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ACETYLATION OF TROPANE DERIVATIVES BY *DATURA INNOXIA* CELL CULTURES

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Key Word Index—*Datura innoxia*; Solanaceae; biotransformation; tissue culture; acetylation; tropane alkaloids; acetyltropine; acetylscopine.

Abstract—Scopine, scopoline, pseudotropine as well as tropine supplied to the culture medium were converted into the corresponding acetates by cell cultures of *Datura innoxia*. Tropine was esterified preferentially with endogenous acetic acid, even if other organic acids in combination with tropine were supplied exogenously. When *D. innoxia* cell cultures were fed with tropine and tropic acid in the presence of different kinds of auxins, no alkaloidal metabolites but acetyltropine were detected in each treatment. Apart from *Datura*, tissue cultures induced from 15 other species belonging to 12 families were incapable of acetylating tropine.

INTRODUCTION

Although the alkaloid content in cultured cells of *Datura* decreases gradually during successive subcultures [1], the ability to esterify tropine with acetic acid is retained even in cell cultures producing only a very small amount of alkaloid [2]. It had been reported by Romeike and Koblitz [3] that tropine fed together with tropic or tiglic acid to tissue cultures of *Datura* is not esterified with these acids but is esterified with endogenous acetic acid. However, it was unknown whether or not *Datura* tissue cultures were able to esterify tropane derivatives other than tropine with endogenous acetic acid. In the present study, we have examined the production of metabolites by *Datura* cells fed with various tropane bases as well as organic acids. The species specificity of tropine acetylation by plant cell cultures and the effect of auxins on the reaction were also studied.

RESULTS

Effect of auxins on acetyltropine formation

D. innoxia cells that had been grown in liquid media supplemented with 1 μM 2,4-dichlorophenoxyacetic acid (2,4-D), 1 μM 1-naphthaleneacetic acid (NAA), 10 μM indole-3-butyric acid (IBA) or 10 μM IAA for 24 days, were supplied aseptically with tropine and DL-tropic acid and incubated for a further 4 days. Alkaloids were detected mostly in the cells with very small amounts being detected in the medium. Qualitative TLC and PC revealed that the only metabolite of tropine in the cells cultured in 2,4-D, NAA, IBA or IAA medium was acetyltropine and that hyoscyamine was undetectable in any cultures (Table 1, experiment 1). The acetyltropine samples (5 mg picrate from 35 flasks) were combined, because of the small quantities and identified by mmp and IR.

Substrate specificity of esterification by Datura *cell cultures*

To examine the possibility of tropine esterification with organic acids other than acetic acid, suspension cultures of *D. innoxia* were supplied with 1 mM tropine and 1 mM of one of the following acids: tiglic acid, DL-mandelic acid, benzoic acid, *iso*-butyric acid, *trans*-cinnamic acid, caffeic acid, DL-lactic acid or DL-β-phenyllactic acid. TLC of the alkaloid fractions revealed that only acetyltropine was synthesized and no unique esters were found (Table 1, experiment 2). Acetyltropine isolated from cultures fed with tiglic acid, benzoic acid or mandelic acid was identified by mmp and IR.

In an attempt to ascertain whether or not cultured cells of *D. innoxia* could transform tropane bases other than tropine to their corresponding acetates, suspension cultures were fed with pseudotropine, scopine or scopoline. Each of the bases was completely absorbed into the cells, and although most remained unchanged, a small amount of metabolite was found in the culture medium. TLC and PC showed that pseudotropine, scopine, scopoline as well as the control substrate, tropine, were converted into their corresponding acetates (Table 1, experiment 3). The acetyl ester was found in both the culture medium and the cells when tropine or scopine were supplied to the culture. Interestingly, the metabolite was detectable only in the medium but not in the cells when the culture was fed with pseudotropine or scopoline. Acetylscopine isolated from the scopine-fed cultures was identified by mmp and IR and mass spectral data. The metabolites of pseudotropine and scopoline were each present in too small an amount for further identification.

