Food Chemistry **28** (1988) 299–309

Study of the Biosynthesis of 3-Isopropyl-2-Methoxypyrazine Produced by *Pseudomonas taetrolens*

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(Received 24 June 1987; revised version received 25 September 1987; accepted 25 September 1987)

ABSTRACT

The biosynthesis pathway of 3-isopropyl-2-methoxypyrazine (1) by Pseudomonas taetrolens *was studied. Cultivation of the bacterium in a broth containing* L-*leucine as a unique source of carbon and nitrogen allowed an improvement of the yield of 1 which was up to 10 mg/litre.* ^{13}C-L-*valine was introduced into the medium. Involvement of the amino-acid in the 1 biosynthesis could be proved by following the incorporation of the labelling by both MS and* ^{1}H *and* ^{13}C-*NMR. Attempts to introduce* ^{13}C-*glycine (which was supposed to be the second precursor) into the 1 molecule were unsuccessful.*

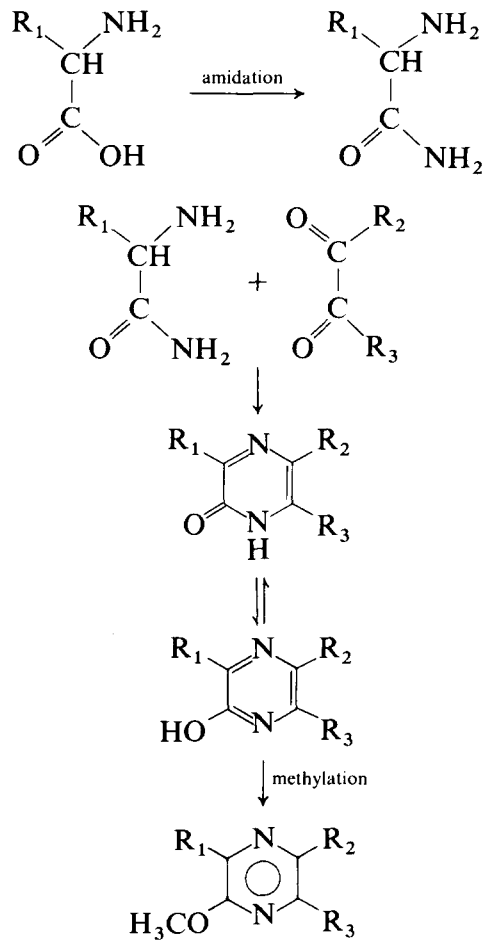
INTRODUCTION

Alkylmethoxypyrazines play a great role in flavour chemistry. Since they are associated with raw foods or produced by microorganisms, it is of interest to elucidate the still unknown pathway involved in their biosynthesis.

Only one hypothesis has been proposed up to now, by Murray *et al.* (1970) for alkylmethoxypyrazines found in vegetables. This pathway suggested condensation of α-amino acids with α,β-dicarbonyl compounds (Scheme 1). According to this scheme, 3-isopropyl-2-methoxypyrazine would thus be derived from valine, with glyoxal as the α,β-dicarbonyl compound.

Nursten & Sheen (1974) disputed this hypothesis, pointing out that neither α-amino acid amides nor glyoxal have ever been isolated from plant

Food Chemistry 0308-8146/88/$03·50



Scheme 1. Pathway formation of alkylmethoxypyrazines (according to Murray *et al.*, 1970).

tissue or microorganisms and suggested glyoxylic acid (which is part of intermediary metabolism in plants) as the dicarbonyl precursor.

Murray & Whitfield (1975) admitted that the role of α-amino acids and the source of the second nitrogen remained uncertain but confirmed the involvement of α,β-dicarbonyl compounds in the pyrazine ring formation.

Since then, however, no other pathway has been suggested. This hypothesis seems disputable. For one thing, the reaction of an amino acid amide (if it could be formed) with a dicarbonyl compound is chemically unlikely; because of the electroattractive effect of the carbonyl group, the amide function is less reactive than the amine one and therefore an α-amino-alcohol cannot be easily obtained.

