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Biosynthetic pathway of 24-membered macrolactam glycoside incednine

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ABSTRACT

Incednine was isolated from *Streptomyces* sp. ML694-90F3 as an inhibitor of anti-apoptotic function of Bcl-2/Bcl-xL oncoproteins. The structure of incednine is quite unique with a characteristic 24-membered macrocyclic lactam aglycone and two unusual aminosugars. To understand its biosynthetic pathway, the incorporation studies were carried out with [1-^{13}C]acetate, [1,2-$^{13}C_2$]acetate, [1-^{13}C]propionate, L-[$^{13}C_5$,^{15}N]glutamate, [1,2,3-$^{13}C_3$]glycerol, D-[6,6-$^{2}H_2$]glucose, and L-[CH$_3$–^{13}C]methionine. As a result, acetate, propionate, and glycerol were well incorporated into the elongation units of the macrolactam moiety, which indicates that its basic skeleton could be constructed by standard polyketide synthase, whereas all atoms of the starter unit were labeled by [$^{13}C_5$,^{15}N]glutamate suggesting that glutamate is somehow decarboxylated and rearranged into 3-aminobutyrate as the unique starter unit. The origins of the sugar moieties and methyl groups were also clarified. Based on the incorporation pattern, a plausible biosynthetic pathway for incednine is proposed.

1. Introduction

Incednine was isolated from the culture broth of *Streptomyces* sp. ML694-90F3 and was shown to inhibit anti-apoptotic function of Bcl-2/Bcl-xL oncoproteins overexpressed in many cancer cell lines.[1] Beside its significant bioactivity, incednine is structurally unique with a characteristic 24-membered macrocyclic lactam aglycone and two unusual aminosugars as shown in Figure 1. Although its aglycone is anticipated to be mostly derived from acetate/propionate precursors through a standard polyketide pathway, two unique biosynthetic features (1) the amine-containing starter unit and (2) a recently identified methoxymalonate extender unit[2] seemed to be involved.

The unique starter unit seemed to be derived from unprecedented biosynthetic pathway of amino acid so that identification of the biosynthetic origin should be an intriguing issue. Incorporation studies of the related macrolactam antibiotics including hitachimycin,[3] lankacidin C,[4] fluvirucin B1,[5] and vicenistatin[6] show that their precursors of the starter unit are amino acids such as phenylalanine, glycine, β-alanine, and glutamate, respectively. The biosynthetic gene clusters of macrolactam polyketides including vicenistatin,[7] salinilactam,[8] and leinamycin[9] also have been identified and indicated that the starter units are generated from amino acids by some characteristic enzymes. Thus, the biosynthetic pathways of starter units of polyketides derived from amino acids seem to contain many interesting enzymatic reactions and can be engineered by gene manipulation of the loading systems to create modified polyketides. However, except for some enzymatic systems such as the aminohydroxybenzoic acid (AHBA) biosynthesis involved in the rifamycin biosynthesis,[10] many of the biosynthetic systems of the starter units derived from amino acids are still uncharacterized so far. Therefore, accumulation of knowledge on such biosynthetic enzymes involved in starter biosynthesis as well as biosynthetic origins of starter units is highly desired. To investigate the biosynthetic origin of incednine, especially the unique starter unit, we carried out a series of feeding experiments with [1-^{13}C]acetate, [1,2-$^{13}C_2$]acetate, [1-^{13}C]propionate, L-[$^{13}C_5$,^{15}N]glutamate, [1,2,3-$^{13}C_3$]glycerol, D-[6,6-$^{2}H_2$]glucose, and L-[CH$_3$–^{13}C]methionine. We then clearly elucidated the precursor–product relationship in the biosynthesis of incednine. Consequently, a plausible biosynthetic pathway for incednine with the unique macrolactam and the unusual aminosugars is proposed.

2. Results and discussion

Since the aglycone of incednine is anticipated to be mostly derived from acetate/propionate precursors through a standard

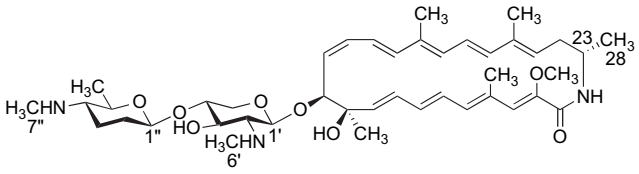

Figure 1. Structure of incednine.

