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ARTICLE

# Isotope Labeling Studies on the Origin of 3,4-Hexanedione and 1,2-Butanedione in an Alanine/Glucose Model System

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Although the importance of α-dicarbonyl compounds as reactive intermediates in the Maillard reaction and as precursors of heterocyclic and odor-active compounds is well-established, however, the detailed origin of many α-dicarbonyl compounds such as 3,4-hexanedione and 1,2-butanedione still remains unknown. Using glucose and glyoxal with labeled [$^{13}$C-1]alanine, [$^{13}$C-2]alanine, [$^{13}$C-3]-alanine, and [$^{15}$N]alanine, the mechanism of their formation was investigated using the label incorporation pattern of the pyrazines derived through the Strecker reaction. Taking into account the non-oxidative mechanism of pyrazine formation, the data indicated that all of the ethyl-substituted pyrazines identified in the glyoxal/alanine model system incorporated C-2′ and C-3′ atoms of alanine, and not that of free acetaldehyde, as the ethyl group carbon atoms. This was achieved through spiking experiments using unlabeled acetaldehyde in the presence of labeled alanine. Furthermore, the data also indicated the occurrence of a chain elongation process of sugar-derived α-dicarbonyl compounds assisted by alanine. On the basis of the proposed mechanism, the glyoxal interaction with alanine through a decarboxylative aldol addition reaction can lead to the formation of 1,2-butanedione with the terminal ethyl carbon atoms originating from C-2′ and C-3′ atoms of alanine, and the similar interaction of 1,2-butanedione with a second molecule of alanine can lead to the formation of 3,4-hexanedione with both terminal ethyl carbon atoms originating from C-2′ and C-3′ atoms of alanine.

**KEYWORDS: isotope labeling; 3,4-hexanedione; 1,2-butanedione; alanine; glyoxal; oxidative and non-oxidative pyrazine formation**

## INTRODUCTION

The origin of many reactive α-dicarbonyl compounds such as glyoxal, pyruvaldehyde, and 2,3-butanedione formed in the Maillard model systems are well-established and can be attributed to the retro-aldol and elimination reactions of different glucosones and other sugar-derived fragments. They play a critical role not only in the generation of different heterocyclic compounds during thermal processing of food but also in the formation of cross-links and advanced glycation end products. However, the formation of a longer alkyl chain containing α-dicarbonyls such as 2,3-pentanedione, 2,3-hexanedione, and 3,4-hexanedione is a less known and a more complex process due to their multiple origins and the involvement of both sugar and amino acid carbon atoms. The 2,3-pentanedione, for example, can be generated in a glucose/alanine model system (*1*) either totally from sugar carbon atoms (10%) or through participation of amino acid carbon atoms (90%). These percentages can vary depending on the reaction conditions (*2*). Isotope labeling studies have revealed that a $C_3$ carbon unit composed of either C1−C2−C3 or C4−C5−C6 (pyruvaldehyde or glyceraldehyde) from D-glucose and a $C_2$ unit either from amino acid or from glucose (C1−C2) are involved in a chain elongation reaction to generate 2,3-pentanedione in an alanine/glucose model system. Although the involvement of 3,4-hexanedione in the formation of pyrazines was predicted from model system studies, to our knowledge, its mechanism of formation has not been reported. Similar to other α-dicarbonyl compounds, 3,4-hexanedione has very strong odor properties described as buttery, cooked, and caramel (*3*). It has been identified in different model systems as a free dicarbonyl compound (*4*−*7*), in bread crust (*8*), or integrated into a pyrazine moiety through Strecker reaction (*9*). On the other hand, 1,2-butanedione has been reported to be formed through aldol condensation of glycolaldehyde with acetaldehyde (*10*). In this study, the formation pathways of 3,4-hexanedione and 1,2-butanedione in alanine model systems were used as an aid in distinguishing between oxidative and non-oxidative pathways of pyrazine formation.

## EXPERIMENTAL PROCEDURES

**Materials and Reagents.** L-Alanine (99%), D-glucose (99.5%), glyoxal trimer dihydrate (95%), acetal (acetaldehyde diethylacetal) (99%), and acetaldehyde (99.5%) were purchased from Sigma-Aldrich Chemical Co. (Oakville, ON, Canada). The labeled [$^{13}$C-1]alanine (98%), [$^{13}$C-2]alanine (99%), [$^{13}$C-3]alanine (99%), and [$^{15}$N]alanine (98%) were purchased from Cambridge Isotope Laboratories (Andover, MI).

