Alkaloids of Anuran Skin: Antimicrobial Function?

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A variety of alkaloids, most of which occur or are structurally related to alkaloids that occur in skin glands of dendrobatid poison frogs, were assayed for antimicrobial activity against the Gram-positive bacterium *Bacillus subtilis*, the Gram-negative bacterium *Escherichia coli* and the fungus *Candida albicans*. Certain pyrrolidines, piperidines and decahydroquinolines, perhydro-histrionicotoxin, and a synthetic pumiliotoxin were active against *B. subtilis*. Only 2-*n*-nonylpiperidine was active against *E. coli*. One pyrrolidine, two piperidines, two decahydroquinolines, and the synthetic pumiliotoxin were active against the fungus *C. albicans*. The results suggest that certain of the skin alkaloids of poison frogs, in addition to being noxious to predators, may also benefit the frog through protection against skin infections.

Key words: Alkaloids, Antibiotics, Antifungals

Introduction

A wide range of biologically active substances are present in skin of amphibians, and include peptides, biogenic amines, bufadienolides, tetrodotoxins, and lipophilic alkaloids (Daly, 1995; Daly *et al.*, 1987). The peptides, amines and bufadienolides are produced by the amphibian, while most of the lipophilic alkaloids are derived unchanged from dietary sources (Daly, 2003). Many such substances in frog skin appear to serve in defense against predation, while others, in particular the peptides, have antimicrobial activity and serve in defense against skin infections. Indeed, a host of antibiotic peptides have been reported from frog skin (Bevins and Zasloff, 1990; Rinaldi, 2002). Antimicrobial activity also has been proposed for alkaloids (Habermehl and Preusser, 1969; Preusser *et al.*, 1975). However, Gram-positive bacteria are present on skin of the European fire salamander (Bettin and Greven, 1986), a species with samandarine alkaloids in the skin. Frogs of certain genera of the families Dendrobatidae, Bufonidae, Mantellidae and Myobatrachidae are characterized by the presence of lipophilic skin alkaloids, which in most cases are sequestered into skin glands unchanged from alkaloid-containing arthropods (Daly, 2003). Most such alkaloids would be merely bitter and unpleasant to predators, but some are quite toxic, consonant with protection against predators and allowing for the bright, aposematic coloration of the diurnal dendrobatid and mantellid poison frogs. Neither biologically active amines nor peptides, including antimicrobial peptides, have been reported for alkaloid-containing dendrobatid (Erspamer *et al.*, 1986; Roseghini *et al.*, 1986) or, apparently, mantellid frogs. The bufonid toads (*Melanophryniscus*) have, in addition to skin alkaloids (Garraffo *et al.*, 1993), both amines (Cei *et al.*, 1968) and bufadienolide-like steroids (Flier *et al.*, 1980). Apparently, no peptides have been detected. The myobatrachid frogs (*Pseudophryne*) do have amines and peptides (Roseghini *et al.*, 1976; Simmaco *et al.*, 1990), in addition to the skin alkaloids (Daly *et al.*, 1990; Erspamer *et al.*, 1985; Smith *et al.*, 2002). Whether or not the skin alkaloids of the poison frogs could serve as antimicrobials and, thus, in lieu of peptides, protect such frogs against infection needed to be investigated. A select group of available compounds, related to ten of the over twenty structural alkaloid classes found in frog skin, were assayed against two bacteria and a fungus. Certain of these showed antibacterial and/or antifungal activity.

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Results and Discussion

Assays were conducted using the paper disc method (Constable and Towers, 1989) in which compounds are loaded onto a paper disc. After 24 h incubation the extent of zone inhibition of the growth of the organism about the disc on the plate was observed.

