The Biosynthesis of Tropane Alkaloids in *Datura stramonium:* The Identity of the Intermediates between *N*-Methylpyrrolinium Salt and Tropinone

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Abstract: The biosynthesis of the tropane alkaloids in transformed root cultures of *Datura stramonium* has been studied using sodium [1,2-$^{13}C_2$]acetate, (*R,S*)-[2,3-$^{13}C_2$]-1-(1-methyl-2-pyrrolidinyl)propan-2-one {(*R,S*)-[2′,3′-$^{13}C_2$]-hygrine}, ethyl (*R,S*)-[1,2-$^{13}C_2$,2-^{14}C]-2-(1-methyl-2-pyrrolidinyl)acetate, and ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate. The incorporation of (*R,S*)-[2′,3′-$^{13}C_2$]hygrine into cuscohygrine and several other condensation products was high (15−40% specific incorporation), but label was not recovered in either tropine or tropine esters (hyoscyamine; 0.0 ± 0.5% specific incorporation). None of the recovered alkaloids was labeled when ethyl (*R,S*)-[1,2-$^{13}C_2$,2-^{14}C]-2-(1-methyl-2-pyrrolidinyl)acetate was fed to the cultures. In contrast, sodium [1,2-$^{13}C_2$]acetate and ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate were incorporated into hyoscyamine (9 and 2% specific incorporation, respectively) and a number of other tropane alkaloids (up to 12% specific incorporation). These data provide further evidence that hygrine is not a direct precursor of tropane alkaloids. ^{13}C-Label from acetate was incorporated symmetrically into the C-2 and C-4 positions of (−)-hyoscyamine. The evidence supports a pathway in which acetoacetate reacts via its C-4 position with *N*-methyl-Δ^1-pyrrolinium salt to give 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate. This intermediate favors cyclization to give 2-carboxytropinone, tropinone being formed by decarboxylation.

Introduction

The biosynthesis of the tropane alkaloids (−)-hyoscyamine, (−)-scopolamine, and (−)-cocaine (Figure 1) has been extensively studied over the last few decades.[1,2] Labeling studies had indicated that hygrine {1-(1-methyl-2-pyrrolidinyl)propan-2-one} was a precursor of the tropine moiety of (−)-hyoscyamine and other tropane alkaloids.[3−5] O'Donovan and Keogh[3] fed (*R,S*)-[*N-methyl*-^{14}C,2′-^{14}C]hygrine to *Datura stramonium* plants and reported an incorporation (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 in the labeled hygrine, indicating that the labeled hygrine had been incorporated intact. McGaw and Woolley[4] reported an incorporation of (2*R*)-[2′-^{14}C]hygrine into the tropane alkaloids in *Datura innoxia* 2.5−10 times greater than for (2*S*)-[2′-^{14}C]hygrine, although they also found that *Hyoscyamus niger* and *Atropa belladonna* utilized both (*R*)- and (*S*)-hygrine equally well in the biosynthesis of (−)-hyoscyamine.[5] In contrast, Liebisch *et al.*[6] reported that labeled hygrine showed a lower incorporation into the tropane alkaloids in *Datura metel* than labeled ornithine, even though hygrine was considered a later intermediate in the biosynthetic pathway. The unsymmetrical incorporation of ornithine into (−)-hyoscyamine in *D. stramonium*[7] and *N*-methylputrescine into (−)-scopolamine in *D. innoxia*[8] requires that only (2*R*)-hygrine and not (2*S*)-hygrine serve as a precursor for the tropane ring. However, hygrine is known to racemize readily in neutral or basic solutions,[2] making it hard to interpret chiral incorporation data.

