THE ISOLATION AND STRUCTURE OF THE NOVEL *CIS*-FUSED INDOLIZIDINE **249H** FROM FROG SKIN: *CIS- VS. TRANS*-FUSED INDOLIZIDINES DISTINGUISHED BY NMR

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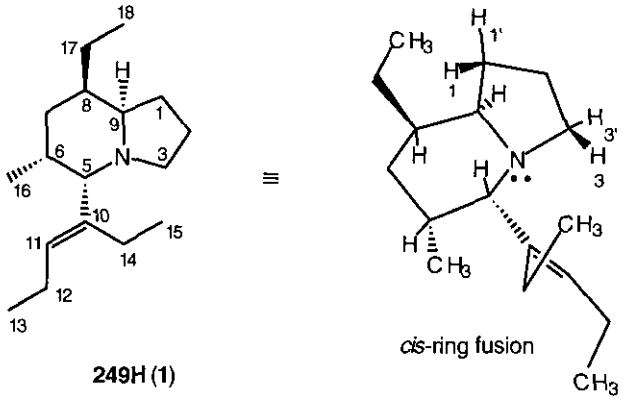
Abstract–A 5,6,8-trisubstituted indolizidine structure (**1**) is proposed for alkaloid **249H** isolated from dendrobatid frogs based upon NMR and IR spectroscopy and MS spectrometry. It has an unusual (*E*)-3-hexen-3-yl side-chain, the first branched substituent reported among the simple indolizidines from frog skin, an unusual *cis*-fused indolizidine ring and finally an atypical *E* configuration of H-5 relative to H-9.

Indolizidines detected in extracts of frog skins consist at present of nearly twenty 3,5-disubstituted indolizidines, about fifty 5,8-disubstituted indolizidines and about ten 5,6,8-trisubstituted indolizidines.[1,2] The first example of a 5,6,8-trisubstituted indolizidine was alkaloid **223A** isolated from skin extracts of the Panamanian poison frog *Dendrobates pumilio*.[3] All of the indolizidines that have been well characterized have had *linear* side-chains with varying degrees of unsaturation.[1,2] Some have hydroxyl or carbonyl substituents in the side-chains.

A minor alkaloid of MW 249 has now been isolated from methanol extracts of skins of the poison frog *Dendrobates auratus* collected in 1976 on Isla Taboga off the Pacific coast of Panama, and is given the designation **249H**. The EIMS with a base peak at *m/z* 166 and a minor fragment ion at *m/z* 110 suggested that it was either a 5,6,8-trisubstituted indolizidine or a 1,4-disubstituted quinolizidine. Further spectral analysis indicated that alkaloid **249H** (**1**) is an additional member of the relatively small group of 5,6,8-trisubstituted indolizidines and is the first example of any of the simple indolizidines in frog skin to have a branched side-chain.



249H (1)

RESULTS AND DISCUSSION

A sample of **249H** containing only traces of impurities was obtained by repeated column chromatography of the methanolic frog skin extract (see Experimental). CIMS with ND_3 indicated that no exchangeable hydrogen was present. HRMS established $C_{17}H_{31}N$ as the formula of the molecular ion, *i.e.* 3 double-bonds and/or rings were present. ^{1}H- and ^{13}C-NMR spectroscopy (Tables 1, 2) and correlation spectra (Tables 2, 4) were obtained that are consistent only with the unusual *cis*-fused trisubstituted indolizidine structure (**1**) above.

