# The role and source of 5′-deoxyadenosyl radical in a carbon skeleton rearrangement catalyzed by a plant enzyme

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**Abstract The last step in the biosynthesis of tropane alkaloids is the carbon skeleton rearrangement of littorine to hyoscyamine. The reaction is catalyzed by a cell-free extract prepared from cultured hairy roots of *Datura stramonium*. Adenosylmethionine stimulated the rearrangement 10–20-fold and showed saturation kinetics with an apparent $K_m$ of 25 μM. It is proposed that *S*-adenosylmethionine is the source of a 5′-deoxyadenosyl radical which initiates the rearrangement in a similar manner as it does in analogous rearrangements catalyzed by coenzyme $B_{12}$-dependent enzymes. Possible roles of *S*-adenosylmethionine as a radical source in higher plants are discussed.**

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*Key words:* Tropane alkaloid; Carbon skeleton rearrangement; *S*-Adenosylmethionine as 5′-deoxyadenosyl radical donor

## 1. Introduction

The last and most intriguing step in the biosynthesis of the tropane alkaloids hyoscyamine and scopolamine is the carbon skeleton rearrangement depicted in Scheme 1 (for a review see [1]). Due to its similarity to the rearrangement of methylmalonyl CoA to succinyl-CoA it was originally speculated that coenzyme $B_{12}$ is involved in the reaction [2–4]. Since, in spite of some claims [5–7], no trace of vitamin $B_{12}$ has ever been found in plants this idea was abandoned [8,9]. More recently it has been surmised that cytochrome P450 is the agent abstracting the benzylic hydrogen atom and redonating a hydroxyl group after the rearrangement [10,11]. Accordingly, clotrimazole, a known P-450 inhibitor, has been said to inhibit the formation of hyoscyamine [1], and the 3-$H_{Re}$ atom abstracted from the benzylic position was lost during the biosynthetic experiment [12]. When the OH group of littorine was labelled with the isotope $^{18}O$, 25–29% of the label was lost in the product [11]. These results were interpreted as the involvement of two enzymes, a mutase and a dehydrogenase, in the conversion of littorine into hyoscyamine, the geminal diol being an intermediate in the reaction. The dehydratation thereof to the aldehyde could be partially stereospecific explaining the less than 50% loss of $^{18}O$ in the overall reaction. Only a few agents are known in biochemistry that are able to abstract hydrogen atoms from non-activated positions. Beside cytochrome P450, nature uses the 5′-deoxyadenosyl radical for this purpose. In animals and bacteria coenzyme $B_{12}$ is the major source of 5′-deoxyadenosyl radical [13]. More recently, *S*-adenosylmethionine (SAM) was also identified to play a similar role [14]. Therefore, SAM has been called the poor man's $B_{12}$ [15].

Here we report on the involvement of SAM in the rearrangement of littorine to hyoscyamine.

## 2. Materials and methods

### 2.1. Chemicals

They were obtained from commercial sources and were the highest purity normally available. SAM and [2,8,5′-$^3$H]ATP (65% $^3$H in position 5′) were obtained from Amersham, Aylesbury, UK, dithiothreitol (DTT) from Promega, ethylene diamine tetraacetic acid (EDTA) from Serva, B5 medium from Bioproduct (Boehringer Ingelheim).

### 2.2. Preparation of the phenyl-lactoyl tropine (littorine)

According to a modified procedure [16] L-(−)-phenyllactic acid (Fluka) (116 mg, 0.7 mmol) dried over phosphorus pentoxide was added to tropine (Acros) (0.5 mmol) previously dried over sodium hydroxide pellets. The mixture was heated to 145°C on an oil bath and dry hydrogen chloride was bubbled through the solution for 3.5 h. (Note that esterification attempts with *para*-toluene sulfonic acid or under montmorrillonite KSF as alternative procedures gave no satisfactory results.) The cooled mixture was dissolved in a 10 ml $H_2SO_4$ solution (50 mM), filtered and made alkaline with a 10% aqueous ammonium hydroxide solution, then extracted into chloroform (6×10 ml). The solvent was removed under reduced pressure and the residue purified by partition chromatography on Celite (Merck) containing 0.5 M phosphate buffer (pH 6.8) eluted with chloroform. GC-MS analysis of the combined fractions (97 mg, yield: 48%) indicated almost pure (98%) littorine.

Retention time (1,4-dioxane) on a Optima 1-02 capillary column (25 m×0.2 mm), ramp 100–300°C at 20°C/min): 12.2 min. MS *m/z* (rel. int.): 361(4), 193(3), 140(4), 125(12), 124(100), 123(1), 96(9), 95(5), 94(12), 91(8), 83(15), 73(19) [17].