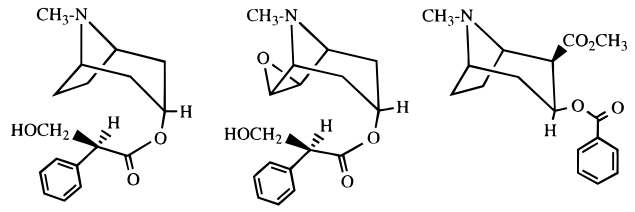

Figure 1. Structures of (−)-hyoscyamine, (−)-scopolamine, and (−)-cocaine

All these studies employed radiolabeled tracers. Recent investigations using heavy isotopes have not only questioned

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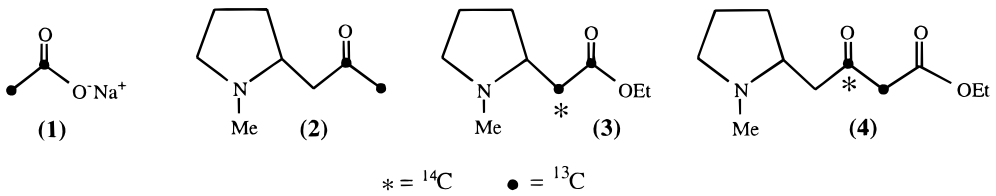

Figure 2. Labeled compounds used in these feeding experiments, showing the points of incorporation of ^{13}C (●) and ^{14}C (*): (**1**) sodium [1,2-$^{13}C_2$]acetate; (**2**) (*R,S*)-[2,3-$^{13}C_2$]-1-(1-methyl-2-pyrrolidinyl)propan-2-one {(*R,S*)-[2′,3′-$^{13}C_2$]hygrine}; (**3**) ethyl (*R,S*)-[1,2-$^{13}C_2$,2-^{14}C]-2-(1-methyl-2-pyrrolidinyl)acetate; and (**4**) ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate.

the preferential incorporation of the (*2R*)-isomer of hygrine relative to the (*2S*)-isomer but have also cast doubts on any role for hygrine as a precursor of (−)-hyoscyamine and (−)-scopolamine.[9−11] In 1990, Sankawa *et al.*[9] reported the nonregiospecific incorporation of sodium [1,2-$^{13}C_2$]acetate into (−)-hyoscyamine and (−)-6β-hydroxyhyoscyamine in *Hyoscyamus albus*. This indicated either that both enantiomers of hygrine were being utilized for the formation of the tropane ring or that other intermediates were involved. Subsequently, in a similar study, Hemscheidt and Spenser[10] demonstrated the nonregiospecific incorporation of sodium [1,2-$^{13}C_2$]acetate into 7β-hydroxytropine in *D. stramonium*. Unfortunately, attempts to incorporate sodium [1,2,3,4-$^{13}C_4$]acetoacetate[10] were not successful, as this was apparently cleaved entirely to acetate. *N*-Methyl-Δ^1-[2-^{2}H]pyrrolinium chloride labeled both C-1 and C-5 of 7β-hydroxytropine equally,[10] indicating that no chiral intermediate was involved in the formation of the tropane ring. Thus, it was suggested[10] that both enantiomers of hygrine or, perhaps, of 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate—an intermediate by this time identified as likely to be formed during the biosynthesis of (−)-cocaine[12] —were being incorporated into the tropane ring. Direct evidence for a new pathway[11] was provided when it was shown that ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate was incorporated into (−)-scopolamine in *D. innoxia* with a specific incorporation of 1.78%. These results also indicated that both enantiomers of 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate were being incorporated into (−)-scopolamine.

Therefore, a new pathway was proposed[10,11] for the biosynthesis of scopolamine in which the *N*-methyl-Δ^1-pyrrolinium salt reacted with two acetate units in a consecutive fashion to give 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate which then cyclized to form the tropane ring. Evidence contrary to this hypothesis was obtained, however, in experiments in which ethyl (*R,S*)-[1,2-$^{13}C_2$,2-^{14}C]-2-(1-methyl-2-pyrrolidinyl)acetate (**3**) was fed to *D. innoxia* and *E. coca* plants.[13] Incorporation of **3** was very low as determined by ^{14}C and undetectable by ^{13}C NMR. Hence, the mechanism for the introduction of the C-2, C-3, and C-4 of the tropane ring remained unresolved.

