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BIOSYNTHESIS OF DIOSCORINE FROM TRIGONELLINE IN *DIOSCOREA HISPIDA**

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Key Word Index—*Dioscorea hispida*; Dioscoreaceae; dioscorine; alkaloid biosynthesis; trigonelline; ^{2}H NMR spectroscopy.

Abstract—[6-^{14}C,2-^{3}H]Nicotinic acid, [6-^{14}C,6-^{3}H]nicotinic acid, [*methyl*-^{14}C,2-^{2}H,^{3}H]trigonelline, and [*methyl*-^{14}C,6-^{2}H,^{3}H]trigonelline were all incorporated into the isoquinuclidine moiety of the alkaloid dioscorine found in the yam *Dioscorea hispida* with complete retention of ^{3}H relative to ^{14}C. A chemical degradation on the dioscorine derived from the labelled trigonellines indicated that all the ^{14}C was located on its *N*-methyl group. ^{2}H NMR spectroscopy established that the samples of dioscorine derived from the [2-^{2}H]- and [6-^{2}H]trigonelline were labelled with deuterium at the 3-*pro-R* and C-1 positions, respectively. These results are consistent with a new hypothesis for the biosynthesis of dioscorine.

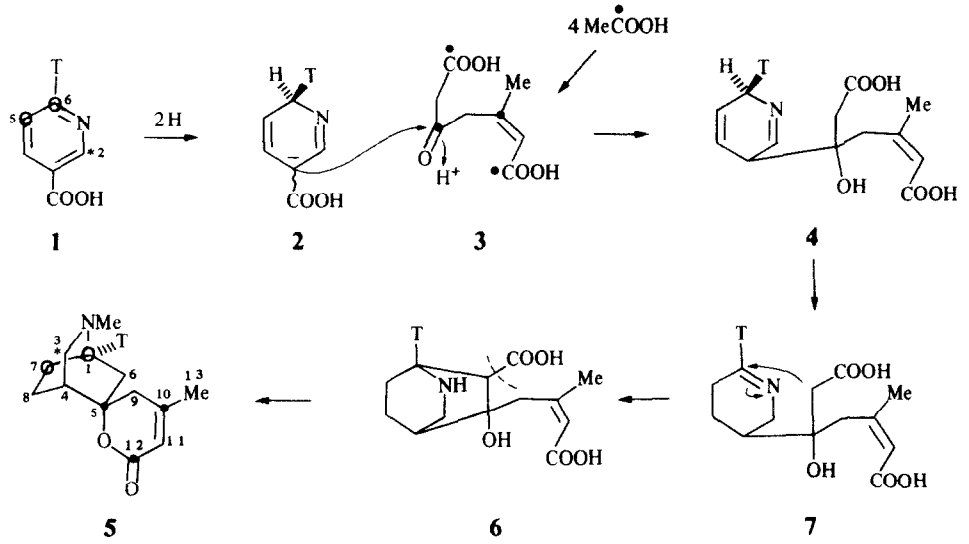
INTRODUCTION

In 1977 [1, 2] we made the unexpected discovery that nicotinic acid (**1**) serves as a precursor of the isoquinuclidine moiety of dioscorine (**5**), an alkaloid found in the yam *Dioscorea hispida* Dennstedt. In particular [5,6-$^{13}C_2$]nicotinic acid afforded dioscorine labelled at C-1 and C-7 (established by ^{13}C NMR). In a complimentary feeding experiment [2-^{14}C]nicotinic acid yielded dioscorine which was labelled at C-3 (established by chemical degradation). It had been previously shown that the other carbons of the isoquinuclidine moiety and those of the unsaturated lactone were derived from acetic acid [3]. Thus, radioactive dioscorine derived from sodium [1-^{14}C] acetate had most of its activity at C-5, C-10, and C-12 and was equally divided between these positions. Scheme 1 is the biosynthetic sequence which was proposed to rationalize these resutlts. The pattern of labelling in the dioscorine from these precursors being illustrated with various symbols. In this scheme, activation of C-3 of nicotinic acid is achieved by reduction to 3,6-dihydronicotinic acid (**2**). This compound, a β-imino carboxylic acid, condenses with the ketone group of **3**, a branched 8-carbon unit derived from four acetate units, possibly with concomitant decarboxylation, to yield **4**. The dihydropyridine moiety of **4** is reduced to a tetrahydropyridine and a double bond migration is proposed to afford **7**. The isoquinuclidine ring system is then generated by an aldol condensation to yield **6**. Final steps to dioscorine involve *N*-methylation (presumably from the *S*-methyl group of methionine), decarboxylation and lactone formation. The present article describes our feeding experiments with multiple labelled nicotinic acid and trigonelline, designed to probe details of this proposed biosynthetic scheme. Some of these results have been reported in a preliminary communication [4].