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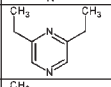
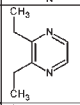
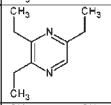
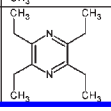
**Table 1.** Pyrazine and Ethyl-Substituted Pyrazines Tentatively Identified in a Glyoxal/Alanine Model System

| Compounds | M.W. | Structure | Retention Time (min) | Confirmation[a] | % abundance |
|---|---|---|---|---|---|
| Pyrazine *(17)* | 80 |  | 10.78 | NIST | 28% |
| 2-Ethylpyrazine *(17, 18)* | 108 |  | 14.34 | NIST & Retention time 14.42 min | 46% |
| 2,6-Diethylpyrazine *(16, 19)* | 136 |  | 17.61 | NIST | 18% |
| 2,3-Diethylpyrazine *(17)* | 136 |  | 17.69 | NIST & Retention time 17.74min | 6% |
| 2,3,5-Triethylpyrazine *(17, 18, 20, 21)* | 164 |  | 20.5 | NIST | 2% |
| Tetraethylpyrazine *(9, 19)* | 192 |  | 22.4 | NIST | 0.06% |

[a] Retention times of the standards; see also **Table 2**, which indicates the presence of two nitrogen atoms in all of the pyrazines.

**Table 2.** Number of Isotopic Atoms Incorporated[a] in Pyrazines Generated from Glyoxal/Alanine Model Systems[2]

| compounds | [$^{13}$C-1′]ala | [$^{13}$C-2′]-ala | [$^{13}$C-3′]ala | [$^{15}$N]ala |
|---|---|---|---|---|
| pyrazine | 0 | 0 | 0 | 2 |
| 2-ethylpyrazine | 0 | 1 | 1 | 2 |
| 2,6-diethylpyrazine | 0 | 2 | 2 | 2 |
| 2,3-diethylpyrazine | 0 | 2 | 2 | 2 |
| 2,3,5-triethylpyrazine | 0 | 3 | 3 | 2 |
| tetraethylpyrazine | 0 | 4 | 4 | 2 |

[a] The number of atoms indicated in the table was incorporated 100 ± 5%. [2] From four separate experiments using glyoxal/[$^{15}$N]alanine, glyoxal/[$^{13}$C-1′]alanine glyoxal/[$^{13}$C-2′]alanine glyoxal/[$^{13}$C-3′]alanine.

**Pyrolysis Gas Chromatography−Mass Spectrometry (Py-GC/MS).** A Varian CP-3800 GC equipped with a sample preconcentration trap (SPT) 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 GC injection port and connected to a CDS Pyroprobe 2000 unit (CDS Analytical, Oxford, PA). The sample separations were carried out on a DB-5MS column (5% diphenyl, 95% dimethyl polysiloxane) with column dimensions of 50 m length × 0.2 mm internal diameter × 33 $\mu$m film thickness (J&W Scientific, ON, Canada) using helium as the carrier gas. One milligram of sample mixture containing 1:1 or 1:3 molar ratio of sugar to alanine in the presence of silica was packed inside a quartz tube (0.3 mm thickness), plugged with quartz wool, and inserted inside the coil probe and pyrolyzed at 250 °C for 20 s under He atmosphere. The volatiles after pyrolysis were concentrated on the sample preconcentration trap (SPT), trapped at 50 °C, and subsequently directed toward the GC column for separation. The GC column flow rate was regulated by an electronic flow controller (EFC) and set at a pressure pulse of 70 psi for 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 at −5 °C for 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 samples were detected by using an ion-trap mass spectrometer. The MS transfer-line temperature was set at 250 °C, manifold temperature was set at 50 °C, and the ion-trap temperature was set at 175 °C. The ionization voltage of 70 eV was used, and EMV was set at 1500 V.

