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THE INCORPORATION OF 3-HYDROXY-3-METHYLGLUTARIC ACID INTO THE LACTONE RING OF DIOSCORINE IN *DIOSCOREA HISPIDA*

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Key Word Index—*Diocorea hispida*; Dioscoreaceae; dioscorine; alkaloid biosynthesis; 3-hydroxy-3-methylgutaric acid; ^{13}C NMR spectroscopy.

Abstract—[3-^{14}C]- and [3,3′-$^{13}C_2$,3-^{14}C]-3-Hydroxy-3-methylglutaric acid were administered to *Dioscorea hispida* plants, resulting in the formation of labelled dioscorine (0.2% absolute incorporation). A chemical degradation indicated that there was a non-random incorporation of ^{14}C into the lactone ring of the alkaloid, the major part of the radioactivity being at its C-10 position. This specific labelling was confirmed by examination of the ^{13}C NMR spectrum of the enriched dioscorine derived from the $^{13}C_2$-labelled precursor. There was some scrambling of label, presumably due to catabolism of the 3-hydroxy-3-methylglutaric acid to acetyl-coenzyme A followed by recycling of this intermediate. However the most significant satellites in the ^{13}C NMR spectrum were those arising from contiguous ^{13}C atoms at C-10 and C-13 of the dioscorine. Ethyl [6-^{14}C] orcellinate failed to serve as a significant precursor of dioscorine. These results indicate that 3-hydroxy-3-methylglutaric acid, or more likely its mono-coenzyme A ester, is an intermediate between acetate and the branched eight-carbon unit required for the biosynthesis of dioscorine.

INTRODUCTION

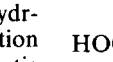
Dioscorine (**5**) is an alkaloid found in the tropical yam *Dioscorea hispida* Dennstedt, the major portion of the alkaloid being present in the tuber. In 1971 [1, 2] it was discovered that the administration of sodium [1-^{14}C] acetate (**1**) to this plant, afforded dioscorine labelled at its C-5, C-10, and C-12 positions, the activity being equally divided between these positions. Recently [3] we have shown that trigonelline (**2**) is a precursor of part of the isoquinuclidine ring of the alkaloid and Scheme 1 illustrates, in abbreviated form, our current hypothesis for the biosynthesis of dioscorine. In this Scheme the branched eight-carbon compound (**3**) (or a suitably activated derivative such as its coenzyme A ester), derived from four acetate units condenses with trigonelline at its C-6 position to form the intermediate **4**. Two decarboxylations, bond formations and reductions, as illustrated, then afford dioscorine.

The present article is concerned with the intermediates between acetate and the branched eight-carbon compound **3**. There are several possible routes to such a compound which are illustrated in Scheme 2. In route A, the first steps from acetyl-coenzyme A (**6**) to 3-hydroxy-3-methylglutaryl-coenzyme A (**8**) via acetoacetyl-coenzyme A (**7**) have been well studied because **8** is the precursor of mevalonic acid (**11**) [4], the universal source of all terpenes. We propose that **8** condenses with another molecule of acetyl-coenzyme A to afford **9** which on dehydration yields the coenzyme A ester of **3**. This dehydration could of course occur at a later stage in the biosynthetic sequence, after condensation with the trigonelline. In route B, three acetyl-coenzyme A units condense in a linear fashion to afford the β, δ-diketo ester (**12**) which then condenses with a fourth acetate unit at its C-5 position to yield **9**. In route C, four acetate units condense in a linear fashion to afford the aromatic compound orcellinic acid (**15**), its biosynthesis by this route having been established [5, 6] via the hypothetical intermediate **14**. An oxidative ring opening of the aromatic ring, which has several biochemical analogies [7], could yield the intermediate **16**. Reduction of the α-keto acid and the alkene affords **13**, the desired hypothetical precursor **3** being formed on dehydration.

We have examined routes A and C to the intermediate **3** by feeding labelled 3-hydroxy-3-methylglutaric acid and ethyl orcellinate to *D. hispida*.

