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The Role of Lysine Amino Nitrogen in the Biosynthesis of Mousy Off-Flavor Compounds by *Dekkera anomala*

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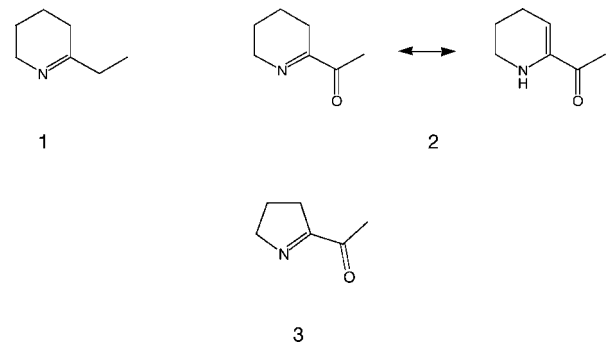
Mousy off-flavor is an insidious and economically disastrous microbiologically derived spoilage characteristic of wine and other fermented beverages. Tainted wines are rendered unpalatable and there is currently no satisfactory procedure for removal of the off-flavor. Here we report the confirmation of that both D- and L-lysine can act as a precursor for the formation of mousy off-flavor *N*-heterocycles. Further, through the use of stable isotope feeding experiments, we could establish that a pentylamine group from lysine is incorporated into the piperideine moiety of two off-flavor *N*-heterocycles. A biochemical pathway for the formation of mousy off-flavor compounds is proposed.

KEYWORDS: Lysine; mousy off-flavor; wine; *Dekkera anomala*; stable isotopes

INTRODUCTION

Mousy off-flavor is an insidious spoilage characteristic of wine and other fermented beverages. We have confirmed and extended earlier reports that mousy off-flavor is associated with yeast of the genus *Dekkera* and its anamorphic form, *Brettanomyces*, as well as with certain strains of lactic acid bacteria and acetic acid bacteria (*1*–*3*). Tainted wines are rendered unpalatable, and such an occurrence can be economically disastrous for the wine producer because no satisfactory procedure for removal of the off-flavor is available.

Previous studies have described the importance of L-lysine to the production of mousy off-flavor by *Dekkera/Brettanomyces*. Tucknott (*4*) observed that addition of L-lysine to a chemically defined culture medium stimulated the development of mousy off-flavor by *B. anomalus* (now classified as *D. anomala*) and inferred a role for L-lysine in the formation of 2-ethyl-3,4,6-tetrahydropyridine (*1*) (**Figure 1**). Subsequent work identified the tautomers 2-acetyl-1,4,5,6-tetrahydropyridine and 2-acetyl-3,4,5,6-tetrahydropyridine (these compounds are also reported in the literature as 6-acetyl-1,2,3,4-tetrahydropyridine and 6-acetyl-2,3,4,5-tetrahydropyridine (1-(3,4,5,6-tetrahydropyrdin-2-yl)ethanone and 1-(1,4,5,6-tetrahydropyrdin-2-yl)ethanone), see ref 9; described together as 2-acetyltetrahydropyridine (**2**); **Figure 1**) as responsible for mousy off-flavor in wine (*5*). Using a chemically defined medium Heresztyn (*6*) showed that the accumulation of **2** by several strains of *B. intermedius* (*D. bruxellensis*) also depended on the presence of L-lysine. This has also been recently confirmed by Lay (*7*). Costello and Henschke (*1*) established the importance of L-lysine for the formation of **2** by *Lactobacillus hilgardii* in a high cell density incubation system. We have also identified a third *N*-heterocycle, 2-acetyl-1-pyrroline (*3*) (**Figure 1**) as important to mousy off-flavor in wine (*8*). The homologue of L-lysine, L-ornithine was found to stimulate the production of **3** by *L. hilgardii* (*1*) and *Bacillus cereus* (*9*). We are aware only of a single report of the production **3** by yeast, in this case, *Kluyvero-*



1 2 3

Figure 1. Chemical structures of *N*-heterocycles: 2-ethyl-3,4,5,6-tetrahydropyridine (**1**), the tautomers 2-acetyl-1,4,5,6-tetrahydropyridine and 2-acetyl-3,4,5,6-tetrahydropyridine (**2**), and 2-acetyl-1-pyrroline (**3**).

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myces marxianus (*10*). For a detailed review of mousy off-flavor in wine see ref (*11*).

The aim of this research was to obtain a more detailed understanding of the metabolic process by which *N*-heterocyclic compounds related to mousy off-flavor are derived from L-lysine. In the investigation reported here, the biosynthesis of **1** and **2** by *Dekkera* was studied by feeding experiments with L-lysine, various acetylated and methylated L-lysine derivatives, D-lysine, and L-lysine, which was uniformly labeled with ^{15}N and ^{13}C and individually labeled α-^{15}N and ϵ-^{15}N.

The use of stable isotope labeling is an elegant technique routinely employed for the elucidation of biochemical transformations in a variety of biological systems, in particular, to investigate lysine catabolism in bacteria, yeast, and other fungi, see for example refs (*12–16*). Further, the biosynthesis of **3** by *Bacillus cereus* has been investigated via the incorporation of ^{15}N-labeled amino acids (*17*).

