Phytochemistry, 1977, Vol. 16, pp. 1705–1709. Pergamon Press Printed in England.

BIOSYNTHESIS OF DIOSCORINE : INCORPORATION OF NICOTINIC ACID INTO THE ISOQUINUCLIDINE MOIETY

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(*Received* 25 *March* 1977)

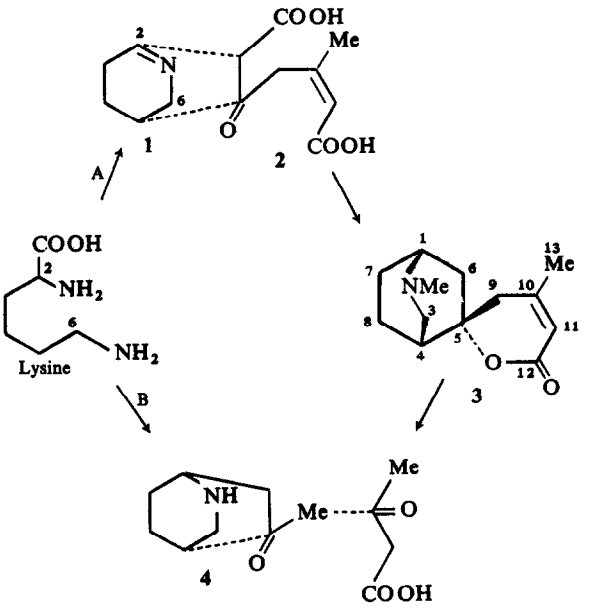
Key Word Index—*Dioscorea hispida*; Dioscoreaceae; dioscorine; alkaloid biosynthesis; nicotinic-[2-^{14}C] acid; nicotinic-[5,6-$^{13}C_2$] acid; ^{13}C NMR spectroscopy.

Abstract—The administration of nicotinic-[2-^{14}C] acid to *Dioscorea hispida* plants afforded radioactive dioscorine (1.9% absolute incorporation) and a systematic degradation of the alkaloid indicated that essentially all the activity was located at C-3. Dioscorine derived from nicotinic-[5,6-^{14}C,$^{13}C_2$] acid was also labelled. Its proton noise decoupled ^{13}C NMR spectrum contained satellites at C-1 and C-7 due to spin–spin coupling of contiguous ^{13}C atoms arising from direct incorporation of the labelled nicotinic acid. A biosynthetic scheme representing a novel utilization of nicotinic acid is proposed.

INTRODUCTION

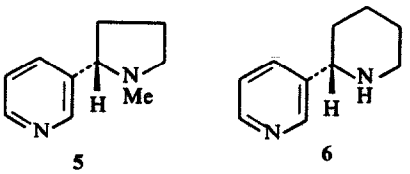
We have previously [1] established that the administration of acetate-[1-^{14}C] to the tropical yam, *Dioscorea hispida*, Dennst., yielded radioactive dioscorine (**3**) which was labelled on alternate carbons of the unsaturated lactone ring (C-5: 31%, C-10: 28%, C-12: 28%). This result led us to suggest that dioscorine is formed by a condensation between a branched 8-carbon unit (**2**) (derived from 4 acetate units) and Δ^1-piperideine (**1**) (formed from lysine in higher plants [2,3]) as illustrated in Scheme 1, route A. Spenser [4] proposed a slight modification in which pelletierine (**4**) (formed from lysine and two acetate units) condenses with acetoacetate (route B). However the administration of DL-lysine-[2-^{14}C] [1] or DL-lysine-[6-^{14}C] (see Experimental) yielded dioscorine with negligible activity (0.003 and 0.007% incorporations respectively). Dioscorine obtained from plants which had been fed Δ^1-piperideine-[6-^{14}C] contained a significant amount of activity (0.03% incorporation). If this compound had been incorporated in accordance with route A all the activity of the dioscorine would be located at C-3. A partial degradation of the labelled dioscorine indicated that the Δ^1-piperideine had not been incorporated specifically. The pattern of labelling was essentially the same as that in the dioscorine derived from acetate-[1-^{14}C] indicating that the Δ^1-piperideine was catabolized to acetate prior to incorporation [1]. More recently Spenser [5] also failed to obtain specific incorporation of labelled Δ^1-piperideine into dioscorine.