RESULTS AND DISCUSSION

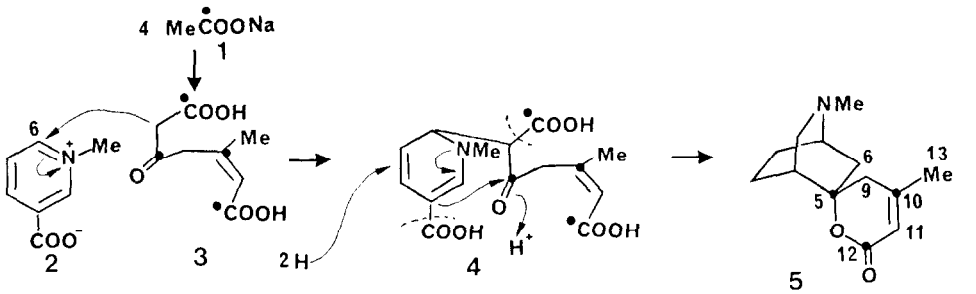
[3,3′-$^{13}C_2$,3-^{14}C]-3-Hydroxy-3-methylglutaric acid was synthesized from a mixture of ethyl [1,2-$^{13}C_2$] acetate and ethyl [1-^{14}C] acetate [8]. Its ^{13}C NMR spectrum illustrated in Fig. 1 is of considerable interest because of the presence of 99% of the $^{13}C_2$ species. The one bond coupling constant between C-3 and C-3′ was 39.9 Hz. The doublet centred at δ 70.0 (C-3) exhibited small signals (about 20% of the main signals, 0.024 ppm upfield) which are assigned to the formation of some



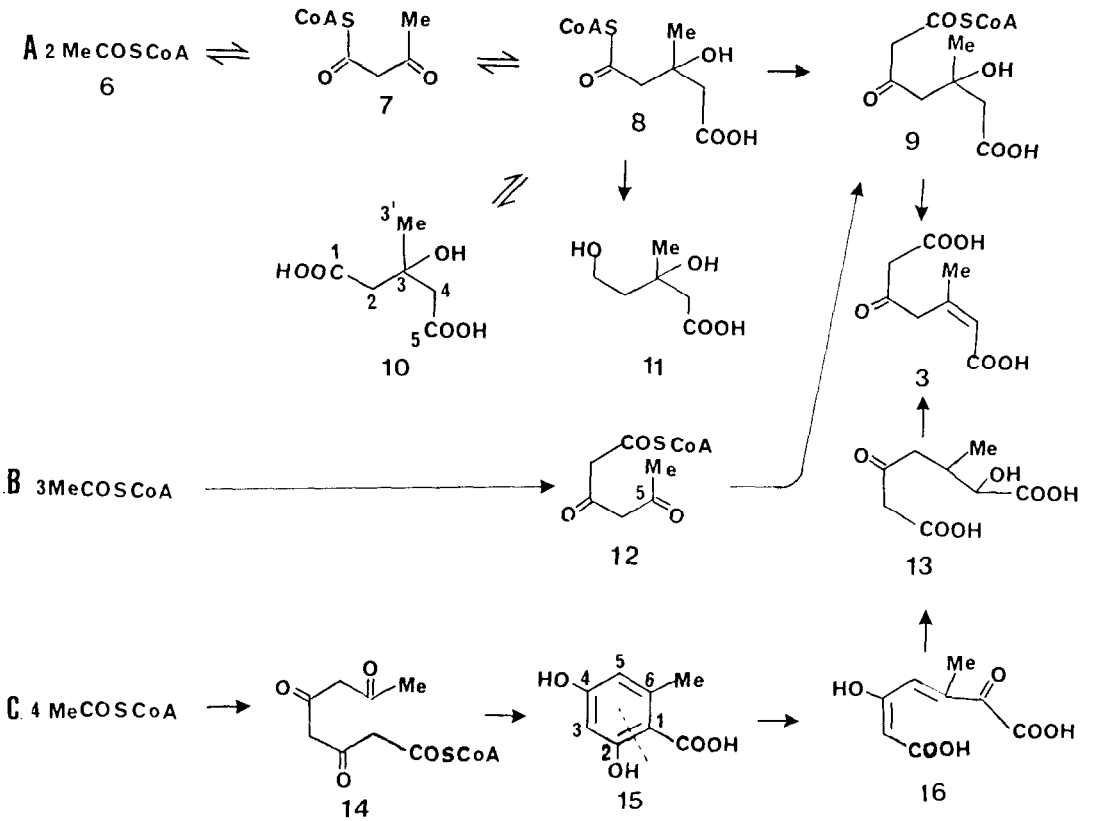
HOOC–CH_2–C(Me)(OD)–CH_2–COOH

by exchange with the solvent (acetone-d_6). Carbons 2 and 4 appear as doublets of doublets due to one and two bond couplings. The carbonyl group of the carboxyl groups appears also as a doublet of doublets due to two and three bond couplings with the highly enriched positions. In a preliminary

†Contribution No. 212 from this laboratory.



