New Intermediate in the Biosynthesis of the Tropane Alkaloids in *Datura innoxia*[1]

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Abstract: The biosynthesis of the tropane alkaloids in *Datura innoxia* was studied using (*R*,*S*)-[2′,3′-$^{13}C_2$]hygrine and ethyl (*R*,*S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate. (*R*,*S*)-[2′,3′-$^{13}C_2$]Hygrine was synthesized from ethyl [3,4-$^{13}C_2$]acetoacetate. Administration of (*R*,*S*)-[2′,3′-$^{13}C_2$]hygrine to *D. innoxia* resulted in a very low incorporation of ^{13}C into (−)-scopolamine and (−)-hyoscyamine. Ethyl (*R*,*S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate was synthesized from potassium [^{13}C,^{14}C]cyanide and diethyl [2-^{13}C]malonate. Administration of ethyl (*R*,*S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate to *D. innoxia* resulted in a high incorporation of both ^{14}C (1.73% specific incorporation) and ^{13}C (1.78% specific incorporation) into (−)-scopolamine. This provides direct evidence for an alternative biosynthetic pathway for the tropane alkaloids in *D. innoxia*.

Introduction

The biosynthesis of the tropane alkaloids has been extensively studied over the last few decades.[2] This is primarily due to the pharmacological importance of compounds such as (−)-cocaine, (−)-scopolamine, and (−)-hyoscyamine (Figure 1). Several intermediates in the biosynthetic pathway to (−)-hyoscyamine and (−)-scopolamine have been identified using labeling as well as enzymatic methods.[2−4] However, several questions remain as to the manner in which the tropane ring is assembled from the 1-methyl-Δ^1-pyrrolinium salt (**1**). For a long time it was believed that the formation of the tropane ring of (−)-scopolamine and (−)-hyoscyamine from 1-methyl-Δ^1-pyrrolinium salt (**1**) occurred as shown in Scheme 1.[5] According to this hypothesis, the 1-methyl-Δ^1-pyrrolinium salt (**1**), which is formed from ornithine, condenses with acetoacetyl CoA (**2**) (which is derived from two molecules of acetyl coenzyme A) to yield the β-keto acid **3**. The β-keto acid **3** then undergoes facile decarboxylation to form hygrine (**4**).

The formation of the tropane ring from hygrine (**4**) was believed to proceed *via* 5-acetonyl-1-methyl-Δ^1-pyrrolinium salt (dehydrohygrine) (**5**). Dehydrohygrine (**5**), which can be formed by oxidation of hygrine, undergoes a Mannich reaction to give tropinone (**6**) (Scheme 1). Dehydrohygrine (**5**) has not been isolated from plant sources, although there is circumstantial evidence for its presence in *Datura innoxia*[6] and its formation during a biomimetic synthesis of tropinone.[7] Tropinone (**6**) is a key intermediate in the biosynthesis of (−)-hyoscyamine and (−)-scopolamine. Tropinone (**6**) undergoes reduction to tropine, which upon esterification with tropic acid results in the formation of (−)-hyoscyamine. (−)-Hyoscyamine is then converted to (−)-scopolamine via (−)-6β-hydroxyhyoscyamine.[8]

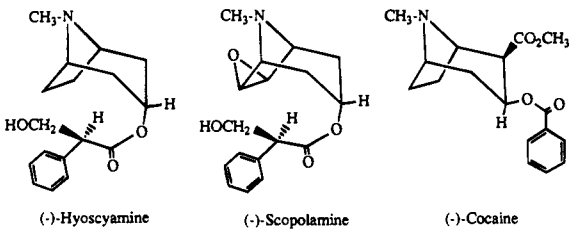
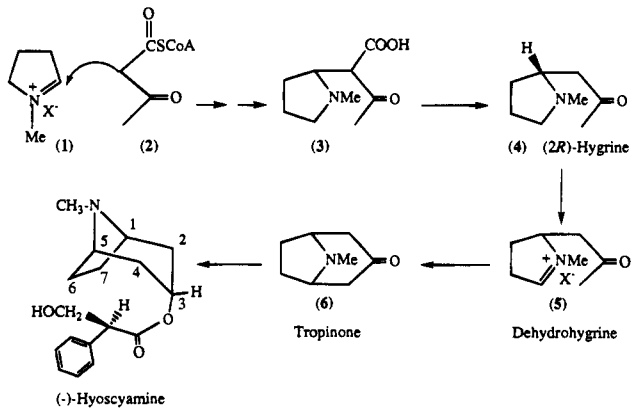


Figure 1. Medicinally important tropane alkaloids.

Scheme 1. Old Hypothesis for the Biosynthesis of (−)-Hyoscyamine in *Datura* Species



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‡ Deceased February 1992. This paper is dedicated to the memory of E.L. by T.W.A.

