Mechanisms of Formation of Alkylpyrazines in the Maillard Reaction

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The formation of alkylpyrazines was investigated in the reaction of glucose and fructose with [3-^{13}C]-alanine and [2-^{13}C]glycine. The reaction systems were heated for 7 min at 180 °C. GC–MS and GC–MS/MS data were used to determine the rate of incorporation and the position of isotopic labeling in the pyrazines formed. The results show that alanine and glycine not only act as the nitrogen source but also contribute to the alkyl side chain of some alkylpyrazines. While glycine was involved in one of the methyl groups of trimethylpyrazine, alanine contributed the C2 element in the ethyl groups of ethylmethyl-, ethyldimethyl- and diethylmethylpyrazines. The proposed reaction routes include the addition of the Strecker aldehydes of alanine and glycine to dihydropyrazines, which are postulated as intermediates.

Keywords: *Maillard reaction; alkylpyrazines; model reaction; formation pathways; GC–MS*

INTRODUCTION

Alkylpyrazines are formed during the Maillard reaction, preferably at temperatures above 100 °C (Koehler and Odell, 1970). They are frequently found in foodstuffs prepared by a heating process. Trialkylated pyrazines such as 2-ethyl-3,5-dimethylpyrazine and 2,3-diethyl-5-methylpyrazine are aroma impact compounds for the flavor of coffee (Blank et al., 1992), roasted sesame seeds (Schieberle, 1992), and roasted beef (Cerny and Grosch, 1993).

The reactions that lead to pyrazines have been extensively studied in a wide range of model systems consisting of different sugars and amino acids [reviewed by Maga (1992)]. The influence of the amino acids on the structure of the pyrazines formed has been investigated by Arnoldi et al. (1988), who heated fructose with different amino acids at 120 °C for 3 h. The α-amino carbonyl compounds, such as α-aminoacetone, are considered to be important intermediates for the formation of dihydropyrazines, which themselves are oxidized to the corresponding pyrazines (Shibamoto and Bernhard, 1977). Recently we found alanine to be an important precursor for the aroma-relevant trialkylated pyrazines 2-ethyl-3,5-dimethylpyrazine and 2,3-diethyl-5-methylpyrazine in a model system which was adapted to the conditions of beefsteak frying (Cerny and Grosch, 1994). While for the first pyrazine this result was in accordance with a proposed mechanism in which acetaldehyde is added to an intermediate dimethyldihydropyrazine (Shibamoto et al., 1979; Milic and Piletic, 1984), the origin of the latter pyrazine was still not completely understood.

Experiments with isotopically labeled precursors are helpful to clarify formation pathways (Tressl et al., 1993). Koehler et al. (1969) used glucose and ^{14}C-labeled amino acids to investigate the origin of the carbon atoms in methylpyrazine and dimethylpyrazine. In these cases the glucose was the exclusive carbon source and the amino acids provided only the nitrogen atoms in the pyrazines. However, for other pyrazines, including the trialkylated pyrazines, no such data were available. Recently Weenen et al. (1994) used ^{13}C-labeled sugars and determined the percentage of ^{13}C incorporation and the position of the labeled carbon atoms in the resulting pyrazines by MS and NMR methods. However, they focused on dimethyl- and trimethylpyrazines in their work. Although some research work has been done to determine the contribution of sugar carbon atoms to Maillard products, little is known about the incorporation of amino acid carbon atoms. Therefore, we performed model reactions based on sugars and the labeled amino acids [2-^{13}C]glycine and [3-^{13}C]alanine, respectively. Our special interest was to determine the extent to which the carbon atoms of the amino acids were involved in the carbon skeleton of the various pyrazines formed.