$^{13}C$ NMR and $^1H$ NMR spectra were recorded on a Bruker DRX 500 spectrometer. Deuteriochloroform was used as the solvent with TMS as an internal reference for $^1H$. The abbreviations used are as follows: δ, shift; s, singlet; d, doublet; t, triplet; m, multiplet; b, broad.

$^1H$ NMR ($CDCl_3$) δ: 1.65 (1H, $H_6$ or $H_7$, d, $J^3_{HH}$ = 15.8 Hz); 1.85 (1H, $H_7$ or $H_6$, d, $J^3_{HH}$ = 15.8 Hz); 1.94–2.20 (2H, $H_6$ or $H_7$, m); 2.00 (N-$CH_3$, s); 2.54–2.72 (4H, $H_4$ and $H_2$, m); 2.78 (1H, $H_5$ or $H_1$, t, $J^3_{HH}$ = 15.8 Hz); 2.80 (1H, $H_1$ or $H_5$, t, $J^3_{HH}$ = 15.8 Hz); 2.98 (2H, $H_{3'}$, d, $J^3_{HH}$ = 6.5 Hz); 3.57 (b, OH); 4.37 (1H, $H_{2'}$, t, $J^3_{HH}$ = 6.5 Hz); 4.99 (1H, $H_3$, t, $J^3_{HH}$ = 4.5 Hz); 7.11–7.22 (5$H_{arom}$, m) [18]. $^{13}C$ NMR ($CDCl_3$) δ: 25.1, 25.3 ($C_7$ and $C_6$); 35.1, 35.2 ($C_4$ and $C_2$); 37.3 ($C_{3'}$); 40.7 ($CH_3$-N); 60.0 ($C_1$ and $C_5$); 71.6 ($C_3$); 74.3 ($C_{2'}$); 126.9 ($C_{7'}$); 128.5, 129.3 ($C_{5',6',8',9'}$); 136.4 ($C_{4'}$); 173.5 ($C_{1'}$).

### 2.3. Preparation of the SAM synthetase

SAM synthetase was purified by the procedure of Markham et al. [19], with some minor modifications: growth in the presence of oxytetracycline (30 μg/ml) and disruption of the cells by sonication (Bandelin Nonopuls HD 2200). The specific activity of the enzyme was 150 000 pmol/min/mg.

### 2.4. Preparation of the S-[2,8,5′-$^3$H]adenosylmethionine

*S*-[2,8,5′-$^3$H]Adenosylmethionine was enzymatically synthesized

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*Abbreviations:* SAM, *S*-adenosylmethionine

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from [2,8,5′-$^3$H]ATP (specific activity: 41 mCi/mmol) and unlabelled methionine, using a SAM synthetase preparation.

The reaction mixture contained 200 μl of 0.1 M diluted solution of [2,8,5′-$^3$H]ATP (specific activity: 424 700 cpm/nmol) in 3 ml Tris-HCl buffer (pH 7.4), adjusted to pH 7,6 by addition of a 2 M KOH solution, 1.5 eq. $MgCl_2$, 5 eq. KCl, 1.2 eq. L-methionine added as solids, 100 μl reduced glutathione (25 mg/ml) and 11 mg of SAM synthetase were incubated for 45 min at 35°C. The reaction was terminated by addition of 40 μl of trichloroacetic acid (6 M). The resulting suspension was centrifuged at 4°C and the extracts pooled and stored at 4°C. After lyophilization, the residue was dissolved in doubly distilled water and applied to a carboxymethyl sepharose high flow column (0.8×20 cm FPLC column, Pharmacia Biotech, Freiburg, Germany) previously equilibrated with water. The column was washed with 2 volumes of water and then *S*-[2,8,5′-$^3$H]adenosylmethionine was eluted with 1 M NaCl gradient (4 ml/min). Fractions containing *S*-[2,8,5′-$^3$H]adenosylmethionine (specific activity: 123 000 cpm/nmol) were mixed, lyophilized and stored at −20°C until used.

*2.5. Plant material*

Roots from *Datura stramonium* D15/5 were a gift from Dr. N.J. Walton (Institute of Food Research, Norwich, UK). Roots cultures were initiated by wounding surface sterilized explants of leaves on the midrib with an overnight suspension of *Agrobacterium rhizogenes*. Tissue bearing emergent roots were excised and placed into 8 ml of Gamborg's B5 medium supplemented by 500 μg/ml ampicillin. Roots for enzyme extraction and purification were grown at 25°C, under stirring at 90 rpm. Rapidly growing roots were subcultured every 2 weeks into 50 ml of the same medium in a 250 ml Erlenmeyer flask. After eight subcultures it should be possible to omit ampicillin from the medium. After this time, roots were harvested by filtration in vacuo, washed twice with water and frozen immediately at −70°C.

