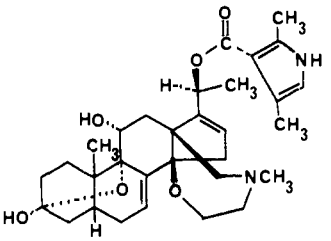
A New Class of Indolizidine Alkaloids from the Poison Frog, *Dendrobates tricolor*. X-ray Analysis of 8-Hydroxy-8-methyl-6-(2′-methylhexylidene)-1-azabicyclo[4.3.0]nonane

J. W. Daly,*[1a] T. Tokuyama,[1b] T. Fujiwara,[1b] R. J. Highet,[1c] and I. L. Karle[1d]

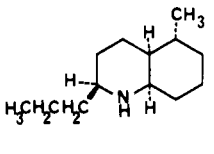
Contribution from the National Institutes of Health, Bethesda, Maryland 20205, Osaka City University, Osaka, Japan, and the Naval Research Laboratory, Washington, D.C. 20390. *Received May 16, 1979*

Abstract: The structure and absolute configuration of a unique alkaloid isolated from an Ecuadoran frog, *Dendrobates tricolor*, have been elucidated by X-ray crystallography and found to be 8-hydroxy-8-methyl-6-(2′-methylhexylidene)-1-azabicyclo[4.3.0]nonane. This indolizidine alkaloid ($C_{16}H_{29}NO$) represents the first structurally defined member of the pumiliotoxin A class of dendrobatid alkaloids. Analysis of mass spectra and proton and carbon-13 magnetic resonance spectra allows the formulation of structures of six further members of this class of alkaloids, including pumiliotoxin A ($C_{19}H_{33}NO_2$) and pumiliotoxin B ($C_{19}H_{33}NO_3$). An allo series of alkaloids in the pumiliotoxin A class is proposed to contain a 7-hydroxy substituent. The pumiliotoxin A class represents the fifth class of pharmacologically active and structurally unique alkaloids to be isolated from defensive skin secretions of Neotropical poison frogs (Dendrobatidae).

Neotropical frogs of the family Dendrobatidae have elaborated a remarkable array of more than 100 alkaloids, none of which have been detected elsewhere in nature (for a recent survey see ref 2). Such alkaloids in skin serve these brightly colored frogs in a passive "chemical defense" against predators. Virtually all of the alkaloids possess high pharmacological activity on nerve and muscle.[3-5] Structures for four classes of dendrobatid alkaloids have been elucidated. The batrachotoxins proved to be complex steroidal alkaloids (see I).[6] All of the other dendrobatid alkaloids that have been structurally defined contained a piperidine moiety. These are the pumiliotoxin C class (disubstituted *cis*-decahydroquinolines; see II),[7,8] the histrionicotoxins (8-hydroxy-1-azaspiro[5.5]-undecanes; see III),[8,9] and gephyrotoxins (perhydropyrrolo[1,2-*a*]quinolines; see IV).[8] One major class of dendrobatid alkaloid remained undeciphered, the pumiliotoxin A class comprising some 24 alkaloids.[2]



I



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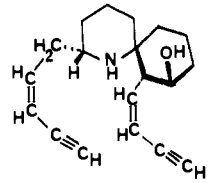

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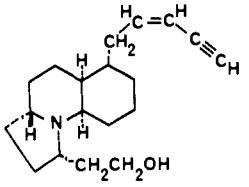

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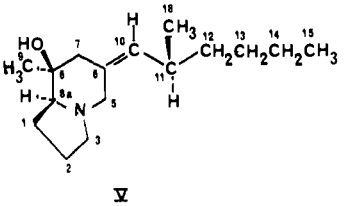

V

A relatively simple member of the pumiliotixin A class has now been isolated from an Ecuadoran frog, *Dendrobates tricolor*. Crystallization and X-ray analysis of this compound (V, *251D*)[10] provide not only its structure but the key to the pumiliotoxin A class of dendrobatid alkaloids. The compound contains an indolizidine moiety in common with gephyrotoxin (IV), but is in certain respects strikingly different from the other classes of dendrobatid alkaloids.

Results and Discussion

Isolation of Alkaloids from *Dendrobates tricolor*. Methanolic extracts from skins of 750 *Dendrobates tricolor* provided 80 mg of alkaloids (see the Experimental Section). Mass spectral-gas chromatographic analysis of the alkaloid fraction indicated the presence of one major alkaloid (V, *251D*), with a molecular weight of 251 and an empirical formula established by high-resolution mass spectrometry as $C_{16}H_{29}NO$. Four minor alkaloids and a number of trace alkaloids were also present. Column chromatography of 60 mg of the alkaloid mixture on silica gel afforded 21 mg of pure V, which was characterized spectrally and after crystallization of the hydrochloride salt by X-ray crystallography.

8-Hydroxy-8-methyl-6-(2′-methylhexylidene)-1-azabicyclo[4.3.0]nonane (V, *251D*[10]). X-ray analysis of a single crystal of the hydrochloride salt of this alkaloid established the molecular structure, absolute configuration, and conformation. The substance crystallizes in space group $P2_1$ with cell dimensions a = 12.079 (4) Å, b = 6.937 (2) Å, c = 10.702 (3) Å, β = 99.54 (3)°, V = 884.3 Å^3, Z = 2, mol wt (with HCl) 287.9, and a calculated density of 1.081 g/cm^3. Intensity data were collected on a needle crystal of dimensions 0.10 × 1.0 × 0.12 mm with Cu Kα radiation (λ 1.5418 Å) on an automatic four-circle diffractometer with a scan of 2.0° + $2\theta(\alpha_2)$ − $2\theta(\alpha_1)$, at a speed of 2°/min, to a maximum scattering angle of 2θ = 120° for a total of 1440 independent reflections. The background was read for 10 s at either end of the scan, and three reflections were monitored at intervals after every 50 scans. The x and z coordinates of the Cl^- ion were determined from a Patterson function computed with ($|E_h|^2 - 1$) coefficients rather than $|F_h|^2$. Phases based on the Cl^- ion were refined and extended with the tangent formula.[11] The E map[12] based on these phases contained peaks for the molecule and its mirror image near the plane at y = 3/4. Several atomic sites

