Biosynthesis of 2-Methoxy-3-isopropylpyrazine in *Pseudomonas perolens*†

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On the basis of the results of labeling experiments, endogenous valine, glycine, and methionine are considered to be the precursors of 2-methoxy-3-isopropylpyrazine (MIPP) produced by *Pseudomonas perolens*. It is postulated that the feeding of [2-^{13}C]pyruvate results in the labeling of C-2 and C-3 of valine and C-2 of glycine, which are subsequently incorporated into MIPP. The feeding of [3-^{13}C]-pyruvate results in the labeling of the two methyl groups of valine, C-1 and C-2 of glycine, and the methyl group of methionine. ^{13}C NMR spectra of the MIPP produced by *P. perolens* in these feeding studies were consistent with the proposed biosynthetic route to MIPP and known metabolic pathways. Methyl-labeled methionine arises from [2-^{13}C]glyoxylate formed from [3-^{13}C]pyruvate via the Krebs cycle and glyoxylate shunt.

INTRODUCTION

The potential pathways for the formation of alkylpyrazines have been discussed by several authors (Hodge, 1953; Koehler et al., 1969; Reineccius et al., 1972; Hodge et al., 1972; Rizzi, 1972, 1988; Manley et al., 1974; Shibamoto and Bernhard, 1977). However, few experiments involving labeled potential precursors of the methoxypyrazines have been carried out. One difficulty in conducting such studies has been the extremely low concentrations (parts per billion level) of methoxypyrazines formed.

2-Methoxy-3-isopropylpyrazine (MIPP) has been found in several plants and microorganisms, but its biosynthesis has remained obscure and controversial. Previously, Murray et al. (1970) proposed a biosynthetic pathway for the formation of alkylmethoxypyrazines in vegetables (Figure 1); however, this mechanism has not been supported by others (Nursten and Sheen, 1974; Morgan, 1976; McIver and Reineccius, 1986). Recently, Gallois et al. (1988) proposed that valine and glycine are possible precursors of MIPP in *Pseudomonas taetrolens*. They obtained MIPP labeled at C-2 when L-[1-^{13}C]valine was fed to this organism, but [^{13}C]glycine was not incorporated into MIPP.

This paper describes our investigation on the biosynthesis of MIPP in *Pseudomonas perolens* grown in a medium containing [2-^{13}C]- or [3-^{13}C]pyruvate as the sole source of carbon.

MATERIALS AND METHODS

Recovery of the MIPP. One-day-old cultures of *P. perolens* [a mutant of ATCC 10757 isolated by McIver and Reineccius (1986)] grown in a nutrient broth was centrifuged at 11000*g* for 10 min, and the sedimented cells were washed three times with sterilized distilled water. One milliliter of this washed cell isolate was added to 100 mL of M-56 minimal salt medium (Carlton and Brown, 1981), yielding a cell density of approximately 10^6 cfu/mL in a 500-mL Erlenmeyer flask which was then sealed by a sterilized sponge plug. The culture was incubated at 25 °C with shaking at 180 rpm. Sodium pyruvate enriched 99% at position 2 or 3 (Isotec Inc., Miamisburg, OH) was dissolved in 5 mL of distilled water and filter-sterilized (Rainin, Inc., Woburn, MA) by using a 0.45-μm filter and then added to the minimal salts medium at a final concentration of 1% as the sole carbon source. During the course of incubation, the flask was connected (1 h every 24 h) to a purge and acid trap device previously described to recover the MIPP (Cheng and Reineccius, 1991). Ten milliliters of 6 N HCl was used to collect the MIPP, which was assayed by ultraviolet (UV) spectroscopy.

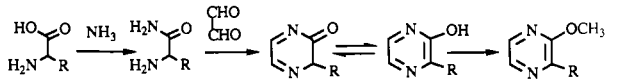

Figure 1. Hypothetical formation of 3-alkyl-2-methoxypyrazines from amino acids and glyoxal proposed by Murray et al. (1970).

