Biosynthesis of Lactacystin

Senji Takahas% Kenichi Uchida and Akira Nakagawa*
Department of Biosciences, Teikyo University,
1-1 Toyosatodai, Utsunomiya 320, Japan

Yoko Miyake and Masatsune Kainosho
Faculty of Science, Tokyo Metropolitan University,
1-1 Minami-Osawa, Hachioji, Tokyo 192, Japan

Keichi Matsuzaki and Satoshi Omura
School of Pharmaceutical Sciences, Kitasato University and The Kitasato Institute,
5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan

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The biosynthesis of lactacystin, a new microbial metabolite which induces differentiation of neuroblastoma cells, was studied by the feeding experiments of various 13C-labeled compounds and NMR spectroscopic analysis. The feeding experiments showed that lactacystin consists of three biosynthetic units, namely isobutyrate (and/or L-valine), L-leucine and L-cysteine. The C10 unit containing γ-lactam moiety arises by a condensation between methylmalonic semialdehyde and Cα position of L-leucine, followed by intramolecular cyclization. Two diastereotopic methyls, C-11 and C-12 of lactacystin were found to originate from the pro-R and pro-S methyls of leucine, respectively, as shown by incorporating a new type of chiral 13C-labeled L-leucine.

Materials and Methods

Microorganism
Streptomyces sp. OM-6519 was used for biosynthetic study of lactacystin.

Labeled Compounds
Sodium [1-13C] acetate (99% 13C enriched), Sodium [1-13C] propionate (99% 13C enriched) were purchased from ISOTEC Inc., U.S.A. Sodium [1-13C] isobutyrate (90% 13C enriched) was purchased from Merck Sharp & Dohme, U.S.A. dl-[2-13C,4-2H] leucine (99% 13C enriched, 80% 2H enriched) were prepared by condensation of a mixture of l-bromo-2-methylpropane5) and [2-13C] ethyl N-(diphenylmethylene) glycinate. [2-13C] Ethyl N-(diphenylmethylene) glycinate6) was prepared from [2-13C] glycine7) which was prepared from [2-13C] acetic acid (99% 13C enriched). The detailed synthetic procedure of dl-[2-13C,4-2H] leucine is described in Scheme 1. L-[2-13C] Leucine (90% 13C enriched) was prepared by the same procedure with that of dl-[2-13C,4-2H] leucine. L,1-[1,1-'13C2] cystine (99% 13C enriched) was synthesized from S-benzyl-L-[1-13C] cysteine which was prepared by optical resolution of N-acetyl-S-benzyl-DL-[1-13C] cysteine by Aspergillus acylase by method of UCHIDA and KAINOSHO8).
Scheme 1. Preparation of dl-[2-13C,4-2H] leucine.

2-Methylpropanal

Reagents: i, D2O; ii, LiAlH4, Et2O; iii, PBr3, CHCl3; iv, LDA, THF; v, HCl

[2-2H] 2-Methylpropanal (1)

A mixture of 2-methylpropanal (12 g, 166 mmol) and 2H2O (32 g, 1.6 mol) in a 100 ml of autoclave was heated at 170°C for 24 hours. The reaction mixture was distilled at 60~63°C (760 mmHg) to yield [2-2H] 2-methylpropanal (9.5 g, 78%). The enrichment factor of deuteration on C-2 position was judged to be 84% from 1H NMR.

[2-2H] 2-Methylpropanol (2)

A solution of 1 (9.5 g, 130 mmol) in dry ether (70 ml) was added dropwise to the suspension of LiAlH4 (1.48 g, 39 mmol) in dry ether (100 ml) at -20°C. The reaction mixture was stirred for overnight at room temperature. The reaction was stopped by adding ice-water (3 ml) and then extracted with ether. The ethereal solution was washed with 1 n HCl, and brine. The solution was dried on anhydrous MgSO4 and then evaporated to dryness. The residue was distilled at 104~106°C (760 mmHg) to yield a colorless oil 2 (8.4 g, 86%).

[2-2H] 1-Bromo-2-methylpropane (3)

A solution of PBr3 (11.0 g, 40 mmol) in dry dichloromethane (6 ml) was added to pyridine (1.7 g) at 0°C for 15 minutes. A mixture of 2 (8.4 g, 112 mmol) and pyridine (0.57 g) was dropped to the solution at -10°C for 2.5 hours. After stirring at room temperature for 48 hours, the solution was distilled gently by rising bath temperature until 120°C (350 mmHg). The crude product was treated with K2CO3, and then redistilled at 91~93°C (760 mmHg) to yield 3 (2.8 g, 51%).

