Biosynthesis of Lactacystin

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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 ^{13}C-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 C_{10} unit containing γ-lactam moiety arises by a condensation between methylmalonic semialdehyde and C_α 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 ^{13}C-labeled L-leucine.

Lactacystin[1], a novel microbial metabolite which induces differentiation of Neuro 2a cells, a mouse neuroblastoma cell line, has been found in the culture broth of *Streptomyces* sp. OM-6519. The structure of lactacystin was determined successfully by NMR spectroscopy and X-ray crystallographic analysis[2]. Lactacystin possesses a unique structural feature which consists of γ-lactam skeleton containing a hydroxyisobutyl and *N*-acetyl-cysteinylthioester. Oxazolomycin[3], a specific inhibitor of cellulose biosynthesis, also contains a similar γ-lactam skeleton in its structure. In this paper we report the biosynthetic origin of carbon atoms of lactacystin by the feeding experiments of ^{13}C-labeled compounds[4]. We also describe the stereochemical assignment of two diastereotopic methyl groups, C-11 and C-12, by NMR spectroscopic analysis of ^{13}C-enriched lactacystin obtained by feeding experiment of ^{13}C-labeled L-leucine which has been obtained from the culture of a leucine-producing microorganism, *Brevibacterium lactofermentum*.

Materials and Methods

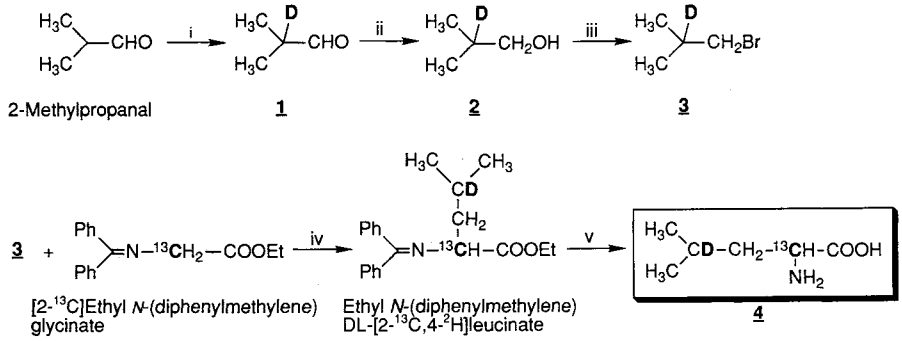
Microorganism

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

Labeled Compounds

Sodium [1-^{13}C] acetate (99% ^{13}C enriched), Sodium [1-^{13}C] propionate (99% ^{13}C enriched) were purchased from ISOTEC Inc., U.S.A. Sodium [1-^{13}C] isobutyrate (90% ^{13}C enriched) was purchased from Merck Sharp & Dohme, U.S.A. DL-[2-^{13}C,4-^{2}H] leucine (99% ^{13}C enriched, 80% ^{2}H enriched) were prepared by condensation of a mixture of 1-bromo-2-methylpropane[5] and [2-^{13}C] 1-bromo-2-methylpropane with [2-^{13}C] ethyl *N*-(diphenylmethylene) glycinate. [2-^{13}C] Ethyl *N*-(diphenylmethylene) glycinate[6] was prepared from [2-^{13}C] glycine[7] which was prepared from [2-^{13}C] acetic acid (99% ^{13}C enriched). The detailed synthetic procedure of DL-[2-^{13}C,4-^{2}H] leucine is described in Scheme 1. L-[2-^{13}C] Leucine (90% ^{13}C enriched) was prepared by the same procedure with that of DL-[2-^{13}C,4-^{2}H] leucine. L,L-[1,1'-$^{13}C_2$] cystine (99% ^{13}C enriched) was synthesized from *S*-benzyl-L-[1-^{13}C] cysteine which was prepared by optical resolution of *N*-acetyl-*S*-benzyl-DL-[1-^{13}C] cysteine by *Aspergillus* acylase by method of UCHIDA and KAINOSHO[8].

Scheme 1. Preparation of DL-[2-^{13}C,4-^{2}H] leucine.



