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Biosynthetic Pathway of Macrolactam Polyketide Glycoside Antitumor Antibiotic Vicenistatins

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Abstract—The biosynthetic studies of antitumor antibiotic vicenistatin and vicenistatin M were undertaken by feeding experiments with [1-^{13}C]- and [1,2-$^{13}C_2$]acetate, [1-^{13}C]propionate, DL-[2,3,3-$^{2}H_3$]glutamate, D-[6,6-$^{2}H_2$]glucose, L-[^{15}N]glutamate, and L-[CH_3–^{13}C]methionine. The elongating units of the macrolactam aglycon were derived from acetate and propionate in a standard manner, whereas the starter unit was not derived from fatty acid, but rather originated from 3-amino-2-methylpropionate or its equivalent, probably formed by the reactions of glutamate mutase and decarboxylase. The sugar units appeared to be biosynthesized through diversed modification of functional groups of a common intermediate.

Biosynthetic pathway, mechanistic enzymology and molecular genetics are key features of present as well as forthcoming interest in engineering microbial secondary metabolism that has long been affording to us vast diversity of chemical structures and important biological activities. Microbial polyketides are among the current targets of major efforts, and significant results have been emerging rapidly. In addition to the regular polyketides such as macrolides and polycyclic aromatics, hybrid-type polyketides seem essentially more intriguing in terms of chemical diversity, in which major metabolites include ansamycins, cytochalasin-type metabolites, acyltetramic and acyltetronic acids, and macrocyclic lactams. Macrocyclic lactam metabolites are interesting because the starter unit of the polyketide skeleton is basically different from those of the regular polyketides. Thus, the precise knowledge of the biosynthesis of hybrid metabolites may provide significant opportunity in engineering of novel secondary metabolites.

Vicenistatin (**1**) is an antitumor antibiotic produced by *Streptomyces halstedii* HC-34, and the structure is actually unique in that it comprises from a 20-membered lactam aglycon having an aminosugar vicenisamine in Fig. 1. Its biological activity is also intriguing since antitumor activity has been particularly shown against xenographed models of certain human colon cancers.[1] Recently, we have isolated a minor congener of vicenistatin in the culture filtrate of the producing microorganism, and named as vicenistatin M (**2**), because the minor congener is substituted by D-mycarose in place of vicenisamine of **1** as shown in Fig. 1.[2]

Our first interest in vicenistatins biosynthesis was focused on that, while the major part of the lactam aglycon appeared to be derived from the polyketide pathway, the starter portion, with a methyl branching, seemed to be irrelevant to the acetate-propionate rule. The same is actually true in most of the macrolactam antibiotics and related microbial metabolites including ansamycins,[3] hitachimycin,[4] fluvirucins,[5] and BE-14106.[6] Although the structure of lankacidins are not really a macrolactam, but rather a 17-membered carbocycle, glycine is known to be the starter unit of lankacidins.[7,8]

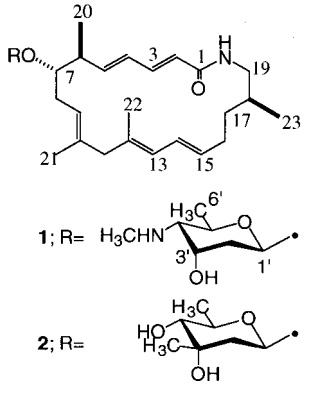

Figure 1. Structure of vicenistatin and vicenistatin M.

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On the basis of the structures of vicenistatins, the elongating units for the formation of the aglycon was expected to be derived from acetate-propionate or acetate-methionine precursors through a rather standard polyketide pathway, and this was in fact proved by a group of Abbott laboratories,[9] but the starter was unsolved. We anticipated that the precursor of the starter unit should be related to an amino acid origin. Further, the carbohydrate moiety of **1** was assigned as a novel aminosugar vicenisamine, while known D-mycarose was included in **2**. The biosynthetic interrelation of these sugars should also be interesting. This paper describes full detail of a series of labeling studies on the precursor–product relationship in the biosynthesis of vicenistatin antibiotics.[10]

