540**, and **556** showed this loss, while PSs **300**, **302**, and **330** did not. PTXs **267C** and **323A** and alloPTX **323B** have been detected as major alkaloids in *Pseudophryne* frogs, while PTX **307A** has not, even as a trace alkaloid.

and **330**. Another species, *Pseudophryne bibroni*, also contained predominantly PTX **267C** (Figure 2E). Trace amounts of PSs were present in all wild-caught *Pseudophryne* frogs, based on the more sensitive GC−MS and LC−MS analyses.

A myobatrachid frog from another genus, *Geocrinia laevis*, did not contain PTXs or PSs, but did contain a small amount of an ant decahydroquinoline, **195A** (Figure 2F), an alkaloid that is frequently found in dendrobatid frogs and occasionally in mantellid frogs.[2] As yet, decahydroquinolines have not been detected in wild-caught *Pseudophryne* frogs (ref 6 and present results). In the original description of *Geocrinia*, the relationship between that *Geocrinia* and *Pseudophryne* was not considered particularly close;[10] however, later extensive comparative studies suggested an intimate relationship.[11] Previous studies on Australian frogs from six other myobatrachid genera did not reveal any skin alkaloids.[6] Thus, even in myobatrachid frogs, PSs are unique to the genus *Pseudophryne*.

Alkaloid extracts from skin of the captive-raised *Pseudophryne semimarmorata* contained a variety of PSs, as identified by GC−MS analysis (Figure 3A−F and traces not shown). The 18 captive-raised frogs (Table 2) represented four different groups differing in parentage and/or age (see Experimental Section). The parental stock were wild-caught frogs from the same State Park. PS levels were higher in most of the captive-raised frogs compared to wild-caught frogs. Levels were higher in the 10 month old frogs of group three (Figure 3A−D) than in the older frogs comprising group four (Figure 3E,F). One frog from group three had very high levels of PS **258A** (Figure 3B).

Table 1. Alkaloids in Skin Extracts of *Pseudophryne semimarmorata* from Wild-Caught Frogs: Pumiliotoxins (PTX) and Pseudophrynamines (PS)

		alkaloid profiles[a]		
wild-caught specimen	relative amount	based on GC−MS analysis (Figure 4)	based on LC−MS analysis (Figure 5)	chromatograms
	major	PTX **267C**	PTX **267C**	Figures 2A, 4A, 5A
I. male	minor	PS **302**	PS **300**, **330** [2], **542**	
	trace	PTX **265G**; PS **330**	PS **526**, **540**, **600**	
	major	PTX **267C**	PTX **267C**	
II. male	minor	PS **302**	PS **300**	
	trace	PTX **265G**; PS **332**	PS **330**, **526**, **542**, **572**, **600**	
	major	PTX **267C**	PTX **267C**	
III. female	minor		PS **300**, **542**	
	trace	PTX **265G**; PS **302**	PS **526**, **556**, **572**	
	major	PTX **267C**	PTX **267C**	
IV. female	minor		PS **258A**, **300**, **330**, **526**	Figures 2B, 4B, 5B
	trace	PTX **265G**; PS **258A**, **302**	PS **302**, **332**, **540**, **542**, **556** [2], **558**	
	major	PTX **267C**	PTX **267C**	
V. female	minor	PS **302**	PS **300**	Figure 2C
	trace	PTX **265G**; PS **332**	PS **330**, **526**, **528**, **538**, **542**, **556**, **572**, **614**	
	major	PTX **267C**	PTX **267C**	
VI. male	minor		PS **300**	
	trace	PTX **265G**; PS **302**	PTX **265G**; PS **258A**, **330**, **526**, **542**, **558**, **572**, **574**	
	major	PTX **267C**	PTX **267C**	
VII. male	minor		PS **526**	
	trace	PTX **265G** [2]	PS **258A**, **300**, **330**, **332**, **526**, **528**, **556**, **558**, **572**	
	major	PTX **267C**	PTX **267C**	
VIII. male	minor			Figure 2D
	trace	PTX **265G** [2]	PS **300**, **330**, **526**, **538**, **542**, **544**, **556**, **558**, **572**	

[a] Alkaloids are designated by bold-face code names based on nominal molecular weight and including, when necessary, a letter to distinguish alkaloids with the same nominal mol wt (see ref 2 for complete listing). The presence of two isomers is indicated by a bracketted [2]. Estimates of total levels, based on GC-FID traces, indicated that all wild-caught frogs had about or more than 150 μg per 100 mg skin. Structurally undefined alkaloids with odd molecular weights occurred in all frogs as trace components. The most common had mol wts of 365, 367, 591, and 607 [2]. Others detected in more than one wild-caught or captive-raised frog had mol wts of 395, 541, 593, 621, and 663.

GC−MS Analysis. Alkaloid extracts from skin of wild-caught frogs on GC−MS analysis (Figure 4A,B and Table 1) contained predominantly PTX **267C**, accompanied in most cases by low levels of PSs **302** and/or **330**. In some cases there was a low level of another PTX, tentatively identified as PTX **265G** (see Figure 1 for structure). PTX **265G** had been detected previously only in mantellid frogs.[2]

Captive-raised frogs had no PTX **267C**, and the levels of PSs were usually greater than in the wild-caught frogs (Figure 4C−F and Table 2). The major alkaloid in captive-raised frogs was PS **258A**, while PS **286A** was usually a minor or trace component, based on GC−MS. Thus, GC−MS analysis clearly demonstrated the lack of PTXs in captive-raised frogs and a marked change in the type of PSs with the ring-oxygenated PSs **302** and **330** being predominant in wild-caught frogs, while the simpler PS **258A** and PS **286A** predominated in captive-raised frogs.