The Gram-positive bacterium *B. subtilis* was sensitive to several of the assayed compounds at 30 to 200 μg/assay (Table I). The results suggested that a heterocyclic ring system with one extended lipophilic side-chain was preferred. Thus, compounds with one such extended side-chain, namely the *cis/trans*-2-ethyl-5-*n*-tridecylpyrrolidines (**2**), the racemic 2-*n*-nonylpiperidine (**4**), the *cis/trans*-2-methyl-6-*n*-undecylpiperidines (**5**), and a synthetic pumiliotoxin (**19**) (Fig. 1), exhibited threshold activities at 30–100 μg/assay. However, one compound with one extended lipophilic side-chain, namely the pyridine **8** was inactive at 200 μg/assay. Three compounds with two relatively short lipophilic side-chains had activity thresholds of 100 or 200 μg/assay. These were two decahydroquinolines (**10** and **11**) and a histrionicotoxin (**16**). Compounds, including two enantiomeric pumiliotoxins (**18A** and **B**), two indolizidines (**13**, **14**), and a synthetic histrionicotoxin analog (**17**), each with one or two relatively short side-chains, were inactive at 200 μg/assay. Further studies will be required to refine structure-activity relationships. Alkaloids with more complex structures, lacking an extended lipophilic side-chain, such as pumiliotoxin **307A** (**20**), pseudophrynaminol (**21**), a spiropyrrolizidine (**22**), and the ant alkaloid tetraponerine I (**23**), were inactive at 200 μg/assay. The highly toxic batrachotoxin (**25**) was inactive at 20 μg/assay, while the less toxic batrachotoxinin-A (**24**) was inactive at 50 μg/assay.

The Gram-negative bacterium *E. coli* was affected at 200 μg/assay by only one compound, namely 2-*n*-nonylpiperidine (**4**) (Table I). But 2-*n*-nonylpiperidine was several-fold more potent against *B. subtilis*.

The fungus *C. albicans* was sensitive to several, but not all the compounds that were active against *B. subtilis* (Table I). One pyrrolidine (**2**) and two piperidines (**4**, **5**) were more potent against *C. albicans* than against *B. subtilis*. The decahydroquinoline **12** with a polar methoxy group of the terminus of the extended side-chain was inactive against the bacteria, but was active against *C. albicans*.

For comparison, two standard antibiotics and an antifungal were tested. Penicillin at 30 μg/assay was active against *B. subtilis*, but not *E. coli*. Tetracycline at 30 μg/assay was active against both bacteria. Neither of these antibacterials were active against the fungus *C. albicans*. Nystatin at 30 μg/assay was active against the fungus, but not against the bacteria.

The results indicate that certain compounds, related to classes of alkaloids found in skin of poison frogs, have significant antibacterial activity against a Gram-negative bacterium. A few had significant antifungal activity. Thus, sequestration and storage of alkaloids derived from dietary arthropods may confer not only a deterrent to predators, but also some protection against infection of wounds, resulting from environment- or predation-linked in-

	Bacterium		Fungus
Agent*	*B. subtilis*	*E. coli*	*C. albicans*
Pyrrolidines			
2	A (30)	I (200)	A (10)
Piperidines			
4	A (50)	A (50)	A (30)
5	A (30)	I (200)	A (10)
Decahydroquinolines			
10	A (50)	I (200)	A (50)
11	A (100)	I (200)	I (200)
12	I (200)	I (200)	A (200)
Indolizidines			
15	A (100)	I (200)	I (200)
Histrionicotoxins			
16	A (200)	I (200)	I (200)
Pumiliotoxins			
19	A (30)	I (200)	A (50)

Table I: Antimicrobial activity of alkaloids. The threshold activity (A) in μg per disc is given or the inactivity (I) is given for the maximal μg per disc tested. Threshold activity is defined as the lowest tested amount giving a 6 mm zone of inhibition.

* Structures are in Fig. 1.