Species specificity of tropine acetylation

To investigate this aspect of tropine acetylation, cell suspension cultures derived from 15 plant species belonging to 12 families (Table 2) were cultured in media

Table 1. Acetylation of tropane derivatives by *D. innoxia* cell suspension cultures

Expt No.	Growth regulator*	Substrate†	Metabolite
1	IAA	Tropine + TA	Acetyltropine
	IBA	Tropine + TA	Acetyltropine
	NAA	Tropine + TA	Acetyltropine
	2,4-D	Tropine + TA	Acetyltropine
2	2,4-D	Tropine + acid‡	Acetyltropine
3	2,4-D	Tropine	Acetyltropine
	2,4-D	Pseudotropine	Acetylpseudotropine
	2,4-D	Scopine	Acetylscopine
	2,4-D	Scopoline	Acetylscopoline

*IAA, 10 μM; IBA, 10 μM; NAA, 1 μM; 2,4-D, 1 μM; TA, DL-tropic acid (2 mM).

†Each substrate was supplied at a concentration of 1 mM except for DL-tropic acid (TA, 2 mM).

‡Tropine, along with one of the following acids: tiglic, DL-mandelic, benzoic, *iso*-butyric, *trans*-cinnamic, DL-lactic or DL-β-phenyllactic acid.

Table 2. Plant cell cultures lacking in the ability to convert tropine into acetyltropine

Origin of cell culture			
Family	Systematic name	Organ	Growth regulator (μM)
(Dicotyledoneae)			
Amaranthaceae	*Achyranthes japonica* Nakai	Leaf	2,4-D (1)
Boraginaceae	*Lithospermum erythrorhizon* Sieb. et Zucc.	Seedling	IAA (1) + kinetin (10)
Compositae	*Atractylodes lancea* DC.	Leaf	2,4-D (1)
Euphorbiaceae	*Mallotus japonicus* (Thunb.) Muell Arg.	Leaf	2,4-D (1)
Labiatae	*Ajuga decumbens* Thunb.	Leaf	2,4-D(1) + kinetin (1)
Leguminosae	*Cytisus scoparius* Link	Stem	2,4-D (10)
Phytolaccaceae	*Phytolacca americana* L.	Leaf	2,4-D (1)
Rubiaceae	*Gardenia jasminoides* Ellis	Flower bud	2,4-D (1)
Solanaceae	*Atropa belladonna* L.	Leaf	NAA (1)
	Nicotiana rustica L. cv Brazilia	Root	2,4-D (1)
	N. tabacum L. cv Bright Yellow	Stem	IAA (10) + kinetin (1)
	Scopolia japonica Maxim.	Rhizome	2,4-D (1)
Umbelliferae	*Bupleurum falcatum* L.	Leaf	2,4-D (1) + kinetin (1)
(Monocotyledoneae)			
Dioscoreaceae	*Dioscorea tokoro* Makino	Seedling	2,4-D (1)
Liliaceae	*Allium cepa* L.	Stem	2,4-D (1)

containing 1 mM tropine. However, acetyltropine was undetectable by TLC and PC in any alkaloid fractions from both cells and media of all these cultures.

Acetyltropine formation by intact plants

Two experiments were conducted to examine the capability of the intact plants or organs of *Datura* to form acetyltropine from externally supplied tropine. In the first, *D. innoxia* plants were cultured in a solution of tropine (250 mg in total) for 6 days. Alkaloids extracted from the plants, and the hydroponic medium, were analysed by TLC and PC, but acetyltropine was undetectable in either extract. In the second, intact *D. innoxia* plants were separated into aerial parts and the roots and these were then treated as mentioned above. Acetyltropine was not detected in the aerial parts and their culture medium. It was, however, present in small amounts in the roots and their medium.