Scheme 1. Schematic biosynthesis of dioscorine from acetate and trigonelline.



Scheme 2. Hypothetical routes from acetyl-coenzyme A to the branched eight-carbon unit 3.

experiment, [3-^{14}C]-3-hydroxy-3-methylglutaric acid was fed to *D. hispida* plants by the wick method. The dioscorine isolated from the plants six weeks later was labelled (0.09% specific incorporation). This material was subjected to a Kuhn–Roth oxidation affording acetic acid, derived from C-10 and C-13 of the alkaloid. A Schmidt reaction on this acetic acid afforded carbon dioxide and methylamine. The specific activities of these degradation products (Table 1) indicated that most of the activity (76%) was located at C-10 of dioscorine. This result is consistent with route A for the formation of the eight-carbon unit **3**. The fact that less than 100% of the activity was found at C-10 is not unexpected, and is rationalized by reversal of the steps outlined in Scheme 2 to generate [1-^{14}C] acetyl coenzyme A, from the [3-^{14}C]-3-hydroxy-3-methylglutaric acid, which can then be recycled producing activity at C-5 and C-12 of the ultimate dioscorine.

The direct incorporation of **10** and partial reversal of its biosynthesis was confirmed by feeding the [3,3'-$^{13}C_2$,3-^{14}C]-**10** to the *D. hispida* plants (Experiment 2). In this experiment the tubers and aerial parts of the plant were worked-up separately. The dioscorine isolated from the tubers had a much higher specific activity than that found in the leaves and stems, strongly indicating that the tubers are probably the primary site of alkaloid biosynthesis. A chemical degradation of the dioscorine isolated from the tubers again indicated a specific labelling (57%) at the C-10 position. A complete reversal of the biosynthesis of **10** to acetyl-coenzyme A and re-synthesis to **10** would have afforded dioscorine with 33% of its activity at C-10. Even though the specific incorporation

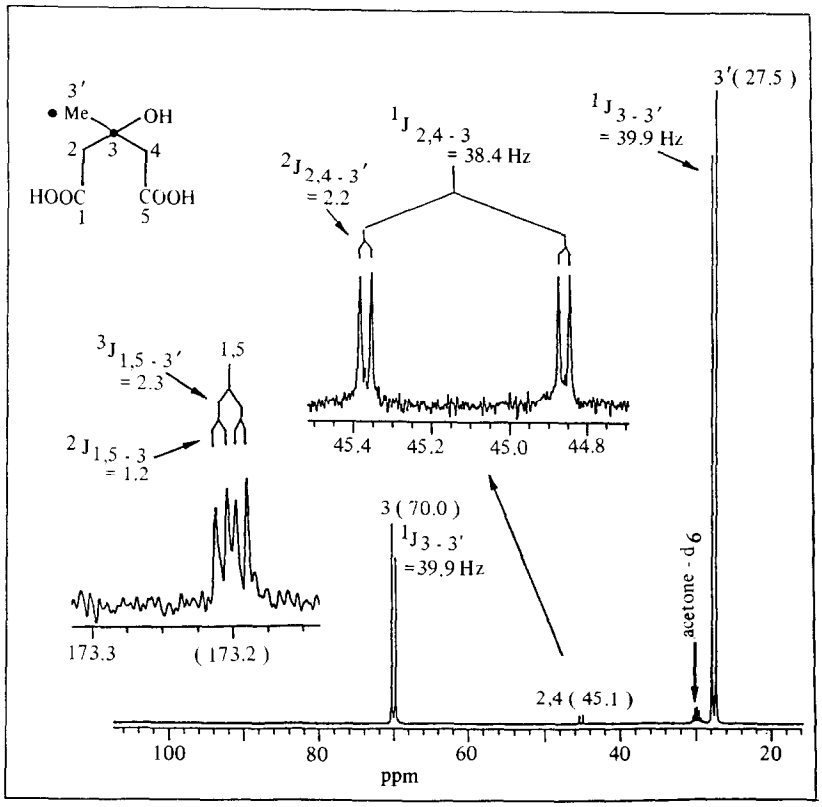


Fig. 1. ^{13}C NMR spectrum of [3,3'-$^{13}C_2$]-3-hydroxy-3-methylglutaric acid (99% $^{13}C_2$) (30 mg in 0.4 ml of acetone-d_6). Chemical shifts (ppm from TMS) are indicated in parentheses. Coupling constants (J) are in Hz.