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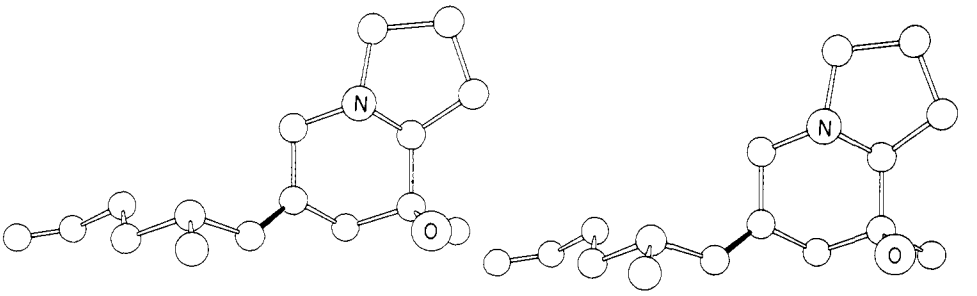


Figure 1. Stereodiagram of the absolute configuration of 8-hydroxy-8-methyl-6-(2′-methylhexylidene)indolizidine, drawn from experimentally determined coordinates by X-ray diffraction. The dark bond indicates a double bond.

Table I. Fractional Coordinates

atom	*x*	*y*	*z*
C(1)	−0.0360 (4)	0.7584 (12)	0.1071 (5)
C(2)	−0.1227 (5)	0.7379 (14)	0.1949 (6)
C(3)	−0.0589 (4)	0.7210 (12)	0.3290 (5)
N(4)	0.0587 (3)	0.7848 (6)	0.3169 (4)
C(5)	0.1492 (4)	0.7270 (11)	0.4235 (4)
C(6)	0.2600 (4)	0.7940 (9)	0.3871 (5)
C(7)	0.2796 (4)	0.7149 (13)	0.2610 (5)
C(8)	0.1853 (4)	0.7793 (10)	0.1538 (5)
C(8a)	0.0752 (4)	0.7039 (11)	0.1895 (5)
C(9)	0.2031 (5)	0.6840 (15)	0.0309 (6)
C(10)	0.3305 (5)	0.9160 (10)	0.4564 (6)
C(11)	0.3209 (5)	1.0176 (12)	0.5768 (6)
C(12)	0.4171 (7)	0.9564 (18)	0.6857 (8)
C(13)	0.4089 (8)	0.7486 (16)	0.7209 (8)
C(14)	0.5092 (15)	0.6696 (33)	0.8189 (11)
C(15)	0.5522 (16)	0.7414 (41)	0.9207 (15)
C(18)	0.3269 (6)	1.2275 (18)	0.5569 (8)
O	0.1833 (3)	0.9797 (7)	0.1351 (4)
Cl	0.0422 (1)	0.2191[a]	0.3167 (1)

[a] This parameter was held constant during the least-squares refinement in order to fix the origin in the *y* direction in space group $P2_1$.

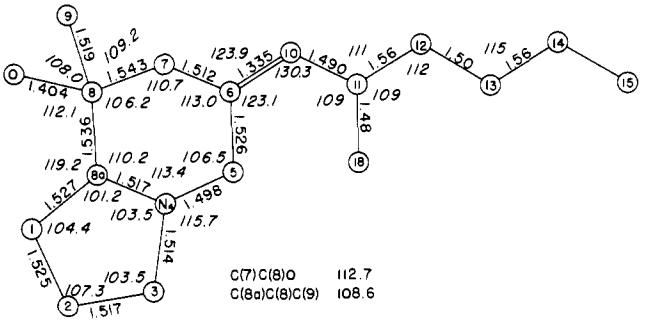


Figure 2. Bond lengths and angles. Standard deviations associated with atoms C(1) to C(11) and O are near 0.008 Å for lengths and 0.5° for angles. For atoms C(12), C(13), and C(16), the standard deviations increase to near 0.013 Å for lengths and 0.7° for angles. The position of atom C(15) is sufficiently uncertain that no value is given for the C(14)–C(15) distance and the C(13)C(14)C(15) angle.

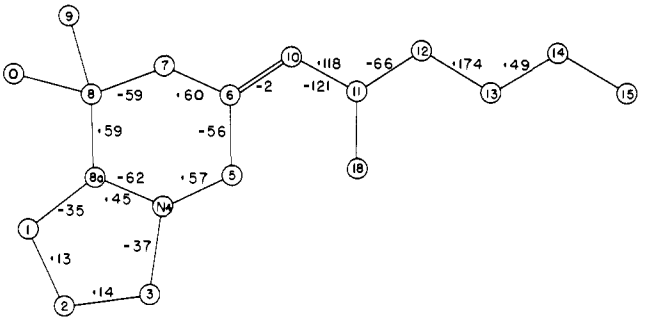


Figure 3. Torsional angles.

were selected that represented atoms in one optical antipode. Phase values based on these atomic sites plus that of the Cl^- ion were extended, but not refined, by the tangent formula. The resulting *E* map showed unambiguously 16 of the 18 atoms of one arbitrarily chosen optical antipode. The remaining two carbon atoms as well as most of the hydrogen atoms were found in difference maps, and the substance was shown to be 8-hydroxy-8-methyl-6-(2′-methylhexylidene)indolizidine (V).