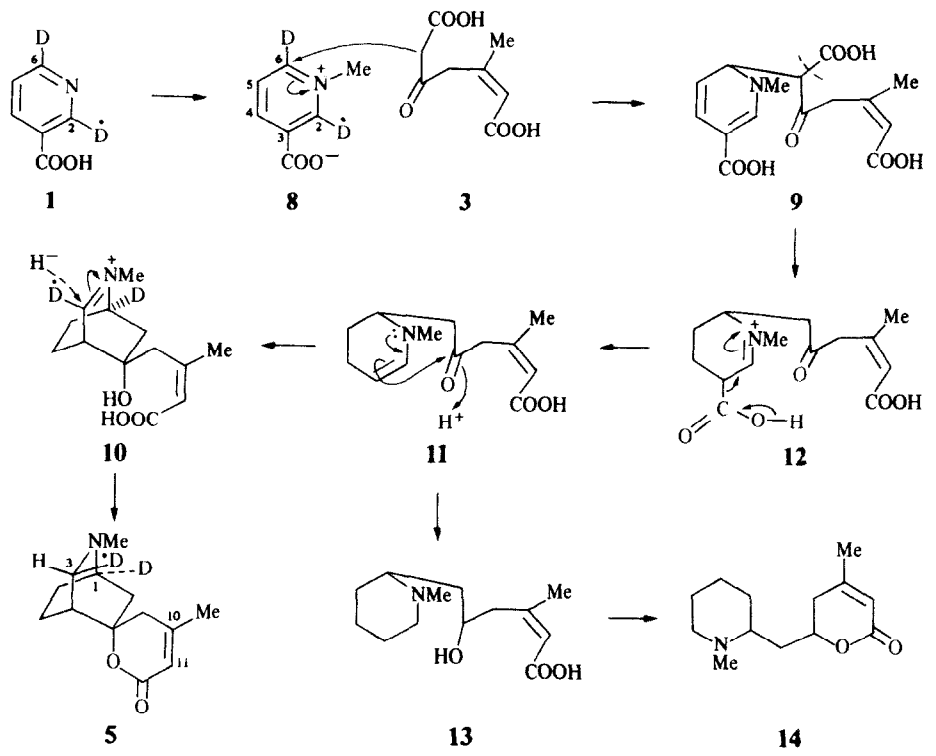
*Contribution No. 209 from this Laboratory.

RESULTS AND DISCUSSION

Dioscorine derived from [6-^{14}C,2-^{3}H] nicotinic acid had the same ^{3}H/^{14}C ratio as in the administered precursor. This complete retention of tritium is consistent with Scheme 1, since no loss of hydrogen from the carbon which was originally C-2 of nicotinic acid seems probable in any of the suggested biosynthetic steps. The same retention of tritium was discovered when [6-^{14}C,6-^{3}H] nicotinic acid was administered to *D. hispida*. However, in order to accommodate this result into Scheme 1 we have to postulate two stereospecific reactions. The first would be a stereospecific reduction of nicotinic acid to **2** in which the tritium is arbitrarily depicted as illustrated in Scheme 1. In the conversion of **4** to **7** the hydrogen introduced in the initial reduction has to be the one lost in the isomerization of a double bond. Such stereospecific reactions are certainly possible in biochemical reactions and have been observed in the conversion of nicotinic acid to anatabine, in which 3,6-dihydronicotinic acid was also proposed as an intermediate [5]. However, these required stereospecific reactions and also the lack of any analogous biochemical reaction for the decarboxylation of **6**, caused us to consider an alternative biosynthesis for dioscorine which is illustrated in Scheme 2. In this scheme nicotinic acid is made susceptible to nucleophilic attack by the 8-carbon unit **3** by formation of trigonelline (**8**) as illustrated, affording **9**. Nucleophilic attack of trigonelline is well known [6], and pyridinium ions react with carbanions under biomimetic conditions at their C-2 and C-4 positions [7]. Intermediate **9** then undergoes reduction of its dihydropyridine ring and shift of a double bond to yield the β-imino acid **12**. Loss of the carboxyl group originally present in nicotinic acid affords the enamine **11**. The isoquinuclidine ring system is then generated by reaction of this enamine with the ketone group to afford **10**. Dioscorine is then formed by reduction of this iminium ion, and lactone formation. Reduction of both the ketone and the tetrahydropyridine ring of compound **11** yields **13**. Lactone formation then affords dumetorine



