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IDENTIFICATION OF HISTRIONICOTOXINS BY GC-MS AND GC-FTIR: PHOTO- AND CHEMICAL-ARTEFACTS AND REVISED ^{13}C NMR ASSIGNMENTS

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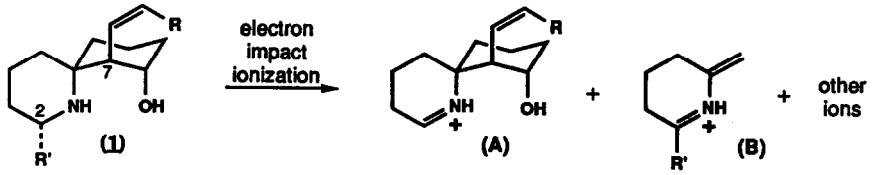
Abstract: GC-mass and GC-FTIR spectral data together permit the rapid identification of the histrionicotoxin (HTX) alkaloids. ^{13}C-NMR chemical shifts (δ_C) are reported for HTX 235A and revised δ_C are tabulated for nine HTXs and a perhydro-derivative. A butylboronic acid HTX derivative offers GC-MS and GC-FTIR advantages. Artefactual trans diene photoisomers of the HTX class are described as are formaldehyde condensation products. Two new HTX alkaloids 261 and 265E, are characterized by mass and FTIR spectra.

Alkaloids from neotropical dendrobatid frogs, having in common the 1-azaspiro[5.5]undecan-8-ol ring system with an unsaturated butyl and an unsaturated pentyl side chain (1), comprise the principal representatives of the class of dendrobatid alkaloids called histrionicotoxins (HTXs). The nature of the side-chain unsaturations (terminal allene or acetylene, cis-diene or cis-enyne or terminal olefin) and sometimes the side-chain length distinguishes one member of the HTX class from another (1). These relatively non-toxic



HTX alkaloid (1)		(A) R	(A) m/z, %	(B) R'	(B) m/z, %
283A*	histrionicotoxin (HTX)	C≡CH	218, 48	(i) $CH_2CH{=}CH{-}C{\equiv}CH$ (Z)	160, 22
285E*	dihydro-HTX	"	218, 100	(ii) $CH_2CH{=}CH{-}CH{=}CH_2$ (Z)	162, ≈0
285A	isodihydro-HTX	"	218, 6	(iii) $(CH_2)_2CH{=}C{=}CH_2$	162, 18
285C	allodihydro-HTX	"	218, 5	(iv) $(CH_2)_3C{\equiv}CH$	162, 17
285B*	neodihydro-HTX	$CH{=}CH_2$	220, 37	i	160, 20
287B*	tetrahydro-HTX	"	220, 43	ii	162, 4
287A	isotetrahydro-HTX	"	220, 30	iii	162, 60
287D	allotetrahydro-HTX	"	220, 24	iv	162, 49

Fig. 1. Structures of HTX alkaloids with two characteristic fragment ions A and B. Those HTX alkaloids with intensity A/B >1 are indicated by an asterisk (see text). Electron-impact (70 e.v.) mass spectral data are taken from ref. 4, where complete tabulations can be found.

compounds bind to acetylcholine receptor channels and to sodium and potassium channels of nerve and muscle (1). ^{1}H-NMR (2,3), ^{13}C-NMR (3) and electron impact (E.I.) mass spectral (4) characterization have been reported. There is, however, still the need for a rapid, reliable identification of these confusingly similar alkaloids at the sub milligram levels encountered in skin extracts. GC-FTIR spectra plus a simple analysis of GC-MS data answers this need.

In the E.I. mass spectrum two fragment ions are particularly descriptive, occurring in each of nine of the HTX alkaloids (4). Ion A (m/z 218 or 220), arising from α-cleavage of the C_5 unit still incorporates the 4-carbon enyne or diene at C-7, while ion B (m/z 160 or 162) contains the 5-carbon (R') enyne (i), diene (ii), allene (iii) or acetylene (iv) moiety originally at C-2 (4). Since two of the five-carbon side chains (i, ii) yield allylic radicals on α-cleavage, while two (iii, iv) do not, the already facile production of ion A is enhanced in the former case, producing the situation where *intensity ion A / intensity ion B > 1* for the four alkaloids **283A**, **285B**, **285E** and **287B**. The four alkaloids with terminal allene (iii) or acetylene (iv) side chains have *intensity A / intensity B < 1*.

Mass spectral data alone can be used to rapidly and unambiguously identify each of the former group (*A/B > 1*), while those of the latter group (*A/B < 1*) require additional data to secure their identification (see Fig. 2), since the pairs **285A/285C** and **287A/287D** have very similar mass spectra. Fortunately, the former alkaloid in each pair has a terminal allene group for which IR absorptions (~1950 cm^{-1}, 850 cm^{-1}) are quite distinctive, consequently GC-FTIR spectra permit an easy characterization.

