JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Double Schiff Base Adducts of 2,3-Butanedione with Glycine: Formation of Pyrazine Rings with the Participation of Amino Acid Carbon Atoms

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ABSTRACT: The 1,2-dicarbonyl compounds are well-known for their ability to undergo a one-to-one interaction with amino acids and generate aroma-active pyrazines through the Strecker reaction. An earlier publication reported the generation of tetrahydropyrazine moiety from the double addition of amino acids to 1,2-dicarbonyl compounds. To evaluate the potential of this intermediate to undergo oxidation and form pyrazines, a model system composed of glycine and 2,3-butanedione was evaluated under pyrolytic conditions at 250 °C, as well as under pressurized high-temperature conditions at 120 °C. These studies have indicated the unexpected formation of 2,3-dimethylpyrazine and 2,3,5-trimethylpyrazine in addition to the expected tetramethylpyrazine. Isotope-labeling studies using [^{13}C-1]glycine (98%), [^{13}C-2]glycine (99%), and [^{15}N]glycine (98%) have shown that, as expected, tetramethylpyrazine was completely unlabeled, whereas 51% of 2,3-dimethylpyrazine incorporated two ^{13}C-2 atoms from glycine, 20% incorporated one atom, and 29% was unlabeled. Furthermore, the label incorporation pattern in the major mass spectral fragment at m/z 67 indicated that the C-2 atoms originating from glycine reside in the ring system of 2,3-dimethylpyrazine. The formation of doubly labeled 2,3-dimethylpyrazine was rationalized through proposition of the double addition of glycine to 2,3-butanedione, and the formation of singly labeled isotopomer was justified by sequential Schiff base formation of 2-amino-butan-3-one first with the Strecker aldehyde and then followed by glycine. This pathway can also generate the double-labeled pyrazine. Finally, the unlabeled pyrazine was proposed to form through the Strecker reaction of 2,3-butanedione and its degradation product glyoxal with glycine. The proposed pathways were also consistent with the observed label distribution patterns of 2,3,5-trimethylpyrazine.

KEYWORDS: *Double-addition reactions, 2,3-butanedione, 2,3-dimethylpyrazine, pyrazine formation mechanism, oxazoles, isotope labeling*

INTRODUCTION

The chemical transformations of 1,2-dicarbonyl compounds in the presence of amino acids are known to generate divers compounds through the Strecker reaction, cross-linking, as well as through quinoxaline formation. Pyrazines derived from such interactions are known to be character-impact compounds in foods, such as coffee[1,2] and chocolate,[3] and are important in many baked products,[4−6] showing low detection threshold values. As such, understanding their various formation pathways represents an important aspect of improving the quality of processed foods. Shibamoto and Bernhard[7] presented detailed schemes on different pathways of formation of α-amino-carbonyls, the main precursors of pyrazines in food. Although the Strecker degradation is the predominant pathway of pyrazine formation during the Maillard reaction,[8,9] there is evidence from the literature that other mechanisms of pyrazine formation may exist.[9] Recently,[10] dimerization of azomethine ylides has been identified as a novel route to pyrazines in the absence of 1,2-dicarbonyl compounds. Furthermore, Chu et al.[11] observed the occurrence of a multiple addition reaction of amino acids with 1,2-dicarbonyl compounds, such as 3-deoxyglucosone, and the formation of a tetrahydropyrazine moiety, a known precursor of pyrazines. The double-addition reaction has also been previously proposed as a mechanism of the formation of pyrazinones and quinoxalinones from 3-deoxyglucosone or cyclotene in the presence of glycine or alanine.[12] To investigate the potential of the double-addition pathway to generate pyrazines, the 2,3-butanedione/glycine model was studied using the isotope-labeling technique to confirm the anticipated incorporation of C-2 carbon atoms of glycine into the ring structure of the pyrazines.

MATERIALS AND METHODS

Reagents and Chemicals. Glycine (99%), glyoxal trimeric dihydrate (95%), pyruvic aldehyde dimethylacetal, 2,3-butanedione (97%), 3,4-hexanedione, 2,3,5-trimethylpyrazine (99%), tetramethylpyrazine (98%), and silica gel (grade 60, 230−400 mesh) were obtained from Sigma-Aldrich Chemical Co. (Oakville, Ontario, Canada). The labeled [^{13}C-1]glycine (98%), [^{13}C-2]glycine (99%), and [^{15}N]glycine (98%) were obtained from Cambridge Isotope Laboratories (Andover, MI).