Table 1. ^{13}C-NMR Shifts (δ) of **249H** (**1**)

Carbon number	δ_C[a]	δ_C[b]
1	20.69 (t)	20.71
2	20.52 (t)	19.81
3	52.32 (t)	53.67
5	61.80 (d)	64.52
6	30.82 (d)	31.19
7	32.73 (t)	31.14
8	32.45 (d)	32.22
9	63.90 (d)	68.14
10	139.21 (s)	135.37
11	127.63 (d)	132.09
12	20.80 (t)	21.93
13	14.62 (q)	14.38
14	23.13 (t)	23.69
15	13.53 (q)	13.40
16	13.70 (q)	13.12
17	26.90 (t)	26.82
18	11.76 (q)	11.58

[a] Free base at 100 MHz, in ppm relative to $CDCl_3$ at 77.03 ppm. Multiplicity from the phase-projection of the H,C-COSY spectrum in parentheses; s, singlet; d, doublet; t, triplet; q, quartet.
[b] Hydrochloride salt at 100 MHz, in ppm relative to CD_3OD at 49.01 ppm.

Both proton and carbon spectra indicated that four methyl groups (C-13, C-15, C-16, C-18) were present. The 9-line proton multiplet between 0.86 and 1.0 ppm can be analyzed as arising from a downfield isolated triplet (CH_3-13) and a multiplet composed of two overlapping triplets and one doublet (see Table 2). An H,C-COSY spectrum indicated that two (C-5, C-9) of the three down-field carbons assigned to *C*-N carbons are CH substituted while the third (C-3) is a methylene, a pattern suggesting an "izidine" alkaloid with one α-substituent. A better separation of the three *C*-N carbons and C*H*N protons is seen

Table 2. ^{1}H-NMR Chemical Shifts (δ) and NOE Correlations for Alkaloid **249H (1)**[a]

H	δ[b]	δ[c]	H,H-COSY cross peaks[d] with H-	H,H-NOESY cross peaks[e] with H-
1	1.71	2.09	1′, 9	**5** (2.4), 17 (2.6)
1′	1.47	2.15	1, 2, 9	17' (2.6), **18** (2.5)
2	1.75	~2.09	1′, 2, 3, 3′	5 (2.5)
2′	1.52	~2.09	2, 3	
3	3.11	3.43	2, 3′	**5** (2.8), 11 (2.9)
3′	2.65	3.35	2, 2′, 3	
5	2.76	3.63	6 (J = 2.5 Hz), 11	**1**, 2, **3**, **7/7′** (2.5), 11 (2.3), **14′** (2.4), **15** (2.4)
6	1.85	2.25	5 (J = 2.5 Hz), 7, 16	**14′**(2.4)
7, 7′	1.42	1.70	6, 8	5, **16** (2.5), **17** (2.6), 17' (2.5), 18 (2.3)
8	2.14	2.25	7, 9, 17/17′	**16** (2.3), **18** (2.5)
9	3.13	3.85	1, 1′, 8	17/17' (2.5), **18** (2.7)
11	5.40 (t, 7.0 Hz)	5.45 (t)	5, 12	3, 5, 13 (2.7), **16** (2.7)
12, 12′	2.09	2.20	11, 13	
CH_3-13	0.98 (t)	1.07	12/12′	11
14	2.19	2.30	14′, 15	
14′	1.65	1.90	14, 15	5, 6
CH_3-15	0.95 (t)	1.05	14, 14′	5
CH_3-16	0.89 (d)	1.03	6	7/7', 8, 11
17	1.27	1.40	8, 18	1, 1', 7/7', 9
17'	1.20	1.31	8, 18	7/7'
CH_3-18	0.92 (t)	0.99[f]	17/ 17′	1', 7/7', 8, **9**

[a] Assignments based on H,H-(DQF) and C,H-COSY spectra. Primed signals are upfield of unprimed. Note, however, that H-1 and H-1' are interchanged in **249H**·HCl.
[b] Free base, ppm downfield in $CDCl_3$ from TMS (0.0 ppm) at 400 MHz. *Multiplicity in parentheses;* d, doublet; t, triplet.
[c] Hydrochloride salt in MeOH-d_4 at 400 MHz.
[d] Phase-sensitive DQF-COSY spectrum in $CDCl_3$ on free base. Digital resolution for J values ≈ 0.3 Hz.
[e] Phase-sensitive NOESY on the free base with a short echo period following the readout pulse. NOE cross peaks on scalar coupling correlations are omitted. Interatomic distance (Å) in parentheses calculated with the Chem3D program for the Macintosh computer. Bold-faced cross-peaks are more intense.
[f] In this solvent, CH_3-18 is the most upfield methyl and a clean triplet separated from the multiplet of the other three methyls. In $CDCl_3$, CH_3-16 is seen as the most upfield methyl.

