ELSEVIER

Journal of Chromatography A, 704 (1995) 483–494

JOURNAL OF CHROMATOGRAPHY A

Hygrine, bona fide alkaloid or artifact: its chemical reduction, novel di-heptafluorobutyrylation and sensitive detection in South American coca leaves using capillary gas chromatography–electron capture detection

James M. Moore*, John F. Casale, Patrick A. Hays, Robert F.X. Klein, Donald A. Cooper

Special Testing and Research Laboratory, United States Drug Enforcement Administration, 7704 Old Springhouse Road, McLean, VA 22102-3494, USA

First received 31 August 1994; revised manuscript received 16 February 1995; accepted 16 February 1995

Abstract

Methodology is described for the isolation of hygrine, along with the related compound cuscohygrine, from South American coca leaf and its major tropane alkaloids. The isolated hygrine was reduced with lithium aluminum hydride to yield diastereomeric alcohols, which were subsequently derivatized with heptafluorobutyric anhydride in the presence of 4-dimethylaminopyridine. The resultant diastereomeric di-heptafluorobutyryl derivatives could be detected on-column at femtogram levels when using a polar fused-silica capillary column interfaced with a ^{63}Ni electron-capture detector. The artifactual formation of hygrine, resulting from the degradation of cuscohygrine, is discussed. Quantitative data are provided for hygrine and cuscohygrine levels in South American coca.

1. Introduction

Most known alkaloids in the leaf of the South American coca plant, *Erythroxylum coca*, possess a tropane moiety [1]. Two exceptions are the N-methylpyrrolidine alkaloids hygrine, 1, and cuscohygrine, 2, seen in Fig. 1. Recently, and for the first time, quantitative data were reported for 1 and 2 in coca leaf, along with the tropane alkaloids cocaine, *cis*- and *trans*-cinnamoylcocaine, the truxillines, tropacocaine and ecgonine methyl ester [2]. In that study, detailed analytical methodology, reaction pathways and derivative structures were not given for 1 and are, therefore, reported here. Furthermore, it was suspected that the presence of 1 in some coca leaf might be in part or in whole artifactual, resulting from the degradation of 2.

In the study described herein, methodology is provided for the isolation of 1 and 2 from coca leaf tissue and the bulk tropane alkaloid matrix. Once isolated, 1 was subjected to chemical reduction using lithium aluminum hydride ($LiAlH_4$), followed by derivatization with heptafluorobutyric anhydride (HFBA)/4-dimethylaminopyridine (4-DMAP) and analysis of the resultant di-heptafluorobutyryl (di-HFB) derivatives using capillary gas chromatography–elec-

* Corresponding author.

