THE ISOLATION AND STRUCTURE OF PUMILIOTOXIN **341A**: A NOVEL CYCLIC ETHER FROM THE FROG *EPIPEDOBATES TRICOLOR*

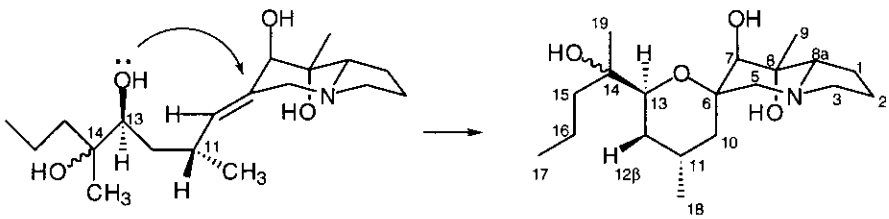
Poonam Jain,[†] Thomas F. Spande, H. Martin Garraffo, and John W. Daly[*]

Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, 20892, USA
[†]Present address: c/o Rahul Jain, Dept. of Med. Chem., Nat. Inst. of Pharmaceutical Education and Research, Sector 67, SAS Nagar (Mohali) 160 062, Punjab, India

Abstract - An alkaloid **341A** isolated by HPLC from skin extracts of the Ecuadoran poison frog *Epipedobates tricolor* was characterized by FTIR, MS and ^{1}H-NMR spectral analysis and assigned a spiro-fused pyranoindolizidine structure (**1**). It represents the first member of a small group of pumiliotoxin alkaloids to contain a cyclic ether moiety.

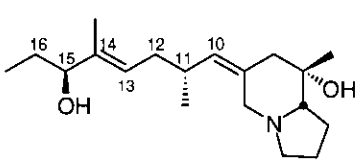
The isoprene-containing pumiliotoxin (PTX) class of alkaloids is one of the largest and most ubiquitous of the poison frog skin alkaloids.[1-3] These are 6-alkylideneindolizidines having a methyl and a tertiary hydroxyl group at C-8 and, in the case of the alloPTX subclass, an additional hydroxyl group at C-7. They have been detected as major or minor alkaloids in frogs from Central and South America, Australia and Madagascar; many have potent myotonic and/or cardiotonic activity. A large variety of alkylidene side-chains have been characterized, comprising alkaloids totaling 13-21 carbons. There is another homologous class, the homopumiliotoxins (homoPTXs), comprising alkaloids of 14-21 carbons having 7-alkylidenequinolizidine structures. Approximately seventy-five members of the PTX/homoPTX classes have been reported, a small number of which lack either the tertiary hydroxyl or the tertiary methyl groups at C-8 in the PTX class or at C-9 in the homoPTX class. The largest subgroup (*ca.* 30) is comprised of 19-carbon alkaloids. Many of this group have additional hydroxyl or carbonyl groups in the side chain, the most common being pumiliotoxin A (**2**) and pumiliotoxin B (**3**), coded as **307A** and **323A**, respectively. An allopumiliotoxin coded as **323B** (**4**), is also widely encountered. Alkaloids **323A** (**3**) and **323B** (**4**) have the three hydroxyl groups apparently essential for maximum cardiotonic activity.[4]

Alkaloids **339A** (**5**) and **339B** (**6**) are 7β- and 7α-hydroxy-substituted alloPTXs, respectively, with the same *threo* 15,16-diol in the side-chain as **3**. Another alkaloid, **341A**, like **339A** and **339B** was shown by HRMS to be a tetra-oxygenated C_{19} alkaloid and, by MS, to be clearly of the PTX class. Alkaloid **341A** had, however, by GC-MS deuterium-exchange measurement, only three exchangeable hydrogens. Since it had no carbonyl absorption in its GC-FTIR spectrum (Figure 1), one oxygen had to be present as an ether or epoxide. The ^{1}H-NMR analysis below locates this oxygen as being in a 6,13-ether linkage, creating a pyran ring spiro-fused to a 6,10-dihydro-7-hydroxylindolizidine nucleus, and can be visualized as arising by intramolecular cyclization of a 13,14-dihydroxy alloPTX as shown below. Uncyclized alloPTX 13,14-diols have yet to be detected in frog skin. A C-13–C-14 double bond is, however, often present in 19-carbon PTXs and alloPTXs.