Pyrolysis−Gas Chromatography−Mass Spectrometry (Py−GC/MS). The Py−GC/MS analysis was performed according to the procedure described by Chu and Yaylayan,[13] with some modifications. A Varian CP-3800 GC equipped with a sample pre-concentration trap filled with Tenax GR was coupled to a Varian Saturn 2000 mass spectrometry detector (Varian, Walnut Creek, CA). The pyrolysis unit included a valved interface (CDS 1500), which was installed onto the

Received: August 23, 2012
Revised: October 23, 2012
Accepted: October 29, 2012
Published: October 29, 2012

dx.doi.org/10.1021/jf303658m | J. Agric. Food Chem. 2012, 60, 11440−11445

Table 1. Intensities of Pyrazines Identified in a Glycine/2,3-Butanedione/Silica Gel Model in Area/Mole of Glycine

Compound	Structure	Time	Area/mol glycine (x10[10]) Pyrolysis[a] 250°C	Dry PHTR[c]	Aq. PHTR[c]
2,3-dimethylpyrazine		11.9	1.8 [b] 0.7 [c]	1.6	0.7
2,3,5-trimethylpyrazine		13.1	4.4 [b] 2.0 [c]	13.6	5.2
Tetramethylpyrazine		14.6	69.4 [b] 53.6 [c]	774.9	220.6

[a]Values are calculated on the basis of duplicates with a percent standard deviation of <15%. [b]Model systems were evaluated in a 1:1:3 proportion. [c]Model systems were evaluated in a 3:1:3 proportion. PHTR = pressurized high-temperature reaction with Q-tube.