The absolute configuration is indicated by the anomalous scattering of the Cl^- ion by Cu Kα radiation.[13] The value for the agreement factor $R = \Sigma||F_o| - |F_c||/\Sigma|F_o|$, where $|F_o|$ are the 1404 experimentally observed values greater than 0 and $|F_c|$ are the calculated structure factors after least-squares refinement when the anomalous scattering of the Cl^- ion is taken into consideration, is 6.5% for the configuration shown in this paper and 6.7% for the other antipode. The difference in the values of the *R* factors indicates the configuration shown in Figure 1 as the absolute configuration at a 99.5% confidence level.[14] Coordinates for this configuration are listed in Table I. The conformation of the piperidyl ring and the configuration at C(8a) are the same as those found in pumiliotoxin C (II) and opposite to that in gephyrotoxin (IV).[8]

Bond lengths and angles, shown in Figure 2, and torsional angles, shown in Figure 3, are within expected values for the indolizidine ring and the adjacent atoms. The six-membered ring is in the chair conformation, while the five-membered ring assumes the half-chair conformation with $\Delta = 2.5°$.[15] Low thermal vibration parameters are associated with atoms C(1) to C(11), N(4), and O. Positional disorder, as evidenced by the size of the experimentally determined thermal parameters for each atom, increases along the 2′-methylhexylidene side chain to such an extent that the values for the bond length for C(14)–C(15) and the bond angle C(13)C(14)C(15) cannot be measured with any reliability. The elongated shape of the anisotropic thermal ellipsoid for C(14) indicates a fair amount of oscillation about the C(12)–C(13) bond. Additional oscillation about the C(13)–C(14) bond contributes to a very large and extremely elongated ellipsoid for C(15) that cannot approximate the average position of C(15). However, the mass spectrum of the compound, including data taken from the crystal used for collecting the X-ray data, shows clearly that the compound does have a saturated terminal $-CH_2CH_2CH_2CH_3$ group.

The 2′-methylhexylidene side chain is not completely extended. After the double bond, which is cis with respect to C(5), the chain folds back at C(11). The only fully extended segment is the sequence C(18)C(11)C(12)C(13)C(14), where C(18) is that of the methyl group. The C(11)–C(18) bond is parallel to the axial C(8)–O bond. The terminal carbon of the hexylidene chain assumes a gauche conformation rather than an anti conformation.

Figure 4 illustrates the packing of the molecules in the

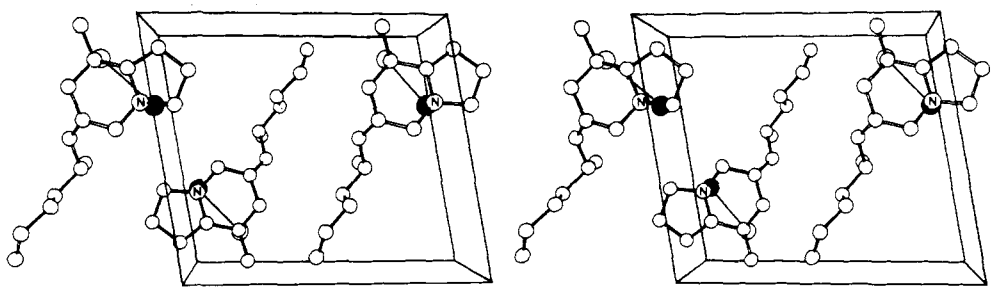


Figure 4. Stereodiagram of the packing in the crystal of the hydrochloride salt. The Cl^- ions are indicated by the darkened circles and the NH···Cl^- and OH···Cl^- hydrogen bonds are indicated by light lines. The axial directions are $a\rightarrow$, $c\uparrow$, and b directed into the plane of the page.

Table II. Carbon-13 Chemical Shifts (ppm)[a]

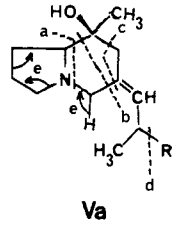
	V *251D*	IX pumiliotoxin A	X pumiliotoxin B
C-1	23.4a	23.2a	23.2a
C-2	21.2a′	21.2a′	21.2a′
C-3	53.3b	53.2b	53.3b
C-5	54.7b′	54.5b′	54.6b′
C-6	130.0	130.0	130.6
C-7	49.0	48.8	48.9
C-8	68.4	68.4	68.5
C-8a	71.8	71.7	71.7
C-9	24.3	24.4	24.4
C-10	134.7	134.2	133.7
C-11	32.1	32.6	32.5
C-12	37.6	35.5	35.6
C-13	29.8	134.2	127.4
C-14	22.9	124.8	135.3
C-15	14.2	79.4	82.9
C-16		27.8	68.9
C-17		10.2	12.2
C-18	21.8	21.1	21.4
C-19		21.1	19.1

[a] Assignments designated a and a′ or b and b′ are tentative and may be interchanged within each column.

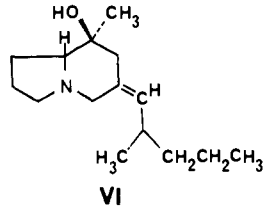
crystal. Each Cl^- ion is associated with a particular molecule by participating in two hydrogen bonds, NH^+···Cl^- and OH···Cl^-, where the N···Cl^- and O···Cl^- separations are 3.02 and 3.25 Å, respectively. The hydrogen bonding scheme is unusual in that there are no hydrogen-bonded links between molecules. Intermolecular contacts between molecules related by screw axes at $x = 0$ have normal van der Waals' values, e.g., C(3)···C(16′) at 3.64 Å and C(3)···C(11′) at 3.74 Å. However, between molecules related by screw axes at $y = 1/2$, all intermolecular attractions are very weak. The closest approaches between molecules in the region near $x = 1/2$ are C(15)···O′ at 3.81 Å, C(14)···O′ at 3.90 Å, C(7)···C(12′) at 4.03 Å, and C(10)···C(14′) at 4.17 Å. All other contacts are greater than 4.2 Å. The positional disorders, especially for C(14) and C(15), can be correlated with the loose intermolecular contacts in the crystal.[16]

The mass spectra of V (see the Experimental Section) can be rationalized with the structure revealed by X-ray crystallography. The base peak at *m/e* 70 (C_4H_8N), which has been the basis of assignment of dendrobatid alkaloids to the pumiliotoxin A class,[2] is accounted for by cleavage a (see Va, R = C_4H_9). In the dihydro derivative this cleavage is still a major pathway, although cleavage b to yield *m/e* 84 ($C_5H_{10}N$) and cleavage presumably via c followed by loss of H_2O to yield *m/e* 110 ($C_7H_{12}N$) now become major pathways (see ref 2). The origin of the major fragment at *m/e* 166 ($C_{10}H_{16}NO$) in V would appear most likely to result from cleavage d (loss of C_4H_9) to yield the major peak at *m/e* 194 ($C_{12}H_{20}NO$), followed by cleavage e (loss of C_2H_4) to yield triene stabilization of the positive charge. As expected, this pathway is not seen in the dihydro derivative.