MATERIALS AND METHODS

Yeast Strain and Media. *Dekkera anomala* AWRI 1101 (CBS 77) was used in all experiments. The yeast were maintained on MYPG agar slants [malt extract (0.3% w/v), yeast extract (0.3% w/v), bacteriological peptone (0.5% w/v), bacteriological agar (2% w/v; Oxoid, U.K.), D-glucose (1% w/v), and calcium carbonate (5% w/v; Sigma-Aldrich, St Louis, MO, U.S.A.] and stored at 4 °C. The chemically defined medium (CDM) was adapted from Heresztyn (*18*). As *Dekkera/Brettanomyces* yeast may have a varying requirement for *myo*-inositol (*19*); this vitamin was included (25 mg/L) in the CDM.

Dose Response Relating L-Lysine and 2-Acetyltetrahydropyridine. The relationship between **2** and the concentration of L-lysine in the growth medium was investigated. A starter culture was inoculated (3 × loop-full) into CDM (60 mL in a 250 mL Erlenmeyer flask plugged with cotton wool) at pH 4.5 and incubated aerobically at 180 oscillations per minute (opm) and 27 °C. The starter culture medium did not contain L-lysine. The starter culture was used when the cell number reached 1×10^8 cells/mL, as determined by microscopic total cell counts.

Experimental cultures were made in 100 mL aliquots of CDM at pH 4.5 in duplicate, cotton wool plugged 250 mL Erlenmeyer flasks that were inoculated to a final cell density of 5×10^6 cells/mL. Media were supplemented with L-lysine·HCl at four different concentrations (10, 50, 100, and 1000 mg/L). The cultures were incubated at 180 opm and 27 °C until the stationary phase was reached, as determined by absorbance at 650 nm measurements on appropriately diluted samples of culture. After removing the cells by centrifugation at 4500 *g* for 10 min, the supernatant was then membrane-filtered (0.2 μm) and stored at −20 °C prior to extraction and analysis.

D-Lysine and L-Lysine Derivatives. The potential of D-lysine and other L-lysine derivatives to act as precursors to *N*-heterocyclic mousy off-flavor compounds was investigated by supplementing CDM with D-lysine·HCl (100 mg/L), α-acetyl-L-lysine, ϵ-acetyl-L-lysine, or ϵ-methyl-L-lysine·HCl (all at an equivalent nitrogen concentration, standardized at 100 mg/L L-lysine·HCl) or L-ornithine·HCl (100 mg/L). Further, this was also examined with L- and D-lysine as the sole source of nitrogen (equivalent total nitrogen of CDM plus 100 mg/L L-lysine·HCl). Uninoculated L-lysine·HCl (100 mg/L) and controls with no lysine added were also included. The amino acids were all purchased from Sigma-Aldrich, St Louis, MO, U.S.A. All treatments were conducted in duplicate. Uptake of lysine from the growth medium was monitored by HPLC analysis (*20*).

Stable Isotopes of L-Lysine. The production of **1** and **2** by *D. anomala* was monitored by the addition of stable isotope labeled L-lysine to culture media.

Chemicals. Initially, L-lysine uniformly labeled with the stable isotopes of ^{13}C and ^{15}N was used in a feeding experiment. The mass labeling increased the molecular weight of L-lysine·HCl from 182.6 to 190.6. In further experiments, the stable isotopes α-^{15}N-L-lysine·2HCl or ϵ-^{15}N-L-lysine·2HCl, both with a molecular weight of 220.1, were also used to determine which nitrogen of L-lysine was incorporated into the mousy off-flavor *N*-heterocycles. The purity of all stable isotope labeled L-lysine compounds was greater than 98%, and the uniformly labeled L-lysine had an isotopic enrichment of greater than 98% for ^{13}C and between 96 and 99% for ^{15}N. The isotopic enrichment for the single nitrogen labeled compound α-^{15}N-L-lysine was between 95 and 99% and greater than 98% for ϵ-^{15}N-L-lysine. The three compounds were purchased from Cambridge Isotope Laboratories, Andover, MA, U.S.A.

Uniformly Labeled L-Lysine (U-$^{13}C_6$-$^{15}N_2$-L-Lysine) and Single Nitrogen Labeled L-Lysine. Following the fermentation protocol described above, *D. anomala* fermentations were supplemented with either U-$^{13}C_6$-$^{15}N_2$-L-lysine·HCl, α-^{15}N-L-lysine·2HCl, or ϵ-^{15}N-L-lysine·2HCl (100 mg/L). Two control fermentations were also incorporated, an addition of unlabeled L-lysine·HCl (100 mg/L) and no addition of L-lysine. All treatments and controls were in triplicate, with U-$^{13}C_6$-$^{15}N_2$-L-lysine·HCl and unlabeled L-lysine·HCl and no addition of L-lysine subsequently repeated in triplicate ($n = 6$).