Scheme 1. Old hypothesis for the biosynthesis of dioscorine.



Scheme 2. New hypothesis for the biosynthesis of dioscorine involving trigonelline as an intermediate.

(14) which is an alkaloid found in *D. dumetorum* [8] along with dihydrodioscorine (**5**, where the $C_{10,11}$-double bond is reduced).

This new hypothesis was tested by feeding [*methyl*-^{14}C,2-^{2}H,^{3}H]- and [*methyl*^{14}C,6-^{2}H,^{3}H]trigonelline to *D. hispida* plants. Both served as precursors of dioscorine (Table 1) with complete retention of tritium relative to ^{14}C. It was established that all the ^{14}C was located on the *N*-methyl group of dioscorine by demethylating with diethyl azodicarboxylate [9]. By this reaction the *N*-methyl group is removed and liberated as formaldehyde which was collected and assayed as its dimedone derivative. The location of the deuterium in the samples of labelled dioscorine was established by ^{2}H NMR spectroscopy. It was necessary to unequivocally assign the ^{1}H NMR of dioscorine, since deuterium NMR chemical shifts are almost identical with proton chemical shifts [10]. The assignments of both the ^{1}H and ^{13}C NMR

Table 1. Precursors fed to *D. hispida* and activity of the resultant dioscorine

Precursor	Date of initial feeding*	Yield of dioscorine (mg)	% Retention of ^3H rel. to ^{14}C	% Incorporation into dioscorine	
				Spec.†	Abs.‡
[6-^{14}C,2-^{3}H]-Nicotinic acid (51.3 mg) ($^3H/^{14}C = 16.5$)	Sept. 16 1985	260	102	0.022	0.056
[6-^{14}C,6-^{3}H]-Nicotinic acid (53.4 mg) ($^3H/^{14}C = 9.53$)	Aug. 14 1985	641	104	0.53	3.51
[*methyl*-^{14}C,6-^{2}H,^{3}H]-Trigonelline (78.5 mg) ($^3H/^{14}C = 1.58$)	Oct. 24 1986	490	99	0.15 [0.14]	0.53
[*methyl*-^{14}C,2-^{2}H,^{3}H]-Trigonelline (49.4 mg) ($^3H/^{14}C = 2.71$)	July 11 1987	65	106	1.62 [1.5]	1.33

*All feedings were done by the cotton wick method, the initial administration of precursor, dissolved in water, being spread over four days, and then continued for a total of five weeks.

†Specific inc. = dpm/mM (^{14}C) in dioscorine/dpm/mM (^{14}C) in precursor. The value indicated in brackets [ ] was calculated from the intensity of the ^2H NMR signals.

‡Absolute inc. = total activity (^{14}C) in dioscorine/total activity (^{14}C) in precursor.

Table 2. Chemical shifts (ppm from TMS) of carbons and their attached hydrogens of dioscorine (in $CDCl_3$)

C	^{13}C NMR (ppm)	^{1}H NMR (ppm)
1	52.23 (1)*	2.36
3	53.65 (2)	2.23 (3b), 2.73 (3a)
4	35.00 (1)	1.70
5	81.40 (0)	—
6	40.77 (2)	1.55 (6 endo), 1.86 (6 exo)
7	20.14 (2)	1.31 (7 endo), 1.74 (7 exo)
8	19.37 (2)	1.25 (8 exo), 1.96 (8 endo)
9	39.34 (2)	2.41
10	155.74 (0)	—
11	116.20 (1)	5.59
12	164.88 (0)	—
13	23.27 (3)	1.79
N-Me	42.52 (3)	2.10

*Number of attached hydrogens determined by the DEPT pulse sequence.

