# Biosynthesis of L-Azetidine -2-Carboxylic Acid in *Actinoplanes ferrugineus*

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L-Azetidine-2-carboxylic acid(A-2-C) is a four-membered cyclic imino acid which was first discovered from *Convalaria majalis* and *Polygonatum officinalis* in 1955 (1, 2). The imino acid A-2-C has been identified in at least 16 species of plants (3) (mostly the families Liliaceae, Agavaceae and Amaryllidaceae); in two marine sponges (*Haliclona sp.* and *Chalinospilla sp.*) (4) ; in the red algae (*Lophocladia lamenandi*) (5) ; in the sugar beet *Beta vulgaris* (6) ; and the microorganism *Actinoplanes ferrugineus* (7).

Since its discovery, the biosynthetic pathway of A-2-C has been investigated in the plants, especially, *C. majalis.* In the plant system, methionine was incorporated into A-2-C (8, 9, 10). However, in the legume, *Delonix regia,* 2, 4-diaminobutyric acid and homoserine were suggested to be possible precursors for the biosynthesis of A-2-C (11). Based on these feeding experiment results, several biosynthetic pathways were postulated such as direct cyclization of S-adenosylmethionine derived from methionine, reductive pathway of dehydroazetidine-2-carboxylic acid derived from 4-amino-2-keto-butyric acid or aspartic-$\beta$-semialdehyde (10).

In *Actinoplanes ferrugineus,* methionine and S-adenosylmethionine were incorporated into A-2-C molecule (12). The biosynthetic pathway and mechanism will be discussed, based on our experimental results.

## A-2-C Biosynthetic Activity (12)

After crushing the cells of *A. ferrugineus* and centrifugation. A-2-C biosynthetic activ-ity was found in the pellet, not in the supernatant solution. The activity in the pellet was sensitive to heat treatment, ultrasonic treatment and ionic detergents(i.e., deoxycholic aicd, alkyl ammonium bromide, etc.). But, the activity in the pellet was retained even after the treatment of nonionic detergents(i.e., Triton X 100) or alkali.

The activity was lost in the treatment of carbonyl group deactivating reagents(i.e., NaCN, $NH_2OH$, $NaBH_4$) (13) and nucleophilic group trapping reagents(i.e., maleimide and N-ethylmaleimide) (14).

On standing at 4℃, the A-2-C biosynthetic activity in the pellet showed the difference in ability to use methionine or S-adenosylmethionine as a substrate. The result is shown in Fig. 1.

These *in vitro* experimental results suggested that the activity resided in the membrane fraction. The biosynthetic activity may contain a carbonyl group cofactor such as pyridoxal phosphate and nucleophilic groups(e. q., $-NH_2$, $-SH$, or imidazole group) which might be essential in the biosynthesis of A-2-C. The substrate specificity indicated that S-adenosylmethionine might be much closer to the product A-2-C than methionine.

## The Biosynthetic Mechanism of A-2-C

Based on the biosynthetic activity *in vitro,* we might postulate a biosynthetic mechanism which contains $\alpha$ -, $\beta$ -ptoton participation during the biosynthesis of A-2-C from substrate S -adenosyl methionine.

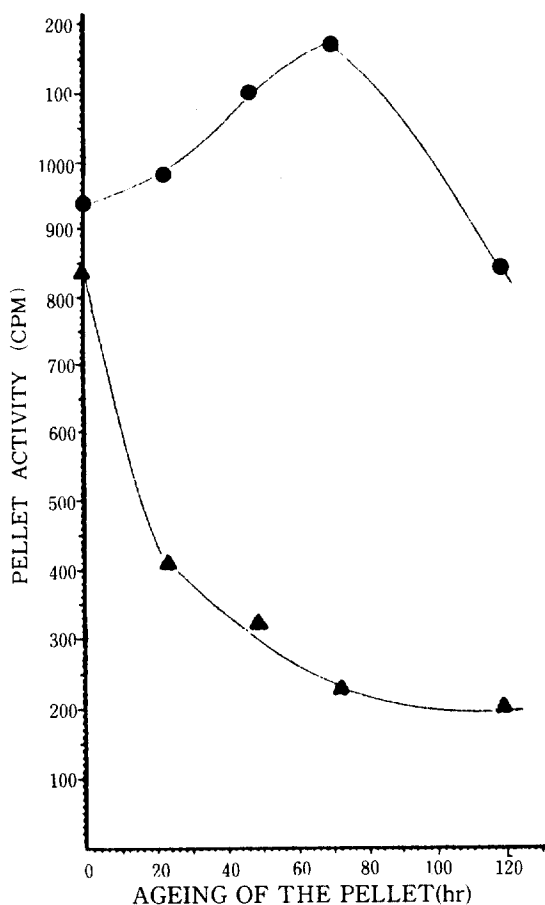To prove the mechanism, $^2$H-labelled meth-

were incorporated into A-2-C molecule, which indicates that the carbon skeleton of methionine molecule is used for A-2-C formation intactly. The proton integrations suggested some loss of deuterium at $\beta$-position of the substrate.

