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A Unique Pathway for the 3-Aminobutyrate Starter Unit from L-Glutamate through β-Glutamate during Biosynthesis of the 24-Membered Macrolactam Antibiotic, Incednine

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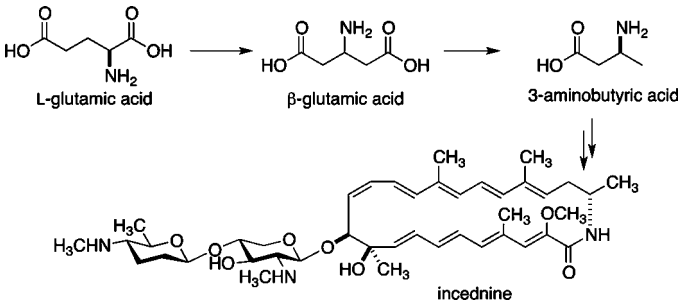
ABSTRACT



Incednine is a 24-membered macrolactam antibiotic produced by *Streptomyces* sp. ML694-90F3. A previous study demonstrated that its unique nitrogen-containing starter unit was derived from L-glutamate. To elucidate the missing link between L-glutamate and the starter unit, deuterium labeled amino acid feeding experiments were conducted. These experiments revealed that 3-[3-^{2}H]aminobutyrate and β-[2,2,4,4-$^2H_4$]glutamate were incorporated into the starter moiety. The results indicate that a novel decarboxylation of β-glutamate to give 3-aminobutyrate is involved in incednine biosynthesis.

Incednine is a 24-membered macrolactam antibiotic isolated from *Streptomyces* sp. ML694-90F3 as an inhibitor of antiapoptotic function in Bcl-2/Bcl-xL oncoproteins (Figure 1).[1] Our previous study on incednine biosynthesis revealed that its 24-membered macrolactam aglycon moiety was biosynthesized through the polyketide pathway using a unique amino acid starter unit, possibly a 3-aminobutyrate equivalent that was derived from L-glutamic acid.[2] In the present study, to elucidate the missing link between L-glutamate and the unique amino acid starter unit, several additional deuterium amino acid feeding experiments were conducted.

First, to determine whether the free form of 3-aminobutyric acid was a direct starter unit of incednine, 3-[3-^{2}H]-aminobutyric acid was prepared from ethyl 3-aminocrotonate by reduction with $NaBD(OAc)_3$, followed by acid hydrolysis to yield 3-[3-^{2}H]aminobutyric acid in its hydrochloride form (see Figure S1).[3,4] The synthesized deuterated 3-aminobutyrate was added to the *Streptomyces* sp. ML694-90F3 culture, and the incednine produced was purified (see Supporting Information) and analyzed by NMR spectroscopy. The ^{2}H NMR spectrum of the incednine obtained showed the specific incorporation of deuterium into the H-23 position (Figure 2). Furthermore, ^{1}H NMR analysis showed that the H-23 signal disappeared and no coupling between H-23 and

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28-CH_3 was observed, indicating a much higher level of incorporation of the deuterated 3-aminobutyrate than its naturally occurring nonlabeled counterpart in the producer strain. This result implies that one of the carboxyl groups in L-glutamic acid is decarboxylated before 3-aminobutyrate formation. Although the incednine biosynthetic gene cluster is not available and the enzymes are not functionally characterized, this mechanism is distinct from the biosynthetic pathway of the similar macrolactam vicenistatin, in which decarboxylation of the free form of 3-methylaspartate derived from L-glutamate by glutamate mutase does not occur.[5] At the start of vicenistatin biosynthesis, 3-methylaspartate is transferred onto an ACP by the ATP-dependent ligase VinN, and the 3-methylaspartyl-ACP formed is then decarboxylated by the PLP-dependent decarboxylase VinO to form 3-aminoisobutyryl-ACP.

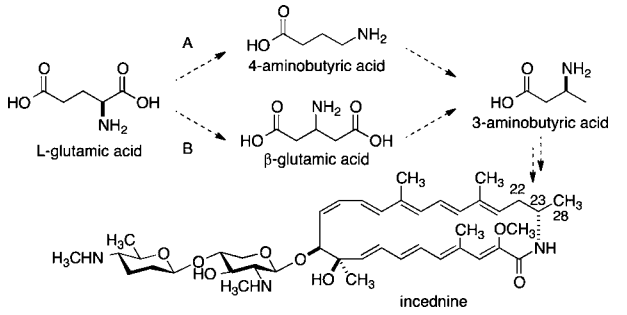

Figure 1. Proposed start of the biosynthetic pathway for incednine biosynthesis.

