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6β-HYDROXYHYOSCYAMINE EPOXIDASE FROM CULTURED ROOTS OF *HYOSCYAMUS NIGER*

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Key Word Index—*Hyoscyamus niger*, Solanaceae; biosynthesis; tropane alkaloids, 6β-hydroxyhyoscyamine, scopolamine, epoxidase.

Abstract—Enzyme preparations from cultured roots of *Hyoscyamus niger* converted 6β-hydroxyhyoscyamine to scopolamine in the presence of the co-factors required by 2-oxoglutarate-dependent dioxygenases, i.e. 2-oxoglutarate, ferrous ion and ascorbate The epoxidase, a soluble enzyme, requires molecular oxygen for the reaction. Incubations with 6β-[6-^{18}O]hydroxyhyoscyamine and 6β-hydroxy[7β-^{2}H]hyoscyamine as substrates demonstrated that the epoxidation reaction proceeds with retention of the 6β-hydroxy oxygen and with loss of the 7β-hydrogen. The epoxidase activity found under the optimal reaction conditions studied was considerably lower than the activity of hyoscyamine 6β-hydroxylase in cultured roots, and the two activities could not be separated during partial purification. The function of this epoxidase in scopolamine biosynthesis is discussed in relation to hyoscyamine 6β-hydroxylase.

INTRODUCTION

Scopolamine is formed by oxidative transformation of hyoscyamine (**1**) in several solanaceous species [1] (Fig. 1). The first step in this epoxidation is the hydroxylation of hyoscyamine to 6β-hydroxyhyoscyamine (HyosOH, **2**), which is catalysed by the 2-oxoglutarate-dependent dioxygenase, hyoscyamine 6β-hydroxylase [2]. No enzyme(s) that catalyses the subsequent step(s) leading to scopolamine (**3**) has yet been found Although 6,7-dehydrohyoscyamine (**4**) is converted to scopolamine when fed to plants [3, 4], as well as acting as a substrate for hyoscyamine 6β-hydroxylase [5], our feeding study with [6-^{18}O]HyosOH [4] established that the unsaturated alkaloid **4** is not a precursor of scopolamine *in vivo*. Independent feeding of [*N*-methyl-^{14}C, 6β,7β-$^{3}H_2$]tropine [6] and [7β-^{2}H]HyosOH [7] to alkaloid-producing plants resulted in the loss of the 7β-hydrogens in scopolamine Therefore, scopolamine must be formed *in vivo* by 7β-dehydrogenation, followed by the epoxide formation of HyosOH.

We here report that enzyme preparations from cultured *Hyoscyamus* roots convert HyosOH to scopolamine with the same stereochemistry reported for *in vivo* feeding experiments

RESULTS

Properties of 6β-hydroxyhyoscyamine epoxidase

When enzyme preparations from root cultures of *Hyoscyamus niger* which had been partially purified by ammonium sulphate fractionation (55–85% saturation) were incubated with HyosOH, 2-oxoglutarate, ferrous ion, ascorbate and catalase under aerobic conditions, a time-dependent formation of scopolamine took place. The formation of radioactive scopolamine when the enzyme preparation was incubated with [*N*-methyl-$^{3}H_3$]HyosOH and the co-factors required by 2-oxoglutarate-dependent dioxygenases is shown in Fig. 2. No radioactive compounds other than scopolamine and the substrate were detected, the formation of scopolamine depending on the presence of the native enzyme and the co-factors. Omission of 2-oxoglutarate, ferrous ion or ascorbate from the reaction mixture markedly reduced the reaction rate (Table 1). The omission of catalase had no effect on the enzyme activity of our relatively crude enzyme preparations, as had been found for the relatively crude hyoscyamine 6β-hydroxylase [2]. The epoxidase reaction required dioxygen; under anaerobic conditions in which the air in the reaction vial was replaced with nitrogen, there was little reaction (Table 2)

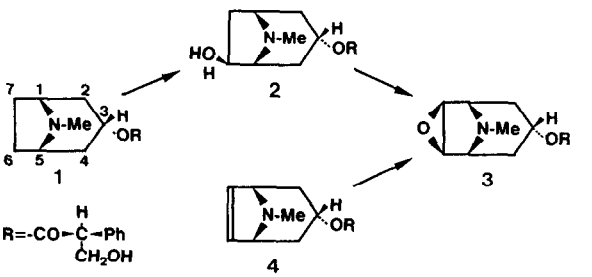

Fig. 1 Biosynthetic pathway from hyoscyamine to scopolamine. The pathway by which 6,7-dehydrohyoscyamine, non-natural alkaloid, is converted to scopolamine is also shown.

