Formation of Sugar-Specific Reactive Intermediates from ^{13}C-Labeled L-Serines

Varoujan A. Yaylayan,* Anahita Keyhani, and Andrzej Wnorowski

Department of Food Science and Agricultural Chemistry, McGill University, 21,111 Lakeshore, Ste. Anne de Bellevue, Quebec, Canada H9X 3V9

Analysis of the pyrolysis products of [1-^{13}C], [2-^{13}C], and [3-^{13}C]-labeled L-serines has indicated the presence of three initial degradation pathways. Decarboxylation followed by deamination produces aminoethanol and acetaldehyde, respectively; a retro-aldol pathway generates formaldehyde and glycine. Dehydration of L-serine can lead to the formation of pyruvic acid, which eventually can be converted into the amino acid alanine. Formation of alanine and glycine was confirmed due to the detection of 2,5-diketo-3,6-dimethylpiperazine and cycloglycylalanine. Most of the advanced decomposition products of L-serine can be rationalized on the basis of these initial degradation products. Label incorporation studies have elucidated the origin of carbonyl precursors of methyl- and 2,3-dimethylpyrazines formed in the thermal decomposition mixture of L-serine. Three mechanistic pathways were identified for the formation of carbonyl precursors of methyl- and 2,3-dimethylpyrazines. The major pathway (70%) for the formation of the precursor of methylpyrazine involved aldol addition of formaldehyde to glycolaldehyde to form glyceraldehyde. On the other hand, the major pathway (60%) for the formation of the precursor of 2,3-dimethylpyrazine involved an aldol condensation of acetaldehyde with glycolaldehyde to form 2,3-butanedione.

Keywords: *Maillard reaction mechanisms; ^{13}C-labeled L-serines; Py/GC/MS; pyrazines; pyruvaldehyde; glyceraldehyde; glycolaldehyde; 2,3-butanedione*

INTRODUCTION

During the Maillard reaction, the formation of most heterocyclic compounds requires the presence, in the reaction mixture, of both sugars and amino acids. However, the unique and interesting case of β-hydroxy amino acids, such as L-serine and L-threonine, is the exception (Reese and Baltes, 1992). These two amino acids can generate, during thermal decomposition, numerous heterocyclic compounds in the absence of a carbohydrate source, indicating their ability to form sugar-specific reactive intermediates such as dicarbonyls. Thermal degradation of L-serine alone (180−360 °C) has been reported to produce a wide range of heterocyclic compounds such as methyl- and ethyl-substituted pyrazines and pyrroles, pyrrolylalkanols, and some fused heterocyclic compounds (Kato et al., 1970; Wang and Odell, 1973; Reese and Baltes, 1992). Kato et al. (1970) pyrolyzed L-serine at 280 °C and identified several methyl- and ethyl-substituted pyrazines and pyrroles in addition to ethylamine, ammonia, aminoethanol, acetaldehyde, and 2,5-diketo-3,6-dimethylpiperazine. Wang and Odell (1973) similarly identified various pyrazines when L-serine was pyrolyzed at 200 °C. More detailed studies on the degradation products of L-serine were carried out by Reese and Baltes (1992). They autoclaved L-serine at 120, 150, and 180 °C for 1 h; however, only at 150 and 180 °C could degradation products be detected. The number of products increased from 58 to 70 at the higher temperature. In addition to methyl- and ethyl-substituted pyrazines and pyrroles, they also detected 2-(1-pyrrolyl)ethanol, pyrrolo[1,2-*a*]-pyrazine, and various carbonyl, dicarbonyl, and α-hydroxycarbonyl compounds including 2,3-butanedione and 1-hydroxy-2-propanone. The origin of such reactive intermediates formed upon thermal degradation of amino acids and sugars is relatively difficult to determine without ^{13}C-labeling studies, due to the multiple origins of these components. By utilizing Py/GC/MS to generate and identify stable end-products formed by the incorporation of these reactive intermediates into their molecular structure using properly ^{13}C-labeled reactants, identification of their origin becomes possible (Yaylayan and Keyhani, 1998; Yaylayan et al., 1998). In this study, using variously ^{13}C-labeled L-serines, the origin of glycolaldehyde, pyruvaldehyde, 2,3-butanedione, and other carbonyl compounds was elucidated. These reactive intermediates lead to the formation of the parent pyrazine and methyl-substituted pyrazines. Studies are underway to identify the origin of other dicarbonyls involved in the formation of ethyl-substituted pyrazines and pyrroles.

