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A NEW SUBCLASS OF ALKALOIDS FROM A DENDROBATID POISON FROG: A STRUCTURE FOR DEOXYPUMILIOTOXIN **251H**

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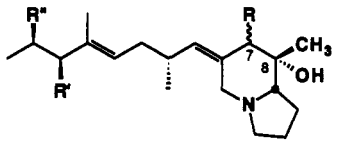
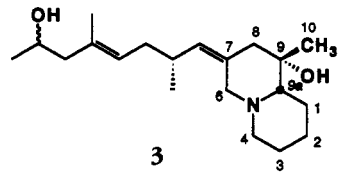
ABSTRACT.—Deoxypumiliotoxin **251H** [**4**], representing a new subclass of the pumiliotoxin-A class of alkaloids, has been isolated from a dendrobatid frog, *Epipedobates tricolor*. The structure, elucidated by nmr, gc-Ftir, and mass spectral analyses, is proposed to be an 8-methyl-6-(5-hydroxy-2-methylhexylidene)-1-azabicyclo[4.3.0]nonane. The absolute stereochemistry and the relative configuration of the hydroxyl group are unknown.

Alkaloids represent one class of toxic or noxious compounds present in the skins of certain amphibians (1), where they can serve as a chemical defense against predators upon being released from cutaneous granular glands onto the skin surface. Recent work indicates that many of the amphibian alkaloids may be sequestered into skin from the insect diet of frogs and toads (2,3). Frogs from four genera of the family Dendrobatidae have the widest variety of alkaloids, known collectively as dendrobatid alkaloids (4,5). One of the major classes of alkaloids from amphibians is the pumiliotoxin-A class found not only in dendrobatid frogs, but also in other frogs and toads from such diverse locations as Australia (5), Madagascar (6), and South America (7). This class has yet to be detected elsewhere in nature and the possibility must be considered that the pumiliotoxin-A class alkaloids are not of dietary origin (2,3), but are instead synthesized by the frogs or toads. The alkaloids belonging to the pumiliotoxin-A class (8–10) have been divided into pumiliotoxins [**1**], allopumiliotoxins [**2**], and homopumiliotoxins [**3**]. The mass spectra of pumiliotoxins show prominent ions of $C_4H_8N^+$ (*m/z* 70) and $C_{10}H_{16}NO^+$ (*m/z* 166). Allopumiliotoxins show corresponding ions of $C_4H_8N^+$ (*m/z* 70) and $C_{10}H_{16}NO_2^+$ (*m/z* 182), while homopumiliotoxins exhibit prominent mass spectral fragment ions of $C_5H_{10}N^+$ (*m/z* 84) and $C_{11}H_{18}NO^+$ (*m/z* 180). The allopumiliotoxins are pumiliotoxins with a hydroxyl group in the 7-position, while homopumiliotoxins have a quinolizidine ring instead of the indolizidine ring of the pumiliotoxins and allopumiliotoxins. All have an alkylidenyl side-chain and methyl and hydroxy substituents at C-8 (pumiliotoxins and allopumiliotoxins) or C-9 (homopumiliotoxins).

This report documents the occurrence of a new alkaloid of the pumiliotoxin-A class, **251H** [**4**], present in trace amounts in the dendrobatid species *Epipedobates tricolor*. This



1 R=H, R′=OH, R″=H or OH
2 R=OH, R′=OH, R″=H or OH



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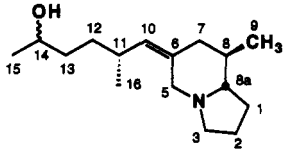

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alkaloid (mol wt 251; $C_{16}H_{29}NO$) exhibits one of the characteristic mass spectral fragment ions of the pumiliotoxin-A class, $C_4H_8N^+$ (*m/z* 70), but the ion $C_{10}H_{16}NO^+$ (*m/z* 166) is replaced by $C_{10}H_{16}N^+$ (*m/z* 150). Because **251H** lacks the characteristic hydroxyl group at C-8 of the pumiliotoxins, as shown by ms, ir, and nmr analysis, it is to be named a deoxypumiliotoxin.

