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Epoxidation *in Vivo* of Hyoscyamine to Scopolamine Does Not Involve a Dehydration Step

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ABSTRACT

Hyoscyamine is epoxidized to scopolamine via 6β-hydroxyhyoscyamine in several solanaceous plants. 6,7-Dehydrohyoscyamine has been proposed to be an intermediate in the conversion of 6β-hydroxyhyoscyamine to scopolamine on the basis of the observation that this unsaturated alkaloid is converted to scopolamine when fed to a *Datura* scion. To determine whether a dehydration step is involved in scopolamine biosynthesis, [6-^{18}O]6β-hydroxyhyoscyamine was prepared from *l*-hyoscyamine and $^{18}O_2$ using hyoscyamine 6β-hydroxylase obtained from root cultures of *Hyoscyamus niger* L. When [6-^{18}O]6β-hydroxyhyoscyamine was fed to shoot cultures of *Duboisia myoporoides* R. B_R., the labeled alkaloid was converted to scopolamine which retained ^{18}O in the epoxide oxygen. It is concluded that 6β-hydroxyhyoscyamine is converted *in vivo* to scopolamine without a dehydration step.

Scopolamine, the epoxide of hyoscyamine, is one of the major alkaloids which accumulate in the family Solanaceae (7). The formation of the epoxide bridge in scopolamine begins with the hydroxylation of L-hyoscyamine to Hyos-OH[1] by a 2-oxoglutarate-dependent dioxygenase, hyoscyamine 6β-hydroxylase (12). The subsequent reaction(s) in scopolamine biosynthesis, *i.e.* the transformation of the hydroxyl group to the epoxide, have not been clarified. In an experiment using the scions of *Datura ferox* L. grafted on *Cyphomandra betacea* cv. Sendtn., Fodor *et al.* (9) demonstrated that De-hyos, exogenously fed to the *Datura* scions, can be converted to scopolamine. Based on this report, it has been proposed that Hyos-OH is first dehydrated to De-hyos, which then is converted to scopolamine (17, 19). This hypothetical unsaturated intermediate, however, has yet to be isolated from plants.

Previously, we (13) reported that hyoscyamine 6β-hydroxylase from root cultures of *Hyoscyamus niger* L. not only hydroxylates various hyoscyamine analogs to their 6-hydroxyl derivatives, but also epoxidizes De-hyos to scopolamine. Thus, the conversion of De-hyos to scopolamine in the *Datura* scions (9) is apparently catalyzed by the hydroxylase. Nevertheless, the question is still open as to whether De-hyos is, in fact, an *in vivo* precursor of scopolamine. To examine this question, we made use of the fact that the proposed dehydration mechanism would result in release of the hydroxyl oxygen whereas an intramolecular epoxidation would not. We prepared [6-^{18}O]Hyos-OH from L-hyoscyamine and $^{18}O_2$ using the reaction catalyzed by hyoscyamine 6β-hydroxylase. Shoot cultures of *Duboisia myoporoides* were chosen to accomplish the transformation since they are not only biosynthetically capable but also do not accumulate alkaloids which would otherwise complicate quantitation. Analysis of the transformed scopolamine by GC-MS can reveal the presence or absence of ^{18}O in the epoxide oxygen. An ^{16}O epoxide oxygen would be expected if epoxidation by 6β-hydroxylase occurs via De-hyos (scheme 1 in Fig. 1); ^{18}O should be retained if dehydration is not involved (scheme 2 in Fig. 1).

[1] Abbreviation: Hyos-OH, 6β-hydroxyhyoscyamine; De-hyos, 6,7-dehydrohyoscyamine; TMS-derivatives, trimethylsilyl derivatives.

MATERIALS AND METHODS

Chemicals. $^{18}O_2$ (>99 atom %) was purchased from CEA, France. L-Hyoscyamine hydrobromide and scopolamine hydrobromide were obtained from Nakarai Chemicals, Kyoto. Hyos-OH hydrobromide was prepared using hyoscyamine 6β-hydroxylase from root cultures of *Hyoscyamus niger* L. (12). De-hyos was synthesized by the method of Sharpless *et al.* (23). Other chemicals were obtained as described previously (12).