MATERIALS AND METHODS

All reagents and chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI). DL-[2-^{13}C]Alanine (92%), [2-^{13}C]-glycine (98%) L-[1-^{13}C]serine (99%), L-[2-^{13}C]serine (99%), and L-[3-^{13}C]serine (98%) were purchased from Cambridge Isotope Laboratories (Andover, MA).

Pyrolysis GC/MS Analysis. A Hewlett-Packard GC/mass selective detector (5890 GC/5971B MSD) interfaced to a CDS pyroprobe 2000 unit was used for the Py/GC/MS analysis. Solid samples (1−4 mg) of L-serine, L-serine/glycine, L-serine/alanine, glycolaldehyde/phenylhydrazine, or L-serine/phenylhydrazine were introduced inside a quartz tube (0.3 mm thickness), which was plugged with quartz wool and inserted inside the

* Author to whom correspondence should be addressed [telephone (514) 398-7918; fax (514) 398-7977; e-mail yaylayan@macdonald.McGill.ca].

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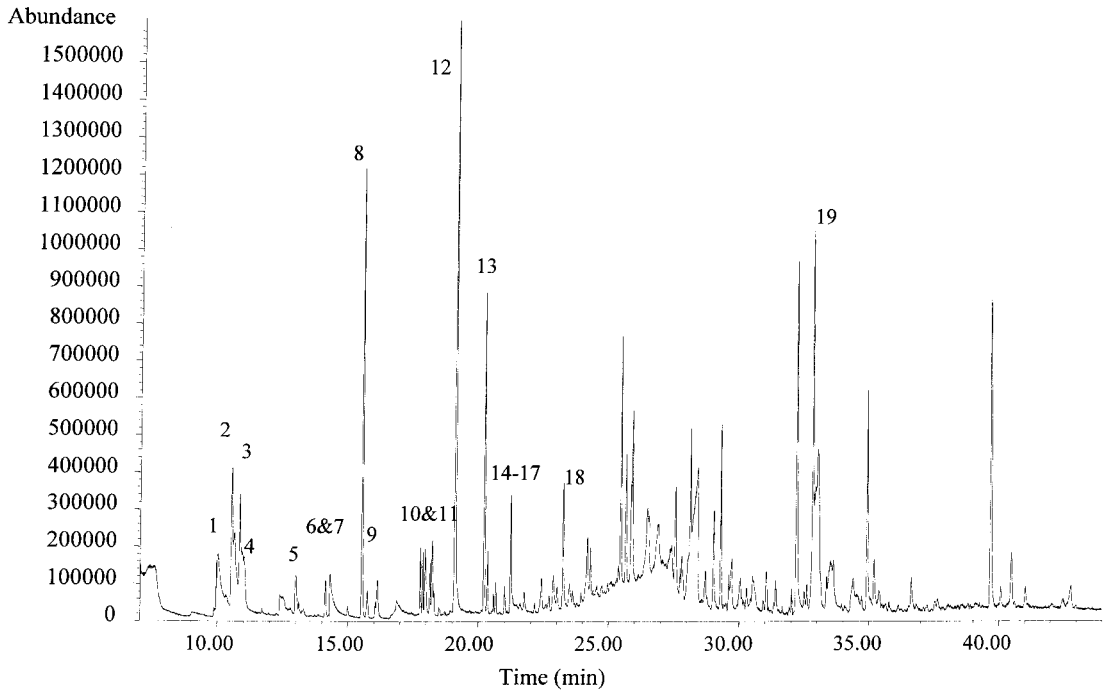