MIPP shows two absorption peaks in the UV range (Gerber, 1977). In this study, the λ maxima of MIPP in 6 N HCl were found at 220 (ε = 9290) and 307 nm (ε = 9270). MIPP was collected from the cultures over an incubation period of 7 days.

Pyrazine Identification. The identification of MIPP from cultures was accomplished by comparing GC retention time and mass spectra to those of authentic MIPP (Pyrazine Specialists, Atlanta, GA). A Kratos MS-25 gas chromatograph–mass spectrometer (GC–MS) (Kratos Analytical, Ramsey, NJ) was used to gather MS data. The GC oven program used was as follows: initial temperature 45 °C, initial time 1 min, program rate 10 °C/min, final temperature I 150 °C, final time I 1 min, program rate II 30 °C/min, final temperature II 250 °C, final time II 5 min. The injection port and flame ionization detector (FID) temperatures were 225 and 275 °C, respectively. The same GC column, a 15 m × 0.32 mm (i.d.) DB-5 fused silica capillary column (J&W Scientific Inc., Rancho Cordova, CA), was used in the GC and GC–MS studies. All MS spectra were obtained at 70 eV. The retention time for MIPP under these conditions was 10.4 min.

Analysis of MIPP by Nuclear Magnetic Resonance (NMR). The collected MIPP·HCl solution was crystallized from solution by evaporating the solvent under vacuum using a Speed Vac (Savant, Inc., Farmingdale, NY) to obtain pure MIPP·HCl. The pure MIPP·HCl was then dissolved in 0.5 mL of D_2O. The amounts of MIPP obtained and examined by NMR were about 0.13 and 0.106 mg in the spectra illustrated in Figures 4 and 5, respectively. These spectra were obtained on a Nicolet NT-300 NMR spectrometer (General Electric, Fremont, CA) operating

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Table I. ^{13}C NMR Chemical Shifts (δ) of Unenriched MIPP in $CDCl_3$, D_2O, and DCl (10%) Solutions

carbon no.	δ (ppm from TMS)		
	$CDCl_3$	D_2O[a]	DCl (10% in D_2O)
2	158.4	157.7	160.1
3	151.5	151.8	147.6
5	135.4	135.9	123.1
6	138.9	138.6	142.9
7	29.6	29.5	28.3
8, 9	20.3	20.8	17.7
10	53.9	53.9	55.1

[a] One drop of dimethyl sulfoxide was added.

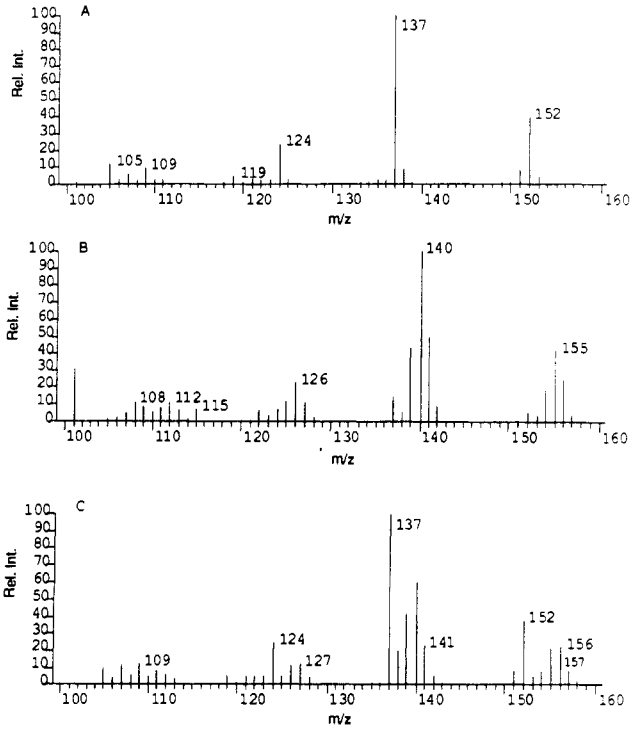

Figure 2. Mass spectra of (A) unenriched MIPP, (B) MIPP resulting from sodium [2-^{13}C]pyruvate feeding experiment, and (C) MIPP resulting from sodium [3-^{13}C]pyruvate feeding experiment.

at 75.46 MHz with the following instrument parameters: 15 000 transients, acquisition time 0.23 s, RG 10 μs, DE 50 μs.