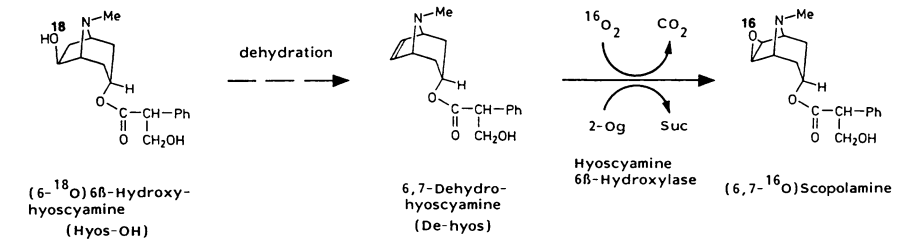
Root and Shoot Cultures. Culture conditions for root cultures of *H. niger* L. have been reported elesewhere (12). Shoot cultures of *Duboisia myoporoides* R. B_R. were initiated according to Endo *et al.* (6) and were subcultured on a Gyrotory shaker (model G10-21, New Brunswick Scientific, Edison, NJ) at 100 rpm and 25°C under light (2,700–4,500 lux) in 100-ml flasks containing 25 ml of liquid B5 medium (10) supplemented with 3% (w/v) sucrose and 10 μM 6-BA. In the feeding experiments, alkaloid solutions were neutralized with HCl when necessary and were sterilized by passing through 0.22 μm membrane filters. These were added to 10 ml of the above culture medium in 50-ml flasks to a final concentration of 0.2 mM. The shoot cultures containing young leaves were inoculated in each flask and cultured under the above conditions.

Partial Purification of Hyoscyamine 6β-Hydroxylase. Hyoscyamine 6β-hydroxylase was partially purified from root cultures of *H. niger*. The hydroxylase in the crude extract was precipitated between 60 and 80% saturation of $(NH_4)_2SO_4$ and subsequently chromatographed on a DEAE-Toyopearl 650M column. The detailed purification procedure has been reported (13). The partially purified enzyme preparation (about 23-fold in specific activity) was concentrated using Amicon YM-10 ultrafiltration membranes to a final volume of approximately 2 ml.

The conditions of the reaction and determination of enzyme activity have been reported (13).

Preparation of [6-^{18}O]6β-Hydroxyhyoscyamine. [6-^{18}O]Hyos-OH was synthesized from L-hyoscyamine and $^{18}O_2$ by the partially purified hyoscyamine 6β-hydroxylase. The reaction mixture contained in a total volume of 69 ml: 50 mM Tris-HCl buffer (pH 7.8), 0.4 mM ferrous sulfate, 4 mM sodium ascorbate, 1 mM 2-oxoglutaric acid, 0.4 mM L-hyoscyamine hydrobromide, 1 mg/ml catalase (C-10; Sigma), and the hydroxylase (8.8 n Kat). A 100-ml Erlenmeyer flask with a side arm, a rubber-stoppered injection port and a gas-introducing port on top, similar to the design of Hayaishi (15), was used for the reaction. The nonprotein components of the reaction mixture were placed at the

Scheme 1



Scheme 2

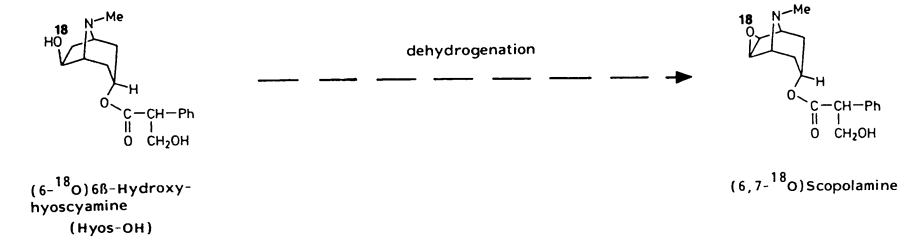


FIG. 1. Two possible routes for the metabolism of [6-^{18}O]Hyos-OH in shoot cultures of *D. myoporoides* (see text for details); 2-Og, 2-oxoglutarate; Suc, succinate.

bottom of the flask and the two protein components in the side arm. Prior to the reaction, the nonprotein components were purged with nitrogen for 10 min, the flask was filled with nitrogen at slightly reduced pressure, then $^{18}O_2$ was introduced to the flask. The reaction was started by the addition of the protein components to the nonprotein components and the combined reaction mixture was incubated with gentle stirring at 30°C for 4 h. The reaction was stopped by injecting 2 ml of 12% (w/v) TCA into the reaction mixture.

