# A Revised Structure for Alkaloid 235C Isolated from Skin Extracts of Mantellid (*Mantella*) Frogs of Madagascar

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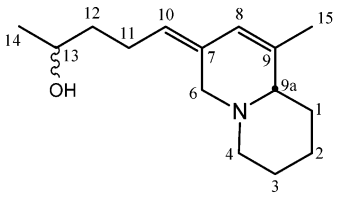
Madagascan frogs of the mantellid genus *Mantella* have been a rich source of alkaloids derived from dietary arthropods. Two species of frogs, inhabiting swamp forest, contain a unique set of alkaloids, previously proposed, based only on GC-MS and GC-FTIR data, to represent dehydro analogues of the homopumiliotoxins. The major alkaloid of this set, alkaloid **235C** (**2**), now has been isolated in sufficient quantities (ca. 0.3 mg) to allow determination of the structure by NMR analysis. The structure of alkaloid **235C** proved to be a 7,8-dehydro-8-desmethylpumiliotoxin. A comparison is presented between the mass, infrared, and $^1$H NMR spectra of **235C** (**2**) and a synthetic dehydrohomopumiliotoxin (**1**), initially proposed incorrectly as the structure for **235C**.

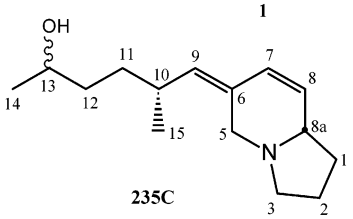
A wide range of alkaloids, representing over 20 structural classes, has been isolated from skin extracts of dendrobatid frogs (*Phyllobates*, *Dendrobates*, *Minyobates*, *Epipedobates*) from tropical Central and South America, bufonid toads (*Melanophryniscus*) from subtropical to temperate South America, and mantellid frogs (*Mantella*) from Madagascar.[1,2] All such alkaloids appear to be derived from dietary alkaloid-containing small arthropods.[3] However, Australian frogs of the myobatracid genus *Pseudophryne* synthesize their unique isoprenylated cyclized *N*-methyl tryptamines, but sequester pumiliotoxins from the diet.[4] The pumiliotoxins are widely distributed in all the above frogs/toads. Approximately 25 pumiliotoxins and five allopumiliotoxins have been found in the *Mantella* frogs, endemic to Madagascar.[5,6] Several homopumiliotoxins also occur in such frogs.[5,6] Structures for many are well-established by NMR analysis and/or by synthesis.[2,7] Some of the major pumiliotoxins, allopumiliotoxins, and homopumiliotoxins of mantellid frogs are shown in Figure 1.

Many alkaloids from frog skin have not been available in quantities sufficient for NMR analysis. Thus, in some cases tentative structures, based only on GS-MS and GC-FTIR data obtained from extracts containing as many as 30 to 80 different alkaloids, have been proposed.[2,7] This is the case for a unique set of alkaloids present in trace to minor amounts in extracts from two Madagascan species, *Mantella aurantiaca* and *M. crocea*, of swamp forest-dwelling frogs.[5,7] A tentative structure **1** was originally proposed for the major alkaloid of this set, observed on GC-MS as a pair of evident diastereomers with identical mass spectra and similar retention times.

This compound has now been synthesized[8] to provide a mixture of two diastereomeric alcohols that, based on spectroscopic properties, clearly differed from the data obtained for the natural alkaloid **235C**. The mass and FTIR spectra (see Figures 2−4) were markedly different, as were the GC retention times (not shown). In the meantime, isolation of a sufficient amount of alkaloid **235C** to allow