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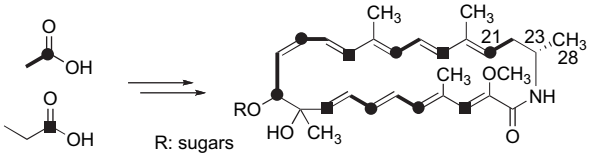
polyketide pathway, standard supplementation culture with labeled acetate or propionate was first carried out. The incedine producer *Streptomyces* sp. ML694-90F3 was cultured according to the previously reported conditions.[1] In this study, at the time of 24 h after inoculation, [1-^{13}C]acetate, [1,2-$^{13}C_2$]acetate or [1-^{13}C]-propionate was supplemented into the culture and the fermentations further continued for 4–5 days. The obtained incedine was further treated with 1.9 equiv of HCl on ice and the HCl salt of incedine was used for the NMR analysis. The ^{13}C NMR spectra of the labeled incedine with [1-^{13}C]acetate, [1,2-$^{13}C_2$]acetate or [1-^{13}C]propionate were compared with the previous data of non-labeled incedine and the present data are summarized in Table 1.

A high degree of incorporation from [1-^{13}C]propionate was found at the C3, C9, C15, and C19. Thus, these units appeared to be derived from methylmalonyl CoA as the extender units for the incedine polyketide synthase. [1-^{13}C]Acetate was well incorporated into the carbons at C5, C7, C11, C13, and C17 with almost same efficiency, while the relative intensity of the carbon at C21 was slightly less than others. The incorporation of intact [1,2-$^{13}C_2$]acetate into C5–6, C7–8, C11–12, C13–14, and C17–18 supported the regular polyketide pathway for these positions. On the other hand, slightly less efficient incorporation of intact acetate into C21 and C22 indicated that this moiety is apart from the regular extension unit and is rather a part of the starter unit, either 3-aminobutyrate or its equivalent. Also, little incorporation of [1,2-$^{13}C_2$]acetate into C23 and C28 excluded the possibility that the starter unit is 3-aminobutyryl CoA biosynthesized from the acetoacetyl CoA derived precursors such as crotonoyl CoA, whose carbons should be all labeled with [1,2-$^{13}C_2$]acetate. As a possible biosynthetic intermediate from acetate to the plausible 3-aminobutyrate starter unit, glutamate was then hypothesized. The C4 and C5 positions of glutamate are known to be labeled by acetate through acetyl CoA, citrate, isocitrate, and 2-oxoglutarate in the TCA cycle. The labeled glutamate could be decarboxylated and rearranged to give 3-aminobutyrate. Similar incorporation from glutamate into polyketide macrolactam can be found in the biosynthesis of vicenistatin, where glutamate is methyl-branched by glutamate mutase and decarboxylated to be aminomethylpropionate.[11,12] This scenario has also been supported with the existence of the genes for the glutamate mutase and decarboxylase in the vicenistatin biosynthetic gene cluster.[7]

To investigate this hypothesis, we carried out a feeding experiment of [$^{13}C_5$,^{15}N]glutamate using the fermentation medium without unlabeled glutamate. As shown in Figure 2, the ^{13}C NMR spectrum of the resulting labeled incedine clearly indicated the intact incorporation of the labeled glutamate into the carbons at C21, C22, C23, and C28 of incedine. The above-mentioned propionate derived units were also labeled with the labeled glutamate probably through the TCA cycle (data not shown). Moreover, ^{15}N INEPT spectrum of the labeled incedine showed a single signal at 122 ppm, which corresponds to chemical shift of an amide nitrogen. This result clearly indicated that the amino group of glutamate

Table 1
The results of ^{13}C NMR of the labeled incedine with [1-^{13}C]acetate, [1,2-$^{13}C_2$]acetate or [1-^{13}C]propionate (125 MHz, CD_3OD–D_2O=7:3)



