NON-STEREOSPECIFIC INCORPORATION OF (1,2-$^{13}C_2$)-ACETATE INTO TROPANE ALKALOIDS IN *HYOSCYAMUS ALBUS*

Ushio SANKAWA,*,[a] Hiroshi NOGUCHI,[a] Takashi HASHIMOTO[b] and Yasuyuki YAMADA[b]

Faculty of Pharmaceutical Sciences, The University of Tokyo,[a] 7-3-1, Hongo, Bynkyo-ku, Tokyo 113, Japan and Research Center for Cell and Tissue Culture, Faculty of Agriculture, Kyoto University,[b] Kitashirakawa, Sakyo-ku, Kyoto 606, Japan

(1,2-$^{13}C_2$) Sodium acetate was incorporated intact into C2 and C3, and C3 and C4 of 6β-hydroxyhyoscyamine in a ratio of 1:1. The result is best explained by assuming that both **R**- and **S**-hygrines are formed from Δ^1-pyrrolinium ions and both of them are converted into tropinone.

KEYWORDS 6β-hydroxyhyoscyamine; hyoscyamine; *Hyoscyamus albus*; biosynthesis; tropane alkaloid; stereospecificity

The biosynthesis of tropane alkaloids has been extensively studied in the last three decades.[1] The intermediates in the pathway leading to hyoscyamine and scopolamine have been clarified by tracer and enzyme studies, yet several problems have been left unsolved. One of the major problems is the condensation reaction between **N**-methyl-Δ^1-pyrrolinium ion and an acetate deriving part (C-2 - C-4 of tropine) to afford a derivative of hygrine. The mechanism proposed earlier was the reaction of **N**-methyl-Δ^1-pyrrolinium ion and acetoacetyl CoA to afford a CoA ester of hygrine-1'-carboxylic acid, a versatile intermediate in the biosynthesis of hyoscyamine and cocaine.[1a] Recently, Leete and Kim proposed another mechanism in which **N**-methyl-Δ^1-pyrrolinium ion reacts successively with two molecules of malonyl CoA instead of acetoacetyl CoA as in polyketide biosynthesis.[2] The new mechanism is based on their observation that the (2-^{13}C, ^{14}C, ^{15}N)-**N**-methyl-Δ^1-pyrrolinium ions labelled C-5 and N of cocaine in *Erythroxylon coca*. If acetoacetyl CoA was involved in the biosynthesis of cocaine, the **N**-methyl-Δ^1-pyrrolinium ions had to react with the methyl but not with the active methylene of the acetoacetyl CoA. It is unknown whether hyoscyamine is formed by the polyketide mechanism or the acetoacetyl CoA mechanism.

Recently we found that the incorporation of acetate into a polyketide is very high, approximately 10% per carbon, in the hairy root cultures of *Cassia torosa*.[3] The root cultures of untransformed *Hyoscyamus albus* which had been used to study the enzymes of tropane alkaloid biosynthesis were expected to have very high metabolic activity.[1a, 4] A feeding experiment with (1,2-$^{13}C_2$)-acetate apparently clarifies the mechanism in question, since the mode of acetate incorporation into C-2 - C-4 would be determined by ^{13}C-^{13}C coupling in ^{13}C-NMR.

