Full Papers

Alkaloid 223A: The First Trisubstituted Indolizidine from Dendrobatid Frogs†

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The structure of alkaloid **223A** (**1**), the first member of a new class of amphibian alkaloids, purified by HPLC from a skin extract of a Panamanian population of the frog *Dendrobates pumilio* Schmidt (Dendrobatidae), has been established as (5*R*,6*S*,8*R*,9*S*)- or (5*S*,6*R*,8*S*,9*R*)-6,8-diethyl-5-propylindolizidine, based on GC−MS, GC−FTIR, and ^{1}H-NMR spectral studies. Three higher homologs of **223A**, namely alkaloids **237L** (**2**), **251M** (**3**), and **267J** (**4**), have been detected in other extracts, and tentative structures are proposed.

Only seven genera of anurans, all from tropical or semitropical regions, are known to contain lipophilic alkaloids in skin glands.[1] It now appears likely that such alkaloids are derived from dietary sources.[2] Eighteen distinct structural classes have been defined, examples being the batrachotoxins, the histrionicotoxins, the pumiliotoxins, the gephyrotoxins, epibatidine, and a variety of simpler alkaloids, including piperidines, pyrrolidines, pyrrolizidines, indolizidines, and quinolizidines. Analytical techniques continue to become more sensitive, and undoubtedly additional structural classes of alkaloids will emerge from further analysis of extracts, which typically consist of complex mixtures of dozens of alkaloids, many present in only minute amounts. One class, commonly occurring among the different species of alkaloid-containing anurans, is that of the indolizidines. Until now, the indolizidines have been encountered as disubstituted at either the 3,5- or 5,8-positions.

The 3,5-disubstituted indolizidines, unlike some other classes of amphibian alkaloids, are not unique to amphibians. Indeed, 3,5-disubstituted indolizidines are common venom constituents in ants of the genera *Monomorium* and *Solenopsis*.[3−5] Six 3,5-disubstituted indolizidines have been reported from frog skin extracts, of which two are known in ants.[1] MS and FTIR data are diagnostic for 3,5-disubstituted indolizidines. The MS show major fragment ions corresponding to the loss of either the 3- or the 5-substituent, both substituents being at positions α to the nitrogen. Cleavage of a methyl group at either position is much less favored compared to larger alkyl substituents. A fragment ion $C_8H_{14}N^+$ at *m*/*z* 124 is often present, being especially prominent in ion-trap MS and arises from a McLafferty rearrangement during cleavage of the second side chain.[6] Intensities of Bohlmann bands in the FTIR spectra can differentiate between the four possible diastereomers of 3,5-disubstituted indolizidines.[6,7]

Thirty aliphatic 5,8-disubstituted indolizidines have been reported in frog skin extracts; none is known elsewhere in nature.[1] A 5-(3-furfuryl)-8-methylindolizidine, however, has been reported as a trace component in extracts from scent glands of the beaver.[8] MS and FTIR are diagnostic for 5,8-disubstituted indolizidines. The MS show a major fragment ion (base peak) corresponding to α-cleavage of the 5-substituent, and, in addition, a diagnostic ion, $C_6H_{10}N^+$, at *m*/*z* 96, arising from the base peak by a retro Diels-Alder process.[6] Analysis of the Bohlmann bands in FTIR spectra of 5,8-disubstituted indolizidines permits the assignment of the relative configurations at C-5 and C-9. The relative configuration at C-8, a position not adjacent to nitrogen, is currently not assignable by FTIR spectra. Some 3,5- and 5,8-disubstituted indolizidines, isolated from frog skin extracts, have been purified and the structures elucidated by NMR spectroscopy.[9−11]

Other disubstituted "izidine" alkaloids have been found in frog skin extracts, including 3,5-disubstituted pyrrolizidines and 1,4-disubstituted quinolizidines. MS and FTIR are diagnostic in their characterization.[6,12] One alkaloid, **223A**, known in frog skin extracts for years, had perplexing properties in that it exhibited a *m*/*z* 124 fragment ion and absence of an *m*/*z* 96 ion typical of the 3,5-disubstituted indolizidines, but had the significant and characteristic Bohlmann band pattern in FTIR (Figure 1) of the 5,8-disubstituted indolizidines.

