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TROPIC ACID MOIETY OF ATROPINE MAY BE RECYCLED IN *DUBOISIA*

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Key Word Index—*Duboisia leichhardtii*; Solanaceae; tropic acid; tropoyl derivatives; tropane alkaloids; root culture.

Abstract—Ester derivatives of labelled tropic acid (denoted tropoyl-A and -D) were isolated as metabolites of atropine from *Duboisia myoporoides* seedlings supplied with [carbonyl-^{14}C]atropine sulphate and were then fed to *D. leichhardtii* root cultures to determine whether they were metabolic end-products or whether they could be metabolized further. Both tropoyl-A and tropoyl-D were incorporated into tropane alkaloids (atropine, 6-hydroxyatropine and scopolamine). Incorporation from tropoyl-D exceeded that from tropoyl-A. This result shows that both tropoyl-A and -D are precursors of tropane alkaloids and that the tropic acid moiety is possibly recycled in plants of *Duboisia*.

INTRODUCTION

The tropane alkaloid atropine [(±)-hyoscyamine] is not necessarily a metabolic end-product in plants from which it is isolated. Scopolamine is the most useful metabolite of atropine and the route has been well established biochemically [1–3]. Atropine is also decomposed to tropine (base) and tropic acid (acid) by atropine esterase [4, 5], and scopolamine may be hydrolysed as well [6]. The fate of tropine and tropic acid after decomposition is very interesting, because it may give us some insight into the physiological role of tropane alkaloids in plants.

In the previous paper [7], we determined some aspects of atropine dynamics in *Duboisia myoporoides* seedlings supplied with [carbonyl-^{14}C]atropine sulphate and showed that atropine was metabolized very rapidly with a half-life of about three weeks. During this process, polar non-alkaloid tropoyl derivatives (tropoyl-A to -D), as well as the alkaloids scopolamine and aposcopolamine, were found as major atropine metabolites [7]. Only a trace of tropic acid was detected, indicating that the free acid produced by the action of atropine esterase must be converted into tropoyl derivatives at once. Although the structures of the tropoyl derivatives have not yet been characterized completely, we have found that they are esters of tropic acid with unknown substances. However, it was not clear whether the tropoyl derivatives were final metabolites or were themselves precursors of further metabolites.

Here, we report that the tropic acid moiety of these tropoyl derivatives is incorporated into tropane alkaloids by a root culture system of *D. leichhardtii*, which has the ability to synthesize tropane alkaloids, but not to decompose them [5]. This is the first proposal of the recycling of the tropic acid moiety of atropine.

RESULTS AND DISCUSSION

Four esters of tropic acid with unknown substances were found as non-alkaloidal metabolites of [carbonyl-^{14}C]atropine in the seedlings of *D. myoporoides* [7]. These compounds were named as tropoyl-A, -B, -C and -D according to their R_f values on TLC with butanol–acetic acid–H_2O (4:1:1); tropoyl-A and -D were major metabolites and tropoyl-B and -C were trace compounds. Therefore, we isolated and radiochemically purified tropoyl-A and -D by TLC using the same solvent system.

In previous work, it was found that *D. leichhardtii* roots started to propagate vigorously seven days after inoculation into fresh medium and a further week was then enough for uptake and incorporation of radiochemicals [6]. Therefore, labelled tropoyl-A and -D were supplied to seven-day-old root cultures and incubation was for a further seven days. In the first feeding experiment, the roots did not grow at all and their tips turned brown. The odour of butanol could be detected in the culture medium, suggesting that the butanol used in the purification of tropoyl-A and -D had been carried over into the culture medium. Therefore, we carefully

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Table 1. Distribution of radioactivity recovered from *D. leichhardtii* root cultures supplied with [^{14}C]-tropoyl-A and [^{14}C]-tropoyl-D