[2-2H] 1-Bromo-2-methylpropane (3) was used in the condensation reaction with [2-13C] ethyl-N-(diphenylmethylene) glycinate and [2-2H] 1-bromo-2-methylpropane (3), followed by hydrolysis with 1 N HCl, in the yield of 42% (50% of glycine was recovered). 1H NMR (400 MHz, 1 n NaOD) δ 0.87 (3H, m, CH3), 0.89 (3H, s, CH3), 1.40 (2H, m, CH2), 1.64 (0.18H, m, CH), 3.24 (1H, m, CH). 13C NMR (100 MHz, 1 n NaOD) δ 23.88 and 24.96 (Cα, s), 26.52 (Cβ, t), 46.83 (Cγ, d), 57.44 (Ca, s), 186.90 (C=O, d). 13C-Labeled l-leucine was obtained from the fermentation broth of l-leucine producing microorganism, Brevibacterium lactofermentum AJ 3918 (FERM-P2516), using a mixture (1:2) of [U-13C6] glucose (98% 13C enriched) and non-labeled glucose as a carbon source.

General Procedure

HPLC analyses were carried out using a Senshu pak N(CH3)2 4251-N (10 x 250 mm) with mobile phase of CH3CN-H2O-AcOH (50:46:4) at a flow rate of 2.5 ml/minute equipped with a Shimadzu LC-9A system, UV detector set at 245 nm. NMR spectra was recorded on a JEOL EX-400 spectrometer at 60°C, using pyridine-d5 δ 8.05 as internal reference for 1H NMR spectra and pyridine-d5 δ 7.55 as internal reference for 13C NMR spectra.

General Procedure

Strain OM-6519 on an agar slant was inoculated into a test tube (2 x 20 cm) containing 10 ml of production medium. The production medium consisted of oatmeal 2.0% w/v; the medium was adjusted to pH 7.0 with 0.1 n NaOH. The fermentation was carried out at 27°C for 132 hours (232 rpm shaking). For the feeding experiments of 13C-labeled compounds, the additions dissolved in distilled water were made at 36 hours after inoculation and the fermentation was continued for 96 hours.

Preparation of 13C-labeled Lactacystins

13C-Labeled precursors, sodium [1-13C] isobutyrate (90% 13C, 0.05% w/v), L-[2-13C] leucine (90% 13C, 0.05% w/v), Sodium [1-13C] propionate (99% 13C, 0.04% w/v), L-L-[1,1-13C2] cystine (99% 13C, 0.03% w/v), 13C-labeled l-valine (33% 13C, 0.03% w/v) and 13C-labeled L-leucine (33% 13C, 0.06% w/v) were used.
in the feeding experiments. Each $^{13}$C-labeled precursor was fed to 36 hours old cultures of Streptomyces sp. OM-6519 grown in 10 ml of oatmeal medium in test tubes (27°C, 232 rpm shaking). The 132 hours old culture broth was centrifuged at 3,000 $\times$ g for 20 minutes and the mycelium was separated. Each supernatant (500~900 ml) was adsorbed to a charcoal (30 ml, activated charcoal, Wako Pure Chemical Ind.) column. After being washed with water, the column was eluted with 80% aqueous acetone. The eluate was concentrated in vacuo to remove acetone. The aqueous solution was passed through Amberlite XAD-2 column (20 ml) and the unbound fraction was collected. The solution was adsorbed to Dowex 1 x 4 (5 ml, 100~200 mesh, OH type) and washed with water. The column was eluted successively with 5% AcOH, 10% AcOH and then 20% AcOH solution. The eluate by 10% AcOH was adsorbed to a charcoal (4 ml) to remove acetic acid and then eluted with 80% aqueous acetone. The eluate was concentrated in vacuo to obtain a white powder. The crude powder was finally purified by preparative HPLC to afford $^{13}$C-enriched lactacystins (2~5 mg).

