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INCORPORATION OF 1-^{13}C-ACETATE INTO TROPANE ALKALOIDS BY HAIRY ROOT CULTURES OF *HYOSCYAMUS ALBUS*

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Key Word Index—*Hyoscyamus albus*; Solanaceae; hairy root culture; feeding experiment; tropane alkaloids; hyalbidone; biosynthesis.

Abstract—Labelled acetate ($C^2H_3{}^{13}COONa$) was fed to fast-growing hairy roots of *Hyoscyamus albus* transformed with *Agrobacterium rhizogenes* MAFF03-01724. The alkaloids were isolated and analysed by ^1H and ^{13}C NMR spectroscopy. [^{13}C]Acetate was found to be incorporated into all tropane alkaloids at the C-3 position of the tropane moiety: the highest incorporation rate was observed in tropinone, followed by tropine, littorine, hyoscyamine, 6β-hydroxyhyoscyamine and scopolamine. In hyalbidone, recently isolated from these hairy roots, a little incorporation of $C^2H_3{}^{13}COO^-$ was observed, indicating that this alkaloid is either not directly derived from tropine or is synthesized at the far end of the pathway. Deuteration of the tropane alkaloids was also observed at both the C-2 and C-4 positions, but to different extents.

INTRODUCTION

Tropane alkaloids are secondary metabolites of several solanaceous plants of the genera *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus* and *Scopolia*. The extracts of the plants and the isolated pure alkaloids atropine and scopolamine (**6**) have been used for medicinal purposes for many centuries. During recent years, the biosynthesis of tropane alkaloids has been intensively studied [for reviews see 1, 2]. The origins of both the acidic and basic moieties have been investigated and some steps in the biosynthesis were examined at the enzymatic level [3]. On the other hand, the last steps of tropic acid biosynthesis, i.e. those of the acidic moiety of the medicinally important alkaloids scopolamine (**6**) and hyoscyamine (**4**), are still not completely understood [4, 5]. The mechanism of esterification of these acids with tropine (**2**) is still unknown. On the other hand, the conversion of the amino acid ornithine into the *N*-methylpyrrolidium ion has been confirmed for many tropane alkaloids [6 and references cited therein]. The origins of the remaining carbons of the tropane moiety are either being derived from acetoacetate, malonate or two units of acetate [1, 2, 7, 8].

In our current investigation of the biosynthesis of tropane alkaloids, we have established a hairy root culture of *Hyoscyamus albus* transformed with *Agrobacterium rhizogenes* MAFF 03-01724 [9]. These fast-growing and highly productive root cultures were used to investigate the incorporation of $C^2H_3^{13}COONa$ into several tropane alkaloids such as tropinone (**1**), tropine (**2**) , littorine (**3**), hyoscyamine (**4**), 6β-hydroxyhyoscyamine (**5**) and scopolamine (**6**) (Scheme 1).

RESULTS AND DISCUSSION

Hairy roots of *H. albus* transformed with *A. rhizogenes* MAFF03-01724 produce several tropane alkaloids at a substantial level [10]. $C^2H_3{}^{13}COONa$ (100 mg l^{-1}) was added to the cultures at day 21, when tropane alkaloid formation started to increase (Fig. 1). Roots were harvested after another seven days of culture. Alkaloids were extracted, isolated by column chromatography and separately analysed by NMR. The labelled acetate was incorporated into the tropane moiety of all tropane alkaloids as tropinone (**1**) , tropine (**2**) , littorine (**3**), hyoscyamine (**4**), 6β-hydroxyhyoscyamine (**5**) and scopolamine (**6**), whereas only little labelling was observed in hyalbidone (**7**). The ^{13}C label appeared only at the C-3 position of tropane, as was expected. According to their integration by ^{13}C NMR, the incorporation of [1-^{13}C]-acetate into the tropane moiety was at a different level for each alkaloid [example given for hyoscyamine (**4**) in Fig. 2a]. The highest rate of ^{13}C-labelling was found in tropinone (**1**), followed by tropine (**2**), littorine (**3**), hyoscyamine (**4**), 6β-hydroxyhyoscyamine (**5**) and scopolamine (**6**) (Table 1). The differences in the incorporation of [1-^{13}C]-acetate into the tropane moiety might reflect their position in the biosynthetic sequence: earlier alkaloids show a higher incorporation than those at later steps. However, we are aware that degradation, turnover and