0021-9673/95/$09.50
SSDI 0021-9673(95)00219-7

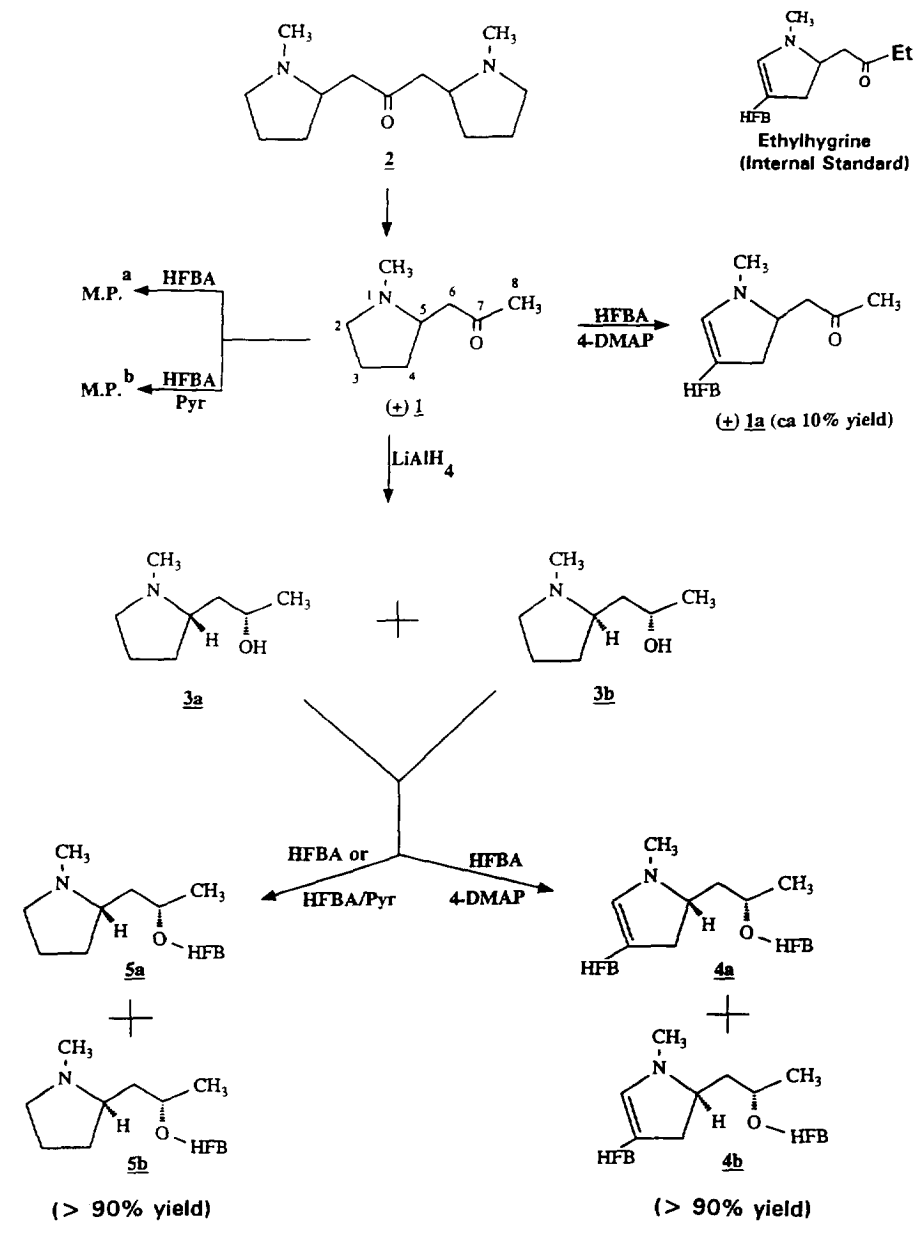

Fig. 1. Formation of hygrine (1) from cuscohygrine (2); lithium aluminum hydride reduction of 1 to yield diastereomeric hygrinols (3a and 3b), followed by heptafluorobutyrylation, with or without catalyst, to yield either heptafluorobutyryl (HFB) derivatives 5a and 5b or di-heptafluorobutyryl (di-HFB) derivatives 4a and 4b. [a] M.P. = Multiple perfluorinated cGC–ECD peaks, none of which appeared analytically useful; [b] M.P. = multiple perfluorinated cGC–ECD peaks, none of which appeared analytically useful; peaks differed from those generated using HFBA without pyridine catalyst.

electron capture detection (cGC–ECD). The relationship between 1 and 2, with respect to the formation of the former from the latter, is discussed.

2. Experimental

2.1. Solvents, chemicals and materials

All solvents were distilled-in-glass products of Burdick and Jackson Labs (Muskegon, MI, USA). HFBA was supplied in 1-ml sealed glass ampules and obtained from Pierce (Rockford, IL, USA). A 1.0 *M* solution of $LiAlH_4$ in diethyl ether and 4-DMAP were supplied by Aldrich (Milwaukee, WI, USA). Celite was provided by J.T. Baker (Jackson, TN, USA) and was used without further treatment. A pH 4.0 acid phthalate buffer was prepared according to the United States Pharmacopeia XIX. All other chemicals were of reagent-grade quality.

2.2. Standards

The syntheses of 1 (Fig. 1) and an internal standard analogue, ethylhygrine (Fig. 1), were accomplished using the methods of Thomsen et al. [3] and Ghirlando et al. [4]. The $LiAlH_4$ reduction of 1 and ethylhygrine yielded diastereomeric hygrinols 3a and 3b, and ethylhygrinols, respectively. The procedure of de Boer and Speckamp [5] was used for the preparation of 2.

2.3. Gas chromatography

Two gas chromatographs (GCs) and three capillary columns were used in this study. The first, a Hewlett-Packard 5880A GC–^{63}Ni ECD interfaced with a Level IV data processor, was used in the initial phase of this study for the determination of 1 in coca leaves. This GC was operated in the splitless mode and was fitted with a 15 m × 0.25 mm I.D. fused-silica capillary column coated with DB-5+ (0.25 μm) (J&W Scientific, Rancho Cordova, CA, USA). The oven temperature was programmed as follows: (level 1) initial temperature, 90°C; initial hold, 5.5 min; temperature program rate, 5°C/min; final temperature, 160°C; final hold, 5 min; (level 2) temperature program rate, 4°C/min; final temperature, 275°C; final hold, 10 min. Injector and detector temperatures were both held at 300°C. Hydrogen was used as a carrier gas at 30–40 cm/s and argon–methane (95:5) as detector make-up gas at 30–40 ml/min.

The second chromatograph was a Hewlett-Packard 5890 Series II GC with dual ^{63}Ni ECD detectors interfaced with two 30 m × 0.25 mm I.D. fused-silica capillary columns, one coated with DB-1701 (0.25 μm) and the other with DB-1 (0.25 μm). The GC oven temperature was programmed as follows for both columns: initial temperature, 90°C; initial hold, 1.0 min; program rate, 6.0°C/min; final temperature, 275°C; final hold, 5 min. Injector and detector temperatures were maintained at 230 and 300°C, respectively. Hydrogen was used as the carrier gas at a velocity of 35–40 cm/s measured at an oven temperature of 90°C. Argon–methane (95:5) was the make-up gas at a flow of 30–40 ml/min.

2.4. Mass spectrometry

A Hewlett-Packard Model 5972 quadrupole mass-selective detector (MSD) interfaced with a Hewlett-Packard 5890 Series II GC was used for the characterization of 1 and 1a, 4a and 4b, and 5a and 5b (Fig. 1). The MSD was operated in the electron ionization mode with an ionization potential of 70 eV, an EMV of 1541 and at 1.2 scans/s. The GC was fitted with a 30 m × 0.25 mm I.D. fused-silica capillary column coated with DB-1 (0.25 μm) and used helium as the carrier gas. The oven was temperature-programmed as follows: initial temperature, 80°C; initial hold, 5 min; program rate, 4°C/min; final temperature, 285°C.

