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Amino acid starter unit in the biosynthesis of macrolactam polyketide antitumor antibiotic vicenistatin

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Abstract—Biosynthetic studies on the starter unit of antitumor antibiotic vicenistatin were undertaken by feeding experiments with (2*S*,3*R*)- and (2*S*,3*S*)-3-[$^2H_3$]methylaspartate, and 3-amino-2-[$^2H_3$]methylpropionate. The starter unit of the macrolactam part of vicenistatin was found to be derived from (2*S*,3*S*)-3-methylaspartate, but not from the (2*S*,3*R*)-isomer. The present as well as the previous results suggest that the starter is formed from L-glutamate through structural rearrangement catalyzed by glutamate mutase to (2*S*,3*S*)-3-methylaspartate, which in turn is loaded to polyketide synthase. Additional epimerization and decarboxylation may take place in the process either of activation or condensation to give the final C-18 stereochemistry.

Vicenistatin is an antitumor antibiotic produced by *Streptomyces halstedii* HC-34, and unique features are found in its polyketide structure of the macrocyclic 20-membered lactam aglycon and an aminosugar vicenisamine as shown in Fig. 1. Its biological activity is also intriguing since antitumor activities were shown particularly against xenographed models of certain human colon cancers.[1] We have been interested in the biosynthesis of vicenistatin because, while the lactam aglycon appears to be mostly derived from the polyketide pathway, the starter portion is irrelevant to the acetate–propionate theory. The same is actually true in most of the macrolactam antibiotics and related microbial metabolites including ansamycins,[2] hitachimycin,[3] lankacidins,[4] Antibiotic TA,[5] epothilone,[6] and the cytochalacin group of antibiotics.[7]

In the previous papers,[8,9] we reported that the elongating units of the vicenistatin aglycon were derived from acetate and propionate in a standard polyketide biosynthetic manner, whereas the starter unit appeared to be derived from 3-methylaspartate or its equivalent, probably formed by the reactions of glutamate mutase and decarboxylase. However, the C-18 stereochemistry of vicenistatin is opposite to the usual product of glutamate mutase, i.e. (2*S*,3*S*)-3-methylaspartate.[10] Therefore, we undertook feeding experiments of deuterium labeled 3-methylaspartate diastereomers in order to confirm whether or not the standard glutamate mutase reaction is involved in the biosynthesis of vicenistatin. In this paper, we describe the synthesis of deuterium labeled 3-methylaspartate isomers and its related compounds, and the feeding experiments of these substrates.

1. Results and discussion

Firstly, the labeled substrates, (2*S*,3*S*)- and (2*S*,3*R*)-3-[$^2H_3$]methylaspartate were prepared according to the method of Chamberlin et al.,[11] as shown in Scheme 1. The protected L-aspartic acid **1** was treated with lithium hexamethyldisilazide (LHMDS) in THF at −23°C to convert to an intermediary enolate, which was then alkylated with [$^2H_3$]methyl iodide (99 atom% ^2H) to give, after chromatographic purification and recrystallization, highly diastereoselectively (2*S*,3*R*)-3-[$^2H_3$]methylaspartate derivative **2a** in 87% yield. On the other hand, its diastereomer, (2*S*,3*S*)-derivative **2b**, was prepared by epimerization of **2a**. Thus, treatment of **2a** with potassium hexamethyldisilazide (KHMDS) in THF at −23°C, and subsequent aqueous quenching gave a separable mixture of (2*S*,3*R*)- and (2*S*,3*S*)-3-[$^2H_3$]methylaspartate derivative **2a** and **2b** in a ratio of 11:10. The desired diastereomer **2b** was obtained after chromatographic separation and crystallization in 36% yield, together with the starting **2a** (29%). Deprotection of **2a** and **2b** was separately carried out by hydrogenation in

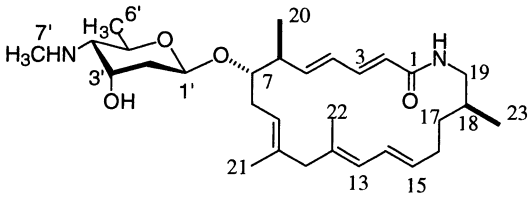

Figure 1. Structure of vicenistatin.

