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Biosynthesis of lactacystin, a novel microbial metabolite which induces differentiation of Neuro 2a cells, a mouse neuroblastoma cell line

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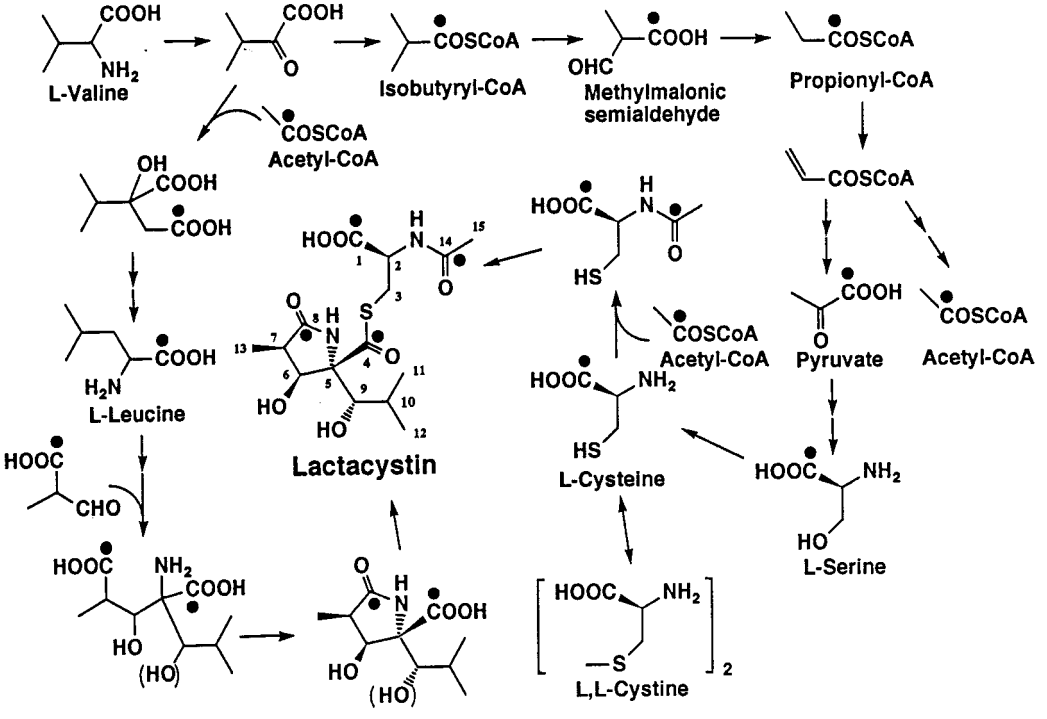
Abstruct: The biosynthesis of lactacystin was studied by feeding experiments of ^{13}C labeled compounds and NMR spectroscopy. The results indicated that lactacystin molecule derives from three biosynthetic units, isobutyrate (or L-valine), L-leucine and cysteine. The γ-lactam skeleton is formed by a condensation between methylmalonic semialdehyde and α-carbon of leucine biosynthetically, followed by intramolecular cyclization. The diastereotopic methyl groups, C-11 and C-12 were assignable as *pro*-R and *pro*-S, respectively, by feeding experiment with chirally ^{13}C-labeled L-leucine.

Lactacystin [1], a novel compound which induces differentiation of Neuro 2a cells, a mouse neuroblastoma cell line, has been isolated from the cultured broth of *Streptomyces* sp. OM-6519. A unique γ- lactam structure for lactacystin has been determined by NMR spectroscopy and X - ray crystallographic analysis [2]. The γ-lactam skeleton of lactacystin containing hydroxyisobutyl and cysteinylthioester moieties led us to study its biosynthesis. In this proceeding we present the biosynthetic pathway and aspects of its stereochemistry deduced from feeding experiments with ^{13}C enriched compounds .

^{13}C Labeled precursors, L-[2-^{13}C] leucine (90% ^{13}C, 0.05% w/v), sodium [1-^{13}C] isobutyrate (90% ^{13}C, 0.04% 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), L-[G-^{13}C] valine (preparation see below, average 33% ^{13}C per carbon, 0.03% w/v), L-[G-^{13}C] leucine (preparation see below, average 33% ^{13}C per carbon, 0.06% w/v), DL-[2 -^{13}C , 4-^{2}H] leucine (99 % ^{13}C, 88 % ^{2}H, 0.07% w/v) were fed to 24 hours old cultures of *Streptomyces* sp. OM-6519 grown in 10 ml of oatmeal medium in test tubes (27℃, 232 rpm , shaking), and fermentations were harvested 96 hours later. Each ^{13}C enriched lactacystin (2-5 mg) was isolated as a white powder from each broth filtrate (total volume ; 500 - 900 ml). The ^{13}C NMR spectra were acquired in $C_5 D_5 N$ at 60℃.