A condensation between two amino-acids seemed to us more likely and

this work was undertaken to study this pathway for 3-isopropyl-2-methoxypyrazine (1) produced by *Pseudomonas taetrolens*, which could thus result from condensation of glycine and valine.

The first step of this study was the enhancement of production of 1 by growing *P. taetrolens* on the most favourable substrates (amino acids and carbon compounds), using nearly the same approach as Morgan (1976). This author tried unsuccessfully to elucidate the mechanism of formation of this pyrazine, produced by the same microorganism grown on milk. All his attempts at growing the bacterium with valine or valine amide, associated with possible C_2 precursors (provided as esters) to obtain a better yield, failed. These results were confirmed later by McIver & Reineccius (1986); studying the influence of the medium composition on the synthesis of 1 by cultures of *P. perolens* (ATCC 10757), they obtained a maximum yield with pyruvate, lactate or nutrient broth as C source (about 50 μg/litre with the parental strain and up to 12·5 mg/litre with a mutant). However, they observed that the nitrogen source had no influence on the production of 1; the addition of N compounds, and especially L-valine, did not increase the amount of 1 produced by the cells. We were more successful, as we obtained a 1000-fold increase of the yield of 1 (up to 10 mg/litre), by feeding *P. taetrolens* with suitable amino acids. In a second step, incorporation of ^{13}C-labelled compounds into 1 was followed by trapping and analysing the molecule by both GC–MS and NMR.

MATERIAL AND METHODS

Bacterial species

The *P. taetrolens* strain used in this study was isolated by Dumont *et al.* (1983) from a Munster cheese (a French smear-coated type). It had been recognized as responsible for a potato-like off-flavour which appeared during ripening. The isolated strain was shown to be similar to the ATCC 4683 type-strain of *P. taetrolens*. Both were Gram negative mobile short rods with polar flagella. They did not produce any pigment, grew in two days at 10°C but did not grow at 41°C. Both were catalase- and oxidase-positive. Their cultural characteristics on API galeries (API-CH: 50 carbon sources, API-AO: 50 organic acids and API-AA: 50 nitrogen sources, API-SYSTEM, La-Balme-les-Grottes, France) were similar.

Cultural conditions

One-litre Erlenmeyer flasks containing 250 ml of (A), (B) or (C) broth (Table 1) autoclaved at 120°C for 15 min were inoculated with 2·5 ml of an

TABLE 1
Composition of the Different Broths used

Broth	*Composition*	*Concentration*[a]
A	Yeast extract (Difco)	5·0
	D (+)-glucose	10·0
	Bacto-tryptone (Difco)	10·0
	Potassium phosphate, dibasic	2·0
B	Sodium phosphate, monobasic	1·5
	Potassium phosphate, monobasic	1·0
	Magnesium sulphate	0·2
	Amino-acid	10·0
	or	
	Carbon compound +	10·0
	Ammonium nitrate	2·0
C	Medium B +	
	L-leucine	10·0

[a] Grams per litre of distilled water.

overnight (A) broth culture of *P. taetrolens*, plugged with card-wool and incubated at 23°C on a rotary shaker (200 rpm) for 90 h.

Plate cultures were incubated aerobically at 23°C after plotting over the surface of agar plates an aliquot of 0·1 ml of an overnight (A) broth culture.

Extraction of volatile compounds

Cultures were centrifuged at 16 300 *g* for 15 min. The supernatant was saturated with sodium chloride (300 g/litre) and extracted with 3 × 25 ml diethylether.

The diethylether extract dried over anhydrous sodium sulphate was concentrated to approximately 100 µl with a Dufton-type reflux column.