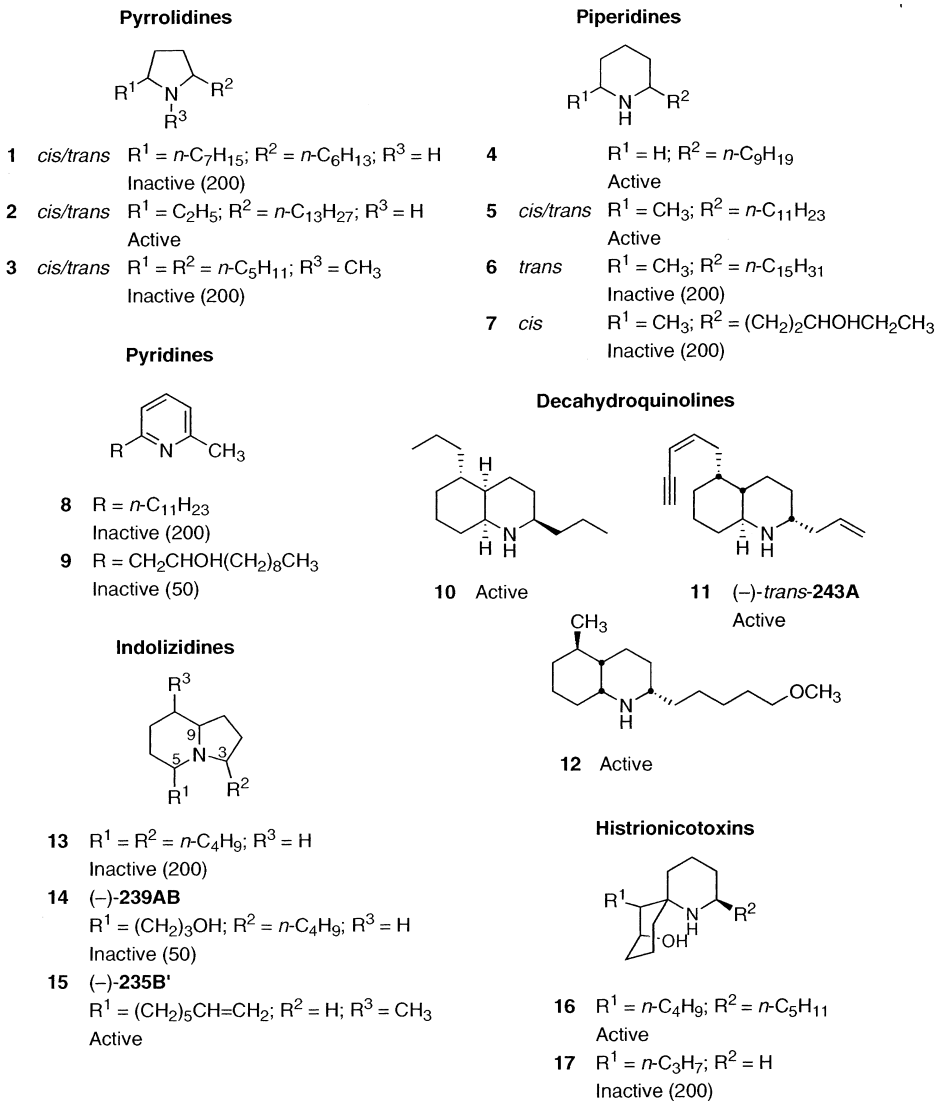
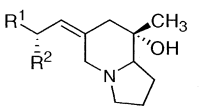


Fig. 1. Structures of alkaloids and alkaloid-like compounds tested as antimicrobials. Compounds are indicated as active (see Table I) or inactive at 200 μg/assay. Sources were as follows: **1**–**9**: Synthetic racemates and in some cases mixtures of *cis*- and *trans*-isomers (T. H. J.). **10**: Synthetic enantiomer (A. G. Schultz, Renssaeler Polytechnic, Troy, NY). **11**: Natural enantiomer. **12**: Synthetic racemate (L. E. Overman, Univ. CA, Irvine, CA). **13**: Synthetic mixture (T. F. S). **14**, **15**: Natural enantiomers. **16**: Perhydro-derivative of natural enantiomer. **17**: Synthetic enantiomer (A. Brossi, NIH). **18**: Synthetic enantiomers: **A**, natural; **B**, unnatural (J. W. D.). **19**: Synthetic enantiomer (T. M. Barger, Dow Elanco, Indianapolis, IN). **20**: Natural enantiomer. **21**, **22**: Synthetic racemates (H. M. G. and J. W. D.). **23**: Natural enantiomer (T. H. J.). **24**, **25**: Natural enantiomers.

juries. However, the active pyrrolidines and piperidines, while often major alkaloids in myrmicine ants, are poorly accumulated into skin by poison frogs (Daly *et al.*, 1994). Thus, the most likely classes of frog skin alkaloids that could provide antimicrobial protection would be decahydroquinolines and izidines, which often have one or two extended lipophilic side-chains (Daly *et al.*, 1987). The indolizidine **235B′** (**15**), with one extended lipophilic side-chain was active at 100 μg/assay. The indolizidine **235B′** is a major alkaloid in skin of certain populations of poison frogs and appears

Fig. 1 (cont.).