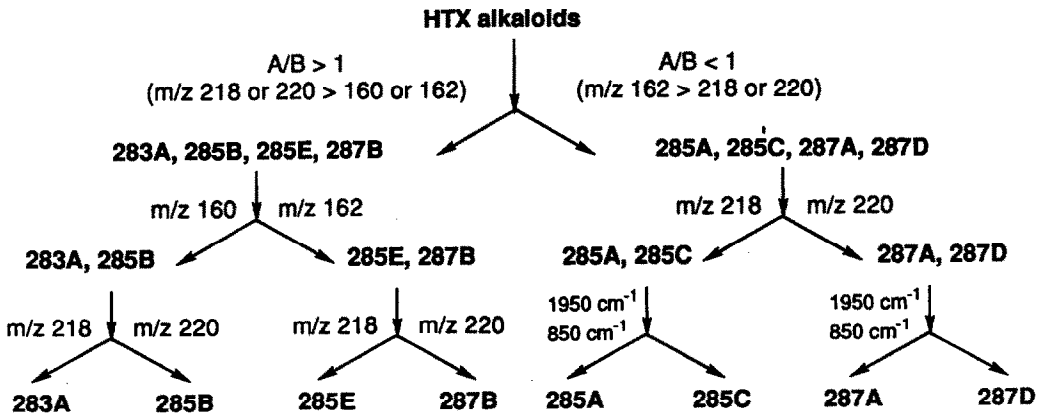


Fig. 2. Scheme for Identification of HTX Alkaloids from Dendrobatid Frogs. The B fragment ions m/z 160 and 162 are invariably accompanied by ions of m/z 65 and 67, respectively.

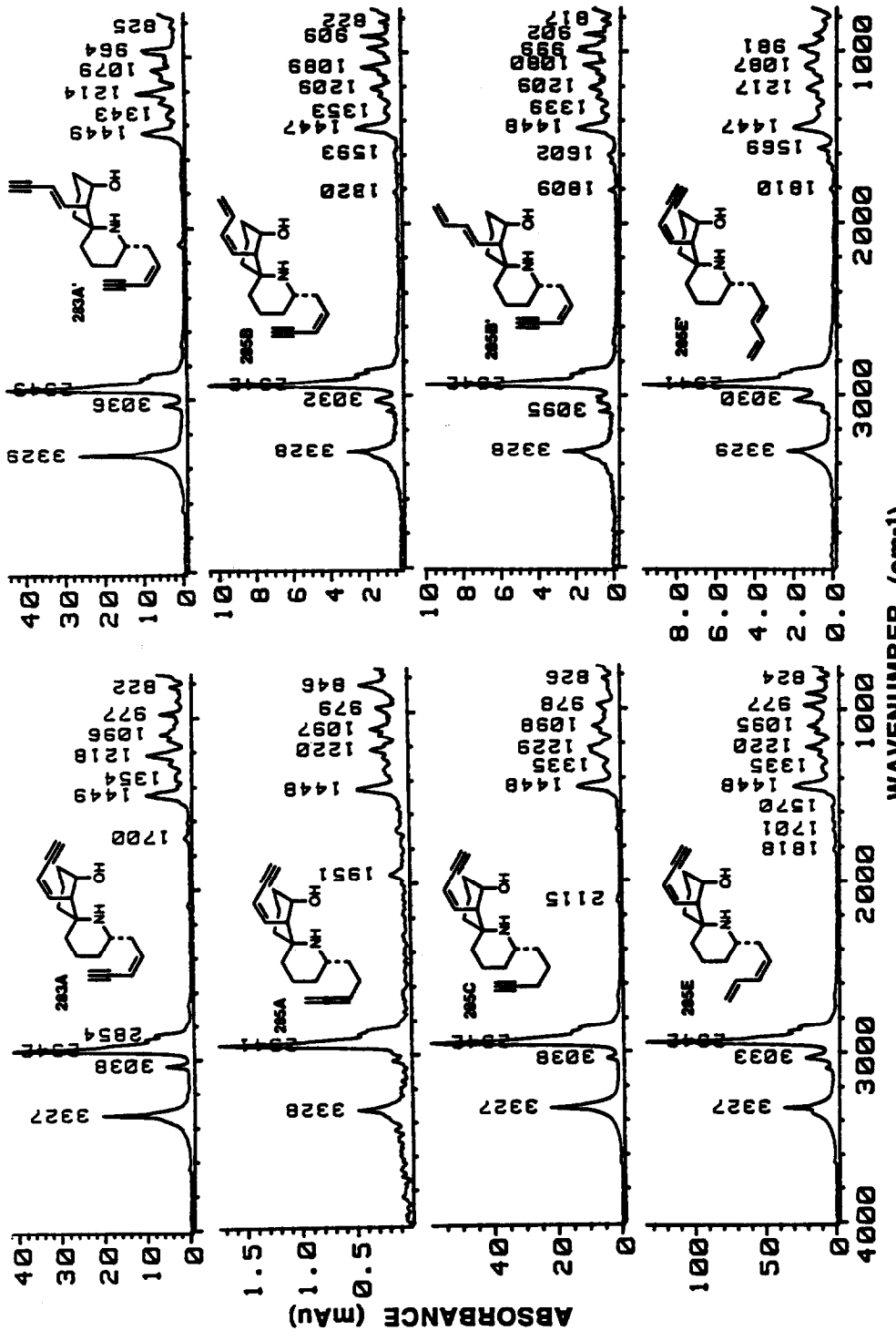


Fig. 3. FTIR Spectra of HTX and DihydroHTXs.