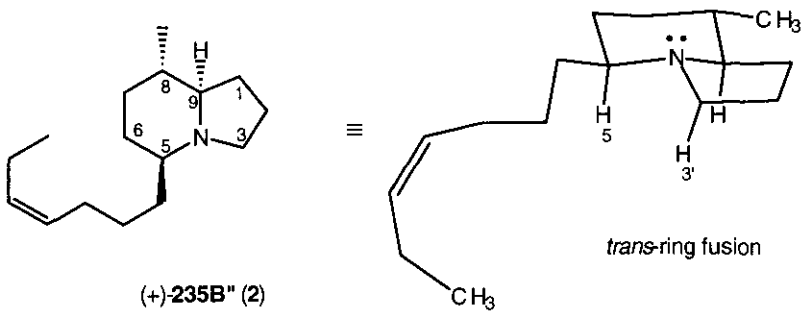
in MeOH-d_4 spectra of the hydrochloride of **249H** (Tables 1, 2). Only one olefinic hydrogen was detected (δ 5.39, t) and it correlated with the olefin carbon signal at 127.6 ppm. The other sp^2 carbon (139.21 ppm) must therefore be C-10 and tetrasubstituted. Alkaloid **249H** is converted to a MW 251 material on hydrogenation, confirming one double-bond. HRMS on the *m/z* 166 peak indicated a $C_{11}H_{20}N$ formula (2 double-bond equivalents, *i.e.* rings) and proved the trisubstituted double bond detected by NMR to be in the side-chain undergoing α-cleavage during MS.

Three sets of connectivities were determined from H,H-COSY (Table 2), H,C-COSY (not shown) and TOCSY (Table 4) spectra. Set A showed the following: N-3-2-1-9(-N)-8(-17-18)-7-6(-16)-5-N; Set B: 11-12-13 and Set C: 14-15. Thus all connectivities sufficient to propose the skeleton of **1** as a 5-(hex-3-en-3-yl)-6-methyl-8-ethylindolizidine were established. The H, H-COSY, showing allylic coupling between H-5 and H-11 and even long-range coupling between H-5 and the H-12s, indicated the missing connection between sets A and B *via* C-10. The only connection possible for sets B and C is also through C-10, but no allylic coupling between H-14 and H-11 was seen. An intense NOESY cross peak between H-1 and H-5 is compatible only with a *cis*-fused indolizidine structure having an axial *N*-lone pair on the piperidine ring and indicates H-5 to be in an axial configuration. For comparison purposes, the H-1–H-5 interatomic distance in **235B"** (**2**), a typical *trans*-fused indolizidine, is 4.7 Å, beyond the range of an NOE interaction. Other ^{1}H- and ^{13}C-NMR data suggested that **249H** did not adopt the *trans* fused conformation usually encountered in indolizidines. In particular, the significantly downfield positions for H-3', H-5 and H-9 (see Table 2) in comparison with the equivalent C*H*N hydrogens in **235B"** (**2**) (see Table 3) are noteworthy and the $^1J_{C\text{-}H}$ values for C-3 (138 Hz), C-5 (130 Hz) and C-9 (139 Hz) in **1**, determined from a coupled DEPT spectrum, are all significantly larger than those of **2** (127, 129 and 128 Hz, respectively).[4] The shielding of H-3', H-5 and H-9 in **235B"** (free base) is traditionally ascribed to their being in a *trans* anti-parallel (TAP) orientation to the neighboring *N*-lone pair.[5] The downfield chemical shift for H-9 (δ 3.13) in **1** is consistent with a *cis*-ring fusion and lack of a TAP effect. Even though H-5 in both **249H** and **235B"** is axial and TAP to the lone-pair, the chemical shift in the latter is 0.9 ppm upfield of H-5 in the former, reflecting the allylic environment of H-5 in **1**.

