Kinetic Study of Littorine Rearrangement in *Datura innoxia* Hairy Roots by ^{13}C NMR Spectroscopy

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The kinetics of tropane alkaloid biosynthesis, particularly the isomerization of littorine into hyoscyamine, were studied by analyzing the kinetics of carbon-13 (^{13}C) in metabolites of *Datura innoxia* hairy root cultures fed with labeled tropoyl moiety precursors. Both littorine and hyoscyamine were the major alkaloids accumulated, while scopolamine was never detected. Feeding root cultures with (*RS*)-phenyl-[1,3-$^{13}C_2$]lactic acid led to ^{13}C spin−spin coupling detected on C-1′ and C-2′ of the hyoscyamine skeleton, which validated the intramolecular rearrangement of littorine into hyoscyamine. Label from phenyl[1-^{13}C]alanine or (*RS*)-phenyl[1,3-$^{13}C_2$]lactic acid was incorporated at higher levels in littorine than in hyoscyamine. Initially, the apparent hyoscyamine biosynthesized rate ($v_{app}hyo = 0.9\ \mu$mol $^{13}C{\cdot}flask^{-1}{\cdot}d^{-1}$) was lower than littorine formation ($v_{app}litto = 1.8\ \mu$mol $^{13}C{\cdot}flask^{-1}{\cdot}d^{-1}$), suggesting that the isomerization reaction could be rate limiting. The results obtained for the kinetics of littorine biosynthesis were in agreement with the role of this compound as a direct precursor of hyoscyamine biosynthesis.

The tropane alkaloids hyoscyamine and scopolamine are secondary metabolites synthesized in several solanaceous plants of the genera *Atropa*, *Datura*, *Duboisia*, and *Hyoscyamus*. These natural products are anticholinergic agents used in medicine as antispasmodics, and in ophthalmology. In plants, hyoscyamine and scopolamine are synthesized in the roots and are translocated into the aerial parts. Transgenic root cultures obtained by transformation via *Agrobacterium rhizogenes*[1−3] provide plant material with a high growth rate on hormone-free media[4,5] and alkaloid yields similar to or higher than in untransformed plants. Recently, transformed root cultures were used to investigate the biosynthesis of tropane alkaloids. This biosynthesis involves the conversion of ornithine into tropine in six steps[6] and of phenylalanine into phenyllactate in two steps.[7−9] Tropine and phenyllactate (Figure 1) then condense into littorine, as reported by Robins et al.[10] The biosynthesis of hyoscyamine involves the molecular rearrangement of littorine. However, the reaction mechanism is still uncharacterized.[11−14] Although significant progress allows a better understanding of tropane alkaloid biosynthesis, little is known about its regulation. If hairy root culture provides a large biomass,[15] the main problem resides in the low accumulation of metabolites. Consequently, some strategies have to be developed to enhance productivity; one of these is to explore the limiting steps in hyoscyamine biosynthesis. Therefore, the activities of several enzymes involved in tropane alkaloid biosynthesis were investigated in order to determine the factors limiting the metabolic flux.[2,16] However, enzymatic activities do not give information about flux rates between biosynthetic intermediates. A kinetic study of hyoscyamine biosynthesis was therefore conducted to explore possible metabolic rate limitations.