Gas chromatography

All analyses were performed on a Girdel 300 gas chromatograph (Delsi, France) equipped with a flame ionization detector and a thermoionic ionization detector. A 39 m × 0·4 mm id glass capillary column bonded with non-polar silicone phase SE 54 was used (film thickness: 0·6 µm). Hydrogen carrier gas linear velocity: 50 cm/s. Oven temperature programme: 40°C to 200°C at 2°C/min. Injector and detector temperatures: respectively, 210°C and 230°C.

Trapping

Isolation of 1 was accomplished on a Varian 1400 gas chromatograph equipped with a 2·4 m × 2·8 mm id glass column, packed with 10% SE 52 on AW, DMCS Chromosorb W (80/100 Mesh). Nitrogen carrier gas flow rate: 7 cm s^{-1}. Oven temperature programme: 110 to 200°C at 2°C/min. Injector and detector temperatures: the same as above. The effluents were split 2:98 between the flame ionization detector and a stainless-steel exit-port through a needle valve (S.G.E., MCVT/100). Chromatographic effluents were collected in a straight glass capillary tube (300 mm × 0·7 mm id – 1·3 mm od), in which a temperature gradient was established. The trap and the trapping conditions are described elsewhere by Gallois (1987).

GC–MS

Mass spectral data were obtained on a Nermag R 10-10 quadrupole mass spectrometer interfaced with a Girdel 31 gas chromatograph. A 60 m × 0·32 mm id fused silica DB5 column (J & W Scientific Inc.) was used under the same conditions as above. Ionization voltage, 70 eV, and source temperature, 150°C. Spectra were scanned from *m/z* 30 to *m/z* 300 in 1·5 s.

FT–NMR

^{1}H and ^{13}C-NMR spectra were acquired at, respectively, 400·13 and 100·62 MHz with a Bruker WM400 spectrometer. Chemical shifts, expressed in ppm and positive downfield were referenced to tetramethylsilane.

Reference compounds

All spectra of pyrazinic compounds were identified by comparison with those of reference compounds (gift from Oril, France). ^{13}C-labelled compounds were supplied by CEA (Saclay, France).

RESULTS

Volatile compounds produced

3-Isopropyl-2-methoxypyrazine (1), methylpyrazine (2), 2,5-dimethylpyrazine (3), trimethylpyrazine (4), and 2,5-dimethyl-3-ethylpyrazine (5) were identified by GC–MS in cultures incubated in (A) broth. Uninoculated

medium was used as blank. (A) Broth had been chosen because of the strong odour *P. taetrolens* developed on it during the isolation step.

Compounds 1 and 3 were the major components but did not exceed a 10 μg/litre level. Compounds 2, 4 and 5 were only obtained at trace level.

Time of production of pyrazines in (A) broth

The production levels of 2, 4 and 5 were too low to allow the determination of the exact moment of their biosynthesis. 3 was synthesized only during the first 24 h of incubation. 1 Appeared after 24 h of incubation, in the middle of the exponential phase which ended towards 50 h. Its production ceased 90 h after the inoculation. At this time, incubation was stopped to avoid losses or degradation of 1.

Enhancement of production of 1

The conditions which led to the best growth (as measured by absorbance values) in medium (A) were shown to be 23°C, aerobic conditions, pH 7·0. However, despite the strong typical odour which developed in (A) broth, the yield of 1 never exceeded 10 μg/litre. In addition, this medium contained a mixture of amino acids too complex to control and understand their utilisation. Consequently, it was replaced by (B) medium, a minimal basal salts medium, to which were added, one by one, at 10 g/litre rate, different substances suspected of having a role in 1 production.

Several carbon compounds (with 2·0 g/litre ammonium nitrate as a source of nitrogen) and different amino acids (which served both as source of carbon and nitrogen) were tested first on agar plates, just to screen substrates which allowed the development of a 1-like odour.