RESULTS AND DISCUSSION

Previous work by Morgan (1976), McIver and Reineccius (1986), and Cheng and Reineccius (1991) has demonstrated that the addition of exogenous amino acids does not improve the yield of MIPP from microbial cultures. Labeled pyruvate, albeit a more distant precursor of MIPP, was used in this study since its incorporation into valine, glycine, and methionine is well documented in the literature (Wagner et al., 1964; Lehninger, 1982). Also *P. perolens* grows well on a medium containing pyruvate as the sole source of carbon.

Feeding experiments were carried out with ^{13}C-labeled sodium pyruvate. The incorporation of labeled carbon into MIPP was then determined by ^{13}C NMR spectroscopy. The ^{13}C chemical shifts [δ ppm with respect to tetramethylsilane (TMS)] and mass spectrum of natural MIPP (isolated from *P. perolens* cultures) are shown in Table I and Figure 2A, respectively.

Two-dimensional NMR techniques were used to assign the chemical shifts of C-5 and C-6 of MIPP. The HETCOR pulse sequence correlated the carbon resonances with the resonances of the attached hydrogens. The chemical shifts of H-5 and H-6 of MIPP have previously been reported (Bramwell and Wells, 1972, 1973).

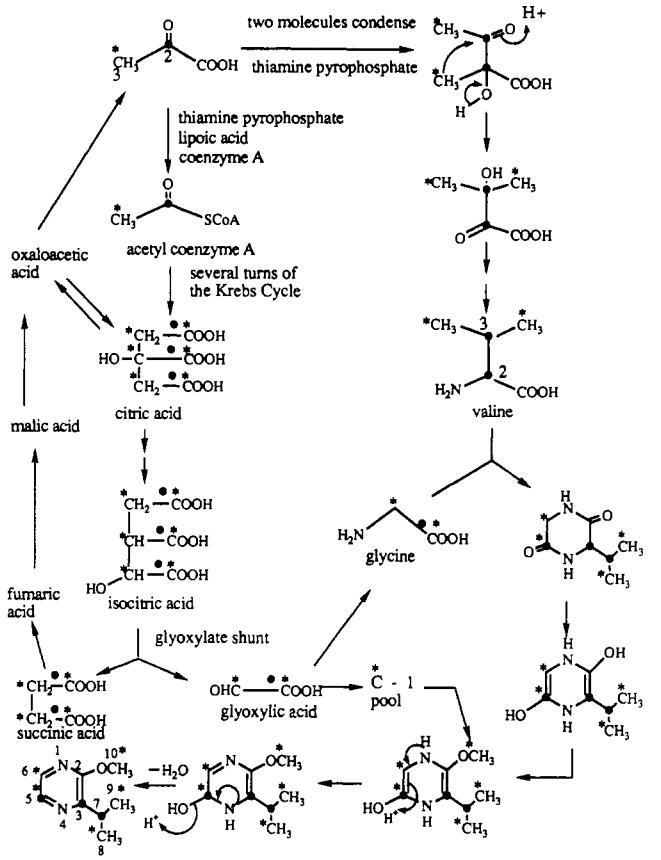

Figure 3. Proposed biosynthesis of MIPP derived from labeled sodium pyruvate feeding experiments. (●) [2-^{13}C]Pyruvate; (*) [3-^{13}C]pyruvate.

The chemical shifts of the pyrazine carbons are quite sensitive to the pH of the solution, and this accounts for the slight differences observed in the spectra of the enriched samples of MIPP. In Table I, the chemical shifts of authentic MIPP in three different conditions are reported. The chemical shifts of MIPP in $CDCl_3$ and D_2O were very close to the data of Gallois et al. (1988). When measured in 10% DCl solution, the chemical shifts of MIPP changed either downfield or upfield depending on the position of the individual carbon atom.