LC−MS Analysis. The LC−MS analysis revealed for all frogs higher molecular weight PSs (Figure 5 and Tables 1 and 2) that were undetected by GC-FID and GC−MS analysis. In most captive-raised frogs, PS **258A** appeared the major alkaloid by LC−MS analysis, as it had been for all captive-raised frogs by GC−MS analysis. However, PS **526**, undetected by GC−MS, was the major alkaloid in two of the 18 captive-raised frogs, based on LC−MS analysis. In addition, in five of the captive-raised frogs and four of the wild-caught frogs PSs **526** and/or **542** were minor components. PS **300**, which is detected poorly on GC analyses, was a major alkaloid by LC−MS analysis in two of the 18 captive-raised frogs. Except for **512**,[5] all of the high molecular weight alkaloids require further characterization (see legend of Figure 1).

High molecular weight compounds with odd rather than even nominal molecular weights were present in trace amounts in almost all alkaloid extracts and probably represent alkaloids with three nitrogens. All have an UV absorption at 260 nm. The most common had apparent nominal molecular weights of 365, 367, 591, and 607 (see legend Table 1). At present, the trace amounts of such odd nominal mass compounds and the instability of some have precluded clear structural insights.

Uptake of Pumiliotoxins. Feeding experiments were conducted in which six captive-raised frogs (see footnote *b*, Table 2) were fed crickets dusted with powder containing the alkaloid mixture described in the Experimental Section for two months. The decahydroquinoline **195A**, an alkaloid known in dendrobatid frogs to be of ant origin,[3,4] was detected at trace levels in one of the six frogs fed this alkaloid. PTXs were detected at trace levels in all six of the alkaloid-fed frogs. Remarkably, not only the PTX **307A** fed to the frogs but apparent metabolites were present; it appeared that reduction of the 13,14-double bond of **307A** had yielded a PTX with a nominal molecular weight of 309, tentatively **309A**, or that 16-hydroxylation had yielded a PTX with a nominal molecular weight of 323, tentatively **323A**. In one frog both reduction and hydroxylation had evidently occurred, yielding a PTX with a nominal molecular weight of 325, tentatively **325A**. All PTXs were present only at trace levels. The springtails, fruit flies, and crickets that comprised the food of captive-raised frogs from metamorphosis to adulthood were analyzed by GC−MS and LC−MS, and no alkaloids were detected.

Discussion

The indolic PSs, as yet known only in Australian myobatrachid frogs of the genus *Pseudophryne*,[2] initially were assumed to be of dietary origin, in analogy with the alkaloids in the dendrobatid and mantellid frogs.[2] However, the present finding of such PSs in captive-raised *Pseudo-*

