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Biosynthesis of the Pipecolate Moiety of Marcfortine A

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Abstract: In this paper, we demonstrate that the marcfortine A (MA) producing *Penicillium* strain incorporates L-lysine, which is metabolized to the pipecolate moiety by losing the α-amino group and not the ϵ-amino group as determined by employing [α-^{15}N]- or [ϵ-^{15}N]-L-lysine in our feeding study. Each of the enriched samples was analyzed by FAB-MS and a number of NMR experiments. The analysis of the ^{1}H NMR spectrum allowed us to estimate the absolute enrichment of the amide nitrogen (N^{β}). Analysis of HMBC spectra allowed us to establish the relative enrichments of the other two nitrogens to N^{β}. To the best of our knowledge, this represents the first time that results from an HMBC experiment have been used to quantitate the relative enrichment of an isotope. The agreement between NMR results and the FAB-MS are excellent, further supporting our conclusions. The knowledge of the specific loss of the α-amino group allows the differentiation of two plausible pathways in converting L-lysine to pipecolic acid.

Introduction

Elucidation of biosynthetic pathways using ^{13}C labels and nuclear magnetic resonance (NMR) analyses has a number of advantages in comparison to traditional experiments, which employ radio label incorporation. As a result, this approach becomes the method of choice when elucidating carbon metabolism.[1] However, researchers have faced a number of obstacles in trying to extend this method to another biologically important nucleus, ^{15}N. Chief among them is the inherent poor signal intensity of the ^{15}N nuclei. The poor S/N of the nitrogen spectrum is due to a number of the inherent properties associated with the ^{15}N nucleus: the low natural abundance, long spin−lattice relaxation time, the negative nuclear Overhauser effect, and the low gyromagnetic ratio.[2] To circumvent this problem, new methods based on reverse polarization transfer have been developed.[3] Despite the wide application of this method in many areas of chemistry[4] and biochemistry[5] in the form of multidimensional NMR spectroscopy, this method has never been applied in biosynthesis studies, especially in providing semi-quantitative information such as enrichment levels. We report here on the successful employment of this method to elucidate

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(1) See: Evans, J. N. *Biomolecular NMR Spectroscopy*; Oxford Press: New York, 1995; pp 256−265. Breitmaier, E.; Voeiter, W. *Carbon-13 NMR Spectrosopy, High-Resolution Methods and Applications in Organic Chemistry and Biochemistry*, 3rd ed.; VCH: New York, 1987; pp 451−459.

(2) See: Levy, G.; Lichter, R. L. *Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy*; John Wiley & Sons: New York, 1979; pp 2−28. Witanowski, M.; Stefaniak, M.; Webb, G. A. *Ann. Rep. NMR Spectrosc.* **1993,** *25*, 15−16.

(3) (a) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986,** *108*, 2093−2094. (b) John, B. K.; Plant, D.; Heald, S. L.; Hurd, R. *J. Mag. Reson.* **1991,** *91*, 664**.**

(4) See: Croasmun, W. R. *Two-Dimensional NMR Spectroscopy: Applications for Chemists*, 2nd ed.; 1994. Sanders, J. K. M. *Modern NMR Spectroscopy: A Guide for Chemists*, 2nd ed.; 1993.

(5) See: Evans, J. N. *Biomolecular NMR Spectroscopy*; Oxford Press: New York, 1995; pp 55−114. Clore, G. M. *NMR of Proteins*; CRC Press: Ann Arbor, 1993.

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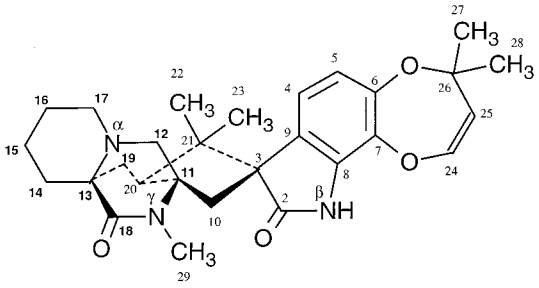
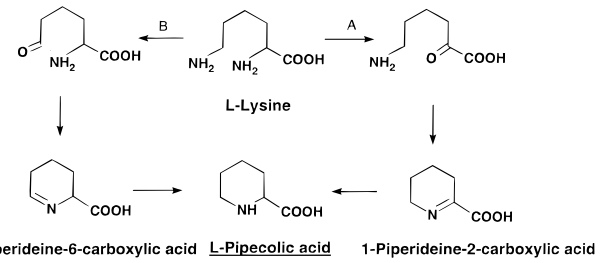


Figure 1. Structure of Marcfortine A.