After removing precipitates by centrifugation, the clear supernatant was made alkaline (pH 10) with 28% NH_4OH and approximately 1.2 M carbonate buffer (pH 10), and extracted three times with three volumes of $CHCl_3$. The $CHCl_3$ extract was concentrated then applied to a preparative TLC plate coated with Silica gel 60 PF_{254}. The chromatography and the subsequent extraction of the alkaloids from the plate were carried out as described previously (12). Purified [6-^{18}O]Hyos-OH was neutralized with HCl and used for the feeding experiment.

Alkaloid Analysis. Alkaloids were extracted and analyzed by GLC and GC-MS as described previously (14).

RESULTS

***Duboisia* Shoot Cultures.** The shoot cultures of *Duboisia myoporoides* grew in suspension as an organized mass of young leaves, shoots, and stems with callus sometimes attached at their bases. Axillary buds often differentiated in these cultures. To test the capacity of the shoot cultures to transform tropane alkaloids, the cultures were fed with L-hyoscyamine, Hyos-OH, De-hyos, and scopolamine at a concentration of 0.2 mM, and incubated at 25°C for 1 week (Table I). In the control experiment, these alkaloids were incubated without the shoot cultures; 100% of the added alkaloids were recovered from the culture medium and no degradation of these alkaloids occurred in the absence of plant cells. When no alkaloids were added, the *Duboisia* shoot cultures accumulated none of the four alkaloids. However, added hyoscyamine was converted to Hyos-OH and scopolamine, and Hyos-OH to scopolamine. It should be noted that we could not detect De-hyos in the cultures when either alkaloid was fed. The unsaturated alkaloid was transformed to scopolamine by the *Duboisia* shoot cultures. The transformation of these alkaloids proceeded only in one direction, from hyoscyamine to scopolamine; no products of the reverse reactions were detected. The metabolites of the added alkaloids were mainly found in the cells. The fair recovery rate of added alkaloids indicates that degradation of tropane alkaloids in the *Duboisia* shoot cultures is not significant. It is concluded that although they do not accumulate any tropane alkaloids, the shoot cultures of *D. myoporoides* have the active pathway for converting hyoscyamine to scopolamine.

Preparation of [6-^{18}O]6β-Hydroxyhyoscyamine. Under the described reaction conditions, the partially purified hyoscyamine 6β-hydroxylase preparation hydroxylated almost all of the L-hyoscyamine in the reaction mixture. The mass spectra of the TMS-derivatives in Figure 2 show that Hyos-OH isolated from the reaction mixture (Fig. 2B) has the parent ion of m/z 451 and a fragment ion of m/z 214, two mass/charge units higher than those corresponding ions in Hyos-OH (m/z 449 and 212, Fig. 2A), thus confirming that the prepared Hyos-OH had 1 atom of ^{18}O at the 6β-hydroxyl position. Based on the ratio of the 214/212 mass/charge peaks, it was calculated that 82.5% of the alkaloid molecules contained one atom of ^{18}O.

Feeding Experiment. The *Duboisia* shoot cultures were incubated with 0.2 mM of [6-^{18}O]Hyos-OH for 6 d, after which alkaloids in the cultures were extracted and analyzed by GLC. The only metabolite of Hyos-OH was scopolamine, which was further analyzed by GC-MS (Fig. 3B). Scopolamine isolated from the shoot cultures showed a parent ion of m/z 377 and a fragment ion of m/z 140, two mass/charge units higher than those corresponding ions in scopolamine (m/z 375 and 138, Fig. 3A). Therefore, the derived scopolamine contained 1 atom of ^{18}O in the epoxide oxygen. Based on the ratio of ions 377/375, it was calculated that 84.6% of the isolated scopolamine molecules contained 1 atom of ^{18}O. This value corresponds very well with the ^{18}O content of 82.5% in the substrate, Hyos-OH. Thus, all ^{18}O in the hydroxyl group of Hyos-OH was retained in the epoxide oxygen of scopolamine after biotransformation *in vivo*.