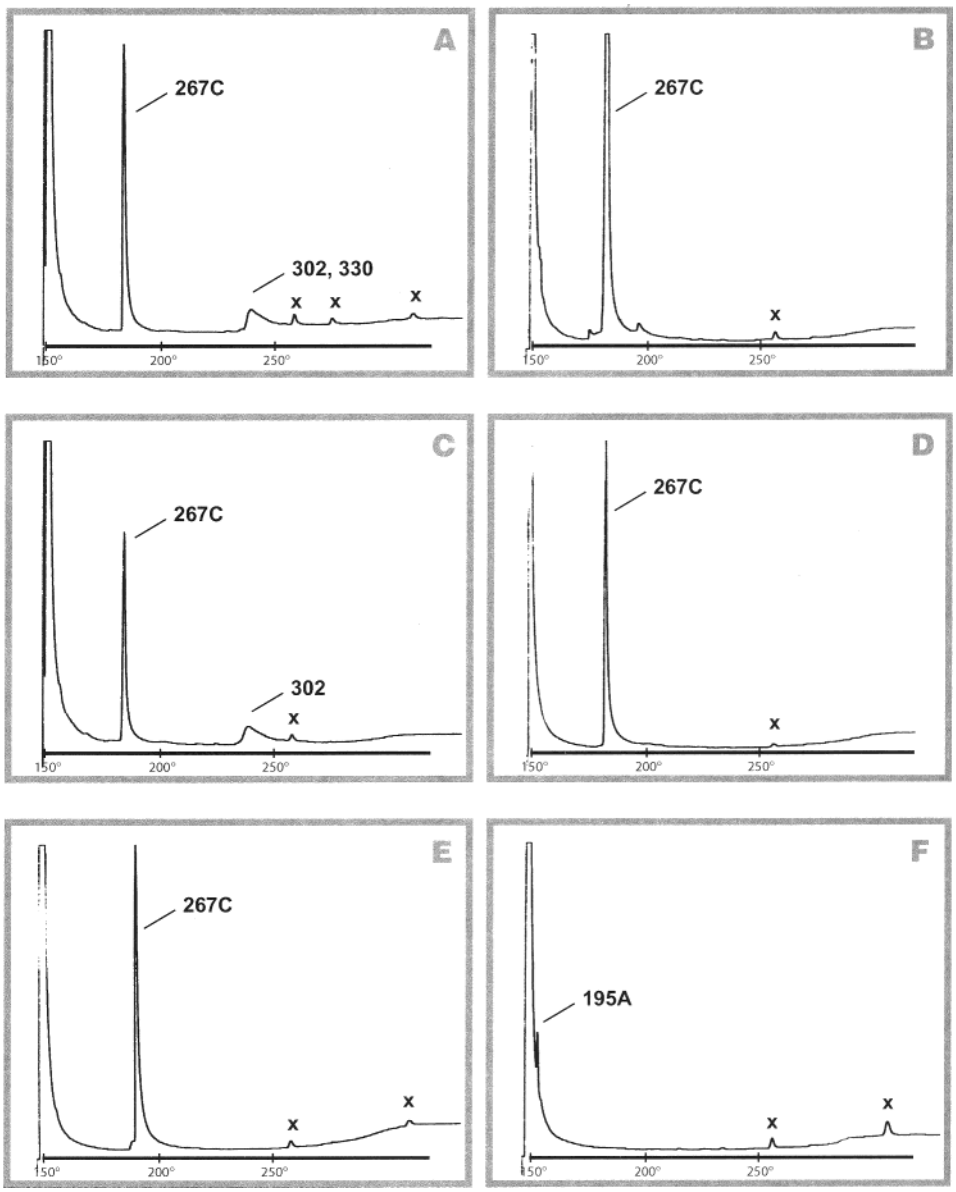

Figure 2. GC-FID profiles of alkaloids from skins of wild-caught myobatrachid frogs. *Pseudophryne semimarmorata*: (A) Male I. (B) Female IV. (C) Female V. (D) Male VIII. Chromatograms for frogs II, III, VI, and VII not shown, but were similar to those shown. *Pseudophryne bibroni*: (E) Two males. *Geocrinia laevis*: (F) Male. The GC-FID chromatograms were obtained as described in the Experimental Section. Alkaloids were then identified by GS−MS analysis (see Figure 4). × = phthalate or other artifact.

phryne semimarmorata indicates that such frogs elaborate these relatively complex indolic alkaloids with isoprenoid side chains. However, the involvement of a symbiotic microorganism cannot be precluded. PSs were detected only in skin and not in muscle, liver, or intestine (data not shown). A closely related frog, *Geocrinia laevis*, did not contain PSs. The *Pseudophryne* frogs seem to be avoided by predator snakes,[12,13] probably due to the presence of skin alkaloids.

Captive-raised frogs, except those that were alkaloid-fed, lacked PTXs, one of which, PTX **267C**, was the predominant alkaloid in wild-caught frogs from the State Park where the parental stock of the captive-raised frogs originated. Thus, like the PTXs of dendrobatid frogs, which apparently are obtained from a dietary arthropod (unpublished results), the PTXs of the *Pseudophryne* frogs also appear to derive from a dietary source. PTX **267C** occurs very rarely in dendrobatid frogs, but occurs frequently in *Pseudophryne* frogs (see Table 3). Extracts of potential dietary arthropods from Holey Plains State Park (see Experimental Section) were examined; PTX **267C** was not detected.

In view of the high levels of PTX **267C** in the wild-caught frogs, it was surprising that frogs fed pumiliotoxin **307A** for two months accumulated only trace amounts (see Results). This is in marked contrast to feeding experiments with captive-raised dendrobatid and mantellid frogs, where the sequestering systems seemed very efficient, yielding intermediate to high levels of a variety of alkaloids in skin.[2,14] Sequestration of alkaloids in the captive-raised *Pseudophryne* frogs appeared quite inefficient for PTX **307A**, while being ineffective for a histrionicotoxin, a 3,5-disubstituted indolizidine, and a decahydroquinoline, all of which are efficiently sequestered by dendrobatid and mantellid frogs. Furthermore, while no evidence for any metabolic alteration in alkaloids fed and sequestered into skin by dendrobatid or mantellid frogs has been obtained, PTX **307A** fed to *Pseudophryne* frogs appeared to be converted to a significant extent to PTXs with nominal molecular weights of 309, 323, and 325, presumably by