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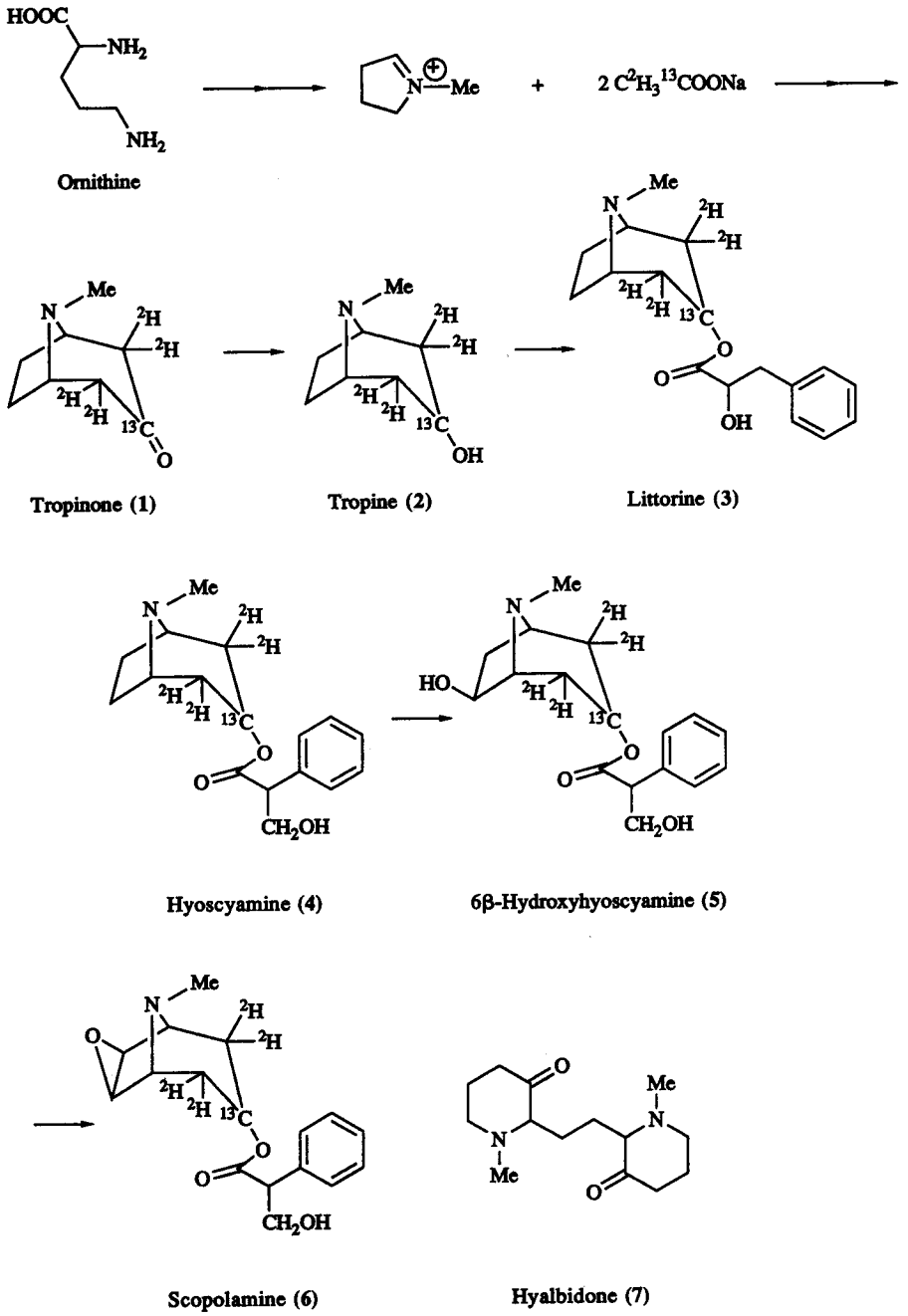

Scheme 1. Apparent biosynthesis of tropane alkaloids in *Hyoscyamus albus* hairy roots fed with labelled acetate.