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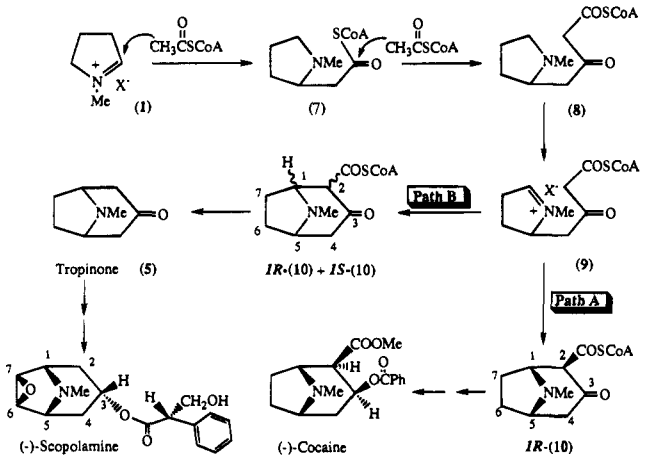
Scheme 2. New Hypotheses for the Biosynthesis of (−)-Cocaine in *E. coca* and (−)-Scopolamine in *D. innoxia*



Hygrine (**4**) has been shown to be a precursor of the tropine moiety of (−)-hyoscyamine and other tropane alkaloids.[9−11] O'Donovan and Keogh[9] fed (*R,S*)-[*N-methyl*-^{14}C,2′-^{14}C]hygrine to *Datura stramonium* plants and obtained an absolute incorporation of 2.1% into (−)-hyoscyamine. The ratio of the radioactivity at the *N*-methyl to that at C-3 in the isolated (−)-hyoscyamine was the same as that for the labeled hygrine, indicating that the labeled hygrine had been incorporated intact into (−)-hyoscyamine without degradation. McGaw and Woolley[10] reported that the incorporation of (2*R*)-[2′-^{14}C]hygrine (**4**) into the tropane alkaloids in *D. innoxia* was 2.5−10 times greater than the incorporation of (2*S*)-[2′-^{14}C]hygrine although hygrine is known to racemize readily in neutral or basic solutions.[5] However, they also found that *Hyoscyamus niger* and *Atropa belladona* utilized both (*R*)- and (*S*)-hygrine equally well in the biosynthesis of (−)-hyoscyamine.[11] Surprisingly, Liebisch *et al.*[12] found that labeled hygrine showed a lower incorporation into the tropane alkaloids in *Datura metel* than labeled ornithine, even though hygrine is a later intermediate in the biosynthetic pathway. The unsymmetrical incorporation of ornithine into (−)-hyoscamine in *D. stramonium*[13] and *N*-methylputrescine into (−)-scopolamine in *D. innoxia*[14] requires that only (2*R*)-hygrine and not (2*S*)-hygrine serves as a precursor for the tropane ring.

However, several observations made over the last few years[15−18] suggest that an alternative pathway may exist for the formation of the tropane ring in (−)-scopolamine and (−)-hyoscyamine. In 1988, Leete and Kim[15] proposed a different mechanism (Scheme 2, path A) for the formation of the tropane ring of (−)-cocaine in *Erythroxylon coca*. This mechanism was based on their observation that [2-^{13}C,^{14}C,^{15}N]-1-methyl-Δ^1-pyrrolinium salt administered to *E. coca* labeled (−)-cocaine at C-5 and N. According to this revised hypothesis for the biosynthesis of (−)-cocaine, the pyrrolinium salt **1** does not condense with acetoacetyl CoA, as had been previously believed (Scheme 1), but condenses successively with two malonyl CoA or acetyl CoA units to give the (*S*)-enantiomers of thio esters **7** and then **8**. The thio ester **8** then cyclizes *via* the pyrrolinium salt **9** to form the thio ester of 2-carboxytropinone, (1*R*)-**10** (path A). (1*R*)-**10** is then converted to the methyl ester (2-carbomethoxytropinone) followed by reduction and benzoylation to yield (−)-cocaine. Since there are several similarities between the biosynthesis of (−)-cocaine and that of (−)-scopolamine and (−)-hyoscyamine, it seemed plausible that the revised biosynthetic pathway for (−)-cocaine could also be operating in the biosynthesis of (−)-scopolamine and (−)-hyoscyamine.

In 1990, Sankawa *et al.*[16] reported the nonstereospecific incorporation of sodium [1,2-$^{13}C_2$]acetate into (−)-hyoscyamine and (−)-6β-hydroxyhyoscyamine in *Hyoscyamus albus*. Although this result did not distinguish between the two mechanisms[5,15] of tropane ring formation, it appeared that both enantiomers of hygrine and/or other intermediates were being utilized for formation of the tropane ring in (−)-hyoscyamine and (−)-6β-hydroxyhyoscyamine. However, Hemscheidt and Spenser[17] fed sodium [1,2,3,4-$^{13}C_4$]acetoacetate and sodium [1,2-$^{13}C_2$]acetate separately to *Sedum sarmentosum* and showed that, at least in the case of *N*-methylpelletierine and *N*-methylallosedridine, the classical biogenetic concept held true. They were able to show that of the four possible modes for formation of the side chains from Δ^1-piperideine and acetate units only one was being utilized for the biosynthesis of *N*-methylpelletierine and *N*-methylallosedridine, i.e. acetoacetate coupled to Δ^1-piperideine via the α-carbon of acetoacetate (similar to that in Scheme 1) and subsequently underwent decarboxylation to form pelletierine. Subsequently, in a similar study, Hemscheidt and Spenser[18] demonstrated the nonregiospecific incorporation of sodium [1,2-$^{13}C_2$]acetate into 6β-hydroxytropine in *D. stramonium*. Also, in the same study, the results from feeding sodium [1,2,3,4-$^{13}C_4$]acetoacetate[18] indicated that acetoacetate was being cleaved to acetate before being incorporated into 6β-hydroxytropine. In addition to this, *N*-methyl-Δ^1-[2-^{2}H]pyrrolinium chloride labeled both C-1 and C-5 of 6β-hydroxytropine equally,[18] indicating that no chiral intermediate was involved in the formation of the tropane ring. These experiments implied that the "cocaine mechanism"[15] and not the "pelletierine mechanism"[17] was functioning in the biosynthesis of 6β-hydroxytropine, and it was suggested[18] that both enantiomers of 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (or of hygrine) were being incorporated into the tropane ring. The above results[18] were in direct contrast to what was expected on the basis of the studies of McGaw and Woolley.[10]