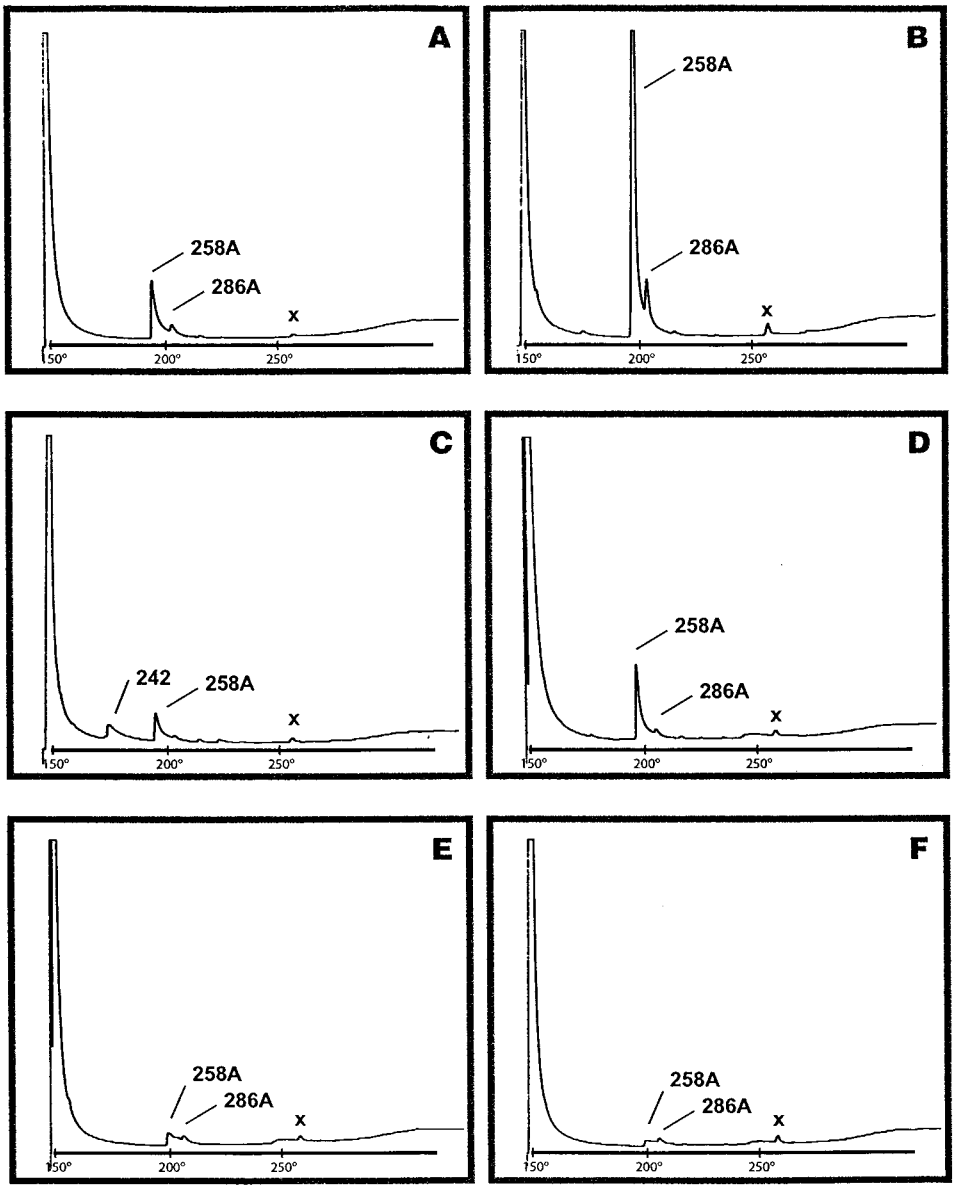

Figure 3. GC-FID profiles of alkaloids from skins of captive-raised myobatrachid frogs *Pseudophryne semimarmorata*: (A) Group three, male XIa. (B) Group three, male XIb. (C) Group three, male XIc. (D) Group three, male XId. (E) Group four, two males XIIa. (F) Group four, male XIId. Chromatograms are not shown for 11 frogs. Levels were low and similar to E and F for the two frogs of group one, for two frogs of group two (Xb, Xf), and for the other two frogs of group four. Levels were modest and similar to A and D for the other four frogs of group two and for the one other frog of group three (see Table 2). For GC-FID details see legend of Figure 2. × = phthalate or other artifact.

enzymatic reduction and/or hydroxylation. Thus, *Pseudophryne* frogs apparently do not have the efficient and relatively nonselective sequestering system of dendrobatid and mantellid frogs, but do have enzymatic systems capable of metabolizing alkaloids. Further studies on uptake of alkaloids by *Pseudophryne* frogs are required to establish structure−activity requirements and how such an apparently low-efficiency system can lead to accumulation of very high levels of PTX **267C** in wild-caught frogs. It is possible that **267C**, but not **307A**, can be readily accumulated; PTXs **267C** and **323A** are often found in wild-caught *Pseudophryne* frogs, but PTX **307A** has never been detected.[6] Supplies of **267C** were insufficient for feeding experiments. The lack of significant uptake of ant alkaloids **223AB** and **195A** explains why ant alkaloids have never been detected in wild-caught *Pseudophryne* frogs (ref 6 and present results), even though such frogs ingest mainly ants.[15,16]

Comparison of levels and nature of PS alkaloids in wild-caught and captive-raised frogs indicated that there are usually much lower levels in wild-caught than in captive-raised frogs. In wild-caught frogs, ring-oxygenated PSs **302** and **330** were predominant, with the simpler PSs **258A** and **286A** being undetectable or trace constituents (Table 1). In contrast, PSs **258A** and **286A** were predominant in captive-raised frogs (Table 2). It appears possible that the accumulation of high levels of a dietary PTX in wild-caught frogs turns off the biosynthesis of PSs **258A** and **286A** and that any PS **286A**, which has been formed, is oxidized further to PSs **302** and **330**. In our previous studies with several *Pseudophryne* species, we found that whenever wild-caught frogs had high levels of PTXs, levels of PSs were usually low.[6]

For the higher molecular weight PSs the distinction between wild-caught and captive-raised frogs was less clear. Based on LC−MS analysis, both wild-caught (4 of 8) and captive-raised (8 of 18) frogs had PSs **526** and/or **542** as significant (even major in two captive-raised frogs) components (see Tables 1 and 2).

The captive-raised frogs represented four different groups and variations in levels, and profiles of PSs between groups