TABLE 2
Odours and Amounts of 1 produced by *P. taetrolens* in (B) Broth Supplemented with Different Substrates (10 g/litre)

Substrate	*Odour*	*Concentration (μg/litre)*
Fructose	Pungent	5
Glycerol	Green peas	50
β-alanine	Very weak green peas	10
DL-α-alanine	Ammonia	10
L-hydroxyproline	Ammonia	10
DL-serine	Green peas	10
DL-valine	Hazelnut	100
L-valine	Green peas	1 000
L-leucine	Green peas	10 000

It seemed impossible to find a medium in which *P. taetrolens* grew without odour production, even if the odour was not always characteristic of 1.

Compounds which led either to a 'green peas-like' odour or to an appreciable growth (i.e. fructose, glycerol, β-alanine, DL-α-alanine, L-hydroxyproline, DL-serine, DL-valine, L-valine, L-leucine) were retained and used in culture flasks with (B) medium (Table 2). In trials using carbon compounds, production of 1 did not exceed 5 μg/litre except with glycerol for which a 50 μg/litre production was obtained. But, when L-valine was unique substrate, *P. taetrolens* synthesised 1 mg/litre and use of L-leucine led to a 10 mg/litre yield.

Incorporation of possible precursors

An addition of precursors of 1, just before the beginning of its biosynthesis, could result in a larger production. Thus, 1 g/litre of each suspected precursor was added to the inoculated (C) broth under sterile conditions after 24 h of incubation (Table 3).

As the best productions were obtained with L-valine and glycine, these two compounds were used in the next step of the study.

With use of (C) broth, a decrease in the production of 3 (which appeared only at trace levels) and a total disappearance of pyrazines 2, 4 and 5 was also noticed.

Use of carbon 13-labelled substrates

One g/litre of L-[1-^{13}C) valine (isotopic enrichment 99%) was added after 24 h to an inoculated flask.

Mass spectral data (Table 4) (especially molecular ions 152–153, base peaks 137–138 and fragment ions 124–125, 105–106) show that a ^{13}C-atom

TABLE 3
Concentration of 1 obtained with Addition of Different Precursors (1 g/litre) after 24 h of Incubation of Inoculated (C) Broth

Precursor	*Concentration (μg/litre)*
2-keto-3-methylbutanoic acid (sodium salt)	100–1000
DL-valine	100–1000
D-valine	1000–10 000
L-valine	> 10 000
Glycine	> 10 000
Glyoxal	no growth, no production of 1
Glyoxylic acid	no growth, no production of 1

TABLE 4
Mass Spectral Data on Reference Compound 1 and Compound 1 produced by *P. taetrolens* Fed with Labelled L-Valine

Compound	*Major Mass fragments: m/z (%[a])*
Reference compound 1	152 (36) 137 (*100*) 124 (*28*) 105 (*17*) 95 (*15*) 41 (*32*) 39 (*24*) 27 (*15*)
Labelled compound 1	153 (*32*) 138 (*100*) 125 (*28*) 106 (*13*) 96 (*14*) 41 (*29*) 39 (*19*) 27 (*12*)

[a] Relative intensities with the base peak taken as 100.

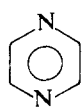
appeared in the molecule of 1: thus proving the incorporation of labelled L-valine in the molecule, at a very high level (almost 100%).

For still unexplained reasons, the yield of 1 was lower with labelled L-valine than with the unlabelled amino acid. So the bacterium was grown with both 0·5 g/litre labelled and 0·5 g/litre unlabelled L-valine, to obtain a sufficient amount of 1 for the ^{13}C-NMR study. Five milligrams of 1 could thus be collected.

NMR data (Tables 5 and 6) indicated the position of the incorporated ^{13}C.

The large enhancement of the signal at δ 158·6 in the ^{13}C-NMR spectrum proved that the labelling had taken place on the quaternary carbon located at the lowest field, i.e. the carbon to which the methoxy group was attached.