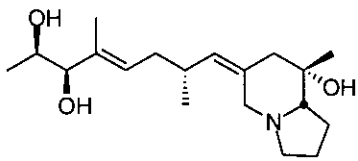


Possible precursor

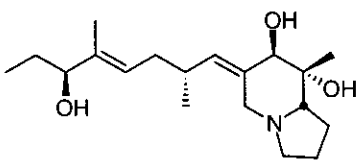
341A (**1**)



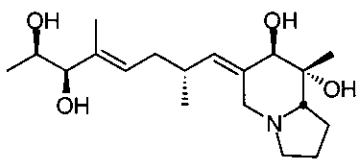
307A (**2**)



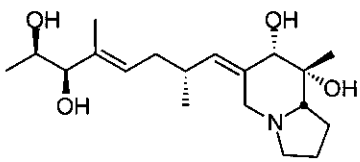
323A (**3**)



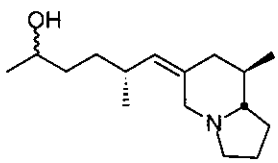
323B (**4**)



339A (**5**)



339B (**6**)



251H (**7**)

RESULTS AND DISCUSSION

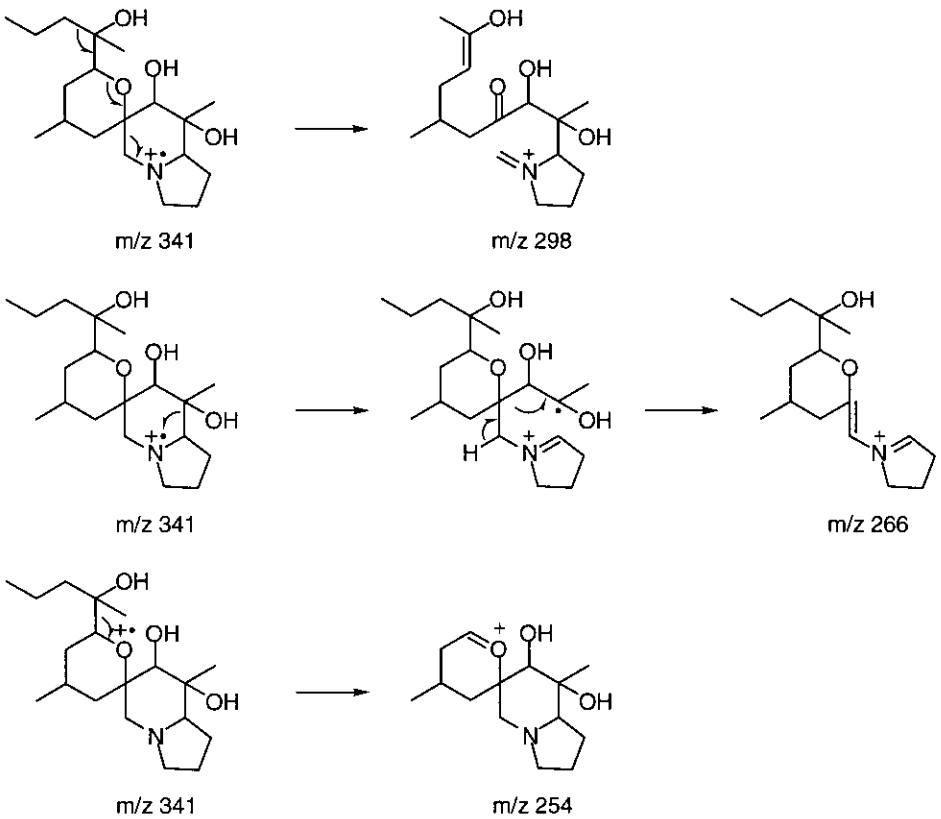
A typical GC-EI-MS spectrum indicated the following molecular and fragment ions with intensities in parentheses (see EXPERIMENTAL for other spectra; these were instrument-dependent suggesting a thermal component): 341 (3), 323 (9), 298 (8), 266 (7), 254 (12), 182 (11), 114 (32), 112 (90), 84 (77), 70 (100). CI-MS with NH_3 gave a protonated parent ion at *m/z* 342, confirming the EI-MS molecular ion of *m/z* 341. CI-MS with ND_3 gave a deuteronated molecular ion of *m/z* 346 indicating three exchangeable hydrogens. EI-MS with an ND_3 leak gave spectra of D-exchanged material where the following maximum numbers of deuterium-exchanged hydrogens are indicated in parentheses for the main EI fragment ions: *m/z* 323 (2D), 298 (3D), 266 (1D), 254 (2D), 184 (2D), 182(2D), 126 (0D), 112 (1D), 84 (0D), 70 (50% 1D). The following molecular and fragment ion formulae were determined by HRMS.