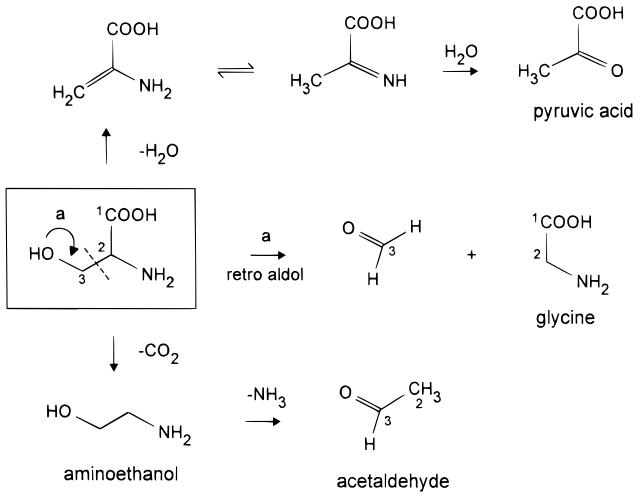
Figure 1. Pyrogram of L-serine (250 °C for 20 s): (1) 2-amino-1-ethanol; (2) pyrazine; (3) pyrrole; (4) 2-methyl-2-oxazoline; (5) methylpyrazine; (6) isoxazole; (7) propaneamide; (8) ethylpyrazine; (9) 2,3-dimethylpyrazine; (10) 2-ethyl-6-methylpyrazine; (11) 2-ethyl-3-methylpyrazine; (12) 2-(1-pyrrolyl)ethanol; (13) 2,6-diethylpyrazine; (14) 2,3-diethyl-5,6-dimethylpyrazine; (14−17) 2,3-diethylpyrazine, 2,5-diethylpyrazine, 2-ethyl-6-vinylpyrazine, and 2,3-dimethyl-5-ethylpyrazine; (18) 2,3-diethyl-5,6-dimethylpyrazine; (19) cyclic dimer of alanine.

coil probe. The Pyroprobe was set at the desired temperature (250 °C) at a heating rate of 50 °C/ms and with a total heating time (THT) of 20 s. The pyroprobe interface temperature was set at 250 °C. The GC column flow rate was 0.8 mL/min for a split ratio of 92:1 and a septum purge of 3 mL/min. Capillary direct MS interface temperature was 180 °C; ion source temperature was 280 °C. The ionization voltage was 70 eV, and the electron multiplier was 1682 V. The mass range analyzed was 30−300 amu. The column was a fused silica DB-5 column (60 m length × 0.25 mm i.d. × 25 μm film thickness; Supelco, Inc.). The column initial temperature (−5 °C) was increased to 260 °C at a rate of 10 °C/min and held at 260 °C for 15 min.

RESULTS AND DISCUSSION

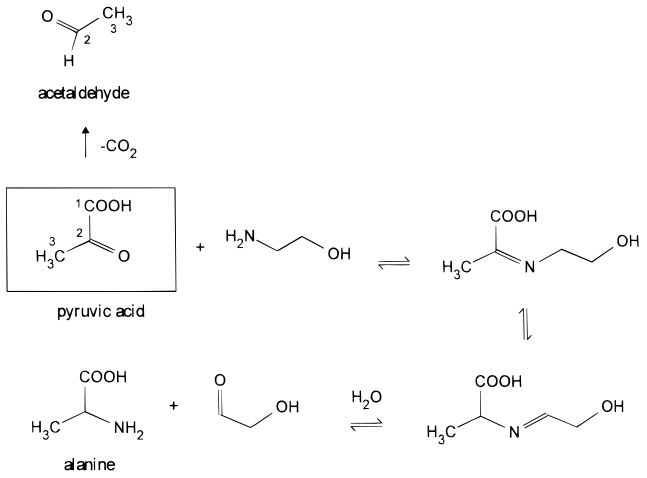
Initial Degradation Products of L-Serine. Pyrolysis of L-serine at 250 °C produced ~70 different products (see Figure 1). A similar number of products was observed during autoclaving of L-serine at 180 °C (Reese and Baltes, 1992). Most of the heterocyclic compounds identified were methyl- and ethyl-substituted pyrazines and pyrroles in addition to 2-(1-pyrrolyl)ethanol. The substitution pattern of the observed pyrazines and pyrroles indicated the formation of different α- and δ-dicarbonyl compounds with three, four, five, and six carbon atoms. Obviously, these dicarbonyl compounds were formed through condensation and chain elongation of shorter carbonyl intermediates formed from the initial degradation of L-serine. The role of amino acids in affecting such chain elongation reactions has been confirmed through labeling studies (Yaylayan and Keyhani, 1998). Glycine, for example, can increase the chain length of α-keto aldehydes or α-hydroxy aldehydes by one carbon atom and L-alanine by two carbon atoms. Aldol condensation between smaller dicarbonyls or aldehydes is another pathway for the formation of longer chains. Scheme 1 summarizes the initial degradation products of L-serine. According to Scheme 1