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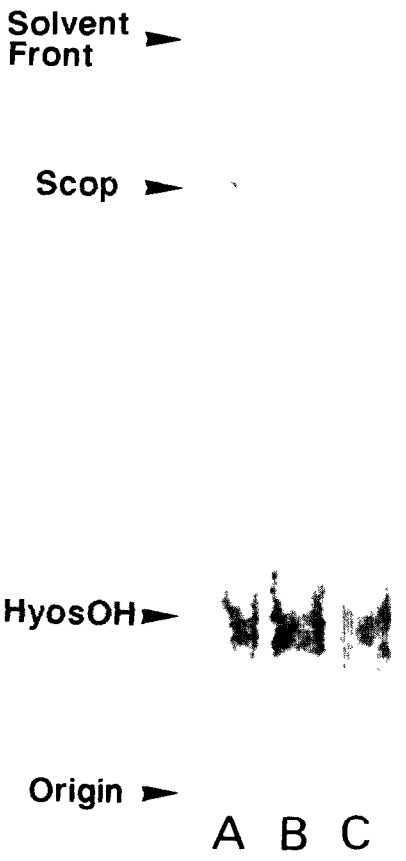


Fig 2 Autoradiography of reaction mixtures after TLC separation Partially purified HyosOH epoxidase (1 1 mg protein) and [*N*-methyl-$^3H_3$]HyosOH (120 nmol, 0 48 μCi) were incubated at 30° overnight with (A), or without (B), the co-factors of 2-oxoglutarate-dependent dioxygenases Boiled enzyme with the co-factors served as the control (C) After termination of the reaction, the alkaloids were extracted and mixed with unlabelled alkaloids The mixture then was chromatographed on a silica gel TLC plate (solvent system A) Alkaloids were located with Dragendorff's reagent, after which the plate was sprayed with EN^{3}HANCE (NEN Research Products) and exposed at −70° for 2 weeks Scop, scopolamine, HyosOH, 6β-hydroxyhyoscyamine

During purification of the epoxidase we found that its activity was rapidly lost even when buffers containing glycerol (up to 30%) or co-factors for the reaction were used. A representative example of our attempted purification is shown in Table 3 The activity of hyoscyamine 6β-hydroxylase was also measured during purification In the crude enzyme extracts, epoxidase activity was three to ten times lower than hydroxylase activity During ammonium sulphate fractionation, Butyl-Toyopearl CC (data not shown), and DEAE-Toyopearl CC, the two enzyme activities were found in the same fractions, but epoxidase activity was generally *ca* 20 times lower than hydroxylase activity after ammonium sulphate fractionation, and *ca* 100 to 200 times lower after the CC steps

Preliminary experiments performed with enzyme preparations partially purified by ammonium sulphate fractionation and Butyl-Toyopearl CC gave a K_m value of 15 μM for HyosOH and an optimal reaction pH of 7 5

Table 1 Co-factor requirements of 6β-hydroxyhyoscyamine epoxidase

Omissions from the complete system	Enzyme activity	
	pkat	% of control
None	1 09	100
6β-Hydroxyhyoscyamine	0 00	0
2-Oxoglutarate	0 06	6
Fe^{2+}	0 00	0
Ascorbate	0 35	32
Catalase	1 15	105

The assay mixtures were incubated for 3 hr at 30° with the partially purified epoxidase (1 75 mg of protein)

Table 2 6β-Hydroxyhyoscyamine epoxidase activity in the presence of air or nitrogen

Experiment	Enzyme activity	
	pkat*	% of control
Control	0 85 ± 0 10	100
N_2→Air	1 03 ± 0 02	121
N_2→N_2	0 03 ± 0 01	3

The standard assay mixture in a stoppered vial was bubbled with nitrogen gas for 10 min, and then partially purified epoxidase (0 56 mg of protein) was injected into the vial and incubated for 2 hr at 30 in air (N_2→air) or in N_2 (N_2→N_2) An assay mixture not subjected to nitrogen bubbling was incubated with the partially purified enzyme preparation in air (control)