The ^{13}C NMR spectra of the enriched MIPP depicted in Figures 4 and 5 are similar to those of the MIPP in D_2O rather than acidified D_2O. MIPP, originally in its protonated form, may have undergone hydrolysis in redissolving the product from lyophilization in D_2O since the pK_a values for pyrazine (Cheeseman and Werstiuk, 1972) are 0.65 and −5.8.

Label at C-2 of Sodium Pyruvate. Pyruvate is a known precursor of valine (Wagner et al., 1964). Sodium pyruvate, labeled with ^{13}C at C-2, will presumably yield valine labeled at C-2 and C-3 as illustrated (Figure 3). The incorporation of this labeled valine into MIPP would then yield MIPP labeled specifically at C-3 and C-7. Examination of the ^{13}C NMR spectrum (Figure 4) of the enriched pyrazine revealed a large enrichment of these positions. Furthermore, the presence of these contiguous ^{13}C atoms in the same molecule resulted in spin–spin coupling and the production of satellite signals on either side of the C-3 and C-7 signals. The remarkably high intensity of these satellite peaks indicated a high level of incorporation of [2,3-^{13}C]valine derived from [2-^{13}C]pyruvate. The observed coupling constant (J = 47.3 Hz) is typical for sp^2–sp^3 coupled carbons (Wray, 1979).

The ^{13}C NMR spectrum of the MIPP derived from [2-^{13}C]pyruvate also showed a significant enhancement of

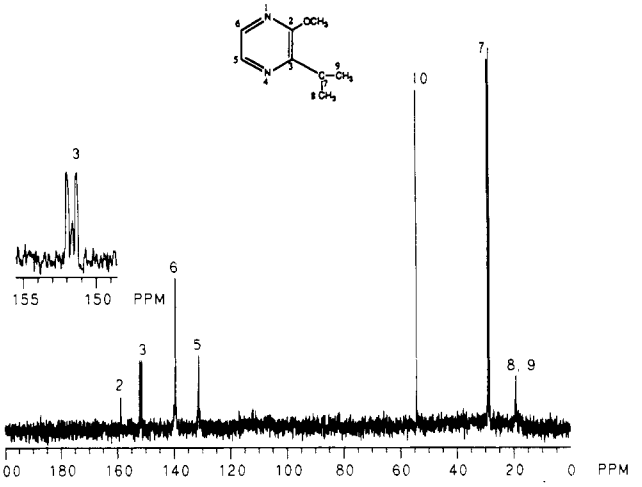

Figure 4. ^{13}C NMR spectrum of MIPP·HCl (in D_2O) resulting from sodium [2-^{13}C]pyruvate feeding experiment.

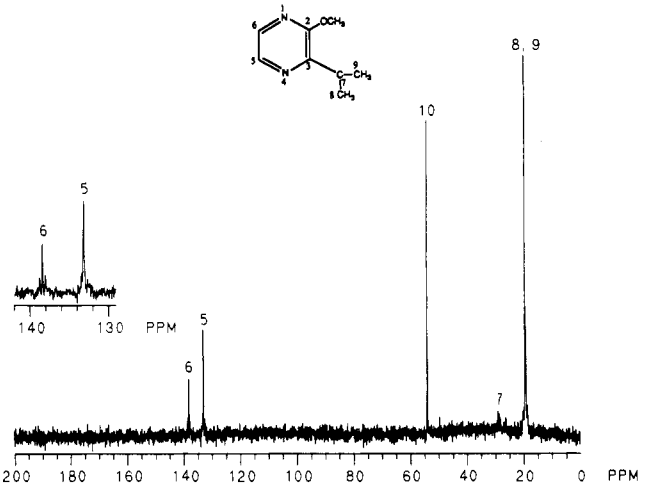

Figure 5. ^{13}C NMR spectrum of MIPP·HCl (in D_2O) resulting from sodium [3-^{13}]pyruvate feeding experiment.