Analysis of Mousy *N*-Heterocycles. A reference sample of **1** was synthesized according to a modified method of (*4*). *N*-Chlorosuccinimide (26.7 g) was added to dried diethyl ether (200 mL). To this slurry, 2-ethylpiperidine (19.6 mL) was added and stirred for 15.5 h at ambient temperature under N_2. Water was then added (200 mL of Milli-Q) and the diethyl ether layer was removed. The aqueous layer was extracted with 2 × 20 mL of diethyl ether and all ether fractions were combined, filtered (Whatman No. 4), and then dried over Na_2SO_4 for 3 h. The combined diethyl ether fractions were then filtered, MeOH (200 mL) was added, and the diethyl ether was removed by rotary evaporation. All reagents were purchased from Sigma-Aldrich, St Louis, MO, U.S.A. To this 1-chloro-2-ethylpiperidine solution (20 mL, 93% purity by GC/MS), ground KOH pellets (3 g) were added and stirred for 4 h. This slurry was then filtered and passed down a K_2CO_3 column (containing about 5 g K_2CO_3, dried for 1 h at 75 °C). The column eluent was then filtered, dried over Na_2SO_4 (1 h), and stored at −20 °C. A sample was analyzed by GC/MS for mass spectral comparison with published data (*4, 21*). Authentic reference samples of **2** and **3** were kindly provided by, respectively, Peter Costello from our laboratory and Dr. Ron Buttery of the Agricultural Research Service, Western Regional Center, U.S. Department of Agriculture, Albany, CA.

The concentration of *N*-heterocycles was determined by the procedures described by refs (*1, 8*). In brief, analysis was undertaken with a Finnigan TSQ 70 mass spectrometer directly coupled to a Varian 3400 gas chromatograph. The chromatograph was equipped with a 30 m J&W Carbowax 20 CAM fused silica column, 0.25 mm i.d, and 0.25 μm film thickness. Injections were splitless (0.5 min), and the column was held at 60 °C for 3 min, programmed at 5 °C/min to 220 °C, and held at 220 °C for 5 min. Electron impact mass spectra (EIMS) were taken at an ionization voltage of 70 eV, and the scanned mass range was *m*/*z* 40–200. The concentrations of **1**, **2**, and **3** were calculated from respective molecular ion responses of **1** (*m*/*z* 111), **2** (*m*/*z* 125), and **3** (*m*/*z* 111) and the internal standard 4-acetylpyridine (Aldrich). Labeled **1** and **2** were identified by comparison of retention times with that of reference samples of unlabeled mousy off-flavor compounds and by predictions of the molecular ion and fragmentation pattern of the labeled compounds. When a single $^{15}N_1$ was incorporated, a molecular ion of *m*/*z* 112 and *m*/*z* 126 for **1** and **2**, respectively, when incorporating $^{13}C_5{}^{15}N_1$, a molecular ion of *m*/*z* 116 and *m*/*z* 131 for **1** and **2**, respectively. Concentrations were calculated from respective molecular ion responses and the internal standard 4-acetylpyridine. The assumption was made that the relative responses for the target compounds and the internal standard was unity. Precision of the analysis was considered to be a higher priority over accuracy. The precision of the analytical method is reflected in the repeat analysis of fermentations samples. The analysis of unlabeled and labeled **2** indicates high precision; unlabeled **2** (*m*/*z* 125), 86.6 ± 4.1 μg/L ($n = 6$) and $^{13}C_5{}^{15}N_1$-labeled **2** (*m*/*z* 131), 87.1 ± 3.0 μg/L ($n = 6$).

Table 1. Production of Mousy Off-Flavor *N*-Heterocycles by *D. anomala* AWRI 1101 Grown in CDM Supplemented with Various Concentrations of L-Lysine[a]

	N-heterocycles (μg/L)	
L-lysine addition (mg/L)	**1**	**2**[b]
10	trace	37.0
50	trace	66.1
100	ND[c]	72.3
1000	trace	82.0

[a] Data presented are the average of duplicate fermentations. [b] The concentration of **2** is expressed as the sum of both tautomers. [c] Not detected.

RESULTS AND DISCUSSION

As described in previous work (*1*, *4*, *6*, *7*) and in the current study, L-lysine plays a central role in the production of **1** (2-ethyltetrahydropyridine) and **2** (2-acetyltetrahydropyridine), although the mechanism by which this occurs in *Dekkera* has not been investigated. The aim of this research was to define the amino nitrogen precursors for the *N*-heterocycle mousy off-flavor compounds produced by *Dekkera* and, through this, investigate the mechanism of their biosynthesis.

Dose Response to L-Lysine. The production by *D. anomala* of mousy off-flavor compounds **1** and **2** relative to the initial concentration of L-lysine were determined and the results are described in **Table 1**. These data show that the production of **2** was stimulated by addition of an increasing concentration of L-lysine up to 100 mg/L in a near linear response. No influence of L-lysine concentration on the amount of **1** produced was found.

The dose-dependent production of **2** indicates a direct relationship between the catabolism of L-lysine and **2** production (**Table 1**). However, when the L-lysine concentration was increased from 100 to 1000 mg/L, there was not a concomitant 10-fold increase in **2**. This result suggests that there may be kinetic control over the biosynthesis of **2**, with a maximum rate of transformation at approximately a precursor concentration of 100 mg/L or that, at higher concentrations (1000 mg/L), other pathways become involved in lysine metabolism. The low efficiency of conversion of L-lysine to **2** was shown, on average 100 mg/L L-lysine produced only 82.0 μg/L **2**.

No dose response between L-lysine and **1** concentration was found, despite the fact that **1** was detected only when L-lysine was present in the growth medium (data not shown). This may be explained by several factors. There is a possibility that **1** is indirectly formed from lysine via **2**, whereby the conditions used may not have been conducive to the production of **1**. The formation of **1** in fermentations that were conducted over a longer period of time indicated that a higher **1** concentration was detected after only 4 days (data not shown). The dose response fermentations were terminated on day 3. The data suggest that the biosynthesis of **1** may be related to a time-dependent biotransformation of **2** or via other intermediates.