2.5. Nuclear magnetic resonance spectrometry

A Varian Unity 500 NMR spectrometer operating at 500 MHz for proton and equipped with a 5 mm I.D. indirect detection probe was used for the structural determination of 4a and

4b. Sample analysis was accomplished at 25°C. The sample was run in deuterated chloroform (Aldrich, Milwaukee, WI, USA), with tetramethylsilane (TMS) added as an internal standard.

2.6. Coca leaf

The leaf material used in this study was cultivated in the South American countries of Bolivia, Peru, Ecuador and Colombia. After harvesting, the leaves were air-dried and then shipped to our laboratory, where they were stored under ambient conditions prior to analysis. At the time of analysis most leaf had an approximate age of 1–2 years from the time of harvesting.

2.7. Isolation of 1 from coca leaf

Dried leaf tissue was powdered in a Wiley mill to pass a 2-mm sieve. About 1–5 g of the powdered coca leaf was accurately weighed, thoroughly mixed with 1–5 ml of saturated aqueous sodium bicarbonate, and placed in a 50-ml centrifuge tube containing an appropriate quantity of ethylhygrine internal standard. For a 1-g sample weight, about 25–30 ml of water-saturated toluene, containing a known quantity of ethylhygrine internal standard, was added and the tube was heated at 60–65°C for 1 h with occasional mixing. The tube was then centrifuged and the toluene layer transferred to a flask. The coca leaf powder residue was re-extracted twice more in the same manner; the combined toluene extracts were transferred to a chromatographic column (260 mm × 22 mm), packed with a mixture of 4 ml of 0.18 *M* sulfuric acid and 8 g of Celite 545. After passing the toluene extracts through the column, an additional 20 ml of water-saturated toluene, followed by 30 ml of water-saturated chloroform, were added; all eluates were discarded. The tropane alkaloids, 1, 2 and the ethylhygrine internal standard were then all eluted from the column with 25 ml of water-saturated chloroform containing 250 μl diethylamine (DEA), followed by 30 ml of water-saturated chloroform.

2.8. Isolation of 1 and 2 from other major coca alkaloids

A 5.0-ml aliquot from the chloroform–DEA eluate above was extracted with 7 ml of a pH 4.0 phthalate buffer and the chloroform phase (containing cocaine and the cinnamoylcocaines) discarded. The buffer (containing 1 and 2) was then back-extracted with 15 ml of chloroform, the latter being discarded. A 1-ml aliquot of 4 *M* sodium hydroxide was added to the buffer, which was then extracted with 3 × 3-ml aliquots of chloroform, filtering the extracts through anhydrous sodium sulfate into a 10-ml volumetric flask. After dilution to volume with chloroform, a 3.0-ml aliquot was transferred to a tube containing 0.3 ml of 0.05 *M* HCl gas in methanol. After vortex-mixing, the solution was evaporated to dryness.

2.9. $LiAlH_4$ reduction of 1

To the residue above was added 1 ml of methylene chloride and the resulting solution heated at 50°C for several minutes with occasional vortex-mixing. Upon cooling, 5 ml of anhydrous ethyl ether was added to the tube, followed by 0.2 ml of 1 *M* $LiAlH_4$ in ethyl ether. After vortex-mixing, the solution was reduced in volume to about 0.5 ml by heating at 50–55°C. About 6 ml of acetone was slowly added to the tube and the contents cooled to 0°C and held there for 10 min. After centrifugation, the acetone supernatant was transferred to another tube containing 5 ml of the methanol–HCl solution and evaporated to dryness.

2.10. HFBA derivatization of diastereomeric 3a and 3b and ethylhygrinols

To the residue above was added 1 ml of acetonitrile, 50 μl of HFBA and 100 mg of 4-DMAP, and the tube was heated at 75°C for 1 h. After cooling, 5.0 ml of isooctane was added to the tube, followed by 5 ml of saturated

aqueous sodium bicarbonate, and the contents mixed vigorously without delay. After centrifugation, the isooctane was back-extracted with 5 ml of 0.18 *M* sulfuric acid (to remove 4-DMAP) and the isooctane layer was then dried over anhydrous sodium sulfate.

2.11. cGC–ECD analysis

About 2 μl of the isooctane extract from above was injected onto the 15 m DB-5+ capillary column using conditions described under Experimental. Also injected was a mixed standard containing the di-HFB derivatives of standard diastereomeric hygrinols and ethylhygrinol internal standards of known concentration. For calculation purposes the peak areas for each diastereomeric pair were summed.