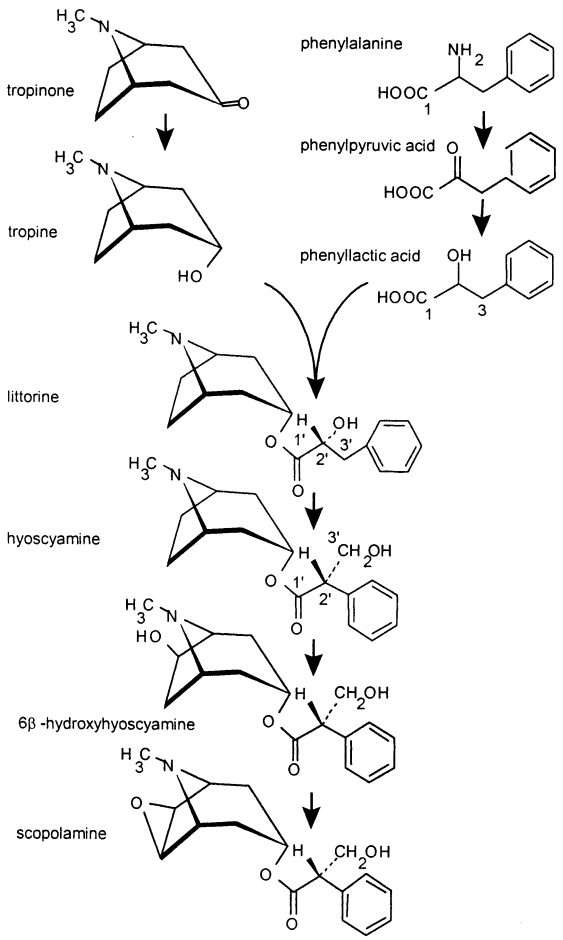

Figure 1. Schematic pathway of hyoscyamine and scopolamine biosynthesis. [1′,3′-^{13}C]littorine is converted into [1′,2′-^{13}C]hyoscyamine. Arrows only represent chemical origins.

In recent years, ^{13}C-labeling strategies have been used to provide further insights into biosynthetic pathways of

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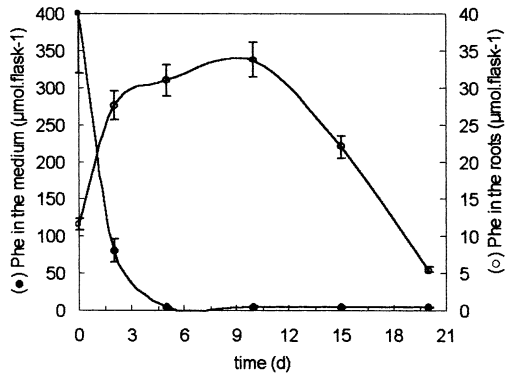

Figure 2. Profile of phenylalanine in the medium (●) and in the roots (○) during feeding with 4 mM phenylalanine.

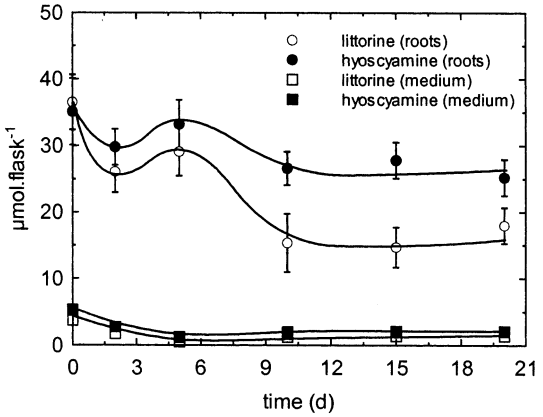

Figure 3. Profile of total littorine and hyoscyamine contained in transformed root cultures during feeding with 4 mM phenylalanine: (○) littorine in the roots, (□) littorine in the medium, (●) hyoscyamine in the roots, (■) hyoscyamine in the medium. Data points represent the mean values of four experiments.

tropane alkaloids and have played a key role in demonstrating the intramolecular rearrangement of littorine into hyoscyamine.[7,10,17] The ^{13}C-labeling technique is powerful in providing kinetic data, including metabolic fluxes, although this has not been applied to the tropane alkaloid pathway. The present work reports on a kinetic study of littorine rearrangement into hyoscyamine using labeled precursors, e.g., phenyl[1-^{13}C]alanine and (*RS*)-phenyl[1,3-$^{13}C_2$]lactic acid.