DISCUSSION

It is well-known that auxins affect the production of some constituents in plant tissue cultures, e.g. nicotine in tobacco callus cultures [4]. Romeike reported that when tropine and tropic acid are supplied to tissue cultures of *D. innoxia* acetyltropine is produced in tissues cultured on a medium containing 2,4-D, while atropine is detectable (TLC and PC) in those grown on a NAA medium [5]. In the present experiments, however, tropine supplied to *D. innoxia* cell cultures was not esterified with exogenous tropic acid. Instead, it was esterified with endogenous

acetic acid to form acetyltropine irrespective of the kind of auxin (2,4-D, NAA, IBA or IAA) in the culture medium. The reason for this discrepancy, however, is unclear.

On the basis of the data on acid substrate specificity, it seems reasonable to assume that tropine added to the culture is esterified preferentially with acetic acid produced by the cell, even when other organic acids are supplied exogenously to the culture. As for the base substrates, three tropane bases in addition to tropine were esterified with acetic acid by *D. innoxia* cell cultures. Although the acetates of pseudotropine and scopoline were formed only in small amounts, 8.8% of the scopine added to the medium was converted to its acetate by the cells. This conversion rate approximates closely that for tropine [2]. These findings suggest that the acetylation of tropine is catalysed by an esterase which is highly specific to acid substrates but much less specific to base substrates.

Formation of acetyltropine has been found in callus cultures derived from various organs of *D. innoxia* [2, 5], *D. metel*, *D. stramonium* [5] and *D. tatula* [2] when they are grown on a medium containing tropine. Although *Atropa belladonna* and *Scopolia japonica* are solanaceous plants containing tropane alkaloids, the present experiments have demonstrated that their callus tissues are incapable of acetylating exogenous tropine. Furthermore, none of the cell suspension cultures listed in Table 2 could convert tropine into acetyltropine. These findings agree with those of Romeike's that *Nicotiana*, *Sphaerophysa*, *Dichroa*, *Ruta* and *Daucus* cells cultured *in vitro* are unable to esterify tropine with acetic acid [3, 5]. Consequently, the reaction leading to acetyltropine must be quite specific to *Datura* cells.

It is interesting that the excised root of *Datura* can esterify tropine with endogenous acetic acid as reported by Romeike and Aurich [6], whereas acetyltropine is undetectable in either the intact plant or the excised shoot fed with tropine. In the intact plant, acetyltropine may be translocated from the root to the aerial parts and converted into other compounds in a way similar to that of tigloyl esters of 3,6,7-tropanetriol in *D. innoxia* [7]. Such metabolism of acetyltropine has been observed in cultured cells of *Datura*. When *D. innoxia* cell suspensions in two flasks precultured for one week were supplied with 72.7 mg acetyltropine, only 15% of the compound was recovered after an incubation period of two additional days. Thus it appears as if the characters of both the intact plant and the excised root were carried into cells cultured *in vitro*, the content of acetyltropine in *Datura* cell cultures fed with tropine might be controlled by a dynamic balance between its synthesis and catabolism.

EXPERIMENTAL

Tissue cultures. Static cultures of *Datura innoxia* Mill. were maintained as previously reported [2]. The other callus tissues were cultured on agar media containing Linsmaier–Skoog's components [8] and the plant growth regulators shown in Table 2. Suspension cultures were initiated from static cultures by transferring them to the corresponding liquid media. These cultures were grown on a reciprocal shaker (90 rpm, 70 mm stroke) in the dark at 25° in 500-ml Erlenmeyer flasks containing 150 ml of medium.

Preparation and administration of substrates. Pseudotropine was prepared from commercially available tropine using Na–*n*-pentanol as equilibrating reagents [9]. Scopine and scopoline were prepared according to ref. [10]. The substrates were dissolved in H_2O or 70% EtOH and added to 14-day-old cultures through a 0.45 μm membrane filter. The final concns of DL-tropic acid and all the other substrates were 2 and 1 mM, respectively. After an incubation period of 7 days, the contents of two culture flasks were harvested and processed as described below. Controls consisted of cultures grown without substrate or substrate suspended in culture media without cells.