Results and Discussion

Isotope-tracer experiments were undertaken to elucidate the biosynthetic pathway of vicenistatins. Standard supplementation culture either with [1-^{13}C]acetate or [1-^{13}C]propionate was first carried out and the labeled vicenistatin (**1**) was purified as reported.[1] The ^{13}C NMR spectra were then analyzed by comparison with the previously assigned chemical shifts,[1] and the results are summarized in Table 1. The labeling pattern with propionate was almost as anticipated. Thus, a high degree of incorporation was found for the most of the methyl-branched units at the C5, C9, and C11. Surprisingly, no incorporation was observed at C17. Although the starter for the polyketide elongation was not really predicted at the beginning, the C17–C18–C23 part was originally expected to be propionate-origin. A plausible rationale to this curious result came from the acetate feeding. C1, C3, C7, C13, and C15 were labeled efficiently again as anticipated. Of significance was that C17 was also labeled with [1-^{13}C]acetate. This result implied that C17 and C18 were acetate-origin. Apparently, this observation was not in accord with the standard polyketide pathway. It was unprecedented that propionate pathway and methylated acetate pathway were to be co-existed in a cell to form an equivalent C_3 intermediate. Accordingly, we envisioned that the starter could constitute the unit from the amide nitrogen to C17, which must not be derived from a simple fatty acid. Vicenistatin is in fact a hybrid polyketide with a non-fatty acyl starter unit vide *post*.

In order to get more insight into the starter unit and the acetate incorporation, a feeding of [1,2-$^{13}C_2$]acetate was next carried out. The results were also described in Table 1. Clear incorporation of intact acetate molecule into the units of C1–C2, C3–C4, C7–C8, C13–C14, and C15–C16 supported the regular polyketide pathway for these positions. In addition, the previous ^{13}C NMR assignment was confirmed. A particularly important observation was the incorporation of an intact acetate unit into C17 and C18, the signals of which appeared as AB-type double-doublet (J=35.1 Hz) accompanied by natural abundance singlet signals in the middle of the doublets as shown in Fig. 2. This fact further implied that C17 cannot be derived from any regular fatty acid precursor of a polyketide starter.

Table 1. ^{13}C NMR results from the incorporation of ^{13}C-labeled precursors

Carbon atom	Chemical shift	Relative ^{13}C intensities[a]		Coupling constant (Hz)
		[1-^{13}C]Acetate	[1-^{13}C]Propionate	[1,2-$^{13}C_2$]Acetate
1	166.3	*3.6*	1.0	64.5
2	124.5	0.6	0.9	64.5
3	140.3	*4.0*	1.4	55.4
4	128.4	0.7	1.0	nd[b]
5	143.3	1.5	*8.2*	
6	46.4	0.7	1.1	
7	86.1	*3.8*	1.4	38.9
8	36.7	0.9	1.2	38.9
9	121.9	1.2	*6.1*	
10	135.0	1.1	1.6	
11	49.2	1.3	*6.2*	
12	134.0	1.1	1.0	
13	128.1	*3.2*	1.1	nd[b]
14	128.4	0.7	1.0	nd[b]
15	132.5	*4.3*	1.4	43.4
16	27.5	1.2	1.5	43.4
17	32.6	*2.7*	1.0	35.1
18	33.5	0.6	1.0	35.1
19	43.0	0.7	1.2	
20	18.7	0.6	1.0	
21	17.9	0.7	0.8	
22	17.4	0.9	0.9	
23	17.6	0.7	1.0	
1′	100.8	0.7	1.2	
2′	39.4	0.8	1.1	
3′	63.1	0.8	1.1	
4′	65.0	1.0	1.5	
5′	70.4	1.1	1.3	
6′	19.5	0.8	1.1	
4′-NHCH3	33.9	1.0	1.0	

[a] Peak intensities were normalized to the carbon signal of 4′-NH*C*H$_3$.
[b] nd: Not determined due to signal overlapping.