RESULTS AND DISCUSSION

An alkaloid fraction prepared from extracts of Ecuadoran frogs of the dendrobatid species *Epipedobates tricolor* was subjected to Si gel cc. The molecular ion, *m/z* 251, was confirmed for the major gc peak in chromatographic fractions 117–120. These fractions containing **251H** were contaminated with neutral impurities that were removed by partitioning between EtOAc and 0.1 N HCl. The acidic layer was neutralized with $NaHCO_3$ and extracted with EtOAc to recover approximately 1 mg of **251H**, homogeneous by gc-ms. Deuteroammonia cims produced a molecular ion at *m/z* 254, indicating that there was one exchangeable hydrogen.

The gc-Ftir spectrum of **251H** (Figure 1) revealed a sharp hydroxyl OH stretching frequency at 3652 cm^{-1}. Two strong Bohlmann bands in the region 2800–2740 cm^{-1}, and a sharp absorption at 965 cm^{-1} (trisubstituted double bond between C-6 and C-10) are typical of alkaloids of the pumiliotoxin-A class. The Bohlmann bands are present in **251H**, but the absorption at 965 cm^{-1} is much weaker than usual. The absorption at 3544 cm^{-1}, due to the hydrogen-bonded tertiary hydroxyl group at C-8 common to other members of the pumiliotoxin-A class, is absent in **251H**.

After the purification of **251H** with acid/base extraction, the sample still contained trace amounts of neutral compounds, detectable in a $CDCl_3$ ^{1}H-nmr spectrum. Therefore, the $CDCl_3$ solution was concentrated to dryness with N_2, and D_2O containing 1 μl of DCl was added. The ^{1}H-nmr spectrum of the DCl salt of **251H** in D_2O was free of impurities. Analysis of the nmr data led to the proposal of structure **4**. All the chemical shifts and almost all the ^{1}H-^{1}H coupling constants were assigned through a 2D ^{1}H COSY

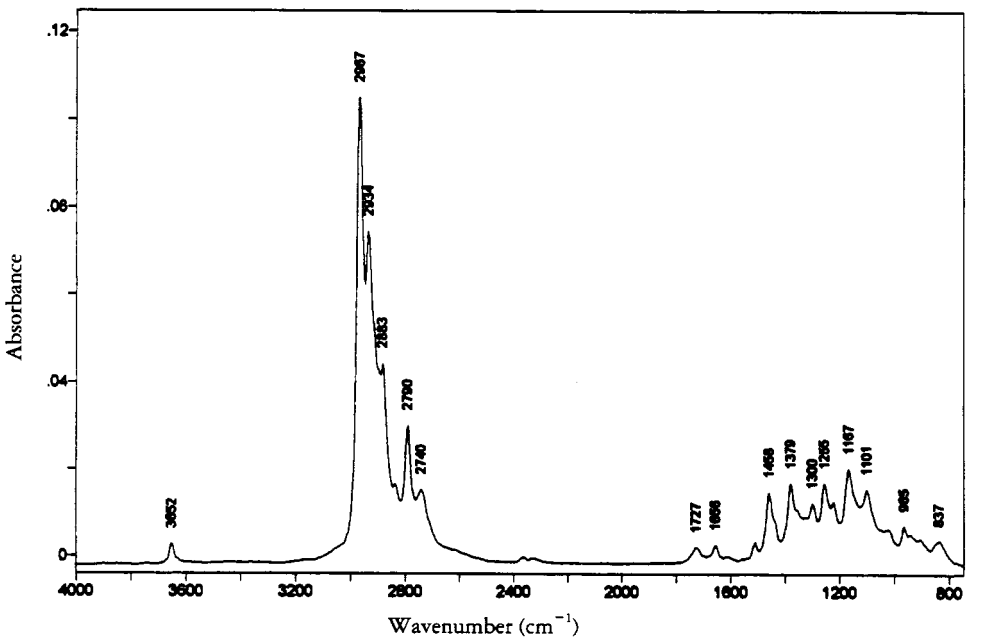


FIGURE 1. Vapor phase Ft-ir spectrum of **251H** [4].