(Table 2) were made by examination of its 2D-HETCOR and 2D-COSY NMR. The 2D-HETCOR spectrum (Fig. 1) revealed that the ^{13}C NMR signals previously [2] assigned to C-6 and C-9 were incorrect and should be reversed. The hydrogens of interest, and the ones expected to be labelled with deuterium from the administered labelled trigonelline, were located at C-1 and C-3 of dioscorine. The region of the ^{1}H NMR spectrum where the signals for these hydrogens occur is illustrated in Fig. 2. The hydrogen at C-1 is a pentet centred at 2.36 ppm. Since the isoquinuclidine moiety of dioscorine is rigid, geminal hydrogens at methylene groups give rise to different chemical shifts and exhibit coupling with each other. The hydrogens at C-3 are labelled H_a and H_b. The $3H_a$ resonance at 2.73 ppm is a doublet of triplets ($^2J_{3a,3b} = 10.7$ Hz, $^3J_{3a,4} = 3.1$ Hz, $^4J_{3a,8\ endo} = 3.1$ Hz). This splitting pattern arises from a 4-bond *W*-coupling between the $3H_a$ and 8H endo, superimposed on the geminal and vicinal couplings. Since the ^3J and ^4J coupling have the same value the expected doublet of doublets of doublets collapses to the observed doublet of triplets. The ^3H_b signal at 2.23 ppm is a doublet of doublets ($^2J_{3b,3a} = 10.7$ Hz, $^3J_{3b,4} = 2.1$ Hz). No 4-bond *W*-coupling is possible for the ^3H_b position since C-5 contains no hydrogens. The ^{2}H NMR spectra of the enriched samples of dioscorine derived from the deuterium labelled trigonelline are illustrated in Fig. 3. The spectra were determined in chloroform, the natural abundance of $CDCl_3$ in this solvent serving as an internal standard with a resonance at 7.26 ppm. The specific incorporation (determined by ^{14}C assay) of the [*methyl*-^{14}C,6-^{2}H,^{3}H]trigonelline into dioscorine was 0.15%. However the only significant ^{2}H NMR signal in this enriched dioscorine was at 2.36 ppm which is the chemical shift of the hydrogen at C-1. This result is thus consistent with the new biosynthetic scheme for dioscorine. The incorporation of the [*methyl*-^{14}C,2-^{2}H,^{3}H]trigonelline into dioscorine was much higher (1.6%) and some $CDCl_3$ was added to the $CHCl_3$ solvent in which its ^{2}H NMR spectrum was determined in order to obtain a significant signal at 7.26 ppm. In this sample the only strong signal was at 2.79 ppm which corresponds to H_a at C-3 (the 3-*pro-R* hydrogen). No deuterium signal was detected at 2.23 ppm (^3H_b). This result indicates that there has been a stereospecific reduction at the position which was originally C-2 of nicotinic acid. In Scheme 2, this result can be rationalized by proposing that the iminium salt **10** is reduced by a hydride anion (from NADH for example) approaching from the less hindered side, i.e. the side remote from where the ultimate lactone ring is located. Integration of the ^{2}H NMR signals at 2.36 and 2.79 ppm in the enriched dioscorine samples relative to known amounts of $CDCl_3$ in the solvent and the intensity of the signal at 7.26 ppm enabled us to estimate the specific