3. Results and discussion

3.1. Isolation of 1 from coca leaf and other coca alkaloids

Toluene was found to be an effective solvent for the high-yield extraction of 1, 2 and other alkaloids from powdered coca leaf tissue [2]. The bulk alkaloid matrix, including 1 and 2, was quantitatively isolated from non-alkaloidal leaf components by acid/Celite column chromatography. After removal from the column, 1 and 2 were isolated from cocaine and the bulk of other tropane alkaloids by partitioning between chloroform and the pH 4.0 phthalate buffer. The higher pK_b of 1 and 2 allowed for their quantitative extraction into the buffer (along with small quantities of isomeric truxillines), while most of the cocaine and other alkaloids were retained in the chloroform phase. Because of its higher volatility, subsequent reduction-in-volume steps involving 1 (as well as 2) were always preceded by its conversion to the hydrochloride ion pairs. It should also be noted that the method recoveries of 1 and the ethylhygrine internal standard were similar and both $>80\%$.

3.2. Reduction and/or chemical derivatization of 1 and diastereomeric 3a and 3b

Given the suspected low levels of 1 in coca leaf, it was believed that the more sensitive ^{63}Ni ECD detector would be required for their analyses, as opposed to the less sensitive flame-ionization detector (FID). This required the high-yield introduction of an electrophilic group onto 1. We have previously described the unusual quantitative attachment of HFB or HFBO groups at carbon sites in other, similar nitrogen heterocycles, including nicotine, itself an N-methylpyrrolidine alkaloid [6–8].

The reactions outlined in Fig. 1 used racemic (synthetic) 1 as the precursor compound. Early in this study, standard 1 was subjected directly to HFBA derivatization in the presence of 4-DMAP, with the intention of incorporating an HFB group into 1; this would allow for its sensitive detection by cGC–ECD. Although this reaction produced the desired product 1a, as seen in Fig. 1, the yield was unacceptably low ($<10\%$). This was surprising, given the intense coloration of the reaction solution, which suggested a higher yield. Furthermore, little or no unreacted 1 was detected. Modest attempts to improve the yield of 1a were unsuccessful. It is also seen in Fig. 1 that the reaction of 1 with HFBA in the presence or absence of pyridine yielded multiple HFB-substituted products, none of which appeared analytically useful. It should be noted that no 1a was produced from these latter reactions; nor was there a significant presence of unreacted 1. Furthermore, the mass spectra of these diverse derivatization products contained no base peak ion at m/z 84, suggesting that modification of the N-methylpyrrolidine ring had occurred.

Given the low yield of 1a, additional attempts were made at providing an improved ECD-responsive derivative from racemic 1. This included the $LiAlH_4$ reduction of 1 to yield diastereomeric alcohols, 3a and 3b (Fig. 1). Subsequent derivatization of these compounds with HFBA in the presence or absence of pyridine yielded two major products in high yield, believed to be the diastereomeric 5a and 5b (Fig.

1). No 4a or 4b were detected as products of this reaction. When 3a and 3b were treated with HFBA in the presence of 4-DMAP, the diastereomeric pair 4a and 4b were afforded in good yield. In fact, the yield of 4a + 4b was markedly greater than that for 1a, obtained by the reaction of 1 with HFBA/4-PMAP (Fig. 1).

It was concluded, as outlined in Fig. 1, that the LiAlH4 reduction of 1 to give 3a and 3b, followed by HFBA/4-PMAP derivatization to yield 4a and 4b, provided the methodology of highest sensitivity for the detection of 1. Not only was the derivatization yield for 4a and 4b high, but the introduction of two HFB groups into a small molecule such as 3a,b provided highly electrophilic derivatives which were ideally suited for sensitive cGC–ECD detection.

3.3. Mass spectral analysis of 1a, 4a and 4b, and 5a and 5b

Given that the structure of 1 was known, characterization of 1a (Fig. 1) could be rationally derived from its EI mass spectrum, seen in Fig. 2a. The molecular ion at m/z 335 supported the introduction of a single HFB group and the concomitant formation of a double bond in 1. This was in agreement with our previous observations regarding the heptafluorobutyrylation of tertiary nitrogen heterocyclic compounds