L-Lysine Derivatives. When L-lysine derivatives where added to the medium, α-acetyl, ϵ-acetyl, and ϵ-methyl-lysine did not act as strong stimulants for the production of **2**. No differences in yeast growth response to the three supplements of L-lysine derivatives were observed. Both ϵ-acetyl and ϵ-methyl analogues produced only a low concentration of **2**, while a higher concentration of **2** was detected when the growth medium was supplemented with α-acetyl-L-lysine (**Table 2**). Proposed analogues of **2**, that is, **2** with additional acetyl or methyl groups, were not detected by GC/MS analysis with selected ion trace monitoring.

Table 2. Production of Mousy Off-Flavor *N*-Heterocycles by *D. anomala* AWRI 1101 Grown in CDM Supplemented with L-Lysine Analogues, D-Lysine, and L-Ornithine[a]

	N-heterocycles (μg/L)		
supplement	**1**	**2**[b]	**3**
no lysine	ND[d]	ND	ND
L-lysine	0.7	83.7	ND
α-acetyl-L-lysine	ND	9.8	ND
ϵ-acetyl-L-lysine	ND	0.7	ND
ϵ-methyl-L-lysine	ND	1.7	ND
D-lysine	ND	83.8	ND
D-lysine (sole N[c])	ND	2.4	ND
L-lysine (sole N)	0.6	38.5	ND
L-ornithine	ND	2.0	0.5

[a] Data presented is an average of duplicate fermentations. [b] The concentration of **2** is expressed as the sum of both tautomers. [c] Sole source of nitrogen. [d] Not detected.

L-Ornithine. The experiment examining the role of L-ornithine in mousy off-flavor compound biosynthesis indicated that a supplementation of 100 mg/L of L-ornithine to a *D. anomala* CDM fermentation could stimulate the production of **3** (**Table 2**). This is the first report of the formation of **3** by *Dekkera* and confirms our proposal that L-ornithine is the probable precursor of this compound in yeast. Previously, the only microorganisms found to produce **3** were bacteria (*1*, *2*, *9*, *17*, *22*) and the recently reported three strains of fungi and one yeast (*10*). Romanczyk et al. (*17*) showed that the compounds L-ornithine, L-proline, and L-glutamic acid could stimulate **3** biosynthesis by *Bac. cereus*. Incorporation of ^{15}N into **3** was also described when either ^{15}N-L-proline or ^{15}N-L-glutamate were added to plate count agar inoculated with *Bac. cereus*, this study did not employ labeled L-ornithine. Under the conditions examined by (*17*) **2** was not detected, however, we observed in *Dekkera* fermentations conducted with L-ornithine as a sole nitrogen source, that **2** was produced (unpublished results) as well as in the fermentations supplemented with 100 mg/L ornithine (**Table 2**). This may indicate stimulation of a low level synthesis of **2** by induction of biosynthetic pathway(s) by L-ornithine, a homologue of L-lysine.

With respect to the production of **3** by *Dekkera*, it required high concentrations of L-ornithine relative to those reported in wine to stimulate its biosynthesis. This may indicate that *Dekkera* yeast are not responsible for the production of this compound in wine. Detection of **3** in a mousy beverage may, therefore, be an indicator of bacterial spoilage, although further investigation of other wine related strains and species of *Dekkera* is required to confirm this.

D-Lysine. *Dekkera anomala* produced **2** when growing in CDM supplemented with D-lysine (**Table 2**). HPLC analysis revealed that, like L-lysine, D-lysine was removed from the growth medium within the first 24 h (data not shown). Growth in the treatments without lysine supplementation, or containing 100 mg/L D- or L-lysine occurred at a similar rate, and all achieved almost equivalent biomass (**Figure 2**). Some **2** was detected when *D. anomala* was cultured in CDM with D-lysine as the sole source of nitrogen (**Table 2**), although growth in this medium was limited (**Figure 2**). Limited growth of *D. anomala* on D-lysine as the sole nitrogen source has not been previously described.

Lysine catabolism via a DL-racemase has been described in bacteria (*16*, *23*–*25*) and fungi (*26*), although no lysine racemase

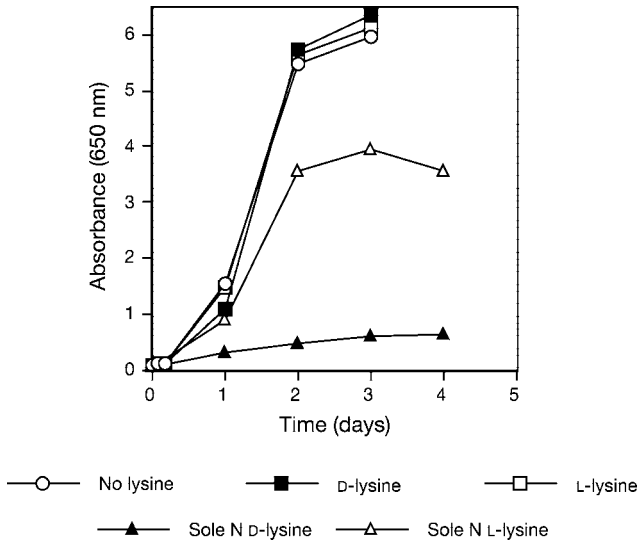

Figure 2. The growth of *D. anomala* AWRI 1101 in CDM: Without lysine, in the presence of lysine enantiomers supplemented into CDM at 100 mg/L, or lysine enantiomers as sole nitrogen sources.

activity has been previously reported for yeast. An alternative to a DL-racemase in mousy off-flavor biosynthesis would be the involvement of a nonstereospecific enzyme(s) in the biotransformation of lysine. An enzyme that catalyzes the transamination of D- and L-ornithine (as well as L-lysine) has been described in *Pseudomonas graveolens* (*27*). The activity of such an enzyme on ornithine as well as lysine is of interest when considered with the data we obtained relating to the biosynthesis of both **2** and **3** from L-ornithine.