Carbon atom	Chemical shift	Relative ^{13}C intensities			Coupling constant (Hz)
		[1-^{13}C]-Acetate	[1,2-$^{13}C_2$]-Acetate	[1-^{13}C]-Propionate	[1,2-$^{13}C_2$]acetate
1	167.8	0.8	0.9	0.5	
2	148.1	0.5	0.8	n.d.	
3	127.4	1.1	2.3	39.6	
4	133.4	0.9	2.6	1.0	
5	138.0	6.1	n.d.	1.8	55 (d)
6	129.1[a]	0.5	n.d.	0.7	n.d.
7	137.6	6.7	n.d.	1.1	57.5 (d)
8	130.5	0.7	n.d.	3.0	57.5 (d)
9	142.2	1.1	2.0	35.5	
10	76.5	0.9	1.3	n.d.	
11	84.3	6.7	7.3	0.8	50.0 (d)
12	129.1[a]	0.5	n.d.	0.7	n.d.
13	131.2	6.3	n.d.	1.0	56.3 (d)
14	125.6	0.7	7.4	0.6	56.3 (d)
15	138.7	1.3	1.8	31.7	
16	136.0	0.7	1.7	n.d.	
17	133.0	6.3	n.d.	0.9	60.0 (d)
18	124.9	0.5	7.3	0.5	60.0 (d)
19	138.7	1.6	1.7	48.3	
20	138.3	0.9	2.7	1.4	
21	131.0	4.3	n.d.	1.1	43.8 (d)
22	38.6	0.5	4.8	0.6	43.8 (d)
23	48.1	0.4	1.9	0.6	
24	14.3	0.7	1.7	0.9	
25	23.5	0.7	1.9	0.9	
26	13.0[a]	0.6	1.4	0.7	
27	13.0[a]	0.6	1.4	0.7	
28	20.6	0.7	2.9	0.8	
2-OCH_3	61.6	0.6	0.7	0.8	
1′	99.7	1.1	1.3	1.1	
2′	62.8	0.8	1.0	0.9	
3′	69.3	1.2	1.9	1.2	
4′	77.3	0.9	0.8	1.0	
5′	63.4	0.9	1.0	0.9	
6′	32.6	1.1	1.1	1.1	
1″	101.3	1.0	0.9	0.9	
2″	30.7	0.9	0.9	0.9	
3″	24.6	1.0	0.9	0.9	
4″	59.9	0.9	0.9	1.0	
5″	73.1	1.0	1.4	1.0	
6″	18.8	1.0	0.9	0.9	
7″	31.1	1.0	1.0	1.0	

Peak intensities were normalized to the carbon signal of 7″-$NHCH_3$. n.d.: Not determined due to signal overlapping.
[a] Signals were overlapped.

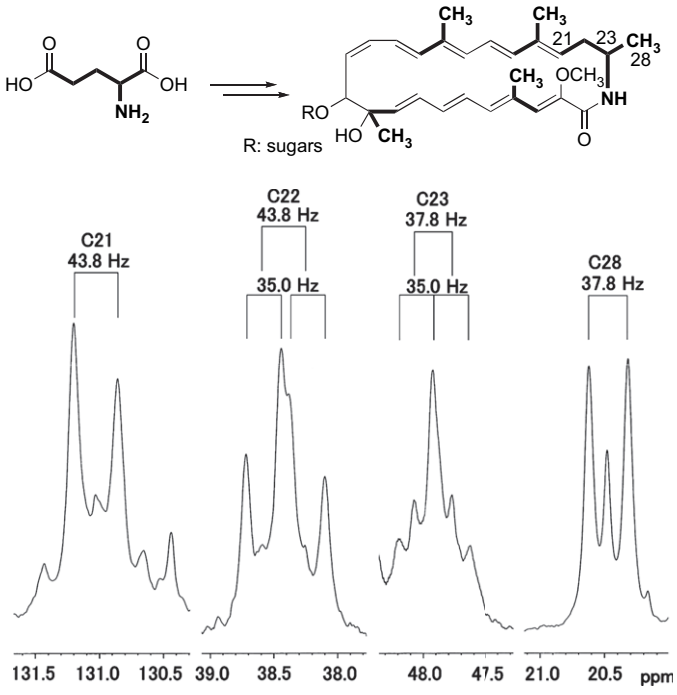


Figure 2. Partial ^{13}C NMR spectra (125 MHz, CD_3OD–D_2O=7:3) of labeled incedine with [$^{13}C_5$,^{15}N]glutamate.

was retained into the amide nitrogen atom at N24 of incednine. Thus, these data clearly demonstrated that the 3-aminobutyrate starter unit is derived from intact glutamate as we hypothesized.