EXPERIMENTAL PROCEDURES

Model Experiments. The reaction partners used were as follows: 1, alanine (2 mmol) + glucose (2 mmol); 2, alanine (2 mmol) + fructose (2 mmol); 3, glycine (2 mmol) + glucose (2 mmol); 4, glycine (2 mmol) + fructose (2 mmol); 5, [3-^{13}C]-alanine (2 mmol) + glucose (2 mmol); 6, [3-^{13}C]alanine (2 mmol) + fructose (2 mmol); 7, [2-^{13}C]glycine (2 mmol) + glucose (2 mmol); 8, [2-^{13}C]glycine (2 mmol) + fructose (2 mmol). Sugar and amino acid were dissolved in 5 mL of sodium/potassium phosphate buffer (pH 5.6; 0.07 mol/L). The solution was adsorbed on kieselguhr (5 g) and heated for 7 min in coconut oil at 180 °C, using the conditions and the apparatus recently described (Cerny and Grosch, 1994). During heating, the water evaporated from the mixture. After heating, the mixture was cooled to 15 °C for 10 min.

Isolation of the Pyrazines. To each of the heated mixtures 1–8 was added diethyl ether (100 mL). The resulting suspension was filtered to remove kieselguhr and insoluble reaction products. The filtrate was distilled under high vacuum (0.1 Pa) by adding it dropwise to the distillation flask (Jung et al., 1992). The distillate was collected in a trap, cooled by liquid nitrogen. A basic fraction was obtained by extracting the distillate with HCl (0.1 mol/L; 3 × 50 mL), adjusting the aqueous phase to pH 12, and reextracting with diethyl ether

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Table 1. Pyrazines Formed in the Model Reactions with Alanine, Glycine, Glucose, and Fructose

no.	compound[a]	RI[b]	% of total pyrazines[c] 1	2	3	4
1	methylpyrazine	1160	8	11	24	12
2	2,5-dimethylpyrazine	1319	25	35	30	33
3	2,6-dimethylpyrazine	1325	4	6	9	11
4	2-ethyl-5-methylpyrazine	1380	10	6	<1	<1
5	2-ethyl-6-methylpyrazine	1386	9	7	<1	1
6	trimethylpyrazine +	1400	12	7	35	42
7	2-ethyl-3-methylpyrazine[d]					
8	3-ethyl-2,5-dimethylpyrazine	1441	20	19	0	0
9	2-ethyl-3,5-dimethylpyrazine	1455	3	2	0	0
10	2,3-diethyl-5-methylpyrazine	1487	6	4	0	0
11	3,5-diethyl-2-methylpyrazine[e]	1505	3	1	0	0

[a] The identification was based on the comparison with reference substance on the basis of retention index as well as mass spectra obtained by MS (EI) and MS (CI). [b] Retention indices on DB-Wax according to the method of van den Dool and Kratz (1963). [c] Based on the comparison of NPD peak areas in one experiment: 1, alanine + glucose; 2, alanine + fructose; 3, glycine + glucose; 4, glycine + fructose. [d] Peaks were not separated; given values represent the sum of both pyrazines. [e] The compound was identified only by comparison with data from the library of mass spectra.

(3 × 100 mL). The organic phase was dried over anhydrous Na_2SO_4 and concentrated to 0.2 mL using first a Vigreux column (50 × 1 cm) and then the microdistillation apparatus described by Bemelmans (1979).

Gas Chromatography. The concentrated samples were analyzed on a Carlo Erba Mega 2 gas chromatograph equipped with a cold on-column injector, a fused silica capillary column (DB-Wax, 30 m × 0.32 mm i.d.; 0.25 μm film thickness; J&W Inc.), a nitrogen–phosphorus detector (NPD) and a flame ionization detector (FID) running simultaneously. Operating conditions were as follows: helium carrier flow, 2.5 mL/min; injected volume, 1 μL; detector temperature, 240 °C; temperature program, 35 °C for 2 min, 40 °C/min to 50 °C, 6 °C/min to 180 °C, 10 °C/min to 240 °C, 240 °C for 20 min. Retention indices were calculated according to the method of van den Dool and Kratz (1963).