spectrum and 1D decoupling experiments. Three regions are present in the ^{1}H-nmr spectrum (Figure 2). The first region from δ 5.6 to 3.0, where the signals are well separated, contains the vinylic H-10, the H-14 on a hydroxylic carbon, and the hydrogens (H-8a, H_2-5, H_2-3) α- to the deuteronated N. The second region, from δ 2.6 to 1.3, contains the methine signals H-8 and H-11 and the remaining methylene signals. The third region, from δ 1.2 to 0.9, contains signals of the three methyls (15, 9, and 16), all appearing as doublets. The ^{1}H-nmr analysis provided the relative configuration of the CH_3-9. The presence of two large coupling constants for H-8a (one of them being the axial-axial interaction with H-8) as well as two large coupling constants for H-7β (the geminal coupling and the axial-axial interaction with H-8) suggests that H-8 is axial and thus, CH_3-9 is equatorial. An attempt was made to ascertain double bond configuration by nOe spectroscopy, but our experiments were inconclusive. Thus, the *Z*-configuration of the double bond as shown in **251H** is tentative, but is supported by the fact that all the other pumiliotoxins previously observed have the same configuration at the double bond between C-6 and C-10. The configurations at C-11 and C-14 can not be discerned

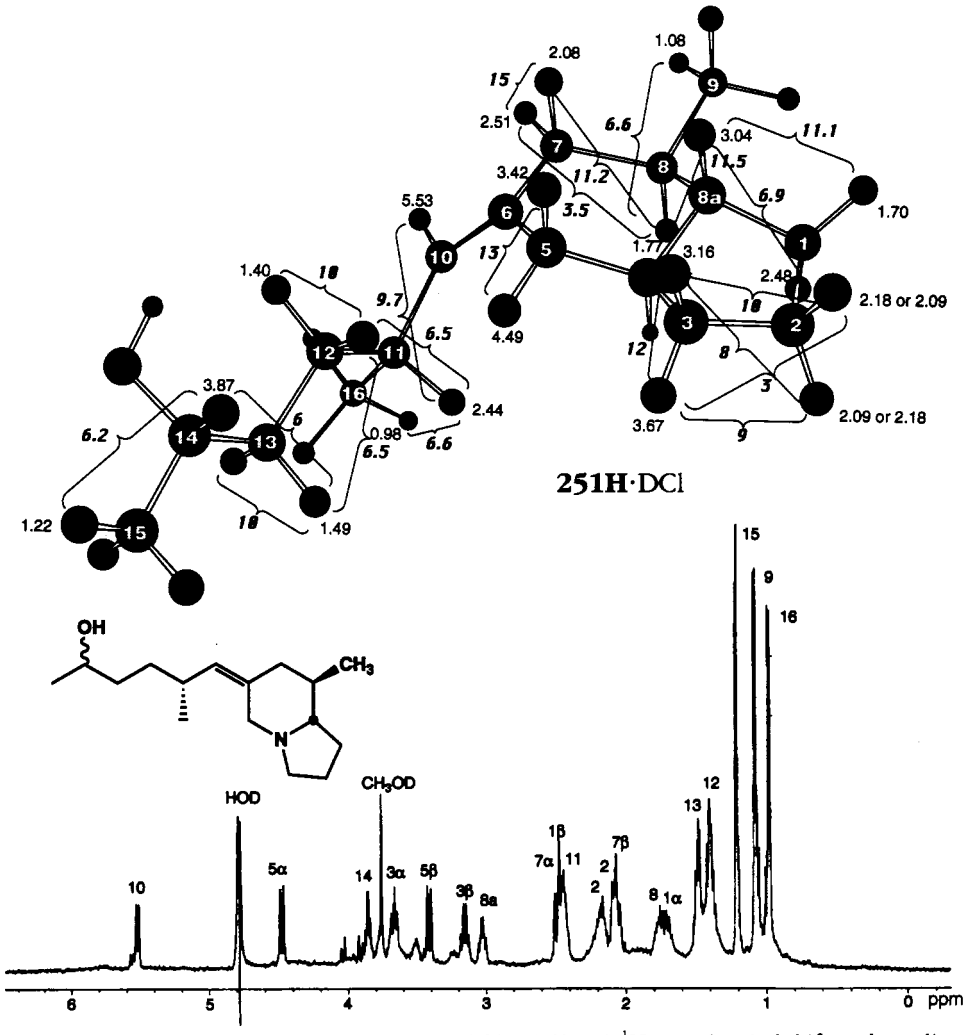
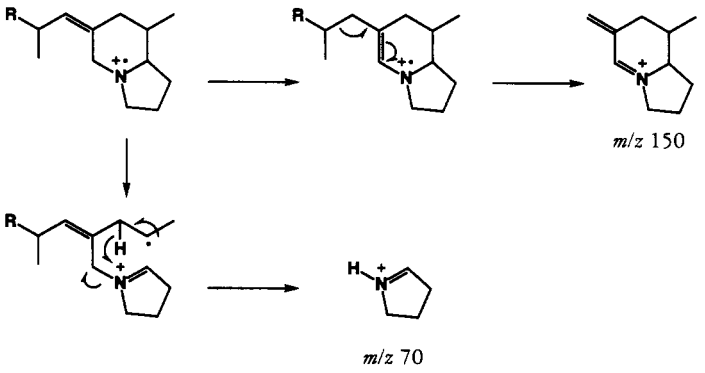