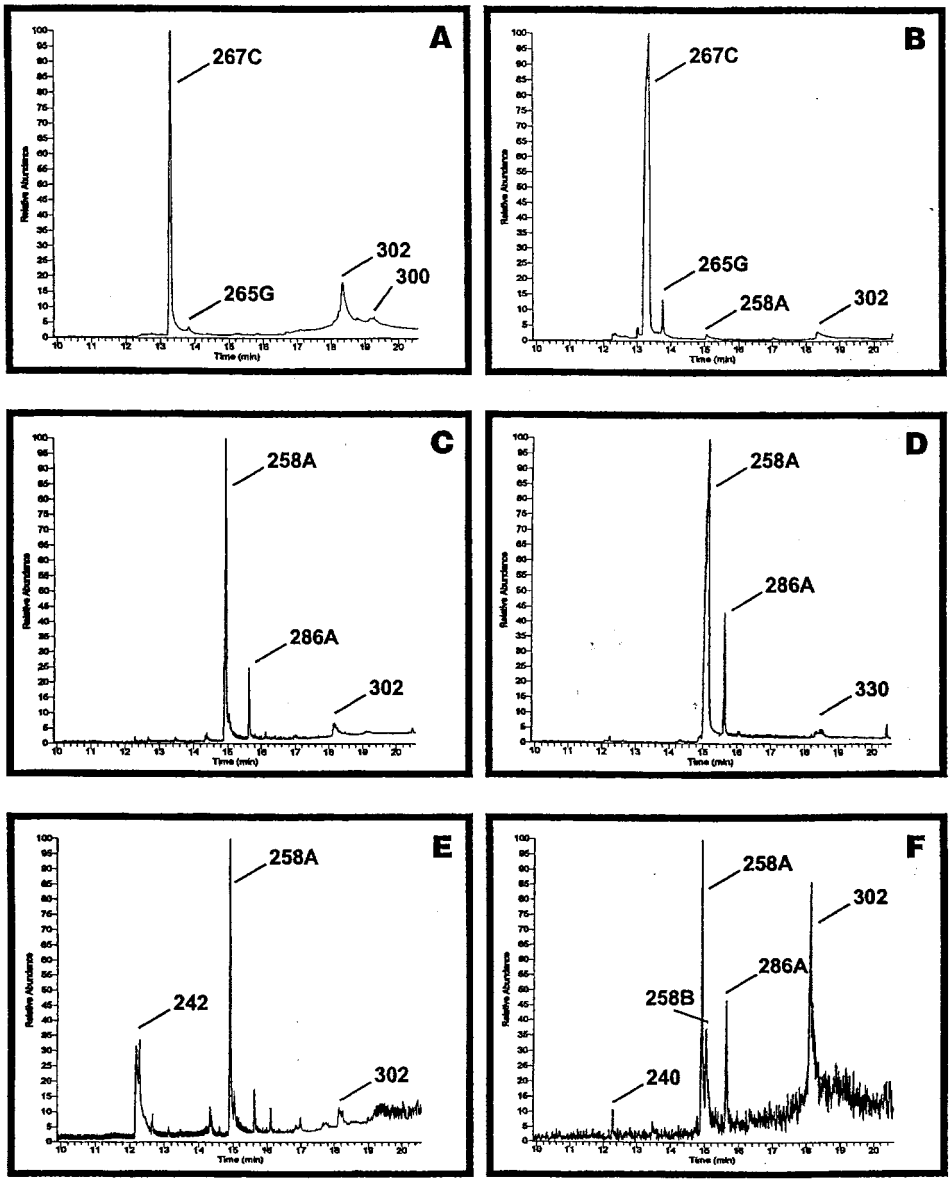

Figure 4. GC−MS analysis of alkaloids from skins of myobatrachid frogs. *Pseudophryne semimarmorata*: (A) Wild-caught male I. Corresponds to Figure 2A, 5A. (B) Wild-caught female IV. Corresponds to Figures 2B, 5B. (C) Captive-raised male XIa. Corresponds to Figures 3A, 5C. (D) Captive-raised male XIb. Corresponds to Figures 3B, 5D. (E) Captive-raised male XIc. Corresponds to Figures 3C, 5E. (F) Two captive-raised males XIIa. Corresponds to Figures 3E, 5F. GC−MS analyses were as described in the Experimental Section.

were not unexpected in view of probable differences in age and in parentage for such groups. Comparison of the two best documented groups, namely, groups three and four, indicates that the younger frogs of group three (Figure 3A−D) had significantly higher levels of PSs than did the older frogs (Figure 3E,F). However, the parentage for the two groups might have been different. The older frogs of group four showed little difference in amounts of PSs. However, in the younger group, one frog had significantly higher levels of PSs **258A** and **286A** (Figure 3B) than any of the other frogs, while another frog had lower levels of PSs **258A** and **286A** (Figure 3C) than the other frogs and instead had significant levels of a PS **242**. PS **242** had not been detected in previous studies on *Pseudophryne* species,[5,6] but a "deoxypseudophrynaminol" that could correspond to it has been synthesized.[17]

The alkaloids detected in previous studies for different species and populations of *Pseudophryne* frogs[6] along with the present results for the wild-caught *Pseudophryne semimarmorata* and *P. bibroni* are listed in Table 3. Only major and minor alkaloids that would be detected under standard GC-FID conditions are listed along with any high molecular weight (>500) alkaloids detected as major or minor alkaloids. High molecular weight PSs were detected in earlier studies[6] by direct probe and thermospray MS, since the more sensitive LC−MS instrumentation was not available to us. It should be noted that, while PTXs are sometimes present at high levels, PSs are usually at relatively low levels in all wild-caught frogs.

In conclusion, profiling of alkaloids in skin extracts of a nocturnal myobatrachid frog, *Pseudophryne semimarmorata*, both with wild-caught and captive-raised frogs suggests the following: (A) PTXs are of a dietary origin and would be expected in each species or population to reflect availability of the prey item. (B) PSs appear to be elaborated by the frogs, certainly independent of a dietary source. Elaboration of these complex indolic alkaloids with isoprenoid side chains appears unique to such myobatrachid frogs. (C) Accumulation of high skin levels of