To identify the link between L-glutamate and 3-aminobutyrate during incednine biosynthesis, two possible decarboxylation and amino group rearrangements were hypothesized, as shown in Figure 1. In pathway A, L-glutamate is first decarboxylated to 4-aminobutyrate by an usual α-amino acid decarboxylase, followed by rearrangement of the amino group to form 3-aminobutyrate, presumably by an aminomutase type enzyme such as lysine-2,3-aminomutase.[6] Another possibility is that rearrangement of the L-glutamate amino group might occur first mediated by a glutamate-2, 3-aminomutase type enzyme to form β-glutamate,[7] which is then converted to 3-aminobutyrate by a β-amino acid decarboxylase such as aspartate 4-decarboxylase (pathway B).[8] To distinguish which of these two pathways was used, deuterium labeled 4-aminobutyrate and β-glutamate were prepared (Figure S1).

4-[4,4-$^2H_2$]Aminobutyrate was synthesized according to the method in literature.[9] In the case of deuterated β-glutamate, reductive amination of dimethyl acetone-1,3-dicarboxylate was carried out using NH_4HCO_3 in CH_3OD, followed by reduction with $NaBH_4$ in CH_3CO_2D to yield dimethyl β-[2,2,4,4-$^2H_4$]glutamate. A subsequent transformation, following methods described elsewhere,[11] gave β-[2,2,4,4-$^2H_4$]glutamic acid in its hydrochloride form.

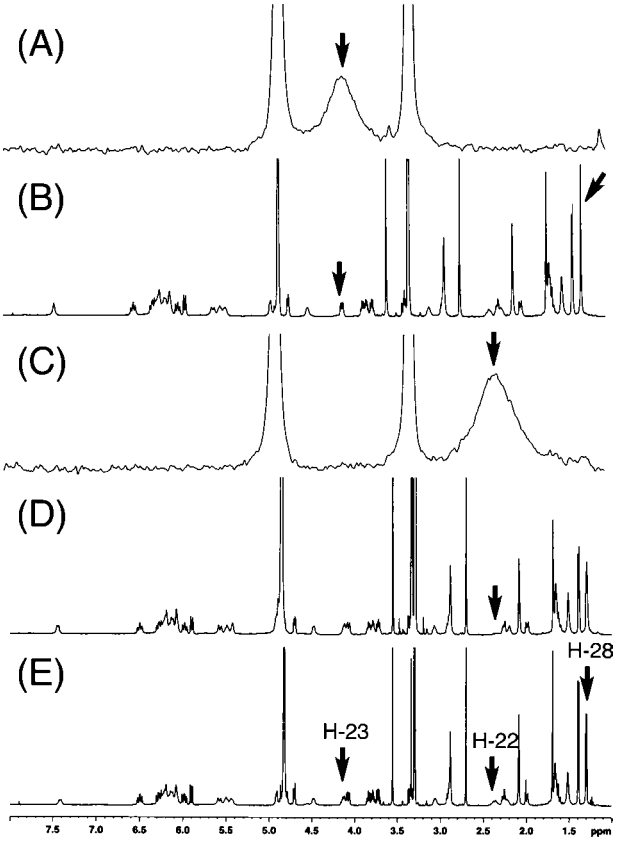

Figure 2. ^2H and ^1H NMR spectra of incednine obtained from feeding experiments. (A) ^2H NMR spectrum (CH_3OH, 76.5 MHz) of labeled incednine with β-[2,2,4,4-$^2H_4$]glutamate, (B) ^1H NMR spectrum (CD_3OD, 500 MHz) of labeled incednine with β-[2,2,4,4-$^2H_4$]glutamate, (C) ^2H NMR spectrum (CH_3OH, 76.5 MHz) of labeled incednine with 3-[3-^2H]aminobutyrate, (D) ^1H NMR spectrum (CD_3OD, 500 MHz) of labeled incednine with 3-[3-^2H]aminobutyrate, (E) ^1H NMR spectrum (CD_3OD, 500 MHz) of nonlabeled incednine.

Feeding experiments using deuterated amino acids were conducted as described previously. Although deuterated 4-aminobutyrate was not incorporated at all (data not shown), deuterated β-glutamate was strongly incorporated into the incednine starter unit. ^1H NMR analysis of the incednine obtained from the deuterated β-glutamate feeding experiment showed that the 22-H signal had disappeared,