A problem with the data so far reported is that experiments have been performed in a range of species and using a variety of techniques (see ref 2 for review). In an effort to clarify the biosynthesis of the tropane alkaloids, a series of uniform experiments have been performed using transformed root cultures of *D. stramonium* growing in sterile conditions[14] to which have been fed sodium [1,2-$^{13}C_2$]acetate (**1**), *(R,S)*-[2′,3′-$^{13}C_2$]hygrine (**2**), ethyl(*R,S*)-[1,2-$^{13}C_2$,2-^{14}C]-2-(1-methyl-2-pyrrolidinyl)acetate (**3**), and ethyl(*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate **(4)** (Figure 2). The findings support a role for 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate as an intermediate but are not consistent with the formation of this compound by the sequential addition of acetate units.

Results and Discussion

Sodium [1,2-$^{13}C_2$]Acetate (1) Feeding. Sodium [1,2-$^{13}C_2$]-acetate (82 mg) was fed to *D. stramonium* root cultures cultivated under standard conditions.[14] After 14 days, the roots (41 g FW) were extracted, yielding a crude alkaloid mixture. GC-MS of this extract clearly showed (Table 1) that the ^{13}C-label was incorporated into the M + 2 and M + 3 ions of a wide range of tropane alkaloids. Acetyl tropine was significantly labeled at the M + 4 level.

The ^{13}C-NMR spectrum of the isolated hyoscyamine (9.2 mg: $[\alpha]^{20}_D = -10°$, *c* 0.46) shows a high incorporation of sodium [1,2-$^{13}C_2$]acetate (**1**). Both the C-4 and C-2 positions of the isolated hyoscyamine have satellites showing coupling to the C-3 position ($J = 36.6$ and 34.2 Hz, respectively). The downfield satellite for C-2 position is directly buried under the natural abundance peak of the C-4 position (see Supporting Information). The upfield satellite for the C-4 position is seen as a shoulder on the C-2 position natural abundance peak. The C-3 resonance (Figure 3) is a doublet symmetrically overlapped with a triplet. Two large satellites ($J = 36.7$ Hz) due to doubly-labeled hyoscyamine, (i.e., C-2/C-3 and C-4/C-3: Figure 4) flank the natural abundance peak. Two small satellites ($J \approx 36$ Hz) are part of a triplet which arises from (−)-hyoscyamine which is labeled at the C-2, C-3, and C-4 positions within the same molecule (Figure 4). Integration yields specific incorporations of 7.22% for the doubly-labeled species and of 2.89% for the triply-labeled species.

These data unequivocally show the C-2 and C-4 positions of hyoscyamine to be equally labeled from sodium [1,2-$^{13}C_2$]acetate (**1**), revoking previous suggestions that the C-2 position is preferably labeled. That this symmetrical labeling was not due to the hyoscyamine being a racemic mixture (i.e., atropine) was apparent from the value of $[\alpha]^{20}_D = -10°$ and was confirmed in a further experiment. Sodium [1,2-$^{13}C_2$]acetate diluted with unlabeled acetate either at a 1:1 ratio or at a 1:3 ratio (12 mg total acetate, fed in 3 aliquots on days 5, 7, and 9) was fed to roots, and the alkaloids extracted 14 days from subculture. The isolated hyoscyamine was shown to be exclusively (−)-hyoscyamine by derivatization as the Mosher acid.[15] NMR analysis confirmed incorporation to be at both the C-2 and C-4 positions, in support of the evidence of the presence of the triply labeled species. The incorporation patterns in the ^{13}C-NMR spectra (125 MHz) were unaltered from the feed made with undiluted sodium [1,2-$^{13}C_2$]acetate, indicating that the incor-

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Table 1. Incorporations of Labeled Precursors (**1**) Sodium [1,2-$^{13}C_2$]Acetate, (**2**) (*R,S*)-[2,3-$^{13}C_2$]-1-(1-Methyl-2-pyrrolidinyl)propan-2-one {(*R,S*)-[2′,3′-$^{13}C_2$]Hygrine}, (**4**) Ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-Methyl-2-pyrrolidinyl)-3-oxoburanoate, and (**3**) Ethyl (*R,S*)-[1,2-$^{13}C_2$,2-^{14}C]-2-(1-Methyl-2-pyrrolidinyl)acetate into Various Alkaloids Extracted from *Datura stramonium* Root Cultures