DISCUSSION

***Duboisia* Shoot Cultures.** To carry out quantitatively the feeding experiment with the ^{18}O-labeled precursor, it was required that the plant material not contain tropane alkaloids yet possess the capacity to transform the added precursor to scopolamine. The main site of tropane alkaloid biosynthesis is in the roots (24), but the aerial parts of several solanaceous plants are known to display at least partial biosynthetic competence. Fodor *et al.* (9) grafted the aerial part of alkaloid-producing *Datura ferox* L.

Table I. *Biotransformation of Tropane Alkaloids in D. myoporoides Shoot Cultures*

Approximately 0.2 g fresh weight of the shoot cultures were incubated with 2 μmol/10 ml of the tropane alkaloid under light for 1 week. Alkaloids in the cells and in the medium were determined by GLC separately and these alkaloid amounts were combined. The values are the mean of three flasks.

Alkaloid Added (2 μmol/flask)	Alkaloids Detected after 1 Week				
	Hyoscyamine	Hyos-OH	De-hyos	Scopolamine	Recovery
		μmol/flask			%
None	ND[a]	ND	ND	ND	
Hyoscyamine	1.60	0.11	ND	0.06	88.5
Hyos-OH	ND	1.23	ND	0.21	72.0
De-hyos	ND	ND	1.75	0.17	96.0
Scopolamine	ND	ND	ND	1.65	82.5

[a] Not detected.

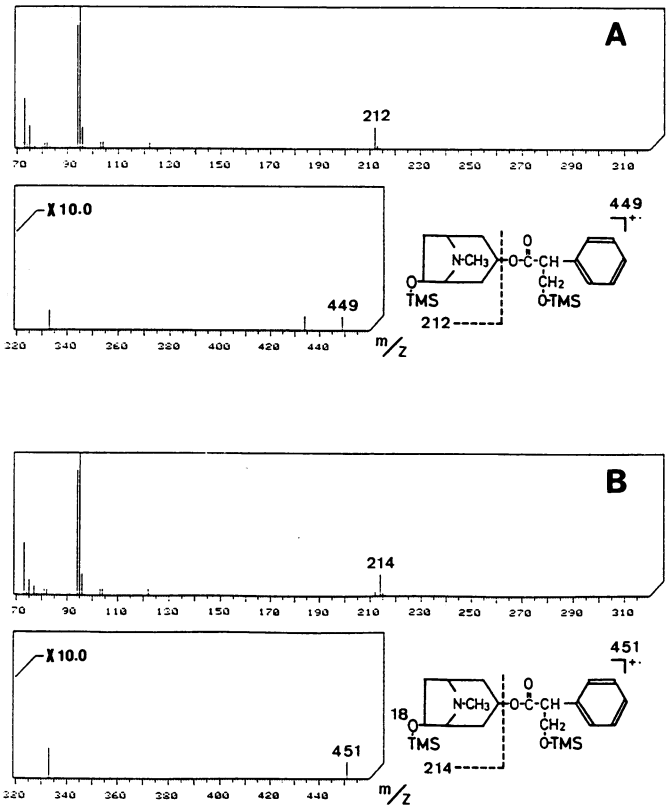


FIG. 2. Mass spectra of the TMS-derivatives of Hyos-OH (A) and of [6-^{18}O]Hyos-OH prepared by the reaction of hyoscyamine 6β-hydroxylase under $^{18}O_2$ (B).