*2.6. Protein extraction*

All procedures were done at 4°C. Roots (6 g) were crushed in a mortar with sea sand, mixed with 15 ml of extraction buffer (100 mM $K_2HPO_4$ pH 8, 3 mM DTT, 5 mM $Na_2$ EDTA and 250 mM sucrose) and then ground during 30 min until a homogeneous suspension was obtained. The homogenate was clarified by passage through Miracloth (Calbiochem, Novabiochem, Frankfurt a/M, Germany) and centrifugation (30 000×*g*, 4°C, 30 min). Low molecular weight compounds were removed using a PD-10 desalting column (Pharmacia Biotech AB, Uppsala, Sweden) equilibrated in a one tenth strength extraction buffer. The proteins were eluted with 3.5 ml of the same buffer. The yellow solution obtained was concentrated to 2–4 mg/ml with centricon 5000 Da (Pharmacia Biotech AB, Uppsala, Sweden).

*2.7. Enzyme assay*

The activity of the enzyme was determined by measuring the conversion of littorine into hyoscyamine by the GC-MS method. The standard reaction mixture, in a volume of 200 μl, contained 200 μM SAM (unlabelled or labelled as prepared above), 100 μl of the protein solution (200–400 μg), 2 mM of littorine, adjusted to 200 μl with the extraction buffer. After 40 min incubation at 33°C, the reaction was terminated by addition of 100 μl 30% $NH_4OH$. Control experiments lacking substrate were routinely included. Reaction mixtures were loaded on an Extrelut-1 column (Merck Art 1.13076, Darmstadt, Germany). After 15 min the alkaloids were eluted with 13 ml of $CHCl_3$. The chloroform fractions were evaporated to dryness at 35°C, dissolved in 200 μl of a fresh solution of 1,4 dioxane/*N*,*O*-bis-(trimethylsilyl)-acetamide (4:1 v/v), analyzed by GC-MS (experiments with unlabelled SAM) and HPLC.

*2.8. Assay of protein*

Protein was assayed by the method of Smith et al. [20] using commercially prepared reagent (bicinchoninic acid) and bovine serum albumin as standard.

*2.9. Extraction and analysis of alkaloids*

Harvested roots were freeze-dried, then powdered and soaked overnight in an EtOH-28% $NH_4OH$ (19:1) mixture. This macerated material was centrifuged for 5 min at 1300×g. Extraction with the basic alcohol was repeated twice and the combined alcohol fractions were evaporated to dryness at 35°C. The dry residue was dissolved in 2 ml of 0.1 N HCl and the acidic aqueous solution was filtered through filter No. 2. One milliliter of the filtrate was made alkaline with 2 ml of 1 M $Na_2CO_3$-$NaHCO_3$ buffer (pH 10) and 1 ml of this alkaline solution was loaded onto a Extrelut-1 column. After 10 min, 6 ml of $CHCl_3$ was passed through the column and the chloroform fractions were evaporated to dryness at 35°C. The dry residue was dissolved in a mixture of 1,4 dioxane/*N*,*O*-bis-(trimethylsilyl)-acetamide (v/v 4:1). Alkaloids were measured with a gas chromatograph coupled with a mass spectrometer model Hewlett Packard 5890 Serie II using a capillary column Optima 1-02 μm (25 m×0.2 mm). The column temperature was 350°C, the carrier gas was $H_2$ at a flow rate of 20 ml/min. The detector FID were used.

The two alkaloids identified for this study were littorine and hyoscyamine [17].

Littorine RT (retention time): 12.2 min. MS *m*/*z* (rel. int.): 361(4), 193(3), 140(4), 125(12), 124(100), 123(1), 96(9), 95(5), 94(12), 91(8), 83(15), 73(19).

Hyoscyamine RT: 12.1 min. MS *m*/*z* (rel. int.): 361(5), 193(0.5), 140(7), 125(10), 124(100), 123(6), 104(6), 96(10), 95(6), 94(13), 83(20), 82(18), 73(15).