		% incorporation[a]											
labeled precursor fed		sodium [1,2-$^{13}C_2$]acetate (**1**)				[2′,3′-$^{13}C_2$]hygrine (**2**)			ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**4**)			ethyl (*R,S*)-[1,2-$^{13}C_2$,2-^{14}C]-2-(1-methyl-2-pyrrolidinyl) acetate (**3**)	
product	mass	M + 1	M + 2	M + 3	M + 4	M + 1	M + 2	M + 3	M + 1	M + 2	M + 3	M + 1	M + 2
hygrine	141					48.7	51.3		15.6	46.0	−0.8		
"hygroline" [b]	143								1.1	4.4	−0.4		
tropinone	139	10.2	6.8			2.4	5.8	3.0					
tropine	141	9.7	7.1	2.2		−1.1	0.03	0.1	1.0	2.4	−0.2		
acetyltropine	183	4.2	12.0	2.9	2.8	−0.3	0.9		3.4	6.5	0.0		
"*Nicandra* 222" [b]	222					7.1	42.8						
N-methylpyrrolidinyl-hygrine. A (frag)[c,d]	224 (152)					13.9	30.0		5.2	28.3			
N-methylpyrrolidinyl-hygrine. B (frag)[c,d]	224 (152)					13.2	34.2		5.1	17.1			
tigloyltropine	223	11.7	7.5	2.3		0.8	0.2		4.0	4.3			
cuscohydgrine (frag)[c]	224 (140)	27.8	10.8	0.3		21.9	14.4	2.9	2.6	28.9	0.0		
3-tigloyl,6-OH-tropine	239					−2.2	0.6		2.2	21.4	0.0		
phenylacetoyltropine	259	11.8	8.3	2.5		0.6	0.9		0.0	4.4	0.0		
apoatropine	271	11.9	9.3	3.1		−0.4	0.5		2.1	1.9	−0.5		
littorine	289	9.9	8.6	2.6					1.7	2.5	−0.7	1.7	−0.1
hyoscyamine	289	8.9	8.8	2.3		−0.7	0.2		1.4	1.8	−0.2	1.3	−0.1
3,6-ditigloyltropine	321	12.7	8.0	5.3					4.2	7.0	−1.0		

[a] Incorporation (%) = 100 × proportion of excess isotopic enrichment at the given mass enhancement in the product. [i.e., $\sum$(M:M + *n*) = 1]. Excess means that the incorporation at M + 1, M + 2 etc. has been corrected for the contribution at the defined mass of the natural abundance isotopic composition, measured on control samples. [b] These compounds are not fully characterized (see refs 16 and 22). [c] The molecular ion is too small to determine accurately in these compounds. The measurements have been made on an identified major fragment (frag), the mass of which is indicated in parentheses. [d] A and B are arbitrary: the assignment to (*R*) and (*S*) has not been made.

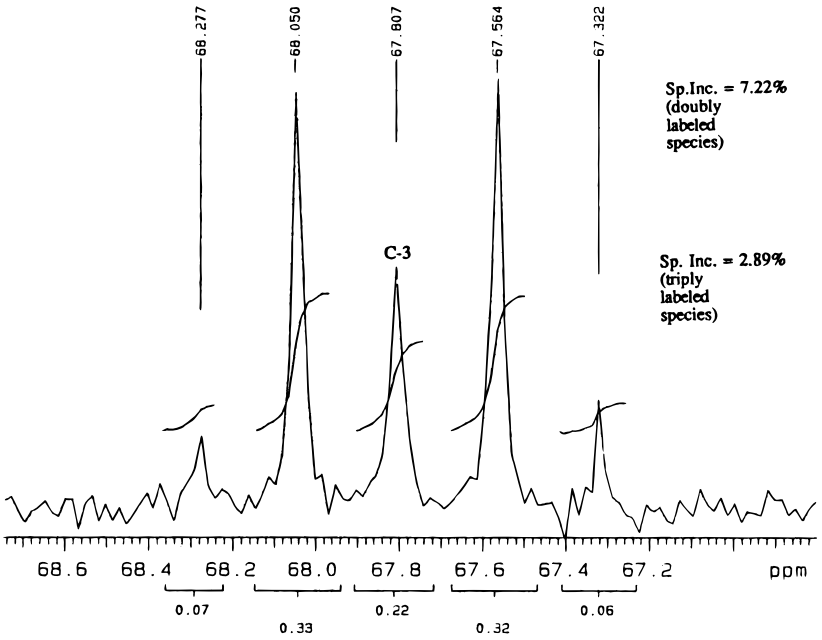


Figure 3. Proton-noise decoupled ^{13}C-NMR spectrum of (−)-hyoscyamine extracted from cultures fed sodium [1,2-$^{13}C_2$]acetate (**1**): expansion of 67−69 ppm region showing the C-3 resonance.