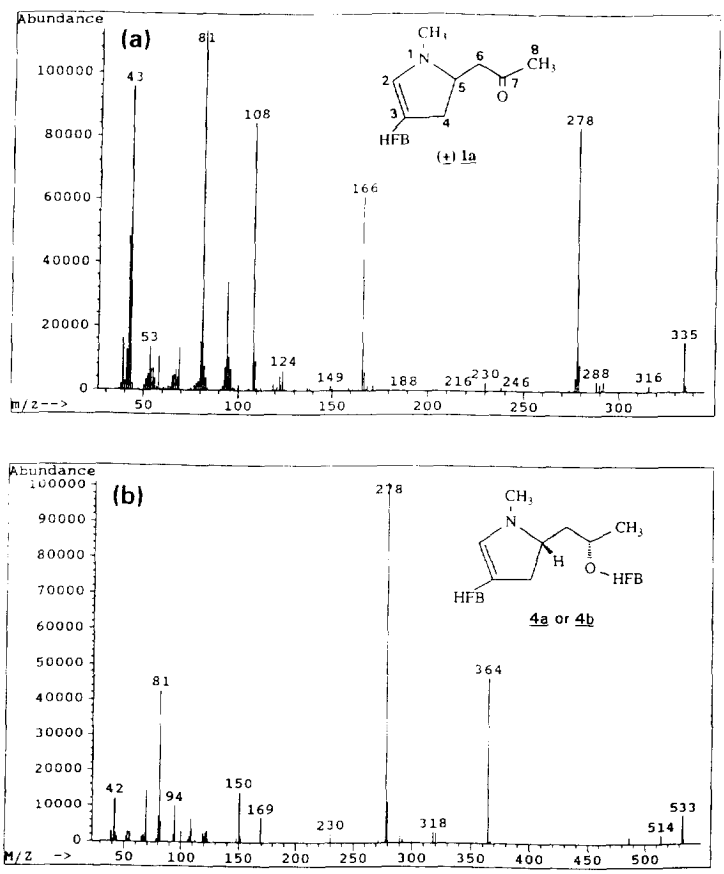

Fig. 2. Electron-ionization mass spectra of (a) derivatization product 1a, obtained by the reaction of hygrine (1a) with heptafluorobutyric anhydride (HFBA) in the presence of 4-dimethylpyridine (4-DMAP) (see Fig. 1) and (b) diastereomeric derivatization product 4a or 4b, obtained by the reaction of hygrinol diastereomers (3a and 3b) with HFBA in the presence of 4-DMAP (see Fig. 1).

[6,7]. The fragment ion at m/z 278 was accounted for by loss of the 2-propanone side chain, also confirming the presence of the HFB/C = C grouping in the N-methylpyrrolidine ring, probably arranged in a vinylogous amide configuration. Supporting this modification of the N-methylpyrrolidine ring was the absence of a fragment ion at m/z 84, which in the mass spectrum of untreated 1 is due to the N-methylpyrrolidinium ion. A prominent ion at m/z 166 is almost certainly due to the loss of the $CF_3CF_2CF_2$ radical. The extraction characteristics of 1a suggested a reduced basicity for this derivative, supporting a vinylogous amide, with (a) the double bond at C2 = C3 and HFB at C3, or (b) double bond at C4 = C5 and HFB at C4, or (c) double bond at C5 = C6 with HFB at C6. The presence of the intense m/z 278 ion (see above and Fig. 2a) discounted proposal (c), as loss of the side chain would not be likely. The intense ion at m/z 108, believed to be due to the loss of $CF_3CF_2CF_2$ and acetone (involving carbon nos. 6, 7 and 8 and the proton at C4) from the molecular ion, discounted proposal (b). The loss of acetone inferred the presence of a proton at C5, which accommodated the structure in proposal (a). The base peak ion at m/z 81 was believed to be due to losses of the HFB moiety and the $-CH_2-CO-CH_3$ side chain from the 1a molecular ion.

The mass spectra of 5a and 5b (see structures in Fig. 1) were virtually identical, with each exhibiting what was believed to be a low-intensity molecule ion at m/z 339, indicating the presence of a sole HFB moiety. A base peak ion at m/z 84 represented loss of the side chain and concomitantly placed the HFB substituent on that leaving group, almost certainly at the secondary alcohol site. The appearance of a low-intensity ion at m/z 126 was probably due to loss of the heptafluorobutyryloxy group from the molecular ion. The presence of a fragment ion at m/z 169 was believed to be due to $[CF_3CF_2CF_2]^+$.

As expected, the EI mass spectra of diastereomeric 4a and 4b, seen in Fig. 2b, were also virtually identical. The molecular ion at m/z 533 necessitated the presence of two HFB groups, with one site of HFB substitution again being at the secondary alcohol. The fragment ion at m/z 364 was due probably to loss of $CF_3CF_2CF_2$ from the molecular ion. The base peak ion at m/z 278 was accounted for by the loss of the CH_2-CH(OHFB)-CH_3 side chain. Amide extraction characteristics were also evident for 4a and 4b and suggested, (a) a double bond at C2 = C3 with HFB attachment at C3, or (b) a double bond at C4 = C5 with an HFB substituent at C4 or (c) a double bond at C5 = C6 with the HFB group at C6. Since 4a and 4b are diastereomers (the result of perfluoroacylation of the diastereomeric precursors 3a and 3b and as evidenced by 2 GC peaks), a proton at C5 was required. This necessitated placement of the double bond at C2 = C3 with HFB attachment at C3.

3.4. Nuclear magnetic resonance spectral analysis of 4a and 4b

NMR spectroscopy verified that two compounds were present in nearly equal amounts and that they were diastereomers. Proton and carbon assignments are found in Table 1, with proton–proton scalar coupling constants in Table 2.

An indirect detection 2D heteronuclear multiple-quantum coherence (HMQC) experiment was used to determine carbon–proton bonding ($^1J_{CH}$). An indirect detection 2D heteronuclear multiple-bond coherence (HMBC) experiment was used to establish skeletal connections between carbons and protons with $^2J_{CH}$ and $^3J_{CH}$ couplings. A 2D correlation spectroscopy (COSY) experiment was used for proton–proton coupling correlations with $^2J_{HH}$ and $^3J_{HH}$ couplings. These 2D experiments and the normal, direct detection proton and carbon spectra allowed for the assignment of all chemical shifts to the two compounds.

The 2D COSY experiment detected a linear five carbon chain CH_2-CH-CH_2-CH-CH_3 for both compounds. This chain corresponds with carbons C4 through C8 respectively. All other protons are isolated from this chain.