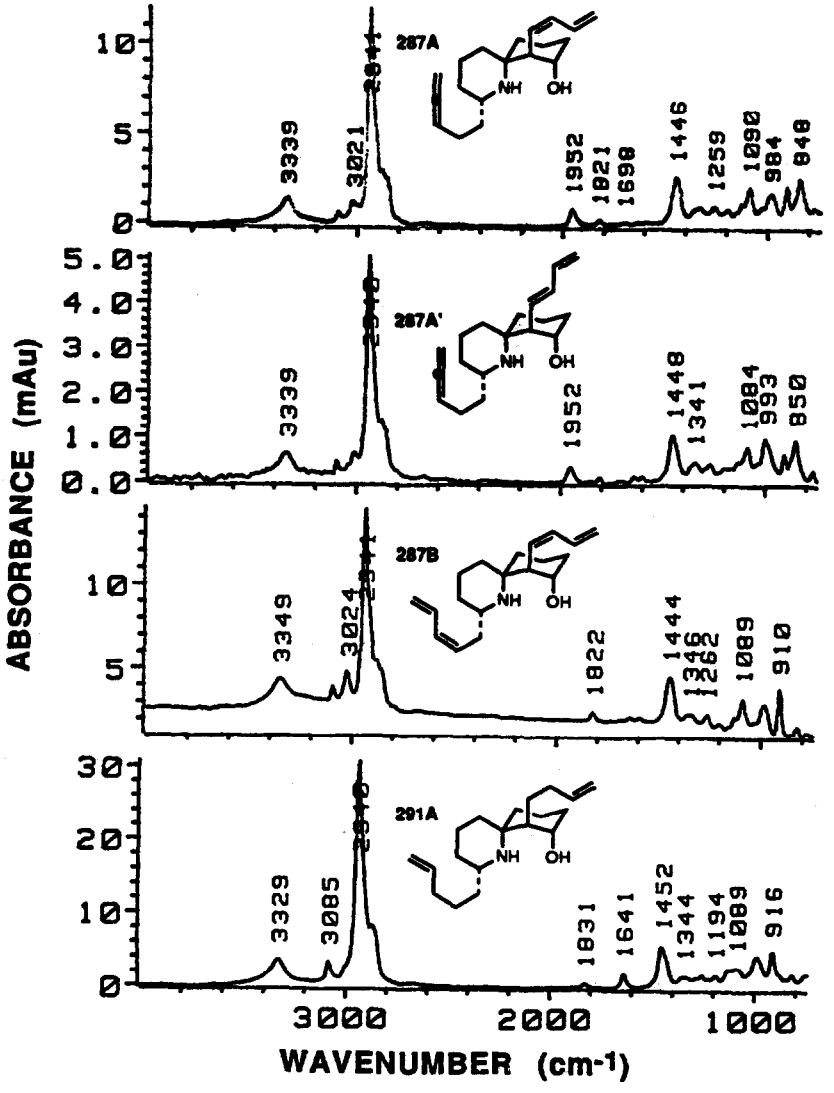


Fig. 4. FTIR Spectra of TetrahydroHTXs and OctahydroHTX.

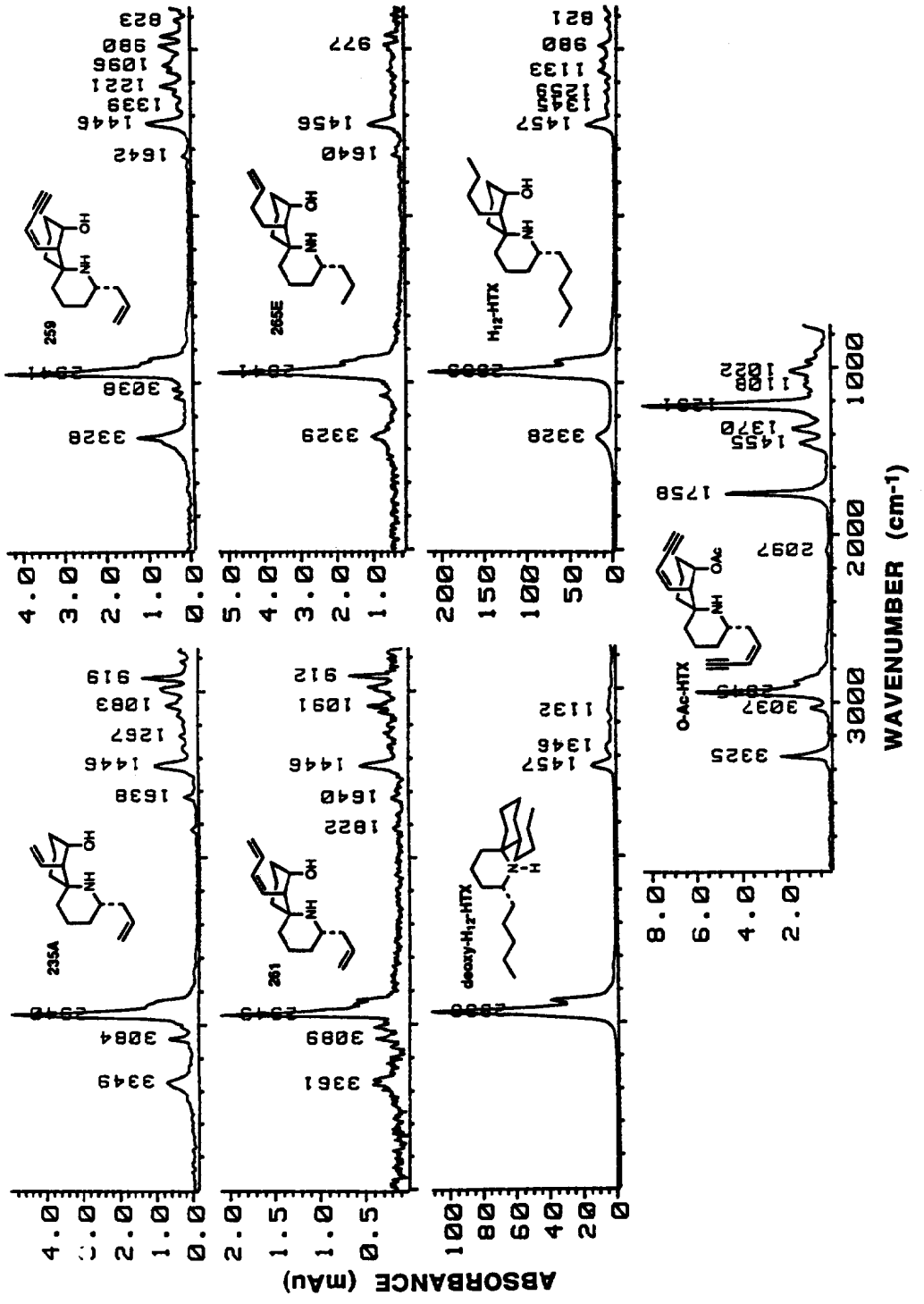
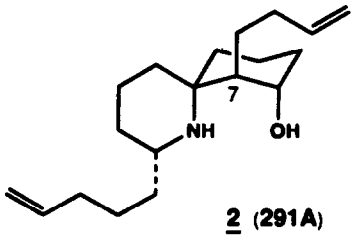