the signal arising from C-5 relative to the signals for C-8 and C-9 that were unenriched. In a typical unenriched ^{13}C spectrum of MIPP, the signal due to C-8 and C-9 was 4–5 times as intense as the C-5 signal. According to the biosynthetic scheme in Figure 3, the C-5 carbon is derived from the carboxyl group of glycine. The observed enhancement of C-5 is consistent with the known metabolism of pyruvate. Oxidative decarboxylation of [2-^{13}C]pyruvate will afford [1-^{13}C]acetyl coenzyme A. Entrance of this material into the Krebs cycle will ultimately, after several turns of the cycle, yield citric and isocitric acid labeled only on its carboxyl groups. Cleavage of this isocitric acid in the glyoxylate shunt affords succinic acid and glyoxylic acid labeled only on their carboxyl groups. Transamination of this glyoxylic acid then affords [1-^{13}C]glycine.

The mass spectrum (Figure 2B) of the MIPP derived from [2-^{13}C]pyruvate is also consistent with these proposed metabolic reactions. The molecular ion (M^+ at 152) in unenriched MIPP is shifted to 155, indicative of a preponderance of molecules containing three ^{13}C atoms. The peak at 137 (M – 15) due to loss of the C-10 methyl group is replaced in the enriched sample with a major peak at 140.

Label at C-3 of Sodium Pyruvate. The results obtained by feeding [3-^{13}C]pyruvate were complementary to those previously described and also yielded information on the source of the *O*-methyl group of MIPP.

Following the pathway illustrated in Figure 3, feeding *P. perolens* [3-^{13}C]pyruvate would yield valine labeled in its two methyl groups. Assuming the incorporation of valine into MIPP, these methyl groups would ultimately become C-8 and C-9 of MIPP. The ^{13}C NMR spectrum of the enriched MIPP (Figure 5) does indeed show enhancement of the signals due to these carbons.

In terms of glycine labeling, entrance of [2-^{13}C]acetyl coenzyme A derived from [3-^{13}C]pyruvate into the Krebs cycle would result in distribution of the label as illustrated in the structures of citric and isocitric acid in Figure 3. The distribution of label that occurs when methyl-labeled acetate enters the Krebs cycle has been discussed in detail by Spenser (1968) and Leete (1971). Thus, the glyoxylic acid and glycine derived by this pathway would be labeled on both carbons and would result in labeling of C-5 and C-6 of MIPP. The coupling of these contiguous carbons was indeed observed in the ^{13}C NMR of the enriched MIPP (Figure 5). The observed coupling of these two carbons (J = 57.2 Hz) is typical for the coupling of adjacent aromatic carbons. The specific incorporation of the two-carbon unit (presumably glycine) into C-5 and C-6 of the pyrazine was significantly less than that of the valine-derived portion as evidenced from the lower intensity of the satellite peaks at C-5 and C-6. This lower specific incorporation is probably due to the fact that glycine formation from pyruvate requires a larger number of metabolic steps than the formation of valine.

The aldehyde carbon of glyoxylic acid would enter the C-1 pool via reaction with tetrahydrofolic acid, reduction, and transfer to the *S*-methyl group of methionine (Staunton, 1978). Thus, the MIPP derived from [3-^{13}C]pyruvate exhibited a significant enhancement of the signal arising from the *O*-methyl group (C-10). Signals arising from enriched carbons (C-2, -3, -7) were not observed or were of very low intensity.

The mass spectrum of the MIPP derived from [3-^{13}C]-pyruvate was less clear-cut than that derived from [2-^{13}C]-pyruvate. However, a significant peak at 157 indicated the incorporation of five ^{13}C atoms (from [4,5-$^{13}C_2$]valine, [1,2-$^{13}C_2$]glycine, and [*methyl*-^{13}C]methionine). A significant peak at 141 that is 4 mass units higher than the natural abundance fragment (M – CH_3) was also present.