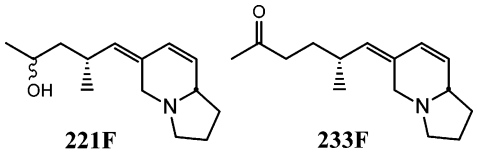

**1**




**235C**

**2**

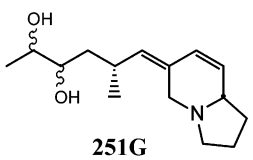
a $^1$H NMR analysis now has revealed its structure to be that of a 7,8-dehydro-8-desmethylpumiliotoxin (**2**). The absolute stereochemistry at C-8a and C-10 remains unknown, and the depicted configuration **2** is based solely on the known absolute stereochemistry of pumiliotoxins.[7] The absolute stereochemistry at C-13 also is unknown in the two naturally occurring diastereomers. GC-MS and LC-MS with an APCI interface revealed two apparent diastereoisomers with closely eluting retention times. Total ion chromatograms on LC-MS indicated for both $[M + H]^+$ ions of *m/z* 236 that lose water, giving ions at *m/z* 218 (20%). The proportions of diastereomers were ca. 1:3 for *M. crocea*




**221F**




**233F**




**251G**

**5**

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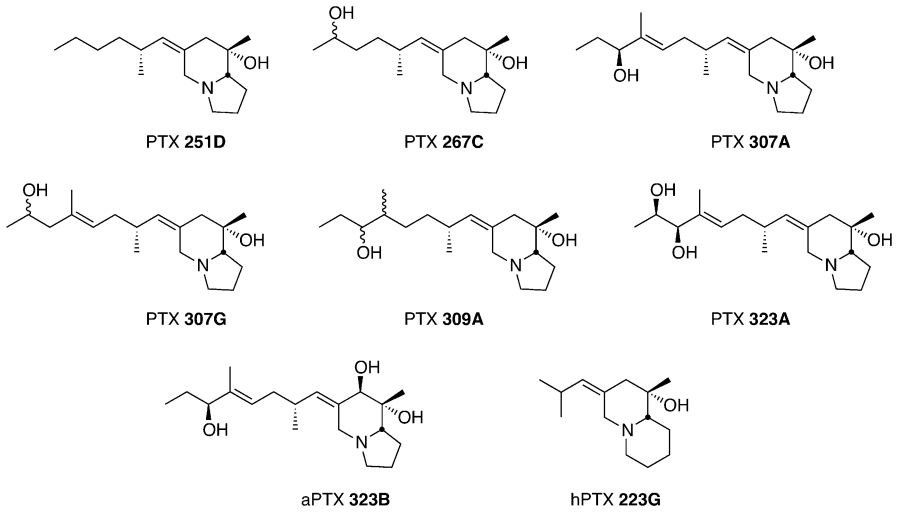


**Figure 1.** Major pumiliotoxins, allopumiliotoxins, and a homopumiliotoxin present in skin extracts of mantellid frogs.[5,6]