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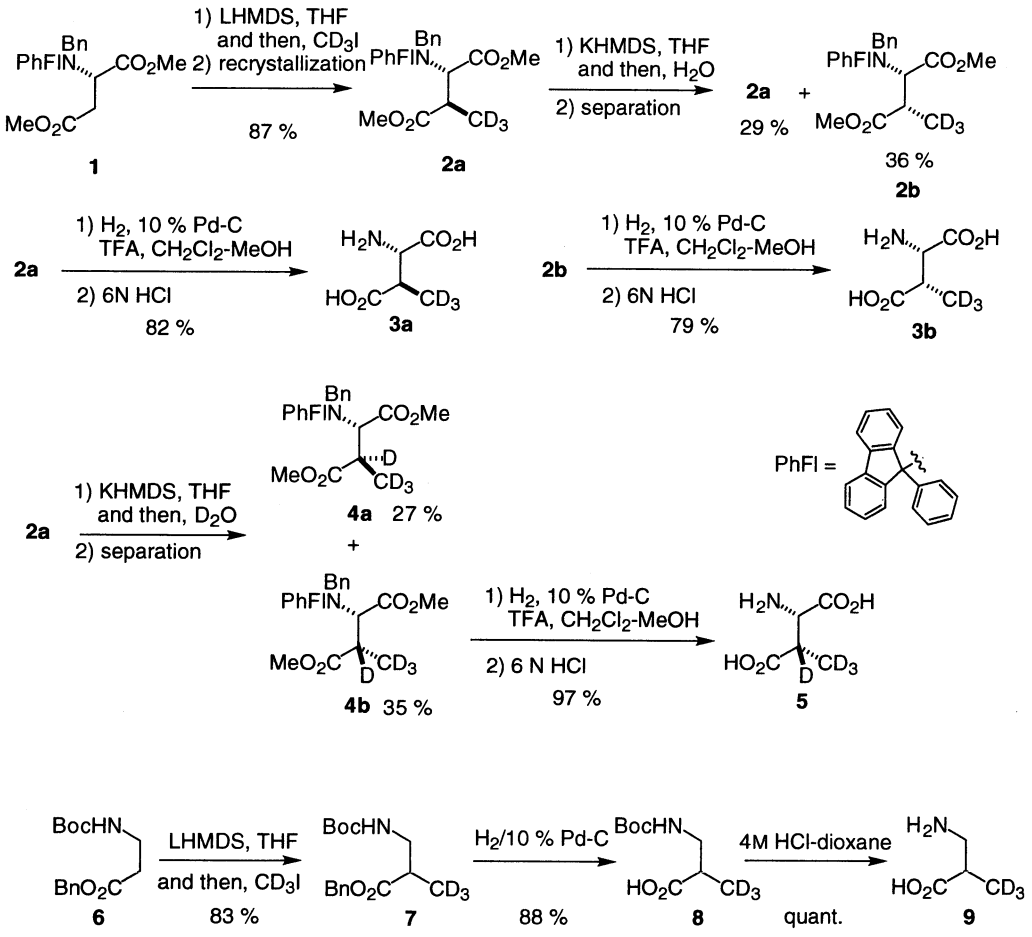

Scheme 1.

the presence of 10% Pd–C catalyst under high pressure of hydrogen (30 atm), and subsequent acidic hydrolysis afforded (2*S*,3*R*)- and (2*S*,3*S*)-3-[$^2H_3$]methylaspartic acid (**3a** and **3b**) in 82 and 79% yield, respectively.

The feeding experiments using these substrates were carried out as reported previously,[8,9] and the labeled vicenistatin was purified. The ^2H NMR spectra of labeled vicenistatin are shown in Fig. 2A and B. No deuterium incorporation was observed in the feeding experiment of (2*S*,3*R*)-3-[$^2H_3$]methylaspartate **3a** (Fig. 2B). In contrast, when (2*S*,3*S*)-3-[$^2H_3$]methylaspartate **3b** was used as substrate, deuterium was efficiently incorporated into all the methyl groups of the aglycon including the C-23 methyl group (Fig. 2A). The incorporation of deuterium into the C-23 methyl group was in fact much higher than that of deuterated glutamate in the previous experiments.[8,9] This observation strongly suggested that (2*S*,3*S*)-3-methylaspartate is the actual precursor of the starter unit of the aglycon. It appears therefore that the starter unit is biosynthesized through (2*S*,3*S*)-3-methylaspartate derived from L-glutamate by the catalysis of glutamate mutase in the usual stereochemistry.