Results and Discussion

We were interested in investigating the formation of the tropane ring in (−)-scopolamine and (−)-hyoscyamine using ^{13}C-labeled precursors. The two halves of the tropane ring of (−)-scopolamine and (−)-hyoscyamine, which are esters of (*S*)-tropic acid, have a diastereomeric relationship. Thus, C-7, C-1, and C-2 are magnetically nonequivalent to C-6, C-5, and C-4, respectively, as a result of the chiral center in the tropic acid moiety. This results in a difference in the chemical shifts of these carbons which is observable in the ^{13}C-NMR spectrum.[19] This difference in chemical shifts allowed us to examine the biosynthesis of the tropane alkaloids using ^{13}C-labeled precursors.

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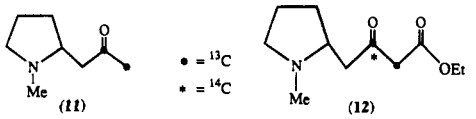

Figure 2. Labeled precursors used in feeding studies.

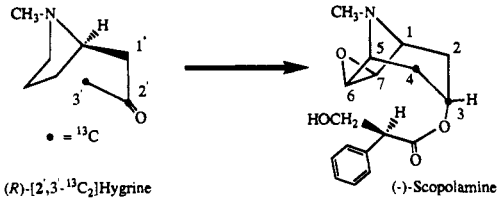

Figure 3. Hypothetical incorporation of (*R*)-[2′,3′-^{13}C$_2$]hygrine into (−)-scopolamine.

(*R*,*S*)-[2′,3′-^{13}C$_2$]Hygrine (**11**, Figure 2) was synthesized with the reasoning that, if the plant utilized only the (*R*)-isomer in the biosynthesis of (−)-scopolamine, then only C-3 and C-4 of the isolated (−)-scopolamine would be labeled with ^{13}C (Figure 3). This labeling would be observed in the ^{13}C-NMR spectrum by the spin−spin coupling between these two carbons. If both isomers, (*R*) and (*S*), are utilized in the biosynthesis of (−)-scopolamine then spin−spin coupling would also be observed between C-2 and C-3. One important condition that needed to be met was that the (−)-scopolamine (or (−)-hyoscyamine) had to be isolated without causing racemization of the tropic acid moiety.

(*R*,*S*)-[2′,3′-^{13}C$_2$]Hygrine (**11**) was synthesized from 5-ethoxy-1-methyl-2-pyrrolidinone and ethyl [3,4-^{13}C$_2$]acetoacetate using minor modifications of previously published procedures.[1,20] In the first feeding experiment, a mixture of (*R*,*S*)-[2′,3′-^{13}C$_2$]-hygrine (**11**) (100.4 mg), DL-[5-^{14}C]ornithine (0.05 mCi), and unlabeled DL-ornithine (5 mg) was fed to *D. innoxia* plants growing in hydroponics. After 2 weeks, the alkaloids were isolated and purified as described previously.[27] The absolute incorporation of DL-[5-^{14}C]ornithine into the tropane alkaloids was low: (−)-scopolamine (0.002%), (−)-hyoscyamine (0.004%), and (−)-3α,6β-bis(tigloyloxy)-7β-hydroxytropane (0.004%). The ^{13}C-NMR spectrum of (−)-scopolamine showed a small pair of satellites for C-4 with a coupling constant of 40.3 Hz (Figure 4a) (specific incorporation 0.22%) and not for C-2. However, no corresponding satellites expected at C-3 were observed.[21]

In another feeding experiment, (*R*,*S*)-[2′,3′-^{13}C$_2$]hygrine (**11**) (104.8 mg) was fed by the wick method to *D. innoxia* plants growing in soil. In this feeding, the ^{13}C-NMR spectrum of the isolated (−)-scopolamine showed a small pair of satellites for C-3 (Figure 4b) with a coupling constant of 36.7 Hz but a specific incorporation of only 0.03%. The satellites for C-2 and C-4 were not well defined as a result of racemization.[21] The scopolamine was then resolved using [(1*S*)-(*endo*,*anti*)]-(−)-3-bromocamphor-8-sulfonic acid.[22] After several recrystallizations from a mixture of ethanol, acetone, and petroleum ether, (−)-scopolamine of 92% optical purity was isolated. However, the numerous recrystallizations depleted the labeled (−)-scopolamine to a level which was not detectable by ^{13}C-NMR.[21]