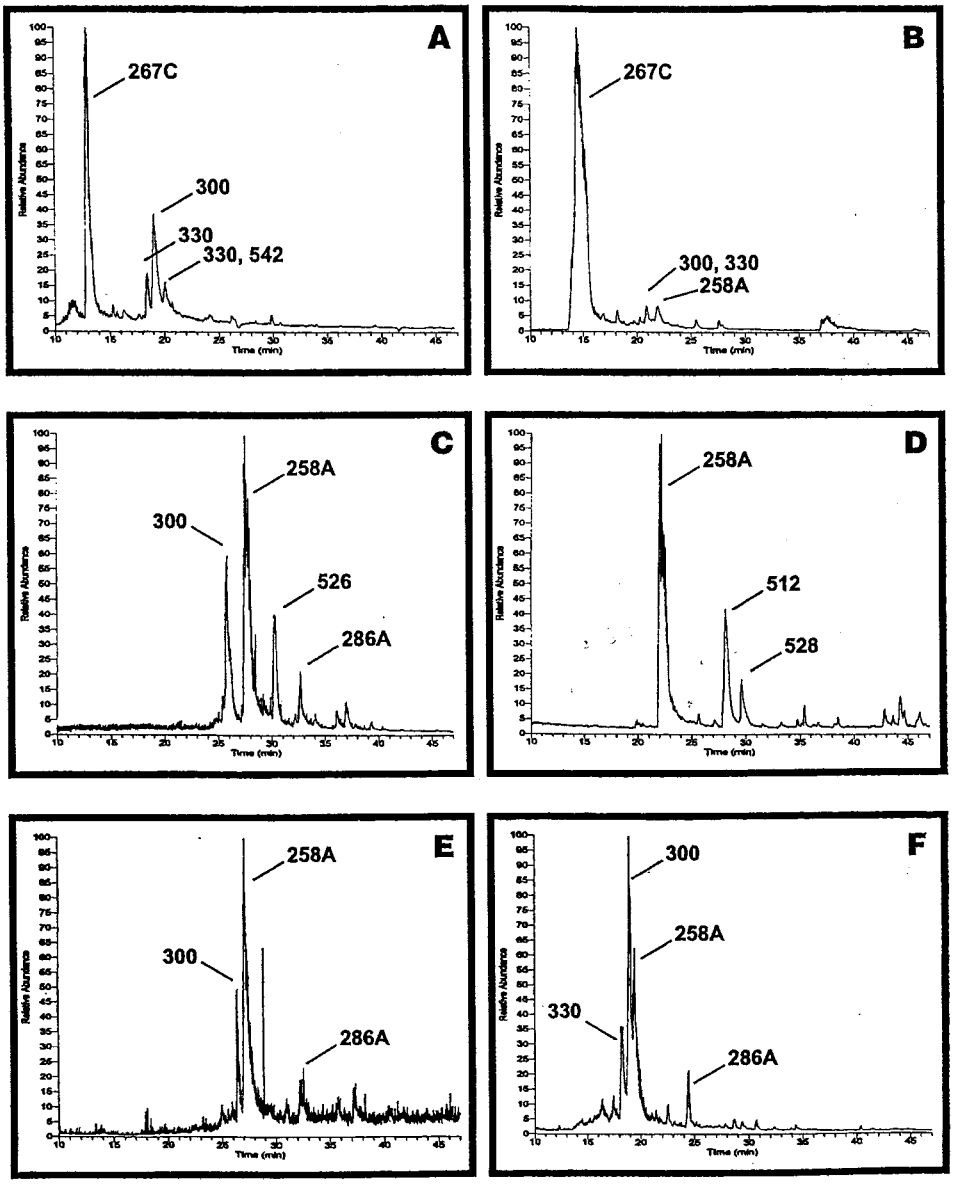

Figure 5. LC−MS analysis of alkaloids from skins of myobatrachid frogs. *Pseudophryne semimarmorata*: (A) Wild-caught male I. Corresponds to Figures 2A, 4A. (B) Wild-caught female IV. Corresponds to Figures 2B, 4B. (C) Captive-raised male XIa. Corresponds to Figures 3A, 4C. (D) Captive-raised male XIb. Corresponds to Figures 3B, 4D. (E) Captive-raised male XIc. Corresponds to Figures 3C, 4E. (F) Two captive-raised males XIIa. Corresponds to Figures 3E, 4F. LC−MS analyses were as described in the Experimental Section; concentration differences and column and elution conditions probably account for varying retention times.

dietary PTXs appears to turn off synthesis of PSs. (D) The alkaloid-sequestering system of *Pseudophryne* frogs is different from the system of dendrobatid and mantellid frogs in being apparently much less efficient, although perhaps selective for certain PTXs, and unable to accumulate effectively indolizidine or decahydroquinoline ant alkaloids. Further studies on structures and apparent synthesis of pseudophrynamines and on sequestration of alkaloids by such vertebrates are warranted along with identification of the dietary arthropod source of the PTXs found in skin of dendrobatid, mantellid, bufonid, and myobatrachid frogs.

Experimental Section

Sources and Preparation of Alkaloid Extracts. Wild-caught *Pseudophryne semimarmorata* Lucas, 1892 were collected from three locations in Holey Plains State Park, Victoria (April 24−25, 1999). Frogs I−IV were from one natural inundation area, V from another near the pipeline, and VI−VIII from a pine plantation. All were adults with snout−vent lengths of 18−26 mm and a wet weight of skin ranging from 180 to 250 mg. Two specimens of *Pseudophryne bibroni* Günther, 1859 (skin wet weight 250 mg each) were from Norton Summit, South Australia (April 14, 2000). The *Geocrinia laevis* Blake, 1973 (skin wet weight 150 mg) was from Mt. Burr near Mt. Gambier, South Australia (August 5, 1999). Skins were placed in 4 mL NUNC vials (Nalge Nunc Internat., Rochester, NY) with MeOH. Voucher specimens are at Adelaide University. Captive-raised frogs (parental stock from the above State Park) were obtained from the Amphibian Research Center, Melbourne, Victoria. The captive-raised frogs can be divided into four groups: (1) two F2 generation frogs (IXa, IXb), analyzed in February 1999; (2) six frogs (Xa−Xf), presumably an F1 generation from one hatching, analyzed in December 1999; (3) five male frogs (XIa−XIe) that underwent metamorphosis in October 1999 and were sacrificed and analyzed August 2000 at an age of 10 months (F2, full