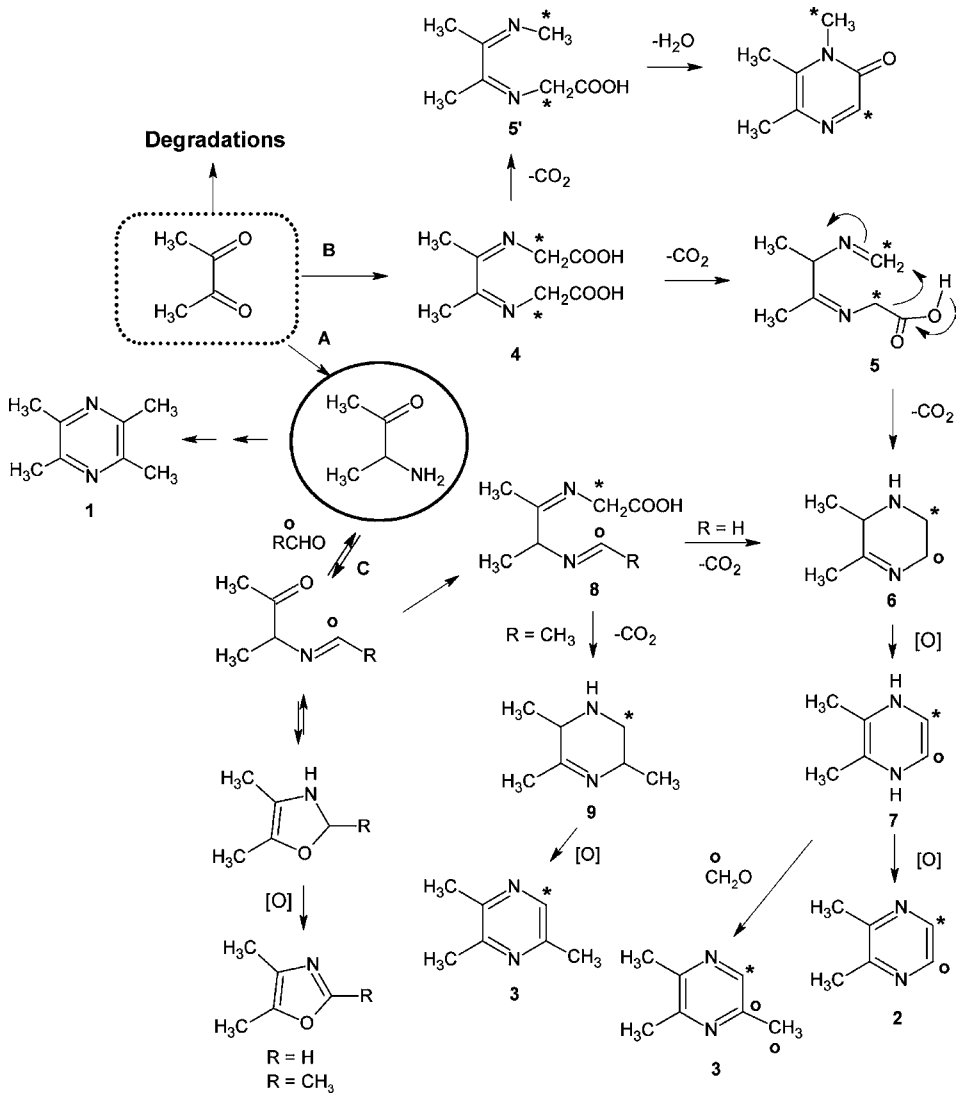

Figure 1. Proposed mechanism of formation of pyrazines through pathways A, B, and C in the model system of 2,3-butanedione in the presence of [^{13}C-2]-glycine. ∗, presence of the C-2 atom of glycine; o, presence of the C-2 atom of glycine depending upon the pathway; [O], oxidation.

GC injection port and connected to a CDS Pyroprobe 2000 unit (CDS Analytical, Oxford, PA). The model systems evaluated consisted of 1:1:3, 2:1:3, 3:1:3, and 7:1:3 ratios of glycine/2,3-butanedione/silica gel. Between 0.9 and 1.5 mg of sample mixtures were packed inside a

dx.doi.org/10.1021/jf303658m | J. Agric. Food Chem. 2012, 60, 11440−11445

quartz tube (0.3 mm thickness), plugged with quartz wool, inserted inside the coil probe, and pyrolyzed at 250 °C for 20 s under a helium atmosphere. Isotope-labeling studies were performed using a 1:1:3 glycine/2,3-butanedione/silica gel model system with labeled glycine. Additional confirmations of the mechanism proposed were performed through the evaluation of the 1:1:3 model system spiked with either glyoxal trimeric dehydrate or pyruvic aldehyde dimethylacetal. After pyrolysis, the volatiles were concentrated on the sample preconcentration trap at 50 °C and, subsequently, directed toward the GC column for separation. The column used was a DB-5MS column (5% diphenyl- and 95% dimethyl-polysiloxane) with dimensions of 50 m length × 0.2 mm internal diameter × 33 μm film thickness (J&W Scientific, Ontario, Canada). The carrier gas employed was helium. Its flow rate was regulated by an Electronic Flow Controller (EFC), set at a delayed (30 s) pressure pulse of 70 psi during the first 4 min, and maintained with a constant flow of 1.5 mL/min for the rest of the run. The GC oven temperature was set to −5 °C during the first 5 min using CO_2 as the cryogenic cooling source and then increased to 50 °C at a rate of 50 °C/min. Then, the oven temperature was again increased to 270 °C at a rate of 8 °C/min and kept at 270 °C for 5 min. The MS detector used was an ion-trap mass spectrometer. The MS transfer-line temperature was set to 250 °C; the manifold temperature was set to 50 °C; and the ion-trap temperature was set to 175 °C. The ionization voltage of 70 eV was used, and the electron multiplier voltage (EMV) was set to 1500 V.

Pressurized High-Temperature Reaction with Q-Tube (PHTR). Samples of glycine/2,3-butanedione/silica gel in a proportion of 3:1:3 were also analyzed using a 12 mL Q-tube reactor from Q Labtech, LLC. A vial with 0.5 g of sample was placed inside the pressure tube reactor. The sample was then heated for 30 min at 110 °C, and then the temperature was raised to 140 °C and kept for 10 min. A part of the sample was analyzed by Py−GC/MS, while the rest was directly analyzed by GC/MS without pyrolysis.

Identification of Pyrazines. Pyrazines were identified by comparison of their retention times to commercial standards and through National Institute of Standards and Technology (NIST) library matches. The data reported in Tables 1 and 4 are based on at least two replicate analyses with a percent standard deviation of <15%.