Analysis of mass spectra indicates that many of the compounds of the pumiliotoxin A class are closely related in structure to V (see ref 2). For example, the major peaks at *m/e* 194 ($C_{12}H_{20}NO$), 166 ($C_{10}H_{16}NO$), and 70 (C_4H_8N) are present in the mass spectra of these alkaloids. After perhydrogenation, peaks at 110, 84, and 70 predominate, as in the dihydro derivative of V. In these alkaloids the structures therefore differ from V only in the nature of the substituent R (see Va). For example, an alkaloid (*237A*[10]) with an empirical formula of $C_{15}H_{27}NO$ (see ref 2) differs from V in having a $-C_3H_7$ rather than a $-C_4H_7$ R substituent. If it is assumed that the $-C_3H_7$ is a straight chain, then the structure is as shown in VI.



Va



VI

The main features of the proton spectrum of V (Figure 5) can be readily accommodated to the structure revealed by X-ray crystallography. The doublet at low field (5.07, $J = 10$ Hz) is that of H-10, coupled with H-11 (2.37). The methyl doublet at high field (0.98, $J = 7$ Hz) is that of H-18 coupled with H-11. The doublet at 3.82 ($J = 12$ Hz) is H-8a, coupled with H-1″. The chemical shifts of H-11 and H-1″ coincide fortuitously at 2.36–7, with the result that irradiation at 2.36 reduces the signals at 5.07, 3.82, and 0.98 to singlets. The multiplet (dd?, $J = 10$ and 4 Hz) at 3.09 is probably that of H-3α and simplifies to a doublet of $J = 10$ Hz by irradiation at 1.73 (H-2). Irradiation at 2.24 (H-3β) simplifies the multiplet at 3.09 to a broad singlet (t?). The broad singlet at 2.16 is apparently that of H-7. The methyl at 1.16 and triplet at 0.89 are readily assigned to H-9 and H-15, respectively. The remaining methylene hydrogens, H-12, H-13, and H-14, are an unresolved multiplet at 1.1–1.3. A methanolic solution of the hydrochloride of V shows H-10 shifted to 5.31 and H-8a to 4.36. Double irradiation shows H-1″ to be at 3.37, a surprising shift of 1.0 ppm. H-11 is little changed at 2.41, confirming the fortuitous character of the coincidence of chemical shifts noted in neutral solution.

In the carbon-13 spectrum (Table II), the peaks appearing as singlets in the off-resonance spectrum are readily assigned to C-6 (130.0 ppm) and C-8 (68.4). Among the doublets, that at 134.7 is the olefinic C-10 and that at 71.8 is clearly C-8a; the very low field of this signal apparently results from the geminal substitution at C-8. The remaining doublet, at 32.1 ppm, must be C-11. Assignment of the triplets is aided by the observation that peaks at 37.6, 29.8, and 22.9 are approximately those observed of C-4, -5, and -6 of 3-methylheptane

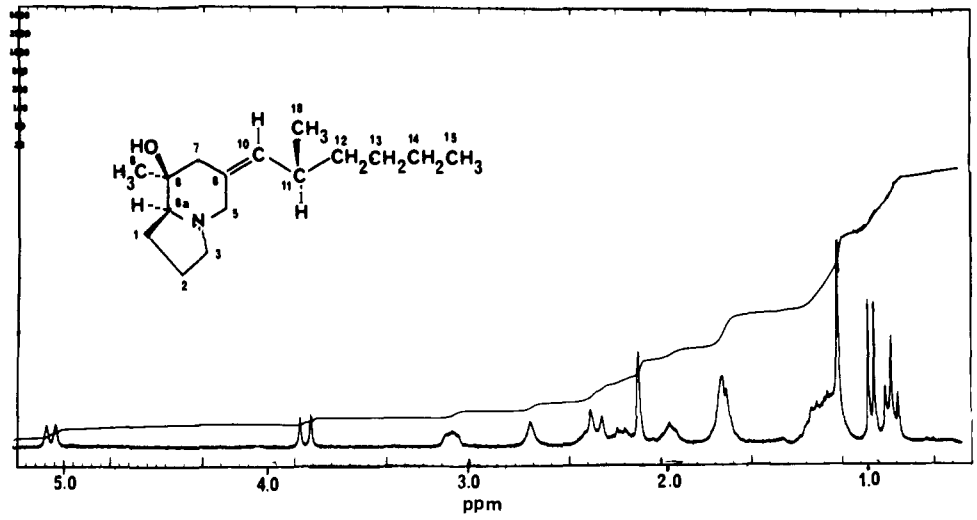
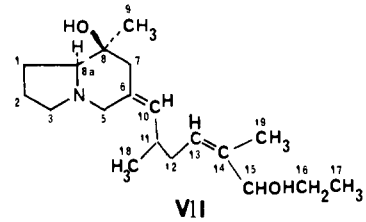


Figure 5. Proton magnetic resonance spectrum of 8-hydroxy-8-methyl-6-(2′-methylhexylidene)indolizidine (V, *251D*). Spectrum in deuteriochloroform at 220 MHz.

(36.8, 29.3, and 23.0). That at 49.0 responds to irradiation of H at 2.16 ppm and must be C-7. The two triplets near 54 ppm, actually observed as doubled doublets at superconducting field (68 kG), correspond to C-3 and C-5, but differentiation is uncertain; irradiation at H = 3.09 produces more response from the signal at 53.3, which is therefore assigned to C-3, leaving that at 54.7 for C-5. The two remaining triplets (23.4, 21.2) have chemical shifts suitable for C-1 and C-2; that at lower field (23.4) is assigned to the carbon with more β substituents, C-1. Three signals appear as clear quartets in the off-resonance spectrum, corresponding to the three methyls, C-9, C-15, and C-18. That at 24.3 responds to irradiation of H at 1.16 ppm and must be C-9, that at 14.2 is approximately that of C-7 of 3-methylheptane and is therefore C-15, leaving that at 21.8 to be C-18.