Nicotinic acid is the established precursor of the pyridine rings of the tobacco alkaloids nicotine (**5**) [6] and anabasine (**6**) [7]. It is also incorporated intact into ricinine (**7**) [8,9]. However, it is not usually considered to be a precursor of piperidine rings, and we would probably not have considered that it could be a precursor of dioscorine if we had not discovered that both rings of anatabine (**8**) are derived from nicotinic acid [10, 11]. A working hypothesis utilizing nicotinic acid (**9**) as a precursor of the isoquinuclidine moiety of dioscorine is illustrated in Scheme 2. It is suggested that nicotinic acid is reduced to 3,6-dihydronicotinic acid (**10**). We currently favour this compound as the activated form of nicotinic acid which is involved in the biosynthesis of nicotine and other tobacco alkaloids. A concerted decarboxylation and condensation with the branched 8-carbon acetate derived unit (**2**) yields compound (**11**). In our preliminary communication [12] it was suggested that decarboxylation occurred after this initial condensation. However, this is a minor modification and we realize that this step and subsequent ones in this biosyn-



Scheme 1. Hypothetical formation of dioscorine from lysine and acetic acid.

* Contribution No 149 from this laboratory.



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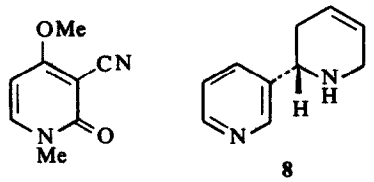

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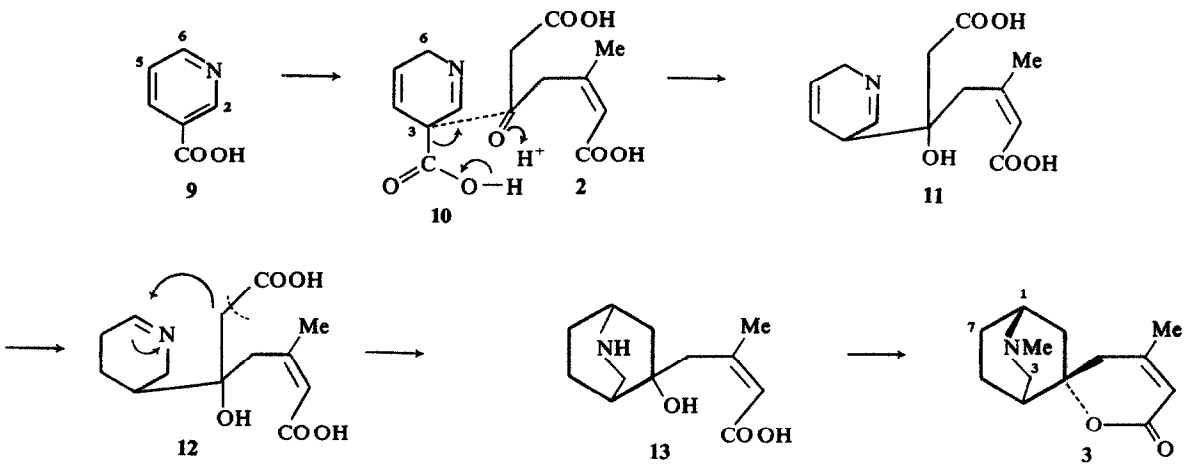
thetic scheme are tentative. Shift of a double bond in the dihydropyridine (**11**) and further reduction of the ring affords (**12**). An aldol condensation, possibly with decarboxylation, leads to (**13**) which contains the isoquinuclidine ring system. Dioscorine then arises by lactone formation and N-methylation.