*2.10. Reverse phase HPLC chromatography*

An Incapharm 100 RP-18 TS (250×4.6 mm) column was equilibrated at room temperature with 0.1% trifluoroacetic acid in water solution. 5 min after sample injection (20 μl in same buffer), a gradient of 0–60% solvent B (0.1% trifluoroacetic acid in acetonitrile) in 40 min was developed by a Hewlett-Packard 1050 T system at a flow rate of 1.2 ml/min. The eluted substances were monitored at 215 and 254 nm. Labelled products were analyzed by liquid scintillation counting (Tri-carb 2100TR, Packard, a Canberra company, Meriben, USA) after adding 15 ml of scintillation liquid (Lumasafe plus–Lumac LSC) to the peak fractions.

*2.11. Determination of the isotope exchange*

Tritium exchange with water was measured by liquid scintillation counting (Tri-carb 2100 TR) after loading 10 ml of scintillation liquid to 10 μl of water resulting from bulb-to-bulb distillations. The difference in emission was measured between *S*-[2,8,5′-$^3$H]adenosylmethionine solution (specific activity: 123 000 cpm/nmol) and the resulting distilled water and between aqueous phase (0.1% trifluoroacetic acid in doubly distilled water) before HPLC analysis and the resulting distilled water.

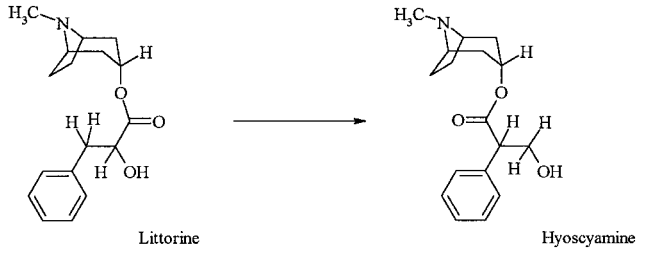
## 3. Results

### 3.1. Dependence of the rearrangement littorine/hyoscyamine on SAM

The experiments were carried out as described in Section 2.7 and are listed in Table 1. The silylated products were detected by GLC coupled with MS. Omission of SAM from the assay mixture resulted in only 3.5% hyoscyamine while 96.5% remained littorine. Addition of SAM (200 μM) increased the conversion leading to 61% hyoscyamine. When the hairy root extract was kept frozen at −20°C for a week

Table 1
Dependence of the rearrangement of tropane alkaloids on SAM

| Hairy roots | SAM (μM) | % GC-MS hyoscyamine | % GC-MS littorine |
|---|---|---|---|
| Fresh extract | 0 | 3.5 | 96.5 |
| Fresh extract | 200 | **61** | 39 |
| One freeze-thawing | 0 | 2.3 | 97.7 |
| One freeze-thawing | 200 | **28** | 72 |




Scheme 1. Carbon skeleton rearrangement of tropane alkaloids.

the conversion under the same conditions without and with SAM decreased to 2.3 and 28% hyoscyamine, respectively. After 4 weeks of freezing little if any mutase activity of the preparation could be detected.

### 3.2. Determination of the Michaelis constant for SAM

As the enzyme source we used a hairy root extract, thawed after 1 week in the deep-freeze. The kinetic experiments were carried out under conditions of the enzyme assay (see Section 2.7), except that the concentration of SAM was varied between 50 and 500 μM. The $K_m$ and $V_{max}$ values were determined using Lineweaver-Burk plot (Fig. 1).

The following values were found: $K_m = 25 \pm 5$ μM, $V_{max} =$ 29 μmol/40 min.

### 3.3. Conducting the rearrangement in the presence of S-[2,8,5′-$^3$H]adenosylmethionine

When the enzymic rearrangement of littorine to hyoscyamine was carried out in the presence of [2,8,5′-$^3$H]SAM (see Section 2.4 for preparation), no incorporation of tritium into the alkaloids was observed. However, about 2% of the tritium (calculated for the 5′ position) was washed out into the medium. Only a trace of tritium was found in the solvent in a control experiment without hairy root extract. HPLC showed no appreciable amounts of 5′-deoxyadenosine after the reaction. If the latter was an intermediate, then only in enzyme-bound catalytic amounts. If the putative 5′-deoxyadenosyl radical abstracted the benzylic *pro-R* H-atom, then this process must have been irreversible. Possible reasons for the loss of tritium from the 5′ position of SAM are given below.

## 4. Discussion

The 10–20-fold enhancement of the carbon skeleton rearrangement, littorine to hyoscyamine (Scheme 1) by SAM in cell-free extracts of a root culture of *Datura stramonium* suggests a new role for this cofactor in plant biochemistry. The well-known function of SAM as methyl donor is operative in both prokaryotic and eukaryotic organisms and requires a heterolytic cleavage of a carbon sulfur bond. More recently, Frey and coworkers discovered and documented [14,15] a case in which SAM is the source of a 5′-deoxyadenosyl radical, the homolytic cleavage of the carbon sulfur bond being promoted reductively by an $Fe_4S_4$ cluster-containing enzyme.