The feeding of L-[2-^{13}C] leucine gave lactacystin which showed a very intense signal (13.2 times the relative ^{13}C abundance of C-3) for C-5, indicating that the C_6-segment (C-4, C-5, C-9, C-10, C-11, and C-12) is derived from L-leucine. The feeding experiment with sodium [1-^{13}C] isobutyrate revealed equal levels of enrichment (each 2.0-3.1 times the relative ^{13}C abundance of C-3) for C-1, C-4, C-8, and C-14. The incorporation at C-8, especially, provided unequivocal evidence that the γ-lactam ring is formed by condensation of a Schiff base of methylmalonic semialdehyde with pyridoxal phosphate cofactor, with C-5 of the C_6 unit arising from L-leucine, followed by intramolecular cyclization, as shown in Scheme 1. The additional incorporation of [1-^{13}C] isobutyrate at C-1, C-4 and C-14 also indicates the presence of metabolic pathways from

isobutyrate *via* propionyl-CoA to acetyl-CoA and to cysteine. Enrichment at C-4 implies that the β-hydroxyleucine moiety was formed by condensation of 2-ketoisovalerate from valine with acetyl-CoA derived from [1-^{13}C] isobutyrate, followed by hydroxylation. This notion was further supported by a low level of enrichment at the C-1, C-4 and C-14 positions observed in the feeding experiment with [1-^{13}C] sodium propionate. Feeding of L, L-[1,1'-$^{13}C_2$] cystine [3] resulted in very high enrichment 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. Thus, the ^{13}C distribution indicates that lactacystin is biosynthesized from the following three units, L-leucine, isobutyrate (and / or L-valine) and L-cysteine, respectively.



Scheme 1. **Incorporation pattern of [1-^{13}C] isobutyrate to lactacystin**

It has been reported that two nonequivalent methyl groups of L-valine and L-leucine give rise to separate signals in the ^{13}C NMR spectra. This stereospecific correlation of the two heterotopic methyl groups of L-valine has been applied to the biosynthesis of β-lactam antibiotics [4-6] by feeding experiment with valine carrying a stereospecific ^{13}C label in one of methy groups. On the other hand, Gould *et al.* [7] and other groups have reported the usefulness of feedings of [U-$^{13}C_6$] glucose followed by analysis of ^{13}C-^{13}C coupling patterns in biosynthetic studies of microbial secondary metabolites. For confirmation of the biosynthetic origin of the above C_6-segment and stereospecific NMR assignment of the two diastereotopic methyl groups (C-11 and C-12) of lactacystin, we carried out a feeding experiment with chiral ^{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 99 % [U-$^{13}C_6$] glucose and non-labeled glucose [8] as a carbon source. The ^{13}C NMR spectrum of lactacystin enriched from the ^{13}C labeled leucine exhibited satellite peaks, Jcc = 34.3 Hz, based on intact ^{13}C -^{13}C coupling between C-10 and C-11 and Jcc = 51.1Hz between C-4 and C-5 and singlet peaks for C-9 and C-12. This corresponds to the spectral pattern of the precursor, leucine; it clearly demonstrates that L-leucine is an intact precursor of the C_6 -segment and that no racemization at C-10 has occurred in the formation of this segment from leucine. The feeding experiment of DL-[2-^{13}C, 4-^{2}H] leucine which was synthesized by condensation of [2-^{2}H]1-bromo-2-methylpropane with [2-^{13}C] ethyl N (diphenyl methylene) glycinate, carried out to determine if C-10 has undergone retention or inversion of configuration. Coupling patterns of the signals for the diastereotopic methyl groups, C-11 and C-12 (δ 1.14 and 1.07, respectively) in the ^{1}H-NMR spectrum of the labeled lactacystin, accompanied by a high ^{13}C enrichment at C-5 in the ^{13}C-NMR spectrum indicated retention of configuration at C-10. Therefore, diastereotopic methyl groups, C-11 and C-12 are assignable as *pro*-R and *pro*-S, respectively. This conclusion is also supported by the finding of no deuterium exchange at C-10 of lactacystin in a fermentation in 50% D_2O. The unequivocal outcome of this feeding experiment with chirally ^{13}C-labeled leucine sheds light on the stereospecific formation of lactacystin. Such experiments in general are valuable means to contribute to the understanding of the biosynthesis of secondary metabolites containing valine and leucine moieties.

References

1) Ōmura, S.; Fujimoto,T.; Matuszaki, R.; Moriguchi, H.; Tanaka, H.; Sasaki,Y. *J. Antibiot.* **1991**, 44, 113 -116.
2) Ōmura, S.; Matuszaki, R.; Fujimoto, T.; Kosuge, K.; Furuya, T.; Fujita, S.; Nakagawa, A. *J. Antibiot.* **1991**, 44, 117 -118.
3) Uchida, K.; Kainosho, M. *J. Labeled Comp. Radiopharm.* **1991**, 29, 867 -874.
4) Baldwin, J. E.; Loliger, J.; Rastetter, W.; Neuss, N.; Huckstep, L. L.; Higuera, de La. *J. Am. Chem. Soc.* **1973**, 95, 3796 -3797.
5) Neuss, N.; Nash, C. H.; Baldwin, J. E.; Lemke, P. A.; Grutzner, J. B. *J. Am. Chem. Soc.* **1973**, 95, 3797 -3798.
6) Kluender, H.; Bradly, C. H.; Sih, J.; Fawcett, P.; Abraham, P. *J. Am. Chem. Soc.* **1973**, 95, 6149 -6150.
7) Gould, S. J .; Cane, D. E. *J. Am. Chem. Soc.* **1982**, 104, 343-346.
8) Kainosho, M.; Kurihara, N.; Nakamatsu, T. *Japan Kokai Patent*, No. 2291284 (Dec., 3, 1990).