Fig. 5. FTIR Spectra of Further HTX Alkaloids and Derivatives.

A tenth HTX alkaloid, octahydroHTX (291A) (2) can be distinguished easily from the others by its molecular ion and major fragments at m/z 250 (88%), 222 (50), 178 (100) and 165 (36). The A fragment equivalent (m/z 222), containing the 3-butenyl moiety is present; however, the corresponding B fragment (m/z 164) with an expected intensity greater than the A fragment is not. As has been postulated earlier (4), an alternative fragmentation intervenes, involving the 3-butenyl moiety at C-7 (M^+ --> 250).



2 (291A)

Figures 3, 4 and 5 depict FTIR absorbance spectra for the majority of natural HTXs and for certain derivatives. Spectra were obtained from 0.1 - 1 μg alkaloid with the Hewlett Packard 5965A infrared detector (4000-750 cm^{-1}) fitted with a fused silica capillary column.

Since many of the dendrobatid alkaloids are saturated alkaloids of the pyrrolizidine, indolizidine or quinolizidine classes (1), their IR spectra, with the exception of the Bohlmann band region (2800-2700 cm^{-1}), tend to be relatively uninformative. This situation is not the case for the HTX class where different patterns of side-chain unsaturations make FTIR spectra uniquely useful in their characterization. Only the HTX pair, 285B and 285E, have virtually identical spectra, a consequence of the unsaturation patterns in the butyl and pentyl side chains being identical, but interchanged.

Comparison of the spectra of H_{12} (perhydro) HTX and O-acetyl-283A (Fig. 5) reveals that the 3300 cm^{-1} absorption from alkaloids with a terminal acetylene, is a composite of the sharper acetylenic ν_{C-H} and the hydrogen-bonded ν_{O-H}. It should be noted that the secondary amine ν_{NH} is not seen in any of these vapor-phase spectra . In general, this absorption is very weak or absent in primary or secondary amines in the vapor phase, but it does appear in less basic amines, such as anilines, or in indoles, pyrroles and amides (5).

Structural conclusions that can be deduced from the spectra of Figs. 3-5 are:

(1) The ratio of the acetylenic ν_{CH} relative to the methylene vibration at 1450 cm^{-1} is greater for 283A and 283A' with respect to the other HTX alkaloids with only one acetylenic group, as expected.

(2) The acetylenic $\nu_{C\equiv C}$ appears weakly at 2101-2114 cm^{-1} in 283A, 283A' and 285C, but is weaker or absent in HTX alkaloids having only one acetylenic group.

(3) The olefinic ν_{CH} appears at 3020-3037 cm^{-1} for an internal conjugated location and at 3080-3093 cm^{-1} for a terminal position, either isolated (see 291A, Fig. 4) or conjugated (see 285B, Fig. 3).

(4) The cis and trans enyne spectra (cf. **283A** and **283A'**, Fig. 3) are distinguishable by the appearance of the 969 cm^{-1} absorption in the latter, a δ_{C-H} out-of-plane vibration arising from a trans disubstituted double bond.

(5) The $\nu_{C=C}$ absorption for terminal olefins is seen at 1638-42 cm^{-1} in spectra of **235A**, **259** and **291A**. The weak absorption at ~1820 cm^{-1} in **285B** or **287B** is an overtone of the 910 cm^{-1} absorption.

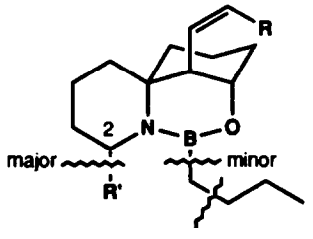
(6) The azaspirodecanol system of the HTX alkaloids is known from X-ray (6) and NMR (7) studies to be present in a conformation where the C-8 hydroxyl and C-7 butyl groups have a trans-diaxial arrangement and are fixed in this conformation by a strong intramolecular hydrogen-bond. The small change observed in the 2870 cm^{-1} region in the IR spectrum of deoxy-H_{12}-HTX *vs* H_{12}-HTX (see Fig. 5) may reflect the absence of a hydrogen-bond and the adoption of a conformation where the C-7 butyl group is now equatorial; however, the effect is admittedly minor.