Reagents : i , D_2O ; ii , $LiAlH_4$, Et_2O ; iii , PBr_3 , $CHCl_3$; iv , LDA , THF ; v , HCl

[2-^{2}H] 2-Methylpropanal (**1**)

A mixture of 2-methylpropanal (12 g, 166 mmol) and $^{2}H_2O$ (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-^{2}H] 2-methylpropanal (9.5 g, 78%). The enrichment factor of deuteration on C-2 position was judged to be 84% from ^{1}H NMR. ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.14 (6H, s, $CH_3 \times 2$), 2.40 (0.16H, m, CH), 9.60 (1H, s, CHO).

[2-^{2}H] 2-Methylpropanol (**2**)

A solution of **1** (9.5 g, 130 mmol) in dry ether (70 ml) was added dropwise to the suspension of $LiAlH_4$ (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 $MgSO_4$ 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%). ^{1}H-NMR (400 MHz, $CDCl_3$) δ 0.93 (6H, s, $CH_3 \times 2$), 1.43 (1H, s, OH), 1.68 (0.16H, m, CH), 3.41 (2H, s, CH_2).

[2-^{2}H] 1-Bromo-2-methylpropane (**3**)

A solution of PBr_3 (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 K_2CO_3, and then redistilled at 91~93°C (760 mmHg) to yield **3** (2.8 g, 51%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.04 (6H, s, $CH_3 \times 2$), 1.99 (0.16H, m, CH), 3.30 (2H, s, CH_2).

DL-[2-^{13}C,4-^{2}H] Leucine (**4**)

DL-[2-^{13}C,4-^{2}H] Leucine was prepared by condensation of [2-^{13}C] ethyl *N*-(diphenylmethylene) glycinate and [2-^{2}H] 1-bromo-2-methylpropane (**3**), followed by hydrolysis with 1 N HCl, in the yield of 42% (50% of glycine was recovered). ^{1}H NMR (400 MHz, 1 N NaOD) δ 0.87 (3H, s, CH_3), 0.89 (3H, s, CH_3), 1.40 (2H, m, CH_2), 1.64 (0.18H, m, CH), 3.24 (1H, m, CH). ^{13}C NMR (100 MHz, 1 N NaOD) δ 23.88 and 24.96 (C_δ, s), 26.52 (C_γ, t), 46.83 (C_β, d), 57.44 (C_α, s), 186.90 (C=O, d). ^{13}C-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*-$^{13}C_6$] glucose (98% ^{13}C enriched) and non-labeled glucose as a carbon source[9].

General Procedure

HPLC analyses were carried out using a Senshu pak $N(CH_3)_2$ 4251-N (10 × 250 mm) with mobile phase of CH_3CN-H_2O-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-d_5 δ_C 135.5 as internal reference for ^{13}C NMR spectra and pyridine-d_5 δ_H 7.55 as internal reference for ^{1}H NMR spectra.

Fermentation

Strain OM-6519 on an agar slant was inoculated into a test tube (2 × 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 ^{13}C-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 ^{13}C-labeled Lactacystins

^{13}C-Labeled precursors, sodium [1-^{13}C] isobutyrate (90% ^{13}C, 0.05% w/v), L-[2-^{13}C] leucine (90% ^{13}C, 0.05% w/v), Sodium [1-^{13}C] propionate (99% ^{13}C, 0.04% w/v), L,L-[1,1'-$^{13}C_2$] cystine (99% ^{13}C, 0.03% w/v), ^{13}C-labeled L-valine (33% ^{13}C, 0.03% w/v) and ^{13}C-labeled L-leucine (33% ^{13}C, 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 × *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 1×4 (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-enriched 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 δ_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 γ-lactam moiety of lactacystin. The ^{13}C NMR spectrum of lactacystin obtained by the feeding of sodium [1-^{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 provided a definitive evidence that isobutyrate was incorporated to γ-lactam moiety as a intact unit. Furthermore, the enrichment at C-8 indicates that the γ-lactam ring was formed by condensation of methylmalonic semialdehyde metabolized from isobutyrate with the C_α of L-leucine. The proposed mechanism for the condensation is shown in Fig. 2. It can be speculated that the γ-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 γ-lactam, followed by intramolecular cyclization. A similar condensation through the intermediacy of a Schiff base has been reported in the biosynthetic study of asukamycin by NAKAGAWA *et al.*[10] 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 β-hydroxyleucine moiety is formed