This report documents the structure of alkaloid **223A** (**1**), of the indolizidine class, isolated as a major component from a skin extract of a Panamanian population of the dendrobatid frog *Dendrobates pumilio* Schmidt (Dendrobatidae). A 6,8-diethyl-5-propylindolizidine structure has now been established for **223A** by ^{1}H-NMR spectroscopy and provides the first example of a trialkyl-substituted indolizidine in frogs. This alkaloid occurs quite commonly in skin extracts of dendrobatid frogs, usually as a minor or trace component of alkaloid mixtures. A tentative 1,4-dipropylquinolizidine structure was proposed earlier for it on the basis of MS and FTIR studies.[1] Three higher homologs of **223A** have been detected in other populations of *D. pumilio*,

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† Dedicated to Professor Eduardo G. Gros of the Universidad de Buenos Aires on the occasion of his 65th birthday.

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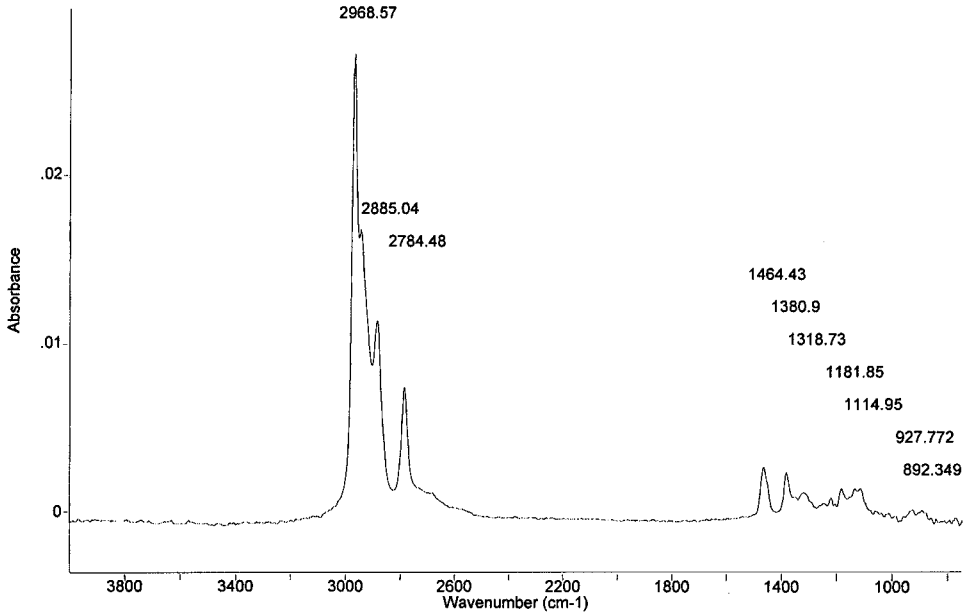
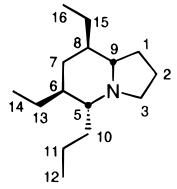


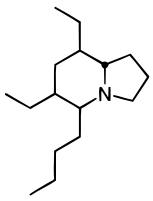
Figure 1.

namely indolizidines **237L** (**2**) and **267J** (**4**), for which the structures 5-butyl-6,8-diethylindolizidine and 6-ethyl-8-(2-hydroxyethyl)-5-pentylindolizidine are proposed, respectively, based on MS and FTIR analysis, and indolizidine **251M** (**3**), which has, based on MS evidence, a 6,8-diethyl-5-pentylindolizidine structure. Such 5,6,8-trisubstituted indolizidines have not been reported elsewhere in nature.



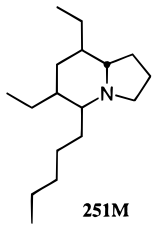
223A

1



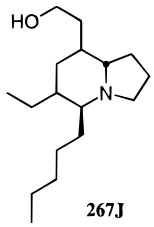
237L

2
Tentative structure based on MS



251M

3
Tentative structure based on MS



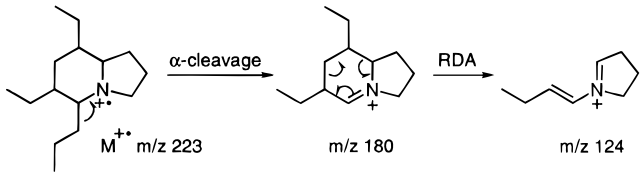
267J

4
Tentative structure based on MS and FTIR

Results and Discussion

An alkaloid fraction, prepared from extracts of *D. pumilio* collected on Isla Cristóbal (see Biological Material), was found to contain mainly **223A**, accompanied by a total of nine minor and trace alkaloids, including a pumiliotoxin, an allopumiliotoxin, a decahydroquinoline, a 3,5-disubstituted pyrrolizidine, 5,8-disubstituted indolizidines, and quinolizidines. Purification by HPLC afforded approximately 1 mg of **223A**, homogeneous by GC−MS. It exhibited a molecular ion of formula $C_{15}H_{29}N^{\bullet+}$ and a major fragment ion $C_{12}H_{22}N^{+}$ at *m/z* 180 (base peak), resulting from an α-cleavage of a propyl