Table 1. Summary of HRMS data on alkaloid **341A** (1)

341	M^+	$C_{19}H_{35}NO_4$	184	$(M^+-C_9H_{17}O_2)$	$C_{10}H_{18}NO_2$
323	(M^+-H_2O)	$C_{19}H_{33}NO_3$	182	$(M^+-C_9H_{19}O_2)$	$C_{10}H_{16}NO_2$
306	(M^+-H_2O-OH)	$C_{19}H_{32}NO_2$	166	$(M^+-C_9H_{19}O_3)$	$C_{10}H_{16}NO$
298	$(M^+-C_3H_7)$[a]	$C_{16}H_{28}NO_4$	126	$(M^+-C_{12}H_{23}O_3)$	$C_7H_{12}NO$
266	$(M^+-C_3H_7O_2)$	$C_{16}H_{28}NO_2$	114	$(M^+-C_{13}H_{23}O_3)$	$C_6H_{12}NO$
254	$(M^+-C_5H_{11}O)$	$C_{14}H_{24}NO_3$	112	$(M^+-C_{12}H_{23}NO_3)$	$C_7H_{12}O$
209	$(M^+-C_7H_{16}O_2)$	$C_{12}H_{19}NO_2$	84	$(M^+-C_{14}H_{25}O_4)$	$C_5H_{10}N$
198	$(M^+-C_7H_{13}NO_2)$	$C_{12}H_{22}O_2$	70	$(M^+-C_{15}H_{27}O_4)$	C_4H_8N

[a]The error here in the best of three trials at peak matching was + 46 ppm; all other fragment ion mass measurements were within ± 5.6 ppm. The *m/z* 298 ion profiled exactly with the total ion current of the **341A** GC peak and also, because of its D-exchange value, is not considered to be an impurity ion. EI-MS/MS on *m/z* 298 shows major ions at *m/z* 280 and 262, consistent with the loss of 2 molecules of H_2O.

The data of Table 1 indicate a loss of water and hydroxyl from the molecular ion, the loss of the elements of a propyl group, and a loss of a pentyloxy moiety. The last two losses, unprecedented in PTX or alloPTX alkaloids, are accomodated by the structural elements of one of the pyran substituents. A $C_3H_7O_2$ loss to give the ion at *m/z* 266 may involve the extrusion of carbons 7-9 with their hydroxyl groups by the fragmentation shown below (see Scheme 1). Fragmentations to give the *m/z* 298 and 254 ions are also proposed. The peaks at *m/z* 182 and 70 are typical of alloPTXs, while a major ion at *m/z* 84 is typical of hydrogenated PTXs and alloPTXs in which the 6,10-double bond has been reduced.[1,5] Peaks at *m/z* 114 and 112 are typical of alloPTXs.[1,5]



Scheme 1. Some MS spectral fragmentations proposed for alkaloid **341A** (**1**).

The FTIR spectrum of **1** (Figure 1) indicated two ν_{OH} absorptions in an approximately 2:1 ratio at 3568 and *ca.* 3536 cm^{-1}, the latter frequency typical of the internally hydrogen-bonded 8-hydroxyl group found in all pumiliotoxins with the exception of the small deoxy class. The 3650 cm^{-1} absorption seen with the side-chain pumiliotoxin C-15 or C-16 hydroxyl absorptions or the 7β-hydroxyl stretching frequency of some allopumiliotoxins is now only a shoulder. The 3568 cm^{-1} absorption may reflect intramolecular hydrogen bonding also. The strong Bohlmann bands at 2817 cm^{-1} is consistent with an indolizidine structure, but is significantly stronger than the Bohlmann bands for alkylidene-containing PTXs. A broad and strong absorption at 1069 cm^{-1}, the position of a C-O stretching vibration, supports one or more hydroxyl or ether groups. No carbonyl absorbance was detected.