Results and Discussion

Biosynthetic origin of each carbon of lactacystin was investigated by $^{13}$C NMR analyses of $^{13}$C-enriched lactacystins which were obtained from the cultured broth of Streptomyces sp. OM-6519 by feeding experiments of $^{13}$C-labeled compounds. Incorporation ratio of various $^{13}$C-labeled precursors to lactacystin molecule is summarized in Table 1. The feeding experiment of L-[2-$^{13}$C] leucine exhibited a high level of incorporation into C-5 at $\delta$C 81.25. This indicates that the biosynthetic origin of C$_6$-segment (C-4, C-5, C-9, C-10, C-11, and C-12) is derived intactly from L-leucine. On the other hand, isobutyrate was assumed as a precursor for the C$_4$ unit (C-6, C-7, C-8, and C-13) from the structural feature of the $\gamma$-lactam moiety of lactacystin. The $^{13}$C NMR spectrum of lactacystin obtained by the feeding of sodium [L-$^{13}$C] isobutyrate showed the enrichment for four carbon signals at C-1, C-4, C-8, and C-14, as shown in Fig. 1. Especially, the enrichment at C-8 provides a definitive evidence that isobutyrate was incorporated to $\gamma$-lactam moiety as a intact unit. Furthermore, the enrichment at C-8 indicates that the $\gamma$-lactam ring was formed by condensation of methylmalonic semialdehyde metabolized from isobutyrate with the C$_4$ of L-leucine. The proposed mechanism for the condensation is shown in Fig. 2. It can be speculated that the $\gamma$-lactam skeleton is formed through the intermediacy of a Schiff base of methylmalonic semialdehyde with a pyridoxal phosphate cofactor, that is, normal pyridoxal phosphate electron cascade would bring about the condensation to give a $\gamma$-lactam, followed by intramolecular cyclization. A similar condensation through the intermediary of a Schiff base has been reported in the biosynthetic study of asukamycin by Nakagawa et al. A low level of enrichment of isobutyrate at the four carbon atoms seems to arise from the consequent label dilution prior to incorporation of the precursor to lactacystin molecule. The incorporation of sodium [1-$^{13}$C] isobutyrate at C-1, C-4 and C-14 also implies the presence of the metabolic pathway of isobutyrate via propionyl-CoA to acetyl-CoA and via pyruvate to cysteine in a lactacystin producing microorganism. Especially, the enrichment at C-4 indicated that the $\beta$-hydroxyleucine moiety is formed in the feeding experiments. Each $^{13}$C-labeled precursor was fed to 36 hours old cultures of Streptomyces sp. OM-6519 grown in 10 ml of oatmeal medium in test tubes (27°C, 232 rpm shaking). The 132 hours old culture broth was centrifuged at 3,000 $\times$ g for 20 minutes and the mycelium was separated. Each supernatant (500~900 ml) was adsorbed to a charcoal (30 ml, activated charcoal, Wako Pure Chemical Ind.) column. After being washed with water, the column was eluted with 80% aqueous acetone. The eluate was concentrated in vacuo to remove acetone. The aqueous solution was passed through Amberlite XAD-2 column (20 ml) and the unbound fraction was collected. The solution was adsorbed to Dowex 1 x 4 (5 ml, 100~200 mesh, OH type) and washed with water. The column was eluted successively with 5% AcOH, 10% AcOH and then 20% AcOH solution. The eluate by 10% AcOH was adsorbed to a charcoal (4 ml) to remove acetic acid and then eluted with 80% aqueous acetone. The eluate was concentrated in vacuo to obtain a white powder. The crude powder was finally purified by preparative HPLC to afford $^{13}$C-enriched lactacystins (2~5 mg).

Table 1. $^{13}$C NMR chemical shifts and $^{13}$C enrichment ratio of lactacystin from $^{13}$C-labeled compounds.

<table>
<thead>
<tr>
<th>Carbon</th>
<th>$\delta$C</th>
<th>L-[2-$^{13}$C] Leucine</th>
<th>[L-$^{13}$C] Isobutyrate</th>
<th>L-L-[1,1-$^{13}$C$_2$] Cystine</th>
<th>[L-$^{13}$C] Propionate</th>
<th>$^{13}$C-labeled L-Leucine</th>
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<tr>
<td>C-1</td>
<td>173.74</td>
<td>1.3</td>
<td>3.1</td>
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<td>1.2</td>
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<td>1.0</td>
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<td>2.1</td>
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<td>C-6</td>
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<td>C-8</td>
<td>181.28</td>
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<td>C-11</td>
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<td>C-13</td>
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<td>1.6</td>
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*a* Relative to the abundance of C-3 signal as 1.0.

*b* Measured in C$_5$D$_5$N at 60 °C.
by condensation of 2-ketoisovalerate arising from L-valine with [1-\(^{13}\)C] acetyl-CoA metabolized from [1-\(^{13}\)C] isobutyrate, followed by hydroxylation. This notion concerning the metabolism of isobutyrate was supported from the incorporation of sodium [1-\(^{13}\)C] propionate to C-1, C-4 and C-14 (Fig 1, Table 1). Although the metabolism of isobutyrate to propionyl-CoA and acetyl-CoA has been reported in polyketide biosyntheses such as macrolide and polyether antibiotics, a tracking of a long metabolic route of isobutyrate via propionyl-CoA to cysteine seems to be a rare case in biosynthetic pathways of microbial secondary metabolites. These results may indicate that the dilution of \(^{13}\)C-labeled isobutyrate with natural carbon supplied from oatmeal which is the production medium is not overwhelmingly significant. Feeding of L,L-[1,1\(^{-13}\)C\(_2\)] cystine resulted in a very high incorporation at C-1, indicating that the C\(_3\) unit (C-1, C-2 and C-3) is derived from l-cysteine, itself formed by an enzymatic reduction of the labeled cystine. The incorporation pattern of \(^{13}\)C-enriched compounds indicates that lactacystin is biosynthesized from three units, L-leucine, isobutyrate (and/or L-valine) and L-cysteine, respectively.