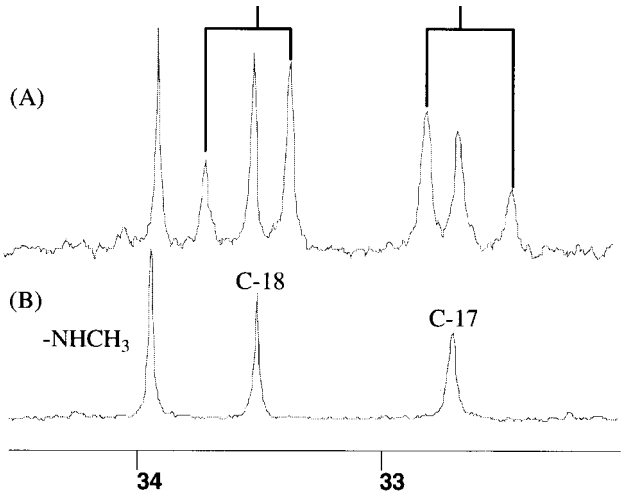


Figure 2. Partial ^{13}C NMR spectra (100 MHz, pyridine-d_5) of (A) non-labeled vicenistatin and (B) labeled vicenistatin by [1,2-$^{13}C_2$]acetate.

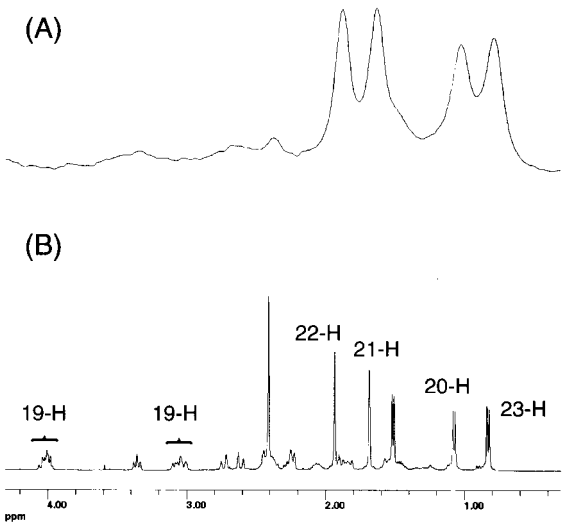


Figure 3. Partial ^1H and ^2H NMR spectra of non-labeled and deuterated vicenistatins: (A) ^2H NMR spectrum (60 MHz, pyridine) of labeled vicenistatin with [2,3,3-$^2H_3$]-glutamate; (B) ^1H NMR spectrum (400 MHz, pyridine-d_5) of non-labeled vicenistatin.

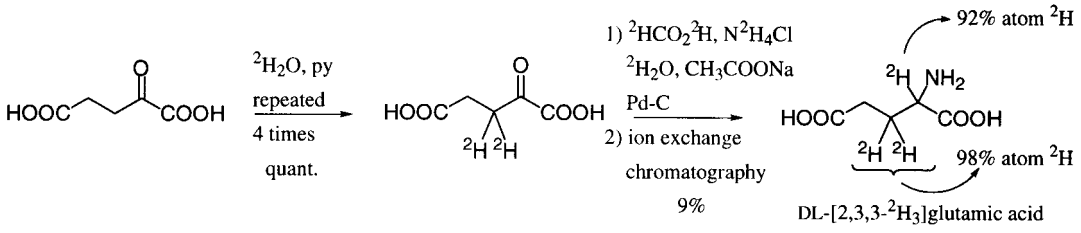
Instead, the starter unit may well be derived from an amino acid origin, the biosynthesis of which should involve the step of acetate incorporation. Perhaps, a 3-amino-2-methylpropionate equivalent is the most plausible. However, a methyl-branched amino acid has not been well established as an efficient metabolic intermediate.

Connection between the acetate metabolism and the amino acid metabolism is found in the TCA cycle. An acetate unit is known to be incorporated into the C4 and C5 positions of glutamate through acetyl-CoA, citrate, isocitrate and 2-oxoglutarate. Structural difference between glutamate and the plausible starter unit, 3-amino-2-methylpropionate, is found in the methyl branching and a loss of carboxyl group. The most plausible scenario for the formation of the starter unit seemed to be less complex. The methyl group extrusion is well predictable, since glutamate mutase is known to convert L-glutamate into 3-methylaspartate with the aid of vitamin B_{12} coenzyme,[11,12] though the stereochemistry at C18 is opposite to that of naturally abundant (2*S*,3*S*)-3-methylaspartate. Decarboxylation of α-amino acid is ubiquitous so that 3-methylaspartate may well be converted into 3-amino-2-methylpropionate unit, probably with the aid of vitamin B_6 coenzyme.