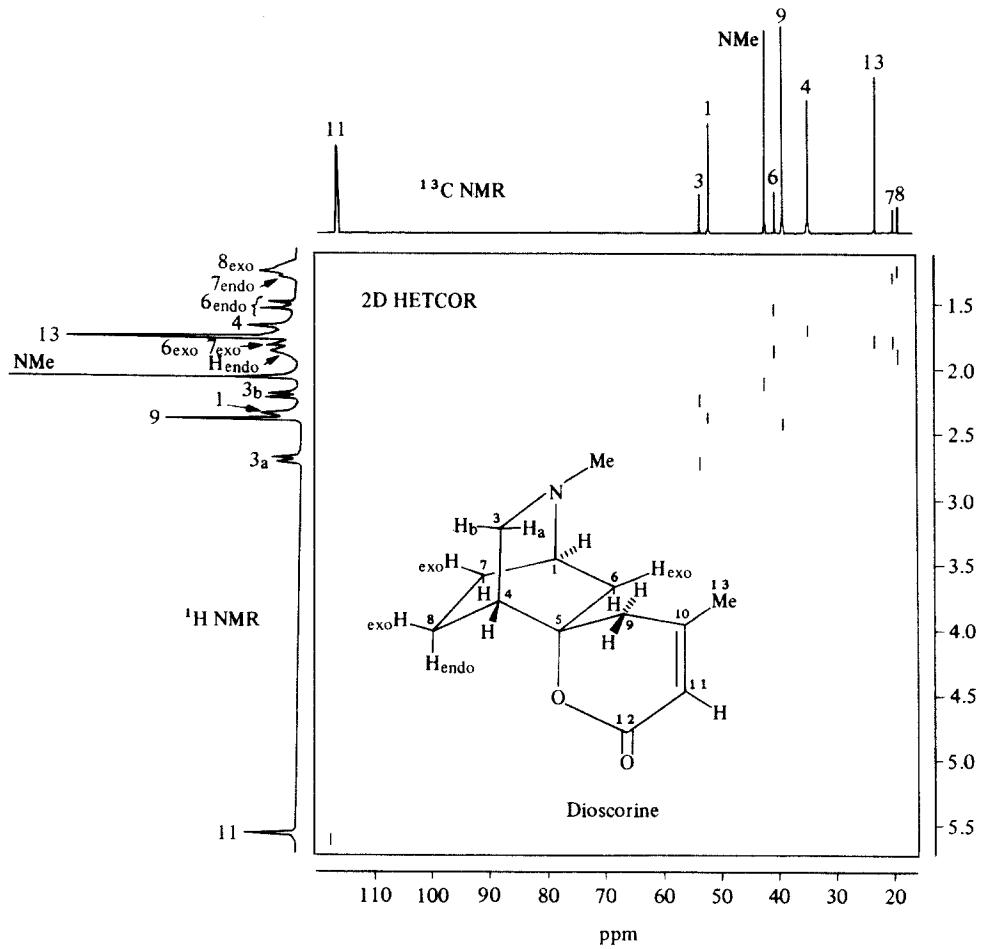


Fig. 1. 2D HETCOR NMR spectrum of dioscorine in $CDCl_3$.

incorporation of these [^{2}H]trigonellines into the alkaloid. The results obtained (Table 1) are in good agreement with the specific incorporations determined by ^{14}C assay. There seems to be no consistency to the degree of incorporation of nicotinic acid and trigonelline into dioscorine (Table 1). In previous work [2] the absolute incorporations of [2-^{14}C] nicotinic acid and [5,6-$^{13}C_2$,^{14}C]nicotinic acid into dioscorine was 1.9 and 0.42%, respectively.

Tritium and deuterium labelled nicotinic acid were made as previously described [11] and were mixed with commercially available [6-^{14}C]nicotinic acid prior to feeding by means of cotton wicks inserted into the stems of the plant. Trigonelline was made by reaction of the [2-^{2}H,^{3}H]- or [6-^{2}H,^{3}H] nicotinic acid with [^{14}C]methyl iodide [12]. The location of the deuterium was confirmed by ^{2}H NMR.

EXPERIMENTAL

General. Radioactive materials were assayed by liquid scintillation counting using dioxane–EtOH as solvent with the usual scintillators [13]. NMR spectra were determined at 300 and 75.5 MHz, respectively, for ^{1}H and ^{13}C, with the assistance of Dr S. B. Philson. All recorded spectra are ppm from TMS. MS were determined by Dr E. Larka and his assistants at the University of Minnesota. GC was carried out on a 25 m glass capillary column coated with cross-linked Me silicone (0.52 μm thick) int diam 0.31 mm, using the following instrument parameters: He 1 ml/min, inj temp 250°, initial oven temp 50°, equilibration time 4 min, rate of temp increase 30°/min, final temp 250°.