Substance*	Experiment	Root growth (g fr. wt)	Radioactivity recovered (kBq)	Distribution of radioactivity (%)					
				Root†			Medium†		
				o.a.	alk.	aq.	o.a.	alk.	aq.
Tropoyl-A	1‡	0.25	1.21	1>	1>	1>	99	1>	1>
	2	3.71	0.73	13	48	39	1>	1>	1>
Tropoyl-D	1‡	0.25	1.05	1>	1>	1>	9	60	31
	2	2.80	0.64	7	89	4	1>	1>	1>

*Radioactive substance (1.2 kBq per culture) was added to 7-day-old root cultures and incubated for an additional 7 days.
†o.a., organic acid fr.; alk, alkaloidal fr.; aq., aqueous residue fr.
‡Root growth was inhibited by butanol contaminated in the process of purification of tropoyl-A and -D.

eliminated butanol from these substances before supplying them to the root cultures in the second feeding experiment.

After one week of incubation, root cultures were harvested and both root extracts and media were fractionated into organic acid, alkaloidal and aqueous residue fractions. The distribution of radioactivity in these fractions was then determined (Table 1). When root growth was inhibited by butanol, all the radioactivity supplied as tropoyl-A and -D was recovered from the media and not from the root tissues. The cultured roots could not retain any labelled compounds under these non-growing conditions. This was consistent with previous observations; under phytostatic conditions cultured roots excreted any alkaloids produced into the cultured medium (unpublished data). On the other hand, in the second experiment, in the absence of growth inhibition, the radioactivity was found only in the root tissues and not in the medium.

Under growing conditions, both tropoyl-A and -D were incorporated into alkaloids: 48 and 89% of total radioactivity, respectively (Table 1). Under phytostatic conditions, 60% of tropoyl-D was still converted into alkaloids, but there was no conversion of tropoyl-A into alkaloid. Organic acid and alkaloidal fractions with determinable levels of radioactivity were further analysed by TLC–autoradiography. A radioactive spot corresponding to free tropic acid was found in the organic acid fraction of the root culture medium supplied with tropoyl-A in the presence of butanol. Under these non-growing conditions, tropoyl-A was apparently hydrolysed, but not converted into alkaloids. In the alkaloidal fraction, labelled atropine, 6-hydroxyatropine and scopolamine were found in the root cultures supplied with tropoyl-A or -D separately (Table 2). Even when tropoyl-D was supplied in the presence of butanol, all the labelled alkaloids above were detected, though scopolamine formation was very small.

Both tropoyl-A and -D, metabolites of atropine by *Duboisia* seedlings, were successfully incorporated into tropane alkaloids of *Duboisia* root cultures under growing conditions, though the rate of incorporation of tropoyl-D was much higher than that of tropoyl-A. Under non-growing conditions only tropoyl-D was converted into tropane alkaloids. These results indicated that both tropoyl-A and -D were precursors of atropine and that the tropic acid moiety of atropine was possibly being recycled in intact plants of *Duboisia*. Tropoyl-D seemed to be a more direct precursor than tropoyl-A. Judging from their behaviour on TLC, tropoyl-D may be a more polar substance than tropoyl-A. The possibility that tropoyl-A and -D may be interconverted has not yet been investigated.

We have previously reported that exogenous tropic acid could not be incorporated into tropane alkaloids in our root culture system [6]. Here, tropoyl derivatives, especially tropoyl-D, were shown to be active precursors for tropane alkaloid formation. The mechanism by which tropoyl-D was converted into hyoscyamine

Table 2. Incorporation of radioactivity from [^{14}C]-tropoyl-A and [^{14}C]-tropoyl-D into tropane alkaloids

Substance*	Experiment	Percentage total incorporated into alkaloid		
		Atropine	6-Hydroxyatropine	Scopolamine
Tropoyl-A	1‡	n.d.	n.d.	n.d.
	2	40	33	27
Tropoyl-D	1‡	19	78	3
	2	13	53	34

*,‡See footnotes to Table 1.
n.d. = not determined.