Since the above scenario seemed promising, we undertook a feeding experiment of DL-[2,3,3-$^2H_3$]glutamate, which had been prepared as shown in Scheme 1. After feeding experiment, the resulting labeled vicenistatin was analyzed with ^2H NMR spectrum as shown in Fig. 3.

Apparently, deuterium was incorporated into all the methyl groups of the aglycon despite the fact that the deuterated substrate has no methyl group in it. Clearly, all the methyl groups were formed through structural rearrangement at least in this case of the supplemented glutamate substrate. The deuterium incorporation into the C23 methyl group strongly supported that the above scenario was in fact involved in the formation of the polyketide starter. Additional incorporation of deuterium into C20, C21, and C22 methyl groups was also rationalized straightforwardly. Glutamate was deaminated by the well-known pyridoxal phosphate mediated transamination into 2-oxoglutarate, which in turn was decarboxylated to succinyl-CoA in the TCA cycle. The well-established rearrangement pathway, again vitamin B_{12} being a cofactor, from succinyl-CoA into methylmalonyl-CoA gave rise to the elongating C_3 unit,[11,13] which was equivalent to an intermediate derived from propionate.

Although the origin of C19 and the amide nitrogen atom had not been determined unequivocally at this stage, a decarboxylated 3-methylaspartate seemed to be the most plausible intermediate of the starter unit in the macrolactam biosynthesis. To clarify the origin of the amide nitrogen, incorporation of L-[^{15}N]glutamate into vicenistatin was examined and the resulting ^{15}N INEPT- and ^{15}N–^1H COSY spectra are shown in Fig. 4. A clear correlation of the single ^{15}N signal with the amide hydrogen but not with



Scheme 1.

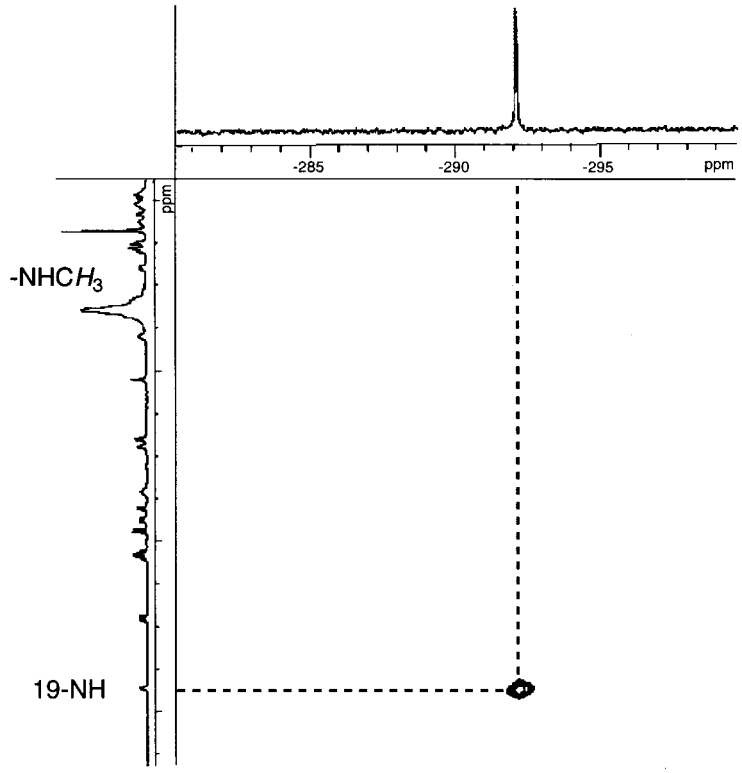


Figure 4. ^{15}N–^{1}H COSY spectrum (40 MHz, DMSO-d_6) of labeled vicenistatin with L-[^{15}N]glutamate.

the methylamino group in the sugar moiety was observed. This result strongly suggested that the amino nitrogen of glutamate was directly incorporated into the starter unit resulting in labeling the amide nitrogen. The results from the above-mentioned ^{2}H and ^{15}N labeling clearly demonstrated that the starter of the macrolactam is in fact derived from glutamate, and the most plausible pathway involves the glutamate mutase reaction and the decarboxylation to form a 3-amino-2-methylpropionate intermediate as depicted in Fig. 5. No incorporation of deuterium at C2 of