Results and Discussion

21-Day-old transformed roots were subcultured in new medium containing labeled or unlabeled phenylalanine (4 mM) and incubated for various periods of time. These conditions were used to study tropane alkaloid biosynthesis with a minimum root growth. After harvesting the roots, phenylalanine was extracted from the tissues as well as from the medium and its concentration was determined (Figure 2). The medium content decreased linearly from day 0 to day 2 with a rate of 160 μmol·flask^{-1}·d^{-1}. During the same period the phenylalanine accumulation in the roots was 8 μmol·flask^{-1}·d^{-1}, indicating that phenylalanine utilization (difference between consumption and accumulation) was 152 μmol·flask^{-1}·d^{-1}. The rate of consumption decreased from day 2 to day 5; during this period phenylalanine was no longer detected in the medium. The rate of phenylalanine accumulation in the roots decreased to 0.8 μmol·flask^{-1}·d^{-1} during this period. Accumulation of phenylalanine in the roots occurred up to day 10. Then,

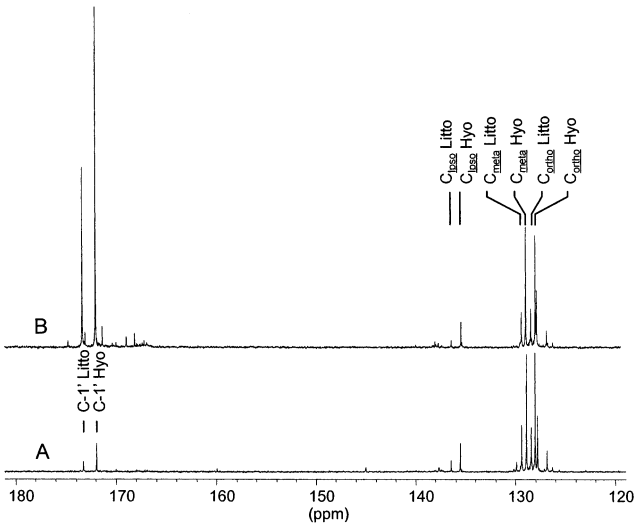

Figure 4. Expansion at 120−180 ppm of ^{13}C NMR spectrum of the alkaloidal extract from root cultures of *Datura innoxia* fed with unlabeled (A) or phenyl[1-^{13}C]alanine (B) showing enrichment in hyoscyamine C-1′ (171.8 ppm) and littorine C-1′ (173.2 ppm).

the phenylalanine root content decreased up to day 20 at a rate of 2.8 μmol·flask^{-1}·d^{-1}. Phenylalanine was efficiently taken up by *D. innoxia* hairy roots and was indeed consumed within 5 days. These results agree with those obtained by Kitamura et al.[18] for *Duboisia leichhardtii*.

Transformed roots of solanaceous species generally accumulate large amounts of hyoscyamine and minor quantities of scopolamine.[19] Tropane alkaloids were extracted and analyzed by HPLC. At first, hyoscyamine (36.5 μmol·flask^{-1}) and littorine (35.1 μmol·flask^{-1}) were the major alkaloids detected. The hairy roots of *D. innoxia* studied here accumulate hyoscyamine (25 μmol/g dry weight) to a larger extent than *D. stramonium* (20 μmol/g dry weight)[14] and *Atropa belladonna* (6 μmol/g dry weight).[20] During the studied period (20 days), roots did not accumulate either scopolamine or 6β-hydroxyhyoscyamine, the other derivatives of hyoscyamine. In contrast, *D. innoxia* hairy roots accumulated more littorine (21 μmol/g dry weight) than other species (3−10 μmol/g dry weight).[14,20,21] The littorine/hyoscyamine ratio was nearly 1 in *D. innoxia* hairy roots, whereas it was in the range 0.2−0.5 for other transformed roots. Further investigation is in progress to determine whether the higher littorine and hyoscyamine contents could be correlated to the absence of scopolamine.

As previously reported by Robins et al.,[2,22] a decrease in tropane alkaloid content was observed after subculturing (Figure 3). Here, the decrease represented 18.4% and 25.6% of the initial contents of hyoscyamine and littorine, respectively. Then, the alkaloid concentration increased from day 2 to day 5 to almost reach the initial content, suggesting the occurrence of alkaloid synthesis. Finally, the alkaloid content decreased again from day 5 to day 20. Losses of alkaloids were 20% and 40% for hyoscyamine and littorine, respectively. There was no alkaloidal release into the medium during the incubation period. It is noteworthy that tropic acid, a usual hydrolysis product of tropane alkaloids, as well as scopolamine or other known alkaloid derivatives, were not detected either in the roots or in the liquid medium.