Acetylation of tropine by plants. In the first expt, 50 *D. innoxia* plants (*ca* 30 cm tall, 200 g fr. wt) at the pre-flowering stage cultivated in the field were harvested and washed with tap H_2O. They were cultivated in a pot containing 50 ml 0.5% soln of tropine which was completely absorbed by plants within 24 hr. H_2O (100 ml) and 0.05% Hyponex soln (300 ml) were supplied on days 2 and 3, respectively. The culture was terminated on day 6 and the alkaloids in the plant materials and hydroponic medium analysed. In the second expt, 22 *D. innoxia* plants were separated into aerial parts and roots. Each group of organs was sterilized with 70% EtOH for 1 min, 3% $Ca(OCl)_2$ for 15 min and finally rinsed with H_2O and incubated with 250 mg tropine in 50 ml H_2O for 6 days. Sterilized H_2O was supplied when necessary.

Extraction and analysis of alkaloids. The dry powdered sample of cultured cells was soaked overnight in MeOH–NH_3 (9:1) and Soxhlet-extracted with $CHCl_3$ for 6 hr. The filtrate was concd to dryness and MeOH was poured onto the residue. After the insoluble portion had been removed by filtration, the MeOH was evaporated. Plant materials were homogenized with MeOH, extracted overnight at room temp. and filtered. The filtrate was then taken to dryness. Each residue obtained from cultured cells, medium or plant materials was shaken in a mixture of $CHCl_3$ and 0.5 N HCl. The aq. layer was made alkaline with 10% NH_3 and then extracted (twice) with $CHCl_3$. The $CHCl_3$ extract was dried with K_2CO_3 and evaporated to dryness. The MeOH soln of the residue was applied to TLC and PC: Si gel G, $CHCl_3$–EtOH–28% NH_3 (85:14:1); Si gel G, $CHCl_3$–Me_2CO–MeOH–28% NH_3 (73:10:15:2) [11]; Si gel G, 70% EtOH–28% NH_3 (99:1) [12]; Toyo Roshi No. 50 filter paper, *n*-BuOH–HOAc–H_2O (50:3:25) [13]. The developed plate or paper was sprayed with Dragendorff reagent. For the detection of acetyltropine in *Nicotiana* cell cultures, the Si gel plate was also sprayed with 20% H_2SO_4, heated at 110–120° for 30 min and sprayed with Dragendorff reagent. By this procedure, acetyltropine was hydrolysed and detected as a purple spot to distinguish acetyltropine from tobacco alkaloids.

Isolation and identification of acetyltropine and acetylscopine. The alkaloid fraction was prepared by the method described above from the medium (870 ml) used for *D. innoxia* stem cell suspension cultures (five 500-ml flasks) fed with 30 mg scopine per flask. The $CHCl_3$ was evaporated to dryness and one drop each of conc. HCl, Me_2CO and hexane was added consecutively to the residue. On standing, the soln gave crystals, which were recrystallized from Me_2CO–hexane to yield colourless needles (18.5 mg) of acetylscopine hydrochloride, mp and mmp 227–229° (decomp.). IR ν_{max}^{nujol} cm^{-1}: 1735, 1235 (ester). EIMS *m/z* (rel. int.): 197 $[M]^+$ (71), 155 (14), 154 (5), 138 (14), 97 (15), 96 (45), 95 (8), 42 (100). Acetylscopine was also isolated as its picrate (5.1 mg) from the cells and identified by TLC, mmp and IR. Acetyltropine was isolated as reported earlier [2], from cultures supplied with tiglic acid, benzoic acid or phenyllactic acid in combination with tropine and identified by comparison with an authentic sample of acetyltropine.

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