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

Carbon	δ_C [b]	^{13}C enrichment ratio [a]				
		L-[2-^{13}C] Leucine	[1-^{13}C] Isobutyrate	L,L-[1,1'-$^{13}C_2$] Cystine	[1-^{13}C] Propionate	^{13}C-labeled L-Leucine
C-1	173.74	1.3	**3.1**	**56.4**	**3.2**	
C-2	52.86	0.9	1.0	1.2	1.1	
C-3	31.28	1.0	1.0	1.0	1.0	
C-4	202.87	1.1	**2.1**	2.1	**1.7**	J=51.1 Hz
C-5	81.25	**13.1**	1.0	1.2	0.9	J=51.1 Hz
C-6	75.94	0.9	0.9	1.0	0.9	
C-7	41.80	1.0	0.9	1.1	1.0	
C-8	181.28	0.9	**2.1**	1.4	1.5	
C-9	79.91	1.0	1.0	1.1	1.1	
C-10	31.99	1.0	1.0	1.0	1.0	J=34.3 Hz
C-11	21.37	1.2	0.8	0.9	0.8	J=34.3 Hz
C-12	19.85	1.0	0.8	1.0	0.8	
C-13	10.12	1.0	0.9	1.1	1.0	
C-14	170.23	1.1	**2.0**	2.0	**2.0**	
C-15	22.99	1.6	1.0	1.1	1.1	

[a] Relative to the abundance of C-3 signal as 1.0.
[b] Measured in C_5D_5N at 60 °C.

Fig. 1. Incorporation pattern of L-[2-^{13}C] leucine and [1-^{13}C] isobutyrate to lactacystin.

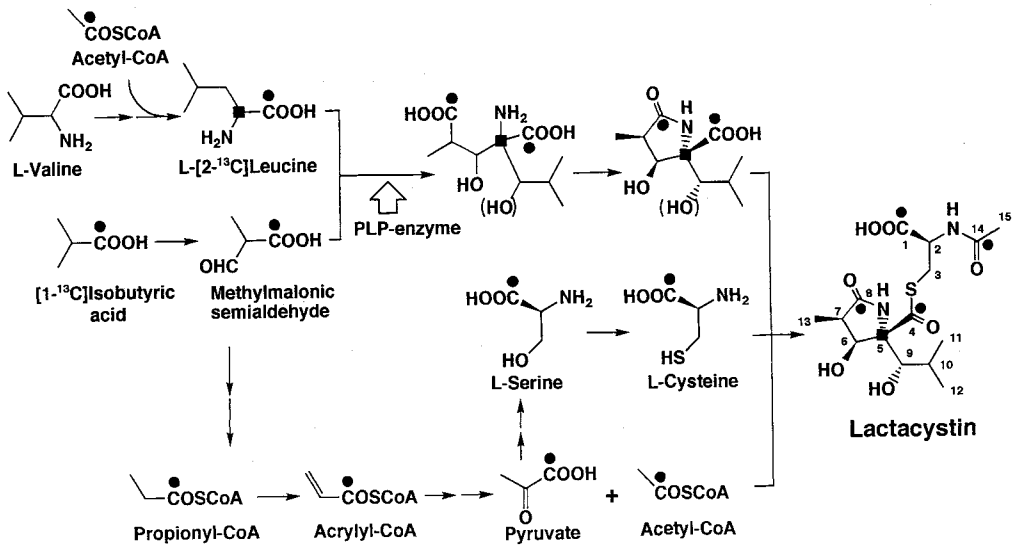
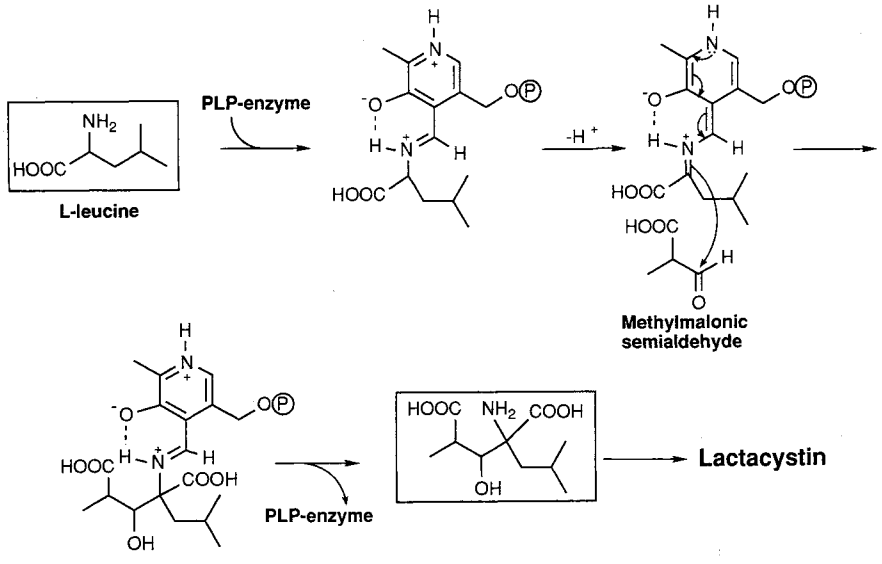