**Oxidative Py-GC/MS.** Pyrolysis under air was achieved through modification of the above-mentioned GC to allow gas stream switching and subsequent isolation of the pyrolysis chamber from the analytical stream. The pyrolysates generated under air were initially collected onto the trap, which retained the organic volatiles and vented the carrier gas (air) and/or moisture. The trap was subsequently flushed with helium and heated to desorb the collected volatiles. For detailed description see (*11*).

**Identification of Pyrazines.** All model systems studied (alanine/sugar or alanine reactive intermediates) were analyzed under both oxidative and non-oxidative conditions, with and without labeling. Pyrazines were identified by their retention times where possible and through NIST library matches in addition to the labeling data (see **Tables 1** and **2**).

## RESULTS AND DISCUSSION

Identification of the origin and the structure of α-dicarbonyl compounds in model systems can be achieved through isotope labeling studies using known precursors such as sugars and amino acids and analyzing for label incorporation in either free α-dicarbonyl compounds formed or in their derivatives such as quinoxalines and pyrazines. A derivatizing agent is needed for the formation of quinoxalines, whereas pyrazines can be considered as "intrinsic derivatives" since their formation does not require the use of a reagent that may interfere with the normal course of the reaction. For example, the presence of 3,4-hexanedione may be inferred in an alanine/glucose model system if either 2,3-diethyl-5-methylpyrazine or 2,3,5-triethyl-6-methylpyrazine is detected, similarly, the presence of 1,2-butanedione may be inferred if 2-ethyl-5,6-dimethylpyrazine is detected (see **Figures 1** and **2**). Although through Strecker reaction (SR) the origin and the identity of α-dicarbonyl compounds can be traced back to the structure of pyrazines, the possibility exists that at the dihydropyrazine stage the enamine moiety can add the Strecker aldehyde or any other aldehyde formed in the system to its ring (see **Figure 3**) and following dehydration can generate a pyrazine through a non-oxidative pathway with one additional alkyl substituent on the pyrazine ring characteristic of the structure of the reacting aldehyde (*12*−*14*). In such cases, only a dihydropyrazine structure can reveal the identity of the α-dicarbonyl species formed in the model system. However, pyrazines formed through such a non-oxidative pathway can also be used as derivatives of α-dicarbonyl compounds if the identity of the aldehyde is known in the model system or the added aldehyde can be distinguished through labeling techniques. In order for pyrazines, therefore, to serve as useful derivatives of α-dicarbonyl compounds and hence be used to trace their origin in model systems, their formation mechanism should be elucidated. In the