Scheme 1. Two Possible Biosynthetic Pathways from Lysine to Pipecolic Acid



the biosynthetic pathway of the pipecolic acid moiety of marcfortine A (MA) (Figure 1).

MA belongs to a class of alkaloids that includes the paraherquamides[6] (PH) and brevianmides[7] (BV). The isolation of MA from a culture of *Penicillium roqueforti* has been reported previously by Polonsky et al. in 1980.[8] The interest in the biosynthetic pathways of this class of compounds has been focused on the formation of the novel bicyclo[2.2.2] ring system.[9,10] Biogenetically, it was reported that the basic skeleton of BV and PH is derived from a dioxopiperazine formed by cyclization of tryptophan and proline.[11] The two isoprene units were presumed to derive from mevalonate[12] for the BV. We demonstrated that other biogenic precursors of MA are lysine, methionine, and tryptophan.[13] The biosynthetic route converting L-lysine to the pipecolic acid has been well documented in the literature since it is widespread in microorganisms, plants, and animals. Basically, there are two general routes leading to pipecolic acid from lysine as indicated in Scheme 1.[14] Mediated by the transamination reaction, each pathway lost a specific amino group resulting two different intermediates: the α-keto-ϵ-amino caproic acid or the L-α-aminoadipic acid semialdehyde.

Microorganisms have been reported to utilize both pathways in converting lysine to pipecolic acid. To clarify this issue, we therefore tried to incorporate L-lysine specifically labeled with ^{15}N at the α or the ϵ nitrogen atom. After the sample was purified by preparative HPLC, it was then subjected to mass spectrometry (MS) and (NMR) studies in order to determine the level of incorporation. This report describes the results of our incorporation experiments to clarify the formation of the pipecolic acid moiety of MA from lysine.

Experimental Section

Materials. All materials are of analytical or HPLC grade.

Microbiological Methods: *Penicillium* sp. UC 7780 was grown in a minimal salts medium with sucrose as the sole carbon source (with the exception of labeled substrates). The composition of this medium was 20 g of sucrose, 100 mL of basal salts, 5 g of MOPS, 50 mg of K_2HPO_4, and 5 mg of $CuSO_4$ per liter of tap water. The pH of this medium was adjusted to 7.0 using NaOH. The basal salts solution contained 46.7 g of NaCl, 10.7 g of NH_4Cl, 4.26 g of Na_2SO_4, 2.03 g of $MgCl_2 \cdot 6H_2O$, 290 mg of $CaCl_2 \cdot 2H_2O$, and 3 mg of $ZnCl_2$ per liter of deionized water. The complete minimal medium was sterilized by autoclaving 100 mL volumes contained in 500 mL large mouth fermentation flasks. The minimal medium was inoculated using seed cultures grown in GS-7F medium. GS-7F contained 25 g of Pharmamedia, 25 g of Cerelose, and 5 g of malt extract per liter of tap water with its pH adjusted to 7.2 using NH_4OH. GS-7F was sterilized in the manner of the minimal salts medium. The seed medium was inoculated using three agar plugs of UC7780 stored over liquid nitrogen per flask. The seed culture was shaken at 200−250 rpm at 24 °C for 36 h and was used to inoculate the minimal salts medium at a 1−2% rate. The inoculated minimal salts medium was shaken in the manner of the seed culture for 24 h before the labeled substrates were added as filter sterilized solutions. The fermentations were continued for an additional 2−3 days before harvest.

Methods for [α-^{15}N]- L-lysine or [ϵ-^{15}N]-L-Lysine(CIL, 99%) Addition. Five hundred mg of filter-sterilized labeled L-lysine in 100 mL of water was added aseptically to 2.0 L of UC7780 at 24 h of fermentation. The fermentation was continued another 72 h before harvest.