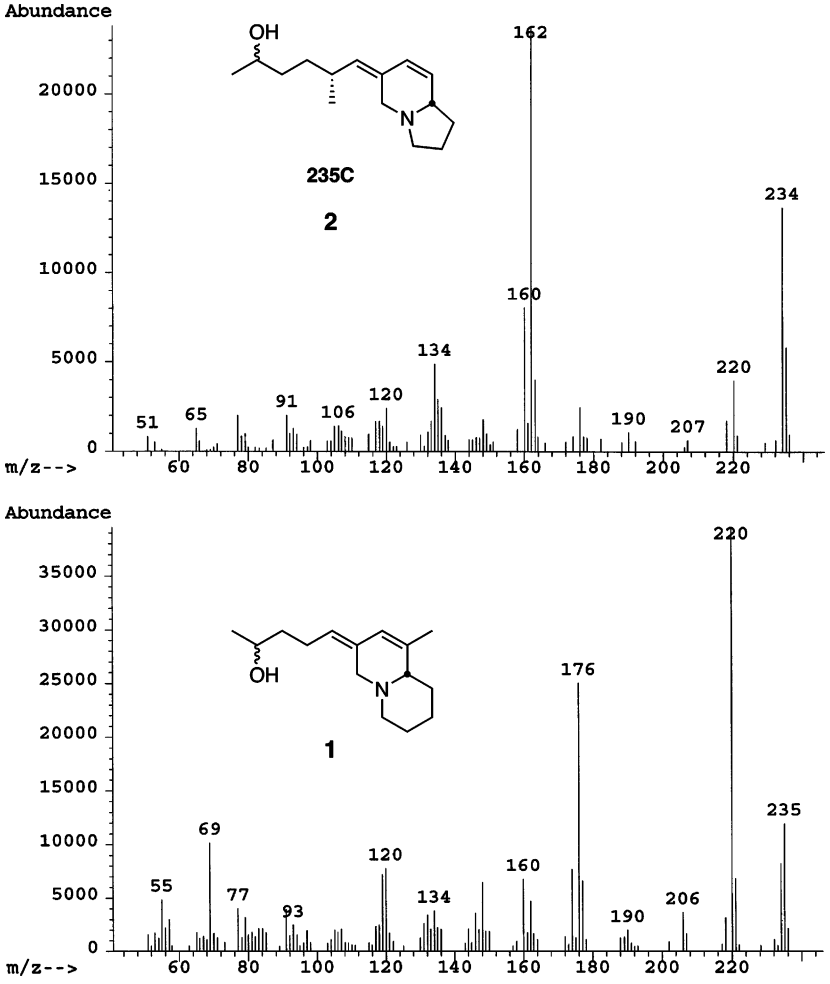


**Figure 2.** EI-mass spectra of alkaloid **235C** (**2**) and synthetic **1**.

and ca. 1:6 for *M. aurantiaca* with the prominent diastereomer at longer retention time. Acetylation of the *M. crocea* extract revealed, on GC-MS, two monoacetylated diastereomers in a 1:2 ratio, of nearly identical EIMS.[5] These likely represent the two C-13-acetoxy diastereomers. Tentative structures for three additional members of a proposed 7,8-dehydro-8-desmethylpumiliotoxin subclass of alkaloids (**3**−**5**) are shown below. All show mass spectral fragmentations (base peak *m/z* 162, accompanied by a major fragment at *m/z* 160) consonant with these structures.[5]

## Results and Discussion

**Isolation.** Alkaloid **235C** (**2**) has been detected as a trace or minor alkaloid in extracts of various populations of two swamp-dwelling mantellid frogs of Madagascar.[5,6] The amounts present in such extracts, namely 20 $\mu$g per extract or less, were sufficient only for GC-MS and GC-FTIR analysis. A tentative dehydrohomopumiliotoxin structure (**1**) was proposed for alkaloid **235C**,[5] but as the present work indicates, this structure is incorrect since synthesis of **1**[8] and comparison of the spectral properties of synthetic