Since simple *cis*-fused indolizidines are rarely encountered in Nature,[6] an effort was made to unambiguously exclude a *trans*-fused conformation for **249H**. Magnetization transfer from H-8 to H-9 or the reverse was slow as noted by the absence of magnetization transfer within 28 msec in a TOCSY spectrum (Table 4) indicating H-8 and H-9 are not in a *trans*-diaxial orientation. A weak coupling between these hydrogens is noted, however, in the phase-sensitive DQF-H,H-COSY. If H-8 were equatorial, a slow transfer between H-8 and H-9 would also be expected. However, the NOESY spectrum

showed a strong CH_3-16—H-8 cross-peak indicating an axial H-8. This and the above data indicated an equatorial configuration for H-9. Thus the TOCSY data provides further confirmation of a *cis*-ring fusion.

NOESY spectroscopy, combined with molecular modeling[7] of a *cis*-fused indolizidine conformation, supplied the interatomic distances of Table 2 for all the major cross-peaks in good agreement with



(+)-**235B"** (**2**)

a *cis*-fused conformation. An MM2 computer program indicated the *cis*-fused indolizidine to be 2.68 kcal/mole more stable than the *trans*-fused indolizidine structure.

The FTIR spectrum (Figure 1) showed weak Bohlmann bands, unlike the spectrum of the 5,6,8-trisubstituted indolizidine **223A,** which exhibited an intense band consistent with H-5 and H-9 having a *Z*-relationship.[3] Thus, the absence of an H-5—H-9 NOESY cross peak and the IR data support **249H** having H-5 and H-9 in an *E*-orientation. One other 5,6,8-trisubstituted indolizidine, **267J**, has been proposed to have an *E*-orientation of H-5 and H-9.[3] The methyl configuration at C-6 in **1** is assigned by the observation in the H,H (DQF)-COSY of the coupling between H-5 and H-6 with a *J* for the H-5 doublet signal estimated as equal to or less than 2.5 Hz. This small coupling constant indicates that H-5 and H-6 are in an axial-equatorial relationship rather than a *trans* diaxial one. The $H\text{-}5_{ax.}\text{-}H\text{-}6_{eq.}$ relationship is also supported by noting that the TOCSY spectrum shows no rapid magnetization transfer between H-5 and H-6 (see Table 4). The chemical shifts for H-12 and H-12′ in **249H** fall together, while the other allylic protons, H-14 and H-14′ are separated by 0.5 ppm, evidence for closer proximity to a chiral center and/or restriction of rotation of the C-14—C-15 bond. The *E*-configuration of the 3-hexen-3-yl side chain of **249H** is indicated by strong NOESY cross peaks between H-11 and each of CH_3-16 and H-3.

The EIMS gives a base peak at *m/z* 166 by the usual α-cleavage and a minor (17%) fragment at *m/z* 110 by retro Diels-Alder fragmentation, typical of 5,6,8-trisubstituted indolizidines.[3] Even though α-cleavage in the case of **249H** is presumably made more difficult by virtue of the olefinic substitutent, it is still the major pathway, as it is in other 5,8- and 5,6,8-substituted indolizidines.