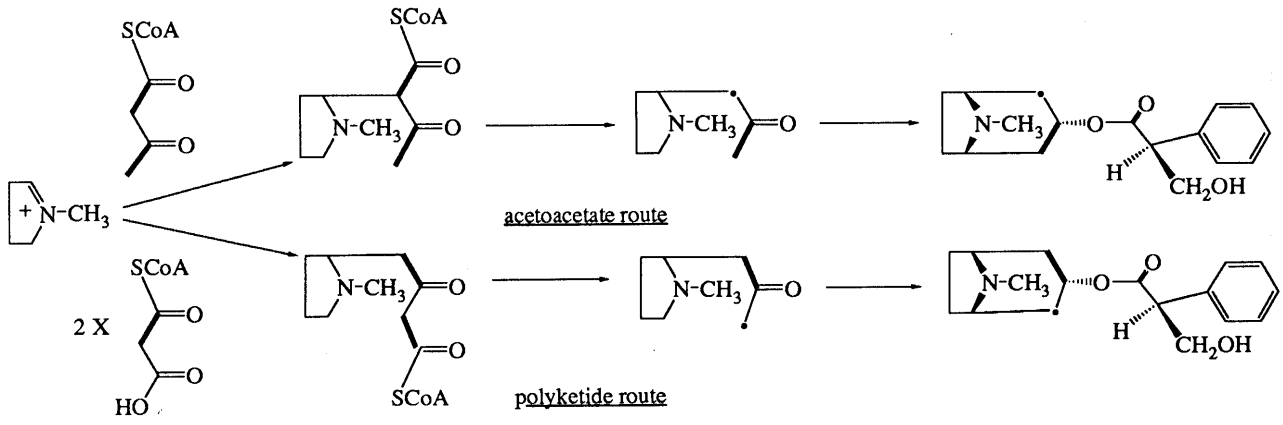

Fig. 1. Two Routes to Hygrine and Hyoscyamine

(1,2-$^{13}C_2$)-Sodium acetate (90 mg 99% enrichment) was administered to the root cultures of *Hyoscyamus albus* three times, on the 4th, 6th and 8th days, during a culture period of 11 days. A mixture of alkaloid hydrochlorides obtained from 53 g fresh weight roots contained hyoscyamine (21.7 mg), 6β-hydroxyhyoscyamine (12.4 mg) and scopolamine (5.84 mg).[4,5] An attempt to detect ^{13}C incorporation directly from the ^{13}C-NMR spectrum of the alkaloid mixture in ^2H_2O solution was unsuccessful, as the spectrum showed a many more peaks than expected. This is due to an equilibrium between the diastereoisomers of different **N**-methyl configurations.[6] 6β-Hydroxyhyoscyamine was isolated along with hyoscyamine to avoid ambiguity arising from the racemization of tropyl residue, since an enantiomer formed by the racemization of 6β-hydroxyhyoscyamine is the enantiomer of 7β-hydroxyhyoscyamine and they are easily separated by HPLC.[7] The labelled alkaloids were separated by HPLC and fractions containing hyoscyamine and 6β-hydroxyhyoscyamine were submitted to the NMR measurements in acidic ^2H_2O solution.[8] The C-3 of hyoscyamine appeared as a quintet-like signal which arose by C-C couplings with both of the adjacent carbons. (Fig. 2a) The intact incorporation of doubly labelled acetate molecule into C-3 and adjacent carbon (C-2 or C-4) caused a C-C coupling to give two satellite signals. Simultaneous labelling of the other adjacent carbon at a significant ratio caused a further C-C coupling in the satellite signals resulting in forming the quintet-like signal, since the values of the two coupling constant were almost identical due to the similarity of the environment of C-2 and C-4. In contrast, as shown in Fig. 2c the C-3 signal of 6β-hydroxyhyoscyamine can be interpreted as two pairs of quintet-like signals arising from the equatorial and axial **N**-methyl isomers. From the intensities of the C-3 signals of hyoscyamine, the incorporation ratio of labelled acetate is calculated to be 8.2 % and the ratio of simultaneous labelling at the other adjacent carbon was ca.18%. These figures are more or less the same in 6β-hydroxyhyoscyamine. C-2 and C-4 signals of 6β-hydroxyhyoscyamine appeared separately as two pairs of triplet-like signals in a ratio of 3:1[9] (Fig. 2d, e). The larger signals are attributable to the equatorial **N**-methyl isomer and the smaller ones to the axial isomer. The signals of C-2 and C-4 of hyoscyamine were not clearly separated and gave a quartet-like signal, which can be regarded as the sum of two triplet-like signals as in 6β-hydroxyhyoscyamine (Fig. 2b). The intensities of the satellite signals of hyoscyamine and 6β-hyoscyamine are approximately 60% of that of the centre signal. This indicate that the labelled acetate molecules were incorporated intact into C-2 and C-3 as well as C-3 and C-4, in a ratio of 1:1.