The methine at C7 and proton and carbon chemical shifts verify that HFB substitution

Table 1
NMR assignments for 4a and 4b

Position	Compound 4a		Compound 4b	
	Proton[a]	Carbon[a]	Proton[a]	Carbon[a]
NCH3	3.11 s	34.9 or 35.1	3.11 s	34.9 or 35.1
2	7.36 s	156.0	7.32 s	156.2
3	–	approx. 108[b]	–	approx. 108[b]
4	2.70 dd, 3.13 dd	33.4	2.76 dd, 3.20 dd	33.6
5	3.75 m	60.8	3.86 m	61.1
6	1.72 ddd, 2.27 ddd	38.4	2.04 ddd, 2.11 p	37.9
7	5.25 ddq	73.2	5.32 as	73.5
8	1.45 d	20.2	1.42 d	19.6

s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, as = apparent sextet.
[a] Chemical shifts are in ppm relative to TMS.
[b] Approximate value. C3 was indirectly detected using HMBC.
Chemical shifts of the carbons in the HFB groups were not determined due to peak splitting from the fluorine atoms.

occurred at the alcohol. The methine at C5 and proton and carbon chemical shifts confirm the nitrogen substitution. The methylene at C4 indicates that the alkene double bond is from C2 to C3. A singlet in the proton spectrum at 3.11 ppm has two corresponding carbon chemical shifts (34.9 and 35.1 ppm) indicating N-methyl groups for 4a and 4b. The remaining olefinic proton is found as a singlet at 7.36 (4a) and 7.32 ppm (4b) with its carbon at 156.0 (4a) and 156.2 ppm (4b). Because this proton is a singlet and is not coupled to any other protons (i.e., the C4 methylene), its position is at C2, verifying HFB substitution at C3.

Table 2
Coupling constants for 4a and 4b ($^{2,3}J_{HH}$ in Hz)

Positions	Compound 4a	Compound 4b
4ab	15.4	15.4
4a,5	6.8	7.1
4b,5	5.1	11.2
5,6a	10.9	3.8
5,6b	2.9	7.5
6ab	14.0	14.6
6a,7	2.6	5.2
6b,7	10.2	7.5
7,8	6.2	6.3

The 5-membered ring is verified by using the 2D HMBC experiment. Through-bond connections are present between the N-methyl protons and C1 and C7. This experiment also confirms a link between the C4 protons and C1 and C2.

Tentative assignments of compounds 4a versus 4b was accomplished using a molecular modelling program. After minimizing energy for these molecules, dihedral angles were determined for H4a/H5, H4b/H5, H5/H6a, H5/H6b, H6a/H7, and H6b/H7, and their coupling constants estimated [9]. These estimates were then compared with experimental values taken from the proton spectrum.

3.5. Yield of 4a and 4b versus derivatization time

At a temperature of 75°C, the diastereomeric mixture of 3a and 3b was subjected to derivatization with HFBA/4-PDAP at reaction times of 10, 20, 45 and 60 min. After summation of the diastereomeric pair peak areas for each of the four reactions, it was determined that the yields

of 4a and 4b did not vary significantly with time. However, it was noted that at the reduced reaction times, the levels of undesirable by-products increased.

3.6. Chromatography and on-column MDQ of 4a and 4b

The chromatography of 4a and 4b, along with the di-HFB derivatives of the corresponding ethylhygrinol diastereomeric internal standard, were investigated using three capillary columns of differing polarity. These included two 30 m × 0.25 mm I.D. fused-silica capillary columns, one coated with DB-1 (0.25 μm) and the other with DB-1701 (0.25 μm). The third column was a 15 m × 0.25 mm I.D. fused-silica capillary column coated with DB-5 + (0.25 μm). Retention time data for 4a and 4b and the di-HFB derivatives of the ethylhygrinol diastereomers for the three columns are presented in Table 3. The most suitable columns were found to be DB-5 + and DB-1701. Fig. 3 illustrates the cGC–ECD chromatography on the DB-5 + column for the determination of 1 in Colombian coca leaves.

The on-column minimum detectability quantity (OC-MDQ) of 4a and 4b was determined on the 30-m DB-1701 column. About 1 mg of 3a + 3b was derivatized with HFBA/4-DMAP and then serially diluted with isooctane until no cGC–ECD response for the derivatives 4a and 4b was observed. The OC-MDQ value for each diastereomer was approximately 400–600 fg.

Table 3
Retention time data for 4a, 4b and the di-HFB derivatives of diastereomeric ethylhygrinol internal standard (see Fig. 1)

Compound	Retention time (min)[a]		
	DB-1	DB-1701	DB-5 +
4a[b]	15.07	19.58	18.62
4b[b]	15.20	19.83	18.85
Ethygr-ISa[c] (di-HFB)	16.23	20.35	20.00
Ethygr-ISb[c] (di-HFB)	16.33	20.58	20.20
Aldrin IS[d]	20.07	21.26	–

[a] See Experimental for column dimensions and oven temperature parameters.
[b] The di-HFB derivatives of diastereomeric hygrinol (see Fig. 1).
[c] The di-HFB derivatives of diastereomeric ethylhygrinol internal standard.
[d] Aldrin used as instrumental internal standard (IS).