The ^{1}H-NMR spectrum obtained after HPLC purification (see Experimental) was taken first in D_2O, then C_6D_6. Both revealed four methyl signals, two singlets at approximately δ 1.15 and 1.34 in D_2O or δ 1.55 and 0.92 in C_6D_6. A doublet (*J*=6.6 Hz) and triplet (*J*=7.2 Hz) were seen at δ 0.96 and 0.87 in D_2O and δ 0.80 and 0.85 in C_6D_6, respectively. The most downfield methyl in C_6D_6 is assigned to the 9-CH_3 and

the other singlet to a methyl attached to another hydroxyl-bearing carbon.

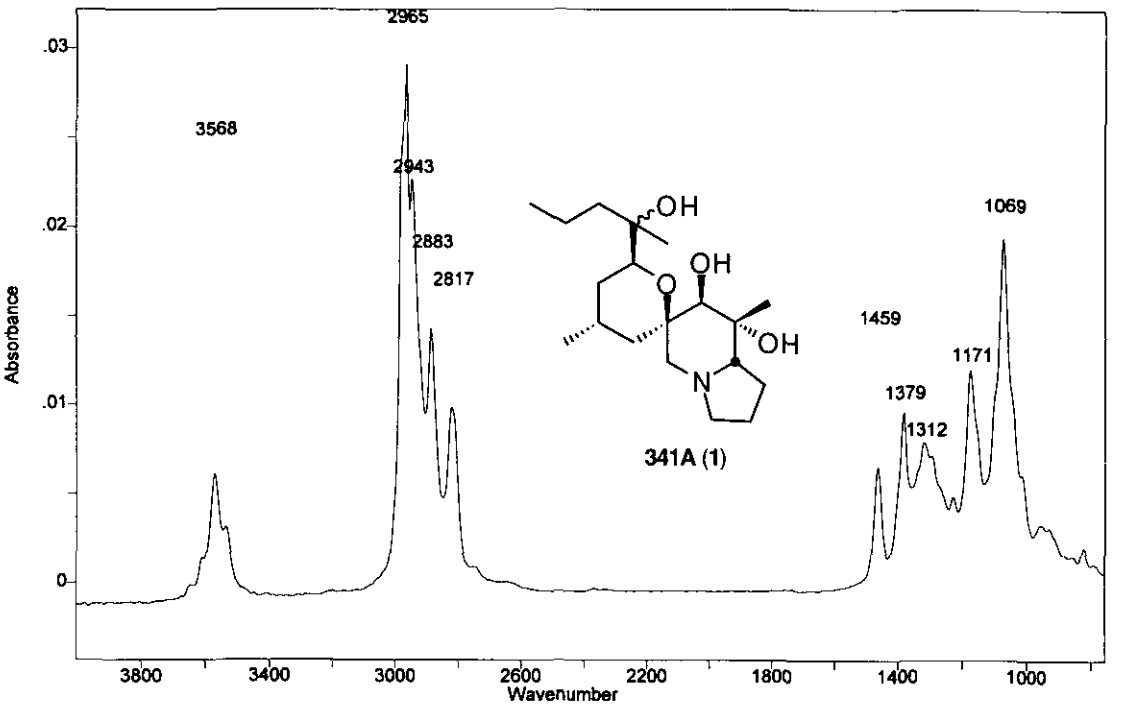


Figure 1. GC-FTIR spectrum of alkaloid **341A** (1).

The most revealing structural details emerged from deuterobenzene spectra and the following discusses that data (see Table 2). The spectrum revealed clearly the distinctive ^{1}H-NMR signals and multiplicities for indolizidine H-3_{ax} and H-5_{eq} protons at δ 2.04 (an apparent q, J=8.8 Hz) and 2.63 (d, J=10.6 Hz), respectively and confirms that ring system.[6] A doublet with a small long-range coupling (*ca.* 1 Hz) is seen at δ 3.73 and is assigned to H-7_{eq} with the long range coupling identified as a "W"-type coupling with H-5_{eq}. A number of other signals not seen in spectra of other pumiliotoxin class alkaloids, were seen in this spectrum that strongly implies a rigid side-chain moiety, *viz.* discrete dd or ddd signals. These are assigned as indicated in Table 2. In particular, the chemical shifts and multiplicities of signals assigned to the H-10α, H-10β, H-11, H-12α, H-12β and H-13 protons all indicate lack of free rotation. For example, the H-10 and H-12 signals show Δδ separations of 0.5 and 1.05 ppm, respectively. The close correspon-dence of measured J values seen in the pyran signals with the dihedral angles calculated for a slightly distorted chair conformation are consistent with their being attached to this rigid six-membered ring. Included in Table 2 are selected dihedral angles calculated with an MM2 computer

program for **341A.** The skewed chair conformation resulted when one dihedral angle ($\theta_{13\text{-}12\alpha}$) was fixed at 40° to agree with the *J* of 7 Hz observed for the coupling between H-13 and H-12α. When this was done and the energy minimized, the other dihedral angles in the pyran ring agreed well with the observed coupling constants.