RESULTS AND DISCUSSION

The sugar-derived 1,2-dicarbonyl compounds are well-known intermediates that can undergo a one-to-one interaction with amino acids during the Maillard reaction, generating reactive precursors to various pyrazines through the Strecker reaction (pathway A in Figure 1). The carbon skeleton of such pyrazines is entirely derived from sugar atoms. On the other hand, double Schiff base formation of amino acids with 1,2-dicarbonyl compounds has been shown (pathway B in Figure 1) to generate a tetrahydropyrazine moiety that, in theory, can be oxidized into pyrazines, incorporating carbon atoms from the amino acids in its ring structure.[11] To investigate the formation of pyrazines through the double-addition pathway, isotope-labeling experiments were performed using a simple 1,2-dicarbonyl compound and an amino acid. The model system was evaluated under pyrolytic conditions at 250 °C and in PHTRs at 120 °C at different ratios and dilutions with silica gel. The reaction of 2,3-butanedione with glycine is expected to generate only tetramethylpyrazine (compound **1** in Figure 1); however, as shown in Table 1, two additional pyrazines 2,3-dimethyl- and 2,3,5-trimethylpyrazines were also detected in addition to trace amounts of 2,5-dimethylpyrazine (not shown), indicating the statistical preference of the major α-amino carbonyl precursor in the reaction pool, 3-amino-2-butanone to interact with itself or with other minor α-amino carbonyls to generate the observed pattern of pyrazines (Figure 2). To investigate the unexpected formation of 2,3-dimethyl- and 2,3,5-trimethylpyrazine, isotope-labeling studies were conducted using variously labeled glycine with unlabeled 2,3-butanedione. Such studies indicated the complete absence of glycine carbon atoms from the tetramethylpyrazine structure; however, 2,3-dimethylpyrazine and 2,3,5-trimethylpyrazines showed various percentages of incorporation of C-2 atoms and 100% incorporation of two nitrogen atoms from glycine (see Table 2), indicating the occurrence of both Strecker and an unknown non-Strecker pathway to generate pyrazines.

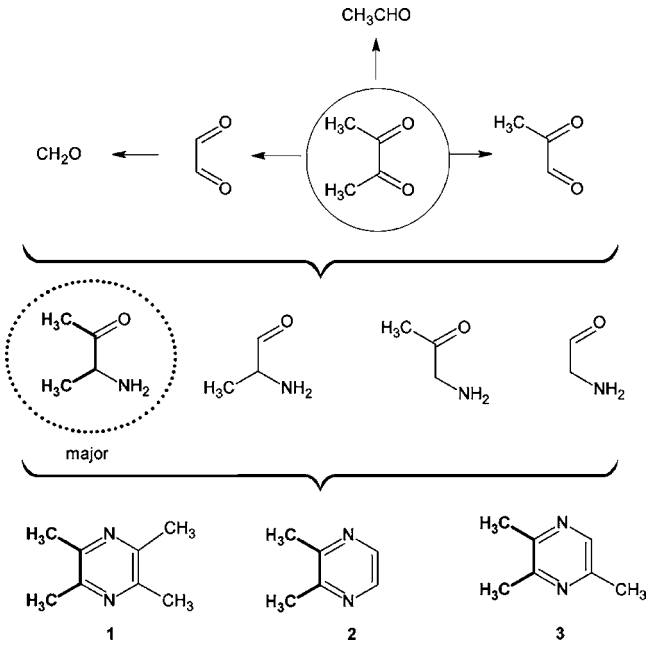

Figure 2. Schematic presentation of thermal degradation products of 2,3-butanedione followed by their Strecker degradation and formation of unlabeled pyrazines.

Table 2. Percent Incorporation of [^{13}C-2]Glycine into Pyrazines Formed in a Glycine/2,3-Butanedione/Silica (1:1:3) Model System[a]

			[^{13}C-2]glycine			
compound	time	MW	M	M + 1	M + 2	M + 3
2,3-dimethylpyrazine	11.9	108.14	29	20	51	0
2,3,5-trimethylpyrazine	13.1	122.16	47	27	8	18
tetramethylpyrazine	14.6	136.19	100	0	0	0

[a]No incorporation of ^{13}C-1 from glycine and 100% incorporation of 2× ^{15}N in all listed compounds.