Stable Isotopes of L-Lysine. Growth of *D. anomala* in CDM in the presence of U-$^{13}C_6$-$^{15}N_2$-L-lysine confirmed that L-lysine was the direct precursor to **2**. Compound **2** incorporating five ^{13}C and one ^{15}N, increasing the molecular mass to 131, was detected in the fermentation extracts. The mass spectra of the two $^{13}C_5{}^{15}N_1$ "heavy" tautomers of **2** are shown in **Figure 3C**,**D**). The identity of the labeled **2** was confirmed by comparison of retention time data with that of unlabeled **2** and from the predicted fragmentation pattern for **2**. The odor of extracts containing labeled **2** had the same intense mousy aroma as unlabeled **2**. The concentration of **2** detected in all treatments is shown in **Table 3**.

This confirmed that L-lysine was directly converted into **2** by *D. anomala*, where five of the six carbon units and one of the two nitrogens of L-lysine were integrated into the ring structure of **2**. Incorporation of exogenously supplied labeled U-$^{13}C_6$-$^{15}N_2$-L-lysine into **2** indicates that this amino acid was the direct precursor and did not act by stimulating a biosynthetic pathway that utilized other substrates for off-flavor compound formation. This showed that the L-lysine derived pentylamine group was exclusively incorporated into the tetrahydropyridine moiety of **2**, with five of the six labeled carbons detected in labeled **2**. A small amount of unlabeled **2** was detected in the labeled L-lysine treatment. This was expected, as there would be a background level of **2** produced from L-lysine present in the amino acid pool of the cell, as well as possibly contaminating unlabeled L-lysine present in the labeled precursor material.

Only five out of the six of the ^{13}C atoms of the uniformly labeled L-lysine precursor were detected in labeled **2**, and this demonstrated that the acetyl side chain of **2** was not provided by L-lysine. Furthermore, the detection of the fragment of mass *m*/*z* 43, that is, the unlabeled acetyl group, occurred in all treatments (**Figure 2**). If ^{13}C were incorporated into the acetyl group, an increase in its mass would have been expected. This supports earlier work that demonstrated the importance of ethanol in the formation of **2** by *Dekkera* and lactic acid bacteria (*6*). In this study, formation of **2** by bacteria did not occur without ethanol present in the growth medium and with the addition of *n*-propanol, both *Dekkera* and bacteria-produced propionyl analogues of **2**. This observation provided evidence for a chain elongation step in the biosynthesis of **2**. To further support the hypothesis that ethanol is responsible for the acetyl side chain of **2**, Costello and Henschke (*1*) showed that *L. hilgardii* produced a deuterated form of **2** in the presence of ethanol-d_6, incorporating three deuterium atoms into the acetyl side chain. In this study, a deuterated equivalent of **3** was also identified, however, no deuterated **1** was detected. These experiments indicate that ethanol is not directly responsible for the side chain of **1** and provide further evidence to suggest that **1** is a result of metabolism of **2**.

The U-$^{13}C_6$-$^{15}N_2$-L-lysine feeding experiment could not be used to show which amino nitrogen of L-lysine was incorporated into **2**, as both nitrogens were ^{15}N-labeled. Initial evidence for which amino nitrogen was incorporated to produce **2** was provided from the L-lysine derivatives study. ϵ-Nitrogen-protected L-lysine derivatives did not act as precursors to **2**. The amount of **2** detected was consistent with results obtained when no L-lysine was added (**Table 2**). The concentration of **2** detected increased, however, when the growth medium was supplemented with α-acetyl L-lysine, suggesting that *D. anomala* catabolism proceeds via removal of the α-amino group of L-lysine, producing **2** and, therefore, incorporating the ϵ-amino nitrogen.

Compound **2** was detected when α-acetyl L-lysine was added to the growth medium, however, it was at a concentration 8.7-fold less than when L-lysine was the supplement. This may reflect a low rate of uptake of the α-acetyl derivative from the medium. Attempts to confirm transport of the L-lysine derivatives from the medium using the same HPLC methodology as used for L- and D-lysine analysis were unsuccessful.

Analysis of extracts of *D. anomala* fermentations conducted with supplementation of single ^{15}N-labeled L-lysine revealed that the ϵ-nitrogen of L-lysine was incorporated into **2**. The mass spectra of this labeled **2**, with the molecular weight increased by 1 mass unit, are shown in **Figure 3A,B**. No incorporation of the α-labeled nitrogen occurred (data not shown). Quantification of the ^{15}N-**2** revealed a slightly lower concentration than expected, when compared to the addition of unlabeled L-lysine or U-$^{13}C_6$-$^{15}N_2$-L-lysine. Reduced concentration of **2** was also found in the α-^{15}N-L-lysine treatment (**Table 3**). This is expected to some extent as both ^{15}N-labeled L-lysine compounds were dihydrochlorides added at 100 mg/L, all other forms of labeled and unlabeled lysine used were monohydrchlorides also supplemented at 100 mg/L.