Our particular interest in lactacystin biosynthesis is to correlate stereochemically two diastereotopic methyl groups, C-11 and C-12, with those of L-Leucine. Baldwin et al.\(^{1,12}\) and the other group\(^{13}\) have reported the correlation of two methyls of penicillin N...
with those of L-valine, by incorporating of (2S,3S)-[4-\( ^{13}\)C] valine into penicillin N. The pro-S methyl of L-valine was found to be correlated with the \( \alpha \)-methyl at C-2 of penicillin N and with the exocyclic methylene (C-17) of cephalosporin C\(^{13}\)). Then, we tried a stereo-specific NMR assignment for the two diastereotopic methyl groups, C-11 and C-12 of lactacystin by the feeding experiment of \( ^{13}\)C-labeled L-leucine which was obtained by fermentation of a leucine-producing microorganism, \textit{Brevibacterium lactofermentum} AJ 3918, on a mixture (1:2) of 98% [U-\( ^{13}\)C\(_6\)] glucose and non-labeled glucose as a carbon source. The \( ^{13}\)C-labeled L-leucine obtained shows a non-random \( ^{13}\)C distribution reflecting the biosynthetic pathway from [U-\( ^{13}\)C\(_6\)] glucose. The \( ^{13}\)C NMR spectrum (in alkaline D\(_2\)O) of \( ^{13}\)C-labeled L-leucine exhibits four peaks (C=O/C\(_{13}\)) and

**Fig. 3.** \( ^{13}\)C NMR spectrum (100 MHz in pyridine-d\(_5\)) of lactacystin enriched from \( ^{13}\)C-labeled L-leucine and \( ^{13}\)C-\(^{13}\)C coupling pattern for the enriched carbon signals.

**Fig. 4.** \( ^{1}\)H NMR spectra of lactacystin labeled from DL-[2-\( ^{13}\)C,4-\(^{2}\)H] leucine and non-labeled lactacystin (400 MHz in DMSO-d\(_6\)).
C/\(C_3\)) with satellite signals, based on intact \(^{13}\text{C}-^{13}\text{C}\) couplings and two singlet peaks (\(C_6/C_8\)). Concerning stereospecific NMR assignment of prochiral methyls of l-leucine, Neri et al.\(^{14}\), have reported that a doublet signal appeared at \(\delta_1^c\) 22.87 and a singlet signal appeared at \(\delta_2^c\) 21.70 observed for both methyls are assignable to pro-R and pro-S, respectively. The \(^{13}\text{C}\) NMR spectrum of \(^{13}\text{C}\)-enriched lactacystin obtained by feeding experiment of \(^{13}\text{C}\)-labeled L-leucine exhibited two intact \(^{13}\text{C}-^{13}\text{C}\) coupling units, C-4/C-5 (\(J_{\text{cc}}=51.1\text{Hz}\)) and C-10/C-11 (\(J_{\text{cc}}=34.3\text{Hz}\)) and two singlet peaks, C-9 and C-12. This spectral pattern corresponds to that of l-leucine, indicating that no racemization at C-10 has occurred in the formation of the segment from leucine. Retention of the configuration at C-10 during formation of lactacystin was evidenced from the NMR spectral data of lactacystin labeled with dl-\([2-^{13}\text{C}, 4-^2\text{H}\] leucine. The \(^1\text{H}\) NMR spectrum of the labeled lactacystin exhibited a high \(^{13}\text{C}\) enrichment at C-5. The labeling pattern means incorporation of the deuterium atom of the precursor to C-10 and retention of the configuration at C-10. The incorporation was also supported from decrease (ca. 20\%) of the intensity for a multiplet signal at H-10 (\(\delta_1^h\) 1.57) in the \(^1\text{H}\) NMR spectrum of labeled lactacystin. Therefore, diastereotopic methyl groups, C-11 and C-12 are assignable as pro-R and pro-S, respectively.

The feeding experiment of a new type of \(^{13}\text{C}\)-labeled l-leucine and l-valine with \(^{13}\text{C}\) distribution reflecting the biosynthetic process from glucose provided us valuable information concerning not only biosynthetic origin of the carbon atoms but also stereospecific formation of secondary metabolites.

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