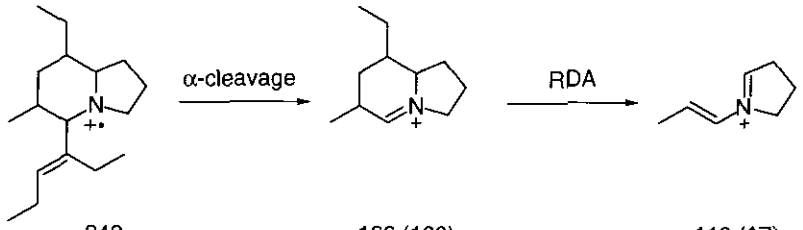


Table 3. ^{1}H-NMR Chemical Shifts (δ) of **235B"(2)**[a]

Free base in $CDCl_3$				**235B**·HCl in CD_3OD			
H	δ	H	δ	H	δ	H	δ
1	1.90	9	1.49*	1	2.37	9	2.87
1'	1.42	10	1.69	1'	1.75	10	1.93
2	1.72	10'	1.32	2	2.09	10'	1.61
2'	1.63	11	1.45	2'	2.09	11	1.55
3	3.25	11'	1.30	3	3.73	11'	1.43
3'	1.96*	12	2.02	3'	3.07	12	2.13
5	1.85*	13	5.33	5	3.07	13	5.35
6	1.73	14	5.34	6	2.15	14	5.44
6'	1.23	15	2.03	6'	1.55	15	2.08
7	1.72	16	0.95	7	1.94	16	0.99
7'	0.95	17	0.86	7'	1.32	17	1.05
8	1.32			8	1.80		

[a] In ppm downfield from TMS (0.0). Primed signals are merely upfield of unprimed and have no stereochemical significance. Asterisks indicate signals discussed in text. Initial NMR studies indicating structure **2**, including complete ^{13}C-assignments and 1D-HOHAHA and 1D–NOE experiments have been reported[8,9] wherein the alkaloid was originally referred to as **235B**. Syntheses proving this structure have been provided.[10] The data of Table 3 have not been published.

Alkaloid **249H** has been detected only very rarely in frog skin extracts. In dendrobatid frogs, it was a trace alkaloid in skin extracts from *Dendrobates auratus* from Isla Taboga and the adjacent Panamanian mainland at Ancon Hill. In both cases it was accompanied by lesser amounts of an apparent congener