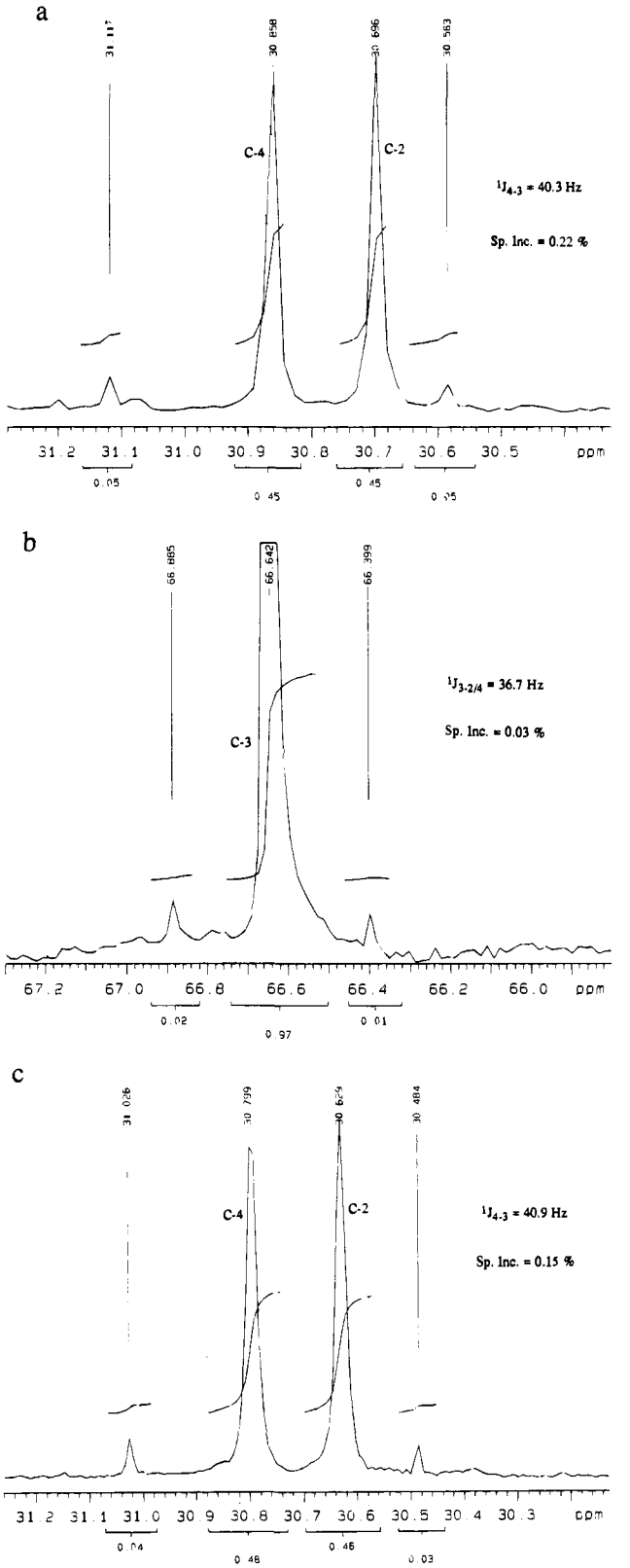

Figure 4. Expansion of ^{13}C-NMR of (−)-scopolamine showing (a) C-2 and C-4 from first hydroponic feeding, (b) C-3 from wick feeding, and (c) C-2 and C-4 from second hydroponic feeding of [2′,3′-^{13}C$_2$]-hygrine.

The feeding of labeled hygrine to *D. innoxia* growing in hydroponics was repeated using a mixture of (*R*,*S*)-[2′,3′-^{13}C$_2$]-hygrine (**11**) (101.6 mg) and (*R*,*S*)-[2-^{13}C,^{14}C]hygrine (DL)-tartrate (34 mg, 9.6 × 10^7 dpm/mmol). Once again, similar to the first feeding experiment, the ^{13}C-NMR spectrum of (−)-scopolamine showed a small pair of satellites with almost the

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same coupling constant (40.9 Hz) and a specific incorporation of 0.15% for C-4 and not for C-2 (Figure 4c). However, no corresponding satellites were seen for C-3.[21] The specific incorporation of radioactive hygrine into (−)-scopolamine was only 0.03%.

In addition to this, (*R,S*)-[2′,3′-$^{13}C_2$]hygrine (**11**) was fed to root cultures of *Hyoscyamus albus*. The ^{13}C-NMR spectra of the isolated (−)-scopolamine and (−)-hyoscyamine showed no incorporation of labeled hygrine.[23a] (*R,S*)-[2′,3′-$^{13}C_2$]hygrine (**11**) was also fed to root cultures of *D. stramonium*. The ^{13}C-NMR spectrum of the isolated (−)-hyoscyamine indicated no incorporation of labeled hygrine.[23b] In another study, (*R*)-[2-^{14}C]hygrine and (*S*)-[2-^{14}C]hygrine were separately fed as their tartrate salts to *D. innoxia* by both the wick and hydroponic methods. The isolated (−)-hyoscyamine and (−)-scopolamine showed very low incorporation of labeled hygrine.[23c] These ambiguous results from the different feeding experiments with labeled hygrine led us to believe that either the hygrine was somehow unable to reach the specific sites of biosynthesis or hygrine was not a true precursor of the tropane alkaloids. The latter suggestion would contradict the results that have been reported in the literature where ^{14}C-labeled hygrine had shown high incorporations (~2%) into (−)-hyoscyamine.[9-11] It was also possible that hygrine was being transformed into another precursor which was then incorporated into the tropane alkaloids, resulting in the low incorporations that were observed in our feeding experiments.