The conversion of exogenous phenylalanine into tropane alkaloids was monitored by stable isotopic experiments (^{13}C NMR). First, roots were incubated for 5 days with 4 mM phenylalanine. ^{13}C NMR spectra resulting from these control experiments (Figure 4A) exhibited two regions of

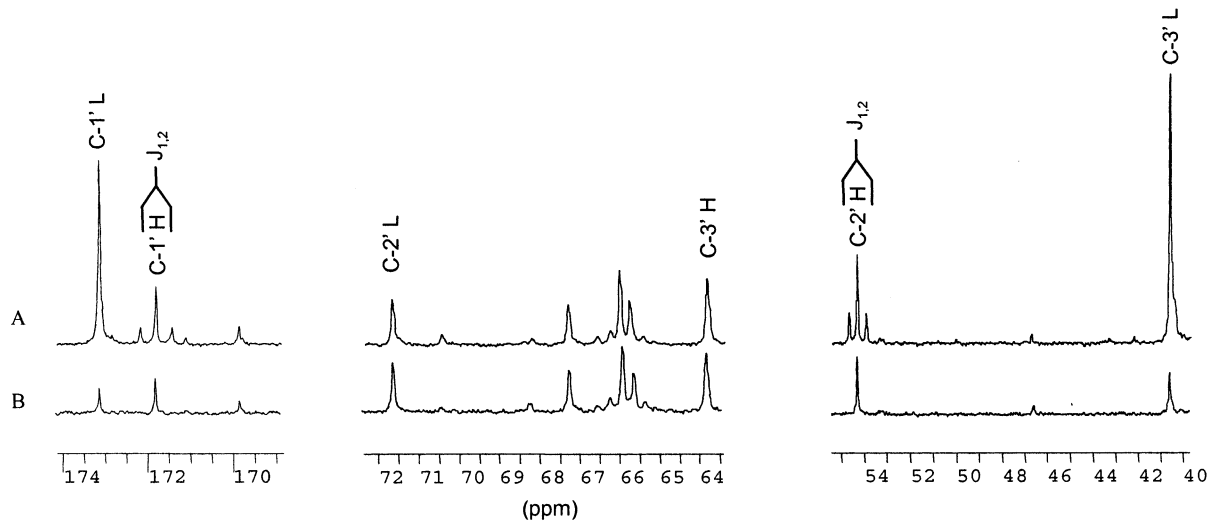

Figure 5. ^{13}C NMR spectrum of the alkaloidal extract from transformed root cultures of *Datura innoxia* fed with (*RS*)-phenyl[1,3-^{13}C]lactic acid (A) or (*RS*)-phenyllactic acid (B). H: Hyoscyamine, L: Littorine, $J_{1,2}$: carbon spin−spin coupling (≈55 Hz). All regions are displayed with the same vertical scale.

interest, one containing the carbons from the aromatic ring (120−140 ppm) and the other containing the carbonyl groups (C-1′ of hyoscyamine = 171.8 ppm, C-1′ of littorine = 173.2 ppm). In agreement with HPLC analyses, hyoscyamine and littorine were the major alkaloids detected, whereas neither scopolamine nor 6β-hydroxyhyoscyamine was detected. The absence of the latter alkaloids was definitively confirmed by capillary electrophoresis (data not shown). In a second experiment, roots were incubated for 5 days with 4 mM phenyl[1-^{13}C]alanine. By comparison with the control spectrum, signals from C-1′ of hyoscyamine and littorine (Figure 4B) had much higher intensities, whereas the signal from other carbons (including those of the aromatic ring) remained constant. This clearly indicated that ^{13}C was incorporated from the exogenous phenylalanine. Fractional enrichments were 18% and 8% for littorine and hyoscyamine, respectively. Moreover, some extra peaks corresponding to ^{13}C enrichment were also observed. Their chemical shifts (172.7−167.9 ppm) could not be correlated to known alkaloids by comparison with commercial standards or literature data.