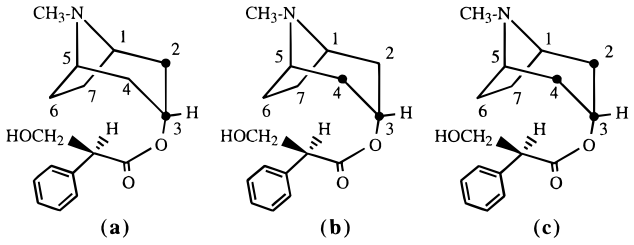


Figure 4. Alternative possible incorporation patterns of sodium [1,2-$^{13}C_2$]acetate into (−)-hyoscyamine.

porations observed were unlikely to be due to over-saturation of the acetate pool in the tissue.

(*R,S*)-[2′,3′-$^{13}C_2$]Hygrine (2) Feeding. (*R,S*)-[2′,3′-$^{13}C_2$]-Hygrine (75 mg in dilute HCl) was fed in parallel with the acetate feeds. After 14 days, the roots (33 g FW) were extracted, yielding a crude alkaloid mixture. GC-MS of the crude alkaloid mixture indicated (Table 1) that no incorporation of labeled hygrine into tropine, apoatropine, or hyoscyamine had occurred.

Although a large amount of unmetabolized labeled hygrine was recovered, high incorporation into cuscohygrine was observed. Two further compounds, previously proposed to be (*R*)- and (*S*)-*N*-methylpyrrolidinylhygrine[16] were also highly enriched, confirming that these compounds are metabolic products of hygrine. Surprisingly, ^{13}C-label was also detected in the M + 2 (5.8%) and M + 3 (3.0%) of tropinone, though not in tropine. Most probably, however, this is due to a minor oxidation product of hygrine co-eluting with this peak.

The ^{13}C-NMR spectrum of the (−)-hyoscyamine isolated after purification showed no measurable incorporation of labeled hygrine.

Ethyl (*R,S*)-[1,2-$^{13}C_2$,2-^{14}C]-2-(1-Methyl-2-pyrrolidinyl)-acetate (3) Feeding. Ethyl (*R,S*)-[1,2-$^{13}C_2$,2-^{14}C]-2-(1-Methyl-2-pyrrolidinyl)acetate (100 mg) was hydrolyzed (2 M HCl; 1 h) and the free acid fed in neutral solution to 4-day-old root cultures. After 14 days, the roots (3.07 g DW) were extracted, yielding a crude alkaloid mixture. An initial analysis by GC/MS showed large amounts of the unmetabolized precursor to be present in the tissue, indicating that it had been effectively absorbed. None of the tropane alkaloids, however, possessed an M + 1 or M + 2 significantly above background (Table 1). Analysis by ^{13}C-NMR of the crude alkaloid extract showed no incorporation into either hyoscyamine or cuscohygrine. Specifically, the resonances at 35.9, 36.0, and 67.8 ppm, due respectively to the C-2, C-4, and C-3 positions of hyoscyamine, showed no detectable coupling. Similarly, there were no doublets centered on 47.9 or 208 ppm, indicating no incorporation into cuscohygrine.

Ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-Methyl-2-pyrrolidinyl)-3-oxobutanoate (4) Feeding. Ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (60 mg) was dissolved in dilute HCl and fed to 4-day-old roots. After 14 days, the roots were harvested (2.37 g DW), and the crude alkaloids isolated. The ^{13}C-NMR spectrum of the crude alkaloid mixture showed clear incorporation into hyoscyamine and cuscohygrine. The alkaloids were further purified by thin layer chromatography.