Additional less efficient incorporation of deuterium into the methyl groups at C-20, C-21, and C-22 can be rationalized straightforwardly. Thus, (2*S*,3*S*)-3-[$^2H_3$]methylaspartate is partially transformed into labeled L-glutamate by the reverse reaction of glutamate mutase, and the labeled L-glutamate is deaminated into 2-oxoglutarate, which in turn is decarboxylated into succinyl-CoA in the TCA cycle. The well-established rearrangement pathway from succinyl-CoA into methylmalonyl-CoA may give rise to the elongating C3 unit,[10,12] being equivalent to an intermediate derived from propionate.

Further manipulation for the starter unit of the aglycon include epimerization at the C-3 position and decarboxylation of (2*S*,3*S*)-3-methylaspartate. To get some insight into the stage and mechanism of epimerization, the feeding experiment of (2*S*,3*S*)-3-[$^2H_3$]methyl-[3-$^2H_1$]aspartate **5** was undertaken. This substrate was synthesized by quenching the enolate derived from **2a** with deuterium oxide and deprotection of the resulting **4b** as described above. The feeding experiment of the synthesized **5** was carried out as described above, and the obtained vicenistatin was analyzed by ^2H NMR as also shown in Fig. 2C. As was expected, the resulted spectrum was quite similar to that of the feeding experiment of (2*S*,3*S*)-3-[$^2H_3$]methylaspartate. The high incorporation of deuterium into the C-23 methyl group was observed and no deuterium signal was shown at the C-18 position (δ_H 1.82). These results clearly demonstrated that the epimerization took place after the glutamate mutase, probably via enolate intermediate. However, consecutive epimerization at the C-3 position immediately after the glutamate mutase reaction must be ruled out, because