Gas Chromatography–Mass Spectrometry (GC–MS). The GC–MS experiments were performed on a HP-5890 gas chromatograph coupled to a Finnigan TSQ 700 mass spectrometer. The GC was equipped with a fused silica capillary column (DB-Wax, 30 m × 0.32 mm i.d.; 0.25 μm film thickness; J&W Inc.) and a cold on-column injector. Operating conditions were the same as described above with the exception of the temperature program of the GC oven: 50 °C for 1 min, 6 °C/min to 150 °C, 30 °C/min to 240 °C, 240 °C for 1 min. Mass spectra were obtained by electron impact (EI, 70 eV) and positive chemical ionization (CI), using ammonia as reactant gas. GC–MS/MS (tandem mass spectrometry) experiments were performed on the same instrument after positive chemical ionization. The collision-induced decomposition spectra (CID) were obtained with a collision energy of 29 eV in the laboratory frame and with argon, set to 0.1 Pa, as collision gas.

RESULTS AND DISCUSSION

Eleven alkylpyrazines have been identified in the reaction mixtures between sugars and unlabeled amino acids (Table 1). These are methylpyrazine (**1**), dimethylpyrazine isomers (**2**, **3**), trimethylpyrazine (**6**), ethylmethylpyrazine isomers (**4**, **5**, **7**), ethyldimethylpyrazine isomers (**8**, **9**), and diethylmethylpyrazine isomers (**10**, **11**). The pyrazines **6** and **7** were not separated on DB-Wax and showed therefore the same retention index (1400). Table 1 shows which pyrazines were formed preferentially. Compound **2** in all experiments was one of the major pyrazines formed. While the nature of the sugar had no pronounced effect, the amino acid used in the experiment showed a great influence on the number of pyrazines formed during the reaction. The pyrazines **8**–**11** were not formed when glycine was used as the nitrogen source. This agrees with the results of earlier experiments (Cerny and Grosch, 1994), in which only mixtures containing alanine gave **9** and **10** in appreciable amounts. When reacting glycerine with alanine or glycine, Wang and Odell (1972) observed that only alanine produced the ethyl-substituted pyrazine **4**, but with glycine higher amounts of **6** were formed. Consequently, the percentage of **6** plus **7** in experiments 3 and 4 (Table 1) is probably mainly due to **6**. Since our attention was more directed toward the ethyl-substituted pyrazines, we did not try to verify this point.

The labeling content of each pyrazine identified was determined by GC–MS after chemical ionization to minimize the fragmentation of the molecule (Table 2). The distribution of the isotope labeling was calculated from the intensities of the ions $[M + 1]^+$ (protonated molecular ion), $[M + 2]^+$ (protonated molecular ion singly labeled), and $[M + 3]^+$ (protonated molecular ion doubly labeled). The results were corrected, on one hand, using the natural ^{13}C content of the corresponding unlabeled reference compounds and, on the other hand, using the small extent of M^+ found in all spectra. As an example, Figure 1 illustrates different labeling contents (0, 30, and 70%) of 2-ethyl-5-methyl-pyrazine.

The labeling position was determined by comparison between the MS/MS spectra of the unlabeled references and the MS/MS spectra of the corresponding labeled compounds. The results are presented in Table 3 summarizing the main diagnostic ions obtained for each labeled pyrazine and its corresponding unlabeled analogue. In the case of, e.g., 3-ethyl-2,5-dimethylpyrazine, the labeling position of the ^{13}C atom was located on the

Table 2. ^{13}C-Labeling Content and Labeling Position of Pyrazines Formed from ^{13}C-Labeled Amino Acids

no.	compound	% of ^{13}C-labeled pyrazine[a] 5	6	7	8	labeling position[b]
1	methylpyrazine	0	0	0	0	
2	2,5-dimethylpyrazine	0	0	0	0	
3	2,6-dimethylpyrazine	0	0	30	25	methyl group (7, 8)
4	2-ethyl-5-methylpyrazine	70	70	30	0	C-2 of ethyl group (5, 6)
5	2-ethyl-6-methylpyrazine	30	20	0	0	C-2 of ethyl group (5, 6)
6	trimethylpyrazine +	50	40	80	100	
7	2-ethyl-3-methylpyrazine[c]					
8	3-ethyl-2,5-dimethylpyrazine	100	100	ND[d]	ND	C-2 of ethyl group (5, 6)
9	2-ethyl-3,5-dimethylpyrazine	70	70	ND	ND	C-2 of ethyl group (5, 6)
10	2,3-diethyl-5-methylpyrazine	90[e]	90[e]	ND	ND	C-2 of one or both ethyl groups (5, 6)
11	3,5-diethyl-2-methylpyrazine	90[e]	90[e]	ND	ND	C-2 of one or both ethyl groups (5, 6)