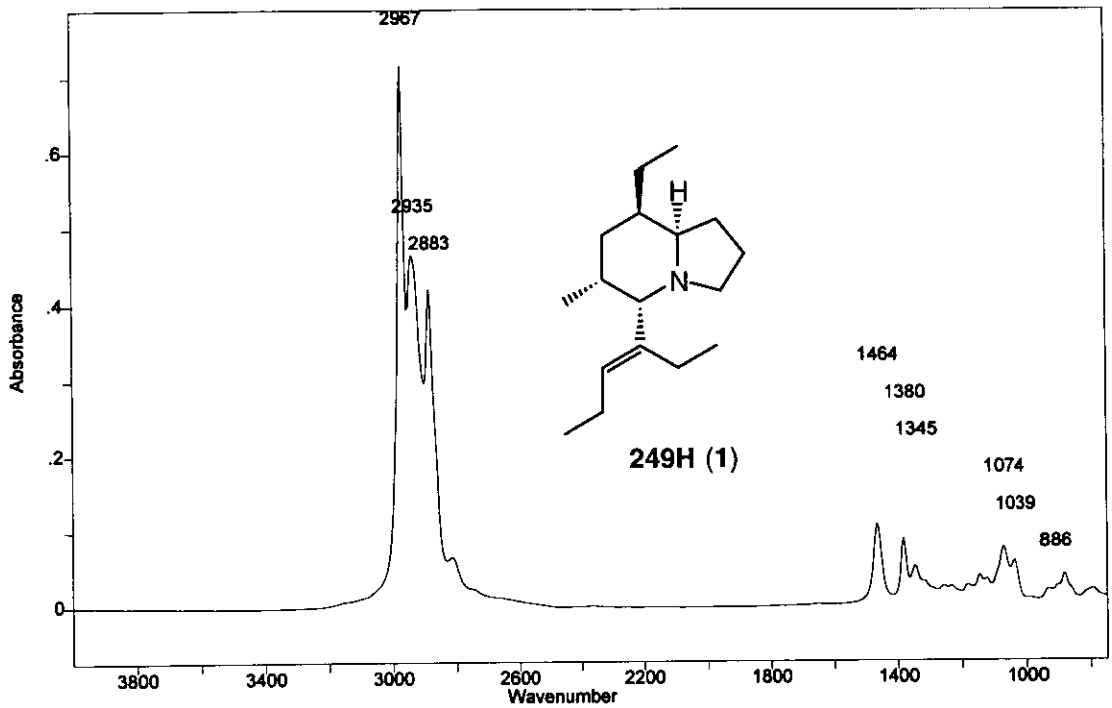


Figure 1. GC-FTIR spectrum of alkaloid **249H (1)**.

Table 4. 2D-HOHAHA Correlation Spectroscopy (TOCSY) on **249H (1)**[a]

Transfer of Magnetization from H-	To H-	To H-
1, 1'→	2, 2'→3, 3'	9
2, 2'→	3, 3'	1, 1'→9
3, 3'→	2, 2'	
5→	—	—
6→	CH_3-16	
7/7'→	8	
8→	7/7'	17→CH_3-18
9→	1, 1'	
12→	11	CH_3-13
CH_3-13→	12→11	
14→	CH_3-15	
CH_3-15→	14, 14'	
CH_3-16→	6	
17→	8→7/7'	CH_3-18
CH_3-18→	17	

[a] In $CDCl_3$ at 400 MHz. Mixing times of 17 and 28 msec were used.

263D with a very similar FTIR spectrum but the MS had a base peak at *m/z* 180 and a fragment at *m/z* 124,[11] suggesting that the 6-methyl group of **249H** is replaced by a 6-ethyl group; the stereochemistry remains unknown. Alkaloid **249H** was also detected as a trace alkaloid in skin extracts from one population of the Madagascan mantelline frog *Mantella betsileo*. A dietary source for this unusual indolizidine alkaloid is unknown.

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 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, EIMS, and FTIR spectra of **249H**. A temperature program of 100°C to 280°C at the rate of 10°C/min was used. A Finnigan GCQ mass spectrometer and chromatograph fitted with the same column as above was used with NH_3 in the CI mode to generate CIMS spectra of **249H** and in the EI mode to generate EIMS spectra. HRMS measurements were made with a JEOL SX 102 instrument fitted with a 15 m × 0.20 mm i.d. HP-5 column. All measurements were within ± 5 ppm. NMR data was obtained with a JEOL GX400 instrument.

Isolation of Alkaloid **249H**: A MeOH extract, prepared from the skins of 1000 *Dendrobates auratus* frogs collected on Isla Taboga, Panama in 1976, was concentrated *in vacuo,* acidified to pH *ca.* 1.5 with dilute HCl and passed through an XAD-2 column (a polystyrene matrix) as previously reported.[8] The column was washed with H_2O and the effluent and wash were made alkaline with aqueous NH_3 and extracted with hexane and then $CHCl_3$. The combined extracts afforded after repeated chromatography a variety of alkaloids including indolizidines, histrionicotoxins, decahydroquinolines and pumiliotoxins as previously reported.[8] The XAD-2 column was then washed with MeOH and finally with CH_2Cl_2, the eluates were combined, evaporated *in vacuo* and then partitioned between CH_2Cl_2 and 0.1 N HCl as previously reported.[8] The acidic layer was made alkaline and extracted with hexane, which yielded after chromatography three decahydroquinolines as previously reported.[8] The CH_2Cl_2 layer was concentrated *in vacuo* to yield 1.2 g of residue. This material was chromatographed on a Merck Lobar LiChroprep silica gel column (Si60, size A) with solvent I, hexane:$CHCl_3$:dioxane:aq. NH_3 (28%) (80:15:5:0.5) to afford three main fractions (A, B and C). The column was then eluted with solvent II, $CHCl_3$:dioxane:*i*-PrOH:aq. NH_3 (80:15:5:1) to afford fraction D. Fraction A was rechromatographed with the same silica gel column with solvent I to yield *ca.* 10 mg of **249H**. Fraction C was further chromatographed on the same silica gel column with solvent III, hexane:$CHCl_3$:*i*-PrOH:aq. NH_3 (70:20:10:1) to yield cholesterol

and 25 mg of crystalline decahydroquinoline *cis*-**243A**. Fraction D was rechromatographed on the same column with solvent II to give two further alkaloids, which are under investigation.