*Each value is the mean of three replicates (± s d)

The inhibitory effects of several metal ions on epoxidase activity were tested at 0.2 mM, $CaCl_2$ (0% inhibition), $MgSO_4$ (24%), $ZnSO_4$ (100%), $MnSO_4$ (100%) and $CuSO_4$ (100%). Pyridine 2,4-dicarboxylate, a potent inhibitor of 2-oxoglutarate-dependent dioxygenase [5, and refs therein], inhibited enzyme activity by 41% at 0 5 mM. Additions of the following compounds (0 1 to 10 mM) to the complete reaction mixture had no marked effect on enzyme activity, NAD^+, $NADP^+$, NADH, NADPH, ATP + $MgSO_4$, FAD, FMN, pyrroloquinoline quinone, acetyl-CoA, 6,7-dimethyl-5,6,7,8-tetrahydrofolate, phenazine methosulphate, 2,6-dichlorophenolindophenol, cytochrome *c* and hydrogen peroxide.

To determine whether the observed low epoxidase activities of crude extracts were caused by inefficient solubilization of the enzyme from root cells, Triton X-100 at 0 1% (v/v) or deoxycholate at 0 05% (w/v) was included in the extraction buffer There was no increase in the epoxidase activity of crude extracts, nor was there any increase when the acetone powder of root cells was the starting material. The association of the epoxidase with the cellular membranes was studied in fractions obtained by ultracentrifugation (Table 4) Ultracentrifugation at 160 000 *g* of a crude cell extract in buffer containing 1 mM $MgCl_2$ separated the soluble enzymes from the membrane fraction, as judged by the distribution of cyto-

Table 3 Partial purification of 6β-hydroxyhyoscyamine epoxidase from cultured *H niger* roots

Purification step	Total protein (mg)	Total activity (pkat)		Specific activity (pkat/mg)	
		Epoxidase	Hydroxylase	Epoxidase	Hydroxylase
Crude extract	1632	3719	8677	2.3	5 3
55–85% $(NH_4)_2SO_4$	290	403	3978	1.4	14
DEAE-Toyopearl	9 7	32	2475	3 3	255

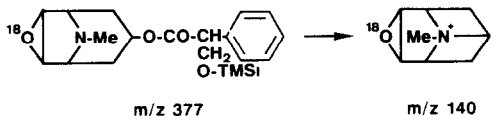
Hyoscyamus roots were cultured and processed as described in Experimental. The enzyme preparation from each purification step was assayed for 6β-hydroxyhyoscyamine epoxidase and hyoscyamine 6β-hydroxylase

chrome *c* reductase, a marker enzyme for microsomes. Both hyoscyamine 6β-hydroxylase and HyosOH epoxidase were recovered in the 160 000 *g* supernatant. Scarcely any activity of either enzyme was associated with the microsomal pellet

Reaction mechanism

Partially purified HyosOH epoxidase was incubated for 4 hr with [6-^{18}O]HyosOH and the co-factors required by 2-oxoglutarate-dependent dioxygenases. The reaction product (scopolamine) was analysed by GC/MS after being converted to its TMSi derivative. It gave a parent ion of *m/z* 377 (rel. int., 8) and a fragment ion at *m/z* 140 (89) (Scheme 1), both of which were two mass/charge units higher than the corresponding ions in authentic scopolamine [*m/z* 375 (5) and 138 (100)]. Based on the ion ratio 377/375 and 140/138, 80% and 78% of the scopolamine molecules contained one atom of ^{18}O in their epoxide oxygen. These values are in good correspondence with the ^{18}O content of 82% found for the substrate, HyosOH. Therefore, all of the ^{18}O in the hydroxyl group of HyosOH was retained in the epoxide oxygen of scopolamine.