Labeled or "heavy" **1** was detected in the ϵ-^{15}N L-lysine and U-$^{13}C_6$-$^{15}N_2$-L-lysine treatments. The identity of these labeled products was confirmed by the same method used for **2**. The concentration of labeled **1** in both treatments was less than 2.0 μg/L. The addition of **1** mass unit was shown in the mass spectrum of **1** formed when ϵ-^{15}N L-lysine was the precursor (**Figure 4A**). The mass spectrum of the labeled **1** produced in the U-$^{13}C_6$-$^{15}N_2$-L-lysine treatment clearly shows the addition of 6 mass units, as found with **2** produced under these conditions (**Figure 4B**). As expected, **3** was not detected in any of the treatments with added labeled or nonlabeled L-lysine.

Retention of the ϵ-nitrogen shows that a deamination of the α-amino group of L-lysine is critical for the biosynthesis of **1**

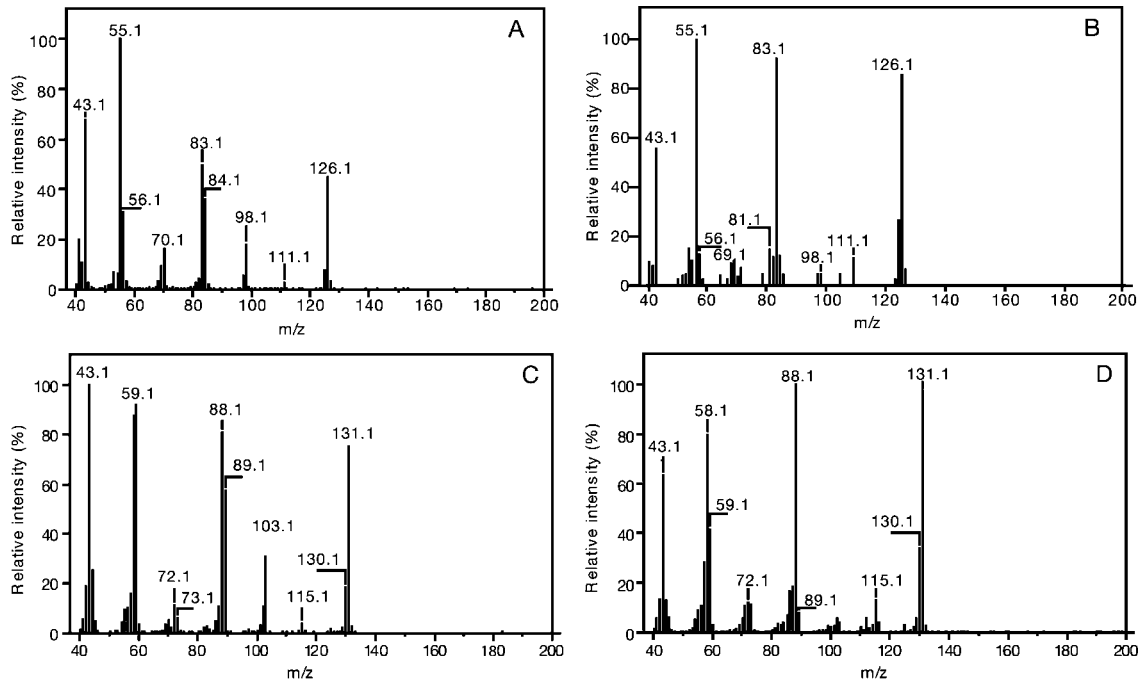

Figure 3. EI mass spectra of labeled 2-acetlytetrahydropyridine; $^{15}N_1$-labeled 2-acetlytetrahydropyridine; (**A**) tautomer I, (**B**) tautomer II and $^{13}C_5{}^{15}N_1$-labeled 2-acetlytetrahydropyridine; (**C**) tautomer I, and (**D**) tautomer II.

Table 3. Concentration of Unlabeled and Labeled 2-Acetlytetrahydropyridine Detected when *D. anomala* (AWRI 1101) was Grown in CDM Supplemented with One of the Following: No Addition, L-Lysine, and U-$^{13}C_6$-$^{15}N_2$-L-Lysine (Data Presented is Mean of Triplicate Fermentations, Repeated), α-^{15}N-L-Lysine, ϵ-^{15}N-L-Lysine (Data Presented is Mean of Triplicate Fermentations)

supplement	unlabeled **2**[a] (μg/L)	labeled **2** (μg/L)
no L-lysine	1.05 ± 0.6	ND[b]
L-lysine	86.6 ± 4.1	ND
U-$^{13}C_6$-$^{15}N_2$-L-lysine	3.2 ± 0.2	87.1 ± 3.0
α-^{15}N-L-lysine	77.6 ± 1.7	ND
ϵ-^{15}N-L-lysine	12.2 ± 2.0	62.4 ± 8.2

[a] The concentration of **2** is expressed as the sum of both tautomers with standard error. [b] Not detected.