In order to investigate whether the biosynthesis of (−)-scopolamine and (−)-hyoscyamine was occurring according to the "cocaine pathway",[15] ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**12**, Figure 2) was synthesized using minor modifications of published procedures.[1,24] Ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**12**) (106 mg, 2.5 × 10^8 dpm/mmol) was fed to 3-month-old *D. innoxia* plants growing in hydroponics. At the end of 2 weeks ~92% of the radioactivity had been taken up by the plants. The crude alkaloids isolated had ~14% of the total radioactivity that was fed. The individual alkaloids isolated after chromatography indicated high levels of incorporation of both ^{13}C and ^{14}C. The absolute and specific incorporations of radioactivity into scopolamine (56.3 mg) were 0.65% and 1.73%, respectively. The ^{13}C-NMR spectrum of the isolated scopolamine showed a pair of large satellites for C-3 (Figure 5b) with a coupling constant of 36.6 Hz. The specific incorporation of labeled ^{13}C at C-3 was 1.78%. Both C-4 and C-2 (Figure 5a) had a pair of satellites with coupling constants of 36.6 and 36.7 Hz, respectively. The specific incorporation of labeled ^{13}C was 1.08% at C-4 and 0.77% at C-2, giving a total specific incorporation of 1.85%. It is clear from looking at the ^{13}C-NMR spectrum of the scopolamine and from the calculated specific incorporations that there appears to be a preference for ^{13}C labeling at C-4 (40% higher) compared to C-2. Unfortunately, the isolation procedure[27] caused racemization of a major portion of the isolated scopolamine, yielding material that was only 19% optically pure. Therefore, the ^{13}C label at C-2 could have been due to one of two reasons: (i) the (*S*)-isomer of the precursor **12** (which was fed as a racemic mixture) was also being incorporated into (−)-scopolamine, although to a lesser extent than the (*R*)-isomer or (ii) only the (*R*)-isomer was being incorporated and the ^{13}C labeling observed at C-2 was due to the partial racemization of the (−)-scopolamine.

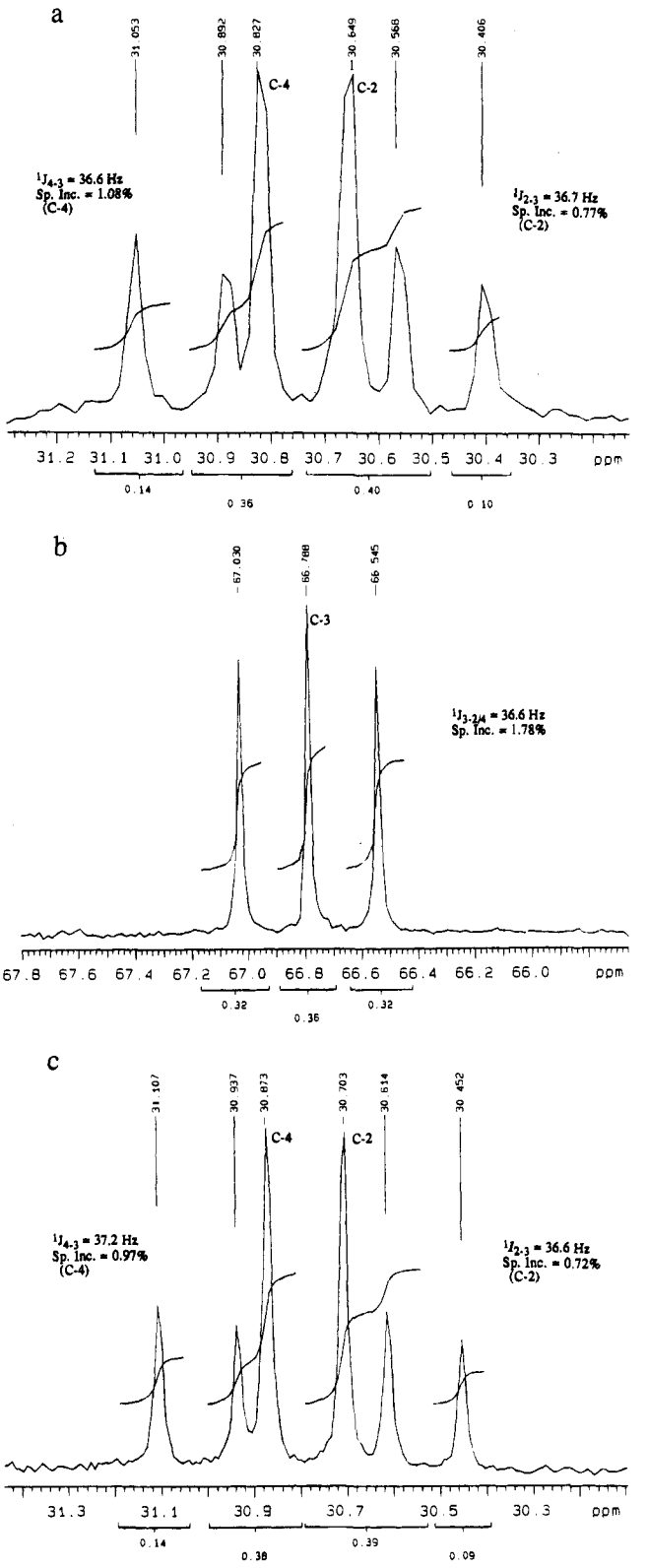

Figure 5. Expansion of ^{13}C-NMR of (−)-scopolamine showing (a, c) C-2 and C-4 and (b) C-3 from feeding ethyl[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate [(a,b) before resolution, (c) after resolution].