Marcfortine Isolation. Two liter fermentation broths, containing MA derived from either [α-^{15}N] or [ϵ-^{15}N]-L-lysine were processed employing solvent extraction and preparatory HPLC methods. Specifically, two extractions using 1 L and 0.5 Ls of methylene chloride were used to remove the marcfortine from the whole beer. The extracts were dried over anhydrous Na_2SO_4 and reduced to dryness. Preparative HPLC was performed using a Waters Prep3000 instrument pumping 24.4 mL/min through three 2.5 × 10 cm cartridges plus a guard cartridge containing μBondapak C-18 resin. The mobile phase consisted of 35% ACN/H_2O (both containing 0.5 mL/L each of concentrated NH_4OH and HOAc) for 5 min, followed by a linear gradient to 65/35 at 35 min. The entire column and mobile phase reservoirs were heated to 60 °C in a large water bath. The effluent was monitored at 240 nm by a Waters 490E detector, and fractions were collected by a Foxy 200 in the peak detect mode. Fractions containing MA were pooled, respectively. The pooled samples were dissolved in 2 mL of methylene chloride and washed twice with 2 mL of 3 M NH_4OH, followed by washing twice with 4 mL of water to remove residual acid and salts. Approximately 30 mg of MA was obtained for each sample.

Mass Spectrometry. The fast atom bombardment (FAB) mass spectra were recorded on the VG ZAB-2F high-resolution mass spectrometer and the data acquired with the UPACS II mass spectrometry data system. The samples were dissolved in a matrix of 2-hydroxyethyl disulfide (2-HED) and introduced into the ion source via the FAB insertion probe. The samples were bombarded with a beam of Xenon atoms of 8 kV energy, and the resulting ions were accelerated into the analyzer at 7 kV. The source and probe temperatures were maintained at 25 °C.

Methods of NMR Spectroscopy. Proton NMR (HMR) spectra were recorded in methylene chloride (d_2, 99.9%D, Cambridge Isotope Laboratories, Cambridge, MA) solutions at 300 K (27 °C) using a Bruker AM-300 spectrometer (Billerica, MA) operating at 300.13 MHz. Sample solution volumes were ca. 500 T. One-dimensional proton spectra were recorded as free induction decays (fids) of 32K complex points and Fourier transformed. A polynomial baseline correction was applied after phasing and before peak integration. Residual methylene chloride solvent peak was used as proton reference at 5.23 ppm.

(6) Yamazaki, M.; Okuyama, E., Kobayashi, M., Inoue H. *Tetrahedron Lett.* **1981**, *22*, 135.

(7) Birch, A. J.; Wright, J. J. *Chem. Soc. Commun.* **1969**, 644.

(8) Polonsky, J.; Merrien, M.-A.; Prange, T.; Pascard, C.J. *J. Chem. Soc., Chem. Commun.* **1980**, 601.

(9) Birch, A. J.; Wright, J. J. *Tetrahedron Lett.* **1970**, *26*, 2329.

(10) Williams, R. M.; Kwast, E.; Coffman, H.; Glinka, T. *J. Am. Chem. Soc.* **1993**, *111*, 3064.

(11) Sanz-Cervera, J. F.; Glinka, T.; Williams, R. M. *Tetrahedron.* **1993**, *49*, 8471.

(12) Baldas, J.; Birch, A. J.; Russell, R. A. *J. Chem. Soc., Perkin Trans. 1* **1974**, 50.

(13) Kuo, M. S.; Wiley: V. H; Cialdella, J. I.; Yurek, D. A.;. Whaley, H. A.; Marshall, V. P. Biosynthesis of Marcfortine A. *J. Antibiot.* **1996**, *49*, 1006.

(14) Luckner, M. *Secondary Metabolism in Microorganisms, Plants, and Animals*, 2nd ed.; Springer-Verlag: New York, 1984; p 372.

Table 1. Mass Spectrometry Experimental Results

compd	peak intensity ratios: $P/P+1/P+2$ $P=478$	peak intensity ratios: $Q/Q+1/Q+2$ $Q=418$	peak intensity ratios: $R/R+1/R+2$ $R=147$
MA, unenriched sample (**1**)	100/32/6	100/60/25	100/44/34
MA derived from [α-^{15}N]-L-lysine (**2**)	100/59/18	100/106/43	100/68/63
MA derived from [ϵ-^{15}N] L-lysine (**3**)	100/148/55	100/143/102	100/172/125