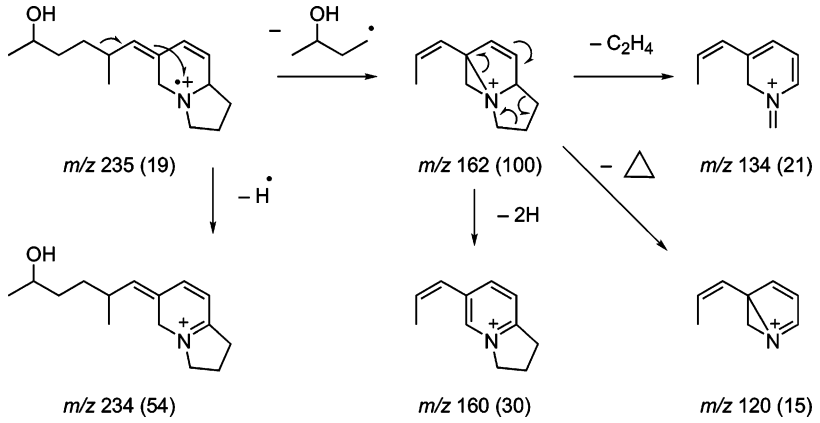


**Figure 3.** Tentative EI-mass spectral fragmentation schemes for alkaloid **235C** (**2**).

**Table 1.** $^1$H NMR Spectroscopic Data (500 MHz, $D_2O$) of Alkaloid **235C**·DCl

| position | $\delta_H$ (ppm) | multiplicity, *J* (Hz) | assignments from 1D- and 2D-NMR irradiations |
|---|---|---|---|
| 1 | $H_\alpha$ 2.20; $H_\beta$ 1.90 | m; m | |
| 2 | $H_\alpha$ 2.0; $H_\beta$ 1.95 | m; m | weak H-1 and H-2 cross-peaks in H,H-COSY |
| 3 | $H_\alpha$ 3.31; $H_\beta$ 3.05 | m; m | H-3 and H-2 cross-peaks |
| 5 | $H_\alpha$ 3.89; $H_\beta$ 3.68 | d, 13.9 | |
| 7 | 6.71 | d, 11.3 | H-7 and H-8 cross-peak |
| 8 | 5.72 | dt, 11.3, ca. 1 | weak coupling with H-8a? |
| 8a | 4.25 | br s | H-8a and H-1$_\alpha$ cross-peak seen, but not H-8a and H-1$_\beta$ |
| 9 | 5.40 | d, 10.1 | H-9 and H-10 cross-peak |
| 10 | 2.68 | m | H-10 and H-15 cross-peak |
| 11 | 1.50 | m | H-11 and H-12 cross-peak |
| 12 | 1.38 | m | irradiation of H-13→t |
| 13 | 3.79 | m | irradiation of H-13→H-14(s); H-13 and H-12 cross-peak |
| 14 | 1.15 | d, 6.3 | irradiation of H-14→effect at $\delta$ 3.79; H-13 and H-14 cross-peak |
| 15 | 1.03 | d, 6.6 | irradiation of H-15→effect at $\delta$ 2.68 |

**1** to those of alkaloid **235C** indicated many differences. A new extract was obtained from 10 skins of the swamp-dwelling mantellid frog *Mantella crocea*, collected in November 1997, north of Andasibe. After preparation of an alkaloid fraction, HPLC purification was conducted as described in the Experimental Section. About 0.3 mg of alkaloid **235C** was isolated. NMR spectroscopic analysis revealed the structure **2** (see below).

**Mass Spectral Analysis.** The EIMS of alkaloid **235C** (Figure 2) is fully consistent with structure **2**. There is a significant loss of a hydrogen radical to afford a pyridinium equivalent at *m/z* 234. The base peak at *m/z* 162 results from allylic cleavage, losing $C_4H_9O$. The loss of 2H then provides a pyridinium ion at *m/z* 160. Losses of $C_2H_4$ and $C_3H_6$ from the base peak would then yield ions at *m/z* 134 and 120, respectively. A postulated fragmentation scheme for **235C** is depicted in Figure 3.

As would be expected, both **235C** (**2**) and **233F** (**3**), the ketone congener of **235C**, give exactly the same fragments (see Experimental Section). The dehydrodesmethylpumiliotoxin structure **2** assigned to **235C** is consistent with an EIMS/MS experiment (see Experimental Section), where collision-activated dissociation (CAD) of the El base peak (*m/z* 162) is examined.

The EIMS of synthetic **1** (Figure 2) is quite different and shows a major loss of 15 mass units to afford the base peak at *m/z* 220. That a fragmentation pathway for apparent loss of a methyl radical should be so prominent with **1** is not easily rationalized. Allylic cleavage of $C_3H_7O$ affords a major ion at *m/z* 176, which loses 2H to afford a stable pyridinium ion at *m/z* 174.

Collision-activated dissociation (CAD) fragments from CI-MS/MS using ammonia reagent gas have been shown to provide structural insights complementary to those obtained from the radical cleavage of EIMS in analysis of alkaloids, particularly those lacking hydroxyl groups or double bonds.[9−11] In the present study of **1** and **2**, this technique gave less useful information. While the fragments resulting from CAD of the *m/z* 236 $[M + H]^+$ ions from **1** or **2** were the same (*m/z* 234 and 218), there was more dehydrogenation and less water loss from **1** relative to **2** (see Experimental Section).

**NMR Spectral Analysis.** The amount of isolated alkaloid was sufficient to provide for detailed $^1$H NMR analysis at 500 MHz that indicated **2** as the structure of **235C**. A summary is presented in Table 1. The following critical elements ruled out the tentative structure **1**, which had been proposed based only upon GC-MS and GC-FTIR spectral data.[5] Two methyl doublets were present, as were three vinyl doublets, two of which are coupled with one another. The third vinyl doublet was coupled with a proton (H-10) that was also coupled with the upfield methyl. Synthetic **1** differed significantly from alkaloid **235C** in NMR, MS, and FTIR spectra. The $^1$H NMR assignments for **1** are presented in Table 2. The relatively high upfield chemical shift ($\delta$ 2.59) of the proton assigned to H-9a is anomalous. It would be expected to be further downfield, as observed for the equivalent H-8a of natural **2**, which is seen at $\delta$ 4.25 (see Table 1). The remaining $^1$H NMR signals for synthetic **1** are consistent with the structure.