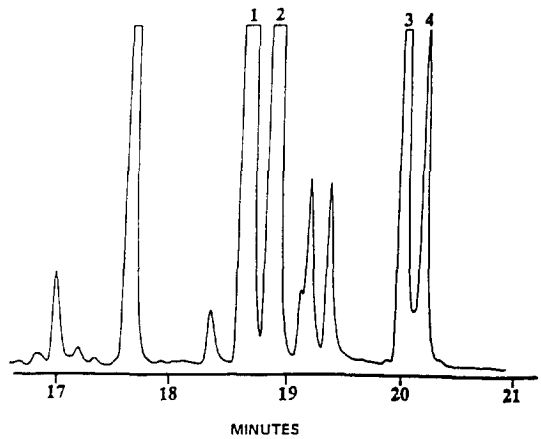

Fig. 3. The cGC–ECD chromatogram of hygrine (1) determined in Colombian coca leaves. Peaks: 1,2 (18.6 min, 18.8 min) = 4a and 4b in Fig. 1; 3,4 (20.0 min and 20.2 min) = di-HFB derivatives of diastereomeric ethylhygrinol (obtained via $LiAlH_4$ reduction and HFBA derivatization of ethylhygrine internal standard).

3.7. Content of 1 in coca leaves–bona fide alkaloid or artifact

The content of 1 in coca leaf from Bolivia, Peru, Ecuador and Colombia was determined soon after the samples were reduced to powder form. After storage of the coca powder under ambient conditions for 2–3 weeks, the analyses were repeated for the Bolivian and Peruvian samples. This data is presented in Table 4. As seen, the low levels of 1 in the Bolivian and Peruvian coca justified the use of the more sensitive cGC–ECD methodology. It is also seen that the same coca leaf powder yielded higher results for 1 after storage under ambient conditions for several weeks. This clearly indicates that 1 can be formed as an artifact in situ. For this reason, the data in Table 4 should be considered the "maximum" level for "true" 1 in the leaf sample. It was surmised that the artifact-

Table 4
Hygrine (1) content of South American coca leaf samples from Bolivia, Peru, Ecuador and Colombia[a]

Country	Hygrine content[b] (%)	
	1 Day[c]	2–3 Weeks[c]
Bolivia	2.3	5.2
Peru	1.4	4.8
Ecuador	4.2	–
Colombia	24	–

[a] Coca leaf samples were harvested and maintained as dry whole leaf under ambient conditions 1–2 years prior to cGC–ECD analyses.
[b] Hygrine (1) content expressed as % (w/w) relative to cocaine content.
[c] Period of time between powdering of the coca leaf and the determination of 1.

ual formation of 1 was enhanced by powdering of the sample and subsequent storage at room temperature (as opposed to sub-ambient temperatures). The preliminary results of a parallel study [10] suggested that keeping the powder at or near 0°C before analysis may retard the increase in 1. However, further work is needed to confirm this observation. It is not known whether 1 is produced in the whole leaf between the time of harvesting and analyses, a period that can be rather lengthy. It is recommended that for maximum accuracy, the determination of 1 and 2 should be carried out soon after harvesting of the leaf. If this is not possible, then the leaf material should be air-dried and stored at or near 0°C over a dehydrating agent prior to reduction to a powder and immediate analysis.

It was concluded that the content of 1 in the Colombian leaf (Table 4) was mostly bona fide. However, although it is not believed that artifactual 1 contributed significantly to this result, it is likely that at least trace levels are present. The level of 1 in the Colombian coca was in general agreement with a relatively high content of 1 in a "fresh" leaf sample harvested in northern Peru [10]. It was estimated that for this latter sample, the time between harvesting and analysis was less than one month; in addition, the sample was analyzed for 1 immediately after powdering, thus establishing 1 as a bona fide alkaloid.

Given the artifactual increase of 1 seen in Table 4, it was reasoned that there should be a concomitant decrease in the levels of 2. To substantiate this hypothesis, a Peruvian coca leaf sample was powdered and then analyzed for 2 over a 2-month period. During this time, the powdered sample was stored under ambient conditions. The results, seen in Table 5, revealed a marked decrease in the level of 2 over the defined time period. As was the case with 1, it is not known if the level of 2 begins decreasing immediately upon harvesting and continues with storage of the whole leaf at ambient temperature.

In addition to the artifactual formation of 1 described above, we have observed that during routine column and thin-layer chromatographic analyses of coca leaf extracts, small amounts of 1 can be created, (again) presumably from the degradation of 2. It is believed that the levels of artifactual 1 formed in this manner are quite small and probably represent $<2\%$ w/w (relative to cocaine). Work is currently on-going in the development of methodology for the isolation of 1 from 2 using column chromatographic techniques. It has also been observed that during cGC–FID analyses of coca leaf for 2, a relationship existed between the formation of 1 from 2 and injection port temperature. This can be seen

Table 5
Degradation of cuscohygrine (2) in powdered Peruvian coca leaf as a function of storage and time[a]

Date of analysis	Cuscohygrine content[b] (%)
3/7/90	95
3/28/90	67
5/8/90	36

[a] Coca leaf sample was harvested and maintained as dry whole leaf under ambient conditions 1–2 years prior to cGC–ECD analyses; sample was powdered prior to the first analysis and subsequently stored at room temperature.
[b] Cuscohygrine content express as % (w/w) relative to the cocaine content.