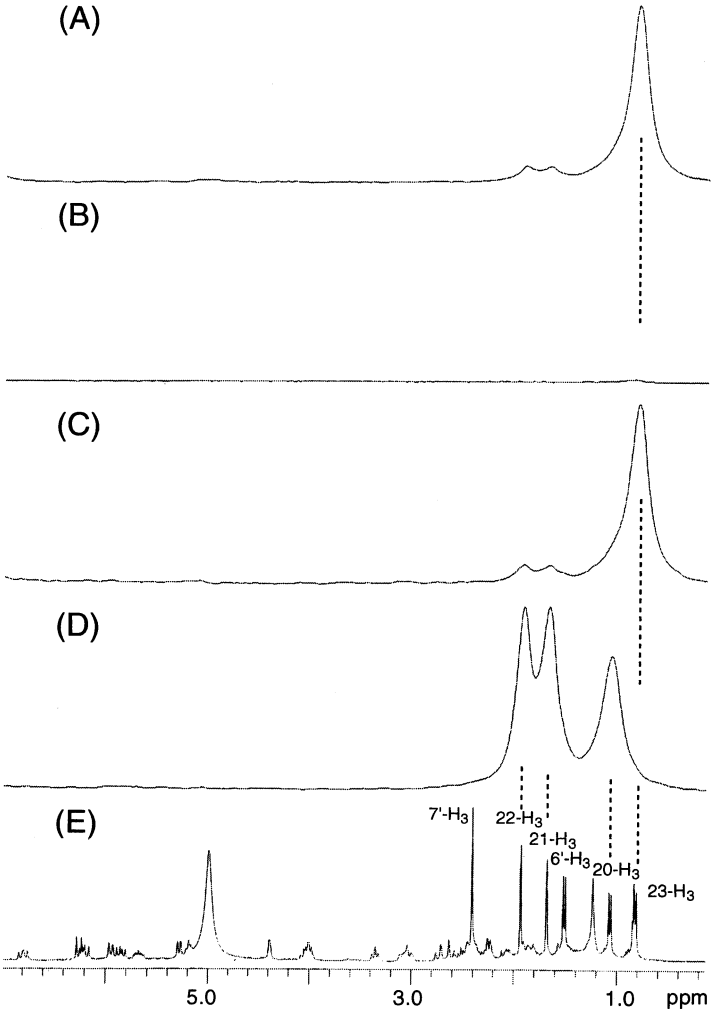

Figure 2. Partial ^{1}H and ^{2}H NMR spectra of non-labeled and deuterated vicenistatins: (A); ^{2}H NMR spectrum (60 MHz, pyridine) of labeled vicenistatin with (2*S*,3*S*)-3-[$^{2}H_3$]methylaspartate **3b**, (B); ^{2}H NMR spectrum of labeled vicenistatin with (2*S*,3*R*)-3-[$^{2}H_3$]methylaspartate **3a**, (C); ^{2}H NMR spectrum of labeled vicenistatin with (2*S*,3*S*)-3-[$^{2}H_3$]methyl-[3-$^{2}H_1$]aspartate **5**, (D); ^{2}H NMR spectrum of labeled vicenistatin with 3-amino-2-[$^{2}H_3$]methylpropionate **9**, (E); ^{1}H NMR spectrum (400 MHz, pyridine-d_5) of non-labeled vicenistatin.

(2*S*,3*R*)-3-[$^{2}H_3$]methylaspartate was not incorporated at all as described above. Therefore, the subsequent reaction could be decarboxylation reaction, probably with the aid of pyridoxal phosphate coenzyme, and a decarboxylated 3-methylaspartate, i.e. 3-amino-2-methylpropionate, seemed to be a candidate for the starter unit in the polyketide elongation.

To examine this possibility, we next undertook a feeding experiment of DL-3-amino-2-[$^{2}H_3$]methylpropionate, which was prepared as also shown in Scheme 1. The protected β-alanine **6** was treated with LHMDS in THF at −78°C to convert to an intermediary enolate, which was quenched with [$^{2}H_3$]methyl iodide to give 3-amino-2[$^{2}H_3$]methylpropionate derivative **7** in 83% yield. Hydrogenation, followed by acid treatment of the resulting acid **8**, gave the desired DL-3-amino-2-[$^{2}H_3$]methylpropionate **9** in high yield. The feeding experiment using this substrate was carried as described above, and the ^{2}H NMR of the obtained vicenistatin was also shown in Fig. 2D. To our surprise, no deuterium incorporation into the C-23 methyl group was observed. Instead, deuterium was apparently incorporated into the methyl groups at the C-20, C-21, and C-22 positions. The results clearly demonstrated that 3-amino-2-methylpropionate was not a precursor of the starter unit and the fed 3-amino-2-methylpropionate was instead converted into the methylmalonyl-CoA. The latter deuterium incorporation into the methyl groups at C-20, C-21, and C-22 can be explainable as follows. Deuterated 3-amino-2-methylpropionate appeared to be deaminated into labeled methylmalonate semialdehyde. The labeled methylmalonate semialdehyde was then converted into methylmalonyl-CoA through methylmalonate or propionate.[13] In any event, since 3-amino-2-methylpropionate is not a precursor of the starter unit of the aglycon. Chain elongation reaction may be the plausible step after the formation of (2*S*,3*R*)-3-methylaspartate by the glutamate mutase reaction.

Several macrolactam antibiotics and related microbial metabolites are considered to be biosynthesized by the polyketide pathway with an amino acid starter unit.[3–7] In rifamycin biosynthesis, the amino acid starter 3-amino-5-hydroxybenzoic acid is directly loaded on a polyketide

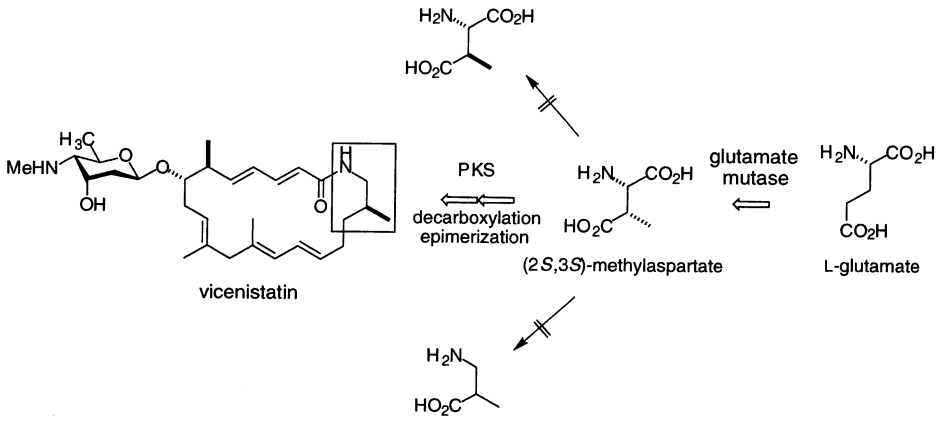

Figure 3.