The structure of V provides an explanation of some of its chemical properties. Thus, the OH function is highly hindered and exists in a gauche configuration with *three* ring atoms, C-1, C-6, and N-4, analogous to the configuration of the unreactive 12-OH of cholic acid. The hindered nature of the OH function is reflected in the difficulty in forming *O*-acetyl or *O*-trimethylsilyl derivatives (see the Experimental Section) and in the fact that the V has a relatively high R_f (0.52) on thin-layer silica gel chromatoplates with $HCCl_3$-MeOH (9:1). The dihydro derivative has a much lower R_f value (0.26) and is somewhat more readily converted to *O*-acetyl and *O*-trimethylsilyl derivatives. Hydrogenation of V produced no more profound change than saturation of the double bond, for the C-13 spectrum of the dihydro derivative closely resembles that of V. The greater reactivity of the hydroxyl may result from a more accessible equatorial conformation, for the anticipated reduction of the double bond on the less hindered side would lead to the dihydro derivative with the 8-methyl axial and the 8-hydroxyl and 6-hexyl groups equatorial.

Pumiliotoxin A (VII, *307A*[10]). Analysis of mass spectra (see ref 2) indicates that pumiliotoxin A differs from V only in the nature of the substituent R (see Va). This substituent appeared to be $-CH_2CH{=}CCH_3CHOHCH_2CH_3$, although the position of the methyl group on the double bond could not be assigned based solely on mass spectral analysis. The structure pumiliotoxin A derived from both mass spectral and nuclear magnetic resonance spectral analyses (see below) is as shown in VII. A dihydro analogue of pumiliotoxin A (*309A*[10]) isolated from extracts of various dendrobatid frogs appears, from mass spectral data, to have a saturated R substituent, perhaps $-CH_2CH_2CHCH_3CHOHCH_2CH_3$.



VII

The structural relation of pumiliotoxin A to V inferred from mass spectral data is supported by the proton NMR spectrum (Figure 6), which is very similar to that of V. Irradiation at 2.38 ppm (H-11) simplifies the doublet at 5.07 ppm (H-10) and that at 0.99 (H-18). The doublet of H-8a appears at 3.76 and is simplified by irradiation at 2.32 (H-1). The singlets of H-7 and H-9 appear within 0.05 ppm of those observed in V. At 84 kG the two protons (H-7′ and -7″) appear as an AB system of 2.11 and 2.12 ppm, J = 14 Hz. The H-3α appears as in V as a multiplet at 3.10, the remaining protons of the indolizidine ring appearing in the complex from 1.2 to 2.7 ppm. The remaining signals in the proton spectrum of pumiliotoxin A provide adequate information to allow a nearly complete structural assignment. The appearance of a triplet at 5.30 (H-13) simplified by irradiation at 1.96 ppm (H-12) requires the moiety $CH_2CH{=}C-$. Both the one-proton triplet at 3.88 (H-15) and the three-proton triplet at 0.84 (H-17) are converted into singlets by irradiation at 1.53, to show the moiety $=CCH(OH)CH_2CH_3$. The remaining substituent on the double bond is revealed by the singlet at 1.55 ppm to be a methyl group (H-19), completing the side chain as shown in VII. The absence of coupling between the vinyl proton H-13 and the C-19 methyl group even at 84 kG suggests that the 13,14-double bond has the Z configuration. Further studies on model compounds will be necessary to confirm this tentative assignment. Nothing is known of the configuration of the OH group at C-15.

These structural assignments are supported by the carbon-13 spectrum of pumiliotoxin A (Table II). The existence of the indolizidine ring with unaltered substitution and stereochemistry is shown by the appearance of signals within 0.2 ppm of those of V for each ring carbon and for the methyl C-9; the alteration of the side chain has produced a change of 0.5 ppm in the signals of C-10 and C-11, and 0.7 ppm in C-18. The remaining peaks possess chemical shifts in accord with their structural situation, those of C-16 and C-17 (27.8 and 10.2 ppm) being quite similar to those observed in 2-methyl-1-penten-3-ol (C-4, 27.7; C-5, 9.7).

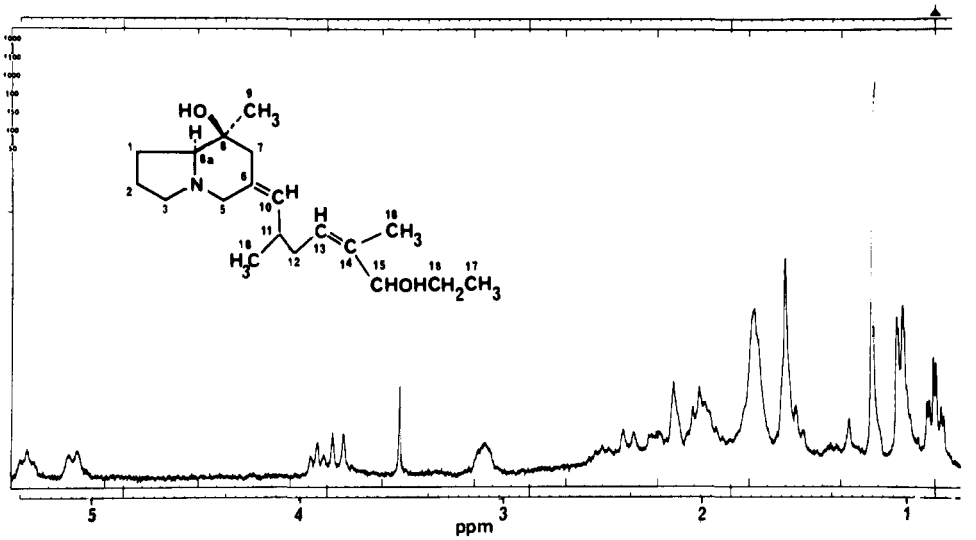


Figure 6. Proton magnetic resonance spectrum of pumiliotoxin A. Spectrum in deuteriochloroform at 220 MHz.