Table 2. ^{1}H-NMR data on alkaloid **341A** (**1**) (500 MHz, C_6D_6)

H-Position	δ (ppm)	multiplicity, *J* (Hz)	θ, Calc'd (°)[a]
1	1.75	m	
1′[b]	1.45	m	
2	1.75	m	
2′	1.45	m	
3ax.	2.04	q, 8.8	$\theta_{3ax\text{-}2\alpha}$(141), $\theta_{3ax\text{-}2\beta}$(21)
3eq.	2.72	td, 8.2, 1.8	$\theta_{3eq\text{-}2\beta}$(99), $\theta_{3eq\text{-}2\alpha}$(20)
5ax.	2.24	dd, 10.6, 1.5	
5eq.	2.63	br d; 10.6	
7eq.	3.73	d, *ca.* 1	
8a	2.58	dd, 9.7, 7.1	$\theta_{8a\text{-}2\alpha}$(161), $\theta_{8a\text{-}2\beta}$(40)
10α	1.35	overlaps H-16	$\theta_{10\alpha\text{-}11}$(65)
10β	1.85	dm, *ca.* 13, m	$\theta_{10\beta\text{-}11}$(47)
11	1.60	m (14 lines)	$\theta_{11\text{-}12\alpha}$(75), $\theta_{11\text{-}12\beta}$(42)
12α	1.80	m	$\theta_{12\alpha\text{-}13}$(40), $\theta_{12\alpha\text{-}11}$(75)
12β	0.75	ddd, 12.8, 10.3, 7.1	$\theta_{12\beta\text{-}13}$(162), $\theta_{12\beta\text{-}11}$(42)
13	3.28	dd, 9.9, 7.1	$\theta_{13\text{-}12\beta}$(162), $\theta_{13\text{-}12\alpha}$(40)
15, 15′	1.55	m	
16	1.30	m, overlaps H-10α	
16′	1.17	m	
CH_3-9	1.55	s	
CH_3-17	0.86	t, 7.2	
CH_3-18	0.79	d, 6.6	
CH_3-19	0.92	s	

[a] Indicated dihedral angles are calculated with the Chem3D program (Cambridge Scientific Computing) for the Macintosh computer.
[b] Primed hydrogens are upfield of unprimed and have no stereochemical significance.

The pyran chair conformation places the C-18 methyl group (δ 0.80; d, 6.6 Hz) in an axial orientation. Note that H-10β shows only one large, geminal, coupling and therefore cannot have any *trans* diaxial couplings. The equatorially disposed pentyloxy side-chain and H-12β are indicated as in *cis* orientation from the highly shielded environment and the couplings observed for H-12β, namely a ddd signal: one doublet, *J*=12.8 Hz is merely a geminal coupling; the other doublets, *J*=10.3 and 7.1 Hz, are assigned to couplings with H-13 and H-11, respectively. The former assignment is secured by noting that the 10.3

Hz coupling is one of the couplings observed in the dd signal of H-13 and is consistent with the calculated $\theta_{12\beta\text{-}13}$ of 162°. The very unusual upfield position for H-12β (δ 0.75) is a consequence of shielding by the C-13 substituent; this significant Δδ cannot be the effect alone of the ether-oxygen lone-pair anisotropy since there is no similarly significant shielding effect observed on H-10β.

The *R* configuration of C-11, is chosen as probable, since that configuration is present in all pumiliotoxins whose structures have been proven either by X-Ray analysis[5] or synthesis.[7] Unfortunately, the complex multiplicity of the H-11 signal prevents extracting the exact couplings between H-11 and the vicinal protons.