was quite low, it was possible to observe the direct incorporation of the [3,3'-$^{13}C_2$]-3-hydroxy-3-methylglutaric acid by ^{13}C NMR spectroscopy. Satellites indicative of one bond spin–spin couplings between C-5 and C-9, C-10 and C-13, and C-11 and C-12 were observable. Figure 2 illustrates the observed satellites for coupled carbons C-10 and C-13 (0.05% enrichment) and the coupled carbons C-11 and C-12 (0.01% enrichment). The specific incorporations, calculated from the magnitude of these satellite signals relative to the intensity of the major signal between the satellites was largest for the satellites of the C-10 and C-13 signals (~0.055%) consistent with the results from the ^{14}C degradation (Table 2). The observed coupling constants are entirely consistent with those previously recorded [9] for these types of adjacent carbon atoms. The assignments of the signals in the ^{13}C NMR spectrum of dioscorine has been previously determined unequivocally [3]. None of the other carbons in the ^{13}C NMR spectrum of this enriched dioscorine exhibited satellites.

The low incorporation of the 3-hydroxy-3-methylglutaric acid may indicate that this molecule is not on the direct pathway between acetyl-coenzyme A and the required eight-carbon units. It is planned to administer thioesters of **10** to *D. hispida* with the expectation of a higher incorporation and less scrambling of label in the ultimate alkaloid. The superior incorporation of thioesters of advanced intermediates in polyketide biosynthesis has been recently reported [10, 11]

Ethyl[6-^{14}C]orsellinate (**15**, COOH = COOEt) was prepared from commercially available ethyl[3-^{14}C]acetoacetate and diketene by modification of a previously described method [12]. Because orsellinic acid rapidly decarboxylates, the labelled ethyl ester was administered to *D. hispida* as a solution in dilute methanol. The dioscorine isolated from the plant six weeks after the initial feeding had negligible activity (0.01% absolute incorporation) rendering pathway C to the eight-carbon unit **3** unlikely.

In conclusion the results reported in this article strongly favour the participation of 3-hydroxy-3-methylglutaric acid, or a suitably activated intermediate such as its coenzyme A ester as an intermediate in the incorporation of acetate into the lactone ring of dioscorine. No evidence has been presented for or against route **B**. However, in the light of the results obtained it seems unlikely, and no close biochemical reactions of this type are currently known.

EXPERIMENTAL

General. Mps are corrected. Radioactive materials were assayed by liquid scintillation counting using the biodegradable Ecoscint A (National Diagnostics) as a scintillation solution. However, this solvent gave very poor ^{14}C counting efficiencies when assaying picrates, and these derivatives were assayed in dioxane–EtOH with naphthalene, PPO and POPOP [13].

Table 1. Activities of dioscorine and its degradation products, derived from labelled 3-hydroxy-3-methylglutaric acid

	Experiment 1		Experiment 2	
Precursor fed	[3-^{14}C]-3-Hydroxy-3-methyl-glutaric acid		[3,3'-$^{13}C_2$,3-^{14}C]-3-Hydroxy-3-methyl-glutaric acid	
Date of inital feeding	4 September 1987		6 September 1988	
Duration of feeding	6 weeks		5 weeks	
	Whole plant		Tubers	Aerial Parts
Dioscorine isolated (mg)	596		910	73
Absolute incorporation*	0.23%		0.21%	0.003%
Specific incorporation†	0.09%		0.10%	0.014%
	Specific activity (dpm/mmol)	RSA‡	Specific activity§ (dpm/mmol)	RSA
Dioscorine	1.64×10^5	100	5.07×10^4	100
Dioscorine picrate	1.67×10^5	102	5.06×10^4	100
Dioscorine methiodide	1.65×10^5	100	not determined	
1-Acetamidonaphthalene‖	1.38×10^5	84	2.98×10^4	59
Barium carbonate¶ [C-10]	1.25×10^5	76	2.89×10^4	57
N-Methylbenzamide¶ [C-13]	0.05×10^5	3	0.07×10^4	1

*Absolute incorporation = total activity (dpm) in the isolated dioscorine/total activity (dpm) administered to the plant.