synthase (PKS) having an amino acid loading domain.[14] In Antibiotic TA biosynthesis, one of the polyketide synthase (PKS) was reported to contain the sequences similar to the large family of non-ribosomal peptide synthethases (NRPS).[5] Further, epimerization of amino acids during the NRPS reactions was reported in several biosynthetic systems.[15] Accordingly, in addition to these precedence, the present study strongly suggests that the glutamate mutase reaction product, (2*S*,3*S*)-3-methylaspartate, may be directly loaded into the corresponding PKS, and the appropriate epimerization and decarboxylation of (2*S*,3*S*)-3-methylaspartate unit take place in the PKS reaction as shown in Fig. 3. While a post-PKS modification cannot be ruled out for these reactions at the moment, genetic as well as enzymatic investigation of vicenistatin biosynthesis should shed light on this interesting biosynthetic problem.

In summary, the present study has clearly demonstrated that the starter unit of the macrolactam part of vicenistatin is derived from (2*S*,3*S*)-3-methylaspartate through the glutamate mutase reaction in the usual stereochemical manner. The glutamate mutase reaction product, (2*S*,3*S*)-3-methylaspartate, was suggested to be directly loaded into the PKS.

2. Experimental

2.1. General

Column chromatography was carried out with a Kieselgel 60 (70–230 mesh or 230–400 mesh, Merck). All reactions, except for catalytic hydrogenation reactions, were carried out in an inert (Ar or N_2) atmosphere. THF was distilled from sodium benzophenone ketyl. ^{1}H-, ^{13}C- and ^{2}H NMR spectra were recorded on a JEOL LA-300, or a LA-400 spectrometer. Data are reported as follows: chemical shift (ppm), multiplicity (s=singlet, d=doublet, t=triplet, q=quartet, m=multiplet, br=broad), coupling constant (Hz), and integration. IR spectra were taken on a Horiba FT-710 Fourier-transform infrared spectrometer. Elemental analyses were performed with a Perkin–Elmer 2400 apparatus. [^{2}H$_3$]Methyl iodide (99 atom% ^{2}H) and deuterium oxide (99.8 atom% ^{2}H) were purchased from Aldrich.

2.1.1. Dimethyl (2*S*,3*R*)-*N*-benzyl-*N*-(9-phenylfluoren-9-yl)-3-[^{2}H$_3$]methylaspartate (2a). To a solution of dimethyl L-*N*-benzyl-*N*-(9-phenylfluoren-9-yl)aspartate **1**[11] (14.7 g, 29.9 mmol) in THF (80 ml) was added dropwise a solution of LHMDS in THF (45.0 ml, 1.0 M solution, 45.0 mmol) at −23°C. After being stirred for 10 min, [^{2}H$_3$]methyl iodide (6.52 g, 45.0 mmol) was added dropwise. The reaction was kept at the same temperature for 1 h and was then quenched with sat. aqueous NH_4Cl. The layers were separated, and the aqueous layer was extracted twice with ether. The combined organic layer was washed with brine, dried over Na_2SO_4, and evaporated. The residual solid was recrystallized from EtOAc–hexane to give **2a** (10.9 g, 71%). The mother liquor was chromatographed on silica gel with hexane–EtOAc (6:1) to give an additional **2a** (2.4 g, 16%); total 13.3 g (87%): mp 151–153°C (lit.[11] 154–155°C for non-labeled 2*R*,3*S*-compound); ^{1}H NMR ($CDCl_3$): δ 2.55 (d, J=11.2 Hz, 1H), 2.91 (s, 3H), 3.54 (s, 3H), 3.79 (d, J=11.2 Hz, 1H), 4.35 (d, J=14.4 Hz, 1H), 4.69 (d, J=14.1 Hz, 1H), 7.21–8.02 (m, 18H); ^{13}C NMR ($CDCl_3$): δ 14.9 (septet, J=18.9 Hz), 4.06, 50.4, 50.8, 51.1, 62.5, 80.2, 119.2, 119.8, 126.6, 127.19, 127.23, 127.3, 127.4, 127.5, 127.7, 128.0, 128.3, 129.0, 139.1, 141.4, 142.1, 144.3, 144.5, 147.2, 171.1, 174.1. Anal. Calcd for $C_{33}H_{28}{}^2H_3NO_4$: C, 77.93; H+^{2}H, 6.14; N; 2.75. Found: C, 77.61; H+^{2}H, 6.04; N; 2.62.