RESULTS AND DISCUSSION

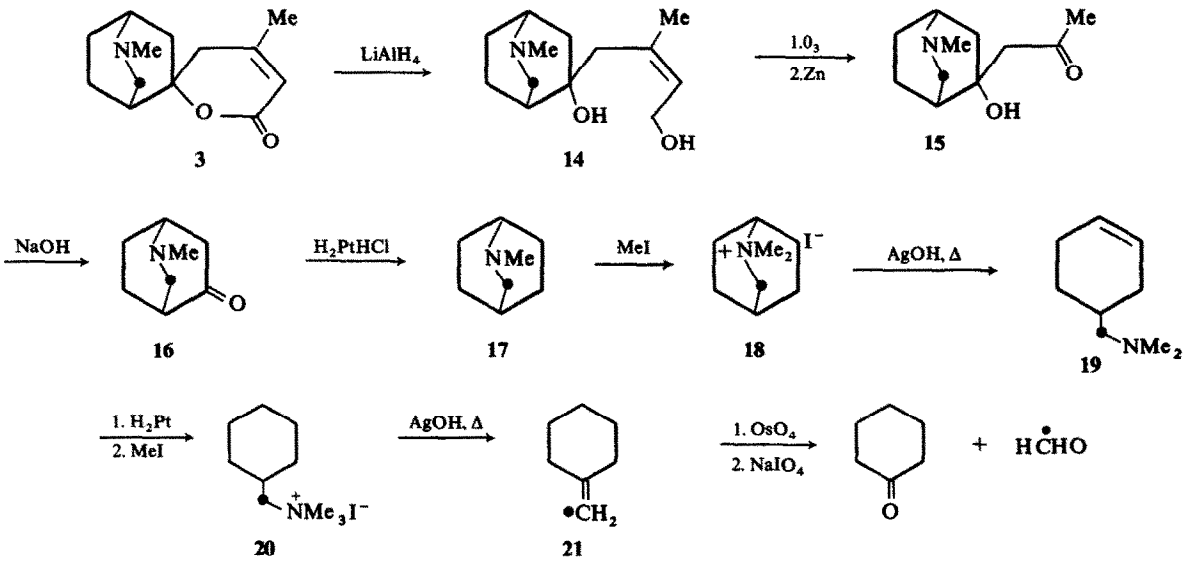
This biosynthetic scheme was initially tested by feeding nicotinic-[2-^{14}C] acid to *D. hispida* plants. In order to maximize incorporation, the tracer was fed (by the wick method) over a period of two weeks and the plants were then allowed to grow for an additional 5 weeks. The dioscorine obtained from the plants was radioactive (1.9% absolute incorporation). If the nicotinic-[2-^{14}C] acid had been incorporated in accordance with the scheme in Scheme 2, activity would be localized at C-3. A degradation (Scheme 3) was carried out to isolate this particular carbon. Reduction of dioscorine with $LiAlH_4$ yielded dioscorinol (**14**) [13], which on ozonolysis and treatment with zinc afforded 5-acetonyl-5-hydroxy-2-methylisoquinuclidine (**15**). This acetonyl derivative undergoes a retroactive aldol condensation with dilute NaOH yielding 2-methyl-5-oxoisoquinuclidine (**16**) [14, 15]. Hydrogenation of this ketone in the presence of HCl yielded 2-methylisoquinuclidine (**17**), which was converted to its methiodide (**18**) [15]. A Hofmann elimination on (**18**) would be expected to yield 4-dimethylaminocyclohexene (**19**). This structure of the Hofmann product was confirmed by its hydrogenation to dimethylaminomethylcyclohexane which was characterized as its methiodide (**20**), identical with an authentic specimen [16]. A Hofmann degradation on this methiodide yielded methylenecyclohexane (**21**), which was converted to the diol with osmium tetroxide, and then cleaved with sodium metaperiodate to yield cyclohexanone (collected as its semicarbazone) and formaldehyde (isolated as its dimedone derivative). The activities of these degradation products of dioscorine are recorded in Table 1, and it is apparent that essentially all the activity was located at C-3.