[a] 5, [3-^{13}C]alanine + glucose; 6, [3-^{13}C]alanine + fructose; 7, [2-^{13}C]glycine + glucose; 8, [2-^{13}C]glycine + fructose. [b] Corresponding experiments are given in parentheses. [c] Peaks were not separated; values represent the average of both pyrazines. [d] Not detected by MS. [e] Including 20% of doubly labeled compound.

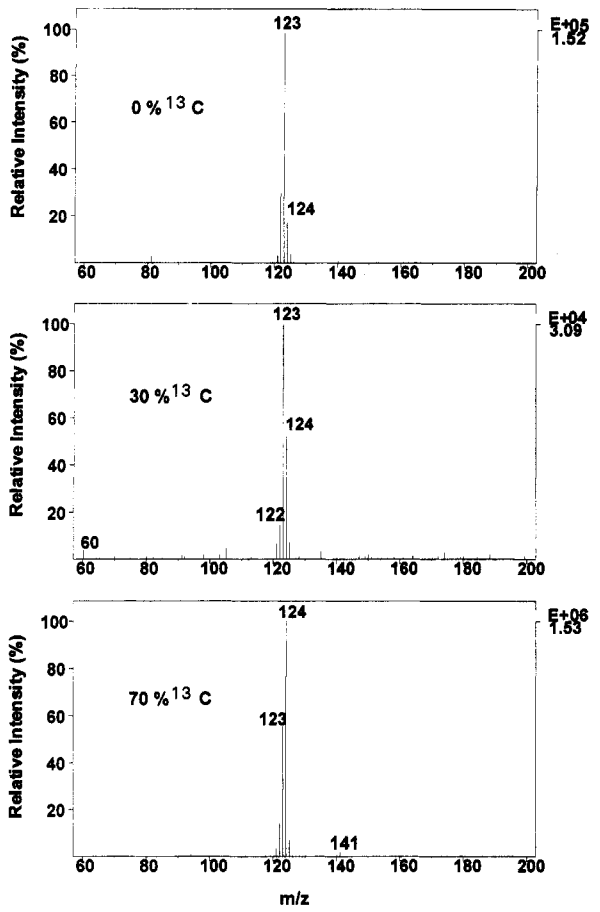

Figure 1. Positive chemical ionization mass spectrum of 2-ethyl-5-methylpyrazine with different contents of labeling. The ion at m/z 123 corresponds to the protonated molecular ion.

C2 of the ethyl group due to the loss of $^{13}CH_3$ in the spectrum of the labeled pyrazine. This fragment comes preferentially from the breakdown of the bond C1–C2 of the ethyl group rather than from the breakdown between the methyl groups and the pyrazine cycle. This is corroborated by the presence of the unlabeled ions at m/z 107, 80, and 42, which do not carry the ethyl group. Similar interpretations can be applied to the other compounds and allowed in most of the cases the location of the ^{13}C position.

The nature of the sugar had no pronounced effect on the ^{13}C-labeling content of the pyrazines formed from [3-^{13}C]alanine. The ethylmethylpyrazines **4** and **5** formed during the reaction with [3-^{13}C]alanine were 20–70% ^{13}C-labeled; pyrazine **8** was even 100% ^{13}C-labeled. Both **10** and **11** were 70% mono-^{13}C-labeled and 20% bi-^{13}C-labeled. The ethyl-substituted pyrazines **4**, **5**, and **8**–**11** were always ^{13}C-labeled at the 2-position of the ethyl group. For **10** and **11** it was not possible to clarify which of the two ethyl groups is concerned. These data show clearly that carbon coming from [3-^{13}C]alanine was incorporated in the course of the reaction. Obviously for the formation of **8** one single reaction route exists, since it was 100% ^{13}C-labeled. In contrast, **4**, **5**, and **9** are formed by at least one additional pathway without the amino acid carbon. The main formation pathway of **10** and **11** includes the incorporation of carbons 2 and 3 from one molecule alanine, while a second minor pathway incorporated two C2 elements from alanine.