To ascertain that littorine conversion into hyoscyamine in *D. innoxia* occurred via the intramolecular rearrangement depicted in *D. stramonium*,[10] root cultures were fed with 4 mM (*RS*)-phenyl[1,3-$^{13}C_2$]lactic acid for 24 h. The ^{13}C incorporation in littorine and hyoscyamine was determined as described in the Experimental Section. Incorporation could be observed in both C-1′ (173.2 ppm) and C-3′ (40.5 ppm) of littorine, indicating that the exogenous phenyllactate was efficiently incorporated and metabolized by roots (Figure 5). Furthermore, two satellite peaks were detected for hyoscyamine C-1′ (171.8 ppm, $J \approx 55$ Hz) and C-2′ (54 ppm, $J \approx 55$ Hz) in addition to the singlets. As previously reported in *D. stramonium* root cultures,[10] the occurrence of such satellites indicated that the two labeled carbons became adjacent during the conversion of littorine into hyoscyamine (Figure 1). The fractional enrichments were 11.8% and 2.1% in littorine and hyoscyamine, respectively.

Kinetics of hyoscyamine synthesis from external phenylalanine was investigated. Two 21-day-old series of hairy roots were subcultured in new liquid medium fed with 4 mM phenyl[1-^{13}C]alanine for various periods of time (ranging from 0 to 20 days), and the isotopic pattern of root alkaloids was examined. To obtain accurate quantitative

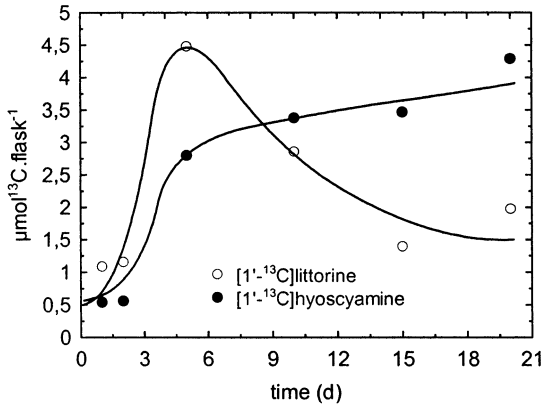

Figure 6. Profile of [1′-^{13}C]littorine and [1′-^{13}C]hyoscyamine contents in transformed root cultures during feeding with 4 mM phenyl[1-^{13}C]-alanine: (○) [1′-^{13}C]littorine, (●) [1′-^{13}C]hyoscyamine. Data points represent the mean values of duplicate experiments; standard error was less than 4% of the mean.

data, all feeding experiments were performed in parallel with either labeled or unlabeled precursors. All ^{13}C NMR spectra were produced with the same acquisition parameters, allowing direct comparison of spectra from labeled and unlabeled experiments. Advantage was also taken from the detection of naturally abundant ^{13}C carbons in the aromatic ring, which offered intramolecular references in all spectra. This strategy enabled an unambiguous quantification of label incorporation at each incubation time.

Kitamura et al.[18] reported the conversion of ^{14}C-labeled phenylalanine into tropane alkaloids as a function of time, but the radioactivity was recovered in the total alkaloidal fraction. In the present work, studies using ^{13}C-labeled substrates were designed to evaluate the kinetics of tropane alkaloid biosynthesis. The kinetics of alkaloid labeling are given in Figure 6. ^{13}C was incorporated into littorine between day 2 and day 5, which agreed with the increase in littorine content mentioned above (see Figure 3). The apparent rate of incorporation into littorine ($v_{app}litto$) was 1.8 μmol^{13}C·flask^{-1}·d^{-1}. The highest enrichment found in littorine (16%) was observed on day 5. After that, the amount of labeled littorine decreased ($v_{app}litto = -0.4$ μmol^{13}C·flask^{-1}·d^{-1}), indicating compound consumption for hyoscyamine biosynthesis. This is consistent with the decrease in littorine content observed in Figure 3. ^{13}C was