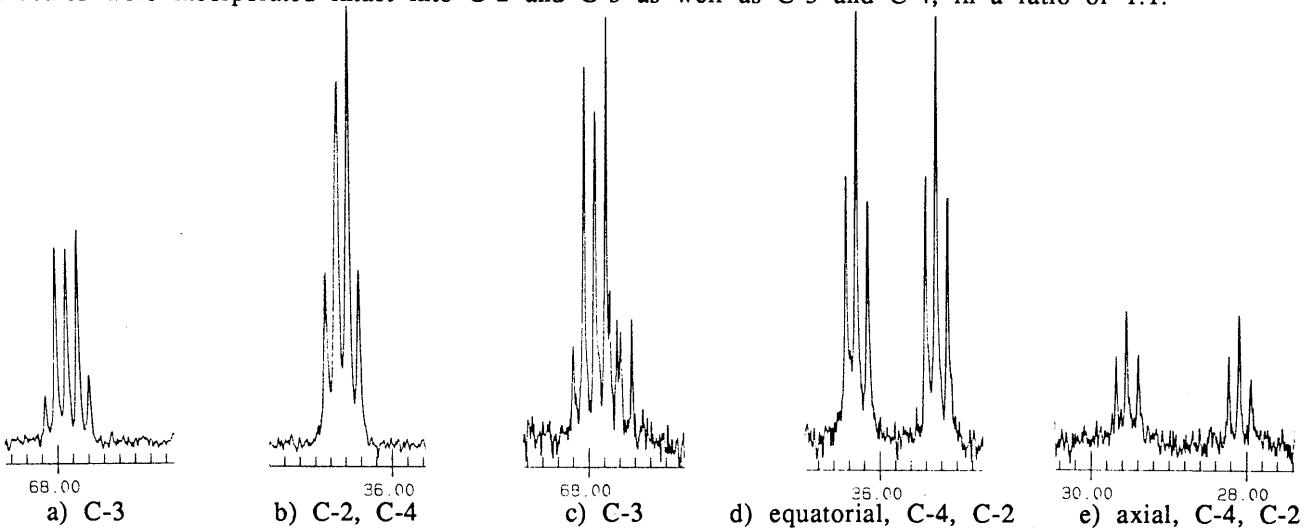

Fig. 2. NMR Spectra of Labelled Hyoscyamine and 6β-Hydroxyhyoscyamine

There are several possible mechanisms to account for the result. In *Datura innoxia* **R**-hygrine incorporated into hyoscyamine much more efficiently than the **S** isomer.[10] From this and other results of incorporation experiments, **R**-hygrine has been regarded as the established intermediate in the biosynthesis of tropane alkaloids in *Datura* species.[1a,b] On the contrary, both **R**-and **S**-hygrines served

as the precursors of tropane alkaloids with an equal efficiency in *Hyoscyamus niger*, *Atropa belladonna* and *Physalis alkekengi*.[11] But this observation was rationalized by a facile racemization of hygrine and the incorporation of S-hygrine was thought to occur via **R**-hygrine formed by racemization.[1a,b] If **R**-hygrine is the sole product formed from **N**-methyl-Δ^1-pyrrolinium ion and the sole substrate for further biosynthetic reactions, the mode of incorporation of acetate is incompatible with the result of our feeding experiment in *Hyoscyamus albus*. On the other hand, if we assume that both **R**- and **S**-hygrines were formed directly from N-methyl-Δ^1-pyrrolinium ion or indirectly by racemization and that both enantiomers were converted into tropinone by dehydration followed by cyclization, the labelling pattern of acetate is compatible with our experimental result.[1c] The labelling pattern of Fig. 3 shows only the case of the acetoacetyl CoA mechanism. The result so far obtained is quite unexpected and inconclusive in regard to the mechanism of hygrine formation and posed another new problem in the biosynthesis of tropane alkaloids.

The biosynthesis of tropane alkaloid in *Hyoscyamus albus* would involve two symmetrical stages. One is the stage of putrescine which is a constitutionally homotopic compound and the other would be that of hygrine in which both enantiomers could serve as intermediates. It became necessary to investigate the incorporation of doubly labelled acetate in *Datura* species and a further incorporation experiment in the hairy root cultures of *Datura innoxia* is in progress.