The 2D ^{1}H-NMR spectra (normal and relay) revealed the positions of all the hydrogens and established all connectivities (*e.g.* C-10-11-12-13; C-8a-1-2-3; C-15-16-17). Chemical shifts, and where possible, their couplings are indicated in Table 2. In 2D spectra (relay 0 and 1), a weak long-range coupling between H-10α and H-5_{eq} and a much stronger one ("W"-type) between H-10α and H-5_{ax}, are observed; a weak cross-peak between H-10β and H-5_{ax} is also observed. These also support a fixed orientation of the H-10 hydrogens relative to the indolizidine ring. Strong four-bond couplings are seen between CH_3-18 and the H-10α, 10β and H-12α protons (H-12β and CH_3-18 practically overlap, so a cross-peak is not seen). Weak long-range couplings are also seen between CH_3-19 and H-13 and one or both H-15 protons. The relative stereochemistry at C-13 in **1** is shown as 13*S* (based on C-11 being in the R-configuration) with C-14 unassigned.

Certain microchemical procedures provided further relevant data. Hydrogenation did not affect **341A**. Acetylation in the presence of pyridine after 16 h at 58° gave a 90% conversion to a monoacetate with 10% recovered starting material. Little reaction was observed at 65-70° for 4.5 h, conditions that resulted in the complete acetylation of **251H** (**7**). The EI-MS spectrum indicated acetylation had occurred at the 7β-hydroxyl group since the *m/z* 254 ion is missing and is replaced by a fragment at *m/z* 296 (5 %). A two-day reaction at 70-75° did give a mixture of mono and diacetates; the second acetyl group is most probably located at the 14-hydroxyl group. Interestingly, both **5** and **6** easily formed triacetates indicating the 7-hydroxyl group in those compounds reacts in either the α or β configuration. Treatment of **341A** with butylboronic acid gave no reaction indicating that no *cis*-diol grouping was present. Under these conditions, synthetic **6**, gave a *bis*-butylboronate.[8]

Alkaloid **341A** occurs in frog skin extracts relatively rarely. Isolated from skin extracts from one population of the Ecuadoran *Epipedobates tricolor*, it has also been detected in certain Central American populations of *Dendrobates auratus, D. pumilio* and *D. granuliferus* and in certain populations of Colombian *D. lehmanni, Minyobates minutus and M. viridis*.[1] Alkaloid **341B**, detected along with **341A** in skin extracts from Colombian *D. lehmanni* and certain populations of Costa Rican *D. pumilio* (ref. 1

and unpublished results), appears to be a diastereomer of **341A**. The mass spectrum is somewhat different from that of **341A**.[2] Alkaloid **357**, detected in *Epipedobates tricolor*, is evidently a hydroxy analog of **341A**.

EXPERIMENTAL

Instrumentation: A Hewlett-Packard model 5890 gas chromatograph having a 25 m × 0.32 mm i.d. HP-5 fused silica-bonded capillary column programmed from 100° to 280° at the rate of 10°/ min, interfaced with a Hewlett-Packard model 5971 Mass Selective Detector and a Hewlett-Packard model 5965B IR instrument with a narrow band (4000-750 cm^{-1}) detector and a Hewlett-Packard ChemStation (DOS based) were used to generate the chromatograms, EI-MS, and FTIR spectra of **341A** (instrument A). Modified EI-MS (ion trap) of GC peaks were generated with a Finnigan Model 800 Ion Trap Mass Detector interfaced with a Varian Model 3400 gas chromatograph fitted with a 30 m × 0.32 mm i.d. RTX-5 (Restek) fused silica-bonded column and using the same temperature program as above (instrument B). A Finnigan 4500 mass spectrometer with a 25 m × 0.25 mm i.d. OV-17 fused silica-bonded column (Supelco) with a 60°-280° program (10°/min or 5°/min) and an INCOS data system was also used for EI-MS (instrument C). HRMS were measured with a JEOL SX 102 instrument fitted with a 15 m × 0.20 mm i.d. HP-5 column. All HRMS measurements were within ± 5.6 ppm except that for the *m/z* 298 ion. Chemical ionization used the ion-trap instrument and NH_3 or ND_3 reagent gases. The Finnigan 4500 was used with a D_2O bleed to obtain deuterium-exchanged EI-MS. The 1D- or 2D-(COSY) ^{1}H-NMR spectra in D_2O or C_6D_6 were measured with either a Varian XL-300 or a Varian VXR-500S spectrometer. Chemical shifts (δ, ppm) in D_2O are referred to HOD at 4.78; those in C_6D_6 to TMS at 0.0 ppm.