Table 2. Alkaloids in Skin Extracts of *Pseudophryne semimarmorata* from Captive-Raised Frogs: Pumiliotoxins (PTX) and Pseudophrynamines (PS)

		alkaloid profiles[a]		
captive-raised specimen	relative amount	based on GC−MS analysis (Figure 4)	based on LC−MS analysis (Figure 5)	chromatograms
group one	major	PS **258A**	PS **258A**	
IXa	minor	PS **302** [2], **316**, **332**	PS **300**, **330**	
	trace	PS **242**, **256A**, **258B**, **300**	PS **556**, **600**	
group one	major	PS **258A**, **332** [2]	PS **330** [2]	
IXb[b]	minor	PS **302**[2]	PS **258A**, **300**	
	trace	PS **240**, **242**, **258B**, **278**, **286A**, **300**, **330**, **346**, **362**	PS **526**, **556**, **600**	
group two	major	PS **258A**	PS **258A**	
Xa	minor	PS **286A**	PS **300**, **526**, **542**	
	trace	PS **240**, **242**, **256**, **258B**	PS **242**, **258B**, **286A**, **330**, **332**, **540**, **556**, **558**, **572**	
group two	major	PS **258A**	PS **258A**	
Xb	minor		PS **286A**, **300**	
	trace	PS **242**, **256**, **286A**	PS **242**, **256**, **274**, **524**, **556**, **572**	
group two	major	PS **258A**	PS **258A**	
Xc[b]	minor	PS **286A**	PS **300**, **526**, **542**	
	trace	PS **242**, **258B**	PS **258B**, **286A**, **332**, **556**, **558**, **572**	
group two	major	PS **258A**	PS **258A**	
Xd	minor	PS **256**	PS **256**, **300**, **526**, **542** [2]	
	trace	PS **242**, **286A**, **316**, **346**	PS **286A**, **332**, **540**, **556**, **558**, **572**	
group two	major	PS **258A**	PS **258A**	
Xe	minor	PS **256**, **300**	PS **300**, **526**	
	trace	PS **242**, **286A**	PS **332**, **542**, **556**, **558**, **572**	
group two	major	PS **258A**	PS **258A**	
Xf	minor	PS **286A**	PS **286A**	
	trace	PS **258B**	PS **274**, **542**, **544**, **560**, **572** [2]	
group three	major	PS **258A**	PS **258A**	Figures 3A, 4C, 5C
XIa male	minor	PS **286A**	PS **286A**, **300**, **526**	
	trace	PS **240**, **242**, **258B**, **302** [2]	PS **270**, **302**, **316**, **512**, **524**, **542**, **556**, **558**	
group three	major	PS **258A**	PS **258A**	Figures 3B, 4D, 5D
XIb male	minor	PS **286A**	PS **512**, **528**	
	trace	PS **218**, **242**, **256**, **270**, **300**, **330**	PS **286A**, **312**, **330**, **524**, **526**, **530**	
group three	major	PS **258A**	PS **258A**	Figures 3C, 4E, 5E
XIc male	minor	PS **242**, **302**	PS **286A**, **300** [2]	
	trace	PS **258B**, **270**, **286A**	PS **242**, **258B**, **526**	
group three	major	PS **258A**	PS **526**	Figure 3D
XId[b] male	minor	PS **286A**, **302**	PS **258A**, **300**	
	trace	PS **240**, **242**, **258B**	PS **286A**, **302**, **512**, **528**, **538**, **542** [2], **556**	
group three	major	PS **258A**	PS **526**	
XIe[b] male	minor	PS **302**	PS **528A**, **300** [2]	
	trace	PS **240**, **242**, **258B**, **286A**	PS **286A**, **302**, **512**, **524**, **542**, **556**, **558**	
group four	major	PS **258A**, **302**	PS **300**	Figures 3E, 4F, 5F
XIIa 2 males	minor	PS **258B**, **286A**	PS **258A**, **286A**	
	trace	PS **240**, **242**, **332**	PS **526**, **542**	
group four	major	PS **302**	PS **258A**, **300** [2]	Figure 3F
XIIb[b] male	minor	PS **258A**, **286A**	PS **286A**	
	trace	PS **240**, **258B**	PS **526**, **542**, **574**	
group four	major	PS **258A**, **302**	PS **300**	
XIIc male	minor	PS **286A**, **332**	PS **258A**	
	trace	PS **258B**	PS **274**, **286A**, **302** [2], **316**, **330**, **332**, **540**, **542**, **572**	
group four	major	PS **258A**, **302**	PS **258A**	
XIId[b] male	minor	PS **286A**	PS **286A**, **300**	
	trace	PS **240**, **242**, **256** [2], **258B**, **332**	PS **242**, **330**, **526**, **542**, **556**, **574**	

[a] For footnote see Table 1. Estimates of total levels based on GC-FID traces indicated that for most captive-raised frogs levels were 30 μg of alkaloids or less per 100 mg skin except for frog XIb, which had about 150 μg per 100 mg skin. [b] Alkaloid mixture was fed to this frog (see Experimental Section). Trace amounts of pumiliotoxins were present in extracts, but are not tabulated (see text).

siblings); (4) five male frogs (XIIa−XIId) that underwent metamorphosis in August 1998 and were sacrificed and analyzed August 2000 at an age of 24 months (F2, full siblings). The captive-raised frogs were maintained for up to two months at the Adelaide laboratory prior to sacrifice, while being fed crickets dusted with either a vitamin-mineral powder or for 6 of the 18 frogs with powder containing 1 wt % of an alkaloid mixture. The mixture consisted of a 3,5-disubstituted indolizidine (**223AB**), a decahydroquinoline (**195A**), and a PTX (**307A**) and in one case a histrionicotoxin (**285E**). Methanol extracts from putative dietary arthropods (ants, termites, spiders, isopods, amphopods, larvae, and species from Hemiptera, Hymenoptera, Coleoptera, and Demoptera) collected in Holey Plains State Park were analyzed by GC−MS for alkaloids, but none were detected.