Recently, many amino acid aminomutases such as lysine-2,3-aminomutase,[13] lysine-5,6-aminomutase,[14] and glutamate-2,3-aminomutase[15] have been identified to catalyze the intramolecular rearrangement of the amino groups. A similar aminomutase might be involved in the biosynthesis of the 3-aminobutyrate starter unit of incednine. Although the free form of 4-aminobutyrate and 3-aminobutyrate might be the possible biosynthetic intermediates, the precursor glutamate is likely activated as its CoA or ACP thioester to enter the specific secondary metabolic pathway. The activation of the precursor amino acid at the early stage of biosynthesis has been often observed in the secondary metabolite biosynthesis including vicenistatin[7] and butirosin.[16]

As the next, to confirm the biosynthetic origin of the glycolate unit at C1 and C2 of incednine, a feeding experiment of [1,2,3-$^{13}C_3$]glycerol has been carried out. It is generally known that the glycolate unit of macrolide polyketides is derived from glycerol.[2] Identifications of the characteristic biosynthetic genes for the glycolate unit in the biosynthetic gene clusters of geldanamycin,[17] FK520,[18] soraphen,[19] ansamitosin,[20] and concanamycin[21] also indicate that the origin of glycolate unit is glycerol. As we anticipated, a significant amount of incorporation of the labeled glycerol into the C1–C2 carbons was observed as shown in Figure 3. This result clearly showed that the glycolate unit of incednine is also derived from glycerol through 1,3-bisphosphoglycerate and methoxymalonyl-ACP (or CoA), which is supposed to be the extender unit of the polyketide synthase.

The ^{13}C label of [1,2,3-$^{13}C_3$]glycerol was also incorporated into the other part of incednine. The glycerol-derived acetate appeared to be incorporated into the aglycone of incednine, since a similar labeling pattern compared with the labeled acetate above mentioned was observed (data not shown). Two aminosugar moieties were also characteristically labeled as shown in Figure 3. The ^{13}C coupling (J_{C-C}) of C1′–C2′ and C4′–C5′ in the *N*-methyl-D-xylosamine moiety, and C1″–C2″ and C4″–C5″–C6″ in *N*-demethyl-D-forosamine was clearly observed as major signals, while the major signals for C3′ and C″3 were singlet. This incorporation pattern suggested that the sugar moieties were biosynthesized from glycerol via gluconeogenesis and the subsequent pentose phosphate pathway (Scheme 1). The fed glycerol could be metabolized to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate, followed by gluconeogenesis to yield glucose 6-phosphate whose C1–C3 and C4–C6 are all labeled with intact glycerol. The labeled glucose 6-phosphate is further converted to sedoheptulose 7-phosphate through the pentose phosphate cycle, followed by condensation with the labeled glyceraldehyde 3-phosphate to yield fructose 6-phosphate and erythrose 4-phosphate. The labeled fructose 6-phosphate is converted to glucose 6-phosphate whose C1–C2, C3, and C4–C6 should be labeled as shown in Scheme 1.

This labeled glucose 6-phosphate can be converted to glucose 1-phosphate, followed by the activation with nucleotide triphosphate to yield nucleotidilyl glucose (NDP-glucose) such as UDP-glucose and dTDP-glucose. UDP-glucose is metabolized to UDP-xylose, which might be converted to UDP-*N*-methyl-D-xylosamine via the oxidation, transamination, and N-monomethylation. UDP-*N*-methyl-D-xylosamine would be used for the glycosylation of the incednine aglycone. The dTDP-D-forosamine biosynthetic pathway from glucose 1-phosphate has been recently characterized in the spinosyn biosyntheis.[22] One of the intermediates in the biosynthesis is dTDP-*N*-demethyl-D-forosamine, which would be used for the glycosylation to complete the incednine biosynthesis. The specific incorporation of D-[6,6-$^2H_2$]glucose into the C6″ position of *N*-demethyl-D-forosamine (data not shown) supported this proposed biosynthetic pathway. On the other hand, no incorporation of D-[6,6-$^2H_2$]glucose into the *N*-methyl-D-xylosamine moiety indicated that it is biosynthesized through the xylose biosynthetic pathway where the C6 carbon of D-glucose is removed. The weak doublet signals for C3′ ($J_{C3'-C2'}$) and C″3 ($J_{C3''-C2''}$) of the labeled incednine with [1,2,3-$^{13}C_3$]glycerol suggested the direct incorporation of glucose produced by gluconeogenesis as shown in Scheme 1. However, the signals were much weaker than those for the pentose phosphate cycle derived sugars (Fig. 3). This result