In conclusion, our results are consistent with the initial condensation of valine and glycine to yield a 2,5-diketopiperazine, which then yields 2-methoxy-3-isopropylpyrazine after enolization, O-methylation from the C-1 pool, and a proton shift and dehydration. Aspergillic acid is produced by a similar condensation of leucine and isoleucine in *Aspergillus flavus* (MacDonald, 1961, 1970). The failure of Gallois et al. (1988) to obtain incorporation of [^{13}C]glycine into MIPP may be due to the fact that the glycine was quickly assimilated by *P. taetrolens* before biosynthesis of MIPP began (suggestion of the authors).

An alternate biosynthetic scheme for the formation of MIPP would be the conversion of 3-isopropyl-2,5-diketopiperazine to 3-isopropyl-2-(1*H*)pyrazinone (or its tautomer 2-hydroxy-3-isopropylpyrazine) which would then undergo O-methylation to yield MIPP. Such methylations have been shown to occur in nonenzymic reactions between 2-(1*H*)pyrazinones and various methylating agents. While we cannot rule this mechanism out, the reaction conditions where this mechanism has been observed were much more severe (temperatures well in access of 100 °C) than those employed in this study.

The biosynthetic pathway of 2-methoxy-3-isopropylpyrazine has been discussed from the aspect of precursors of the MIPP structure; however, formation of the ring structure of pyrazine via enzyme-mediated reactions or purely chemical reactions was not investigated.

Griffith and Hammond (1989) studied the generation of Swiss cheese flavor by the reaction of amino acids with

carbonyl compounds and reported that several alkylpyrazines can be formed from a mixture of lysine–acetoin, lysine–acetol, or ornithine–dihydroxyacetone at room temperature (25 °C). They suggested that the final stages of flavor formation in Swiss cheese were dominated by chemical reactions rather than enzymic or microbiological processes. Work done by Reps et al. (1987) has shown that low molecular weight carbonyl compounds are produced by *Lactobacillus bulgaricus* in an amino acid growth media. Several monocarbonyls (acetaldehyde, butyraldehyde, and valeraldehyde) and dicarbonyls [glyoxal, methylglyoxal, and (hydroxymethyl)glyoxal] were identified. Rizzi (1988) has provided further support for the hypothesis that pyrazines can be formed under very mild reaction conditions. He found the formation of alkylpyrazines from acyloin and ammonium salts under mild in vitro conditions (22 °C) and was successful in isolating individual pyrazines depending on the acyloin and ammonium salt precursors used.

On the basis of the findings and suggestions of Griffith and Hammonds (1989), Reps et al. (1987), and Rizzi (1988), the possibility exists that the formation of MIPP is due, in part, to chemical reaction rather than microbial action. However, the precursors used to form pyrazines in test tubes (in vitro) (Griffith and Hammond, 1989; Rizzi 1988) are different from the precursors (valine and glycine) we found in biological systems (in vivo) for MIPP biosynthesis. Under the same mild conditions (Griffith and Hammond, 1989; Rizzi, 1988), amino acids, by themselves, cannot form pyrazines and become aromatic resonance structures. While we have not demonstrated that amino acids were directly incorporated into MIPP, our data strongly suggest that pyruvate was metabolized to amino acids which were then converted to MIPP. Enzymes of microbial origin must be involved in mediating the MIPP ring-closure mechanism of these amino acids.

Additionally, MIPP has never been produced in vitro (in the absence of enzymes) by mixing together amino acids and carbonyl compounds. The methylation of the enol of the amide

does not occur under so-called "biomimic condition" and needs an enzyme to catalyze the transfer of a methyl group from *S*-adenosyl-L-methionine. Thus, we believe that MIPP production is an enzyme-catalyzed reaction rather than a chemical reaction.

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Registry No. MIPP, 25773-40-4; valine, 72-18-4; glycine, 56-40-6; methionine, 63-68-3; pyruvate, 127-17-3.