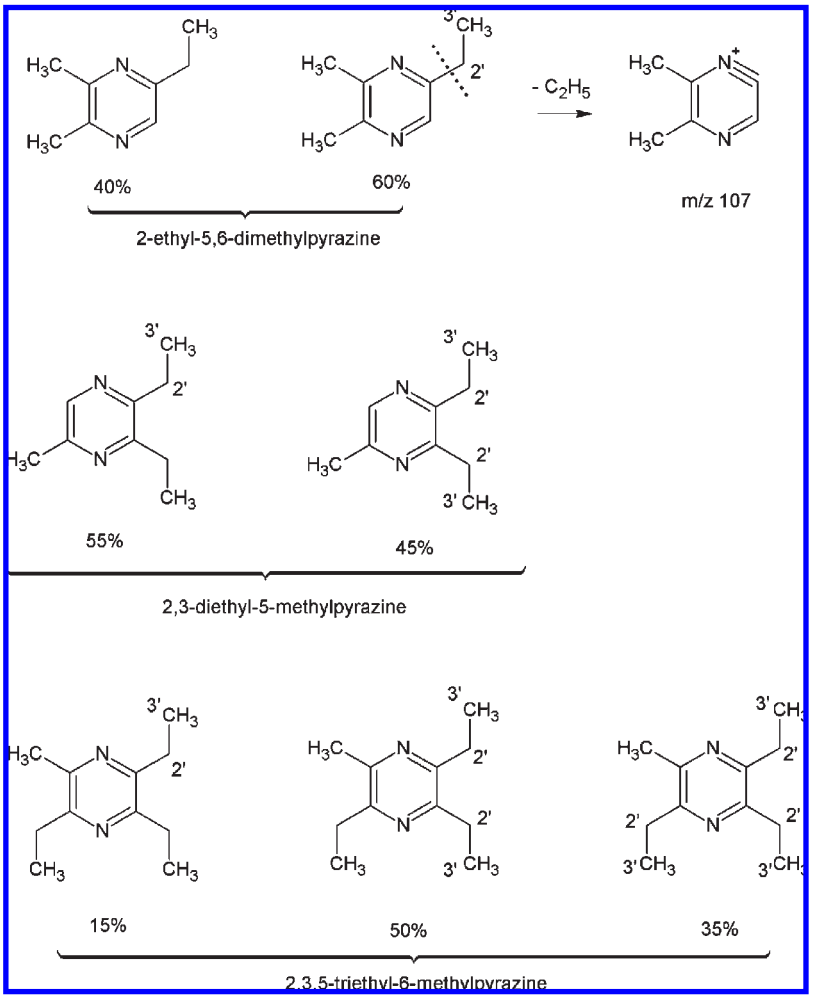



**Figure 1.** Ethyl-substituted pyrazines tentatively identified in a glucose/alanine model system and percentage incorporation of alanine C-2′ and C-3′ atoms.

glucose/alanine model system studied, the reaction generated 2,3-diethyl-5-methylpyrazine, 2,3,5-triethyl-6-methylpyrazine, and 2-ethyl-5,6-dimethylpyrazine (**Figure 1**), among others. Label incorporation studies indicated that alanine played a major role in providing the two carbon atoms of the ethyl groups (see **Figure 1**). The 2-ethyl-5,6-dimethylpyrazine exhibited 60% incorporation of alanine carbon atoms, and the 2,3-diethyl-5-methylpyrazine or 2,3,5-triethyl-6-methylpyrazine incorporated at least one ethyl group from alanine. The origin of the ethyl groups was confirmed through analysis of the presence or absence of a label in the mass fragment generated by the loss of an ethyl radical such as formation of an ion at *m*/*z* 107 in 2-ethyl-5,6-dimethylpyrazine (see **Figure 1**). The origin of the unlabeled ethyl groups can be ascribed to α-dicarbonyls formed through an aldol condensation pathway from glucose precursors, as shown in **Figure 5**.

The presence of the above-mentioned pyrazines in the glucose/alanine model system may indicate the formation of 1,2-butanedione and 3,4-hexanedione in addition to other required α-dicarbonyls such as 2,3-butanedione, 2,3-pentanedione, and pyruvaldehyde. As mentioned above, the structure of a given pyrazine may not necessarily allow deduction of the type of α-dicarbonyl compound needed to give rise to its formation due to the possibility of non-oxidative pathway, as shown in **Figure 3**. However, one limitation of this pathway is the fact that a given dihydropyrazine moiety can add only one aldehyde at a time to form a pyrazine with one additional alkyl group. For example, the dihydropyrazine I in **Figure 3** can only form ethylpyrazine through a non-oxidative pathway and not diethylpyrazine; as a result, the pyrazines listed in **Figure 1** with multiple ethyl groups originating from alanine can only arise from preformed 1,2-butanedione and 3,4-hexanedione molecules irrespective of their method of formation (oxidative vs nonoxidative), as illustrated in **Figure 2**. Furthermore, due to the incorporation of a significant percentage of alanine carbon atoms into the pyrazines, the above α-dicarbonyls should arise through chain elongation processes (*1*) of smaller sugar-derived α-dicarbonyl compounds with the assistance of alanine.

**Chain Elongation Reactions of α-Dicarbonyl Compounds.** To justify the label incorporation patterns of the pyrazines shown in **Figure 1**, the four-carbon α-dicarbonyl compound, 1,2-butanedione, should have two of its carbon atoms originating from alanine and the six-carbon α-dicarbonyl compound, 3,4-hexanedione, should have four of its carbon atoms originating from alanine, thus pointing to glyoxal as the possible sugar-derived α-dicarbonyl precursor needed for the conversion into four-carbon and six-carbon analogues through chain elongation reactions involving alanine. When alanine was reacted with glyoxal, in addition to the parent pyrazine, many ethyl-substituted pyrazines (see **Tables 1** and **2**), pyrroles, pyridines, and pyrazinones were also detected (see **Figure 4**), the major group being the pyrazine derivatives. Since glyoxal, the only carbohydrate source in the model system, can only generate the parent pyrazine through the Strecker reaction, all other substituted pyrazines in the glyoxal alanine model system should therefore arise either through chain elongation reactions of glyoxal with alanine to form longer