Similar to the experiments with ^{13}C-labeled alanine, the nature of the sugar, whether glucose or fructose, did not much affect the ^{13}C-labeling content in the reaction with [2-^{13}C]glycine. Compound **6+7** from the reaction with [2-^{13}C]glycine was 80–100% ^{13}C-labeled, and **3** was 25–30% ^{13}C-labeled. The labeling position of **3** is in the side chain, at one of the two methyl groups. These data suggest that for **6** there exists a reaction route with incorporation of the C2 from glycine, while for the formation of **3** there are at least two routes, one with and another without incorporation of the amino acid carbon.

Dihydropyrazines are postulated in the literature as intermediate products of pyrazine formation (Shibamoto and Bernhard, 1977; Milic and Piletic, 1984; Weenen and Tjan, 1992). According to Shibamoto et al. (1979), 2,5-dimethyldihydropyrazine combines with acetaldehyde, which finally results in 3-ethyl-2,5-dimethylpyrazine. We suggest that acetaldehyde and other aldehydes for the reaction with dihydropyrazines result from a Strecker degradation of the corresponding amino acids. The dihydropyrazines can be formed through condensation of two α-aminocarbonyl compounds, which result from the Strecker reaction of amino acids and α-dicarbonyl compounds such as 2-oxopropanal.

In accordance with the formation pathway for 3-ethyl-2,6-dimethylpyrazine proposed by Shibamoto et al. (1979), we suggest a similar reaction route for pyrazine **6** (Figure 2). Formaldehyde, which comes from the Strecker reaction of glycine, combines with 2,5-dimethyldihydropyrazine, finally forming **6**.

The proposed reaction route to the ethylmethylpyrazines **4**, **5**, and **7** from alanine is illustrated in Figure 3. In the first step methyldihydropyrazine is formed from aminoacetone and aminoacetaldehyde, which result from the Strecker reaction of 2-oxopropanal and glyoxal,

Table 3. Characteristic Ions in the Mass Spectra of the Labeled Pyrazines and Their Corresponding Unlabeled Analogues

compound	no.	$[M + H]^+$ diagnostic ions,[a] m/z (intensity, %)
unlabeled 2-ethyl-5-methylpyrazine	**4**	na[b]
labeled 2-ethyl-5-methylpyrazine		124 (70), 109 (30), 108 (100), 107 (40), 83 (10), 80 (15)
unlabeled 2-ethyl-6-methylpyrazine	**5**	123 (100), 108 (39), 107 (3), 82 (18), 80 (5), 67 (7), 42 (26)
labeled 2-ethyl-6-methylpyrazine		124 (71), 109 (100), 108 (29), 83 (11), 80 (4), 68 (8), 42 (18)
unlabeled 3-ethyl-2,5-dimethylpyrazine	**8**	137 (39), 122 (100), 121 (70), 107 (8), 94 (9), 80 (20), 42 (11)
labeled 3-ethyl-2,5-dimethylpyrazine		138 (31), 122 (100), 121 (70), 107 (8), 95 (11), 80 (22), 42 (13)
unlabeled 2-ethyl-3,5-dimethylpyrazine	**9**	137 (37), 122 (100), 121 (67), 107 (7), 94 (6), 80 (20), 42 (12)
labeled 2-ethyl-3,5-dimethylpyrazine		138 (30), 122 (100), 121 (71), 107 (5), 95 (7), 80 (22), 42 (13)
unlabeled 2,3-diethyl-5-methylpyrazine	**10**	151 (27), 136 (34), 135 (100), 134 (0), 121 (7), 108 (26), 107 (16), 82 (3)
labeled 2,3-diethyl-5-methylpyrazine		152 (41), 137 (39), 136 (100), 135 (36), 121 (38), 108 (17), 107 (23), 82 (9)
unlabeled 3,5-diethyl-2-methylpyrazine	**11**	na[c]
labeled 3,5-diethyl-2-methylpyrazine		152 (24), 137 (20), 136 (100), 135 (63), 121 (61)