Isolation of **341A**: The usual acid/base partitioning procedure of methanolic extracts of skins of 750 *Epipedobates tricolor* frogs collected 16 km west of Santa Isabel, Azuay province in southwestern Ecuador[9] yielded 60 mg of an alkaloid mixture.[5] The alkaloid mixture was chromatographed on a Merck prepacked silica gel 60 (1.0 x 24 cm) column with 500 mL of $CHCl_3$:CH_3OH:6 N NH_3 (800:10:0.1) followed by 1.0 L of 100:10:0.2 and collecting 5 mL fractions. Compound **341A** (*ca.* 9 mg) appeared in fractions 13-17, but these fractions also contained substantial amounts of pumiliotoxin **251D** and minor or trace amounts of other alkaloids, including quinolizidine **207I**, indolizidine **207A**, deoxypumiliotoxin **251H** (2 isomers), pumiliotoxins **265G**, **267C** (2 isomers) and **307A**, allopumiliotoxin **323B** and epibatidines **208/210** and **308/310**, as well as minor amounts of pumiliotoxin **357**, evidently a hydroxy congener of **341A** (EI-MS data below). The fractions containing **341A** were concentrated, dissolved in

methanol and purified using reverse phase HPLC with a C-18 column and a flow rate of 1 mL/min and the solvent system HOAc:CH_3CN:H_2O. After isocratic conditions (2:6:92) for the first 5 min, a gradient from 2:6:92 to 2:68:30 over 20 min was employed. Thirty fractions (1 mL each) were collected and analyzed by GC-MS after evaporation and redissolution in methanol. Additional purification of **341A**-containing fractions was performed with an Asahipack ODP-50 column (available from Hewlett-Packard; 4.6 mm x 25 cm) and the solvent system CH_3CN:H_2O with a gradient of 10:90 to 90:10 over 30 min and a flow rate of 0.5 mL/min. Thirty fractions (0.5 mL each) were collected with pure **341A** appearing in fractions 10 and 11 (*ca.* 1 mg total as an oil).

Characterization of **341A**:

EI-MS: Instrument A: 341(6), 323(10, probably a thermal loss of H_2O), 306(2), 298(16), 266(9), 254(19), 236(3), 198(3), 184(7), 182(6), 180(5), 166(3), 164(3), 126(16), 125(15), 114(28), 112(100), 97(14), 96(11), 87(13), 84(61), 70(98), 55(18). Instrument B (Ion trap pseudo EI-MS): 342(14), 323(7), 306(4), 298(5), 254(7), 184(5), 182(3), 126(15), 125(15), 114(23), 112(40), 84(79), 70(100). Instrument C: 341(8), 323(8), 306(1), 298(10), 266(7), 254(13), 184(8), 182(8), 180(4), 126(12), 125(8), 114(16), 112(76), 97(8), 87(14), 84(50), 82(30), 70(100). The first EI-MS of **341A** was obtained with an LKB 300 spectrometer with a 1.5% OV-1 packed column (10). The EI-MS was as follows: 341(4), 324(3), 323(1), 306(1), 298(3), 266(4), 254(7), 114(10), 112(60), 84(42), 70(100).

IR: (see Figure 1) 3569(23), 3536 (13), 2965(100), 2943(77), 2883(49), 2818(33), 1459(22), 1379(33), 1313(27), 1300(24), 1226(17), 1171(40), 1070(65), 821(12) cm^{-1}.

Optical rotation was not measured.

Mono-O-acetate of **341A**; *EI-MS*: M^+ not detected, 340(3), 324(4), 306(3), 296(5), 280(4), 266(3), 236(3), 217(3), 166(3), 126(5), 102(7), 83(100), 70(34), 55(10).

Characterization of **357**:

EI-MS: 357(6), 339(6), 322(2), 314(8), 270(4), 184(6), 182(6), 128(12), 126(8), 125(8), 114(15), 110(74), 100(10), 96(8), 95(8), 87(16), 84(48), 83(28), 70(100), 55(16).

IR: 3684(5), 3657(5), 3575(28), 3536(13), 2972(100), 2942(83), 2885(50), 2816(42), 1458(25), 1380(42), 1307(35), 1225(28), 1173(52), 1071(87), 951(27), 824(13) cm^{-1}.

ACKNOWLEDGMENT

We thank Drs. L. K. Pannell and H. J. C. Yeh (NIH) for HRMS and the 2D ^{1}H-NMR data, respectively.

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Received, 17th August, 1998