Labelled precursors. [2-^{2}H, ^{3}H]- and [6-^{2}H, ^{3}H]nicotinic acids were made from 2-bromo-3-methylpyridine and 2-bromo-5-methylpyridine, respectively. The organo-Li derivatives derived from these bromo compounds were quenched with D_2O or triturated H_2O. The resultant [^{2}H] and [^{3}H]-labelled samples of 3-methylpyridine were oxidized with $KMnO_4$ to yield the labelled nicotinic acids as previously described [11]. The [6-^{14}C] nicotinic acid was purchased from Amersham.

[*methyl*-^{14}C,6-^{2}H,^{3}H]*Trigonelline.* [^{14}C]MeI (0.2 ml, 3 mmol, nominal activity 4.4×10^8 dpm) was added to a soln of [6-^{2}H]nicotinic acid (260 mg, 2.1 mmol) and [6-^{3}H]nicotinic acid (21.3 mg, 0.17 mmol, 6.6×10^8 dpm) in *n*-BuOH (0.5 ml) and the mixt heated in a sealed tube at 100° for 3 hr. The contents of the tube, dissolved in H_2O (50 ml) was shaken with freshly prepd AgOH (from 10% NaOH and 3.4 g $AgNO_3$). The filtered soln was treated with H_2S to remove traces of Ag^+ and again filtered. The residue obtained on evapn of the filtrate was crystallized from EtOH-Et_2O to afford colourless prisms of [*methyl*-^{14}C,6-^{2}H,^{3}H]trigonelline. (0.23 g, 73%, sp. act. ^{14}C. 1.27×10^8 dpm/mmol, sp. act. ^{3}H: 2.01×10^8 dmp/mmol, ^{2}H content by MS and ^{2}H NMR spectroscopy: 92%), mp 216° (dec), lit. [11]

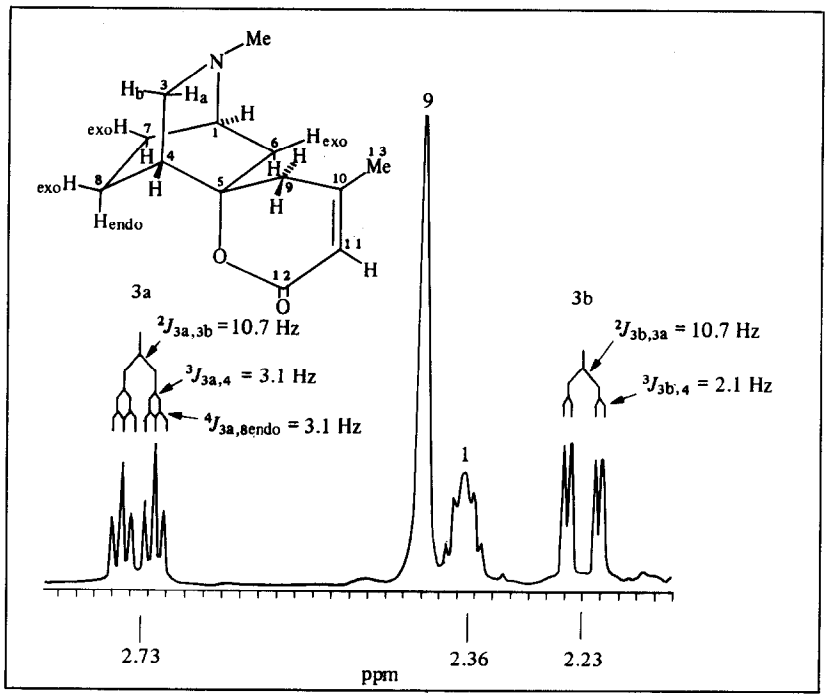


Fig. 2. ^{1}H NMR spectrum (2.0–3.0 ppm) of dioscorine in $CDCl_3$.