Table 2. ^{1}H−^{15}N HMBC NMR Experimental Results for **1**−**3**

		cross peak assignments,[a] intensities,[b] and (percentages of control,[c] %)											
compd	atom	N−H 9.21, 9.00	12$_\alpha$−H 3.68, 3.64	N−CH$_3$ 3.09	20-H 3.01[d]	10$_\alpha$-H 2.69, 2.64	12$_\beta$-H 2.41, 2.37	14$_\alpha$-H 2.06[d]	10$_I$-H 1.91, 1.86	19$_\alpha$-H 1.70[d]	19$_\beta$−H 1.58[d]	relative enrichment (to N$^\beta$) (%)	absolute enrichment (%)
1	N$^\alpha$	0	160[f]	0	0	0	0	14[h]	0	36[h]	115[f]		
	N$^\beta$	100[e]	0	0	0	0	0	0	0	0	0		
	N$^\gamma$	0	165[f]	44	43[h]	81[f]	26[f]	0	27[f]	0	0		
2	N$^\alpha$	0	18[f] (11)	0	0	0	0	2[h] (14)	0	5[h] (14)	20[h] (17)	14[i]	1.8[k]
	N$^\beta$	100[e]	0	0	0	0	0	0	0	0	0		13[l]
	N$^\gamma$	0	97[f] (59)	29 (66)	29[h] (67)	57[f] (70)	20[f] (77)	0	20[f] (74)	0	0	69[i]	9.0[k]
3	N$^\alpha$	0	2144[f,g] (1340)	0	0	0	0	293[h] (2092)	0	733[h] (2036)	2167[h] (1884)	2000[j]	54[k]
	N$^\beta$	100[e]	0	0	0	0	0	0	0	0	0	0	2.7
	N$^\gamma$	0	76[f,g] (46)	56 (127)	63[h] (147)	126[f] (156)	38[f] (146)	0	41[f] (152)	0	0	146	3.9[k]

[a] ^{1}H NMR chemical shift values are relative to TMS as ppm's and reported under the assignments. [b] Intensities are obtained by measuring peak heights and normalized relative to the sum of the amide NH doublet. [c] Obtained by dividing the intensity with its counterpart in the control sample (1). [d] The reported δ_H is the center of a triplet. [e] Sum of intensities of an NH doublet, arbitrarily assigned as 100 for each compound. [f] Sum of intensities of an HH doublet. [g] The peak intensity is underestimated due to significant broadening caused by 15N coupling and not used for averaging. [h] Sum of intensities of an HH triplet. [i] Obtained by averaging of all nonzero intensities (in percentages of control). [j] Obtained by averaging of all nonzero (except 12I−H, see footnote *g*) intensities (in percentages of control). [k] Obtained by multiplying the absolute enrichment of N$^\beta$ by the relative enrichment. [l] Obtained by measuring the amide proton signal intensity to its NH satellites in the HMR spectrum.

The 2D ^{1}H−^{15}N heteronuclear multiple bond correlation (HMBC) NMR spectra were recorded on a Bruker ARX-400 spectrometer equipped with a 9.4 T magnet, operating at 400.13 MHz for ^{1}H and 40.55 for ^{15}N nuclei. A Bruker 5 mm broadband inverse gradient probe was used for these experiments. HMBC data were acquired using a pulse sequence developed by Bax et al.[3a] The 2D transformations were accomplished using Bruker UXNMR software on an SGI Indy computer. All samples were dissolved in CD_2Cl_2. Spectra were recorded in the magnitude mode using a 1K × 512 data table with 160 pulses per 256 increments in F1 and domain and no zero-filling in F2 domain. Sweep widths were 10.1 ppm for proton and 308 ppm for ^{15}N dimensions. All spectra were acquired at 300 K. Relaxation delays typically were 1.1 s. Intensities were measured from expanded contour plots using the cross hairs to locate the maximum signal for each cross-peak. One-dimensional ^{13}C NMR (CMR) spectra were obtained on a Bruker AM 300 spectrometer. In experiments to obtain the shift assignments, a delay of 1 s was used to obtain the broad-band and DEPT spectra. The center of the quintet of deuterated methylene chloride at 53.7 ppm was used as the carbon signal reference. For experiments where the relative intensities are measured, the reverse gated-decoupling pulse sequence was used in order to minimize relaxation and NOE effects. A delay of 15 s was used on all reverse gated-decoupling experiments. Under such experimental conditions, the control sample (unenriched MA) gave essentially equal peak intensity for all signals.