^{13}C-NMR Assignments for Histrionicotoxins:

Although most of the histrionicotoxins have been isolated in quantities sufficient for ^{1}H-NMR (2) and ^{13}C-NMR (3) characterization, this was not initially the case with several minor HTX alkaloids, including **235A**. Recently, sufficient **235A** was isolated from *Dendrobates auratus* so as to permit HH- and CH-COSY NMR spectra to be obtained and to confirm the original structure (2-allyl-7-vinyl-1-azaspiro[5.5]undecan-8-ol), proposed based on mass spectral analysis (1,8). The data led to the unambiguous assignment of all carbon resonances to **235A** (see Table 1); it, however, necessitated the revision of resonances previously assigned to carbons 3, 5, 9 and 11 of the other HTXs. Revised chemical shift data are presented in Table 1.

Boronic Acid Derivative:

The butylboronic acid derivative (3) of HTX alkaloids forms easily at room temperature with a 2-3 fold excess of reagent (n-BuB$(OH)_2$) in dimethoxyethane. It offers advantages of three types:



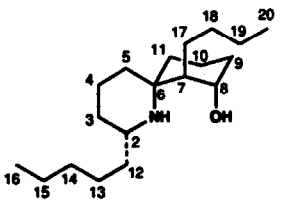
3

1) Chromatographic. Better separations on GC columns of complex mixtures of HTX alkaloids are achieved after conversion to butylboronides. Due to the less polar nature of derivatives, GC peaks of the butylboronides are somewhat sharper than the underivatized alkaloids (Fig. 6). It should be pointed out that the boronic acid derivatives are stable only in the absence of hydroxylic solvents (9).

Table 1. Carbon-13 NMR Chemical Shifts (δ_C) for Histrionicotoxins[1]

	235A[2]	259	283A (HTX)	283A'[3]	285A	285B	285C	285E	287A	291A	H_{12}-HTX
2	49.8	49.6	49.7	50.0	49.7	50.0	49.6	50.1	51.8	49.8	50.3
3	32.2	32.5	32.8	32.8	33.0	32.9	32.7	32.6	32.8	33.4	32.7
4	19.0	19.6	19.5	19.1	19.5	19.5	19.2	19.7	20.3	19.6	19.5
5	37.3	38.1	37.8	37.6	37.1	37.5	37.8	38.2	37.5	37.1	37.4
6	54.3	54.4	54.1	54.2	54.3	54.4	54.3	54.4	56.1	55.0	55.8
7	45.6	41.6	41.4	44.9	41.6	38.8	41.2	41.7	39.5	37.7	38.1
8	73.1	71.5	71.3	72.6	71.5	72.4	71.2	71.6	73.6	69.6	69.7
9	28.6	29.1	29.0	28.8	29.1	28.7	28.8	29.2	29.1	27.8	27.6
10	15.3	15.3	15.0	15.2	15.3	15.2	15.0	15.3	16.2	15.2	15.2
11	37.0	37.0	36.8	37.1	36.8	36.8	36.3	37.1	36.2	37.1	36.6
12	41.6	41.6	37.9	37.9	38.4	38.0	36.5	35.3	37.0	37.2	36.6
13	134.7	134.6	141.6	141.6	24.5	141.7	24.5	128.1	25.7	25.2	25.6
14	117.5	117.5	110.0	110.8	89.8	110.6	18.3	131.6	90.3	33.9	32.1
15			80.3	82.1	208.4	80.5	83.9	132.0	209.7	138.6	22.5
16			81.7	81.9	75.2	81.9	68.5	117.8	75.2	114.6	14.1
17	136.7	143.1	142.9	144.1	143.1	129.8	142.6	143.1	129.9	27.4	27.6
18	117.5	110.2	110.3	110.8	110.1	131.8	110.0	110.2	132.8	32.2	30.3
19		79.9	79.7	82.1	79.9	130.8	79.6	80.0	132.4	138.6	23.0
20		82.6	82.6	76.9	82.5	118.6	82.6	82.7	119.3	114.9	14.1

[1] See structure below for numbering system used. The original assignments are found in ref. 3. Spectra are in $CDCl_3$ except for 287A where CD_3OD was used. See ref. 3 for instrument and conditions.
[2] Assignments for 235A are based on homo- and hetero-nuclear shift correlation spectroscopies.
[3] Δ^{17}-trans HTX.



2) Mass spectral. After derivatization, the mass spectra of HTX alkaloids are much simpler and their identification is much easier. The α-cleavage at C-2 now greatly dominates other cleavage pathways; the major fragmentation producing a m/z 96 ion, for example, is not seen. Minor but characteristic cleavages of the boron-butyl group are seen ($M^+ - 43$ and $M^+ - 57$), which are very useful in establishing the molecular weight (see below). With such information and GC-FTIR data, it is usually possible to identify the HTX alkaloids, without recourse to GC retention times and/or comparison with reference HTXs.

Since the butylboronides of the HTX alkaloids are 66 mass units greater than the alkaloids themselves and since α-cleavage to nitrogen gives loss of side chains of masses 65, 67 or 69 (or 41 for the simpler HTX alkaloids **235A** and **259**), the mass spectra of the butylboronide (BuB) derivatives exhibit base peaks at either m/z 260 (from **235A**); m/z 284 (from **259**, **283A**, **283A'**, **285C**, **285E**); m/z 286 (from **285B**, **287A**, **287B**, **287D**) or 288 (**291A**), respectively. Note that **285B** is the only dihydro HTX with an m/z 286 fragment.