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indicating much higher level of incorporation of deuterated β-glutamate compared to the naturally occurring nonlabeled one. Furthermore, incorporation of β-[2,2,4,4-$^2H_4$]glutamate deuterium atoms was clearly detected at the C-22 position, but not at the C-28 position by ^2H NMR analysis (Figure 2B). Therefore, the β-[2,2,4,4-$^2H_4$]glutamate deuterium atoms in one of the enantiotopic methylene groups was probably exchanged with protium during the decarboxylation reaction. Our previous [1,2-$^{13}C_2$]acetate feeding experiment revealed intact incorporation of acetate into the incednine C21−C22 moiety.[2] Thus, the prochirality of β-glutamate appeared to be distinguished by the putative β-decarboxylase. Although the enzymatic decarboxylation of β-glutamate has not been reported, some β-amino acid decarboxylases such as aspartate 4-decarboxylase (E.C. 4.1.1.12), which catalyzes the PLP-dependent decarboxylation of L-aspartate to L-alanine, have been well described.[8] A similar PLP-dependent decarboxylation of β-glutamate seems to be involved at the start of incednine biosynthesis. In the PLP-dependent enzymatic reaction, after decarboxylation, protonation of the enamine intermediate is required to complete the reaction. Thus, during the presumably rapid equilibrium that is reached between the enamine and iminium intermediates, the deuterium labels of the 3-aminobutyrate intermediate terminal methyl moiety might be exchanged with protium from exterior molecules such as water (Figure S2).

A structure that is similar to the incednine nitrogen-containing starter unit can be found in salinilactam, which was isolated from the marine actinomycetes *Salinispora tropica* (Figure 3).[12] The number of salinilactam PKS modules suggested that the nitrogen-containing C_6 molecule (i.e., 5-aminohex-2-enoate) might be its starter unit, which is thought to be derived from lysine (Figure 3A). In the salinilactam biosynthetic gene cluster, an aminomutase (Strop_2771) and a PLP-dependent enzyme (Strop_2772) are encoded that seem to be involved at the start of biosynthesis. Strop_2771 belongs to a family of radical SAM amino acid aminomutases such as glutamate 2,3-aminomutase, which converts L-glutamate to β-glutamate. As discussed above, β-glutamate can be decarboxylated to 3-aminobutyrate by a PLP-dependent enzyme. Thus, we propose that the salinilactam 3-aminobutyrate moiety can also be generated by Strop_2771 and Strop_2772 during the start of salinilactam biosynthesis (Figure 3B). If this is the case, one salinilactam PKS module must be utilized iteratively in polyketide elongation; alternatively, a hidden PKS module is required. Feeding experiments similar to the present study using the salinilactam producer *Salinispora tropica* will provide final proof of the biosynthetic pathway.

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In conclusion, the incednine nitrogen-containing starter unit appears to be derived from 3-aminobutyrate, which is biosynthesized from L-glutamate through β-glutamate (Figure 1B). It is noteworthy that a novel β-decarboxylation of β-glutamate should be involved at the start of incednine biosynthesis. This newly proposed 3-aminobutyrate formation is totally different from the well-known 3-aminobutyryl-CoA biosynthesis from L-lysine and from acetoacetyl-CoA.[13] To obtain greater detail about incednine biosynthesis, genetic analysis of the incednine biosynthetic gene cluster is currently underway in our laboratory.

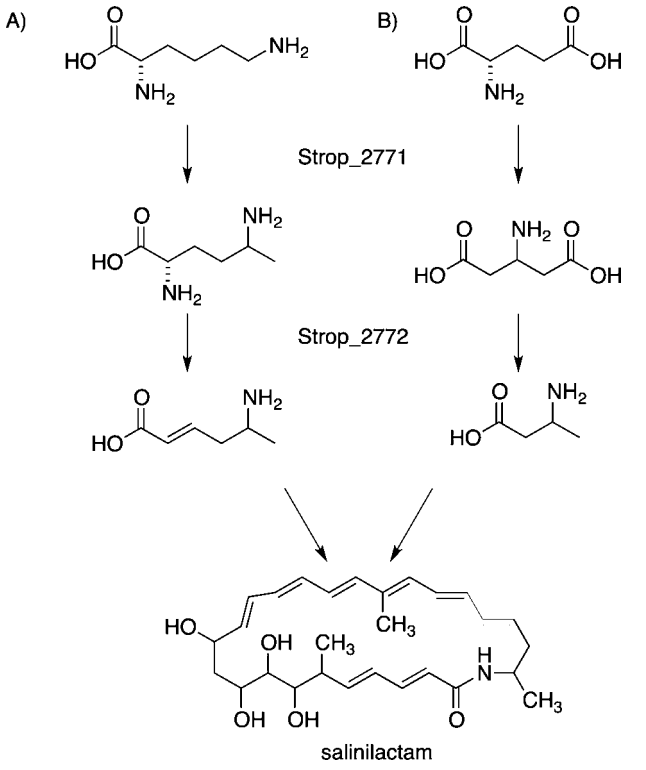

Figure 3. Two possible biosynthetic pathways for salinilactam.

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Supporting Information Available. Experimental procedures; ^1H NMR, ^{13}C NMR, and FAB mass spectra of deuterium-labeled amino acids and incednine. This material is available free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.