Results

Study of the Control Sample (1). We first acquired the FAB-MS spectrum of the control sample, which was obtained by fermentation followed by purification. The results are listed in Table. 1. The ratios of the abundances of the pseudomolecular ion $(M+H)^+$, or P, at *m*/*z* 478, to the P + 1 peak at *m*/*z* 479 and P + 2 peak at *m*/*z* 480 are very close to the theoretical values (100:32 and 100:6, respectively). The peak intensity data are listed in Table 1. It is important to note that all important fragment ions such as *m*/*z* 418 and 147 have a much higher isotopic abundances than the theoretical values, rendering these cluster ions unsuitable for the purpose of this study. The NMR experiments conducted for the control sample are the 1D proton, proton-decoupled ^{15}N and 2D ^{1}H−^{15}N HMBC experiments. As expected, the signal-to-noise ratio of the one-dimensional ^{15}N NMR spectrum that was acquired over an 80 h period was too poor to allow clear assessment of intensity ratios. The problem was further complicated by the fact that the two downfield nitrogen resonances are very close to each other and can cross over as a result of minute changes in experimental conditions. We then employed the 2D HMBC method to our sample. The HMBC experiment allowed us to detect all three nitrogen atoms within a 12 h period. Furthermore, the HMBC spectrum also unambiguously identified all three nitrogen signals since the proton signals all have been assigned previously.[13] Thus, the nitrogen resonance at 30 ppm is assigned as the pipecolate (henceforth referred to as N$^\alpha$), the 110 ppm signal as the indoloxyl (N$^\beta$), and the 111 ppm signal as the methylamido nitrogen (N$^\gamma$) resonances. The intensity of each cross-peak varies as a function of its *J* value.[15] The assignments and intensities of all cross-peaks are listed in Table 2. We repeated this experiment and found the relative intensities were to be reproducible (within 5%).

Results from Incorporation of [α-^{15}N]-L-Lysine. The sample derived from [α-^{15}N]-L-lysine incorporation (**2**) was subjected to a number of NMR experiments including ^{1}H, ^{13}C, and ^{15}N nuclei. The HMR experiment was conducted first. The only readily available information from this spectrum is that the N$^\beta$ amide nitrogen is significantly enriched as its attached proton displayed a set of satellite peaks due to ^{15}N−^{1}H coupling ($J^1 = 96$ Hz). The intensity ratio of the center peak to the sum of its two satellites is 6.66:1, indicating that N$^\beta$ is approximately 13% enriched. This represents a 35-fold enrichment over the background as the natural abundance of ^{15}N is 0.36%.[16] On the basis that those carbons with significantly enriched ^{15}N as their neighbors would also display detectable satellites in the ^{13}C

(15) Levy, G.; Lichter, R. L. *Nitrogen-15 Nuclear Magnetic Resonance Spectroscopy*; John Wiley & Sons: New York, 1979; pp 109−118.

(16) Mann, B E. In *NMR and the Periodic Table*; Harris R. K., Mann B. E., Eds.; Academic Press: New York, 1978; p 96; pp 120−122.

NMR spectrum, we next obtained the ^{1}H-decoupled CMR spectrum. Inspection of the spectrum revealed that the signals corresponded to C-2 and C-8, two immediate neighbors of N^{β} displayed satellites with similar ratios as those displayed in the proton spectrum. The coupling constant is 9 Hz for both sets of satellites. Further inspection of the ^{13}C NMR spectrum showed that all three carbons connecting to N^{γ} are either broad or with a detectable satellite. The broadenings of the two signals are due to the fact that the $^{13}C-^{15}N$ coupling constants approach the peak widths of these two signals. The ratio of the central signal of C-11 to the sum of the satellites is visibly lower than that of C-3, indicating that N^{γ} is less enriched than N^{β}. Equally important, no satellites or broadening of signals were detected for two of the three carbons connecting to N^{α} in the ^{13}C NMR spectrum, suggesting that this nitrogen is not significantly enriched. The signal of the third neighbor (atom number 13) of N^{α} is broader than its counterpart in the control spectrum as a result of the two-bond coupling between C-13 and N^{β}. The two-bond coupling constant can be large (7−9 Hz) for this type of coupling. In summary, the findings from the HMR and CMR spectra suggest that the ^{15}N absolute enrichment is the highest for N^{β} (13%), followed by N^{γ}, then distantly by N^{α}. However, no quantitative information can be obtained on the basis of the CMR spectrum alone as a result of the small coupling constants between ^{13}C and ^{15}N nuclei. To obtain enrichment levels of the other two nitrogen atoms, an HMBC experiment was conducted. The results are summarized in Table 2 where the relative intensity of each cross-peak is expressed as a percentage of the sum of intensity of the cross-peak $N^{\beta}-H$ doublets. These percentages vacillate as functions of $^{1}H-^{15}N$ coupling constants and the ^{15}N enrichment levels. To remove their dependence on coupling constants, the relative intensities are further normalized by the relative intensities of its counterpart in the control sample 1 and are expressed as percentages in parentheses. Since the absolute enrichment of N^{β} has been obtained unambiguously by integration of its satellites in the HMR spectrum, we can use the relative enrichment obtained in the HMBC experiment to find the absolute enrichments. Thus, the ^{15}N content of N^{α} and N^{γ} of **2** are found to be approximately 14% and 69%, respectively, of that of N^{β}. Recalling that N^{β} is 13% enriched, we therefore deduce that N^{α} and N^{γ} are 1.8% and 9.0% enriched, respectively, as summarized in Table 2. The total enrichment percentage of **2** is therefore assessed to be 23.8% by this method.