The ^{13}C-NMR of the isolated hyoscyamine showed a high incorporation of doubly-labeled ^{13}C (Figure 5a). The C-3 position (67.79 ppm) had large satellites (J = 35.9 Hz) and a specific incorporation of 1.91%. Both the C-2 (35.82 ppm) and C-4 (36.02 ppm) positions also show satellites (J = 36.6 and 35.1 Hz respectively), indicating that both the (*R*)- and (*S*)-isomers of the precursor had been incorporated. Due to the overlap of these two resonances in hyoscyamine, it was not possible to calculate the specific incorporations at these carbons. It would appear from the outermost satellites, however, that the labeling at C-2 and C-4 is equivalent. This could arise from equal incorporations of the two isomers of the precursor or from the hyoscyamine produced being a racemate. The chirality of the hyoscyamine was not determined due to a paucity of material. However, in a previous experiment,[11] (−)-scopolamine extracted from *D. innoxia* plants fed ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate had been confirmed to be 77% optically pure, indicating both the (*R*)- and (*S*)-isomers of the precursor to have been incorporated.

A very high incorporation of ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate **(4)** into cuscohygrine was also measured (Figure 5b). Both the C-6 and C-6′ positions (47.89 and 47.95 ppm, unassigned; J = 39.0 Hz) are labeled, with mean specific incorporations of 6.1 and 9.3%, indicating again that both isomers of the precursor had been metabolized,

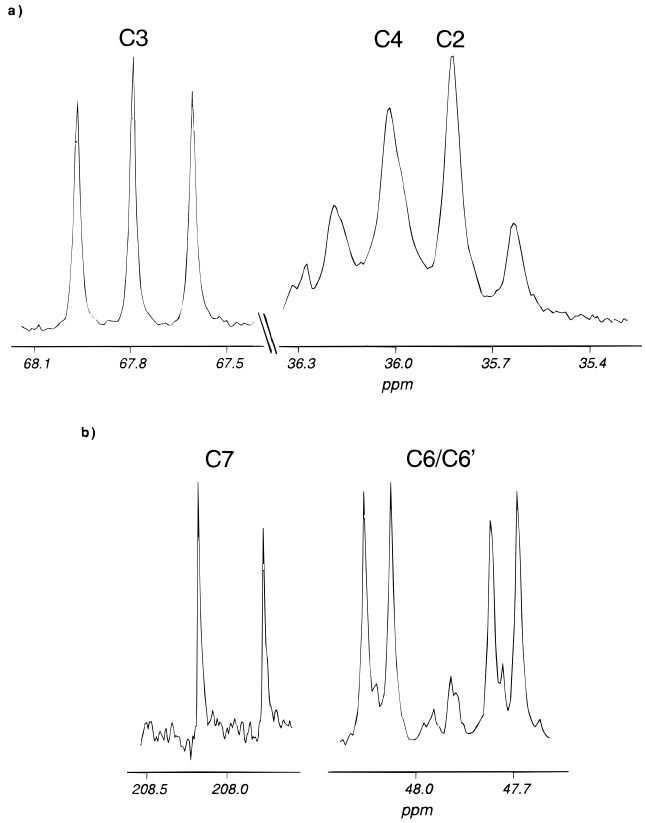


Figure 5. Proton-noise decoupled ^{13}C-NMR spectrum of a crude alkaloid extract isolated from cultures fed ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**4**): (a) C-3, C-4, and C-2 resonances of hyoscyamine and (b) C-7, C-6, and C-6′ of (*R,R*)- and (*R,S*)-cuscohygrine.

yielding a mixture of (*R,R*)- and (*R,S*)-cuscohygrines. The C-7 is also strongly labeled.

Conclusions

Four possible routes have been proposed (Scheme 1) for the addition of acetate to *N*-methyl-Δ^1-pyrrolinium salt (**5**) to form the tropane skeleton[10,13] or to Δ^1-piperideine salt to form the pelletierine skeleton.[17]