The **235C** sample isolated by HPLC was contaminated with approximately 2 equiv of glycerol, which we concluded was present in the original methanol extract of *M. crocea*. We suspect that some sort of complex allowed glycerol (perhaps originating from hydrolysis of a glyceride) to be eluted with **235C** in the nonpolar fractions of the HPLC chromatography. Efforts to remove the glycerol were unsuccessful. The glycerol partially obscured the doublet for H-5$\beta$ and totally obscured the multiplet for H-13, but an H,H-COSY revealed cross-peaks for these signals. A 1D

**Table 2.** $^{1}H$ and $^{13}C$ NMR Spectral Data of **1** in $CDCl_3$

| position | $\delta_H$ (ppm) | multiplicity, $J$ (Hz) | $\delta_C$ (ppm) | assignments from 1D and 2D irradiations |
|---|---|---|---|---|
| 1 | 1.2−1.3 | m | 25.4 | H-9a COSY cross-peaks |
| 2 | 1.8 | m | 29.0 | H-1 cross-peaks |
| 3 | 1.5−1.6 | m | 25.0 | H-4 and H2 cross-peaks |
| 4 | 2.91 and 2.14 | m; m | 56.1 | HMQC confirmed two protons on same C. Strong COSY cross-peaks to each other and H-3 |
| 6 | 3.58; 2.82, | d, 10.8; m | 53.6 | HMQC confirmed two protons on same C. Strong COSY cross-peak between these two protons |
| 7 | | | 128.4[a] | |
| 8 | 5.86 | s | 125.5 | low-intensity cross-peak with H-15 |
| 9 | | | 133.5[a] | |
| 9a | 2.59 | m | 64.0 | H-1 cross-peak |
| 10 | 5.15 | t, 7.4 | 123.8 | H-11 cross-peak |
| 11 | 2.07−2.12 | 2 m | 23.6 | H-10 and H-12 cross-peaks |
| 12 | 1.4−1.5 | m | 39.0 | H-13 and H-11 cross-peaks |
| 13 | 3.80 | sextet, 6.2 | 67.7 | H-14 and H12 cross-peaks |
| 14 | 1.19 | d, 6.2 | 23.8 | H-13 cross-peak |
| 15 | 1.71 | s | 19.6 | low-intensity cross-peak with H-8 |

[a] Could be interchanged. A 4,13-diketo intermediate in the synthesis of **1** showed an NOE of 9.7% in the signal of H-10 when H-8 was irradiated, also supporting the stereochemistry indicated for **1**.