[a] The spectra were obtained by GC–MS/MS after collision-induced dissociation of the protonated molecular ion generated by positive chemical ionization (see Experimental Procedures). [b] Not analyzed. Diagnostic ions of unlabeled 2-ethyl-6-methylpyrazine were used. [c] Not analyzed. Diagnostic ions of unlabeled 2,3-diethyl-5-methylpyrazine were used.

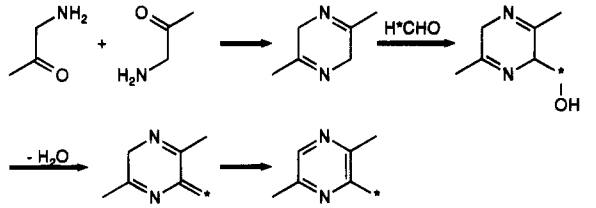

Figure 2. Proposed reaction route to [^{13}C]trimethylpyrazine in hexose/[^{13}C]glycine systems. Asterisks indicate ^{13}C-labeled carbon atoms.

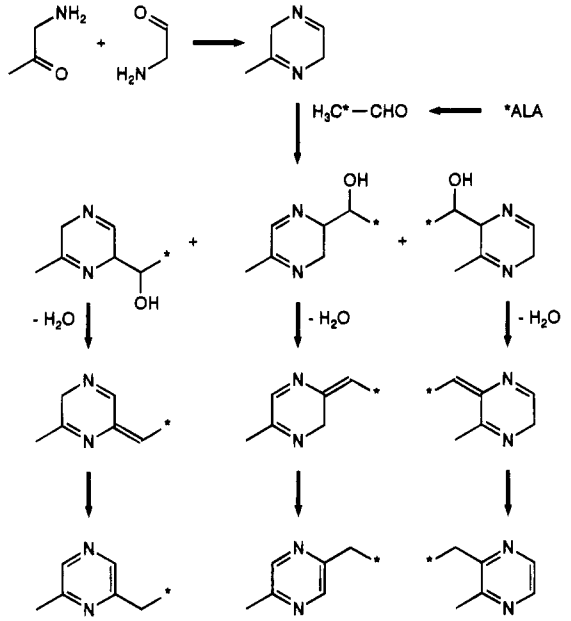

Figure 3. Proposed reaction routes to [^{13}C]ethylmethylpyrazine isomers in hexose/[^{13}C]alanine systems. Asterisks indicate ^{13}C-labeled carbon atoms.

respectively. Acetaldehyde reacts with the methyldihydropyrazine, finally resulting in three ethyldimethylpyrazine isomers. Carbon 5 in the methyldihydropyrazine is the preferred reaction site for acetaldehyde, since 70% of the resulting pyrazine **4** is ^{13}C-labeled (Table 2), while 20−30% of the pyrazine **5** is ^{13}C-labeled.

By analogy, **8** and **9** can be formulated via 2,5-dimethyldihydropyrazine and 2,6-dimethyldihydropyrazine, respectively, as intermediates (Figure 4). The fact that the ethyl group comes 100 and 70%, respectively, from alanine confirms this formation pathway proposed earlier (Cerny and Grosch, 1994).