different pool sizes may influence this pattern significantly. It has been shown that dual labelled littorine (**3**), 3α(2-hydroxy-3-phenylpropionyloxy- [1-^{14}C]-tropane-[3β-^{3}H], is easily hydrolysed in *Datura stramonium* plants and the phenyllactic acid undergoes rearrangement to tropic acid, the latter then esterified with tropine (**2**) to hyoscyamine (**4**) . In the same experiment it has been demonstrated that the hydrolysed parts of labelled littorine (**3**) (tropine- [3β-^{3}H] and [1-^{14}C] -phenyllactic acid) do not rearrange with endogenous unlabelled phenyllactic acid or tropine to again yield littorine (**3**) [11]. Because littorine (**3**) shows a higher incorporation of [1-^{13}C]-acetate (Table 1) it may be synthesized earlier than hyoscyamine (**5**). Circumstantial evidence also supports this view. In the time course of alkaloid production by hairy roots the amount of littorine (**3**) decreased after 24 days, whereas the amount of the alkaloids hyoscyamine (**4**), 6β-hydroxyhyoscyamine (**5**) and scopolamine (**6**) increased (Fig. 1). We assume from these observations that littorine (**3**) might be a biosynthetic intermediate of hyoscyamine (**4**), 6β-hydroxyhyoscyamine (**5**) and scopolamine (**6**). In hyalbidone (**7**), a little labelling was

Table 1. Incorporation of [1-^{13}C]-acetate into tropane alkaloids as analysed by ^{13}C NMR spectroscopy

Alkaloid	Amount [mg]	C-3 shift [ppm]	Normalized on [area = 100%]	C-3 [% peak area]
1	22	178.1	C-2′ of 4 added	921
2	11	56.9	C-2′ of 4 added	874
3	550	69.1	C-2′	419
4	930	68.3	C-2′	306
5	160	68.0	C-2′	252
6	75	66.8	C-2′	177

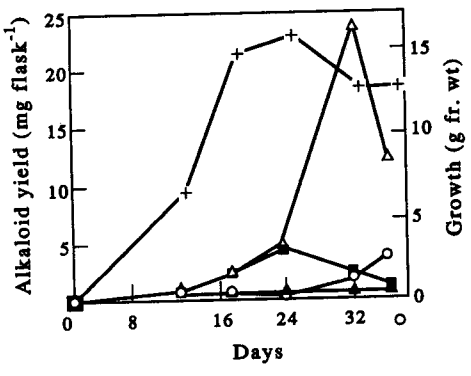

Fig. 1. Growth and alkaloid production in *Hyoscyamus albus* hairy roots cultured in WP liquid medium.

observed, indicating that this alkaloid is either not related to the tropane alkaloids or is synthesized at a later growth stage when the [^{13}C]-acetate was already consumed.

Concomitant to the enhancement of the signal for C-3 in the ^{13}C NMR spectrum, the signals for H-2 and H-4 decreased in the ^{1}H NMR spectra of all the tropine moieties compared with those in authentic samples. This indicates the incorporation of deuterium at C-2 and C-4 derived from $C^2H_3{}^{13}COO^-$. As the diminution of the signals for H-2 and H-4 was not identical at both positions, we suppose a sequential incorporation of labelled acetate (example given in Fig. 2b). From this data it must be assumed that the biosynthesis from *N*-methylpyrrolidinium ion to tropine is a two-step process which does not involve a four carbon unit (acetoacetyl coenzyme A) but two units of acetyl coenzyme A, which were added sequentially as has been suggested for the biosynthesis of cocaine [2, 8]. On the other hand it has been reported that [1,2-$^{13}C_2$]-acetate was incorporated with an equal efficiency at C-2 and C-4 by normal root cultures of *H. albus* [7]. The results obtained in this study are condictory to ours. Further investigations are required to clarify this matter.

EXPERIMENTAL

Plant material. The hairy roots were induced by co-culture of leaf discs with *Agrobacterium rhizogenes* strain MAFF 03-01724. The axenic hairy roots thus obtained were subcultured in hormone-free Woody Plant [12] liquid medium with 3% sucrose and kept on a rotary shaker (100 rpm) in the dark [9].

Feeding experiments. $C^2H_3^{13}COONa$ (Sigma) (200 mg) was added aseptically to hairy roots cultured in a 2l air-lift type fermenter with hormone-free WP liquid medium for 21 days (6 times). After an additional 7 days of culture, the hairy roots were harvested. From the lyophilized roots (300 g dry wt) alkaloids were isolated as described in ref. [10]. After final purification of the alkaloids by CC on silica gel (EtOAc–MeOH–NH_3 gradient) ^{1}H and ^{13}C NMR spectra, were recorded at 300 and 75.5 MHz, respectively, locked to the major deuterium resonance of the solvent ($CDCl_3$). The intensity of the enhanced signal at C-3 of the tropane moiety of each alkaloid was normalized to the signal of the methine in the side chain of the respective alkaloid itself or, in the case of **1** and **2**, to that of a known amount of unlabelled hyoscyamine added to the sample. Due to the overlapping of the signals in some cases the diminution of deuterated C-2 and C-4 was not quantified.