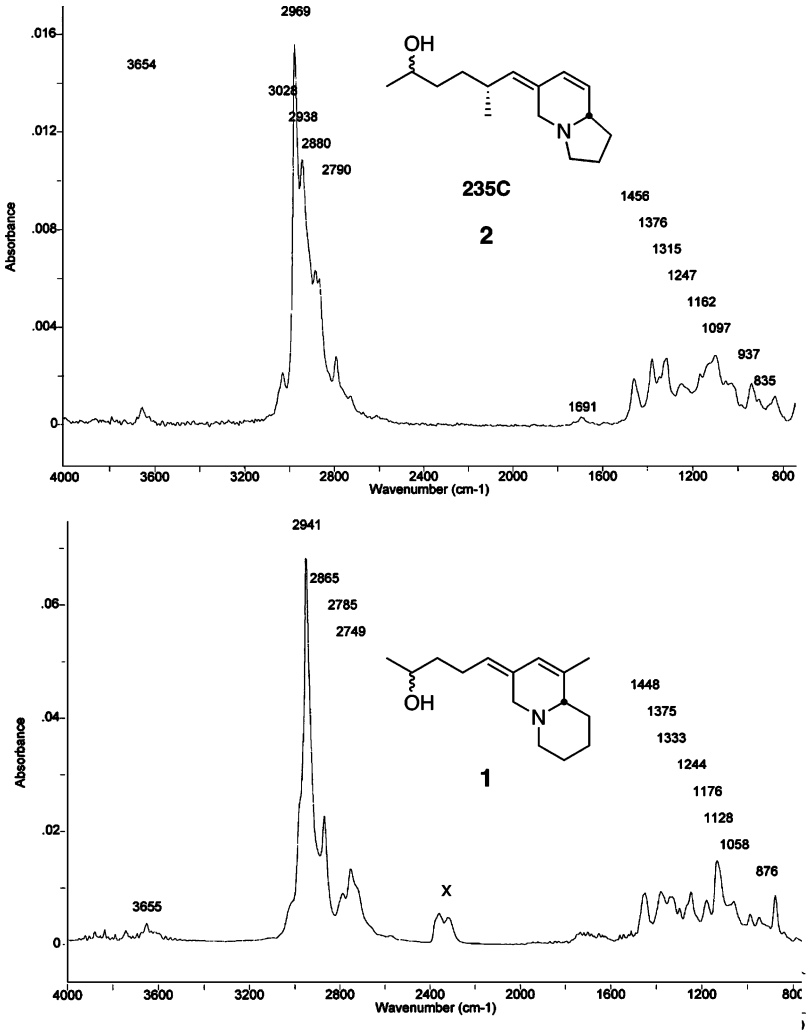


**Figure 4.** GC-FTIR spectra for alkaloid **235C** (**2**) and synthetic **1**. "×" indicates an artifactual absorption.

irradiation of the multiplet from the C(2)H-OD signal of glycerol showed a change in the signal at $\delta$ 1.4 assigned to H-12. Irradiation of the H-14 methyl also changed the multiplet at $\delta$ 3.8, showing that H-13 lies under the downfield glycerol multiplet. Fortunately, the ABC pattern of the glycerol C(1)H2-OD signals, consisting of a total of two pairs of dd signals, did not obscure any other signals. The glycerol signals were shifted in $d_6$-acetone, but certain signals of **235C** deuterochloride were still obscured.

The H-8a signal was found at $\delta$ 4.25, considerably downfield from the usual position at ca. $\delta$ 3, reflecting its allylic position and also lack of shielding from the usual adjacent methyl at C-8 in most pumiliotoxins.

Molecular modeling revealed that the dihedral angle ($\theta$) between H-8 and H-8a was 80°, leading to negligible coupling. No coupling was also seen in an H,H-COSY between H-8a and H-1$\beta$, where $\theta$ is modeled to be 82°, again leading to a $J \approx 0$ Hz. A coupling was seen between

H-8a and H-1α where $\theta$ is 39°. This dihedral angle results from an energy-minimized structure (22.97 kcal/mol) when a *cis*-ring fusion was modeled. A *trans*-ring fusion model where the *N*-lone pair and H-8a are on opposite faces had a slightly higher energy (23.12 kcal/mol) than the *cis*-ring model. While no coupling would also be predicted between H-8 and H-8a (calculated $\theta = 88°$), couplings of medium *J*'s are predicted for H-8a and *both* protons at C-1 ($\theta_{8a-1\alpha} = 163°$; $\theta_{8a-1\beta} = 42°$). For these reasons we prefer the less common *cis*-ring-fused indolizidine. The diene moiety and lack of the intramolecular hydrogen bond that is present with the usual pumiliotoxin structures presumably are responsible for this preferred conformation in the case of **235C**.