Scheme 1. Initial Thermal Degradation Products of L-Serine



aminoethanol can be formed by the decarboxylation of L-serine and acetaldehyde by the subsequent deamination of aminoethanol. Ammonia, aminoethanol, and acetaldehyde have all been detected during the decomposition of L-serine (Kato et al., 1970), supporting the presence of this pathway. Another decomposition pathway consistent with the observed product distribution, is the retro-aldol reaction of L-serine to produce formaldehyde and glycine. On the other hand, dehydration of L-serine can lead to the formation of pyruvic acid and eventually the formation of alanine as shown in Scheme 2. The formation of glycine and alanine was verified by the detection of cycloglycylalanine and 2,5-diketo-3,6-dimethylpiperazine (cyclic dimer of alanine) in the pyrolysis mixture of L-serine. In addition, the detection of unlabeled dimers in the model systems containing L-serine and labeled L-alanine or glycine indicated that

Scheme 2. Proposed Mechanism of Formation of Glycolaldehyde and D,L-Alanine[a]



[a] Numbers indicate original carbon atom locations of L-serine.

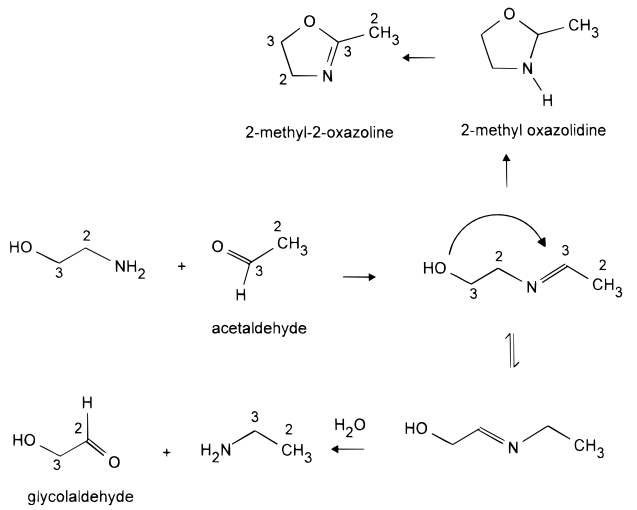
Table 1. Percent Incorporation of ^{13}C-Labeled Carbon Atoms in Cycloglycylalanine and 2,5-Diketo-3,6-dimethylpiperazine (Alanine Cyclic Dimer)

	M	M + 1	M + 2
2,5-diketo-3,6-dimethylpiperazine			
L-[1-^{13}C]serine	0	0	100
L-[2-^{13}C]serine	0	0	100
L-[3-^{13}C]serine	0	0	100
L-serine/[2-^{13}C]alanine (1:3)	20	50	30
cycloglycylalanine			
L-serine/[2-^{13}C]glycine (1:3)	5	95	

these amino acids can also be formed from the thermal degradation of L-serine (see Table 1). However, the observed percentage of unlabeled glycine in this mixture indicates that it is produced in much smaller amounts relative to alanine. Baltes (1990) predicted the formation of similar initial degradation products from L-serine.

Formation of Glycolaldehyde, Alanine, and Ethylamine. The reaction of aminoethanol with carbonyl compounds is a common method of synthesis of oxazolidines (Acheson, 1976) as shown in Scheme 3. Detection of 2-methyl-2-oxazoline (see Figure 1) in the pyrolysate indicates the interaction of acetaldehyde with aminoethanol during the pyrolysis of L-serine. Analysis of label incorporation in 2-methyl-2-oxazoline also indicated the presence of two C-2 and two C-3 atoms of L-serine, consistent with the proposed mechanism. Isomerization of the intermediate imine thus formed (Scheme 3) and its subsequent hydrolysis can generate ethylamine and glycolaldehyde. Ethylamine has been detected in the pyrolysis mixture of L-serine (Kato et al., 1970). Similarly, formaldehyde and pyruvic acid can be converted into their corresponding amines through their interaction with aminoethanol. Formaldehyde can generate methylamine containing the C-3 atom of L-serine, and pyruvic acid can generate D,L-alanine (see Scheme 2). The presence of 2,5-diketo-3,6-dimethylpyrazine (cyclic alanine dimer) in the pyrolysis mixture (see Table 1) confirms the formation of alanine from L-serine. Kato et al. (1970) also isolated 2,5-diketo-3,6-dimethylpiperazine from the thermal degradation of L-serine. Utilization of L-[3-^{13}C], L-[2-^{13}C], and L-[1-^{13}C]serines produced 100% doubly labeled 2,5-diketo-3,6-dimethylpiperazine in all cases. A mixture of L-serine with L-[2-^{13}C]alanine produced 20% unlabeled and 50% singly labeled dimer,