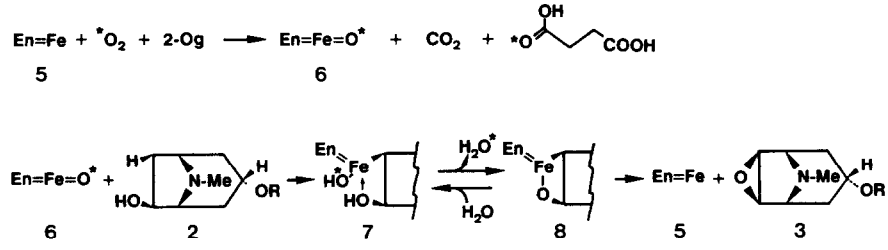
When a large excess of the partially purified epoxidase was incubated overnight with 20 nmol of [7β-^{2}H]HyosOH and the cofactors, all the labelled substrate was converted to scopolamine. GC/MS analysis of the scopolamine formed showed that the reaction product had a mass spectrum identical with that of authentic scopolamine. Thus, the 7β-deuterium in the substrate was lost during epoxidation



Scheme 1

DISCUSSION

A 2-oxoglutarate-dependent dioxygenase, HyosOH epoxidase, from cultured *H. niger* roots converts HyosOH to scopolamine. Although dioxygen is required for the reaction, it is not incorporated into scopolamine, the epoxide oxygen of the scopolamine being derived from the 6β-hydroxyl oxygen of HyosOH. Based on our comparisons of reactions catalysed by other 2-oxoglutarate-dependent dioxygenases [8, 9], we propose the following reaction mechanism for the epoxidation catalysed by HyosOH epoxidase (Scheme 2). The reaction of a ferrous ion-enzyme complex (**5**) with dioxygen and 2-oxoglutarate decarboxylates the keto acid. Simultaneously a highly reactive ferryl enzyme species (**6**) is formed, which we postulate to be a species common to all the dioxygenases of this class. This oxidizing species could be inserted into the C-7-Hβ bond of HyosOH with retention of configuration to give an iron–carbon species



R=-COCH(CH$_2$OH)Ph
En; 6β-Hydroxyhyoscyamine Epoxidase
2-Og; 2-Oxoglutarate

Scheme 2

Table 4 Association of 6β-hydroxyhyoscyamine epoxidase and hyoscyamine 6β-hydroxylase with microsomes

Fraction	Enzyme activities					
	Epoxidase*		Hydroxylase*		CR*	
	(pkat)	(pkat/mg)	(pkat)	(pkat/mg)	(A_{550})	(A_{550}/mg)
270 *g* supernatant (crude extract)	37 2	0 6	528	7 8	417	6 1
160 000 *g* supernatant (soluble enzymes)	28 4	0 7	687	17 0	81	2 0
160 000 *g* pellet (microsome fraction)	0	0	24	1 7	398	19 1

*Epoxidase 6β-hydroxyhyoscyamine epoxidase, hydroxylase hyoscyamine 6β-hydroxylase, CR cytochrome *c* reductase CR was used as the marker enzyme for microsomes

Experiments were repeated twice, all gave similar results

(7) Formation of such an iron–carbon species has been suggested as the explanation of the ring expansion of penicillin N by deacetoxycephalosporin C/deacethylcephalosporin C synthetase, another 2-oxoglutarate-dependent dioxygenase [9] In the subsequent reaction step, intramolecular attack of the 6β-hydroxyl oxygen on the adjacent iron atom from the same exo side of HyosOH would produce, with release of water, a metallocycle (8) which would collapse to scopolamine and the initial ferrous enzyme In such a scheme, iron–carbon species **7** might undergo hydroxylation, to give 6β,7β-dihydroxyhyoscyamine In none of our experiments, did we detect any formation of the dihydroxylated alkaloid, but, the low epoxidase activity and the unavailability of an authentic sample of the alkaloid prevented the detection of any rare hydroxylation reaction

In cultured roots of *H niger*, from which our enzyme preparations were obtained, the most abundant alkaloid is scopolamine (0.1–0 2% dry wt), followed by hyoscyamine (0 03–0 07%) then HyosOH (*ca* 0 01%) [5, 10] As judged by the high ratio of scopolamine to HyosOH and of hyoscyamine to HyosOH, our cultured roots should have higher HyosOH epoxidase activity than hyoscyamine 6β-hydroxylase activity The low epoxidase activity found in the crude enzyme extracts is not the result of inefficient solubilization of the enzyme The rapid loss of enzyme activity during purification may indicate instability of the epoxidase, which might account for the loss of enzyme activity during the preparation of crude extracts from cultured roots Alternatively, optimal expression of the epoxidase activity may require another unknown factor (enzyme?) which is eliminated (or inactivated) during the extraction and purification of the epoxidase. Our reconstitution experiments, in which partially purified epoxidase preparations with reduced activity were mixed with crude extracts, did not, however, restore enzyme activity to the expected levels