The $\alpha$-proton participation was confirmed through the feeding experiments of 2, 3, 3-[$^2$H$_3$]methionine in $H_2O$ medium or S-adenosylmethionine in $D_2O$ medium. Almost all of $\alpha$-$^2$H of 2, 3, 3-[$^2$H$_3$]methionine was devoid in the resulting A-2-C. Reverselv, the A-2-C obtained from the incubation mixture of S-adenosylmethionine in $D_2O$ medium gave rise to little proton integration at $\alpha$-proton signal, which suggests that $\alpha$-proton of the substrate was exchanged with solvent deuterium. A preferential deuterium loss or incorporation at the most upfield signal in deuterium NMR spectra was observed in the two A-2-C's.

Using the substrate of which stereochemistry in known, it is possible to analyze the stereochemical events during the biosynthesis, if the stereochemistry of product can be also analyzed. The stereochemistry of A-2-C can be assigned based on the known proton assignment in a proton NMR of A-2-C(12). Therefore, the substrates of which stereochemistry is known(i.e., 4$S$-, 4$R$-, 3$S$- and 3$R$-methionine) were fed and the resulting A-2-C's were isolated. The $^1$H- and $^2$H-NMR spectra of these A-2-C's suggested that the reaction at the $\beta$-carbon center was~80% stereospecific with overall inversion mode. The reaction at the $\gamma$-carbon center was~70% stereospecific with overall inversion mode.

From the data discussed above, we might postulate the biosynthetic mechanism of A-2-C in *A. ferrugineus* as follows.

1. The abstration of the $\alpha$-hydrogen atom of S-adenosyl-L-methionine seems to be essential to A-2-C biosynthesis. This possibility is supported by the observation that $\alpha$-methyl-S-adenosyl-D, L-methionine inhibited the A-2-C biosynthesis and did not result in the



**Fig.1.** Time Dependence of the A-2-C Biosynthetic Activity of the Pellet to the Substrates: S-Adenosyl-L-methionine(●—●) and Methionine(▲—▲).

ionines in $H_2O$ solvent or S-adenosylmethionine in $D_2O$ medium were added to the cells of *A. ferrugineus* and incubated. The resulting product A-2-C's were isolated and analyzed by $^1$H- or $^2$H-NMR spectroscopy. The typical examples of various feeding experiments are shown in Fig.2 and Fig.3. The deuterium content at each proton signal could be obtained by comparing proton integrations in a $^1$H-NMR spectrum. The A-2-C obtained from 3, 3, 4, 4-[$^2$H$_4$]methionine suggested a couple of points about the reaction mechanism of A-2-C biosynthesis. First, the four $^2$H of methionine

corresponding cyclized product 2-methyl-A -2-C via a direct displacement reaction of the $\gamma$ -leaving group by the amino group of $\alpha$-methyl -S-adenosylmethionine(12). In the plant system *C. majalis,* the A-2-C derived from 2-[³ H₁]-methionine was devoid of ³H(10), which also suggest $\alpha$-proton participation-exchange during A-2-C biosynthesis.

2. At the $\beta$-position of the substrate, deprotonation and reprotonation occurs. During the biosynthesis of A-2-C, the site of deprotonation seems to be pro $S$ proton at $\beta$
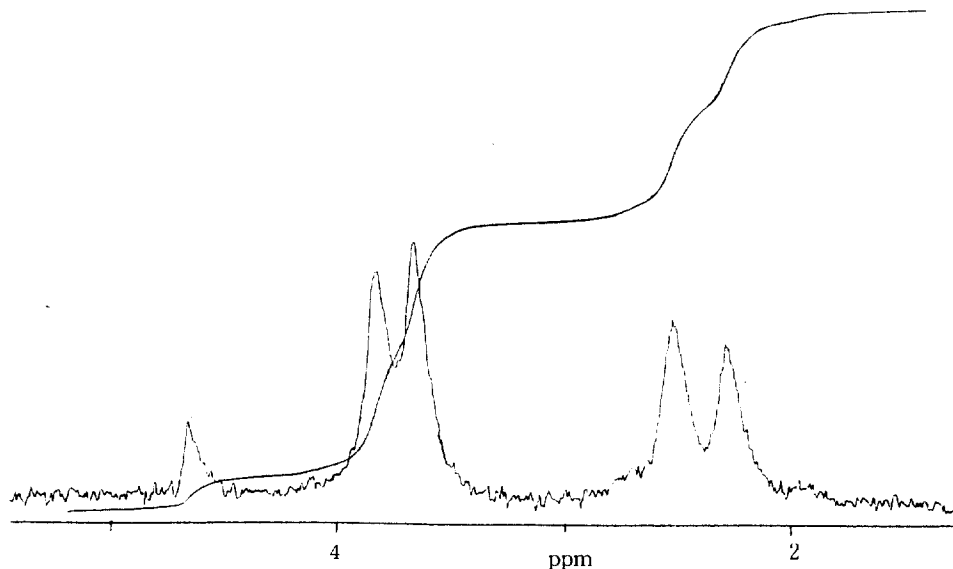