Many of these butylboronides show weak peaks (2-20%) in their mass spectra due to cleavage of a propyl or butyl fragment from the boron-butyl group. These peaks are easily detected in an uncluttered region of the spectrum at masses greater than the base peak. The propyl cleavage, often more significant than the butyl cleavage, gives rise to ions at m/z 308 or 310 with dihydro-(285) or tetrahydro-(287) HTXs, respectively. No peak at m/z 306 is observed for **283A**, probably a consequence of the very facile α-cleavage of the C_5 enyne side chain at C-2. Table 2 summarizes our results using a Finnigan Model 800 ion trap mass spectrometer.

Table 2. Butyl boronate (BuB) Derivatives of HTX alkaloids in Order of Elution from an HP-5 GC Column

HTX Alkaloid or Derivative	Molecular Weight BuB Derivative	M^+ (%) (if detected)	M^+-43	M^+-57	base peak (100%)
235A	301				260
259	325				284
285E	351	351 (<1)	308 (25)	294 (30)	284
291A	357		314 (<1)		288
285C	351	351 (1)	308 (10)	294 (3)	284
283A	349				284
287D	353	353 (<1)	310 (15)	296 (10)	286
285B	351		308 (25)	294 (35)	286
287B	353		310 (15)	296 (10)	286
285A	351		308 (5)	294 (5)	284
287A	353	353 (<1)	310 (6)	296 (10)	286

It might be expected that parent ions and cleavages from the boron-butyl group would be more significant in HTX alkaloids that do not generate allylic radicals on α-cleavage (**285A**, **285C**, **287A**, **287D**), than in those that do (**235A**, **259**, **283A**, **285E**, **285B**, **287B**). This prediction is borne out only for **235A**, **259**, **283A** and **287D**.

3) Infrared spectral. In the boronic acid derivative, the ν_{OH} is now absent, no longer overlapping the $\nu_{\equiv CH}$ absorption. Consequently, the acetylenic moiety is now easily quantitated in the HTX alkaloids (two (e.g. 283A), one (e.g. 285E) or none (e.g. 287B, H_{12}-HTX) (see Fig. 7).

The butyl boronyl moiety is largely non-interfering in regions of the infrared domain most useful for characterizing the HTX alkaloids (4000-3000, 2800-1600 and 1200-750 cm^{-1}). The FTIR spectrum of the phenylboronic acid derivative of H_{12}-HTX (Fig. 7) reveals that the phenylboronyl moiety also is relatively free of interference in the detection of allenes and terminal double bonds; however, the aromatic ν_{CH} at 3061 cm^{-1} would make it difficult to use this region to detect the internal or terminal double bond carbon-hydrogen stretching vibration.

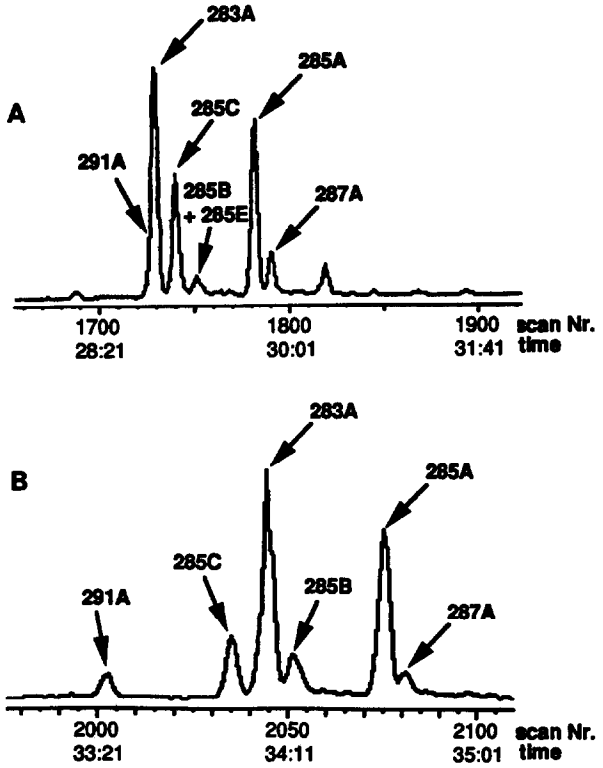


Fig. 6. Total Ion Current Mass Spectra Chromatograms of A) Mixture of histrionicotoxins from *Dendrobates histrionicus*, Guayacana, Colombia; B) Same mixture after conversion to butylboronides. Note particularly the improved separation of 291A after derivatization. HP-5 fused silica capillary column (25 m x 0.35 mm) Program 100-280°, 1 min at 100° then 5°/min to 280°.