Skins were placed in MeOH and later cut into small pieces and triturated with MeOH (three times with at least 10 volumes). The MeOH extract was diluted with an equal volume of H_2O, and the aqueous MeOH was then extracted three times, each time with an equal volume of $CHCl_3$. The combined $CHCl_3$ layers were concentrated to a small volume in vacuo at 30 °C, followed by addition of 10−20 mL of hexane. The hexane solution was extracted three times, each time with a half volume of 0.1 M HCl. The combined acid layers were made basic with 1 M NH_4OH, and alkaloids were extracted three times, each time with a half volume of $CHCl_3$. The combined $CHCl_3$ layers were dried over Na_2SO_4 and carefully evaporated

Table 3. Alkaloids from Skin of Myobatrachid Frogs of the Genus *Pseudophryne*[a]

Pseudophryne (Ps.) species	collection site and date	number frogs	GC-FID total amount[b]	alkaloids[c]
Ps. australis	Pearl Beach, NSW 9/87	3	+ + +	major: PTX **323A**. minor: PS **258A**, **300**, **302**, **346**
Ps. bibroni	Yarrangobilly, NSW 3/81	1	+	major: PS **258A**. minor: **286A**
	Nortin Summit, SA 4/00	2	+ + +	major: PTX **267C**. minor: PS **346A**[d]
Ps. coriacea[e]	North of Childers, Qnd 1/87	3	+ +	major: PS **302**. minor: PTX **323A**; PS **258A**, **316**, **330**, **332**
	Esk, Qnd 1/87	2	+ +	major: PS **258A**. minor: PTX **323A**; PS **302**
	Daisy Hill, Qnd 1/87	3	+ +	major: aPTX **323B**. minor: PS **258A**, **286A**, **330**, **332**
	Nerang, Qnd 1/87	3	+	major: PS **258A**. minor: PS **286A**, **302**, **316**, **330**, **332**
	Grafton, NSW 1/87	2	+ +	major: PS **258A**, **286A**. minor: PS **302**
	Buladelah, NSW 1/87	5	+ +	major: PS **258A**. minor: PTX **267C**; PS **286A**, **512**, **528**
Ps. corroboree	Round Mtn. NSW 1/87	5	+ + +	major: PTX **323A**. minor: PTX **267C**, **277**; aPTX **323B;** PS **302**.
Ps. guentheri	Walyunga Nat. Park, WA 6/87	5	+	major: PS **258A**
	Walyunga Nat. Park, WA 6/87	10	+	major: PS **258A**
	Goomalling, WA 6/87	6	+	major: PS **258A**
	Brookton, WA 6/89	9	+	major: PS **258A**
Ps. occidentalis	Merridin, WA 6/87	10	+ +	major: PS **258A**
Ps. pengilleyi[f]	Yarrangobilly, NSW 1/87	1	+ +	major: PS **286A**. minor: PTX **267C**, **323A**; PS **302**, **512**, **528**
Ps. semimarmorata	Gembrook State Forest, Vic 3/81	1	+	major: PTX **267C**. minor: PS **258A**
	Holey Plains State Park, Vic 4/99	8	+ + +	major: PTX **267C**. minor: PS **302**
	Victoria 85	8	+ +	major: PS **258A**, **286A**. minor: PTX **267C**; PS **528**

[a] Based on data in ref 6 and the present results. [b] Estimated levels based on GC-FID chromatograms: + + + > 100 μg/100 mg skin; + + 30−100 μg; + < 30 μg. [c] Major and minor alkaloids reported. Trace alkaloids are not reported (see Table 1 and ref 6). The absolute amount of the major alkaloid varies, being high in extracts having high total amounts (+ + +) of alkaloids to quite low in extracts having low total amounts (+). [d] PSs **258A** and **332** were detected as trace alkaloids. [e] *Ps. coriacea* probably represents more than one species. The Childers, Esk, and Daisy Hill populations are most likely *Ps. coriacea*. The Nerang population may be *Ps. raveni*.[18] [f] Referred to in ref 6 as *Ps. corroboree*, population A; now classified as separate species *Ps. pengilleyi*.[19]

in vacuo at 30 °C. The residue was dissolved in MeOH so that 1 μL of the alkaloid extract was equivalent to 1 mg wet weight of skin.

Analysis of Alkaloid Extracts. Gas chromatographic (GC) analyses were conducted using a flame ionization detector (GC-FID analysis, Figures 2 and 3, Tables 1 and 2) and a 6 ft × 2 mm i.d. OV-1-coated (1.5%) column on 80−100 mesh Gas Chrom Q with He carrier at a flow rate of about 30 mL/min. An injection of 2 μL of the methanolic alkaloid extracts was made onto the column at an initial temperature of 150 °C. An injection of 2 μL of standard alkaloid fraction from a dendrobatid frog was used in order to adjust sensitivity so that the GC-FID traces are comparable to previously published alkaloid traces from *Pseudophryne*.[6] After the maximum of the solvent peak had passed (0.3 min) the column was heated to 280 °C at 10 deg per min. This GC-FID procedure has been used routinely for over two decades for semiquantitative analysis of alkaloid extracts from frog skin.[2]

GC−mass spectral analyses of alkaloid extracts were performed with a Finnigan GCQ instrument with a 25 m × 0.25 mm i.d. Rtx-5 Amine column (using a temperature program from 100 °C (1 min) to 280 °C at a rate of 10 °C/min (Figure 4, Tables 1 and 2). Samples of 1 μL were injected. Liquid chromatographic MS analyses were performed using a Hewlett-Packard model 1100 liquid chromatograph interfaced with a Finnigan LCQ mass spectrometer operating in the atmospheric pressure chemical ionization (APCI) mode (Figure 5, Tables 1 and 2). The ultraviolet detector was set at 260 nm, the maximum long wavelength absorbance of pseudophrynamines. An HP Zorbax column (SB-C-18 column, 25 cm × 4.6 mm i.d., 5 μm particle size) was used at a 0.5 mL/min flow rate with a solvent gradient from 9 parts of 1% HOAc in H_2O to 1 part of 1% HOAc in CH_3CN to a 1:1 mixture over 40 min. Samples of 10 μL were injected.

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