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(28) Leete, E. *J. Am. Chem. Soc.* **1960**, *82*, 612−614.

In order to distinguish between these two possibilities, the isolated partially racemic (−)-scopolamine was resolved using [(1*S*)-(*endo*,*anti*)]-(−)-3-bromocamphor-8-sulfonic acid.[22] After several recrystallizations from a mixture of ethanol−acetone−petroleum ether, 77% optically pure (−)-scopolamine was reisolated. The ^{13}C-NMR spectrum of the resolved (−)-scopolamine showed specific incorporations of 0.97% at C-4 and 0.72% at C-2 (Figure 5c), which are similar to those of the (−)-scopolamine before resolution. This indicated that the ^{13}C labeling at C-2 was not due to the partial racemization of the (−)-scopolamine during isolation but due to the incorporation of the (*S*)-isomer of the precursor that was fed. Therefore, it appears that both the (*R*)- and (*S*)-isomers of ethyl [2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**12**) were incorporated into (−)-scopolamine.

The above results provide direct evidence for the revised pathway[18] for the biosynthesis of the tropane skeleton from 1-methyl-Δ^1-pyrrolinium salt (**1**) in *Datura* species (Scheme 2, path B). The pyrrolinium salt **1** does not react with a four-carbon unit (acetoacetyl CoA) but condenses successively with two acetyl CoA or malonyl CoA units to form the thio ester **7** with a two-carbon side chain first and then the thio ester **8** with a four-carbon side chain. The latter (**8**) then cyclizes *via* the pyrrolinium salt **9** to a racemic mixture of the thio ester of 2-carboxy-3-tropinone, (1*R*)-**10** and (1*S*)-**10** (actually four stereoisomers are possible if the stereochemistry at C-2 is also considered) (path B). The thio esters (1*R*)-**10** and (1*S*)-**10** can hydrolyze to the free acid which on undergoing decarboxylation form tropinone (**5**). Tropinone (**5**) then goes on to form (−)-hyoscyamine and (−)-scopolamine (Scheme 1). The incorporation of ethyl (*R*,*S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**12**) into (−)-scopolamine can be explained according to the revised hypothesis. The *D. innoxia* plants were able to transesterify the ethyl ester **12**, which was fed, to the thioester **8** containing the ^{13}C and ^{14}C labels. This intermediate was then incorporated into the tropane alkaloids via path B shown in Scheme 2. The possibility that ethyl (*R*,*S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**12**) could have undergone decarbethoxylation to (*R*,*S*)-[2′,3′-$^{13}C_2$,2′-^{14}C]hygrine within the plant and that the latter was the actual precursor to the tropane alkaloids seems very unlikely, since the feeding experiments with (*R*,*S*)-[2′,3′-$^{13}C_2$]hygrine did not show good incorporations.

Recent studies[16,18] not only cast doubt on the preference for (*R*)-hygrine over (*S*)-hygrine that had been reported by McGaw and Woolley[10] but also questioned whether hygrine itself was an actual intermediate in the biosynthetic pathway of the tropane alkaloids.[18] As mentioned earlier, it is possible that the incorporation of hygrine observed by others[9−11] and us is due to hygrine being metabolized into another intermediate which then gets incorporated into the tropane alkaloids. One possibility is for hygrine (**4**) to undergo a retro-Mannich type reaction to form the pyrrolinium salt **1** and acetone, the latter then being metabolized to acetate before being incorporated into the tropane alkaloids according to the "cocaine" pathway. Another possibility is for the enol form of hygrine to condense directly with carbon dioxide to form the butanoate **8**, which is then incorporated as described above. On the other hand, it is also possible that our observations are a result of species-dependent biosynthesis which has previously been observed[29] in this family, i.e. the tropane alkaloids in *D. innoxia* may be biosynthesized according to the "cocaine" pathway in contrast to the "pelletierine" pathway observed in *H. albus*,[11] *A. belladona*,[11] and *D. stramonium*.[9]

(29) Hashimoto, T.; Yamada, Y.; Leete, E. *J. Am. Chem. Soc.* **1989**, *111*, 1141−1142.