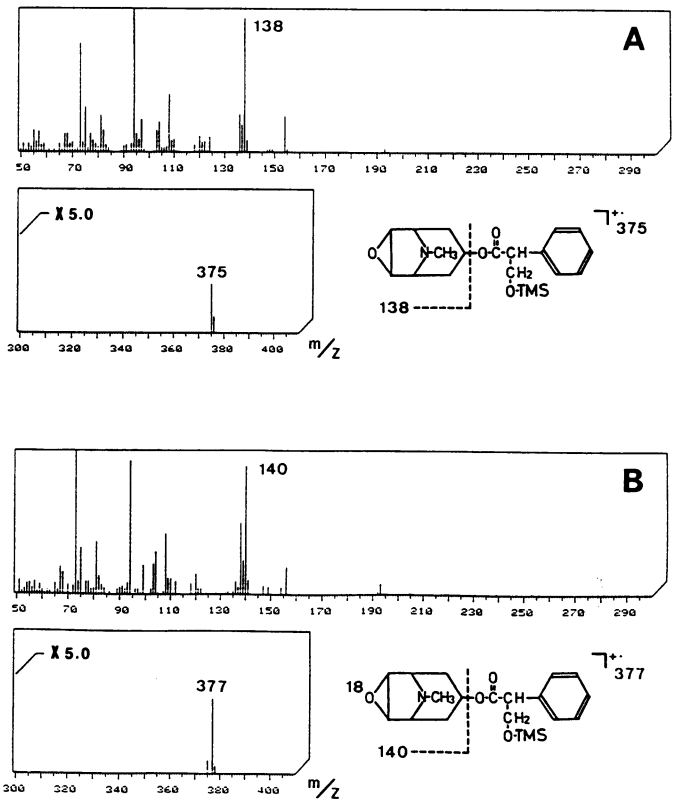


FIG. 3. Mass spectra of TMS-derivatives of scopolamine (A) and of scopolamine formed *in vivo* from [6-^{18}O]Hyos-OH (B).

on to the root stock of alkaloid-nonproducing *Cyphomandra betacea* cv Sendtn. to obtain practically alkaloid-free, yet synthetically capable, *Datura* scions. Shoot cultures of alkaloid-producing plants are also excellent materials for feeding experiments (25). We have previously shown that shoot cultures of *D. myoporoides* contain no tropane alkaloids, but have a weak hyoscyamine 6β-hydroxylase activity (12). The shoot cultures metabolized hyoscyamine and Hyos-OH to scopolamine without significant degradation of the alkaloids (Table I). These results suggest that the *Duboisia* shoot cultures are fully capable of converting hyoscyamine to scopolamine but their inability to synthesize hyoscyamine, or some earlier precursors of hyoscyamine, prevent accumulation of tropane alkaloids. Shoot cultures of *Duboisia leichhardtii* L. (25) and of a hybrid of *D. leichhardtii* and *D. myoporoides* (11) are also reported to synthesize scopolamine from added hyoscyamine despite the absence normally of these alkaloids in culture.

Hyoscyamine 6β-Hydroxylase. Hyoscyamine 6β-hydroxylase, a 2-oxoglutarate-dependent dioxygenase, uses molecular oxygen for the hydroxylation of the substrate. The requirement of molecular oxygen for the hydroxylation reaction was previously demonstrated (12), and the incorporation of ^{18}O from $^{18}O_2$ into the hydroxyl oxygen (Fig. 2) provides further evidence for this requirement. The other atom of molecular oxygen is incorporated into succinate, the decarboxylation product of 2-oxoglutarate, in a reaction typical of those catalyzed by 2-oxoglutarate-dependent dioxygenases (1). Simultaneous incorporation of $^{18}O_2$ into a hydroxyl group and into succinate has been reported for two 2-oxoglutarate-dependent dioxygenases; γ-butyrobetaine hydroxylase (20) and prolyl 4-hydroxylase (3).

Biosynthesis of Scopolamine. Retention of ^{18}O during the *in vivo* conversion of Hyos-OH to scopolamine argues against the proposed biosynthetic pathway of scopolamine (17, 19) in which De-hyos is a precursor of scopolamine (scheme 1 in Fig. 1). The conversion of added De-hyos to scopolamine in the *Datura*

scions (9) and in our *Duboisia* shoot cultures (Table I) is probably catalyzed by hyoscyamine 6β-hydroxylase which can epoxidize De-hyos to scopolamine (13). That isolation of the unsaturated alkaloid from plants has not been reported, together with our inability to detect this alkaloid in precursor-fed *Duboisia* shoot cultures (Table I), supports our conclusion that De-hyos is not involved in the biosynthesis of scopolamine.

With regard to stereochemistry of the reaction sequence, Leete and Lucast (18) reported that both tritium atoms in [N-methyl-^{14}C, 6β,7β-$^{3}H_2$]tropine were lost during conversion to scopolamine in *Datura* plants. Given that dehydration does not occur, these results can only be taken to imply that scopolamine must be formed *in vivo* by a *cis*-dehydrogenation of Hyos-OH (scheme 2 in Fig. 1). The dehydrogenation herein described is a unique reaction since all known epoxidations incorporate molecular oxygen into unsaturated compounds (2, 4, 5, 8, 16, 21, 22). We are now searching for the enzyme(s) that converts Hyos-OH to scopolamine by a *cis*-dehydrogenation reaction.

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