FIGURE 2. A 3D structure of deoxypumiliotoxin **251H**·DCl with ^{1}H-nmr chemical shifts and coupling constants, a planar representation of deoxypumiliotoxin **251H** [4] and the 500 MHz ^{1}H-nmr spectrum of **251H**·DCl in D_2O.

through ^{1}H-nmr experiments. However, we have tentatively assigned the indicated relative configuration at C-11 because that configuration is conserved in the previously known pumiliotoxins, whose structures have been rigorously proved. On the other hand, the configuration at C-14 of **251H** is not assigned, because it is a highly variable position in the known pumiliotoxins (11). Hydrogens at C-12 are not equivalent but are very similar and overlap as do the hydrogens at C-13 (Figure 2). Couplings between hydrogens at C-1 and C-2 could not be discerned due to the limited sample size of **4** and the congestion of signals. Chemical shifts of H-1α and H-1β (δ 2.48 and δ 1.70, respectively) were assigned after decoupling of H-8a, which removed a large *J* from the signal of H-1α, but not from H-1β. Assignment of H-7α and H-7β (equatorial and axial respectively) were done similarly by decoupling of H-8, which removed a large *J* from the signal of H-7β and a small *J* from H-7α.

We prepose here a mass spectral fragmentation pathway for **4** (Scheme 1) which can be easily extrapolated to all pumiliotoxin-A class alkaloids.



SCHEME 1. Proposed mass spectral fragmentation of **251H** [**4**].

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—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 programmed from 100° to 280° at the rate of 10°/min, 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 series) was used to generate the chromatograms, eims, and Ft-ir spectra of gc peaks. A Finnigan 4500 mass spectrometer with a 25 m×0.25 mm OV-1 fused silica-bonded column was used for the cims studies with NH_3 or ND_3 as reagent gases. Hrms used a JEOL SX 102 instrument fitted with a 15 m×0.20 mm HP-5 column.

^{1}H-Nmr spectra in D_2O were measured with either a Varian XL-300 or a Varian VXR-500S spectrometer. Chemical shifts (δ, ppm) are referred to HOD at 4.78. ^{1}H 2D-COSY experiments were carried out on a Varian VXR-500S spectrometer.

ANIMAL MATERIAL.—An alkaloid fraction (60 mg) was prepared from MeOH extracts of skins of 750 frogs of the dendrobatid species *Epipedobates tricolor* from southwestern Ecuador (16 km W. Santa Isabel, Azuay, Ecuador) by acid-base extractions as previously described (8). Voucher specimens are located in the collections of the American Museum of Natural History, New York.