Scheme 3. Proposed Mechanism of Formation of Glycolaldehyde and Ethylamine[a]

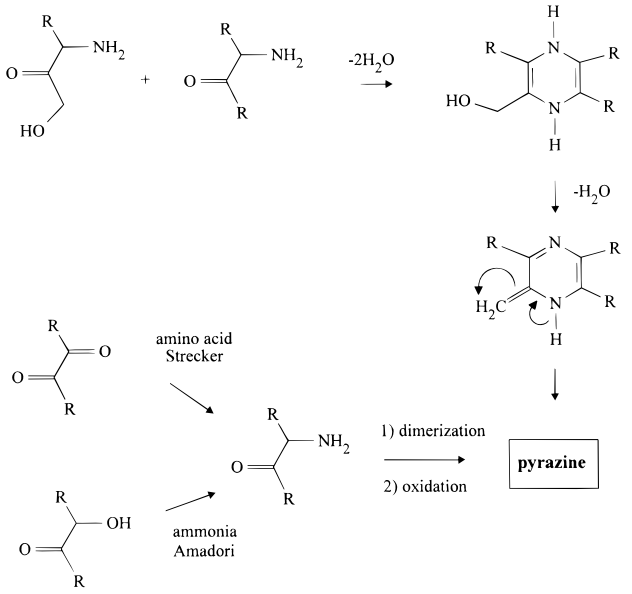


[a] Numbers indicate original carbon atom locations of L-serine.

indicating an abundance of an alternative source of alanine formation in the system. Therefore, under pyrolytic conditions L-serine could be considered to be an efficient producer of alanine. Although glycolaldehyde is not reported as a degradation product of L-serine, evidence for its formation can be provided through trapping experiments with phenylhydrazine. *N*-Ethylidene-*N*′-phenylhydrazine was the major reaction product of phenylhydrazine in the presence glycolaldehyde as well as in the presence of L-serine. Interestingly, performing the pyrolysis of L-serine in the presence of phenylhydrazine prevented the formation of most heterocyclic compounds observed during the pyrolysis of L-serine alone, and the only major product detected was *N*-ethylidene-*N*′-phenylhydrazine. This observation indicates the important role of glycolaldehyde as the major precursor of other carbonyl compounds during the pyrolysis of L-serine. Furthermore, a similar effect was observed when L-serine was pyrolyzed in the presence of excess glycine. The intensities of pyrazine and pyrrole peaks were reduced significantly, and the major products were pyrazinones, which are known to require glycolaldehyde as their important precursor (Keyhani and Yaylayan, 1996).

Advanced Degradation Products of L-Serine. Incorporation of reactive carbonyl compounds into stable end-products can be utilized to investigate their origin in the reaction mixture (Yaylayan et al., 1998). Useful and stable end-products that form abundantly in Maillard systems are the different pyrazines. Pyrazines can be formed by the dimerization of α-aminocarbonyl compounds followed by oxidation, except when one of the reactants is an α-amino-α′-hydroxyl derivative, and then a dehydration step instead of oxidation will generate pyrazines (see Scheme 4). α-Aminocarbonyl compounds, on the other hand, can be formed either by Strecker reaction or by Amadori rearrangement of an α-hydroxycarbonyl compound with ammonia (see Scheme 4). Consequently, reactive precursors of pyrazines can also include α-hydroxycarbonyls and α,β-dihydroxycarbonyls in addition to α-dicarbonyl compounds (Scheme 4). Using ^{13}C-labeled L-serines at the different carbon atoms and analyzing the label incorporation in pyrazines, the origin of such precursors can be predicted (see Figure 2). For example, analysis of label distribution

Scheme 4. Carbonyl Precursors and Proposed Pathways of Formation of Pyrazine



in the parent pyrazine formed in the L-serine pyrolysate allowed unambiguous determination of the origin of its carbon atoms. Such analysis confirmed 100% incorporation of two C-2 and two C-3 atoms of L-serine into the pyrazine structure, indicating the reaction of glycolaldehyde with ammonia through the Amadori rearrangement to form 2-aminoacetaldehyde as the precursor of parent pyrazine (see Figure 2).