Whilst this degradation was in progress it seemed feasible to investigate the biosynthesis of dioscorine utilizing nicotinic-[5,6-$^{13}C_2$] acid [17]. The direct incorporation of this compound, containing contiguous ^{13}C atoms, into dioscorine should afford satellite peaks in its ^{13}C NMR, due to spin–spin coupling, symmetrically* located about the corresponding singlet peaks arising from isolated ^{13}C atoms. This method has been used to elucidate pathways for the biosynthesis of several microbial metabolites derived from acetate-[1,2-$^{13}C_2$] and has been reviewed [19, 20]. The proton noise decoupled spectrum of dioscorine is illustrated in Fig. 1, and the chemical shifts recorded in Table 2. Chemical shifts were assigned by continuous wave off resonance decoupling and by comparison with model compounds [21, 22]. Spectra were run in both $CDCl_3$ and benzene-d_6, the latter solvent being preferable since it afforded a better lock signal. The only significant difference (1.3 ppm) in chemical shifts was for the carbonyl group (C-12). Similar shifts have been previously observed [23]. The dioscorine obtained from plants which had been fed nicotinic-[5,6-^{14}C, $^{13}C_2$] acid was labelled with ^{14}C (2.9% absolute

* The satellites are not completely symmetrical, the inner peaks are closer to the central singlet peak than the outer satellite peaks. This effect is due to an approach to an AB spin system, and is especially pronounced when the difference in chemical shifts of the coupled carbons is of the same magnitude as the coupling constant of the contiguous carbons. This effect has been previously observed [18].



Scheme 2. Hypothetical formation of dioscorine from nicotinic acid and acetic acid.



Scheme 3. Degradation of dioscorine derived from nicotinic-[2-^{14}C] acid (heavy dots indicate the location of ^{14}C).

Table 1. Activities of dioscorine derived from nicotinic-[2-^{14}C] acid, and its degradation products

Compound	Specific activity (dpm/mmol × 10^{-7})
Dioscorine picrate	1.03
2-Methyl-5-oxoisoquinuclidine picrate	0.97
2-Methylisoquinuclidine methiodide	0.97
Dimethylaminomethylcyclohexane methiodide	0.97
Formaldehyde dimedone	0.94
Cyclohexanone semicarbazone	< 0.05

Table 2. ^{13}C NMR spectra of dioscorine

Carbon no.	Multiplicity*	Chemical shift (ppm from Me_4Si) in $CDCl_3$	Chemical shift (ppm from Me_4Si) in benzene-d_6
1	doublet	52.4	52.5
3	triplet	53.8	53.7
4	doublet	35.2	35.5
5	singlet	81.6	81.0
6	triplet	39.5	39.4
7	triplet	20.3	20.4
8	triplet	19.5	19.8
9	triplet	40.9	41.0
10	singlet	155.9	155.4
11	doublet	116.5	116.7
12	singlet	165.1	163.8
13	quartet	23.3	23.0
N-Me	quartet	42.6	42.4

* In off resonance decoupled spectrum.