**Infrared Spectral Analysis.** The GC-FTIR spectrum of natural **235C** (Figure 4) showed a modest, broad Bohlmann band at 2790 $cm^{-1}$, consonant with structure **2**. Pumiliotoxins show slightly stronger, broad Bohlmann bands at 2798 $cm^{-1}$.[7] The *trans*-fused model for **235C** had the calculated *trans-anti*-parallel $\theta$ of 178° (with H-5$\beta$), 178° (with H-8a), and 167° (with H-3$\beta$) and would have been predicted to have an intense, sharp Bohlmann band typical of an indolizidine. Bohlmann bands were modest, but were not absent for **235C**, as would have been predicted from only a single *trans-anti*-parallel H-3$\beta$-N dihedral angle of 160° calculated for the *cis*-fused model. On the basis of the IR spectrum and the NMR analysis discussed above, the *cis*-fused structure does appear to be the preferred configuration of **235C**. The OH group of **235C** was indicated by the absorbance at 3654 $cm^{-1}$ and a *cis*-HC=CH by the $\nu_{C-H}$ absorbance at 3028 $cm^{-1}$ and absence of an absorption at ca. 965 $cm.^{-1}$ The GC-FTIR spectrum of synthetic **1** (Figure 4) had much more pronounced Bohlmann bands at 2785 $cm^{-1}$ and a weak broad hydroxyl $\nu_{O-H}$ at ca. 3655 $cm^{-1}$. A shoulder slightly above 3000 $cm^{-1}$ indicated an aliphatic *cis* double bond.

**Summary.** The structure of alkaloid **235C** has now been established as **2**, in accordance with MS, NMR, and FTIR spectra. Compound **1**, tentatively proposed[5,7] as a structure for **235C** and now synthesized,[8] has a mass and infrared spectra greatly differing from that of **2**, illustrating the inherent danger of proposing structures based solely on MS and FTIR, in this case, along with analogies to known congeneric compounds. The pharmacological properties of synthetic **1** and natural **2** warrant study. Pumiliotoxins have marked cardiotonic and myotonic activity,[12,13] but in addition have convulsant and hyperalgesic effects.[14,15]

## Experimental Section

**General Experimental Procedures.** Mass spectral data (EIMS, EIMS/MS, CIMS ($NH_3$), and CI-MS/MS ($NH_3$)) were obtained with a Finnigan Thermoquest GCQ instrument, having a Restek RTX-5MS capillary column (30 m, 0.25 mm i.d.) programmed from 100 to 280 °C at 10 deg/min. GC-FTIR and EIMS spectra were obtained with a Hewlett-Packard model 5890 gas chromatograph, having an HP-5 fused silica-bonded capillary column (30 m, 0.32 mm i.d.) programmed from 100 to 280 °C at a rate of 10 deg/min and interfaced with a Hewlett-Packard model 5971 mass selective detector and a Model 5965B IRD with a narrow band (4000−750 $cm^{-1}$) detector. A Hewlett-Packard ChemStation was used to generate EIMS and FTIR spectra of **1** and **2**. The $^1H$ NMR spectra of **235C**·DCl in $D_2O$ were measured with a Varian VXR-500X spectrometer. Molecular modeling used Chem 3D-Pro, version 5.0 (Cambridge Soft Corp.).

**Extraction and Isolation.** A methanol extract obtained from skins (ca. 1 g wet weight) of 10 *Mantella crocea* frogs that were collected in November 1997 from a swamp forest north of Andasibe was subjected to acid−base partitioning as described.[5] The resultant alkaloid fraction contained about 7 mg of alkaloids. GC-MS analysis indicated pumiliotoxins **267C**, **307A**, and **323A** and allopumiliotoxin **323B** (see Figure 1) as major components, with alkaloid **235C** and its keto-congener **233F** (ketone $\nu_{C=O} = 1730$ $cm^{-1}$) as minor components. Alkaloids **392** and **434** of unknown structures were also minor components. Pumiliotoxins **265G**, **305G**, and **307B**, decahydroquinoline **195A**, pyrrolizidine **223H**, and quinolizidine **231A** were detected as trace components. The most recent general listing of frog skin alkaloids is provided in ref 2.