Time course and HPLC analysis. Two cultures were harvested and the dry weight (after lyophilization) of the tissues was determined individually. *Ca* 50 mg of each sample was extracted with 5 ml $CHCl_3$–MeOH–NH_4OH (15:5:1). Further sample preparation and HPLC conditions were the same as in ref. [10].

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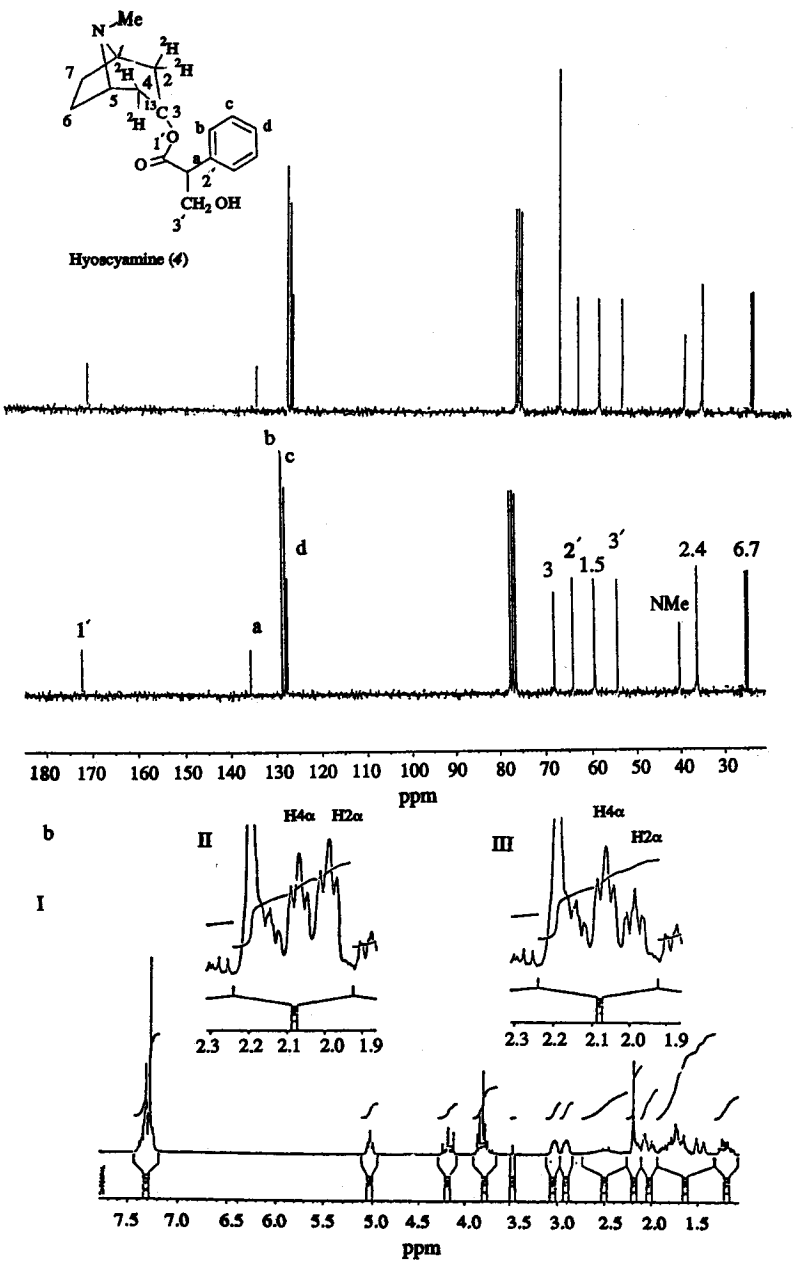

Fig. 2. Incorporation of $C^2H_3^{13}COO^-$ in hyoscyamine by hairy root cultures of *H. albus*. Numbers indicate the signals of the tropane moiety. (a) ^{13}C NMR (I) after feeding, (II) control; (b) ^1H NMR (I) after feeding, (II) control, enlargement of H2α, H4α, (III) after feeding, enlargement of H2α, H4α, partially deuterated.

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