Properties of Alkaloid **249H**:

GC-FTIR: (see Fig. 1). *EIMS*: 249(8), 234(4), 220(10), 206(2), 192(2), 190(2), 178(5), 166(100), 148(4), 136(5), 124(4), 122(4), 110(17), 95(5), 70(5), 67(5).

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REFERENCES AND NOTES

1. J.W. Daly, H.M. Garraffo, and T.F. Spande, 'The Alkaloids', Vol. 43, ed. by G.A. Cordell, Acad. Press, San Diego, 1993, p. 185.
2. J.W. Daly, H.M. Garraffo, and T.F. Spande, 'Alkaloids: Chemical and Biological Perspectives', Vol. 13, ed. by S.W. Pelletier, John Wiley and Sons, New York, 1999, p. 1.
3. H.M. Garraffo, P. Jain, T.F. Spande, and J.W. Daly, *J. Nat. Prod.*,1997, **60**, 2.
4. Y. Takeuchi, P.J. Chivers, and T.A. Crabb, *J. Chem. Soc., Chem. Commun.*, 1974, 216.; G.V. Binst and D. Tourwe, *Heterocycles*, 1973, **1**, 257.
5. T.A. Crabb, 'Ann. Reports on NMR Spectroscopy', Vol 6A, ed. by E.F. Mooney, Academic Press, London, 1975, p. 323.
6. The only other *cis*-fused simple indolizidine we are aware of is dendroprimine, 5*R*(5α,7α,9α)-5,7-dimethylindolizidine, where, as is the case with **249H**, a severe steric interaction in the *trans*-fused conformation is relieved in adopting the *cis*-fused conformation. B. Lüning and C. Lündin, *Acta Chim. Scand.*, 1967, **21**, 2136; L. Blomqvist, K. Leander, B. Lüning, and J. Rosenblom, *Acta Chim. Scand.*, 1972, **26**, 3203.
7. Chem3D for the Macintosh computer (Cambridge Scientific Computing).
8. T. Tokuyama, T. Tsujita, A. Shimada, H.M. Garraffo, T.F. Spande, and J.W. Daly, *Tetrahedron,* 1991, **47**, 5401.
9. T. Tokuyama, N. Nishimori, A. Shimada, M.W. Edwards, and J.W. Daly, *Tetrahedron,* 1987, **43**, 643. Enantioselective syntheses of **235B** by R.P. Polniaszek and S.E. Belmont (*J. Org. Chem.*, 1991, **56**, 4868) and Y. Shisido and C. Kibayashi (*J. Org. Chem.*, 1992, **57**, 2876) indicated that our originally

proposed structure is probably enantiomeric to that of the natural material. Structure **2** is therefore represented as a mirror image of the originally proposed structure.

10. A.L. Smith, S.F. Williams, A.B. Holmes, L.R. Hughes, Z. Lidert, and C. Swithenbank, *J. Am. Chem. Soc.,* 1988, **110**, 8696.; A.B. Holmes, A.L. Smith, S.F. Williams, L.R. Hughes, Z. Lidert, and C. Swithenbank, *J. Org. Chem.,* 1991, **56**, 1393.; I. Collins, M.E. Fox, A.B. Holmes, S.F. Williams, R. Baker, I.J. Forbes, and M. Thompson, *J. Chem. Soc., Perkin Trans. I,* 1991, 175.
11. Alkaloid **263D**. *EIMS: m/z* 263(6), 248(2), 234(10), 229(2), 180(100), 178(11), 164(10), 150(3), 148(3), 136(4), 124(13), 109(3).

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