The alkaloid fraction in methanol was concentrated to 125 $\mu$L, and 25 $\mu$L portions were purified by HPLC. A reversed-phase column (Phenomenex column AQUA-125A, $C_{18}$, 4.6 mm i.d. × 250 mm with particle size 5 $\mu$m) was used with $CH_3CN$ (0.1% HOAc)−$H_2O$ (0.1% HOAc) and a gradient from 10:90 to 90:10 over a 30 min period and with a flow rate of 0.5 mL/min. Thirty fractions of 0.5 mL were collected, and alkaloid content was assayed by GC-MS. Fraction 14 contained mainly alkaloid **235C**. The combined **235C** fractions were used for NMR spectroscopic analysis. Alkaloid **235C** was freed from trace amounts of neutral compounds in the $CDCl_3$ solution by extraction with $D_2O$ containing DCl, followed by concentration of the $D_2O$ to dryness to afford **235C**·DCl salt for $^1H$ NMR spectroscopic analysis in $D_2O$. Roughly 2 equiv of glycerol was, however, present and could not be removed on additional HPLC or extractions. An LC-MS (Finnigan Thermoquest LCQ) used the same column and solvents and a gradient from 10:90 to 50:50 over 40 min. The atmospheric pressure chemical ionization (APCI) interface (vap temp = 480 °C) showed alkaloids as their $M + H^+$ ions and in the case of the two diastereomers of **235C** showed *m/z* 236 and a 218 fragment (ca. 20%) at retention times of 17.4 min (minor diastereomer) and 17.9 min. (major diastereomer). These did not absorb UV at 260 nm.

**EIMS of 235C (2)** (see Figure 2): *m/z* 235 (19), 234 (54), 220 (18), 190 (8), 176 (12), 162 (100), 160 (30), 148 (10), 146 (10), 135 (15), 134 (21), 120 (15), 106 (8), 105 (8), 91 (8), 79 (8), 77 (7).

**CIMS ($NH_3$) of 2:** *m/z* 237 (15), 236 (100), 235 (10), 162 (3).

**EIMS of 233F (4):** *m/z* 233 (13), 232 (27), 218 (15), 196 (5), 190 (7), 176 (4), 162 (100), 160 (31), 148 (7), 146 (7), 134 (16), 120 (11), 106 (11), 98 (10), 91 (12), 78 (5), 76 (5), 55 (14).

**EIMS/MS of 235C (2):** For the *m/z* 234 ion: *m/z* 234 (63), 218 (22), 160 (6), 146 (12), 133 (100), 105 (8). For the *m/z* 162 ion (excitation voltage 1.30): *m/z* 158 (16), 147 (46), 146 (42), 134 (92), 133 (40), 132 (52), 130 (26), 120 (100), 117 (44), 106 (18), 105 (28), 103 (20), 92 (8), 91 (18). For the *m/z* 162 ion with excitation voltage 1.20, only the ions *m/z* 160 (100), 147 (12), 134 (38), and 120 (28) were observed. The EIMS/MS of **233F** (**4**) was virtually identical to that of **2** at 1.30 V.

**CI-MS/MS on 235C (2):** (excitation voltage, 3.0) for the *m/z* 236 ion: *m/z* 236 (100), 234 (44), 218 (28).

**EIMS of synthetic 1** (see Figure 2): *m/z* 235 (29), 234 (21), 220 (100), 206 (9), 190 (5), 176 (63), 174 (18), 162 (13), 160 (18), 148 (17), 146 (9), 134 (9), 120 (20), 119 (19), 107 (5), 106 (5), 105 (5), 97 (5), 93 (7), 91 (12), 79 (10), 77 (12), 69 (25), 57 (8), 55 (13).

**EIMS of the synthetic ketone analogue of 1:** *m/z* 233 (30), 232 (15), 219 (9), 218 (22), 204 (8), 190 (6), 176 (100), 175 (30) [$M^+$ − acetone], 174 (26), 160 (20), 148 (17), 120 (26), 119 (18), 43 (26).

**EIMS/MS of synthetic 1** (diastereomer of retention time 13.57 min) (excitation voltage 1.20): for the *m/z* 234 ion: *m/z* 234 (100), 216 (14), 133 (45); for the *m/z* 220 ion (retention time 13.57 min): *m/z* 220 (36), 218 (100), 202 (36), 162 (20), 147 (26), 134 (12); (retention time 13.49 min): *m/z* 220 (42), 218 (100), 202 (30), 162 (22), 147 (16), 134 (7).

**CI-MS/MS on synthetic 1 diastereomers** (retention times 13.59 and 13.70 min; with excitation voltage, 3.0): Both had identical fragments from the *m/z* 236 ion: *m/z* 236 (20), 234 (100), 218 (8).

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