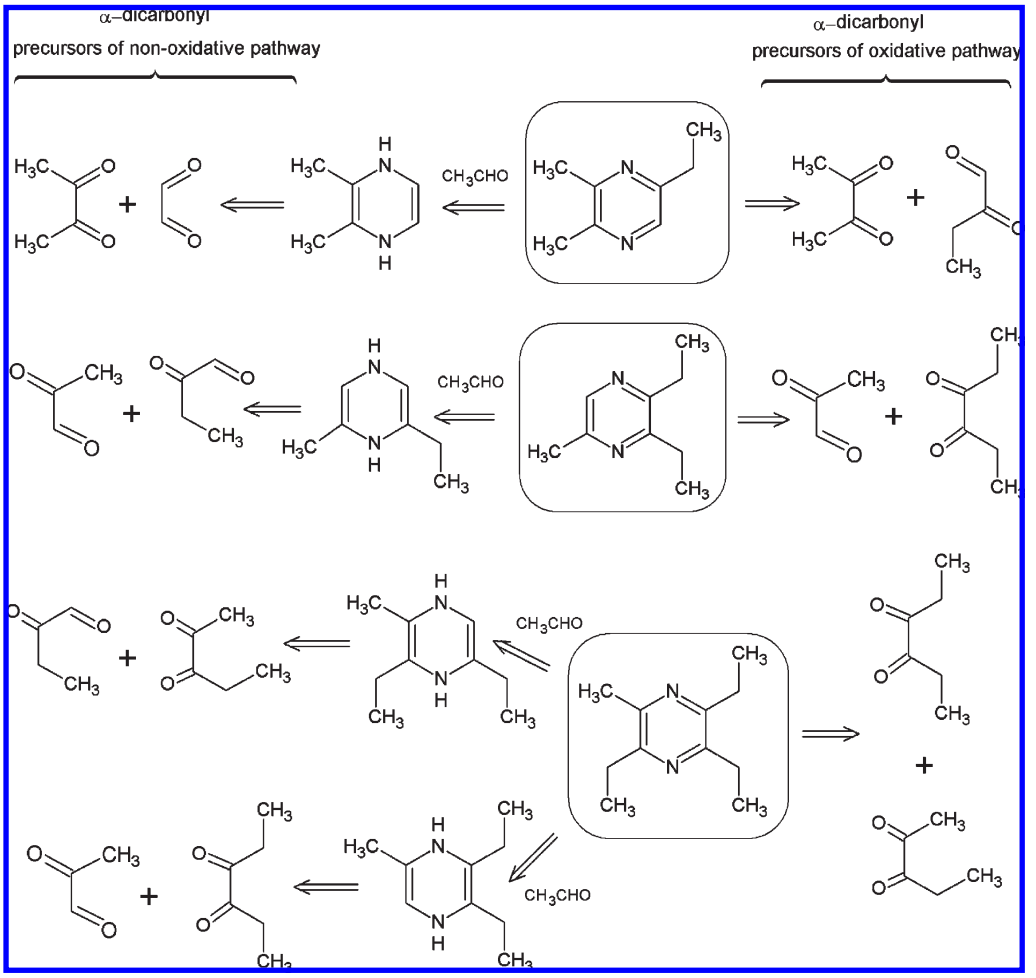



**Figure 2.** α-Dicarbonyl precursors required for oxidative and non-oxidative pyrazine formation through the Strecker reaction in the glucose/alanine model system.