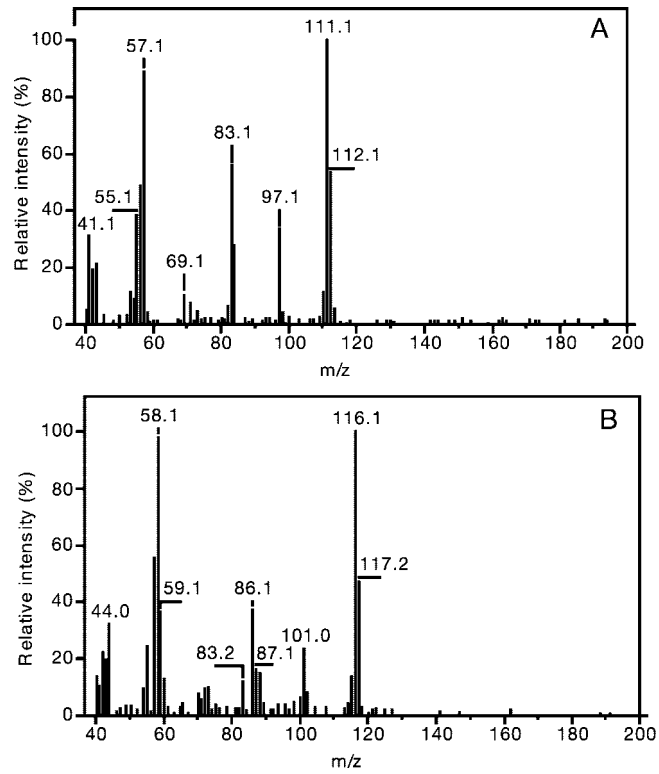

Figure 4. EI mass spectra of labeled 2-ethyltetrahydropyridine; (**A**) $^{15}N_1$-labeled and (**B**) $^{13}C_5{}^{15}N_1$-labeled.

and **2**. While investigating L-lysine catabolism in yeast, Hammer et al. (*28*), found that the principle pathway of L-lysine catabolism in *D. anomala* was via ϵ-aminotransferase, however, the stable isotope study above indicated that this enzyme was not involved in **2** synthesis. The removal of the α-nitrogen of L-lysine has been reported in other biological systems. A deaminating α-oxidase has been described in the fungi *Trichoderma viride* (*29*) and in chicken liver microsomes (*30*). The incorporation of the ϵ-^{15}N amino group of lysine in the production of the lysine catabolic intermediate L-pipecolic acid has been reported in range of organisms (*12, 31–36*). The incorporation of ϵ-^{15}N amino group into pipecolate by the fungi *Rhizoctonia leguminicola* was reported by Guengerich and Broquist (*37*), however, this finding has been disputed (*15*). There have not been any reports of α-amino deamination of lysine in yeast, except after ϵ-N-acetylation (*28, 37–41*). However, no activity of the enzyme responsible for ϵ-*N*-acetylation of L-lysine in *D. anomala* was detected (*28*). If the biosynthesis of **2** occurred via *N*-acetylation, it would be expected that the supplementation with the *N*-acetyl lysine derivatives results in the production of a typical concentration of **2** (that is, **2** concentration produced from 100 mg/L L-lysine). As only a low concentration of **2** was produced from the *N*-acetyl L-lysine derivatives, this suggests that *N*-acetylation does not play a role in mousy off-flavor biosynthesis, although uptake of these compounds was not confirmed.

The stable isotope feeding experiments unequivocally identified L-lysine as the key precursor for **1** and **2** biosynthesis. It was determined that C_5N tetrahydropyridine ring structure of **1** and **2** was provided by L-lysine. Furthermore, the ϵN of L-lysine was characterized as the nitrogen atom incorporated into these heterocycles. These findings provided the necessary data to