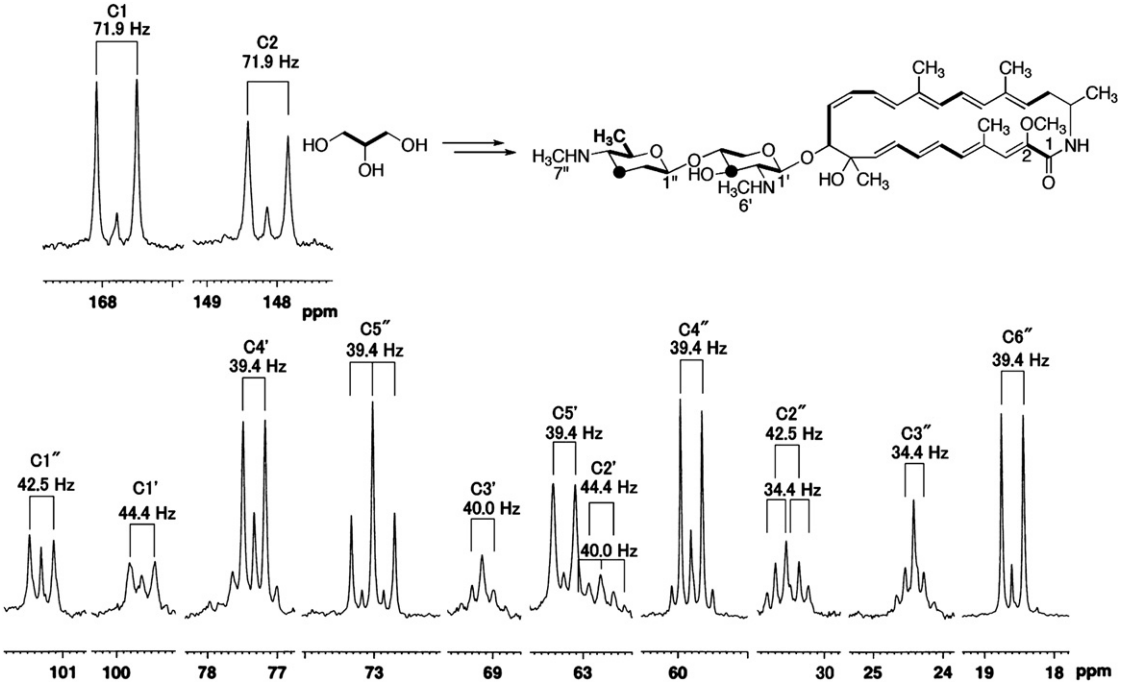
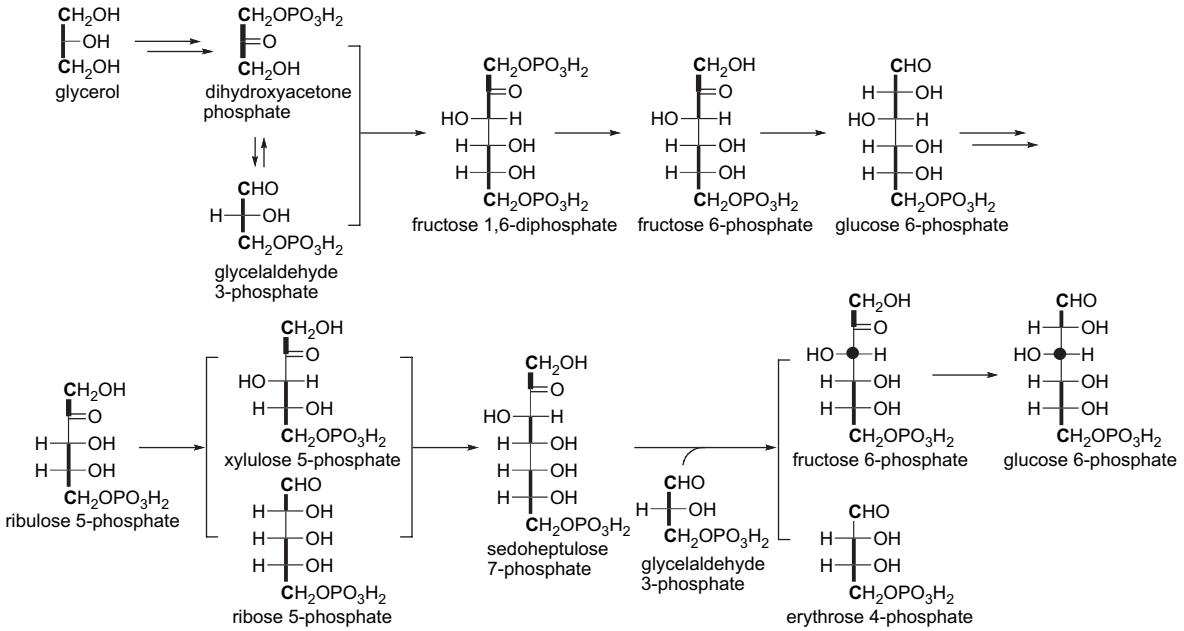