without involvement of free tropic acid is a very important question. It has been shown that phenyllactic acid is an intermediate in the biosynethesis of the tropic acid moiety of atropine [8, 9]. Furthermore, in transformed root cultures of *Datura stramonium* it has now been reported that littorine, the ester of tropine with phenyllactic acid, is converted into hyoscyamine by an intramolecular rearrangement [10, 11] and it has been demonstrated in these cultures that tropic acid is not an intermediate in hyoscyamine biosynthesis [12]. Both results obtained from *Datura stramonium* and *D. leichhardtii* root cultures support the view that free tropic acid is not incorporated into tropane alkaloid. However, it is unknown how the recycling of the tropic acid moiety indicated in the present work relates to the mechanism of *de novo* biosynthesis of hyoscyamine via littorine demonstrated to occur in *D. stramonium* [10, 11].

From our experimental results, we propose a possible recycling pathway for the tropic acid moiety of atropine in intact plants of *Duboisia* (Fig. 1). Although atropine and tropoyl derivatives are readily extractable from aerial tissues of the intact plants [7], their biosynthesis and degradation are thought to occur exclusively in the root [5, 7]. Therefore, prior to degradation these compounds must be returned to the roots from the aerial parts.

EXPERIMENTAL

Chemicals. Tropoyl derivatives. [^{14}C]-Tropoyl-A and -D were isolated from *D. myoporoides* seedlings supplied with [carbonyl-^{14}C]atropine sulphate and cultured for 7–11 weeks as described previously [7]. Lyophilized plant material was powdered and extracted 4× with 80% MeOH, the extract evapd to dryness and the residue dissolved in H_2O. This soln was sepd into 3 frs: (i) organic acid, (ii) alkaloidal and (iii) aq. residue, as follows. The aq. soln was acidified with 10% HCl and extracted 3× with Et_2O. The aq. layer was then adjusted to pH 9 with 7% NH_4OH and extracted 3× with $CHCl_3$. The resulting aq. residue fr. from which organic acids and alkaloids were eliminated was neutralized with HOAc, concd under red. pres. below 60° and subjected to TLC–autoradiography (silica gel GF_{254}, BuOH–HOAc–H_2O, 4:1:1). 4 radioactive bands (R_f values: 0.89, 0.68, 0.59, 0.44, 0.44) were observed; the 2 major bands (R_f values: 0.89, 0.44) were removed and eluted 3× with 80% MeOH. This soln was resubjected to TLC–autoradiography to achieve complete sepn. The substances were then dissolved in H_2O and supplied to the root cultures.

Standard. Atropine (Nacalai Tesque), scopolamine hydrobormide (Nacalai Tesque) and 6-hydroxyatropine sulphate synthesized in our laboratory according to the