Scheme 1



side chain at C-5. A fragment ion $C_8H_{14}N^{+}$ at *m/z* 124 was present, arising from the base peak ion after a retro Diels-Alder process (see Scheme 1). CIMS with deuteroammonia produced a molecular ion at *m/z* 225 indicating no exchangeable hydrogens.[13] The GC−FTIR spectrum of **223A** (Figure 1) showed a sharp, intense Bohlmann band at 2784 cm^{-1}, characteristic of 5,8-disubstituted indolizidines with hydrogens H-5 and H-9 in a *cis* relationship (5,9 *Z*), and *trans*-antiparallel to the *N*-lone-pair.

Hydrogenation of a portion of the crude extract showed **223A** to be unchanged, indicating the absence of double or triple bonds.

After HPLC isolation of **223A**, the sample still contained trace amounts of neutral compounds, detectable in a $CDCl_3$ ^{1}H-NMR spectrum. When this $CDCl_3$ solution was concentrated to dryness with N_2 and D_2O containing 1 μl of DCl was added, the resulting ^{1}H-NMR spectrum of purified **223A**·DCl in D_2O permitted the assignment of structure **1** to **223A** (see Figure 2). Most of the chemical shifts were determined through a ^{1}H−2D COSY spectrum. After computer modeling of the structure, the coupling constants were estimated from the calculated dihedral angles and compared with the observed values. Three regions were distinguishable in the ^{1}H-NMR spectrum. The first, between 3.7 and 2.5 ppm, displayed well-separated signals, corresponding to hydrogens H-9, H-3α, H-3*β*, and H-5, all α to the deuteronated N. The second region, between 2.5 and 1.0 ppm, included signals for hydrogens H-1 (2H), H-2 (2H), H-7α, H-7*β*, H-6, as well as the remaining methylenes. The third region, between 1.0 and 0.86 ppm, showed on expansion three overlapping triplets corresponding to the three methyls (CH_3-12, -14, and -16). These signals indicated a trisubstituted alkaloid and

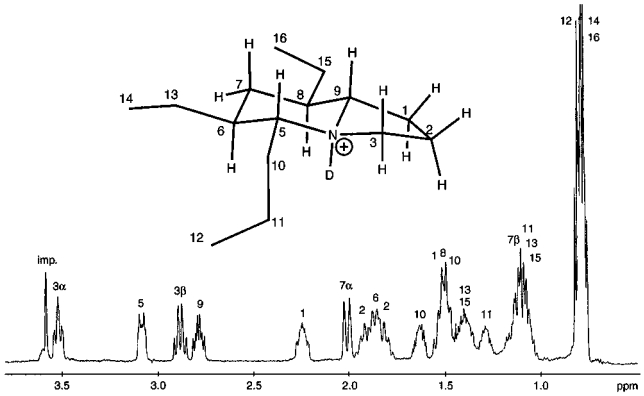


Figure 2.

forced us to abandon the tentative disubstituted quinolizidine structure postulated earlier. The presence of a propyl side chain, as indicated from the EIMS, and two other methyls bonded to methylenes required at least seven carbons in side chains, leaving an indolizidine with a propyl and two ethyl groups as the only possibility. The three methyl triplets were centered at δ 1.04, 1.02, and 1.01. Irradiation at δ 1.52 (H-11), altered the signal at δ 1.04, indicating this to be the CH_3-12 signal. The other two methyls could not be unambiguously assigned because the signals for H-13α and H-15α as well as H-13β and H-15β overlapped. The detection of two large and one small coupling constant for H-9 (with H-8, H-1α and H-1β, respectively) indicated that H-8 was axial. The detection of one large and two small coupling constants for H-5 (with H-6 and the two H-10s, respectively) indicated that H-5 and H-6 were also axial.