Formation of Unlabeled Pyrazines in a [^{13}C-2]Glycine/2,3-Butanedione Model System. In the above model system studied, the only expected pyrazine is the completely unlabeled tetramethylpyrazine because the only α-dicarbonyl present in the model system was unlabeled 2,3-butanedione. As mentioned above, not only were other pyrazines detected but they also exhibited various percentages of ^{13}C-2 atom incorporation patterns, as presented in Table 1. The formation of completely unlabeled pyrazines can only be explained by the degradation of 2,3-butanedione into glyoxal and pyruvaldehyde, the two unlabeled α-dicarbonyls needed for their formation (see Figure 2). To our knowledge, there are no published reports that document the formation of glyoxal or pyruvaldehyde from 2,3-butanedione, although generation of acetaldehyde and formaldehyde has been reported.[14] Replacing 2,3-

butanedione with 3,4-hexanedione in the glycine model system also generated pyrazines that could only be justified if the degradation of 3,4-hexanedione into shorter chain α-dicarbonyl is assumed, similar to the 2,3-butanedione case. Additional confirmation of the occurrence of glyoxal and pyruvaldehyde was provided by spiking the model system with glyoxal and pyruvaldehyde, which, as expected, resulted in the increased levels of all of the pyrazines (see Table 3). Although the above

Table 3. Intensities of Pyrazines after Spiking a Model System of Glycine/2,3-Butanedione/Silica (1:1:3) with Glyoxal Trimeric Dihydrate and Pyruvic Aldehyde Dimethylacetal

		area/mol of glycine ($\times 10^{10}$)		
pyrazine	time	parent model	spiked with glyoxal	spiked with pyruvaldehyde
2,3-dimethyl-	11.9	1.8	42.0	23.6
2,3,5-trimethyl-	13.1	4.4	82.0	57.3
tetramethyl-	14.6	69.4	127.9	197.7

results demonstrate that glyoxal and pyruvaldehyde are formed from the pyrolytic degradation of 2,3-butanedione, the mechanism of this reaction still remains unknown.

Formation of Labeled Pyrazines in a [^{13}C-2]Glycine/2,3-Butanedione Model System. 2,3-Dimethylpyrazine, which was detected in the above model system, is an important aroma contributor in Parmigiano-Reggiano cheese, with nutty and coffee aroma qualities,[15] as well as in other foods. Therefore, it is important to understand the different pathways that can lead to its formation in addition to the Strecker reaction and dimerization of the azomethine ylides.[10] As was mentioned above, this pyrazine was formed in the 2,3-butanedione/glycine model system with the contribution of C-2 atoms from glycine, where-isotope labeling studies have indicated the incorporation of one (20%) and two (50%) such carbon atoms from ^{13}C-2 glycine; similarly, 53% of trimethylpyrazine was also found to be formed from the contribution of multiple C-2 atoms from glycine (Table 2). The Strecker mechanism cannot justify the incorporation of ^{13}C-2 atoms into the ring system of the pyrazines. There is evidence from the mass spectral data of 2,3-dimethylpyrazine and 2,3,5-trimethylpyrazine that the label incorporation occurred at the ring carbon atoms of the pyrazines (see Figure 3). Under the electron impact conditions, methyl-substituted pyrazines undergo a well-established fragmentation through the loss of an acetonitrile molecule,[16] generating an ion at m/z 67 in the case of 2,3-dimethylpyrazine and the ion at m/z 81 in the case of 2,3,5-trimethylpyrazine. Because of the symmetrical nature of 2,3-dimethylpyrazine, this loss can occur from both methyl groups equally. Any label incorporation in the methyl groups should register a 50% loss in the label incorporation in m/z 67. However, as shown in Figure 3, m/z 67 retained the percent distribution of labels in the original parent ion, indicating the incorporation of the amino acid carbons into the pyrazine ring structure and not as methyl substituents. On the other hand, in the case of 2,3,5-trimethylpyrazine, the label incorporation pattern should slightly deviate from the distribution of the parent ion because of the possibility of a loss of an acetonitrile molecule and formation of an ion F1 at m/z 81 that partially carries the label as a side chain. As a result, a slight variation is expected to be observed in the label incorporation pattern, particularly in the intensity of ion M + 3 because of the simultaneous loss of two labels.