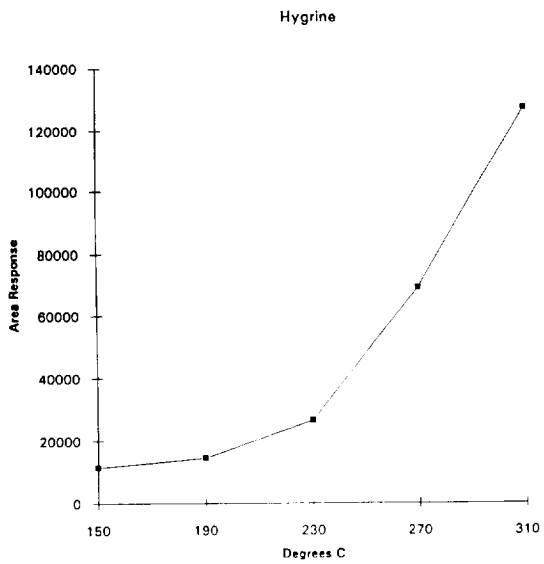

Fig. 4. Artifactual formation of 1 after cGC–FID injection of chloroform solution of standard 2 at increasing injection port temperatures; injections carried out in fused-silica injection port liner at a split ratio of 20:1.

in Fig. 4 for standard 2, from which marked increases of 1 were observed at injection port temperatures in excess of 230°C. It should be emphasized, however, that for the cGC–ECD methodology described herein, the injection port conversion of 2 to 1 is a non-factor, since 1 is first reduced and then heptafluorobutyrylated prior to chromatographic determination.

In summary, it is clear that 1 is a bona fide alkaloid in some coca leaf. It is also apparent that this compound can be produced artifactually, due to improper storage of the coca powder (and perhaps of the whole leaf as well). It also appears that trace levels of 1 can be formed during routine acid–base extractions. Artifactual 1 almost certainly results from the degradation of 2. As mentioned previously, it is not known whether significant amounts of 1 are produced from 2 during prolonged storage of the whole, dry coca leaf under ambient conditions. It is recommended, therefore, that for the determination of both 1 and 2, the analyses be carried out soon after harvesting of the leaf. If this is not feasible, then storage of the air-dried whole leaf over a dehydration agent at or near 0°C is recommended. Although it appears that by storing powdered coca leaf at 0°C the degradation of 2 to 1 was retarded, it is recommended that determination of these alkaloids in stored leaf be done immediately after the sample is powdered.

4. Conclusions

Sensitive methodology has been described for the detection and quantitation of hygrine in cocaine-bearing plants of South America. After isolation of hygrine and cuscohygrine from the leaf and other alkaloids, it is reduced with lithium aluminum hydride and the resultant diastereomeric hygrinols derivatized with heptafluorobutyric anhydride in the presence of 4-dimethylaminopyridine. The resulting di-heptafluorobutyryl derivatives could be detected on-column at femtogram levels when using a cGC–ECD equipped with a fused-silica capillary column. Hygrine levels of 1% w/w or less (relative to cocaine) in coca leaf could be determined when using a structurally-related internal standard. Although hygrine has been confirmed as a bona fide alkaloid of coca leaf, the storage of leaf in the powdered form at room temperature can result in the artifactual formation of this compound, due probably to the degradation of cuscohygrine. It is not known if artifactual hygrine is produced when whole coca leaf is stored for prolonged time periods at room temperature. Hygrine can also be produced as an artifact during work-up of the coca leaf sample and in the injection port of the gas chromatograph, due to the thermal degradation of cuscohygrine.

References

[1] C.E. Turner, B.S. Urbanek, G.M. Waller, in Cocaine–An Annotated Bibliography, Vols. I and II, Research Institute of Pharmaceutical Sciences, University of Mississippi, Oxford, MS, 1988.

[2] J.M. Moore, J.F. Casale, R.F.X. Klein, D.A. Cooper and J. Lydon, J. Chromatogr. A, 659 (1994) 163–175.

[3] I. Thomsen, K. Clausen, S. Scheibye and S.O. Lawesson, Org. Synth., 62 (1984) 158–163.
[4] R. Ghirlando, A.S. Howard, R.B. Katz and J.P. Michael, Tetrahedron, 40 (1984) 2879–2884.
[5] J.J. de Boer and W.N. Speckamp, Proc. 11th IUPAC Int. Symp. Chem. Nat. Prod., 1978, Vol. 3, pp. 129–132.
[6] J.M. Moore, A.C. Allen and D.A. Cooper, Anal. Chem., 56 (1984) 642–646.
[7] J.M. Moore, A.C. Allen, D.A. Cooper and S. Carr, Anal. Chem., 58 (1986) 1656–1660.
[8] J.M. Moore, D.A. Cooper, T.C. Kram and R.F.X. Klein, J. Chromatogr., 645 (1993) 273–281.
[9] R.M. Silverstein, G.C. Bassler, T.C. Morrill, in Spectrometric Identification of Organic Compounds, 4th edn., John Wiley and Sons, New York, NY, 1981.
[10] J.M. Moore and J.F. Casale, unpublished results.