Origin of Pyruvaldehyde and Three-Carbon Precursors of 2-Methylpyrazine. Methylpyrazine can be used to determine the origin of pyruvaldehyde or an equivalent of three-carbon precursor unit. Labeling studies have indicated the incorporation of all the three-carbon atoms of L-serine in different ratios into the methylpyrazine (see Table 2 and Figure 2). Analysis of the labeling data in Table 2 confirmed the existence of three pathways (designated A−C in Scheme 5) of formation of carbonyl precursors of methylpyrazine. According to the labeling data in Table 2, the major pathway (pathway A in Scheme 5, 70%) involves aldol addition of formaldehyde to glycolaldehyde to generate glyceraldehyde with two C-3 atoms and one C-2 atom of

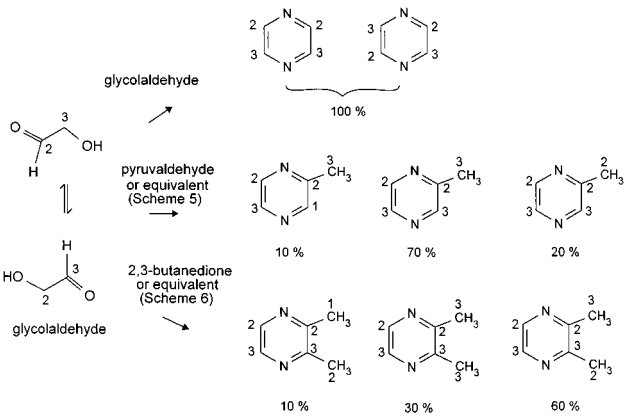


Figure 2. Carbonyl precursors and percent isotopomers of pyrazine and methyl- and 2,3-dimethylpyrazines (see Tables 2 and 3). Similar to parent pyrazine, the positions of the C-2 and C-3 atoms originating from glycolaldehyde in methyl- and 2,3-dimethylpyrazines can be interchanged. Numbers indicate original carbon atom locations of L-serine.

Table 2. Percent Incorporation of ^{13}C-Labeled Carbon Atoms in Methylpyrazine

	% label incorporation				
model	M	M + 1	M + 2	M + 3	M + 4
L-[1-^{13}C]serine	90	10	0	0	0
L-[2-^{13}C]serine	0	0	80	20	0
L-[3-^{13}C]serine	0	0	30	70	0
L-serine/[2-^{13}C]glycine (1:3)	25	75			

Table 3. Percent Incorporation of ^{13}C-Labeled Carbon Atoms in 2,3-Dimethylpyrazine

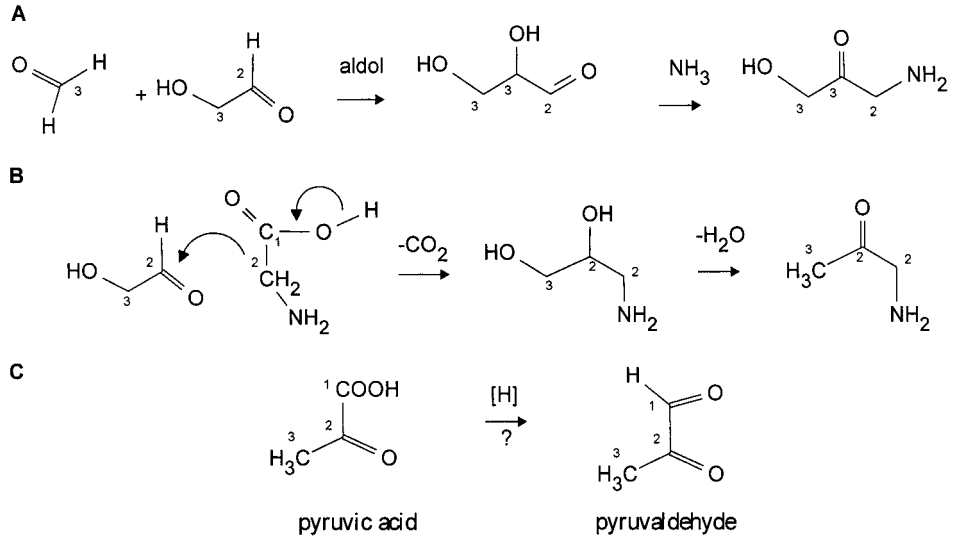
	% label incorporation				
model	M	M + 1	M + 2	M + 3	M + 4
L-[1-^{13}C]serine	90	10	0	0	0
L-[2-^{13}C]serine	0	0	30	70	0
L-[3-^{13}C]serine	0	0	10	60	30
L-serine/[2-^{13}C]glycine (1:3)	10	30	60		
L-serine/[2-^{13}C]alanine (1:3)	60	40			