Figure 3. Partial ^{13}C NMR spectrum (125 MHz, CD_3OD–D_2O=7:3) of labeled incednine with [1,2,3-$^{13}C_3$]glycerol. The signals for the C1–C2 unit and sugar moieties are shown. The major labeling pattern of the sugars with the labeled glycerol would be derived via the pentose phosphate cycle through gluconeogenesis (see Scheme 1).

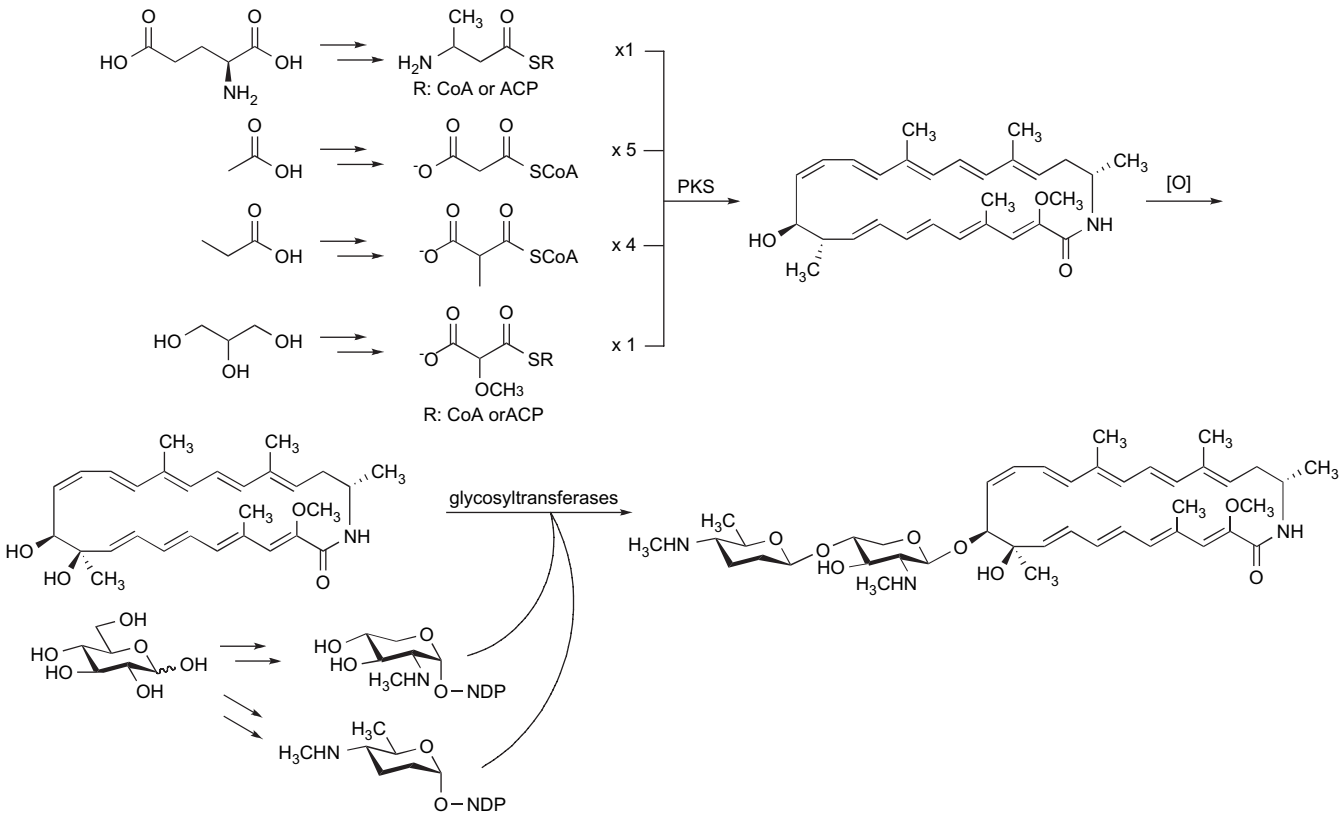


Scheme 1. A possible biosynthetic pathway of glucose from glycerol based on the incorporation pattern into the aminosugar moieties in incednine. Upper: gluconeogenesis, bottom: pentose phosphate pathway. The major labeling pattern of the sugars with the labeled glycerol would be derived via the pentose phosphate cycle through gluconeogenesis.

indicates that most of the glucose is consumed until the stationary phase and the pentose phosphate cycle derived glucose would be a sole source during the secondary metabolic sugar biosynthesis.

Finally, a feeding of L-[CH_3–^{13}C]methionine into the culture was carried out to determine the carbon source of one *O*-methyl and two *N*-methyl groups. As we expected, significant ^{13}C incorporation was observed at the methyl groups in the ^{13}C NMR spectrum (data not shown). Thus, the methyl groups appeared to be transferred from *S*-adenosyl L-methionine (SAM) by SAM dependent methyltransferases.

In the present study, the precursor–product relationship in the biosynthesis of incednine has been elucidated as mentioned above. Based on the incorporation pattern, a plausible biosynthetic pathway for incednine is proposed as shown in Scheme 2. The



Scheme 2. A plausible biosynthetic pathway of incednine.

amine-containing starter unit, probably 3-aminobutyrate generated from glutamate, could be loaded onto the first module of the PKS and condensed with five malonates, four methylmalonates, and one methoxymalonate extender unit derived from glycerol to construct the macrolactam skeleton. The oxygen at C10 of incednine could be introduced by a certain oxygenase. At the last stage of biosynthesis, two aminodeoxysugars derived from D-glucose would be transferred onto the aglycone by glycosyltransferases. In this proposed