2.1.2. Dimethyl (2*S*,3*S*)-*N*-benzyl-*N*-(9-phenylfluoren-9-yl)-3-[^{2}H$_3$]methylaspartate (2b). To a solution of **2a** (80.0 mg, 0.158 mmol) in THF (2.0 ml) was added dropwise a solution of KHMDS in THF (0.50 ml, 0.5 M solution, 0.25 mmol) at −23°C. The mixture was stirred at the same temperature for 10 min, and then at 0°C for 12 h. The reaction was quenched with sat. aqueous NH_4Cl. The layers were separated, and the aqueous layer was extracted twice with ether. The combined organic layer was washed with brine, dried over Na_2SO_4, and evaporated. Flash silica gel column chromatography with hexane–EtOAc (6:1) and recrystallization from EtOAc–hexane gave the more polar product **2a** (23 mg, 29%) and the less polar product **2b** (29 mg, 36%): mp 179–181°C (lit.[11] 184–185°C for non-labeled 2*R*,3*R*-compound); ^{1}H NMR ($CDCl_3$): δ 2.26 (d, J=11.5 Hz, 1H), 2.96 (s, 3H), 3.35 (s, 3H), 3.54 (d, J=11.2 Hz, 1H), 4.16 (d, J=13.4 Hz, 1H) 4.40 (d, J=13.4 Hz, 1H), 7.22–7.78 (m, 18H). Anal. Calcd for $C_{33}H_{28}{}^2H_3NO_4$: C, 77.93; H+^{2}H, 6.14; N, 2.75. Found: C, 77.79; H+^{2}H, 6.19: N, 2.65.

2.1.3. (2*S*,3*R*)-3-[$^2H_3$]Methylaspartic acid hydrochloride (3a). A mixture of **2a** (4.0 g, 7.9 mmol), trifluoroacetic acid (1.72 g, 15.1 mmol), and 10% Pd–C (1.0 g) in CH_2Cl_2–methanol (1:2, 45 ml) was stirred for 13.5 h under 31 atm pressure of hydrogen. The mixture was filtered through a pad of Celite, which was then washed with methanol. The filtrate and washings were combined and concentrated. The residue was chromatographed with $CHCl_3$–methanol (5:1) to give oily residue, which was used for the next step without further purification. A solution of the obtained residue in 6 M HCl (30 ml) was stirred at 60°C for 16 h. After concentration of the mixture, the residue was dissolved in a minimal amount of water and loaded to a cation-exchange resin column (Dowex 50W-X2, H^+ form). The column was washed with water, and eluted with 5% aqueous pyridine. Ninhydrin-positive fractions were combined and evaporated. To the residue, was added 4N HCl and the solution was evaporated to give 1.2 g of **3a** as a HCl salt (82%, 2 steps): mp 149–151°C; ^1H NMR (D_2O): δ 3.20, (br d, J=3.7 Hz, 1H), 4.17 (d, J=4.1 Hz, 1H). Anal. Calcd for $C_5H_7{}^2H_3NO_4Cl$: C, 32.18; H+^2H, 5.41; N; 7.51. Found: C, 32.26; H+^2H, 5.45; N, 7.33.

2.1.4. (2*S*,3*S*)-3-[$^2H_3$]Methylaspartic acid hydrochloride (3b). Compound **2b** (1.95 g, 3.83 mmol) was treated in the same manner as described for the preparation of **3a** to give **3b** as a HCl salt (563 mg, 79%): mp 155–160°C; ^1H NMR (D_2O): δ 3.11, (br d, J=2.4 Hz, 1H), 4.21 (d, J=3.7 Hz, 1H). Anal. Calcd for $C_5H_7{}^2H_3NO_4Cl$: C, 32.18; H+^2H, 5.41; N, 7.51. Found: C, 32.32; H+^2H, 5.36; N, 7.21.