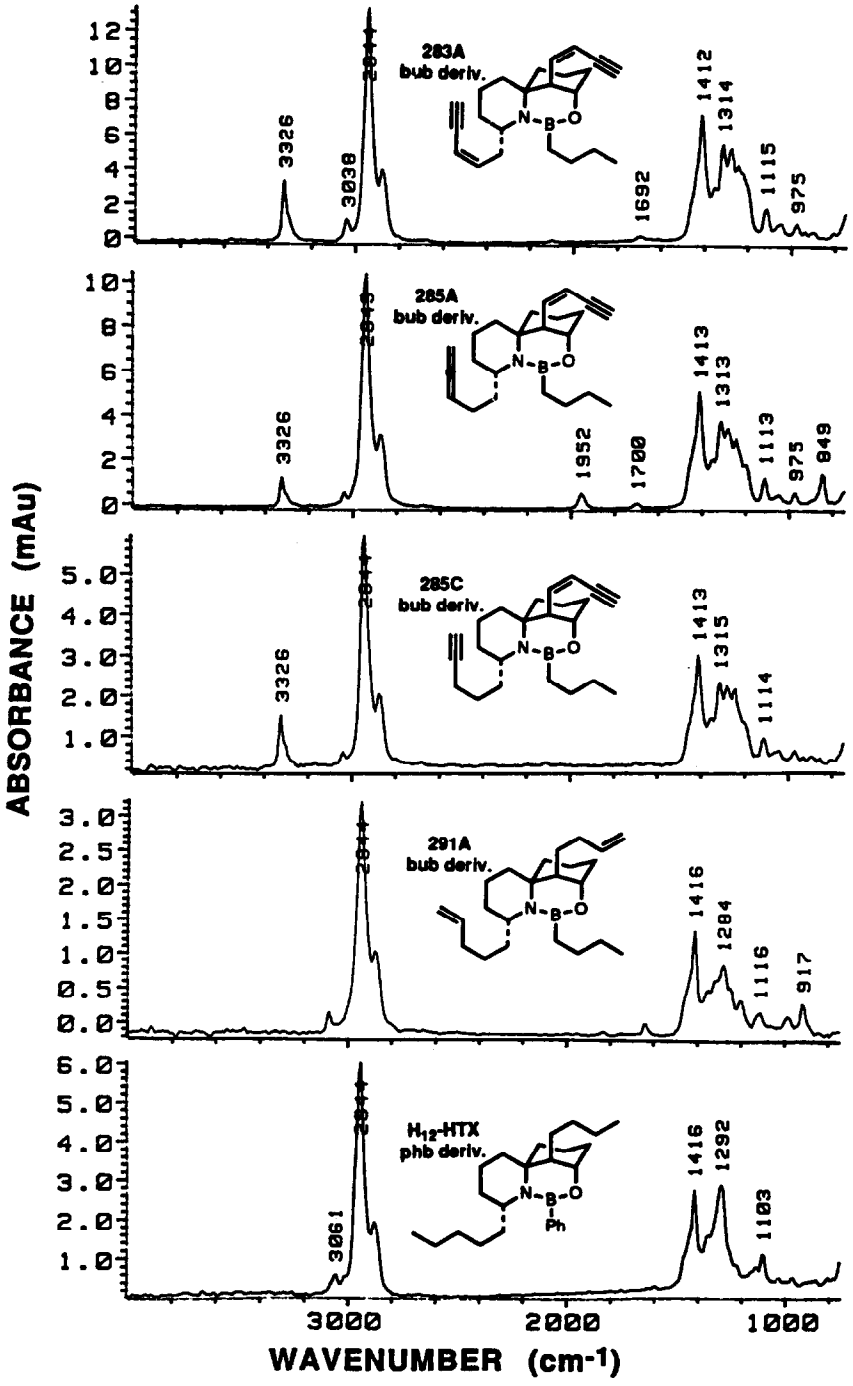


Fig. 7. Representative FTIR Spectra of HTX Boronides.

Photoisomerization of HTX Alkaloids:

During GC-MS and GC-FTIR analyses, a minor peak was detected at a retention time slightly greater than that of the major peak in reference samples of **285B**, **285E** and **287A**. In the former two, the minor peak was only a few percent of the intensity of the major, while with **287A**, it approached 25%. These had mass spectra virtually identical with the major component; however, the FTIR spectra of each pair showed slight differences in the ratio of the 910 to 990 cm^{-1} absorptions. These arise in terminal (conjugated or non-conjugated) double bonds from out-of-plane C-H bending vibrations (see **291A**, Fig. 4; **235A**, 259 (Fig. 5)).

In a cis diene (e.g. cis 1,3-pentadiene (5)), the 910 cm^{-1} absorption is usually more intense than the 990 cm^{-1} absorption, whereas in a trans diene (see trans 1,3-pentadiene (5)), the reverse is true. Since the 990 cm^{-1} absorption is enhanced relative to the 910 cm^{-1} absorption in these three minor HTX isomers, we have assigned to them a trans diene structure and refer to these as **285B'**, **285E'** and **287A'** (see Figs. 3,4).

We suspected that these trans diene stereoisomers were artefacts of a photochemical isomerization, analogous to the well studied cis-trans photoisomerization of 1,3-pentadiene (10). The trans enyne **283A'**, previously reported (3), might likewise be a photoisomerization artefact. We find, indeed, that dilute methanol solutions of the diene-containing HTX alkaloids, **285B**, **285E**, **287A** or **287B** exposed out of doors for two to eight hours to diffused daylight in borosilicate glass vials show significantly increased proportions of the trans diene isomers. A sample of **287A** after 15 h of daylight exposure contained approximately 55% of **287A'**. Additional light exposure caused little change in this ratio, indicating that this photostationary state is close to the 45:55 cis:trans ratio reported (10a) for 1,3-pentadiene. These photoisomerizations are promoted by the addition of 2% acetone as photosensitizer (10a). Compound **287A** is approximately 33% isomerized after 5 h in the presence of acetone, but only 25% in its absence. That the photoisomerization involves only the C_4-diene unit of **287A** is indicated by the following:

1) Both GC peaks still show the same terminal allene IR absorption.

2) On hydrogenation (5% Rh/Al_2O_3, 2 atm. H_2, 1 h) a single product (MW 295) is observed, identical in mass spectrum and retention time to perhydro HTX, the same reduction product as obtained from **283A**.