Double Schiff Base Formation of Amino Acids with 1,2-Dicarbonyl Compounds: Pathway B. As mentioned above, the evidence from label incorporation into the mass spectral fragments at m/z 67 and 81 arising from the loss of acetonitrile moieties from both pyrazines indicates the involvement of C-2 carbon atoms from glycine in the process

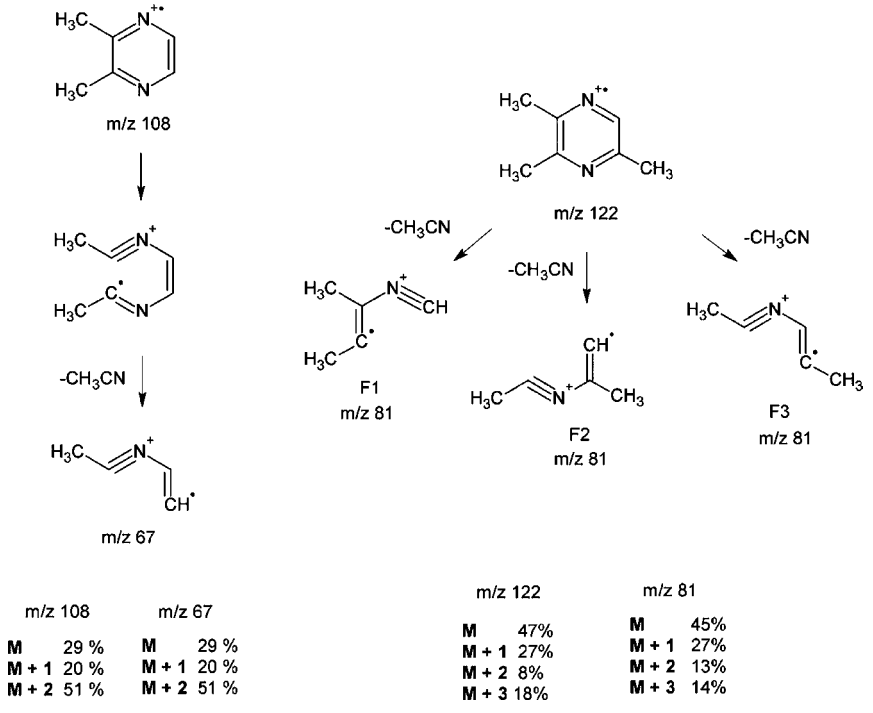

Figure 3. Mass spectral fragmentation of 2,3-dimethyl- and 2,3,5-trimethylpyrazins.

of pyrazine ring formation, consistent with the proposed mechanism of tetrahydropyrazine formation by Chu et al.[11] (see pathway B in Figure 1). According to this mechanism, one of the double Schiff base adducts (**4**) can undergo decarboxylation through the 5-oxazolidinone intermediate[17] to generate two isomeric Schiff bases[18] **5** and **5′**. Intermediate **5** can cyclize, as shown in Figure 1, to generate 2,3-dimethyltetrahydropyrazine structure **6**, incorporating two C-2 carbon atoms. The structure **6** can become oxidized into dihydropyrazine (**7**) and finally into 2,3-dimethylpyrazine (**2**). The formation of double Schiff base adduct **4** can also be inferred from the fact that 1,5,6-trimethylpyrazin-2(1*H*)-one was detected in the same model system. The latter has been stipulated to arise from double Schiff base adduct **4** through intramolecular cyclic amide formation,[12,19] as shown in Figure 1. Although the double-addition pathway B can explain the formation of doubly labeled 2,3-dimethylpyrazine, it cannot explain the formation of either singly labeled 2,3-dimethylpyrazine or any of the labeled 2,3,4-trimethylpyrazines, which are considered important aroma compounds found in many food products, such as popcorn[20] and cocoa beans.[21]