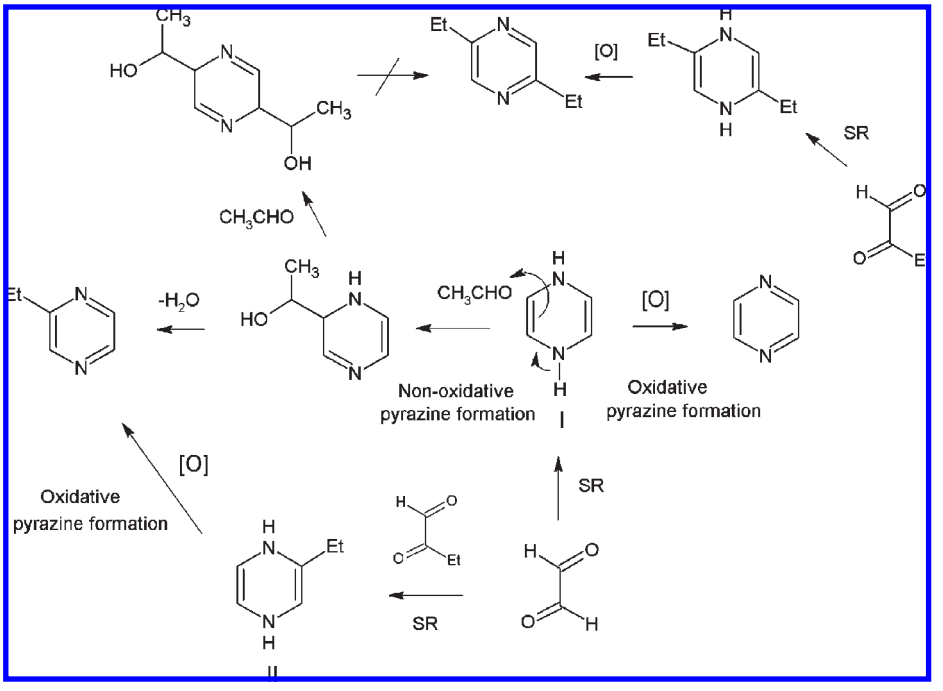



**Figure 3.** Proposed oxidative and non-oxidative mechanisms of pyrazine formation. SR = Strecker reaction; [O] = oxidation.

α-dicarbonyl compounds as was demonstrated before in the case of 2,3-pentanedione (*1*) or through aldol condensation of acetaldehyde (the Strecker aldehyde) to glycolaldehyde to generate the same precursors (*10*) (see **Figure 5**). Although in the glyoxal/alanine