incorporated into hyoscyamine between day 2 and day 5, although at a lower rate ($v_{app}hyo = 0.9$ μmol $^{13}C \cdot flask^{-1} \cdot d^{-1}$). After day 5, the amount of labeled hyoscyamine tended to a plateau ($v_{app}hyo = 0.09$ μmol $^{13}C \cdot flask^{-1} \cdot d^{-1}$). As a result, the fractional enrichment in hyoscyamine increased from 8% on day 5 to 17% on day 20. The latter value was similar to the highest fractional enrichment observed in littorine, suggesting that no dilution posterior to littorine occurred. The highest enrichment found in littorine was observed on day 5, whereas it was on day 20 for hyoscyamine. These results indicate that the labeling transfer occurred until day 20. The apparent rates reported for the biosynthesis of tropane alkaloids suggest that the isomerization reaction could be rate limiting. Considering the complex mechanism of littorine isomerization,[23] it is suggested that this is a limiting step for hyoscyamine biosynthesis.

Incorporation of (*RS*)-phenyl[1,3-$^{13}C_2$]lactic acid in hyoscyamine was lower than that of littorine after 24 h. Similar results were observed with phenyl[1-^{13}C]alanine on day 5, when maximal labeling of littorine was observed. After 5 days, with the latter substrate, labeling of hyoscyamine increased, whereas that of littorine decreased. All these results are consistent with the role of littorine as intermediate in the biosynthesis of hyoscyamine in *D. innoxia*.

Whereas the maximum rate of phenylalanine consumption occurred very early (see Figure 2), incorporation of ^{13}C into tropane alkaloid was important after day 2, i.e., when the intracellular pool of phenylalanine had significantly increased (by a factor 2). Labeled hyoscyamine, 4 μmol $^{13}C \cdot flask^{-1}$, represented approximately 1% of the initial labeled phenylalanine fed in the medium and 10% of phenylalanine observed in the roots, indicating that the majority of phenylalanine is used for other processes. These data suggest that the competition between tropane alkaloid synthesis and other phenylalanine utilizing pathways is widely in favor of the latter.

The intensities of unassigned peaks corresponding to ^{13}C enrichment already observed in Figure 4B increased until day 5, further decreased up to day 10, and were no longer detected thereafter. No signal from apoatropine, 6β-hydroxyhyoscyamine, or scopolamine was detected in the course of the experiments. Moreover, losses of alkaloids were observed in Figure 3. In the whole plant, tropane alkaloids are normally synthesized in the roots and are then translocated into the aerial parts. In contrast, transformed roots constitute both the synthesis and storage organ. Thus, a possible turnover could avoid a toxic alkaloid accumulation for the cell. It has been found that exogenous tropic acid is converted into glucose esters in *D. innoxia* hairy roots.[24] A possible recycling of tropane alkaloids in *Duboisia* root cultures has been discussed by Kitamura et al.[25] These authors suggest a hypothetical pathway of tropic acid recycling originating in hyoscyamine. However, further investigations with labeled substrates are still required to better understand tropane alkaloid metabolism in hairy roots. Also, the enzymes involved in the last steps of alkaloid biosynthesis must be characterized.

Experimental Section

General Experimental Procedures. NMR spectra were obtained in $CDCl_3$ at 300 MHz using a Bruker 300 MHz spectrometer. Phenyl[1-^{13}C]alanine was provided by Eurisotop (St. Aubin, France) (99% specific enrichment). (*RS*)-Phenyl[1,3-$^{13}C_2$]lactic acid (80.9% specific enrichment) was kindly provided by J. G. Woolley (Faculty of Applied Sciences, De Monfort University, Leicester, UK).

Hairy Root Cultures. Transformed root cultures of *D. innoxia* Mill. were obtained and maintained as described by Boitel-Conti et al.[26] They were cultured in 250 mL Erlenmeyer flasks containing 100 mL of Gamborg's B5 culture medium[27] supplemented with 30 g/L sucrose for 21 days. Hairy roots were grown on a rotary shaker (80 rpm) at 27 ± 1 °C under cool light fluorescent lamps (35 $\mu E \cdot m^{-2} \cdot s^{-1}$), with a 16 h light/8 h dark period. The culture conditions were determined in order to study tropane alkaloid biosynthesis with a minimum root growth. This was obtained by subculturing a 21-day-old entire root culture in new B5 medium. The roots occupied all the available volume in the flask and, under such conditions, remained in a stationary phase. Solutions for feeding experiments were prepared from neutral aqueous stock solutions (13 mg/mL) at appropriate concentrations and were filter-sterilized directly into the flasks. Transformed roots were subcultured in new media containing 4 mM of labeled or unlabeled phenylalanine.