Pyrazines **10** and **11** have similar formation pathways, because both of them are 70% mono-^{13}C-labeled and 20% bi-^{13}C-labeled, the labeling position being at the C2 of the ethyl group. Bemis-Young et al. (1993) proposed that **10** is formed from 2-aminopropanal and 2-hydroxy-3-amino-4-hexanone, the latter being formed in a multistep reaction involving 2-hydroxybutanal and acetaldehyde and including a dehydration reaction, keto−enol tautomerisms, and an Amadori rearrangement. This pathway, however, cannot explain the formation of **11**. On the other hand, the proposed mechanism in Figure 5, which includes 2-ethyl-5-methyldihydropyrazine as intermediate product, explains the formation of both **10** and **11** and is in accordance with the experimental data. 2-Ethyl-5-methyldihydropyrazine could form from aminoacetone and 1-amino-2-butanone. The formation of the latter compound from glucose and fructose, however, is not clear.

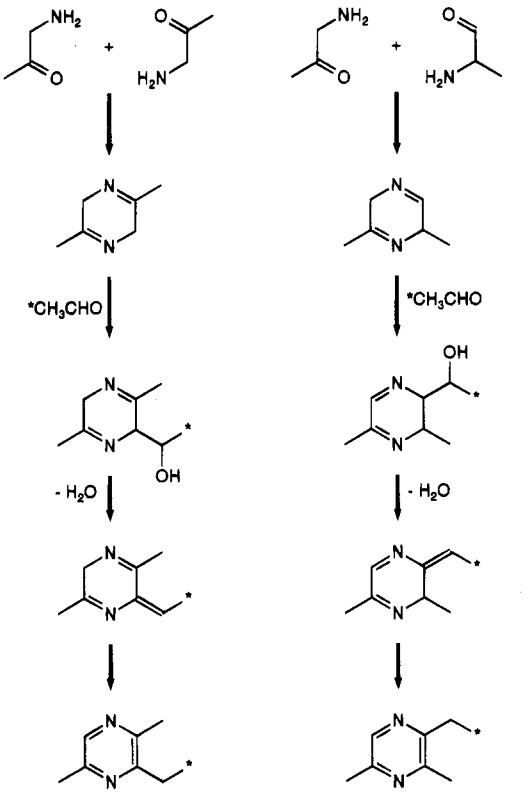

Figure 4. Proposed reaction routes to [^{13}C]-2-ethyl-3,6-dimethyl- and 2-ethyl-3,5-dimethylpyrazine in hexose/[^{13}C]-alanine systems. Asterisks indicate ^{13}C-labeled carbon atoms.

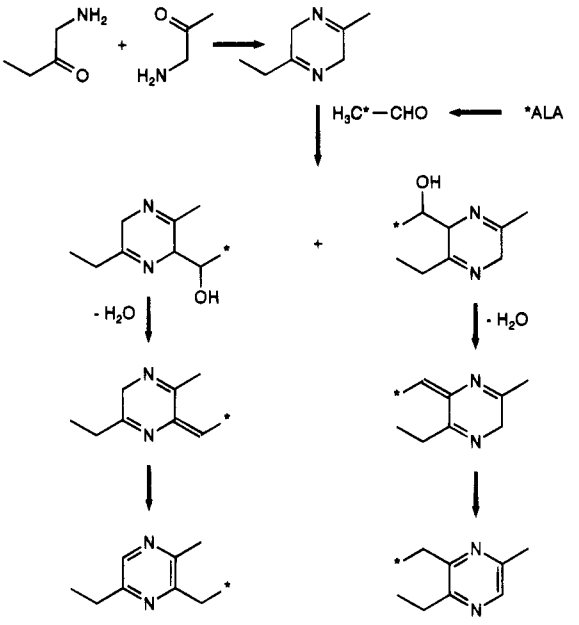

Figure 5. Proposed reaction routes to [^{13}C]diethylmethylpyrazine isomers in hexose/[^{13}C]alanine systems. Asterisks indicate ^{13}C-labeled carbon atoms.

In model reactions between hexoses and the ^{13}C-labeled amino acids glycine and alanine, it was shown that carbon atoms from the amino acids are incorporated during the formation of different pyrazines. For these pyrazines, reaction routes were proposed, which involve the Strecker aldehydes formaldehyde and acetaldehyde, respectively. However, for most pyrazines there are additional formation pathways not using the amino acid as a carbon source. Further investigations will be needed to clear up these mechanisms.

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