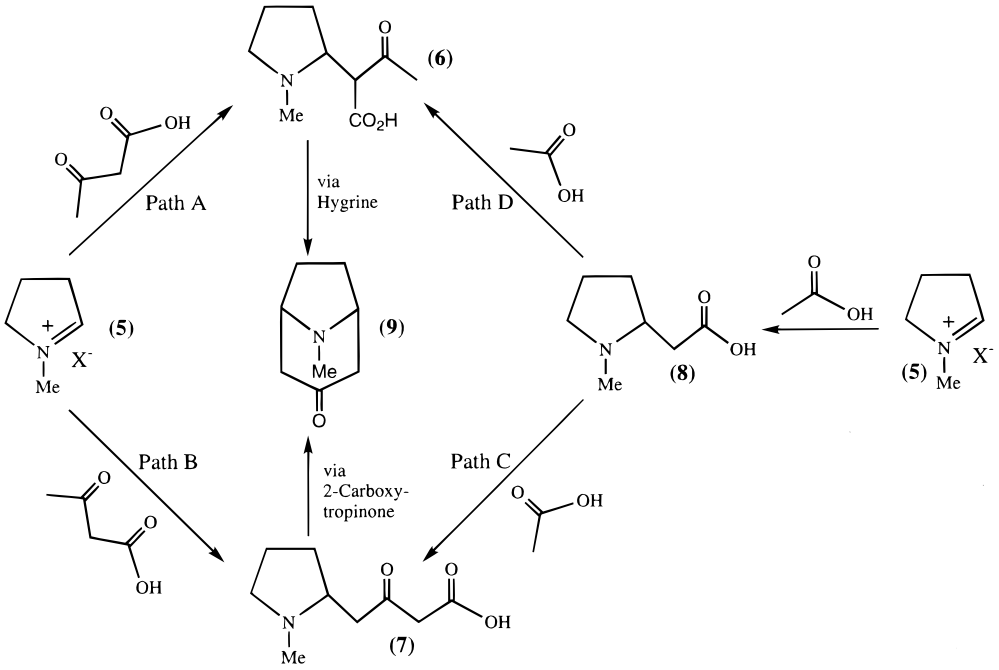
In the earliest hypothesis,[1−3] path A (Scheme 1), in which a four carbon unit (acetoacetate) adds to *N*-methyl-Δ^1-pyrrolinium salt (**5**) to form 2-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**6**), was proposed for the biosynthesis of all the tropane alkaloids. While previous results[3−6] had indicated the apparent intermediacy of hygrine, recent studies[10,11] and the present results discount this proposal. Path A (Scheme 1) was, however, unequivocally demonstrated to hold true for the biosynthesis of *N*-methylpelletierine, the higher homologue of hygrine, in *Sedum* plants.[17]

It was later postulated, from results obtained by feeding [2-^{13}C,^{14}C,^{15}N]-1-methyl-Δ^1-pyrrolinium chloride and (*R,S*)-[1,2-$^{13}C_2$,1-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate to *E. coca*,[12] that (−)-cocaine was biosynthesized along path C (Scheme 1). In the light of these findings, a revised hypothesis[10,11] for the biosynthesis of (−)-hyoscyamine and (−)-scopolamine also proposed path C (Scheme 1), wherein the consecutive addition of two acetate units to *N*-methyl-Δ^1-pyrrolinium salt (**5**) gives firstly 2-(1-methyl-2-pyrrolidinyl)-acetate (**8**) and then 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**7**) which, following ring closure and decarboxylation, leads to

(16) Witte, L., Müller, K, Arfmann, H.-A. *Planta Medica* **1987**, *53*, 192−197.

(17) Hemscheidt, T.; Spenser, I. D. *J. Am. Chem. Soc.* **1990**, *112*, 6360−6363.

Scheme 1. Four Possible Routes for the Biosynthesis of Tropinone (**9**) from *N*-Methyl-Δ^1-Pyrrolinium Salt (**5**)



tropinone (**9**) via the intermediacy of 2-carboxytropinone (or an ester thereof). The failure, however, to obtain any incorporation of ethyl (*R,S*)-[1,2-$^{13}C_2$,2-^{14}C]-2-(1-methyl-2-pyrrolidinyl)-acetate (**3**) makes the intermediacy of **8** improbable, although it cannot be ruled out that **8** is a sequestered intermediate, as occurs during the sequential addition of acetate-derived units in flavonoid[18] and polyketide[19] synthesis. Similar conclusions that 2-(1-methyl-2-pyrrolidinyl)acetate, or a thioester thereof, is not an efficient precursor of either (−)-cocaine,[12] (−)-scopolamine, or (−)-hyoscyamine[11,12] have been reached in other species, making both paths C and D (Scheme 1) improbable.