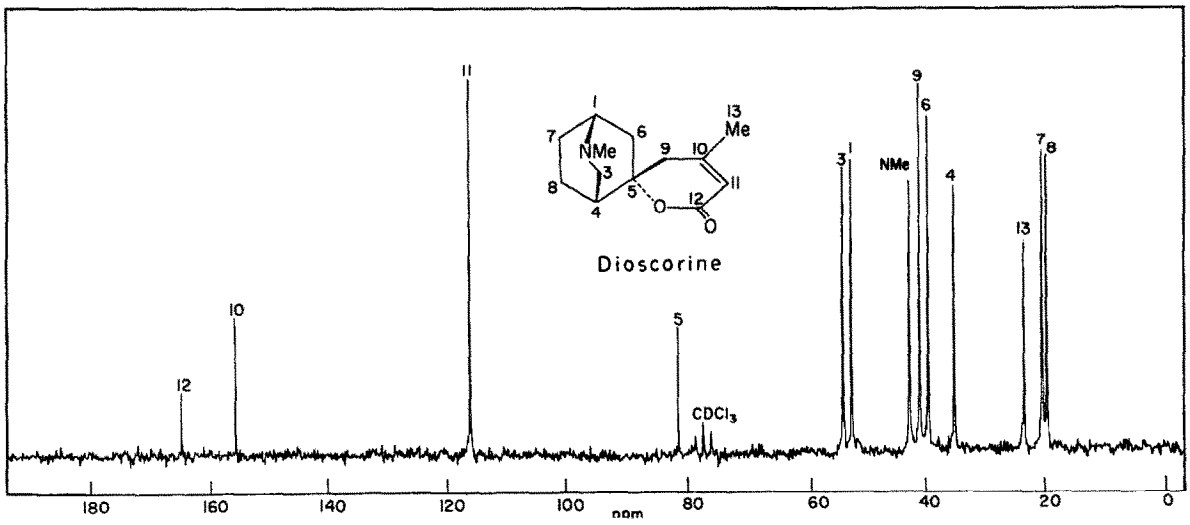


Fig. 1. Proton noise decoupled ^{13}C NMR spectrum of unenriched dioscorine.

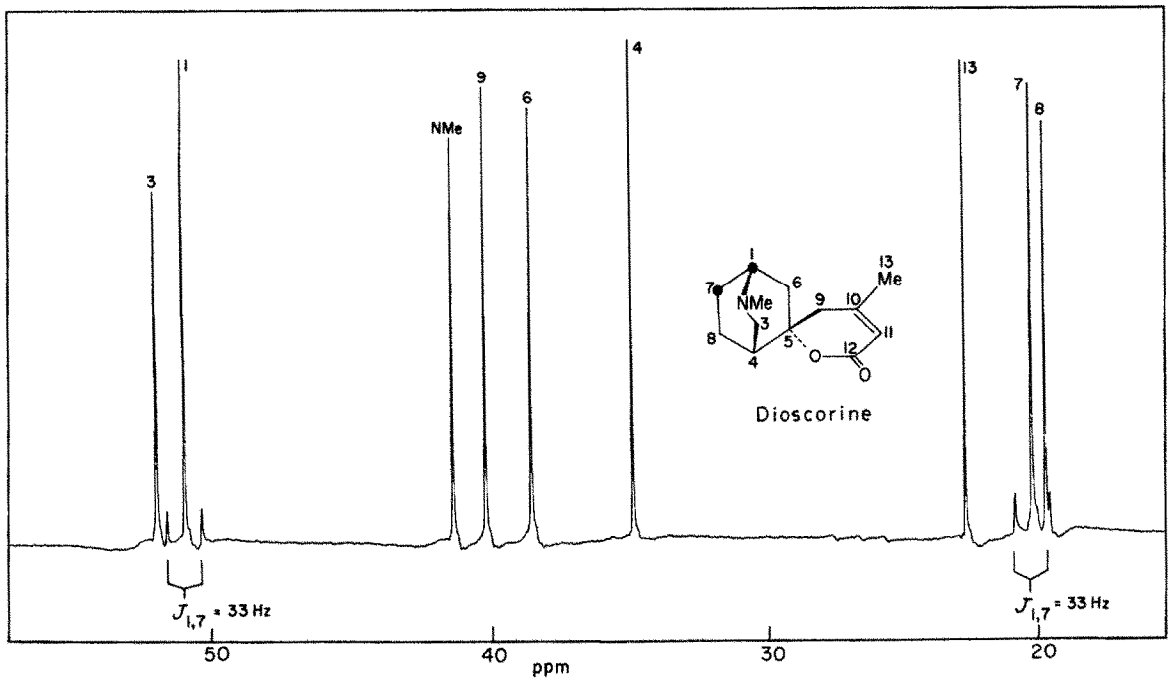


Fig. 2. Proton noise decoupled ^{13}C NMR spectrum of the aliphatic region of enriched dioscorine derived from nicotinic-[5,6-$^{13}C_2$] acid.

incorporation, 0.42% specific incorporation) and its ^{13}C NMR spectra (Fig. 2) clearly showed satellites at C-1 and C-7 having coupling constants of 33.4 ± 0.2 and 33.3 ± 0.2 Hz respectively. This is the expected coupling for an sp^3–sp^3 bond [24]. The specific incorporation of nicotinic-[5,6-$^{13}C_2$] acid into dioscorine, determined from the intensity of the satellite peaks relative to the centrally located singlet peaks, and calculated as previously described [25] was 0.37%, in excellent agreement with the incorporation determined by radioactive assay.