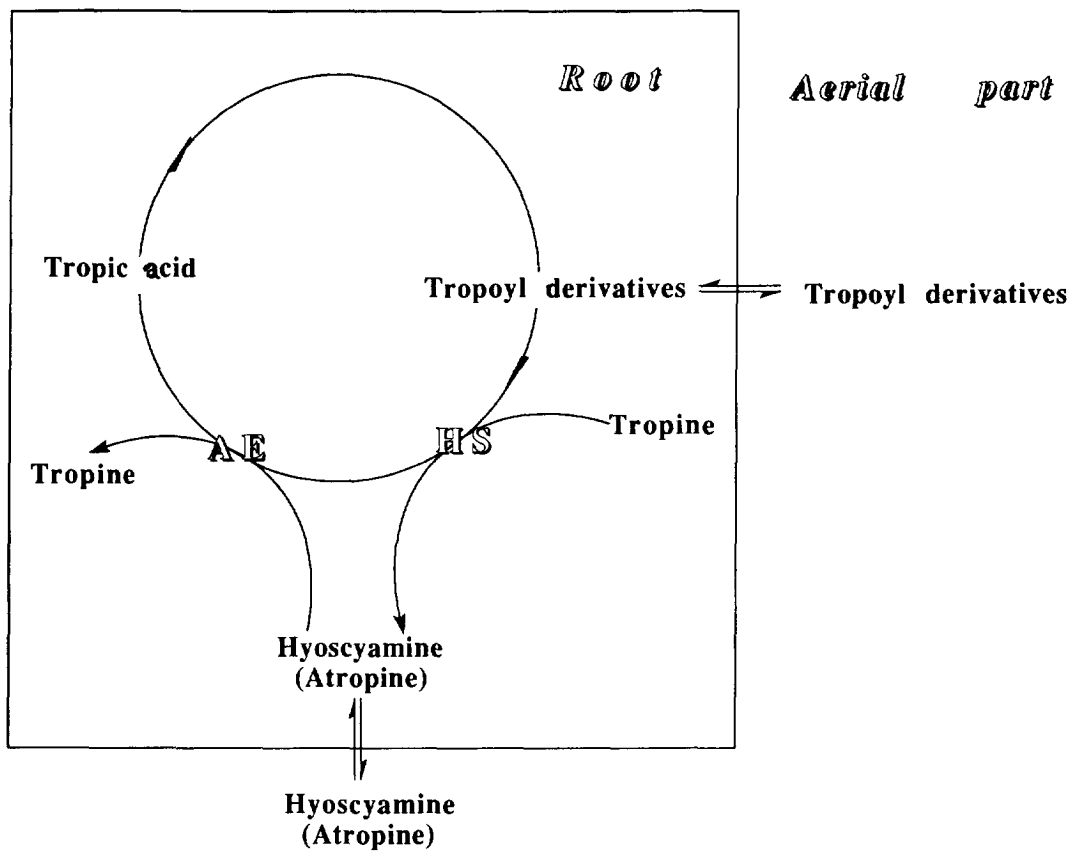

Fig. 1. Hypothetical pathway of recycling of tropic acid moiety of atropine in *Duboisia*. HS, hyoscyamine synthetase; AE, atropine esterase.

method of ref. [13] were used as authentic alkaloids. As authentic acids, (±)-phenyllactic acid (Sigma), phenylpyruvic acid (Sigma) and [carboxyl-^{14}C] tropic acid, prepd from [carbonyl-^{14}C]atropine sulphate by enzymic digestion [6], were used.

Feeding experiment. Root cultures of *D. leichhardtii* F. Muell were maintained in Murashige–Skoog basal medium [14] containing indole-3-butyric acid (2 mg^{-1}), gibberellic acid (1 mg^{-1}) and sucrose (70 g^{-1}) as described in ref. [6]. The cultured roots (0.3 g. fr. wt) were inoculated into fresh medium (25 ml) in a 100-ml conical flask and cultured for 7 days on a rotary shaker (80 rpm) at 25° in continuous dim light. Then 0.5 ml of an aq. soln of [^{14}C]-tropoyl-A or -D (1.2 kBq) was added separately to the cultures, which were then incubated for an additional 7 days under the same conditions.

Extraction and analysis of products. Cultured roots were harvested by filtration, lyophilized and then extracted 4× with 80% MeOH. The 80% MeOH extract and filtered medium were further divided into organic acid, alkaloidal and aq. frs as described above. Both the organic acid and alkaloidal frs were evapd to dryness and then redissolved in MeOH. Radioactivity of each fr. was determined by liquid scintillation counting (Beckman LS 6000 TA). Organic acid and alkaloidal frs were subjected to TLC–autoradiography [silica gel GF_{254}; MeOH–C_6H_6, (1:1) and $CHCl_3$–EtOH–NH_4OH (85:14:1), respectively; R_f-values of tropic, phenyllactic and phenylpyruvic acids were 0.67, 0.59 and 0.85 and those of 6-hydroxyatropine, atropine and scopolamine were 0.11, 0.25 and 0.68]. Radioactive spots corresponding to atropine, scopolamine and 6-hydroxyatropine were removed, the alkaloids were eluted from the gel with MeOH and their radioactivities were again measured by liquid scintillation counting.

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