The cis enyne moiety of **283A**, **285A**, **285C** and **285E** is isomerized much more slowly than the cis diene. In the presence of 2% acetone, **285C** does produce a longer retention time minor isomer (~15%) after 13 h light exposure. This peak had an identical mass spectrum to **285C** but, as with **283A'**, showed an IR absorption at 968 cm^{-1} in its FTIR spectrum, a spectrum otherwise very similar to **285C**. Only a trace of this material was detected in the absence of sensitizer.

Assignment of structures to many of these photoisomers requires further study. For example, **287B**, which has both a C-4 and a C-5 diene, gives after 12 h of daylight exposure, four major GC peaks in addition to starting material, whereas only four isomers total are

predicted on the basis of a simple cis-trans photoisomerization. Photoisomerization of HTX (283A) likewise should produce a mixture of four isomers, although only three were detected after acetone-sensitized light exposure.

New HTX Alkaloids and Artefacts:

An HTX alkaloid of molecular weight 261 had been reported earlier in two species of Peruvian dendrobatid frogs, *Dendrobates quinquevittatus* and *Dendrobates trivittatus* (1). This HTX 261 now has been detected in one more species of Peruvian dendrobatid frog (unpublished results). The electron impact mass spectrum of 261 indicated an allyl cleavage (m/z 261-->220). Since an FTIR spectrum of this material (see Fig. 5) indicated no acetylenic absorption, it is postulated that the enyne moiety at C-7 of HTX 259 has been replaced by a diene moiety in HTX 261.

An extract of skin from the specimen of the Surinam dendrobatid frog *Dendrobates azureus* (11) was found to contain a very minor HTX alkaloid 265E of molecular weight 265 (CI (NH_3), m/z 266), exhibiting a major fragment ion at m/z 96 in its E.I. mass spectrum. High resolution mass measurements (12) of 265E established the formula $C_{17}H_{31}NO$ for the molecular ion (M^+) (265, 5%) and the following formulae for major fragments: $C_{17}H_{30}N$ (248, 10%, M^+-OH); $C_{14}H_{26}NO$ (224, 48%, M^+-C_3H_5), $C_{14}H_{24}NO$ (222, 20%, M^+-C_3H_7); $C_{10}H_{18}N$ (152, 100%), $C_9H_{17}N$ (139, 63%) and $C_6H_{10}N$ (96, 95%). Evidently the m/z 152 and m/z 139 fragments are analogous to the major fragments with 178 and 165 observed in the E.I. mass spectrum of 291A, suggesting that a similar fragmentation pathway is operative here and supporting the presence of a 3-butenyl moiety at C-7. A terminal double bond is supported by its FTIR spectrum (see Fig. 5, 265E), which shows 916, 977 cm^{-1} and 3084 cm^{-1} absorptions.

An extract from skins of a Costa Rican population of *Dendrobates pumilio* (13), showed in addition to substantial amounts of HTX alkaloids, a trace GC-MS peak at a retention time slightly greater than those of the HTX alkaloids with an evident molecular ion at m/z 297, confirmed by chemical ionization. The molecular ion had no exchangeable hydrogens as determined by chemical ionization with ND_3 (MD^+ = 299). Tentatively, we concluded that this peak could be a formaldehyde condensation product of a dihydro-HTX. In addition, another GC peak of equivalent intensity was seen at a slightly greater retention time with m/z 299 as its molecular ion, perhaps the analogous formaldehyde reaction product from a tetrahydro HTX. Reaction of a reference sample of 285A with a 100-fold excess of formalin (37.5% aqueous, stabilized with methanol) in methanol for 24 h gave approximately 5% of an m/z 297 product at approximately the same retention time and having virtually the same mass spectrum: 297 (80), 254 (20), 230 (90), 204 (33), 200 (30), 176 (45), 162 (72), 148 (20), 134 (25), 120 (30), 109 (50), 96 (100) as seen for the compound detected in the alkaloid fraction from *Dendrobates pumilio*. Addition of a trace of formic acid increased the proportion of the 297 product to 35% after another 24 h. Unfortunately for direct comparison purposes, the alkaloid fraction from *Dendrobates pumilio* determined by GC-MS to have the m/z 297/299 materials in September 1988, now no longer showed these compounds to be present when

reanalyzed in 1990. We have concluded that these materials most probably arose from **285A** (or **285C**) and **287A** and are artefacts of reaction with traces of formaldehyde present in the reagent grade methanol used in the extraction procedure. The possibility does remain, however, that these are naturally occurring derivatives.

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8. **235A** ($C_{15}H_{25}NO$): m/z 235 (9), 220 (5), 218 (10), 194 (68), 176 (33), 150 (25), 96 (100). $[\alpha]_D$ - 38.6 (c, 1.75 $CHCl_3$).
9. The following is a representative procedure: To ~80 µg **285A** in 100 µl freshly purified (Aluminum Oxide - Super 1) 1,2-dimethoxyethane (DME) are added 10 µl (3.5 eq.) of n-$BuB(OH)_2$ [Alltech Assoc. Inc., Deerfield, IL 60015] in DME (10 mg/ml). The solution is stirred 1 h at rt, then 20 µl of N-methylaminoethanol (Aldrich) in DME (7.5 mg/ml) are added to react with the excess reagent and stirred for 1 h at rt. One µl portions of the resulting solution are used for GC-MS; 5 µl for GC-FTIR.
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11. Skin sample provided by the National Aquarium in Baltimore, MD. Analysis of alkaloids in this sample will be published separately.
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13. Analysis of alkaloids in this sample from a population of *Dendrobates pumilio* collected in June, 1988 in the Rio Sarapiqui drainage, Heredia, Costa Rica, will be published separately.