The alkaloid fraction from frogs collected at Macca Bite (see Biological Material), contained alkaloids **223A**, **237L**, and **267J**, as minor alkaloids. A total of 15 other alkaloids were present. The alkaloid fraction from frogs collected at Carbón (see Biological Material) contained alkaloid **223A** as a minor alkaloid together with trace amounts of an isomer of **223A** and alkaloids **251M** and **267J**. A total of 27 other alkaloids was detected. Alkaloids **251M** and **267J** were found to be present along with alkaloid **223A** in two other populations of *D. pumilio*. Alkaloid **267J** was found, together with alkaloid **223A**, in two additional populations of *D. pumilio*. Alkaloid **223A** was present in a total of 32 of 42 populations of *D. pumilio* sampled in the past two decades in Panama and Costa Rica. Alkaloids **223A**, **237L**, and **251M**, displayed very similar MS fragmentation patterns, with a 14 unit difference between the parent peaks. All three showed *m/z* 180 as the base peak, and *m/z* 124 as the next most important peak, which suggested that they produce the former fragment ion by an α-cleavage of propyl, butyl, or pentyl side chains at position C-5, and then, the peak *m/z* 124 by retro Diels−Alder fragmentation, with the loss of butene. Thus, the tentative structures **2** and **3** were assigned to the alkaloids **237L** and **251M**. FTIR spectra could not be obtained due to the very limited amount of these compounds. Alkaloid **267J** produced a mass spectral fragmentation pattern indicating α-cleavage of a pentyl side chain to yield a base peak at *m/z* 196, suggesting the presence of an oxygen that is eliminated as butenol during a retro Diels−Alder process, yielding the *m/z* 124 ion. The oxygen was confirmed by HRMS data and the observation of a characteristic FTIR stretching absorption at 3651 cm^{-1} indicated that the oxygen was present in a non-hydrogen-bonded hydroxyl group. Therefore, the tentative structure **4** was assigned to alkaloid **267J**. Alkaloids **223A** and **267J** showed dissimilar FTIR spectra in the Bohlmann band region and thus, for the moment, we are assigning a tentative 5,9-*E* relative stereochemistry to alkaloid **267J**.

Experimental Section

General Experimental Procedures. GC−MS analysis used an RTX-5 fused silica-bonded capillary column (Restek, 30 m × 0.25 mm i.d.) in a Varian model 3400 gas chromatograph programmed from 100 to 280 °C at a rate of 10°/min, interfaced with a Finnigan ion-trap model 800 to obtain EIMS, or CIMS with NH_3 or ND_3. GC−MS−FTIR spectra were obtained using a Hewlett-Packard model 5890 gas chromatograph having a 25 m × 0.32 mm HP-5 (polymer of 5% diphenylsiloxane and 95% dimethylsiloxane) fused silica-bonded capillary column with the same program as used above for the GC−MS analysis, interfaced with a Hewlett-Packard model 5971 series mass selective detector and a Hewlett-Packard model 5965B IR instrument with a narrow band (4000−750 cm^{-1}) detector. A Hewlett-Packard MS/IR ChemStation (DOS based) was used to generate the chromatograms, and the EIMS and FTIR spectra of GC peaks. HRMS used a JEOL SX 102 instrument fitted with a 15 m × 0.20 mm HP-5 column. ^{1}H-NMR and ^{1}H−2D COSY spectra in D_2O were measured with Varian XL-300 and Varian VXR-500 spectrometers. Chemical shifts (δ) are referred to internal HOD at 4.78 ppm.

Biological Material. The frogs (*Dendrobates pumilio*) were from the following collection sites: (a) 20 skins on Isla Cristóbal, Bocas, Panama in October 1983; (b) 20 skins at Macca Bite, Isla Bastimentos, Bocas, Panama, in February 1992; and (c) 4 skins collected at Carbón, Limón, Costa Rica, in June 1990. Voucher specimens of the frogs are at the American Museum of Natural History, New York.