The same sample was subjected to the FAB-MS study as described for the control sample. As indicated in Table 1, the $(P + 1):P$ and $(P + 2):P$ values are 59:100 and 18:100, respectively, significantly higher than the control values. It can be rationalized that the higher $P + 1$ and $P + 2$ peak intensities are due to ^{15}N enrichment with 22% excess[17] of the control background, assuming that the excess $P + 2$ peak intensity is contributed mainly by a singly enriched nitrogen atom. However, as the percentage of the enrichment becomes higher, the contribution of the doubly enriched ^{15}N species also becomes higher, causing underestimation of the total enrichment. In our case, since the distribution of the enrichment among the three nitrogen atoms in the molecule is known, it can be estimated that an additional 1.6% ($0.13 \times 0.09 + 0.13 \times 0.018 + 0.09 \times 0.018$) should be added to the total enrichment percentage, bringing the total to 23.6% in excellent agreement to the NMR method.

Results from Incorporation of [ϵ-^{15}N]-L-Lysine. The MA sample (**3**) obtained from the [ϵ-^{15}N]-L-lysine incorporation experiment[16,17] was subjected to the same NMR and FAB-MS studies as described above. The HMR signal corresponding to the N^{β} amide proton also displayed a pair of satellites. The intensity ratio of the main signal to its satellites is 37:1, which means the ^{15}N content of N^{β} is 2.7%, a 7-fold enrichment. As a result of the small enrichment, no detectable satellites were observed for the C-2 and C-8 signals in the CMR spectrum of **3**. Similarly, the C-11 signal has a very weak satellite, suggesting N^{r} is enriched trivially also. On the other hand, the C-12 and C-17 signals displayed large satellites, suggesting that N^{α} is enriched significantly. Again, the coupling constant is not large enough to allow accurate measurement of peak ratios. To have a clear assessment of the relative enrichment of the three nitrogen atoms, an HMBC experiment was conducted for **3**, and the results are summarized in Table 2. As discussed previously, the normalized values (against N^{β} of **3**) of cross-peak intensities, when compared to their counterparts in the control sample, provided us the relative enrichment of each nitrogen atom. Examination of Table 2 reveals that cross-peaks associated with N^{α} have extremely high intensities, ranging from 1340% to 2092%. Note that the cross-peak intensity corresponding to $N^{\alpha}/12_{\alpha}$-H is smaller than the rest of the N^{α} cross-peaks. This abnormally low value is caused by the significant peak broadening of the 12^{α}-H signal as a result of substantial enrichment of the N^{α} nucleus. We therefore excluded this cross-peak in our estimation of the relative enrichment. Using the other three cross-peak ratios, the relative enrichment of N^{α} to N^{β} is calculated to be 2000%. Similarly, the relative enrichment of N^{γ} to N^{β} is estimated to be 146%, disregarding the 12_{α}-H cross-peak. Since the absolute enrichment of N^{β} has been determined to be 2.7%, it can easily be calculated that the absolute enrichment for the N^{α} and N^{γ} are 54% and 3.9%, respectively, as summarized in Table 2. The total enrichment percentage of **2** is therefore assessed to be 60.6% by this method.