†Specific incorporation = activity of dioscorine (dpm/mmol)/activity of the precursor (dpm/mmol).

‡RSA = relative specific activity.

§Activity of the dioscorine isolated from the tubers.

‖Derived from the acetic acid produced by a Kuhn–Roth oxidation of dioscorine.

¶Derived from the carbon dioxide and methylamine produced by a Schmidt reaction in the acetic acid.

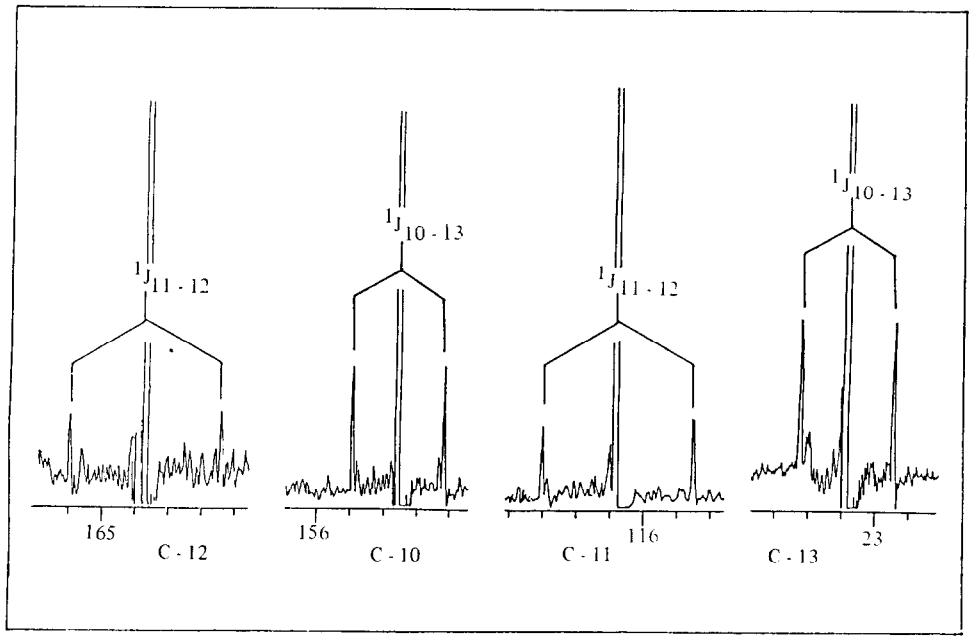


Fig. 2. Details of the ^{13}C NMR spectrum of the enriched dioscorine at carbon signals where satellites were observed.

[3,3'-$^{13}C_2$,3'-^{14}C]-3-*Hydroxy*-3-*methylglutaric acid* (**10**). Ethyl [1,2-$^{13}C_2$] acetate [99.6% ^{13}C at C-1, 99.3% ^{13}C at C-2 - Aldrich] (1.0 g, 11.1 mmol), ethyl [1-^{14}C]acetate (nominal activity 5.5×10^8 dpm, 4.5 mg, Sigma) and allyl bromide (2.7 ml, 31.2 mmol) dissolved in THF (10 ml) was added to magnesium (0.95 g, 40.1 mg-atoms) suspended in ET_2O (3 ml) and the mixture stirred under N_2 for 2 hr, the mixture refluxing due to the exothermic reaction. Et_2O (100 ml) was then added, followed by ice and 1 M H_2SO_4. The separated organic layer was washed with 10% Na_2CO_3 and then dried (Na_2SO_4). The residue obtained on evapn of the Et_2O was dissolved in CH_2Cl_2 (30 ml) and acetic acid (3 ml), cooled to $-78°$ and ozone passed in slowly for 30 min until the soln became permanently blue. The soln was allowed to warm to room temp., evapd on a rotary evaporator, and the residual oil refluxed with a mixture of HOAC acid (17 ml) and 30% H_2O_2 (7 ml) for 8 hr. The residue