Extraction of Alkaloids and Purification of 1. Alkaloid fractions from MeOH skin extracts were prepared as described.[14] The alkaloid fraction from Isla Cristóbal, containing mainly alkaloid **223A**, was concentrated to 125 μL, and a sample of 25 μL was purified by HPLC on an Asahipak ODP-50 column (5 μm, 250 × 4 mm) with a mixed solvent of CH_3CN-H_2O and a gradient from 10:90 to 90:10 from 1 to 30 min with a solvent flow rate of 0.5 mL/min. Thirty fractions of 0.5 mL were collected, dried under N_2, and tested for the presence of **223A** using GC−MS. Fraction 12 contained mainly **223A**. This procedure was repeated four times, each time with 25 μL of the concentrated alkaloid fraction. Purified **223A** fractions were pooled to yield approximately 1 mg of **223A**.

The properties of **223A**, **237L**, **251M**, and **267J** are as follows, with the molecular formula determined by HRMS; the EIMS with intensities relative to the base peak set equal to 100; the number of exchangeable hydrogens expressed as 0D, 1D, and so on; the hydrogenation data as H_0, H_2, and so on, derivatives; the FTIR data with frequencies in cm^{-1} and intensities relative to the maximum absorbance set equal to 100; and the ^{1}H-NMR data with chemical shifts (δ) in ppm and coupling constants (J) in Hz (see also Figure 2).

Indolizidine 223A (**1**): $C_{15}H_{29}N$; EIMS *m*/*z* 223 (1), 222 (1), 194 (2), 180 (100), 152 (2), 124 (11), 96 (6), 70 (9), 55 (7); ion trap EIMS *m*/*z* 223 (5), 222 (5), 208 (1), 194 (2), 180 (100), 166 (1), 150 (2), 138 (7), 124 (26), 110 (1), 96 (10), 84 (2), 70 (10); 0D; H_0; FTIR 2968 (100), 2946 (61), 2885 (42), 2784 (27), 1464 (12), 1380 (11), 1318 (6), 1220 (5), 1181 (7), 1133 (7), 1114 (7) cm^{-1}; ^1H NMR for **223A**·DCl (500 MHz, D_2O), δ 3.75 (1H, ddd, J = 11.5, 8.9, 3.1, H-3α), 3.30 (1H, dt, J = 10.6, 4.7, 4.7, H-5), 3.11 (1H, ddd, J = 11.5, 10, 9.4, H-3β), 3.00 (1H, ddd, J = 12, 11, 6.5, H-9), 2.47 (1H, dddd, J = 13.5, 10, 6.5, 3, H-1β), 2.22 (1H, dt, J = 14.3, 2.6, 2.6, H-7α), 2.00−2.18 (3H, m, H-2α, H-2β, H-6), 1.87 (1H, m, H-10a), 1.68−1.80 (3H, m, H-1α, H-8, H-10b), 1.58−1.68 (2H, m, H-13a, H-15a), 1.52 (1H, m, H-11a), 1.27−1.43 (4H, m, H-7β, H-11b, H-13b, H-15b), 1.04 (3H, t, J = 7.5, CH_3-12), 1.02 (3H, t, J =7.5, CH_3-14 or CH_3-16), 1.01 (3H, t, J = 7.5, CH_3-16 or CH_3-14); structure, (5*R*,6*S*,8*R*,9*S*)- or (5*S*,6*R*,8*S*,9*R*)-6,8-diethyl-5-propylindolizidine.

Indolizidine 237L (**2**): $C_{16}H_{31}N$; ion trap EIMS *m*/*z* 236 (1), 208 (2), 180 (100), 164 (4), 124 (21), 96 (4), 70 (14); 0D; tentative structure, a 5-butyl-6,8-diethylindolizidine.

Indolizidine 251M (**3**): $C_{17}H_{33}N$; ion trap EIMS *m*/*z* 251 (2), 250 (2), 222 (1), 180 (100), 164 (4), 136 (3), 124 (20), 110 (6), 96 (5), 70 (7); 0D, tentative structure, a 6,8-diethyl-5-pentylindolizidine.

Indolizidine 267J (**4**): $C_{17}H_{33}NO$; EIMS *m*/*z* 267 (1), 266 (1), 196 (100), 180 (3), 152 (4), 138 (3), 124 (19), 96 (3), 70 (14), 55 (10), ion trap EIMS *m*/*z* 196 (100), 124 (72), 96 (4), 70 (18); 1D; FTIR 3651 (6), 2968 (100), 2939 (71), 2884 (63), 2807 (17), 1464 (13), 1378 (13), 1280 (11), 1164 (11), 1121 (11), 1066 (11), 1000 (11) cm^{-1}; tentative structure, a 6-ethyl-8-(2-hydroxyethyl)-5-pentylindolizidine.

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