The direct incorporation of nicotinic acid into dioscorine has thus been clearly established, and the pattern of labelling obtained is consistent with the hypothetical scheme illustrated in Scheme 2.

EXPERIMENTAL

General methods. Radioactive materials were assayed in duplicate in a liquid scintillation counter, using dioxane–EtOH, with the usual scintillators [26]. The color quenching of picrates was reduced by the addition of a drop of 2N HCl to the scintillation vial. The yellowing of methiodide samples was inhibited by the addition of NH_2NH_2.

Labelled precursors. DL-Lysine-[6-^{14}C] was purchased from Schwartz/Mann. Nicotinic-[2-^{14}C] acid was prepared from aniline-[1-^{14}C] (ICN, California) [27]. Nicotinic-[5,6-^{14}C, $^{13}C_2$] acid was prepared from a mixture of Me-[^{14}C] I and Me-[90% ^{13}C] I [17]. Analysis of this compound by MS (carried out by Dr. Roger Upham at the University of Minnesota) indicated the presence of 55% [5,6-$^{13}C_2$], 38% of the singly labelled species, [5-^{13}C] and [6-^{13}C], and 7% of unenriched nicotinic acid.

Administration of tracers to Dioscorea hispida *and isolation of the dioscorine.* DL-Lysine-[6-^{14}C] (0.23 mg, nominal activity 0.1 mCi) dissolved in H_2O was fed to *D. hispida* plants growing in a greenhouse (July, 1972) by painting on the leaves. After 5 weeks the whole plant (fr. wt 420 g) was extracted as previously described [1] and afforded dioscorine (69 mg) which was purified to constant activity as its picrate, 4.9×10^4 dpm/mmol. Nicotinic-[2-^{14}C] acid (32.6 mg, 8.05×10^8 dpm/mmol) was dissolved in H_2O (30 ml) and fed to two plants via cotton wicks inserted in the stems (July, 1976). Each day 1 ml of the tracer soln was fed to each plant. After 15 days, when the feeding was complete the plants were allowed to grow for an additional 5 weeks. The whole plants (fr. wt 1.1 kg) afforded dioscorine (85 mg) which yielded a picrate, 1.03×10^7 dpm/mmol (1.9% absolute incorporation, 1.3% specific incorporation). Nicotinic-[5,6-^{14}C, $^{13}C_2$] acid (28.7 mg, 6.52×10^8 dpm/mmol) was fed (Sept., 1976) to one large plant during 20 days by the wick method. Two weeks later the plant was harvested and the roots and tubers (1.9 kg) yielded dioscorine (348 mg) whose picrate had an activity of 2.75×10^6 dpm/mmol (2.9% absolute incorporation, 0.42% specific incorporation).