Table 2. Observed coupling constants between contiguous ^{13}C atoms in enriched dioscorine derived from [3,3'-$^{13}C_2$]-3-hydroxy-3-methylglutaric acid (in $CDCl_3$)

Coupled carbon atoms	Chemical shift (ppm from TMS)	Coupling constant (Hz)	Enrichment* %
5 }	81.23	37.8	0.021
9 }	39.27	38.2	0.023
10 }	155.51	41.7	0.054
13 }	23.14	42.0	0.057
11 }	116.16	68.0	0.012
12 }	164.70	68.0	0.015

*Calculated from: area of satellite signals/area of central signal −0.012 (natural abundance of contiguous ^{13}C atoms). The above data were obtained in a Nicolet 300 NMR spectrometer operating at 75.46 MHz on 100 mg of enriched dioscorine in $CDCl_3$ (0.4 ml) with the following instrument parameters: 21 304 transients, acquisition time 0.819 sec, RG 40 μsec, DE 50 μsec.

obtained on evapn was dissolved in dioxane (4 ml) and then C_6H_6 (60 ml) was added, when colourless plates of [3,3'-$^{13}C_2$,3-^{14}C]-3-hydroxy-3-methylglutaric acid separated (1.15 g, 67%) having a specific activity of 5.02×10^7 dpm/mmol, expected: 4.95×10^7 dpm/mmol, mp 110–111°, lit [8] mp 110–112°. Its ^{13}C NMR spectrum (in Me_2CO-d_6) is illustrated in Fig. 1. The [3-^{14}C]-hydroxy-3-methylglutaric acid used in feeding Experiment 1 was prepared similarly starting with ethyl [1-^{14}C] acetate.

Ethyl[6-^{14}C]*orsellinate.* A 50% oil suspension of NaH (0.24 g, 5 mmol) was washed with hexane to remove the oil and then added to a solution of ethyl[3-^{14}C]acetoacetate (0.64 ml, 5 mmol, nominal activity 1.1×10^9 dpm, Amersham) in THF (10 ml) at room temp. After 30 min, when the evolution of H_2 had ceased, a soln of freshly distilled diketene (0.47 ml, 6 mmol) in THF (5 ml) was slowly added over 10 min. The mixture was then refluxed for 4 days in a N_2 atmosphere. The soln was then cooled and 1 M H_2SO_4 (50 ml) was added. The mixture was extracted with Et_2O (3 × 50 ml). The residue obtained on evapn of the dried ($MgSO_4$) extract was crystallized from a mixture of $CHCl_3$ and hexane to afford crude ethyl [3-^{14}C]orcellinate. Sublimation (120°, 10^{-3} mm Hg) afforded colourless plates (0.38 g, 39%) mp 131–132°, ref. [12] mp 132°, specific activity 1.89×10^8 dpm/mmol, expected: 2.2×10^8 dpm/mmol.

Administration of precursors to Dioscorea hispida *and isolation of the dioscorine.* All feedings were carried out by the wick method, cotton wicks being inserted into the long vines of this plant near to ground level. Several plants, growing in a greenhouse, were used for each feeding experiment. The dioscorine was isolated as previously described [14] and purified by distillation and formation of its picrate. The degradations carried out on the labelled dioscorine from Experiments 1 and 2 were done as previously outlined [2].

In Experiment 1, [3-^{14}C]-3-hydroxy-3-methylglutaric acid (172.8 mg, 1.07 mmol, 1.86×10^8 dpm/mmol), was dissolved in H_2O (20 ml) and fed to 3 plants. The fresh plants (tubers, roots, and aerial parts) at the time of harvesting weighed 870 g. In Experiment 2 [3,3'-$^{13}C_2$,3-^{14}C]-3-hydroxy-3-methylglutaric acid (300 mg, 1.83 mmol, 5.02×10^7 dpm/mmol) was fed in a similar fashion. At harvest time the fresh tubers weighed 1320 g, and the leaves and stems had a fresh wt of 450 g. Ethyl [6-^{14}C] orcellinate (155 mg, 0.79 mmol, 1.87×10^8 dpm/mmol) was dissolved in MeOH (2 ml) which was then diluted with H_2O (10 ml) and fed to 3 plants. The fresh wt. of the harvested plants was 794 g and this material afforded 439 mg of dioscorine, having a specific activity of 7.4×10^3 dpm/mmol.

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