2.1.5. Dimethyl (2*S*,3*R*)-*N*-benzyl-*N*-(9-phenylfluoren-9-yl)-3-[$^2H_3$]methyl-[3-^2H]aspartate (4a) and dimethyl (2*S*,3*S*)-*N*-benzyl-*N*-(9-phenylfluoren-9-yl)-3-[$^2H_3$]methyl-[3-^2H]aspartate (4b). To a solution of **2a** (15.0 g, 29.5 mmol) in THF (450 ml) was added dropwise a solution of KHMDS in THF (65.0 ml, 0.5 M solution, 32.5 mmol) at −23°C. After being stirred at the same temperature for 15 min, D_2O (10 ml) was added. The mixture was warmed to room temperature and sat. aqueous NH_4Cl was added. The layers were separated, and the aqueous portion was extracted twice with ether. The combined organic layer was washed with brine, dried over Na_2SO_4, and evaporated. Silica gel column chromatography with hexane–EtOAc (4:1) gave a mixture of **4a** and **4b**. This isomerization and deuterium introduction procedure was repeated three times. Flash silica gel column chromatography of the mixture of **4a** and **4b** with hexane–EtOAc (6:1) gave the more polar product **4a** (4.05 g, 27%) and the less polar product **4b** (5.31 g, 35%): **4a**; mp 152–155°C; ^1H NMR ($CDCl_3$): δ 2.90 (s, 3H), 3.54 (s, 3H), 3.78 (s, 1H), 4.35 (d, J=14.1 Hz, 1H) 4.68 (d, J=14.4 Hz, 1H), 7.14–7.98 (m, 18H); ^2H NMR ($CHCl_3$): δ 0.58, 2.53. Anal. Calcd for $C_{33}H_{27}{}^2H_4NO_4$: C, 77.77; H+^2H, 6.13; N, 2.75. Found: C, 77.67; H+^2H, 6.19; N, 2.68. **4b**; mp 180–182°C; ^1H NMR ($CDCl_3$): δ 2.96 (s, 3H), 3.35 (s, 3H), 3.49 (s, 1H), 4.16 (d, J=12.9 Hz, 1H) 4.41, (d, J=13.4 Hz, 1H), 7.22–7.95 (m, 18H); ^2H NMR ($CHCl_3$): δ 1.11, 2.27. Anal. Calcd for $C_{33}H_{27}{}^2H_4NO_4$: C, 77.77; H+^2H, 6.13; N, 2.75. Found: C, 77.61; H+^2H, 6.04; N, 2.62.

2.1.6. (2*S*,3*S*)-3-[$^2H_3$]Methyl[3-^2H]aspartic acid hydrochloride (5). Compound **4b** (1.80 g, 3.53 mmol) was treated in the same manner as described for the preparation of **3a** to give **5** as a HCl salt (639 mg, 97%): mp 158–162°C; ^1H NMR (D_2O) 4.21. Anal. Calcd for $C_5H_6{}^2H_4NO_4Cl$: C, 32.01; H+^2H, 5.37; N, 7.41. Found: C, 32.22; H+^2H, 5.26; N, 7.31.

2.1.7. Benzyl 3-(*N*-*tert*-butoxycarbonyl)amino-2-[$^2H_3$]-methylpropionate (7). To a solution of **6**[16] (8.50 g, 30.4 mmol) in THF (130 ml) was added dropwise a solution of LHMDS (67.0 ml, 1.0 M solution in THF, 67.0 mmol) at −78°C. After being stirred for 10 min, [$^2H_3$]methyl iodide (4.84 g, 33.4 mmol) was added dropwise. The reaction was kept at the same temperature for 10 min and was then quenched with sat. aqueous NH_4Cl. The layers were separated, and the aqueous portion was extracted with EtOAc. The combined organic layer was washed with brine, dried over Na_2SO_4, and evaporated. Flash silica gel column chromatography with hexane–EtOAc (4:1) provided 7.50 g of **7** (83%) as an oil: ^1H NMR ($CDCl_3$) 1.43 (s, 9H), 2.72 (br t, J=5.9 Hz, 1H), 3.19–3.40 (m, 3H), 4.94 (br, 1H), 5.14 (br, 2H), 7.35 (m, 5H); ^{13}C NMR ($CDCl_3$) 13.4 (septet, J=19.7 Hz), 27.9, 39.4, 42.6, 65.8, 78.6, 127.5, 127.7, 128.1, 135.5, 155.5, 174.6; ^2H NMR ($CHCl_3$) 1.16. Anal. Calcd for $C_{16}H_{20}{}^2H_3O_4N$: C, 64.85; H+^2H, 7.82; N, 4.73. Found: C, 64.55, H+^2H, 7.70; N, 4.68.