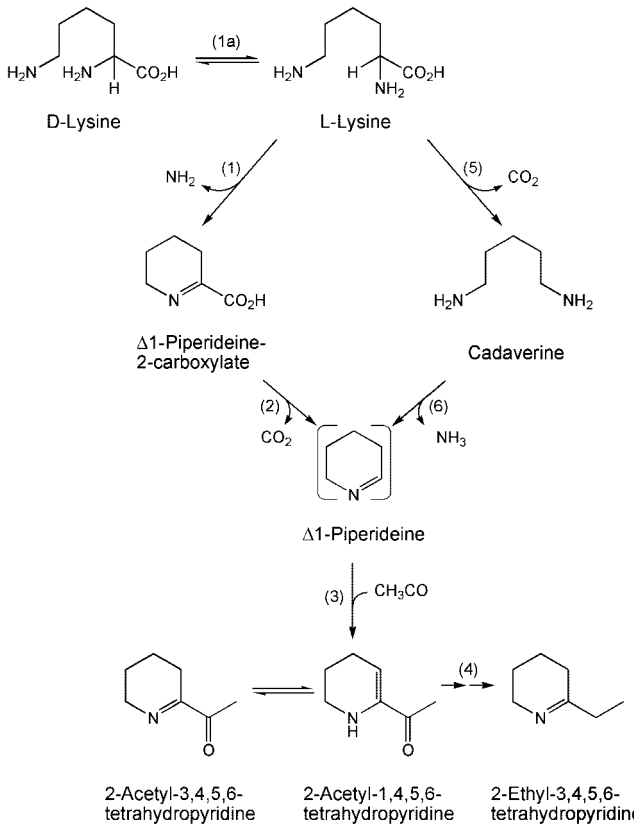

Figure 5. Proposed pathway(s) of biosynthesis of 2-acetyl-1,4,5,6-tetrahydropyridine/2-acetyl-3,4,5,6-tetrahydropyridine and 2-ethyl-3,4,5,6-tetrahydropyridine. Pathway A: reactions (1)→(2)→(3)→(4). Pathway B: (5)→(6)→(3)→(4). L-Ornithine can be substituted for L-lysine to produce the C_4N intermediate required for the biosynthesis of 2-acetyl-1-pyrroline. Proposed enzyme-mediated reactions: (1) L-lysine α-aminotransferase or α-oxidase (deaminating), (1a) lysine racemase, (2) Δ^1-piperideine-2-carboxylic acid decarboxylase, (3) acylase via acetyl-CoA or other condensation reaction, (4) dehydrogenase, (5) L-lysine decarboxylase or L-ornithine decarboxylase, (6) diamine aminotransferase or putrescine oxidase (deaminating).

propose a biosynthetic pathway(s) for the formation of these compounds (**Figure 5**). That D-lysine can also act as a precursor for **1** and **2** biosynthesis must also be considered.

Provision of the C_5N backbone structure of **1** and **2** by L-lysine suggests that biosynthesis would proceed via the synthesis of the piperideine or tetrahydropyridine ring through intermediates such as Δ^1-piperideine or Δ^1-piperideine-2-carboxylic acid (P2C; **Figure 5**). Δ^1-Piperideine can be formed by the deamination of cadaverine, which is produced by an initial decarboxylation of L-lysine (**Figure 5**; reaction (5)), while P2C can be produced directly from the α-*N*-deamination of lysine (**Figure 5**; reaction (1)). Therefore, two distinct initial steps of lysine catabolism could be involved in the biosynthesis of the tetrahydropyridine ring. In the same way, ornithine can be transformed to produce the proposed **3** biosynthetic intermediate, the pyrroline ring, through either Δ^1-pyrroline or Δ^1-pyrroline-2-carboxylic acid. Δ^1-Pyrroline as the intermediate has been previously proposed, in both nonenzymatic Maillard studies (*44*) and investigations of bacterial metabolism (*1, 9*).

Which Pathway. Evidence for the role of the critical intermediate Δ^1-piperideine, in either pathway, was obtained from the stable isotope study that also determined that only the ϵ-amino nitrogen of L-lysine was incorporated into **1** and **2**. This data eliminates other possible routes for tetrahydropyridine biosynthesis. That D-lysine can also act as a precursor suggests that pathway B is the least likely biosynthetic route, due the stereospecificity described for biogenic amine metabolism (*45*). Although cadaverine/putrescine analysis was not undertaken, the distinctive and recognizable aroma of these compounds was not detected in any fermentation or extract, however, these compounds could have been present below their sensory detection thresholds. This indicates further that pathway B is a less likely option. The possibility of an enantiomeric conversion of D-lysine via a racemase (**Figure 5**; reaction (1a)) must be verified experimentally before this pathway can be completely discounted.

The initial reaction of pathway A (**Figure 5**; reaction (1)) is proposed to occur via an α-aminotransferase or by an α-oxidase. L-Lysine α-aminotransferase has not been widely described, and it has been suggested that this transformation is unlikely to occur (*46, 47*). However, an enzyme with some L-lysine α-aminotransferase activity (with L- and D-ornithine as well) has been described (*42, 43*). While α-aminotransferase activity is common in the catabolism of a wide variety of amino acids and a number of alternatives to this ubiquitous reaction for L-lysine catabolism have been described in nature. This suggests that this is the unlikely option in the biosynthesis of **2**.

The alternative, via an α-oxidase, may be the more likely as these enzymes require oxygen and all studies we conducted were aerobic. Specific lysine or ornithine enzymes are probably not involved; otherwise, the production of these compounds would be more favorable. A question remains regarding the stereospecificity of α-oxidase enzymes. In general, oxidases are stereospecific (*48*), however, the induction of L-amino acid oxidase activity by D-amino acids has been reported and could explain **2** biosynthesis from D-lysine. Alternatively, as postulated in pathway B, a lysine racemase may function.

In summary, pathway A, which utilizes an α-amino acid oxidase, would appear the most likely approach for the biosynthesis of mousy off-flavor compounds. This choice best fits the available evidence, although the other options of an α-aminotransferase or via pathway B cannot be completely eliminated. The ratio of conversion of L-lysine to **1** and **2** indicated that this biotransformation was not efficient and suggested that biosynthesis of these off-flavor compounds was not the principal pathway of L-lysine catabolism at least under the conditions examined. The addition of acetaldehyde (**Figure 5**; reaction (3)) to produce **2** might involve an "Umpolung"-type step similar to decarboxylation of alpha-keto acids or acyloin condensations, and it may occur either before or after cyclization of the heterocyclic ring.

The catabolism of the amino acids, L-lysine, D-lysine, and L-ornithine, has provided confirmation that a biological transformation is required for mousy off-flavor production, rather than a nonenzymatic chemical synthesis. Although previous studies have provided strong evidence for the biological nature of mousy off-flavor spoilage, this study has provided conclusive evidence of an enzyme-mediated synthetic pathway in *Dekkera* yeast.

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