HPLC Alkaloid Analysis. To analyze the alkaloids, the roots were dried at 60 °C overnight. The powdered, dried material (40 mg) was soaked in 4 mL of methanol for 5 h. After evaporation, the residue was dissolved in an appropriate volume of mobile phase, filtered through a Millex 0.22 μm (Millipore, Bedford, MA) and analyzed by HPLC (Waters 510 apparatus). The volume injected was 20 μL (Rheodyne, Rohnert Park, CA). Hyoscyamine and littorine were quantified with an ODS-AQ (YMC, Kyoto, Japan) column (5 μm, 250 × 4.6 mm) at ambient temperature with UV detection at 204 nm (Waters 484 UV detector; Milford, MA). The mobile phase was $H_2O-CH_3CN-H_3PO_4$ (89:10.8:0.2) with 1 g/L KH_2PO_4 supplemented with triethylamine at pH 3.1. The flow rate was 0.8 mL/min. Quantification was achieved by reference to external standards of hyoscyamine, littorine, scopolamine, tropic acid, phenyllactic acid, and phenylpyruvic acid (Sigma-Aldrich, St. Louis, MO) and expressed in $\mu mol \cdot flask^{-1}$. Phenylalanine analyses were carried out on a Beckman 6300 amino acid analyzer.

Spectroscopic Analysis. For NMR alkaloid analysis, 1 g of dried plant material was extracted overnight in 500 mL of $MeOH-CHCl_3-28\%$ NH_4OH (50:50:1.4). This extract was filtered, evaporated under reduced pressure, and rinsed three times with 20 mL of 0.1 N HCl. After addition of 3 mL of 28% NH_4OH, alkaloids were extracted with 3 × 30 mL of $CHCl_3$. The organic phase was reduced to dryness, and the residue was collected in $CDCl_3$. NMR analyses were acquired using acquisition parameters as follows: spectral width, 15 000 Hz; flip angle 90°; data size, 16 K; number of scans, 10 000; proton decoupling was carried out during acquisition (1.08 s). A 3 Hz line broadening was applied prior to Fourier transform. Chemical shifts were expressed as ppm relative to $CDCl_3$. The assignments of NMR resonances were made on the basis of literature data[9,28,29] and were confirmed by spiking root extracts with reference samples.

Specific Enrichment Calculations. The ^{13}C signals detected in samples from unlabeled experiments corresponded to the 1.1% ^{13}C naturally occurring in each carbon position. In samples from labeled experiments, ^{13}C signals corresponded to the naturally occurring level plus the level incorporated (when occurring) from the exogenous labeled precursor. Then, the differences in intensity observed for a given signal between spectra from labeled/unlabeled experiments represented the incorporation rate of the labeled precursor in the corresponding carbon position. Because the precursors were labeled in the C-1′−C-3′ positions only, no label could be incorporated in the phenyl moiety or in the tropane moiety of tropane alkaloids. Thus, signals from the aromatic moiety could be used as intramolecular references to estimate the natural abundance of ^{13}C. Quantitative analysis of label incorporation was performed as described below: C_i fractional enrichment (%) = $[(I_{ci}/I_{ar \cdot c})\text{labeled}/(I_{ci}/I_{ar \cdot c})\text{unlabeled}] \times 1.1$, where I_{ci} is the intensity of the signal from "*i*" carbon (C-1′ to C-3′) in the alkaloid including satellite peaks, $I_{ar \cdot c}$ is the intensity of the signal from the aromatic carbon, and 1.1% is the correction for the ^{13}C natural abundance. This method compensates for small instrumental variations.

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