We surmise a similar role for SAM in the rearrangement of littorine to hyoscyamine. Indeed, addition of SAM to the assay mixture not only increased the conversion of littorine to hyoscyamine, but showed saturation kinetics with a $K_m$ of 25 μM (Fig. 1).

The low activity of the hairy root extracts without addition of SAM may be due to trace amounts of endogenous SAM that remained in the extract after the PD-10 desalting column. Indeed, in previous studies no SAM was added to the cell-free system, but much more extract and longer incubation times were used. Moreover, most of the incorporation experiments were carried out with growing root cultures and the incubation times were 10–28 days. In these cases endogenous SAM must have been provided by the in vivo system.

Preliminary experiments using [2,8,5′-$^3$H]SAM showed no incorporation of tritium into littorine or hyoscyamine but some loss of the radioactivity to water. This is in agreement with the observed loss of the benzylic *pro-R* H-atom of littorine during the rearrangement [12]. Since all work has been done with root cultures or crude cell-free extracts it is possible that the migrated H-atom is washed out by redox enzymes. This could also explain the 25–29% loss of $^{18}$O of littorine during the rearrangement [11] since the intermediate aldehyde could be in equilibrium with its hydrate.

The steric course of the migration at the relevant centers is also of importance. After contradictory results [2,3,21] it has been established that the substitution occurs with inversion at both migration centers [12]. The same steric course has been determined for the lysine 2,3-aminomutase reaction [22]. In the latter, however, the migrating hydrogen atom is not lost

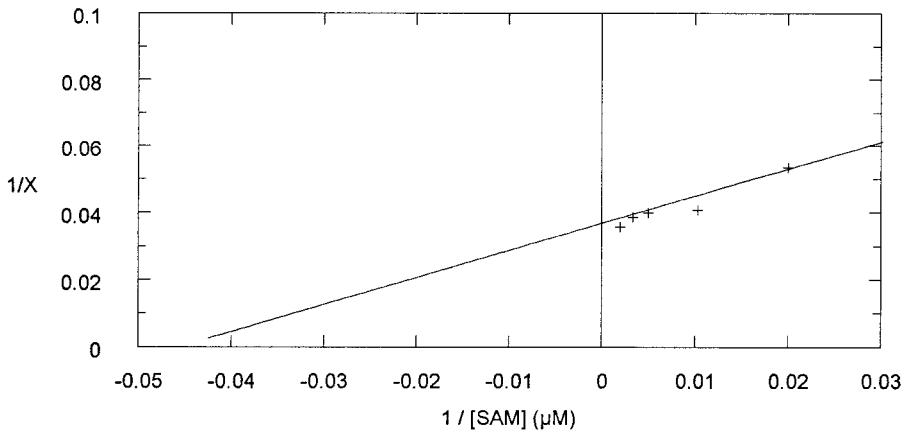



Fig. 1. Lineweaver-Burk determination of $K_m$ and $V_{max}$. X = % GC-MS hyoscyamine.

to the medium. This discrepancy can only be clarified when pure littorine mutase is available. Another plant enzyme that catalyzes the rearrangement of α-phenylalanine to β-phenylalanine is the phenylalanine aminomutase. This reaction is part of taxol biosynthesis. Recently it has been established that the substitution occurs with retention of configuration at both migration centers [23]. This fact does not rule out the possibility that the same cofactor, namely SAM, is operative also in the phenylalanine aminomutase reaction. Precedence for opposite steric courses is found in the coenzyme $B_{12}$-dependent carbon skeleton rearrangements. Methylmalonyl-CoA mutase operates with retention [24,25], methylene glutarate mutase [26] and glutamate mutases [27] with inversion of configuration. Even the same enzyme, the coenzyme $B_{12}$-dependent ethanolamine ammonia-lyase, operates with opposite steric courses, inversion and retention with (2*R*)- and (2*S*)-propanolamines, respectively [28]. We thus propose that SAM may be the cofactor in the $B_{12}$-like rearrangements occurring in plants and the reaction is initiated by the 5′-deoxyadenosyl radical derived from SAM.

That plants use SAM as the source of 5′-deoxyadenosyl radical has been established for the last step of biotin biosynthesis in *Arabidopsis thaliana* [29]. A similar mechanism operates in *Bacillus sphaericus* and *Escherichia coli* [30,31]. We thus believe that while bacteria are able to use both coenzyme $B_{12}$ and SAM as sources of 5′-deoxyadenosyl radical, animals and humans inherited from them the former and higher plants the latter.

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