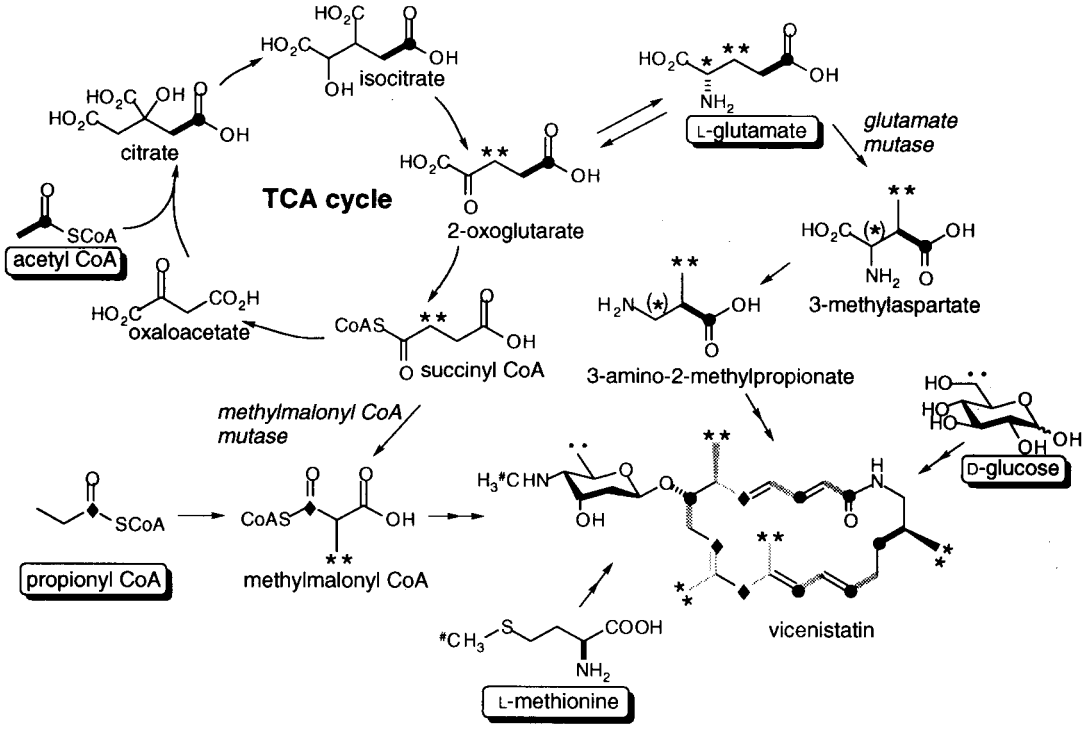


Figure 5. Precursor–product relationship in the biosynthesis of vicenistatin.

glutamate into C19 amidomethylene group was observed. This could probably be due to rapid deprotonation–reprotonation equilibrium of a Schiff base intermediate with pyridoxal phosphate in the glutamate metabolism catalyzed by aminotransferase and/or decarboxylase mentioned above. As to the biosynthesis of related lactam antibiotic, a similar scenario appears to be operative in the biosynthesis of the 14-membered lactam aglycon of fluvirucin A1.[4] While the authors did not discuss in detail, the reported labeling pattern obtained by [^{13}C]acetate feeding could be rationalized by a plausible starter being a decarboxylated aspartic acid, i.e. β-alanine. The plausible biosynthetic pathway of vicenistatin is summarized in Fig. 5.

In view of the biosynthesis of vicenisamine in vicenistatin (**1**) and D-mycarose (rather than L-mycarose found in various secondary metabolites) in vicenistatin M (**2**),[2] labeling with D-[6,6-$^{2}H_2$]glucose and L-[$CH_3-^{13}C$]methionine was carried out. Significant deuterium incorporation at the C6′ position of the vicenisamine moiety in **1** was confirmed by a strong signal at 1.5 ppm in the ^{2}H NMR spectrum (which corresponds to a doublet signal observed at 1.54 ppm in the ^{1}H NMR spectrum). Further, efficient incorporation of the methyl group of methionine was secured by the observation of a strong single signal at 33.9 ppm (CH_3NH-) in the ^{13}C NMR spectrum of **1**. These observation were in good accord with precedence that the novel aminosugar vicenisamine is actually biosynthesized from D-glucose via 2,6-dideoxy-4-ketohexose intermediate,[14] followed by transamination and methyl transfer to the resulting amino group from methionine. As to the biosynthesis of D-mycarose,[14] L-[$CH_3-^{13}C$]methionine supplementation allowed to observe extremely high incorporation of the label at 27.44 ppm in the ^{13}C NMR spectrum of **2**. Further, a doublet methyl signal (J^{13}_{C-H}=125 Hz) centered at 1.59 ppm was observed in the ^{1}H NMR spectrum. This was attributable to the branching methyl group on C3′.[2] Thus, the most plausible precursor to the *C*-methylation seems to be a 2,6-dideoxy-3-ketohexose intermediate, which is a highly related isomer of 2,6-dideoxy-4-ketohexose intermediate as mentioned above as a transamination precursor. It appears therefore that these two sugars found in vicenistatin antibiotics are biosynthesized by branching the pathway from a common intermediate as depicted in Fig. 6. Genetic as well as enzymological regulation as to a control of flow, if any, should be interesting.