Fig. 2. Proposed mechanism for the condensation of L-leucine with methylmalonic semialdehyde.



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 pathway 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.*[11,12], and the other group[13] have reported the correlation of two methyls of penicillin N

with those of L-valine, by incorporating of (2*S*,3*S*)-[4-^{13}C] valine into penicillin N. The *pro*-S methyl of L-valine was found to be correlated with the α-methyl at C-2 of penicillin N and with the exocyclic methylene (C-17) of cephalosporin C[13]. Then, we tried a stereospecific 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, *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_2O) of ^{13}C-labeled L-leucine exhibits four peaks (C=O/C_α 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.

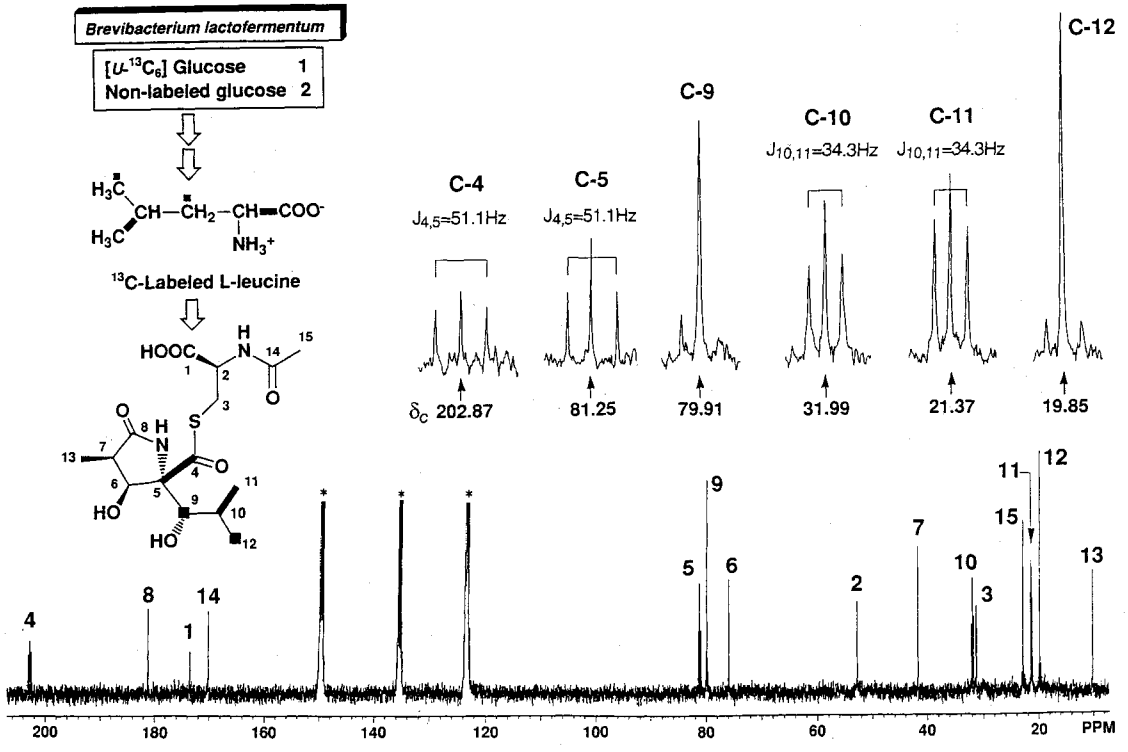
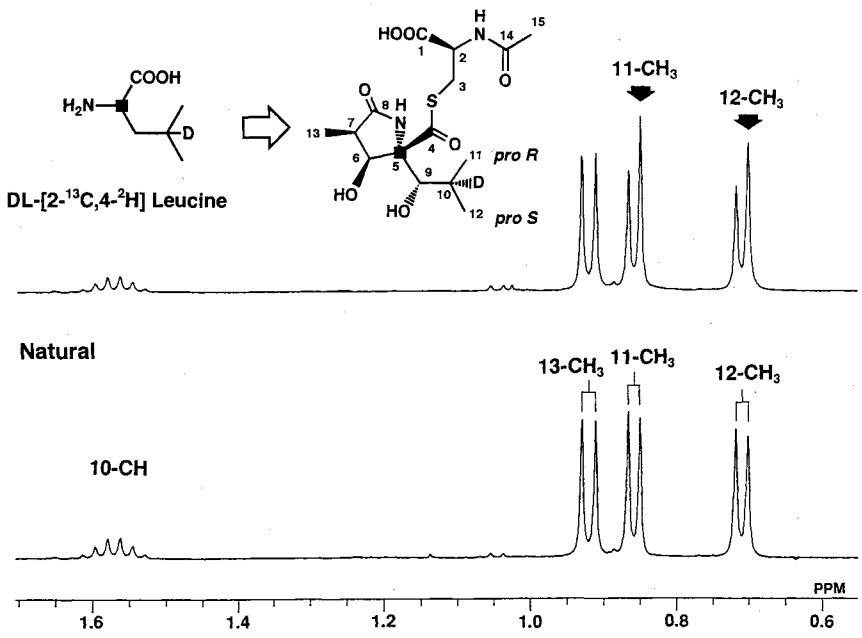


Fig. 4. ^{1}H NMR spectra of lactacystin labeled from DL-[2-^{13}C,4-^{2}H] leucine and non-labeled lactacystin (400 MHz in DMSO-d_6).