biosynthetic pathway, the starter unit biosynthesis from glutamate seemed to be the most interesting on the point of view of biosynthetic engineering, because modification/swapping of the loading system by gene manipulation may result in creation of novel polyketides. Also, this starter biosynthetic system appears to include some unprecedented enzymes such as an aminomutase type protein, which catalyzes an intramolecular rearrangement of the amino group of glutamate. Therefore, much of the enzymatic and genetic information on the biosynthesis of incednine is highly required to understand its biosynthetic system in detail. We are now trying to identify the biosynthetic gene cluster of incednine from the producer microorganism.

3. Experimental

3.1. Chemicals and reagents

Isotope-labeled precursors used were sodium [1-^{13}C]acetate (99 at% enriched), sodium [1,2-$^{13}C_2$]acetate (99 at% enriched), sodium [1-^{13}C]propionate (99 at% enriched), [$^{13}C_5$,^{15}N]glutamate (98 at% enriched), and L-[CH_3–^{13}C]methionine (99 at% enriched). These were purchased from Isotec Co. D-[6,6-$^{2}H_2$]Glucose was prepared according to the reported method.[23]

3.2. Culture conditions

Streptomyces sp. ML694-90F3 was maintained on a slant of yeast–starch agar (0.2% yeast extract, 1% soluble starch, 1.5% agar, pH was adjusted to 7.3 with NaOH) at 28 °C. A scraping from the slant was used to inoculate 100 mL of seed medium (2% galactose, 2% dextrin, 1% bactopeptone, 0.5% potato starch, 0.1% $(NH_4)_2SO_4$, 0.01% $CaCO_3$, 0.01% silicon antifoam, and pH was adjusted to 7 with NaOH before autoclaving) in a 500 mL baffled flask equipped with a cotton plug and incubated for 4 or 5 days at 30 °C with shaking at 220 rpm. This preculture (1 mL) was used to inoculate each 100 mL vegetative medium (0.5% glucose, 0.5% glycerol, 0.15% sodium L-glutamate, 0.5% soybean powder, 0.1% $CaCO_3$, and pH was adjusted to 7.4 with NaOH before autoclaving) in a 500 mL baffled flask equipped with a cotton plug, and all of which were incubated for 4 or 5 days at 30 °C with shaking at 220 rpm. The shaker was kept shaded due to the sensitivity of incednine to light.