In summary, the precursor-product relationship in the biosynthesis of vicenistatins, 20-membered macrolactam antitumor antibiotics, has been elucidated. The elongating units of the aglycon formation involves acetate and propionate according to the standard polyketide pathway, and the starter unit appears to be derived from glutamate through a 3-amino-2-methylpropionate equivalent. The sugar, vicenisamine and D-mycarose, moiety of vicenistatin (**1**) and vicenistatin M (**2**) seemed to share a common pathway but with branching at the later stage of the biosynthesis.

Experimental

Instrumentation

^{1}H and ^{13}C NMR spectra were recorded on a JEOL EX-270, a JEOL Lambda-300 and/or a Bruker DRX-500 spectrometers in a deuteriopyridine solution (99.8% atom enriched, Aldrich). Tetramethylsilane (δ_H=0.0 ppm) was used as an internal standard. Deuterium oxide (99.9% atom enriched, Aldrich HOD (δ_H=4.8 ppm)) was also used as NMR solvent. ^{2}H NMR spectra were recorded on a JEOL EX-270 or a JEOL Lambda-400 spectrometer. FAB-MS spectra were recorded with a JEOL JMS-AX 505HA spectrometer, using glycerol as matrix. Chromatographic separation was carried out with Merck Kieselgel 60 (70–230 mesh).

Chemicals and Reagents

Isotope-labeled precursors used were L-[^{15}N]glutamic acid (98 atom% enriched, Aldrich), sodium [1-^{13}C]acetate (99 atom% enriched, Isotec Co.), sodium [1,2-$^{13}C_2$]acetate (99 atom% enriched, Isotec Co.), sodium [1-^{13}C]propionate (99 atom% enriched, Isotec Co.), and L-[CH_3-^{13}C]methionine (99 atom% enriched, Isotec Co.). D-[6,6-$^{2}H_2$]glucose was prepared as already reported.[15]

Preparation of DL-[2,3,3-$^{2}H_3$]glutamic acid

To a solution of 25 ml of pyridine and 10 ml of heavy water ($^{2}H_2O$) was added 3.6 g (24.7 mmol) of 2-oxoglutarate, and the mixture was stirred overnight at room temperature. The whole was then evaporated to dryness. To the residue were added 25 ml of pyridine and 10 ml of heavy water

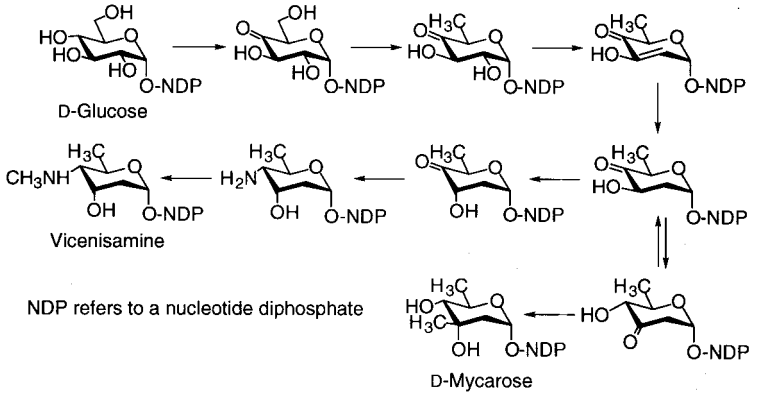


Figure 6. Plausible biosynthetic pathway from D-glucose to vicenisamine and D-mycarose.