Degradation of the dioscorine derived from nicotinic-[2-^{14}C] acid. The labelled dioscorine was converted to (+)-2-methyl-5-oxoisoquinuclidine picrate as previously described [1]. This picrate was diluted ×10 with racemic material [28]. The diluted picrate (276 mg) was dissolved in hot 2N HCl (10 ml), cooled, extracted with Et_2O till colorless, then concd HCl (5 ml) added. The mixture was hydrogenated in the presence of PtO_2 (0.1 g) at 3 kg/cm² for 16 hr. The filtered mixture was made basic with NaOH and continuously extracted with Et_2O for 10 hr. MeI (1 ml) was added to the dried (K_2CO_3) extract. On standing 18 hr 2-methylisoquinuclidine methiodide (170 mg, 85%) separated. This methiodide (165 mg) was dissolved in H_2O (10 ml) and shaken with AgOH (from 0.5 g of $AgNO_3$). The residue obtained after lyophilization of the filtered mixture was distilled (170°, 10^{-2} mm) into a U-tube cooled in dry ice. The contents of the U-tube were washed out with MeOH (10 ml) and hydrogenated in the presence of PtO_2 (0.1 g) for 6 hr. MeI (1 ml) was added to the filtered reaction mixture. After standing 18 hr the soln was evapd, and the residue crystallized from EtOH–EtOAc affording dimethylaminomethylcyclohexane methiodide (116 mg, 66%) mp 222–224°, lit. [16] mp 226–227°, having an IR spectrum identical with an authentic specimen. This methiodide (100 mg) in H_2O (5 ml) was shaken with AgOH (from 0.5 g of $AgNO_3$), filtered, lyophilized, and distilled (170°, 10^{-2} mm). The distillate was dissolved in Et_2O (10 ml) and extracted with 2N HCl (2 × 5 ml) to remove Me_3N. OsO_4 (110

mg) and a drop of C_5H_5N were added to the dried Et_2O soln. After standing 18 hr the dark brown reaction mixture was evapd and the residue boiled with Na_2SO_3 (1 g) in MeOH–H_2O (1:1) (20 ml) for 90 min. The filtered mixture was evapd. redissolved in a little H_2O and extracted with Et_2O. $NaIO_4$ (100 mg) in H_2O (10 ml) was added to the residue obtained on evapn of the Et_2O. After standing for 1 hr at room temp. the soln was extracted with Et_2O (2 × 20 ml). The residual aq. soln was distilled into a soln of dimedone (100 mg) in H_2O. The white solid which deposited overnight was crystallized from MeOH affording formaldehyde-dimedone (30 mg, 29%) identical with an authentic specimen. The Et_2O extract which contained cyclohexanone was shaken with an aq. soln of semicarbazide HCl and NaOAc. The Et_2O was evapd, and after standing 18 hr the aq. soln was extracted with EtOAc. The residue obtained on evapn of the dried (K_2CO_3) extract was sublimed (150°, 10^{-2} mm) yielding cyclohexanone semicarbazone (24 mg, 44%) which was crystallized from hot H_2O.

Determination of ^{13}C NMR spectra. The spectra were obtained on a Varian XL-100-15 spectrometer (25.2 MHz), equipped with a VFT-100 Fourier transform accessory. Broad band proton decoupling was effected by 200 Hz square wave modulation centered 1.4 ppm from Me_4Si. The unenriched dioscorine (200 mg in 0.3 ml of $CDCl_3$ in a 5 mm tube) was run with an acquisition time of 0.7 sec (1.5 Hz/data point) for 4500 transients. The enriched dioscorine (240 mg in 0.3 ml of benzene-d_6 in a 5 mm tube) was run with an acquisition time of 2 sec (0.5 Hz/data point) for 5000 transients. To obtain greater digital resolution, a sweep offset was chosen such that the carbonyl resonance (C-12) and the solvent resonance would not interfere with the resonances of interest, when a spectral window of 2000 Hz was employed to examine the aliphatic region (0–80 ppm from Me_4Si) of the spectrum. In the enriched dioscorine (Fig. 2) the observed distances of the satellite peaks from the central singlet peaks were as follows: C-1 +17.0, −16.4; C-7 +16.1, −17.1 Hz (positive downfield, negative upfield). The calculated [29] value of the internal satellite distance for an AB spectrum where J_{AB} = 33.35 Hz and V_{AB} = 815.5 Hz is 16.3 Hz.

Acknowledgements—I thank Professor A. R. Pinder, Clemson University, South Carolina, for generous supplies of dioscorine, compound (**16**), and the *Dioscorea hispida* plants which were obtained from Malaya via the Tropical Products Institute, London. The ^{13}C NMR spectra were determined by Dr. Robert Riddle (University of Minnesota) whose advice on the interpretation of spectra I greatly appreciate. This investigation was supported by a research grant GM-13246 from the U.S. Public Health Service.

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