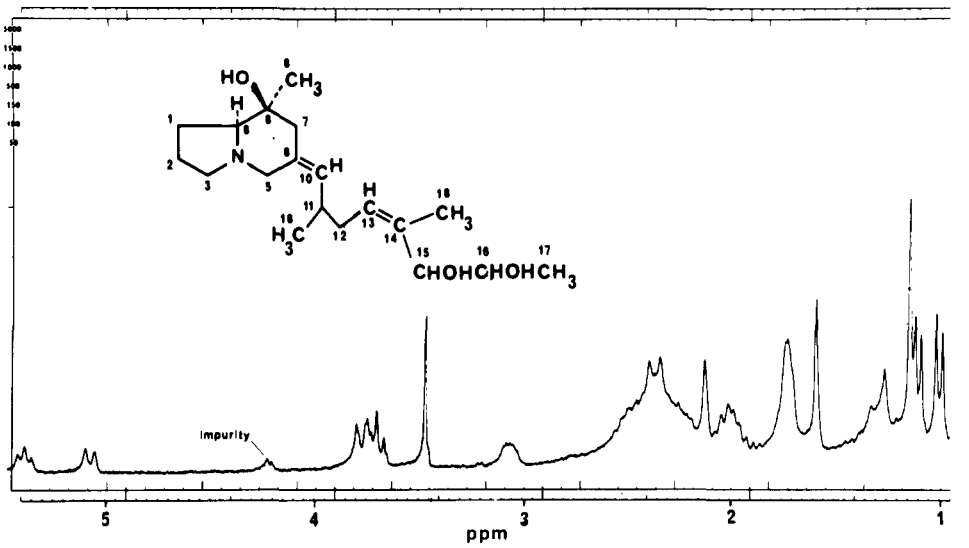
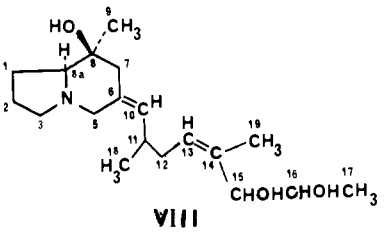


Figure 7. Proton magnetic resonance spectra of pumiliotoxin B. Spectrum in deuteriochloroform at 220 MHz.

Pumiliotoxin B (VIII, *323A*[10]). The mass spectrum (see ref 2) indicates that pumiliotoxin B differs from pumiliotoxin A only in the presence of an additional hydroxyl group in the R substituent (see Va). The structure of pumiliotoxin B derived from mass spectral and nuclear magnetic resonance spectral analyses (see below) is as shown in VIII.



VIII

The proton spectrum of pumiliotoxin B (Figure 7) confirms the close relation of this material to V and pumiliotoxin A. Peaks for the discrete signals of H-7, -8a, -9, -10, -13, -18, and -19 show similar chemical shifts and respond similarly to double irradiation experiments. The protons of the terminal carbon C-17 now appear as a doublet shifted downfield by 0.25 ppm. It is clear that the added oxygen is situated at C-16, also producing a slight shift at C-15 (0.18 ppm). The *Z* configuration of the 13,14-double bond is a tentative assignment and requires further investigation. Nothing is known of the configurations of the hydroxyls at C-15 and C-16. Boronate and dimethylsilanate derivatives are readily formed (data not presented).

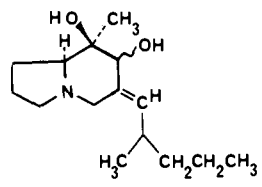
The carbon-13 spectrum of pumiliotoxin B (Table II) again confirms the retention of the unaltered indolizidine system, peaks appearing within 0.2 ppm of those of C-1 to C-12 with the exception of those of the olefin, which are shifted slightly more (0.5 and 0.6 ppm). Addition of the oxygen to C-16 has substantially altered the shift of the δ-13 olefin. C-15 and C-17 show suitable β effects, and C-19 a reasonable γ effect.

Allo Series of Pumiliotoxin A Class Alkaloids. Certain compounds of the pumiliotoxin A class, an allo series, have a major peak at *m/e* 182 ($C_{10}H_{16}NO_2$) in the mass spectrum rather than that at *m/e* 166 ($C_{10}H_{16}NO$) (see ref 2). Thus, an additional OH moiety appears to be present in the indolizidine ring system. The lack of a significant loss of CH_2OH in the allo series of alkaloids rules out the presence of the hydroxy moiety at the 9 (methyl) position. Since the additional OH moiety in the indolizidine ring system can be readily acetylated, it is a secondary alcohol (see ref 2). Its position is defined by comparison of mass spectra of dihydro compounds of the allo series to the mass spectrum of the dihydro derivative of V. All dihydro derivatives of the allo series have major peaks at *m/e* 70 (*m/e* C_4H_8N). Thus, the OH moiety cannot be in the five-

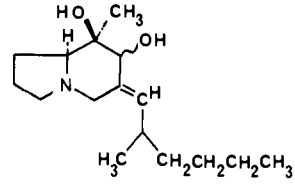
membered ring (Va, cleavage a). All dihydro derivatives have major peaks at 84 (*m/e* $C_5H_{10}N$). Thus, the OH moiety cannot be at position 5 (Va, cleavage b). This leaves only position 7, which is compatible with the major peak in the dihydro derivatives at *m/e* 110 ($C_7H_{12}N$) (Va, cleavage c, followed by loss of H_2O). Certain allo compounds (*253*, *267A*, *297A*[10]) show major 7,8-glycol cleavage peaks at *m/e* 112 ($C_6H_{10}NO$) and 114 ($C_6H_{12}NO$) (see ref 2).

The structures of three members of the allo series of pumiliotoxin A class alkaloids can be postulated based on mass spectral data. An alkaloid (*253*[10]) with a molecular weight of 253 and an empirical formula of $C_{15}H_{27}NO_2$ (see ref 2) apparently differs from VI only in having the 7-hydroxy typical of the allo series. The structure is shown in IX with the stereochemistry of the 7-hydroxy undefined. Another dendrobatid alkaloid of the allo series with a molecular weight of 267 and empirical formula of $C_{16}H_2NO_2$ (*267A*, see ref 2) appears to be the 7-hydroxy analogue of V and would have the structure shown in X. Finally, allopumiliotoxin B (*323B*[10]) is a member of the allo series, which, based on mass spectral data (see ref 2), probably represents the 7-hydroxy analogue of pumiliotoxin A as shown in XI.

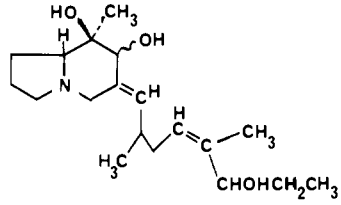
Further nuclear magnetic resonance studies will be required before the structures for the remaining alkaloids of the pumiliotoxin A class can be formulated. Such studies will also be necessary to confirm the postulated structures VI, IX, X, and XI.