EXTRACTION AND ISOLATION.—The alkaloid fraction was chromatographed on a prepacked Si gel 60 column (Merck 1.0×24 cm) with $CHCl_3$-MeOH-NH_3 (6 N) (500 ml of 800:10:0.1 followed by 1000 ml of 1000:100:2) as described (8). Fractions of 5 ml were collected. Pumiliotoxin **251D** (21 mg) eluted in fractions 6–10, allopumiliotoxin **341A** (ca. 9 mg) and quinolizidine **217A** (ca. 3 mg) eluted together in fractions 13–17, indolizidine **203A** (ca. 1 mg) eluted in fractions 18–20, epibatidine (**208/210**) (ca. 1 mg) eluted in fractions 108–111, allopumiliotoxin **323B** (ca. 13 mg) eluted mainly in fractions 112–116, and

alkaloid **251H** (ca. 1 mg) eluted in fractions 117–120. Other trace alkaloids included pumiliotoxin **237A**, pumiliotoxin A **307A**, allopumiliotoxins **267A** and **357**, and quinolizidines **231A** and **231B**. Structures of most of these alkaloids have been reported (11). The chromatographic fractions 117–120 containing **251H** were combined, concentrated, dissolved in EtOAc, and then extracted with 0.1 N HCl. The acidic layer was neutralized with saturated $NaHCO_3$ and then extracted with EtOAc. The EtOAc portion was dried over anhydrous Na_2SO_4 and concentrated to yield less than 1 mg of **251H**.

The properties of deoxypumiliotoxin **251H** [**4**] are as follows with the molecular formula determined by hrms, the eims with intensities relative to the base peak set equal to 100, the Ft-ir data with selected frequencies in cm^{-1} and intensities in parentheses relative to the maximum absorbance set equal to 100, and the ^1H-nmr data with chemical shifts and coupling constants (see also Figure 2).

*Deoxypumiliotoxin **251H*** [**4**].—$C_{16}H_{29}NO$; eims *m/z* 251 (3), 250 (4), 236 (3), 222 (1), 208 (1), 194 (1), 178 (10), 162 (1), 150 (100), 136 (5), 122 (3), 108 (3), 93 (3), 79 (5), 70 (20); Ft-ir 3652 (6), 2968 (100), 2935 (72), 2883 (43), 2790 (28), 2741 (15), 1459 (15), 1379 (16), 1300 (13), 1256 (18), 1170 (24), 1101 (15), 965 (7) cm^{-1}. A minor diastereomer, that emerged after **251H** [**4**] on gc, was also seen, but is not described here. ^1H-Nmr data for **251H**·DCl (500 MHz, D_2O) δ 5.53 (1H, d, J=9.7 Hz, H-10), 4.49 (1H, d, J=13 Hz, H-5α), 3.87 (1H, m, J=6.2 and 6 Hz, H-14), 3.67 (1H, ddd, J=12, 9, and 3 Hz, H-3α), 3.42 (1H, d, J=13 Hz, H-5β), 3.16 (1H, ddd, J=12, 10, and 8 Hz, H-3β), 3.04 (1H, ddd, J=11.5, 11.1, and 6.9 Hz, H-8a), 2.51 (1H, m, J=15 and 3.5 Hz, H-7α), 2.48 (1H, m, H-1α), 2.44 (1H, m, J=9.7, 6.6, and 6.5 Hz, H-11), 2.18 (1H, m, H-2α or H-2β), 2.09 (1H, m, H-2β or H-2α), 2.08 (1H, m, J=15 and 11.2 Hz, H-7β), 1.77 (1H, m, J=11.5, 11.2, 6.6, and 3.5 Hz, H-8), 1.70 (1H, m, H-1β), 1.49 (2H, m, J=10, 6.5, and 6 Hz, H_2-13), 1.40 (2H, m, J=10, 6.5, and 6.5 Hz, H_2-12), 1.22 (3H, d, J=6.2 Hz, H_3-15), 1.08 (3H, d, J=6.6 Hz, H_3-9), 0.98 (3H, d, J=6.6 Hz, H_3-16).

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