2.1.8. 3-(*N*-*tert*-Butoxycarbonyl)amino-2-[$^2H_3$]methylpropionic acid (8). A mixture of **7** (6.96 g, 23.5 mmol) and 10% Pd–C (427 mg) in dioxane (70 ml) was stirred for 8 h under an atmospheric pressure of hydrogen. The mixture was filtered through a pad of Celite and concentrated. Recrystallization from $CHCl_3$–hexane provided 4.28 g of **8** (88%): mp 88–90°C; ^1H NMR ($CDCl_3$, ca. 2:1 mixture of conformers) major: 1.44 (s, 9H), 2.69 (br, 1H), 3.25 (br dd, J=6.1, 13.4 Hz, 1H), 3.34 (br m, 1H), 5.05 (br, 1H), 10.7 (br 1H), minor: 1.47 (s, 9H), 2.65 (br, 1H), 3.26 (br, 1H), 3.39 (br m, 1H), 6.39 (br, 1H); ^2H NMR ($CHCl_3$) 1.18. Anal. Calcd for $C_9H_{14}{}^2H_3O_4N$: C, 52.42; H, 8.31; N, 6.79. Found: C, 52.39, H, 8.61; N, 6.99.

2.1.9. 3-Amino-2-[$^2H_3$]methylpropionic acid hydrochloride (9). A solution of **8** (1.20 g, 5.82 mmol) in 4 M HCl–dioxane (2:1, 20 ml) was stirred for 1 h. The mixture was evaporated to yield 824 mg of **9** as a HCl salt (quant.): mp 119–123°C; ^1H NMR (D_2O) 2.68 (br dd, J=5.0, 8.2 Hz, 1H), 2.91 (dd, J=5.0, 13.0 Hz, 1H), 3.03 (dd, J=8.2, 13.0 Hz, 1H). Anal. Calcd for $C_4H_7{}^2H_3O_4NCl$: C, 33.69; H+^2H, 7.07; N, 9.82. Found: C, 33.54, H+^2H, 7.00; N, 9.84.

2.2. Culture of *Streptomyces halstedii* HC34 for feeding experiments

An autoclaved 100 ml seed medium (potato starch 3%, soya flake 1.5%, yeast extract 0.2%, corn steep liquor 0.5%, NaCl 0.3%, $MgSO_4{\cdot}7H_2O$ 0.05%, $CaCO_3$ 0.3%, and $CoCl_2{\cdot}6H_2O$ 0.0005%, pH adjusted to 7.1 with 3 M NaOH) in a 500 ml baffled flask equipped with a cotton plug was inoculated with a scraping from an agar slant of the producing *St. halstedii* HC 34. The culture was grown on a shaker at 27°C and 200 rpm for 48 h. Vegetative cultures (100 ml×12) were initiated by inoculation of the vegetative medium (potato starch 3%, soya flake 1.5%, yeast extract 0.2%, corn

steep liquor 0.5%, NaCl 0.3%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.3%, and $CoCl_2 \cdot 6H_2O$ 0.0005%, pH adjusted to 7.1 with 3 M NaOH) with 0.5 ml of a seed culture and were grown under the same conditions for 72 h. Isotopically labeled substrates were added twice to the culture at 24 and 48 h after inoculation.

2.3. Isolation and purification of vicenistatin

The fermentation broth (1.2 l) was centrifuged (7,000 rpm, 30 min) to give a mycelium cake. The mycelium cake was extracted overnight with acetone (2.0 l). The extracts were filtered and concentrated in vacuo to an aqueous state. The aqueous solution was extracted three times with EtOAc. The combined organic layer was dried over Na_2SO_4 and evaporated. The residue was applied to a silica gel column chromatography with EtOAc, and then $CHCl_3$–methanol (5:1). Vicenistatin containing fractions were collected and concentrated to dryness. The residue was further purified by flash silica gel column chromatography with $CHCl_3$–MeOH (9:1) to give a colorless powder of vicenistatin (ca 10 mg).

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