C_γ/C_δ) with satellite signals, based on intact ^{13}C-^{13}C couplings and two singlet peaks (C_β/C_δ). Concerning stereospecific NMR assignment of prochiral methyls of L-leucine, NERI *et al.*[14], have reported that a doublet signal appeared at δ_C 22.87 and a singlet signal appeared at δ_C 21.70 observed for both methyls are assignable to *pro*-R and *pro*-S, respectively. The ^{13}C NMR spectrum of ^{13}C-enriched lactacystin obtained by feeding experiment of ^{13}C-labeled L-leucine exhibited two intact ^{13}C-^{13}C coupling units, C-4/C-5 ($J_{cc}=51.1$ Hz) and C-10/C-11 ($J_{cc}=34.3$ 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}C,4-^{2}H] leucine. The ^1H NMR spectrum of lactacystin labeled with DL-[2-^{13}C, 4-^{2}H] leucine indicated the appearance of two singlet signals to overlap with each signal at the right side of doublet signals of the diastereotopic methyl groups at C-11 (δ_H 0.86) and C-12 (δ_H 0.71), as shown in Fig. 4. The ^{13}C NMR spectrum of the labeled lactacystin exhibited a high ^{13}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 (δ_H 1.57) in the ^1H 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}C-labeled L-leucine and L-valine with ^{13}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.

Acknowledgments

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