There is some circumstantial evidence supporting the "cocaine" mechanism[15,18] for the biosynthesis of the tropane alkaloids. Leete[25] used a trapping experiment to isolate labeled 2-(1-methyl-2-pyrrolidinyl)acetic acid from coca plants which had been fed [2-^{14}C]-1-methyl-Δ^1-pyrrolinium chloride. Also, Brenner *et al.*[26] isolated methyl 2-(1-methyl-2-pyrrolidinyl)-acetate from *Solanum sturtianum*, a *Solanaceae* plant native to Australia. According to the old hypothesis (Scheme 1), when the pyrrolinium salt **1** condenses with acetoacetate, it is the C-1−C-2 bond of the ultimate tropane skeleton that is formed before the C-5−C-4 bond. Our results indicate that there is no such preference for bond formation, since both enantiomers of the oxobutanoate **12** appear to be utilized in the formation of the tropane ring.

Conclusion

In conclusion, direct evidence for the revised hypothesis[18] for the biosynthesis of the tropane moiety in *Datura* species is provided. (*R*,*S*)-[2′,3′-$^{13}C_2$]hygrine (**11**) did not serve as a good precursor for the tropane alkaloids in *D. innoxia*. However, ethyl (*R*,*S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**12**) showed a high incorporation into (−)-scopolamine in *D. innoxia*, indicating the essential role of this intermediate in the biosynthetic pathway.

Experimental Section

General Experimental Procedures. NMR spectra were obtained on Brüker/IBM NR-300 and Varian VXR-300 spectrometers. All ^{1}H-NMR spectra were obtained at 300 MHz. All ^{13}C-NMR spectra were obtained at 75 MHz. Radioactivity measurements were carried out using a Tracer Analytic Mark III 6881 liquid scintillation system. The scintillation fluid used was Ecoscint A from National Diagnostics. Radioactivities were determined using calibrated quench curves. Optical rotations were measured on a Perkin-Elmer model 241 polarimeter. Preparative TLC was performed using Merck kieselgel 60 PF_{254}, rolled to a thickness of 1.5 mm on 20 × 20 cm glass plates. Ethyl [3,4-$^{13}C_2$]acetoacetate (99 atom % ^{13}C), potassium [^{13}C]cyanide (99 atom % ^{13}C), and diethyl [2-^{13}C]malonate (99 atom % ^{13}C) were purchased from Isotec Inc.

General Feeding Methods. (i) **Hydroponic Feeding.** The labeled precursor was dissolved in some water with the aid of a few drops of acetic acid, and equal amounts of the resulting solution were added to the beakers in which the plants were growing. Typically, the plants were 3−4.5 months old and had a large mass of healthy roots, growing in the nutrient solution through which air was bubbled continuously. The beakers containing the roots were kept in the dark and filled with water everyday. The uptake of radioactive precursors was monitored every 24 h for the first 3−4 days and at the end of the feeding experiment. After a 2-week period the entire plants were used for isolation of the alkaloids.

(ii) **Wick Feeding.** The main stem of each 3−4-month-old plant was threaded twice with a needle and cotton so that the ends of the thread emerged from the same side of the stem. Just below the emerging ends of the thread was attached a 5 mL beaker with the help of masking tape. The labeled precursor was dissolved in a little water with the help of a few drops of acetic acid, and equal amounts were placed in each of the beakers. The emerging ends of each thread were dipped into the solution in the beaker. At the end of 2 weeks the plants were carefully uprooted, trying to retain as much of the roots as possible. The roots were washed with water to remove the soil clinging to it, and the entire plants were then used for the extraction of the alkaloids.

General Method for the Isolation and Purification of the Tropane Alkaloids. The literature procedure of Leete[27] was used for the isolation of the tropane alkaloids. The crude alkaloids were dissolved in chloroform and separated by preparative thin layer chromatography on silica gel, developing with $CHCl_3$:EtOH:NH_4OH (100:20:1). The R_f values for (−)-hyoscyamine, (−)-scopolamine, and (−)-3α,6β-bis-(tigloyloxy)-7β-hydroxytropane were 0.09, 0.48, and 0.71, respectively. The zones of silica gel corresponding to the above three compounds

were carefully scraped off the plates and eluted with methanol to obtain the individual alkaloids. Further purification of (−)-scopolamine by chromatography on a Celite column[28] was necessary in order to obtain samples good enough for NMR spectroscopy.

Individual Feeding Results. (*R,S*)-[2′,3′-$^{13}C_2$]Hygrine (11) and DL-[5-^{14}C]Ornithine Feeding (Hydroponic Method). (*R,S*)-[2′,3′-$^{13}C_2$]-Hygrine (**11**) (100.4 mg), DL-[5-^{14}C]ornithine (0.05 mCi), and DL-ornithine (5 mg) were dissolved in water (20 mL) containing 5 drops of acetic acid. This solution was fed to four *D. innoxia* plants growing in hydroponics. At the end of 2 weeks, 97% of the radioactive ornithine had been taken up by the plants. The plants (200 g) were then removed from the nutrient solutions and the crude alkaloids (0.6412 g) isolated and purified as previously described. (−)-Hyoscyamine (20.3 mg), (−)-scopolamine (30 mg), and (−)-3α,6β-bis(tigloyloxy)-7β-hydroxytropane (10.2 mg) were isolated. The ^{13}C-NMR spectrum of (−)-scopolamine showed a pair of satellites for C-4 at 30.86 ppm (coupling constant = 40.3 Hz) but not for C-2 at 30.69 ppm (Figure 4a). However, no corresponding pair of satellites, expected from the spin−spin coupling, was seen for C-3.[21] The specific incorporation of labeled hygrine calculated on the basis of the satellites for C-4 was 0.22%.