The co-purification of the epoxidase and the hydroxylase during partial purification and their requirement for the same co-factors raise the question as to whether the two enzyme activities are located on the same polypeptide In terms of the reaction mechanism, the active site of the hydroxylase might also catalyse the epoxidation reaction, although probably less efficiently Because of steric hindrance between the 6β-hydroxyl group of HyosOH and the ferryl oxygen of the oxidizing species **6**, HyosOH might bind at the active site of the hydroxylase in an orientation that forces the ferryl oxygen to a position closer to the 7β-hydrogen than it takes with the normal substrate hyoscyamine If so, species **6** might be inserted into the C-7-Hβ bond of HyosOH at a low rate, with formation of an epoxide taking place by the mechanism described in Scheme 2 Although this bifunctional nature of the hydroxylase is theoretically possible, an unknown factor for epoxidase activity still must be postulated to explain the stability of the epoxidase being lower than that of the hydroxylase during partial purification

EXPERIMENTAL

Materials Root cultures of *Hyoscyamus niger* L were initiated and subcultured as described in ref [10] Prior to enzyme extraction, the roots were transferred to 300 ml flasks containing 75 ml of B5 medium [11] then cultured for 1 week Butyl-Toyopearl 650 M and DEAE-Toyopearl 650 M were purchased from TOSOH, Japan

6β-[6-^{18}O]*Hydroxyhyoscyamine* [6-^{18}O]HyosOH was prepared from hyoscyamine and $^{18}O_2$ by hyoscyamine 6β-hydroxylase as described in ref [4] GC/MS analysis showed that the alkaloid has 82 atom % of ^{18}O at the 6β-hydroxyl oxygen

6β-*Hydroxy*[7β-^{2}H]*hyoscyamine* Scopolamine was converted to 6,7-dehydrohyoscyamine as described in ref [6] The unsaturated alkaloid, identified by ^{1}H NMR and MS, was hydrogenated in EtOAc with $^2H_2$ (>99 75%, CEA, France) in the presence of 10% Pd/C to give [6β,7β-$^2H_2$]hyoscyamine ^{1}H NMR (400 MHz, $CDCl_3$) on unlabelled hyoscyamine δ2 20 (3H, s, N-Me), 2 92 and 3 04 (1H, *br t*, J = 3 4 Hz, H-1 and H-5), 1 47 and 1 68 (1H, *br d*, J_{gem} = 15 0 Hz, H-2$_{ax}$ and H-4$_{ax}$), 2 03 and 2 11 (1H, *br dt*, J_{gem} = 15 0 Hz, J = 3 4–5 2 Hz, H-2$_{eq}$ and H-4$_{eq}$), 5 03 (1H, *t*, J = 5 2–5 5 Hz, H-3), 1 18 and 1 76 (1H, *ddd*, J_{gem} = 13 2 Hz, J = 4 0 Hz, 9 2 Hz, H-6$_{endo}$ and H-7$_{endo}$), 1 74 and 1.86 (1H, *m*, H-6$_{exo}$ and H-7$_{exo}$) In the [^{2}H]hyoscyamine, the broad triplet signals at δ2 92 and 3 04 changed to broad singlets, and the doublets of double doublets at δ1 18 and 1 76, to broad doublets (J = 9 8) The multiplets at δ1 74 and 1 86 also were reduced to small signals The TMSi derivative of the labelled hyoscyamine gave MS (70 eV), *m/z* (rel int) 363 [M]$^+$ (5 5), 126 [M − tropic acid moiety]$^+$ (100), 94 [$C_5H_5N^+Me$] (22) The HBr salt had $[\alpha]_D^{20}$ −25 1 (H_2O, *c* 3 09) Comparisons of NMR

and mass spectra data for the labelled hyoscyamine with the data for the unlabelled alkaloid confirmed the location of the two deuterium atoms