As summarized in Table 1, the FAB-MS results indicated that the $(P + 1):P$ and $(P + 2):P$ values are 148:100 and 55:100, respectively, significantly higher than the control values. Again, assuming that the excess of $P + 1$ and $P + 2$ ion intensities are caused by species containing only one ^{15}N nucleus, the enrichment is calculated to be 54.5%. The small difference between the FAB-MS and NMR results can be explained again by the presence of a doubly enriched species that was omitted in the calculation. The percentage of the doubly enriched species is estimated be 3.5% for **3**, bringing the total ^{15}N enrichment to 58%, agreeing well with the NMR results.

Discussion

Our NMR and MS results indicate that the [α-^{15}N]-L-lysine was incorporated to MA with a rate of 1.8%, 13%, and 9% for the α, β, and γ nitrogen, respectively. In sharp contrast, the incorporation rate for the [ϵ-^{15}N] L-lysine was found to be 54%, 2.7%, and 3.9%, respectively, for the three nitrogens. In comparing the two sets of data, it can be easily concluded that the [ϵ-^{15}N]-L-lysine incorporation gave a more specific pattern with the enrichment concentrated on the pipecolate nitrogen. The low-level enrichments of the other two nitrogens by [ϵ-^{15}N]-L-lysine are probably due to the recycling of nitrogen atom through lysine catabolism. At the outset of this study, we suspected that feeding the fermentation with a highly enriched amino acid such as L-lysine is likely to result nonspecific labeling. The primary recycling pathway is the exchange of amino groups due to the action of transaminase. As a coenzyme for the aminotransferase, pyridoxal phosphate (PLP) can easily be enriched with ^{15}N and therefore exchanges isotopes between amino acids. Alternatively, the nitrogen atom can be recycled

(17) Obtained by finding the ratio of excess isotope ions to the total: $(59 - 32 + 18 - 6)/(100 + 59 + 18)$.

through ammonia, albeit resulting lower level of enrichment. The two nitrogens of MF derived from tryptophan can be further traced to their biogensis from anthranilic acid and L-serine, two distinct pathways.[18] Since anthranilic acid is an adduct of ammonia and chorismic acid,[19] our [ϵ-^{15}N]-L-lysine feeding results suggest that the recycling through ammonia provides about 3−4% nonspecific enrichment for this microorganism under our fermentation conditions. On the basis of those arguments, it is concluded that the enrichment of nitrogen at the pipecolate nitrogen of MA by [ϵ-^{15}N]-L-lysine is *specific*, an observation that can be rationalized only by the "A" pathway in Scheme 1. In support of this conclusion is the enrichment pattern of the [α-^{15}N]-L-lysine feeding results. The fact that **2** has the lowest enrichment at the pipecolate nitrogen clearly rule out the possibility of the "B" pathway for the pipecolate biosynthesis. The 1.8% enrichment of the pipecolate nitrogen could well be the result of recycling through ammonia as eluded earlier. The higher enrichment at the N^{β} and N^{γ} positions of MA by the [α-^{15}N]-L-lysine suggests to us that the catabolism pathways of the two amino groups of L-lysine are quite different in this *Penicillium* species with the α amino group heavily mediated by PLP, which provides a more efficient process for nitrogen turnover.

(18) Luckner, M. *Secondary Metabolism in Microorganisms, Plants, and Animals* 2nd ed.; Springer-Verlag: New York, 1984; p 385.

(19) Metzler, D. E. *Biochemistry*; Academic Press: New York, 1977; p 853−855.

Conclusion

In summary, we have incorporated L-lysine individually labeled with ^{15}N at the α or ϵ positions into MA. Each of the enriched samples was analyzed by FAB-MS and a number of NMR experiments. The analysis of the HMR spectrum allowed us to estimate the absolute enrichment of the amide nitrogen (N^{β}). Analysis of the HMBC spectra allowed us to establish the relative enrichments of the other two nitrogens to N^{β}. To our knowledge, this represents the first time that results from an HMBC experiment have been used to quantitate the relative enrichment of an isotope. The agreement between the NMR and FAB-MS results are excellent, further supporting our conclusions. The knowledge of the specific loss of the α-amino group allows the differentiation of two plausible pathways in converting L-lysine to pipecolic acid. Our results strongly suggest that the MA-producing *Penicillium* strain incorporates L-lysine to the pipecolate moiety via the α-keto-ϵ-amino caproic acid pathway.

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