Schiff Base Formation of α-Amino Carbonyl Intermediates with Aldehydes: Pathway C. The formation of singly labeled 2,3-dimethylpyrazine and all of the labeled isotopomers of 2,3,5-trimethylpyrazine listed in Table 2 can be justified by assuming the ability of reactive aldehydes, such as Strecker aldehyde, to form Schiff base adducts (pathway C in Figure 1) with 3-amino-butan-2-one, the precursor of tetramethylpyrazine, generated through the Strecker reaction (pathway A). In fact, the formation of Schiff base adducts with formaldehyde and acetaldehyde is verified in this model system through the detection of their corresponding cyclization products 4,5-dimethyl-1,3-oxazole and 2,4,5-trimethyl-1,3-oxazole, as shown in Figure 1 and as reported earlier.[14] The oxazolines, the precursor of oxazoles, have also been observed as products from the reaction of 2,3-butanedione with amino acids.[22] Oxazoles have been identified as important aroma compounds in baked potatoes[23] and roasted peanuts.[24] Oxazoles, therefore, can be used as chemical markers not only for the presence of α-amino carbonyl intermediates in the Maillard reaction but also for the presence of small reactive aldehdyes trapped by them. The two detected oxazoles, therefore, indicate the formation of formaldehyde and acetaldehyde in the model system studied, and according to Table 4, 70% of formaldehyde generated in the system is labeled and 30% is unlabeled. These percentages depend upon the reaction conditions used, such as the presence of silica. Acetaldehyde generated from the degradation of 2,3-butanedione on the other hand should be unlabeled, generating unlabeled trimethyloxazole. Labeled formaldehyde can be formed through the Strecker degradation of labeled glycine, resulting in a singly labeled 4,5-dimethyl-1,3-oxazole, and the unlabeled formaldehyde and acetaldehyde can arise from the degradation of the only source of unlabeled carbons, which is 2,3-butanedione, as proposed in Figure 2. The oxidative α-dicarbonyl cleavage could also result in the formation of formic acid or acetic acid, according to the mechanism proposed by Davídek et al.[25] Recently, Granvogl et al.[26] demonstrated the ability of oxazolines to release back the Strecker aldehydes under hydrolytic conditions.

Table 4. Percent Incorporation of [^{15}N]-, [^{13}C-1]-, and [^{13}C-2]Glycine into 4,5-Dimethyl-1,3-oxazole and 2,4,5-Trimethyl-1,3-oxazole in a Glycine/2,3-Butanedione/Silica (1:1:3) Model System

	4,5-dimethyl-1,3-oxazole			2,4,5-trimethyl-1,3-oxazole		
label	M	M + 1	M + 2	M	M + 1	M + 2
[^{13}C-1]-	100	0	0	100	0	0
[^{13}C-2]-	30	70	0	100	0	0
[^{15}N]-	0	100	0	0	100	0

Formation of Pyrazines through Pathway C. As shown above, 3-amino-butan-2-one not only can dimerize into tetramethylpyrazine (**1**) but also can form Schiff base adducts with simple aldehydes (pathway C in Figure 1). These intermediates can cyclize to generate oxazoles or react further with amino acids to form a second Schiff base adduct **8**, which is chemically identical to the intermediate **5** (if R = H) and can undergo similar cyclization and form intermediate **6** if the initial aldehyde was formaldehyde and generate intermediate **9** if the initial aldehyde was acetaldehyde. Tetrahydropyrazine structures similar to intermediate **6** or **9** generated from alanine and 3-deoxyglucosone has also been identified earlier.[11] Intermediates **6** and **9** can undergo further oxidation steps to generate dihydropyazines and eventually pyrazines **2** and **3** with various degrees of label incorporation depending upon the origin of formaldehyde. Structure **6** originating from intermediate **5** will always be doubly labeled in the [^{13}C-2]glycine models, whereas structure **6** originating from Schiff base adduct **8** could be singly or doubly labeled depending upon the origin of formaldehyde. Structure **6** can therefore generate singly labeled or doubly labeled pyrazine **2**. Finally, the addition of formaldehyde to either singly labeled or doubly labeled dihydropyrazine **7** can justify the formation of doubly labeled or triply labeled pyrazine **3**.

The isotope-labeling approach allowed for the identification of two distinct pathways for the generation of aroma-active pyrazines, one initiated through double Schiff base formation between the 1,2-dicarbonyl compounds and the amino acids and the other, similar to the Strecker reaction, initiated by the formation of α-amino carbonyls followed by sequential Schiff base formation first at the amino group with an Strecker aldehyde and then followed at the carbonyl group by an amino acid. The consequences of such pathways could be important in food, where the diversity of pyrazine formation can be rationalized not only by the type of α-dicarbonyl compounds formed from sugar but also by the type and number of amino acids present. Furthermore, the process of double Schiff base formation can be viewed as a model for cross-linking reactions occurring in food during processing, and pyrazines themselves could be viewed as cross-linked structures.

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Funding

The authors acknowledge funding for this research from the Natural Sciences and Engineering Research Council of Canada (NSERC).

Notes

The authors declare no competing financial interest.

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