The deuterated hyoscyamine was hydroxylated at the 6β-position by hyoscyamine 6β-hydroxylase partially purified from cultured *H niger* roots ^{1}H NMR (400 MHz, $CDCl_3$) on unlabelled HyosOH δ2 48 (3H, *s*, N-Me), 3.09 (1H, *br t*, $J = 3$ 0 Hz, H-1), 1.22 and 1 59 (1H, *br d*, $J_{gem} = 15$ 6 Hz, H-2_{ax} and H-4_{ax}), 2 06 and 2 16 (1H, *br dt*, $J_{gem} = 15$ 6 Hz, $J = 3$ 6–5 4 Hz, H-2_{eq} and H-4_{eq}), 5 04 (1H, *t*, $J = 5$ 2–5 5 Hz, H-3), 2 96 (1H, *br s*, H-5), 4 32 (1H, *dd*, $J = 2$ 4 Hz, 7 0 Hz, H-6_{endo}), 1 75 (1H, *dd*, $J_{gem} = 13.7$–14 0 Hz, $J = 7$ 0–7 3 Hz, H-7_{endo}), 1 5–1 6 (1H, *m*, H-7_{exo}) In the [^{2}H]HyosOH, the broad triplet signal at δ3 09 changed to a broad singlet, and the doublets of doublets at δ4 32 and 1 75, to doublets (each with $J = 7$ 6) The signal at δ 1 5–1 6 disappeared The TMSi derivative of the labelled HyosOH gave MS (70 eV), *m/z* (rel int) 450 $[M]^+$ (1 2), 435 $[M - Me]^+$ (1 2), 213 [M − tropic acid moiety]$^+$ (17), 207 (18), 95 $[C_5H_6N^+Me]$ (100), 94 $[C_5H_5N^+Me]$ (74) These spectral data confirmed the location of ^{2}H Mass fragmentography with two selected sets of ions (*m/z* 212 − 215 and *m/z* 449 − 452) indicated that the alkaloid has 85 and 84 atom % of ^{2}H at the 7β-position

6β-Hydroxy[*N*-methyl-$^3H_3$]*hyoscyamine* [*N*-methyl-$^3H_3$] atropine (0 75 mCi, 87 Ci/mmol, NEN Research Products) was converted to [*N*-methyl-$^3H_3$]HyosOH by the hyoscyamine 6β-hydroxylase partially purified from cultured *H niger* roots The hydroxylase is active only for the *S* isomer in [*R, S*]hyoscyamine (i e atropine) [2]. After the reaction, the radioactive HyosOH was mixed with unlabelled HyosOH, purified by prep TLC, and the mixture recrystallized as an HBr salt from EtOH–Et_2O (see ref [2]) This HBr salt (4 0 Ci/mol) was dissolved in 50% EtOH and stored at −20° until used

Partial purification of 6β-hydroxyhyoscyamine epoxidase Root cultures were harvested on a suction filter, frozen with liquid N_2, then homogenized in a Waring blendor The frozen homogenate was kept at −20° until use Subsequent procedures were done at 4° The cell homogenate was suspended in 2 vol of Pi buffer (100 mM, pH 7 8) containing 0.2 M sucrose and 3 mM dithiothreitol This suspension was ground thoroughly in a mortar with sea sand then mixed with 10% (w/v) insoluble polyvinylpyrrolidone. After being kept at 4° for 30 min with occasional stirring, the suspension was passed through a composite cheesecloth–Miracloth–cheesecloth filter and centrifuged at 12 000 *g* for 30 min The resulting supernatant was used as the crude extract

The HyosOH epoxidase in the crude extract was pptd between 55 and 85% saturation by $(NH_4)_2SO_4$ The ppt obtained after centrifugation at 12 000 *g* for 30 min was dissolved in a minimum vol of 40 mM Tris–HCl buffer (pH 7.5) containing 0.2 M sucrose, 1 mM dithiothreitol and 30% (v/v) glycerol (buffer A), this buffer having been adjusted to 21% saturation with solid $(NH_4)_2SO_4$

The enzyme soln was loaded on a butyl-Toyopearl column (2 6 × 10 cm) that had been equilibrated with buffer A saturated (21%) with $(NH_4)_2SO_4$ The column was treated in steps with buffer (100-ml portions) containing 21, 14, 7 and 0% $(NH_4)_2SO_4$ in buffer A, at a flow rate of 1 ml/min Most of the active fractions were eluted with 7% $(NH_4)_2SO_4$