TABLE 5
^{13}C-NMR Data for Reference Compound ($CDCl_3$)

Carbon designation	*Shifts*
$\begin{matrix} CH_3 \\ \diagup \\ \diagdown \\ CH_3 \end{matrix}$ (isopropyl group)	20·98
$-CH\begin{matrix} \diagup \\ \diagdown \end{matrix}$ (isopropyl group)	30·21
$-CH_3O$	53·9
	135·9–138·9–152·9–158·6[a]

[a] Increase of the 158·6 ppm signal in molecule obtained with labelled L-valine.

TABLE 6
^{1}H-NMR Data Obtained for Reference Compound 1 and Labelled Compound 1

Carbon designation	*Reference compound 1*	*Labelled compound 1*
aromatics	1H,d,7·92 1H,d,8·06	1H,m,7·92 1H,m,8·06
CH_3O	3H,s,4·00	3H,d + s,4·00
—CH<	1H,sept,3·37	1H,sept,3·37
$C(CH_3)_2$	6H,d,1·27	6H,d,1·27

The appearance of a doublet (due to a long distance coupling ^{13}C-^1H, $J^3 = 3{\cdot}8$ Hz) in addition to the singlet at δ 4·00 in the ^{1}H-NMR spectrum confirmed this fact. Areas of both singlet and doublet were identical. As these two signals correspond to the incorporation of, respectively, unlabelled and labelled valine, this is an additional proof of the 100% incorporation of the labelling.

The second step was addition of [1-^{13}C] glycine (1 g/litre) (isotopic enrichment 99%) together with unlabelled L-valine (1 g/litre). A 10 mg/litre production of 1 was obtained. MS fragments, which could have been due to a labelling of the molecule, were present at a very low level (1% or 2%). Although this enhancement of the fragment ions could be attributed to the presence of a ^{13}C and not to parasite background fragments (as checked by a medium resolution mass spectral analysis), the labelling level did not exceed the isotopic natural abundance. So it could not be concluded that glycine was really incorporated in compound 1.

DISCUSSION

Like many odorous biosynthesised molecules, 1 appeared at the late exponential phase, when most of the substrates had been consumed. A similar phenomenon can be observed with *Aspergillus parasiticus* which produces 2-hydroxy-3,6 substituted pyrazines via the condensation of amino acids, only after depletion of the carbon source (Buchanan & Houston, 1982), and with *P. perolens* as reported by McIver & Reineccius (1986).

Both L-leucine and 2-keto-3-methylbutanoic acid were favourable

substrates. They probably acted as valine precursors. 2-Keto-3-methylbutanoic acid is used for the leucine synthesis and is also the immediate precursor of valine. A feedback inhibition could occur here (as it does in *Neurospora* for instance), the first enzyme in the sequence of the leucine synthesis being repressed by leucine. This would result in an accumulation of 2-keto-3-methylbutanoic acid which can only lead to valine. So, even if the initial medium does not contain valine (but contains leucine or 2-keto-3-methylbutanoic acid), *P. taetrolens* is able to synthesize valine during incubation to produce 1.

The position of the ^{13}C-atom incorporated in 1 by labelled L-valine seems to prove that L-valine was incorporated without any breakage or recombination of the molecule.

Although the involvement of an amino-acid in the biosynthesis of 1 seemed disputable to Murray & Whitfield (1975), it has been clearly proved by this experiment.

However, the second precursor remains unknown. Glycine is probably quickly assimilated by *P. taetrolens*, perhaps before biosynthesis of 1 begins, and its possible role in the biosynthesis will be difficult to prove. On the other hand, the more recent publication of Mottram *et al.* (1984), who identified 2,6-dimethyl-3-methoxypyrazine as produced by an aerobic bacterium, invalidated our hypothesis; the condensation of two amino acids (in this case, two molecules of alanine) would have led to the 2,5-dimethyl isomer. We are now working with resting cells of the bacterium. We have already succeeded in stopping the formation of 1 by washing and suspending cells in a phosphate buffer (a result that Morgan (1976) could not obtain). Incubation with dicarbonyl compounds (such as glyoxal or glyoxylic acid which could not be assimilated by metabolizing cells of *P. taetrolens*) will now be investigated.

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