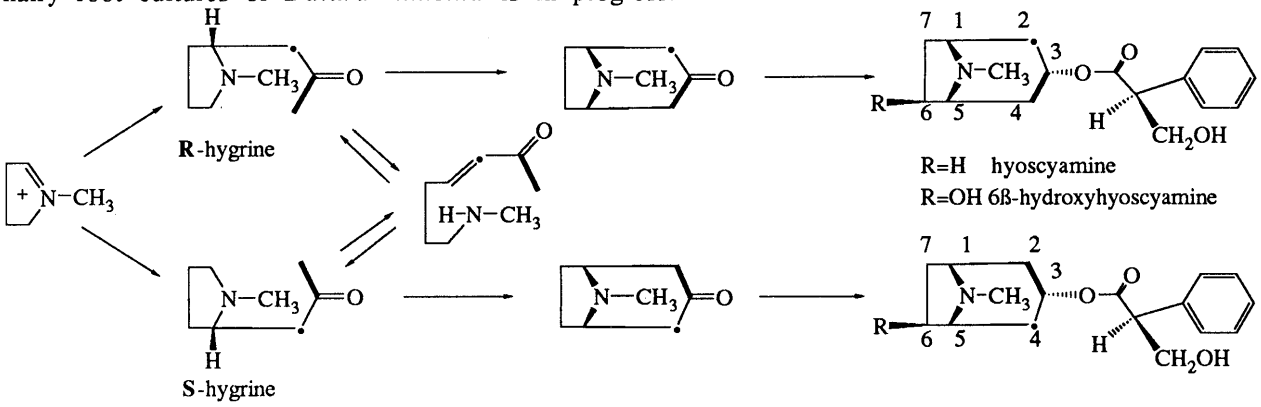

Fig. 3. Mode of Incorporation of Doubly Labelled Acetate into Hyoscyamine and 6ß-Hydroxyhyoscyamine

REFERENCES AND NOTES

1) a) E. Leete, Planta Medica, **36**, 97 (1979); b) E. Leete, Planta Medica, in press; c) M. Lounasamaa, in "The Alkaloids", ed. by A. Brossi, pp 2-81, Academic Press, New York, 1988; d) Y. Yamada, T. Hashimoto, Y. Endo, Y. Yukimune, J. Kohno, N. Hamaguchi and B. Draeger, in "Secondary Products from Plant Tissue culture", ed. by B. V. Charlwood, pp 225-239, Oxford Press, Oxford, 1990. The authors thank Prof. E. Leete for his preprint manuscript.
2) E. Leete and S. H. Kim., J.Am.Chem.Soc., **110**, 2976 (1988).
3) K. S. Ko, Y. Ebizuka, H. Noguchi and U. Sankawa, Chem.Pharm.Bull., **36**, 4217 (1988).
4) T. Hashimoto, Y. Yukimune and Y. Yamada, J.Plant Physiol., **124**, 61 (1986).
5) Tropane alkaloids were quantified with GLC.[4]
6) R. Glaser, Q.-J. Peng and A. S. Perlin, J.Org.Chem., **53**, 2172 (1988).
7) 7β-Hydroxyhyoscyamine was recently isolated from *Datura innoxia* ; K. Ishimaru and K. Shimnomura, Phytochemistry, **28**, 3507 (1989). The authors thank Dr. K. Shimomura for his kind supply of a sample.
8) The alkaloids were separated on a reverse phase HPLC column with a solvent system, 0.05M NaH_2PO_4-H_3PO_4 (pH 2.0):MeOH, 2:1. The residue obtained upon removal of the solvent was dissolved in ^2H_2O and submitted to NMR measurements.
9) The ratio of equatorial and axial isomers of atropine sulphate in ^2H_2O has been reported to be 7:1, while that of scopolamine hydrobromide is 1:18. See reference 6.
10) B. A. McGaw and J. G. Wooley, Phytochemistry, **17**, 257 (1978).
11) B. A. McGaw and J. G. Wooley, Phytochemistry, **18**, 189 (1979).

(Received May 14, 1990)