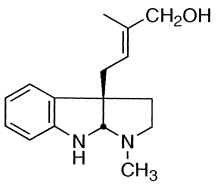
Pumiliotoxins



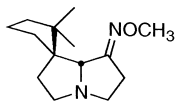
18 A&B (+)- & (–)-**251D**
$R^1 = n\text{-}C_4H_9$; $R^2 = CH_3$
Inactive (50)

19 $R^1 = n\text{-}C_6H_{13}$; $R^2 = H$
Active

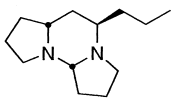
20 (+)-**307A**
$R^1 = CH_2CH{=}CH(CH_3)CH_2OHCH_3$; $R^2 = CH_3$
Inactive (50)



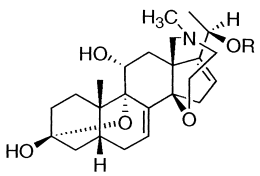
21 (±)-Pseudophrynaminol
Inactive (50)



22 (±)-Spiropyrrolizidine **236**
Inactive (200)

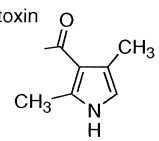


23 Tetraponerine I
Inactive (200)



24 Batrachotoxinin-A
R = H
Inactive (50)

25 Batrachotoxin
R =



Inactive (20)

to be obtained from dietary leaf-litter arthropods (Daly *et al.*, 2002). Whether any of the present alkaloids would have activity against the chytrid fungus that is decimating many amphibians (Daszak *et al.*, 1999) is unknown. A peptide from a ranid frog has been reported to have activity against the chytrid fungus *Batrachochytrium dendrobatidis* (Rollins-Smith *et al.*, 2002). In addition to decimation of certain Central American bufonid toads, the dendrobatid frogs have also suffered from chytrid infections (Pessier *et al.*, 1999).

The antibacterial/antifungal activity found for certain pyrrolidines (**2**), piperidines (**4**, **5**), indolizidines (**15**), decahydroquinolines (**10**, **11**), and pumiliotoxins (**19**), all but the decahydroquinolines with one extended lipophilic side-chain, could provide lead structures for the development of new classes of antibiotics.

Experimental

The structures of the compounds are depicted in Fig. 1 and the sources are provided in the footnote. Racemic 2-*n*-nonylpiperidine (**4**) was prepared starting from 2-picoline using previously described methodology (Jones *et al.*, 1990). Hydrogenation of the intermediate 2-*n*-nonylpyridine in 95% ethanol, acidified with HCl, was carried out under 300 Pa of H_2 with a 5% Rh on Al_2O_3 catalyst. MS (EI): *m/z* (% of base peak) = 211 (1), 210 (1), 85

(5), 84 (100). High resolution-MS: Exact mass of $[M+1]^+$ = 212.2382; calcd. for $C_{14}H_{30}N^+$ = 212.2378.

The assay of antimicrobial activity was as follows: The microorganisms used were obtained from Ward's Natural Science Inc. The two bacteria, *E. coli* and *B. subtilis* were grown in Tryptose agar solid medium and transferred to liquid nutrient broth for 48 h at 30 °C. The fungus *C. albicans* was grown in Sabouraud dextrose solid medium and transferred to liquid Sabouraud broth for 24 h at 37 °C. Aseptic conditions were used to dilute the microorganisms spectrophotometrically to 10^6 spores/ml, and 1 ml aliquots of each poured onto a petri dish containing the above solid media, and a sterile glass spreader used to ensure uniform growth of the inoculum. Different amounts of the test compounds in methanol were bioassayed for antimicrobial activity using the paper disc method (Constable *et al.*, 1989) by injecting samples into 6 mm diameter sterile discs. Standard antibiotic discs containing penicillin, tetracycline and nystatin were used for comparison. The petri dishes were then incubated at 4 °C for 2 h, and placed in an incubator for 24 h at 37 °C for the bacteria and 24 °C for the fungus. At the end of this period, inhibition zones were evaluated in mm and compared with those of the reference discs. A threshold effect was recorded if the zone of inhibition was greater than 6 mm at that concentration of compound.

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