(*R,S*)-[2′,3′-$^{13}C_2$]Hygrine (11) Feeding (Wick Method). (*R,S*)-[2′,3′-$^{13}C_2$]Hygrine (**11**) (104.8 mg) was dissolved in water (20 mL) with the help of some acetic acid (42 mg) and fed by the wick method to ten, 4-month-old plants growing in soil. After 2 weeks, the plants (890 g) were uprooted and the crude alkaloids (0.5664 g) isolated and purified as previously described. (−)-Scopolamine (84.5 mg), (−)-hyoscyamine (71 mg), and (−)-3α,6β-bis(tigloyloxy)-7β-hydroxytropane (18.4 mg) were isolated.

The ^{13}C-NMR spectrum of (−)-scopolamine showed a small pair of satellites for C-3 at 66.6 ppm with a coupling constant of 36.7 Hz and a specific incorporation of only 0.03% (Figure 4b). The satellites for C-2 and C-4 were not well defined as a result of racemization.[21] The scopolamine was resolved using [(1*S*)-(*endo,anti*)-(−)-3-bromocamphor-8-sulfonic acid[22] with a mixture of absolute ethanol, acetone, and petroleum ether as the solvent. Obtained (−)-scopolamine was 92% optically pure. However, the labeled (−)-scopolamine was depleted to a level not detectable by ^{13}C-NMR.[21]

(*R,S*)-[2′,3′-$^{13}C_2$]Hygrine (11) and (*R,S*)-[2-^{13}C,^{14}C]Hygrine DL-Tartrate Feeding (Hydroponic Method). (*R,S*)-[2′,3′-$^{13}C_2$]Hygrine (**11**) (101.6 mg), (*R,S*)-[2-^{13}C,^{14}C]hygrine DL-tartrate (34 mg, 9.6 × 10^7 dpm/mmol), and acetic acid (40 mg) were dissolved in water (35 mL) and fed to 4-month-old plants growing in hydroponics. Within 48 h, about 95% of the radioactive hygrine had been taken up by the plants. After 2 weeks, the plants (575 g) were removed from the nutrient solution and the crude alkaloids (482.1 mg) isolated and purified as previously described. (−)-Scopolamine (57.9 mg) and (−)-hyoscyamine (20.6 mg) were isolated. Similar to the first hydroponic feeding experiment with [2′,3′-$^{13}C_2$]hygrine, the ^{13}C-NMR spectrum of (−)-scopolamine showed a pair of satellites for C-4 and not for C-2 (Figure 4c). However, once again no corresponding satellites expected at C-3 were observed.[21] The satellites for C-4 showed a specific incorporation of 0.15% with a coupling constant of 40.9 Hz. The specific incorporation of radioactive hygrine into (−)-scopolamine was only 0.03%.

Ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-Methyl-2-pyrrolidinyl)-3-oxobutanoate (12) Feeding (Hydroponic Method). Ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**12**) (106 mg, 2.5 × 10^8 dpm/mmol) was dissolved in water with the help of acetic acid (40 mg) and fed to five, 3-month-old plants growing in hydroponics. At the end of 2 weeks, when about 92% of the radioactivity had been taken up, the plants (400 g) were removed from the nutrient solution and the crude alkaloids (0.3667 g) isolated and purified as previously described. The crude alkaloids contained over 14% of the radioactivity that was fed. A high incorporation of radioactivity into (−)-scopolamine (56.3 mg, 1.73% specific incorporation) was observed. The ^{13}C-NMR of (−)-scopolamine also showed a high incorporation of doubly-labeled ^{13}C. C-3 had a pair of satellites almost as tall as the natural abundance peak (coupling constant = 36.6 Hz (Figure 5b). The specific incorporation at C-3 was 1.78%. Surprisingly, both C-2 and C-4 had satellites (coupling constant = 36.7 and 36.6 Hz, respectively) (Figure 5a). Therefore, the (−)-scopolamine had either racemized during isolation or both the (*R*)- and (*S*)-isomers of the precursor were incorporated into (−)-scopolamine. A careful look at the integration for the two pairs of satellites for C-4 (1.08% specific incorporation) and C-2 (0.77% specific incorporation) showed about a 40% preference for labeling at C-4. The rotation observed for the isolated (−)-scopolamine, $[\alpha]_D^{20} = -5.33°$ ($c = 0.1$), indicated that it was only about 19% optically pure. The partially racemized (−)-scopolamine was resolved using [(1*S*)-(*endo,anti*)]-(−)-3-bromocamphor-8-sulfonic acid.[22] After six recrystallizations, from a mixture of ethanol−acetone−petroleum ether, the free base (19 mg) was reisolated; $[\alpha]_D^{20} = -21.5°$ ($c = 0.95$, $CDCl_3$), i.e., 77% optically pure. The ^{13}C-NMR spectrum (Figure 5c) showed a 35% preference for labeling at C-4 compared to C-2 which is not much different from that before resolution.

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Supporting Information Available: ^{13}C-NMR spectra of isolated (−)-scopolamine showing the other labeled carbons (5 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, can be ordered from the ACS, and can be downloaded from the Internet; see any current masthead page for ordering information and Internet access instructions.

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