L-serine. Reaction of ammonia with glyceraldehyde can generate the Amadori rearrangement product able to form methylpyrazine by reaction with 2-aminoacetaldehyde. Pathway B (20%) shows the interaction of glycine with glycolaldehyde (Yaylayan and Keyhani, 1998) to form 1-aminoacetone, incorporating two C-2 and one C-3 atoms of L-serine. The minor pathway (pathway C, 10%) proposes reduction of pyruvic acid to pyruvaldehyde to account for the incorporation of C-1, C-2, and C-3 atoms of L-serine into methylpyrazine.

Origin of 2,3-Butanedione and Other Four-Carbon Precursors of 2,3-Dimethylpyrazine. Similar to methylpyrazine, 2,3-dimethylpyrazine can be used to determine the origin of 2,3-butanedione formed in the pyrolysis products of L-serine. Again, labeling studies have indicated the incorporation of all three carbon atoms of L-serine, in different ratios, into the 2,3-dimethylpyrazine structure (see Table 3 and Figure 2). Analysis of the data in Table 3 indicated incorporation of four C-3 and only three C-2 atoms of L-serine into 2,3-dimethylpyrazine. In addition, 10% of the 2,3-dimethylpyrazine detected was formed from 2,3-butanedione or its equivalent, incorporating one C-1, one C-3, and two C-2 atoms of L-serine. On the other hand, 30% was formed by the incorporation of one C-2 and three C-3 atoms of L-serine and 60% by the incorporation of two C-2 and two C-3 atoms (see Figure 2). Scheme 6 summarizes the possible mechanistic routes of formation of 2,3-butanedione or its equivalent, consistent with label incorporation patterns of 2,3-dimethylpyrazine. Aldol condensation between glycolaldehyde and acetaldehyde can generate 2,3-butanedione, incorporating two C-2 and two C-3 atoms (Scheme 6, mechanism A). On the other hand, pyruvaldehyde, produced as shown in Scheme 5, can undergo chain elongation through glycine reaction (Yaylayan and Keyhani, 1998) to produce 2,3-butanedione having two C-2, one C-1, and one C-3 L-serine atoms (see Scheme 6, mechanism B). Aldol condensation of glyceraldehyde with formaldehyde produces 1-hydroxy-2,3-butanedione, incorporating one C-2 and three C-3 atoms of L-serine (see Scheme 6, mechanism C). Unfortunately, the mass spectral fragmentation patterns of the above pyrazines cannot be used to extract information related to the sequence of the serine atoms incorporated into the pyrazines to confirm the exact locations of the labeled atoms to further support the proposed pathways.

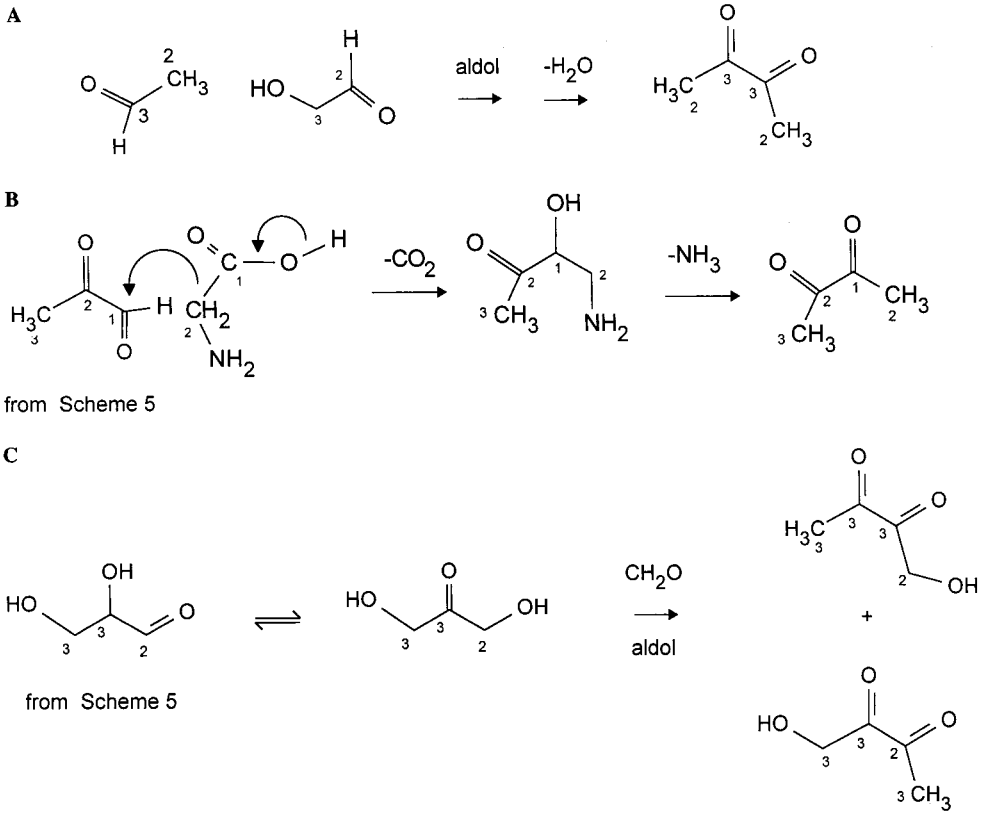
Confirmation of the Proposed Pathways. The proposed mechanisms shown in Schemes 5 and 6 are based on