**Fig.2.** The ²H-NMR Spectrum of the A-2-C Obtained from D,L-3, 3, 4, 4-[²H₄]Methionine Feeding Experiment.
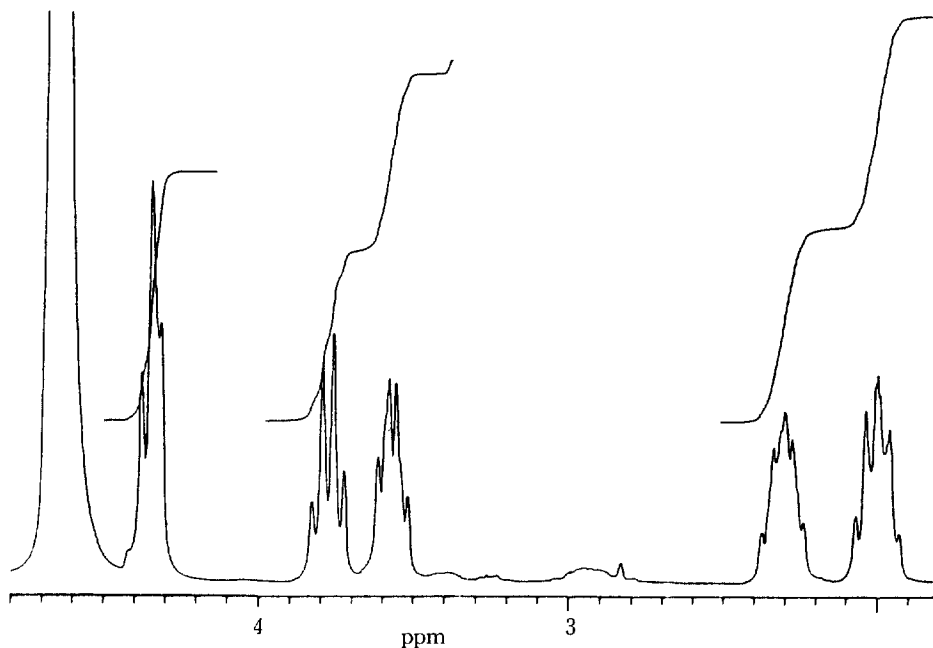


**Fig.3.** The ¹H-NMR Spectrum of the A-2-C Obtained from D,L-3, 3, 4, 4-[²H₄]Methionine in the Presence of Eu(III).

-carbon of substrates. The reprotonation occurs at the same site, which results in $3R$-A-2-C from unlabelled substrate in $D_2O$ medium.

3. Therefore, the biosynthetic mechanism might be illustrated in terms of $\alpha$-deprotonation, $\beta$, $\gamma$-elimination and cyclization. The stereochemical data suggest that $\beta$, $\gamma$-elimination consists of the cisoid mode because the deprotonation site(pro $S$) on the $\beta$-carbon of the substrate is cis to the leaving group(thioether) on the $\gamma$-carbon of the substrate. However, the ring formation reaction consists of the transoid mode, because the $NH_2$-attacking site on the $\gamma$-carbon is trans to the reprotonation site(pro-$R$) on the $\beta$-carbon of the product.

4. The A-2-C synthetic enzyme has to have at least two active site basic groups involved in the proton transfer from substrates to intermediates and nascent products. One basic group is needed for deprotonation and reprotonation of the $\alpha$-carbon. Another basic group abstracts the pro-$S$ proton of the $\beta$-carbon, trans to $\alpha$-proton, of the substrate and later returns it to the vinylglycine intermediate during the ring formation.

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