(^2H_2O) and the same operation was repeated four times. The resulting residue was further mixed with 2.0 ml (53 mmol) of $^2HCOO^2H$, 3.0 g (52 mmol) of N^2H_4Cl, 3.0 g of 10% Pd–C, 3.0 g of CH_3COONa and 50 ml of ^2H_2O, and the mixture was stirred at 40°C for 3 days. After filtration and evaporation, the mixture was subjected successively to ion-exchange chromatography (Dowex 50W-X8 [H^+cycle] eluted with 1 M HCl, and Amberlite IRA410 [OH^- cycle] eluted with 1 M HCl) to yield 0.40 g (8.7% yield) of DL-[2,3,3-$^2H_3$]glutamic acid hydrochloride, FAB-MS (positive): *m/z* 150 (M^+; 3.56), 151 ($[M+1]^+$; 5.30), ^1H NMR (D_2O); 2.42 ppm (br. s); $^{13}C\{^1H\}$-NMR (D_2O, dioxane=δ_C 66.5): 24.1 (quintet), 29.2 (s), 51.7 (t), 171.6 (s), 176.2(s).

Culture conditions

The producing strain *Streptomyces halstedii* HC-34 was maintained on a slant of Bennet's agar. Preculture and production culture were carried out with a same liquid medium comprising from 3% potato starch, 1.5% soya flour A, 0.2% yeast extract, 0.5% corn steep liquor, 0.3% NaCl, 0.05% $MgSO_4/7H_2O$, 0.0005% $CoCl_2/6H_2O$ and 0.3% $CaCO_3$, pH 7.2. An aliquot (0.5 ml) of 2-day preculture was inoculated to a 100 ml of the medium in a 500 ml Erlenmeyer flask and culture was carried out with 200 rpm shaking at 27°C for 3–5 days. Supplementation of isotope-labeled compounds was carried out as follows; 2.0 g of sodium [1-^{13}C]acetate and 1.0 g of sodium [1,2-$^{13}C_2$]-acetate were separately dissolved in 20 ml of water, and each solution was added independently to a 72-hour culture (total 2 L); sodium [1-^{13}C]propionate (1.0 g) and D-[6,6-$^2H_2$]glucose (1.0 g) were separately dissolved in 40 ml of water, and each solution was added to a culture (total 2 L) independently by pulse labeling at the time of 24 and 48 h after inoculation; DL-[2,3,3-$^2H_3$]]glutamic acid/HCl (0.8 g) was dissolved in 32 ml of water, and the solution was added to a culture (total 1.6 L) by pulse labeling at the time of 36 and 48 h after inoculation; L-[^{15}N]glutamic acid (0.5 g) and L-[CH_3-^{13}C]methionine (0.5 g) were separately dissolved in 20 ml of water, and each solution was added to a culture (total 1 L) independently by pulse labeling at the time of 24 and 48 h after inoculation.

Isolation and purification of vicenistatin (1) and vicenistatin M (2)

Vicenistatin (**1**) and vicenistatin M (**2**) produced by supplementation culture were isolated and purified as follows. A culture of *Streptomyces halstedii* HC-34 was centrifuged at 7000 rpm for 30 min. The sedimented mycelia was suspended in acetone and was extracted overnight at room temperature. After filtration to remove mycelia and evaporation of the filtrate, the aqueous residue was adjusted to pH 10 and then extracted twice with ethyl acetate. After removal of the solvent by evaporation, the targeted metabolites were isolated and purified through repeated chromatography as already reported.[1] The yields of each purified labeled **1** was 29 mg (from sodium [1-^{13}C]acetate supplementation, 4 mg (from sodium [1,2-$^{13}C_2$]acetate supplementation), 13 mg sodium ([1-^{13}C]propionate supplementation), 14 mg (from D-[6,6-$^2H_2$]glucose supplementation), 19 mg (from DL-[2,3,3-$^2H_3$]-glutamic acid/HCl supplementation), 20 mg (from [^{15}N]glutamic acid supplementation), and 15 mg (from L-[CH_3-^{13}C]methionine supplementation). The yield of labeled **2** was 1.4 mg (from L-[CH_3-^{13}C]methionine).

Acknowledgements

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