IX



X



XI

Pharmacological Aspects. Initial studies indicated that pumiliotoxin A and B were relatively toxic substances, comparable in potency to strychnine.[4a] It was not until quite recently that the extremely potent cardiotonic and myotonic activities of pumiliotoxin B were recognized.[4b,c] Such activity, probably involving calcium-dependent mechanisms, makes this class of dendrobatid alkaloids invaluable research tools with potential for clinical development. In view of the limited supplies that will be available from nature, synthesis of such alkaloids deserves a high priority.

Experimental Section

High-resolution mass spectral data were obtained on AEI MS-30, VG Micromass 7070F, or JEOL 01SG-2 mass spectrometers. Nuclear magnetic resonance spectra were obtained on approximately 0.1 M deuteriochloroform solutions. Proton spectra were determined with Varian HR-220 and Bruker 360-MHz spectrometers, and the chemical shifts are expressed in parts per million in the text and figures. Carbon-13 spectra chemical shifts and single-proton decoupled spectra were obtained on a JEOL FX60 using a 16 K Fourier transform and 4 kHz spectral range for a digital resolution of 0.5 Hz (0.03 ppm). Typically, 50 000 free induction decays from a 30° pulse were collected at 1-s intervals. Multiplicities consistent with the assignments were observed on off-resonance spectra determined at 68 MHz on a Nicolet-Bruker hybrid spectrometer using similar collection parameters.

Isolation of Alkaloids. Skins from 750 *Dendrobates tricolor* from southwestern Ecuador[17] were minced and extracted with trituration three times with methanol (total volume 1.5 L). After concentration of the methanol extract in vacuo to 500 mL and dilution with an equal volume of water, alkaloids were extracted three times into equal volumes of chloroform. The chloroform-soluble alkaloids were then extracted four times into one-half volumes of 0.1 N HCl. The combined 0.1 N HCl solutions were adjusted to pH 9 with 1 N aqueous ammonia, followed by extraction of alkaloids three times into equal volumes of chloroform. The combined chloroform solutions were dried over sodium sulfate and concentrated in vacuo to dryness to yield 80 mg of crude alkaloids. Mass spectral–gas chromatographic analyses on a Finnigan 1015 mass spectrometer with methane as a carrier gas indicated the presence of one major (mol wt 251), three minor (mol wt 217, 341, 323), and a number of trace alkaloids. High-resolution mass spectral–gas chromatographic analyses indicated the following empirical formulas: $C_{16}H_{29}NO$ (*251D*[10]); $C_{15}H_{23}N$ (*217*[18]); $C_{19}H_{35}NO_4$ (*341A*[10]); and $C_{19}H_{33}NO_3$ (*323B*,[10] allopumiliotoxin B). Gas chromatography was on a 1.5% OV-1 on Chromosorb G AW-DMCS (80–100 mesh, Applied Science Laboratories, State College, Pa.) with programming at 10 °C per min from 150 to 280 °C. Further details on analysis of dendrobatid alkaloids by combined mass spectral–gas chromatography are in ref 2.

Sixty milligrams of alkaloids from *Dendrobates tricolor* was dissolved in 0.5 mL of chloroform and applied to a prepacked silica gel 60 column (Merck 1.0 × 24 cm) and eluted with 500 mL of chloroform–methanol–aqueous 6 N ammonia (800:10:0.1) and then with 1 L of chloroform–methanol–aqueous ammonia (1000:100:0.2). Five-milliliter fractions were collected. Twenty-one milligrams of pure V (*251D*[10]) was eluted in fractions 6–10. Two of the minor alkaloids (mol wt 217 ~3 mg and mol wt 341 ~9 mg) eluted only partially separated in fractions 13–18 while the other minor alkaloid eluted in fractions 113–118 (mol wt 323 ~13 mg). The latter alkaloid was significantly contaminated by trace alkaloids. Full details of gas, thin-layer, and column chromatographic analyses of extracts from *Dendrobates tricolor* will be published elsewhere.

The following mass spectral data pertain for V. Only ions of 4% of base peak or greater at 70 eV are reported. Empirical formulas are based on data accurate to within 10 ppm. Intensities are in parentheses and are relative to base peak equal to 100: *m/e* 251 ($C_{16}H_{29}NO$, 20), 250 ($C_{16}H_{28}NO$, 12), 236 ($C_{15}H_{26}NO$, 8), 234 ($C_{16}H_{28}N$, 4), 233 ($C_{16}H_{27}N$, 5), 218 ($C_{15}H_{24}N$, 4), 208 ($C_{14}H_{26}N$, 13), 206 ($C_{14}H_{24}N$, 9), 194 ($C_{12}H_{20}NO$, 20), 176 ($C_{12}H_{18}N$, 10), 166 ($C_{10}H_{16}NO$, 85), 150 ($C_{10}H_{16}N$, 18), 148 ($C_{10}H_{14}N$, 6), 112 ($C_6H_{10}NO$, 15), 84 ($C_5H_{10}N$, 6), 70 (C_4H_8N, 100).

The following proton chemical shifts (parts per million) pertain for V: H-1′, 1.73; H-1″, 2.36; H-2, 1.73; H-3α, 3.09; H-3β, 2.24; H-7, 2.16; H-8a, 3.82; H-9, H-13, H-14, ~1.16; H-10, 5.07; H-11, 2.37; H-15, 0.89; H-18, 0.98.

The extraction and isolation of alkaloids from skins of *Dendrobates pumilio*[17] from Isla Bastimentos, Panama, have been briefly described.[7] From 250 skins, 17 mg of pumiliotoxin A ($C_{19}H_{33}NO_2$), 20 mg of pumiliotoxin B ($C_{19}H_{33}NO_3$), and 16 mg of pumiliotoxin C ($C_{13}H_{25}N$) were isolated. Full details on the isolation of alkaloids from extracts of *Dendrobates pumilio* will be published elsewhere.