When DEAE-Toyopearl chromatography was used for the purification, the $(NH_4)_2SO_4$ ppt dissolved in buffer A was desalted by passing it through a Sephadex G-25 M (Pharmacia) column, after which it was loaded on a DEAE-Toyopearl 650 M column (2 6 × 20 cm) that had been equilibrated with buffer A containing 50 mM NaCl After the column had been washed with 150 ml of the same buffer, the enzyme was eluted at a flow rate of 1 ml/min with a linear gradient of 50 and 200 mM NaCl (150-ml portions) dissolved in buffer A

In both systems the active fractions were pooled and concd with an Amicon YM-10 ultrafiltration membrane. The concentrate was passed through a PD-10 column (Pharmacia), and the enzyme eluted with buffer A Glycerol was added to the eluted enzyme to a final concentration of 50% (v/v) The enzyme was stored in small portions at −20° until assayed

Protein concns were determined by the method of ref. [12] with bovine serum albumin as the standard

Enzyme assay. HyosOH epoxidase activity was assayed by measuring the formation of scopolamine by GC The complete assay mixture, in a total vol of 1 ml, contained 50 mM Tris–HCl buffer (pH 7 5 at 30°), 0.4 mM $FeSO_4$, 4 mM Na ascorbate, 1 mM 2-oxoglutaric acid, 0 2 mM HyosOH HBr, 2 mg/ml catalase C-10 (Sigma) and the enzyme Incubation was at 30° for 2 hr The reaction was started by the addition of the enzyme and was stopped by the addition of 0.1 ml of *ca* 1 2 M Na-carbonate buffer (pH 10 5) The subsequent procedure used to extract the reaction product and the conditions for derivatization are reported in ref [4] TMSi derivatives of alkaloids were analysed with a gas chromatograph equipped with a fused silica capillary column CBP-1 (25m × 0.2mm, Shimadzu, Kyoto) Conditions for GC· detection, FID, carrier gas, He at 1 ml/min, split ratio, 50 · 1, column temp, 250°, injection temp, 300°, injection vol, 2 μl.

In the study with [*N*-methyl-$^3H_3$]HyosOH as the substrate, the epoxidase activity was determined by measuring the radioactivity of scopolamine that had been separated from the substrate by silica gel TLC ($CHCl_3$–EtOH–28% NH_4OH 85 15 4, solvent system A) After detection under UV_{254}, the portion containing scopolamine was scraped from the plate, dissolved in 1 ml of MeOH then mixed with 10 ml of Univer-gel II (Nacalai tesque, Kyoto). The enzyme activities determined in several samples by both the GC and RI methods were the same

Hyoscyamine 6β-hydroxylase activity was measured as described in ref [2].

Identification of the reaction product The reaction product was identified as scopolamine based on the following data· (i) R_t (5 5 min) on GC under the conditions described (ii) R_f (0 81) on silica gel TLC (solvent system A) with detection under UV_{254} or by Dragendorff's reagent (iii) GC/MS of the TMSi derivative (70 eV) *m/z* (rel int.)· 375 $[M]^+$ (9 6), 138 [M − tropic acid moiety]$^+$ (100)

Fractionation of microsomes Freshly harvested cultured roots of *H niger* (25 g) were suspended in 45 ml of Tris–HCl buffer (0 2 M, pH 7 2) containing 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 10 mM dithiothreitol and 20% (w/v) sucrose (buffer B), after which they were ground gently in a mortar then mixed with 1 g of insoluble polyvinylpyrrolidone. This suspension was passed through 4 layers of cheesecloth then centrifuged at 270 *g* for 10 min The resulting crude extract was centrifuged at 160 000 *g* for 1 hr, after which the ppt. obtained was washed once with buffer B then suspended in buffer A. The protein in the supernatant was precipitated by adding $(NH_4)_2SO_4$ to 85% saturation The ppt obtained after centrifugation at 15 000 *g* for 20 min was dissolved in a minimum vol of buffer A, then passed through a PD-10 column and the enzyme eluted with buffer A.

Cytochrome *c* reductase was used as the marker enzyme for microsomes Its activity was measured as described in ref [13]

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