In contrast, the high specific incorporations of ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**4**) into (−)-cocaine,[12] (−)-scopolamine,[11] and hyoscyamine (present data) strongly support a conclusion that either path B or C of Scheme 1, with the intermediacy of (**7**) or a thioester thereof, is involved in the biosynthesis of (−)-hyoscyamine, while paths A and D are not. To form **7** directly requires that acetoacetate reacts *via* its C-4 position with *N*-methyl-Δ^1-pyrrolinium salt (**5**) (path B, Scheme 1), one of the options considered for the formation of pelletierine.[17] Cyclization and decarboxylation will give tropinone. While condensation via the C-4 position is less favored than *via* the C-2 position, this reaction can take advantage of the activated state of the C-2 carbon in *N*-methyl-Δ^1-pyrrolinium salt (**5**), leaving the reactive C-2 position of **7** available to carry out the second condensation with the C-5′ position of the heterocycle. This hypothesis is compatible with the biosynthesis of both (−)-hyoscyamine and (−)-cocaine.

The incorporation of an intact 4-carbon unit is directly supported by the results from feeding sodium [1,2-$^{13}C_2$]acetate **(1)**. Not only is symmetrical incorporation of ^{13}C-label into the C-2 and C-4 positions of (−)-hyoscyamine obtained but also there is considerable formation (2.9% specific incorporation) of a triply-labeled species in which equal incorporation into both the C-2 and C-4 positions has occurred. These data may indicate that there is no preference for the (*R*)- isomer over the (*S*)-isomer of the intermediate [4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate (**7**) or its thioester] that cyclizes to form the tropane ring. It may also be interpreted, however, as indicating that the acetate-derived carbon atoms have not been incorporated stepwise, but rather that they are incorporated as a single unit. Thus, indirectly, the labeling with sodium [1,2-$^{13}C_2$]acetate (**1**) can be seen to support the proposed path B of Scheme 1. While this high incorporation into a triply-labeled species is in contrast to the data of Hemscheidt and Spencer[10] in *D. stramonium*, it may be that the level of incorporation measured in 7β-hydroxytropinone (5−10 times lower) was insufficient to detect this species. Recently, the methyl ester of the putative intermediate 2-carboxytropinone has been reported[20] in extracts of *D. stramonium* root cultures, adding weight to the likely intermediacy of (**7**) which, on ring closure will form 2-carboxytropinone.

Experimental Section

Sodium [1,2-$^{13}C_2$]acetate was obtained from Aldrich Chemical Co. and by a generous gift from Dr. T. Hemscheidt (HI). The syntheses of *(R,S)*-[2′,3′-$^{13}C_2$]hygrine, ethyl (*R,S*)-[1,2-$^{13}C_2$,2-^{14}C]-2-(1-methyl-2-pyrrolidinyl)acetate, and ethyl (*R,S*)-[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate were reported previously.[11−13]

GC-MS were recorded on a VG TRIO-1S mass spectrometer fitted with a Hewlett Packard 5890 series II gas chromatograph, as described previously.[21]

^{1}H and ^{13}C NMR spectra were recorded variously on Varian 300, Varian 500, or Bruker 400 MHz spectrometers.

Root cultures were grown as described previously.[14,21] Additions were made as described in the text. All solutions were filter-sterilized directly as they were introduced into the culture flasks.

Alkaloids were extracted by homogenization of freeze-dried tissue with 0.1 N sulfuric acid. Following removal of debris by filtration, the solution was basified (35% ammonium hydroxide solution) and applied to an Extrelute column. Elution with dichloromethane−methanol (95:5) yielded a crude total alkaloid fraction. Alkaloids

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were separated by GC on a 30 m DB-17 capillary column (J&W Scientific) and identified by GC-MS, essentially as described previously.[14,21]

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Supporting Information Available: ^{13}C NMR spectra from extracts of root cultures fed labeled **1**, **2**, and **3** (5 pages). See any current masthead page for ordering and Internet access instructions.

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