The following proton chemical shifts (parts per million) could be assigned to pumiliotoxin A (VII): H-1, 2.32; H-3α, 3.10; H-7, 2.11; H-8a, 3.76; H-9, 1.12; H-10, 5.07; H-11, 2.38; H-12, 1.96; H-13, 5.30; H-15, 3.88; H-16, 1.53; H-17, 0.84; H-18, 0.99; H-19, 1.55.

The following proton chemical shifts (parts per million) could be assigned to pumiliotoxin B (VIII): H-1, 2.36; H-3α, 3.05; H-7, 2.12; H-8a, 3.77; H-9, 1.13; H-10, 5.07; H-11, 2.47; H-13, 5.39; H-15, 3.69; H-16, 3.75; H-17, 1.09; H-18, 0.99; H-19, 1.58.

Reduction of alkaloids was with 3 atm of hydrogen with 10% palladium on charcoal catalyst in methanol for 16 h. A quantitative conversion of V to a dihydro derivative pertained based on mass spectral–gas chromatographic analyses. Pumiliotoxin A and B were converted to a mixture of dihydro and tetrahydro derivatives.[2]

Acetylation of alkaloids was with acetic anhydride in acetone with anhydrous sodium acetate at room temperature for 16 h. Under such conditions about 3% of V was converted to an *O*-acetyl derivative. The dihydro derivative of V gave a 20% conversion to an *O*-acetyl derivative under the same conditions. Pumiliotoxin A was quantitatively converted to the 15-*O*-acetyl derivative and pumiliotoxin B in 50%

yield to a 15,16-di-*O*-acetyl derivative with the remaining 50% present as a mixture of mono-*O*-acetyl derivatives.

Trimethylsilylation of alkaloids was conducted in pyridine with bis(trimethylsilyl)fluoroacetamide for 1 h at room temperature. Under these conditions, V was converted in only 30% yield to an *O*-trimethylsilyl derivative, while the dihydro reduction product was converted in almost quantitative yield to an *O*-trimethylsilyl derivative.

All reactions were carried out with 10–100 μg of alkaloid.

Crystals of V were prepared as follows: The free base (11 mg) was converted to the hydrochloride salt with a stoichiometric amount of methanolic HCl. After concentration in vacuo, it was crystallized at room temperature from ethyl acetate–cyclohexane.

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Supplementary Material Available: Listings of observed and calculated structure factors as well as tables of anisotropic thermal parameters for the nonhydrogen atoms and coordinates for hydrogen atoms (8 pages). Ordering information is given on any current masthead page.

References and Notes

(1) (a) Laboratory of Bioorganic Chemistry, National Institute of Arthritis Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Md. (b) Faculty of Science, Osaka City University, Sugimotocho, Syuiyoski-Ku, Osaka, Japan. (c) Laboratory of Chemistry, National Heart, Lung, and Blood Institute, National Institutes of Health. (d) Laboratory for the Structure of Matter, Naval Research Laboratory, Washington, D.C. 20390.
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(10) The nearly 100 dendrobatid alkaloids reported in ref 2 were assigned numerical designations based on molecular weight and, in the case of more than one alkaloid with the same molecular weight, by a letter (A, B, C, etc.). For example, the alkaloid (V) isolated from *D. tricolor* and characterized by X-ray crystallography in the present paper was designated *251D* based on its detection in a number of other species of dendrobatid frogs. The designations from ref 2 are cited as appropriate throughout the present paper.
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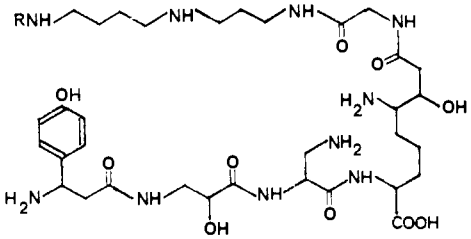
(16) See paragraph at end of paper regarding supplementary material.
(17) Voucher specimens of *Dendrobates tricolor* and *Dendrobates pumilio* are in the collections of the American Museum of Natural History, New York.
(18) This compound will be designated *217* according to the numbering system for dendrobatid alkaloids introduced in ref 2 (see footnote 10).

Communications to the Editor

Biosynthesis of Amino Acids. Investigation of the Mechanism of β-Tyrosine Formation

Sir:

Cultures of *Bacillus brevis* Vm4 produce two peptide antibiotics, edeine A and edeine B (**1** and **2**), that contain a group



1, R= H

2, R= $H_2N\overset{|}{C}=NH$

of novel amino acids.[1] One of these amino acids is β-tyrosine (**3**), which is formed by the isomerization of L-α-tyrosine (**4**)

4 3

catalyzed by the enzyme tyrosine α,β-mutase.[2] The mutase enzyme is unusual in that it requires ATP, is inhibited by reagents reacting with carbonyl groups, and has no requirement for pyridoxal phosphate. To provide additional information concerning the mechanism of the mutase reaction, we have examined the conversion of doubly labeled forms of α-tyrosine into β-tyrosine.

3(*R,S*)-[3-^{3}H,3-^{14}C]-DL-α-Tyrosine[3] was administered to cultures of *B. brevis* Vm4 and the β-tyrosine produced was isolated and purified by paper chromatography. Further purification was accomplished by dilution and repeated recrystallization to constant specific activity and constant $^3H/^{14}C$ ratio. The results of this experiment are outlined in Table I (expt 1). The $^3H/^{14}C$ ratio of the purified β-tyrosine clearly shows that amino group migration is accompanied by the stereospecific removal of one hydrogen atom from C-3 of α-tyrosine (expected loss is 50%). Additional experiments were carried out to define the stereospecificty of this hydrogen loss. Samples of (3*R*)-[3-^{3}H]- and (3*S*)-[3-^{3}H]-α-DL-tyrosine were prepared,[4] mixed with [3-^{14}C]-DL-α-tyrosine, and administered to *B. brevis* cultures. Interpretable results were not obtained in these in vivo experiments, presumably because of metabolism of the α- or β-tyrosine via pathways unrelated to the migration process. The samples of chirally labeled α-tyrosine were therefore converted into β-tyrosine in vitro, using the purified mutase enzyme.[2] The β-tyrosine isolated in these experiments gave the $^3H/^{14}C$ ratios shown (Table I, expt 2, 3). The data indicate clearly that the migration of the amino

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