Scheme 5. Proposed Pathways of Formation of Pyruvaldehyde or Its Equivalents[a]



[a] Numbers indicate original carbon atom locations of L-serine.

Scheme 6. Proposed Pathways of Formation of 2,3-Butanedione or Its Equivalent[a]



[a] Numbers indicate original carbon atom locations of L-serine.

the assumption that the main source of formaldehyde is the C-3 atom of L-serine as shown in Scheme 1. This assumption can be justified on the basis of the limited amount of glycine produced from L-serine degradation, thus preventing an extensive Strecker reaction from occurring to produce significant amounts of formaldehyde (having the C-2 atom of L-serine) from glycine. Furthermore, the preference of glycine to undergo double addition rather than Strecker reaction with α-dicarbonyl compounds has been verified (Keyhani and Yaylayan, 1996). However, to confirm the participation of glycine in the formation of precursors of pyrazines as depicted in Schemes 5 and 6, L-serine was reacted with excess [2-^{13}C]glycine. The presence of excess [2-^{13}C]-glycine in the mixture can lead to the formation of formaldehyde as a Strecker aldehyde (incorporating the labeled C-2 atom) and, consequently, generate according to Scheme 5 unlabeled and singly labeled methylpyrazines. Unlabeled methylpyrazine can be produced through pathway C, where there is no glycine involvement, and singly labeled methylpyrazine can be formed through pathways A and B. On the other hand, according to Scheme 6, unlabeled and singly and doubly labeled 2,3-dimethylpyrazines should be formed in the reaction mixture containing [2-^{13}C]glycine. Pathway A should produce unlabeled isotopomer because there is

no glycine or formaldehyde involvement, pathway B should produce singly labeled pyrazine, and pathway C should produce singly and doubly labeled pyrazines depending on the origin of formaldehyde. Inspection of Tables 2 and 3 shows data consistent with the above predictions. Furthermore, to confirm the major pathway (pathway A in Scheme 6) of formation of 2,3-butanedione, which involves aldol condensation of acetaldehyde, the Strecker aldehyde of L-alanine, L-serine, was also pyrolyzed in the presence of L-[2-^{13}C]alanine. L-Alanine is known to efficiently produce acetaldehyde in the presence of dicarbonyls. Consequently, if acetaldehyde is indeed involved in the formation of 2,3-butanedione, then the mixture should produce singly labeled 2,3-dimethylpyrazine through pathway A and unlabeled 2,3-dimethylpyrazine through pathways B and C. The latter two pathways do not involve acetaldehyde. Again, the data presented in Table 3 confirm the above predictions.

Conclusion. Unlike other amino acids, degradation of L-serine in the absence of sugars can lead to the formation of a multitude of heterocyclic compounds. In addition, it can also produce two other amino acids, glycine and alanine. Analysis of the label distribution in pyrazine, methylpyrazine, and 2,3-dimethylpyrazine generated from the various model systems indicated the importance of glycolaldehyde in the formation of longer chain reactive dicarbonyl compounds.

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