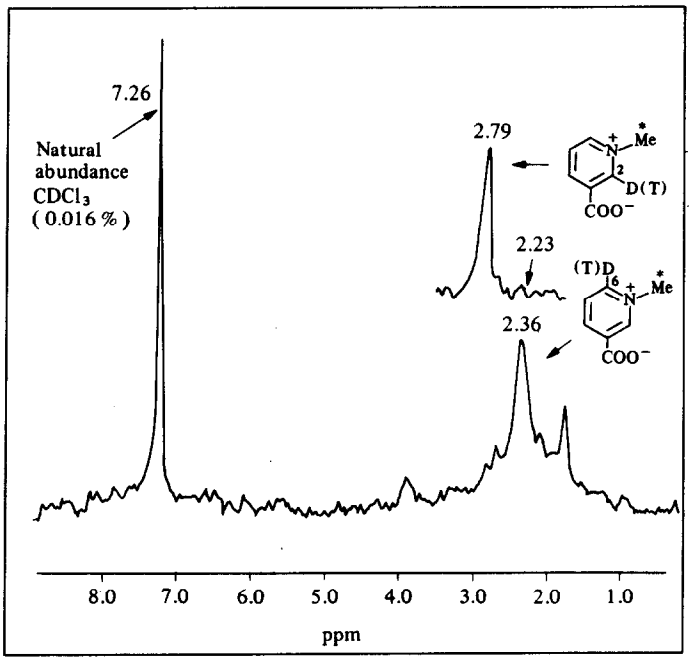


Fig. 3. ^{2}H NMR spectra (in $CHCl_3$) of dioscorine derived from [2-^{2}H]- and [6-^{2}H] trigonelline.

mp 218° (dec.), ^{1}H NMR (D_2O) δ 4.57 (*s*, 3H, NMe), 8.06 (*d*, 1H, $J_{5,4}$ = 8.02 Hz, H-5), 8.75 (*d*, 1H, $J_{4,5}$ = 8.02 Hz, H-4), 9.03 (*s*, 1H, H-2), the signal at 8.83 in unlabelled trigonelline (due to H-6) was missing; also the H-5 signal in unlabelled material was a triplet. ^{13}C NMR (D_2O) δ 170.4 (C=O), 148.6 (C-2), 147.5 (C-4), 139.7 (C-3), 130.4 (C-5), 51.0 (NMe). ^{2}H NMR (H_2O) δ 8.83 (*s*, D-6) the signal due to HO*D* being at 4.84 ppm; MS (70 eV) *m/z* (rel. int.) 139 ($[M + 1]^+$, 11.8), 138 ($[M]^+$, 4.5), 96 (18), 94 (16), 44 (100). TLC on silica gel, developing with MeOH–conc. NH_3 (9:1) indicated that > 99% of its radioactivity was at a spot (R_f 0.3)

coincident with authentic trigonelline. The [*methyl*-^{14}C, 2-^{2}H,^{3}H]trigonelline was prepared in a similar fashion from [2-^{2}H,^{3}H]nicotinic acid.

Administration of precursors and isolation of dioscorine. The amounts and activities of precursors fed are recorded in Table 1. Dioscorine was isolated as previously described [2] and purified by dist. and crystallization of its picrate. The free base has a GC R_t of 14.45 min.

^{2}H NMR spectra. Spectra of enriched dioscorine (100 mg) derived from [6-^{2}H]trigonelline were determined in $CHCl_3$ (458 mg) with the following instrument parameters: frequency 30.72 MHz, no. of transients 13 000, acquisition time 1.66 sec. (total time 6 hr), spectral window 615.7 Hz, spectrometer offset −324.0 Hz, line broadening 1.0. Less time was required to obtain the ^{2}H NMR spectrum of the dioscorine derived from [2-^{2}H] trigonelline since the specific incorporation was much higher.

Demethylation of dioscorine. Dioscorine (138 mg, ^{14}C: 2.16 × 10^{6} dpm/mmol, ^{3}H/^{14}C = 2.87) derived from [*methyl*-^{14}C,2-^{2}H,^{3}H] rigonelline was dissolved in C_6H_6 (7 ml) and diethyl azodicarboxylate (0.3 ml) added. After 22 hr at 18° the soln was evapd and the residue dissolved in 1 N HCl (20 ml) and dist. into a soln of dimedone (200 mg) in H_2O (100 ml). H_2O was added to the dist. flask to maintain the vol. and dist. continued until 60 ml of distillate was obtained. On standing for 18 hr at 4° the dist deposited crystals of formaldehyde-dimedone (36 mg) which were crystallized from MeOH, sp. act. ^{14}C: 2.21 × 10^{6} dpm/mmol, no ^{3}H).

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