Supplementation of isotope-labeled compounds was carried out as follows: 500 mg of sodium [1-^{13}C]acetate in distilled water was filter sterilized and equally added to a culture (total 1 L) at the time of 24 h after inoculation; [1,2-$^{13}C_2$]acetate (500 mg) in water was filter sterilized and equally added to a culture (total 1 L) at the time of 24 h after inoculation; sodium [1-^{13}C]propionate (250 mg) in water was filter sterilized and equally added to a culture (total 1 L) at the time of 24 h after inoculation; L-[CH_3–^{13}C]methionine (500 mg) in water was filter sterilized and equally added to a culture (total 1 L) by pulse feeding at the time of 24 and 48 h after inoculation; [$^{13}C_5$,^{15}N]glutamate (250 mg) in water was adjusted to pH 7 with NaOH before filter sterilized and equally added to an autoclaved medium (total 0.5 L), which was prepared without unlabeled sodium glutamate; [1,2,3-$^{13}C_3$]glycerol (500 mg) in water was filter sterilized and equally added to an autoclaved medium (total 1 L), which was prepared without unlabeled glycerol; D-[6,6-$^{2}H_2$]glucose (3.0 g) in water was filter sterilized and equally added to an autoclaved medium (total 1 L), which was prepared without non-labeled glucose.

3.3. Isolation and purification

Incednine produced by supplementation culture was isolated and purified as follows. A culture of *Streptomyces* sp. ML694-90F3 was adjusted to pH 10 by 1 M NaOH aq and was left overnight at 4 °C. After filtration under reduced pressure, the solid residue was washed with a mixed solvent of $CHCl_3$–CH_3OH (1:4), and the eluted filtrate was collected. After removal of the solvent, the residual liquid was dissolved in water and methanol and the solution was adjusted to pH 9 with $NaHCO_3$. The mixture was mixed with a solution ($CHCl_3$–CH_3OH–H_2O=5:6:4) and the $CHCl_3$ layer was then extracted for six times. After removal of the solvent by evaporation, the residue was purified through silica gel column chromatography ($CHCl_3$–CH_3OH=7:1–5:1) with Silica Gel 60N (100–210 μm). The obtained incednine was dissolved in CH_3OH and the solution was treated with 1.9 equiv of HCl (0.005 M) on ice. The solution was evaporated to give incednine as the HCl salt. The product was further washed with $CHCl_3$ and CH_3CN to remove impurities. The solid material was dissolved in CH_3OH and the supernatant was evaporated to yield pure incednine. The yields of each purified labeled incednine were 25 mg from the sodium [1-^{13}C]acetate supplemented culture (1 L), 15 mg from the sodium [1,2-$^{13}C_2$]-acetate supplemented culture (1 L), 9.3 mg from the sodium [1-^{13}C]-propionate supplemented culture (1 L), 3.0 mg from the [$^{13}C_5$,^{15}N]glutamate supplemented culture (0.5 L), 10 mg from the L-[CH_3–^{13}C]methionine supplemented culture (1 L), 4.3 mg from the [1,2,3-$^{13}C_3$]glycerol supplemented culture (1 L), and 15 mg from the D-[6,6-$^{2}H_2$]glucose supplemented culture (1 L).

3.4. NMR analysis

All NMR spectra were recorded on a Bruker DRX500 spectrometer. ^{1}H, ^{2}H, and ^{13}C NMR spectra were analyzed in a CD_3OD (99.8 at% enriched, ACROS) or CD_3OD–D_2O (99.8 at% enriched, ACROS)=7:3. Chemical shifts are reported in parts per million relative to the solvent peaks (3.3 ppm for methanol in ^{1}H NMR, 49.0 ppm for methanol in ^{13}C NMR). ^{15}N INEPT spectrum was analyzed in a CD_3OH (99 at% enriched, Aldrich). Chemical shift of ^{15}N INEPT spectrum is reported in parts per million relative to the external formamide standard (112.4 ppm).

Acknowledgements

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