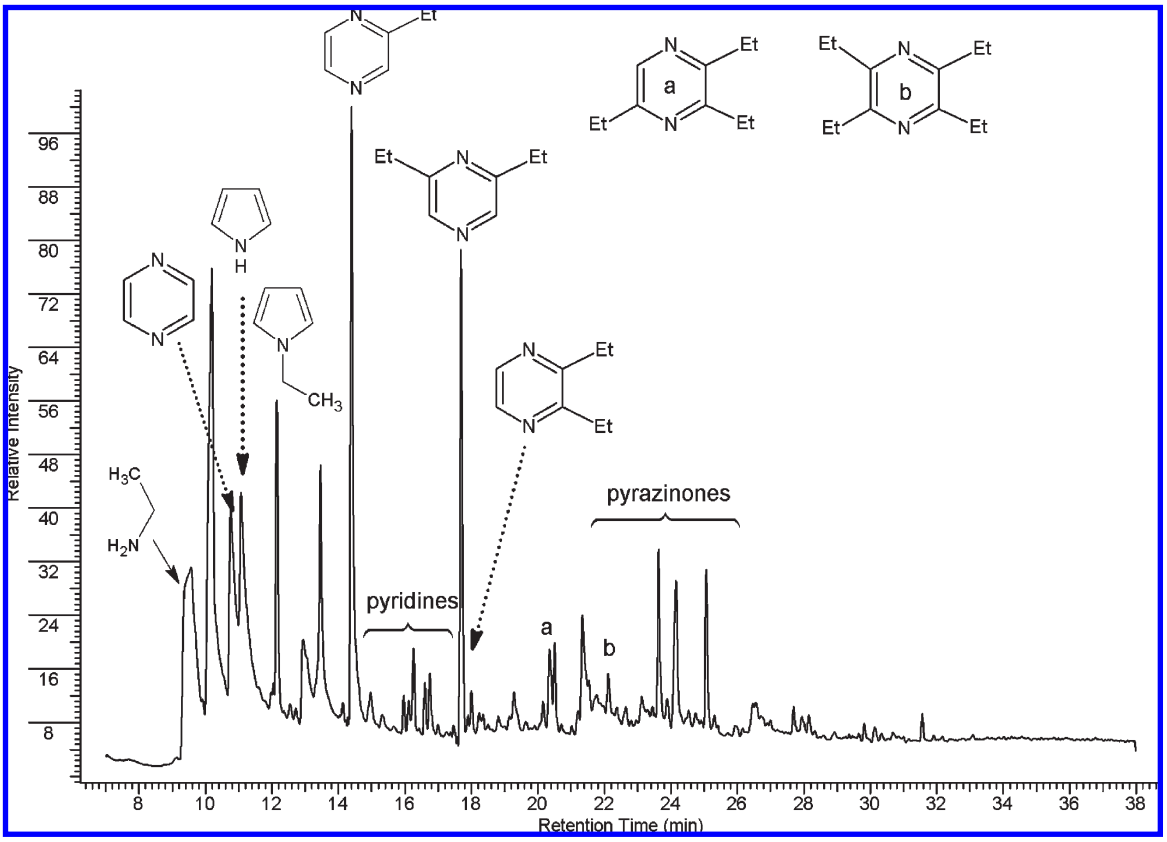



**Figure 4.** Chromatogram generated at 210 °C by pyrolysis of the glyoxal/alanine model system.

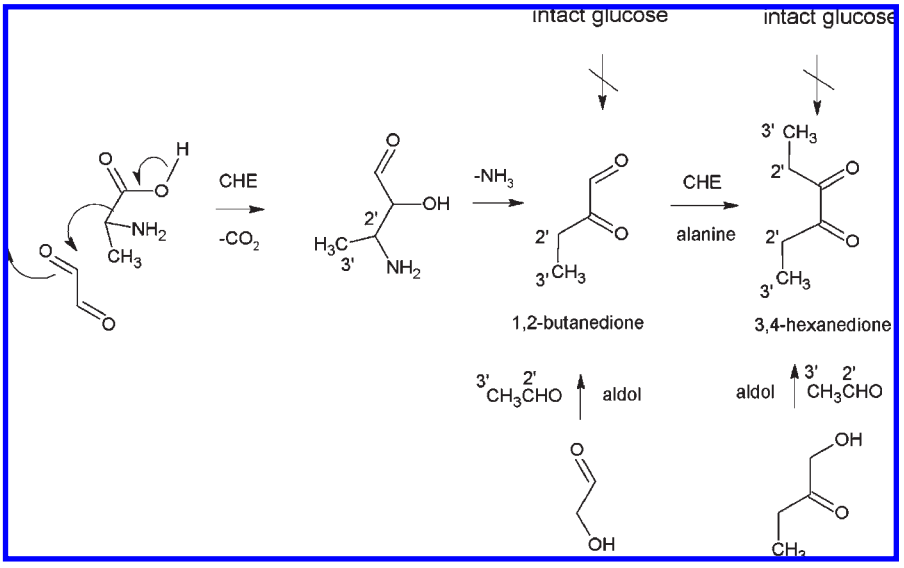



**Figure 5.** Proposed mechanisms of formation of 1,2-butanedione and 3,4-hexandione. CHE = chain elongation; aldol = aldol condensation.

model system some glycolaldehyde may be formed, the main contribution to the formation of substituted pyrazines should come from glyoxal, the major constituent of the model system. In addition, spiking the glyoxal/alanine model with glycolaldehyde (20% relative to glyoxal content) did not increase the intensities of the pyrazine peaks; on the contrary, they were decreased, indicating its preference to react with alanine to form Amadori product rather than to undergo aldol condensation with acetaldehyde. According to **Table 2**, all of the carbon atoms of the ethyl groups of all the detected pyrazines originated 100% from C-2′ and C-3′ atoms of alanine. This pattern of isotopic substitution is consistent with both mechanisms proposed in **Figure 5**. Although the addition of glycolaldehyde to the model system did not increase the intensities of pyrazines, to further confirm that aldol addition of acetaldehyde to glycolaldehyde does not contribute significantly to the formation of 1,2-butanedione, the [$^{13}$C-3′]alanine/glyoxal and [$^{13}$C-2′]alanine/glyoxal model systems were also analyzed in the presence of increasing concentrations of unlabeled acetaldehyde to estimate the percentage of acetaldehyde incorporation into the structure of pyrazines through aldol reaction. Adding a molar or less than a molar equivalent of acetaldehyde to the above model systems did not alter at all the isotopic distribution

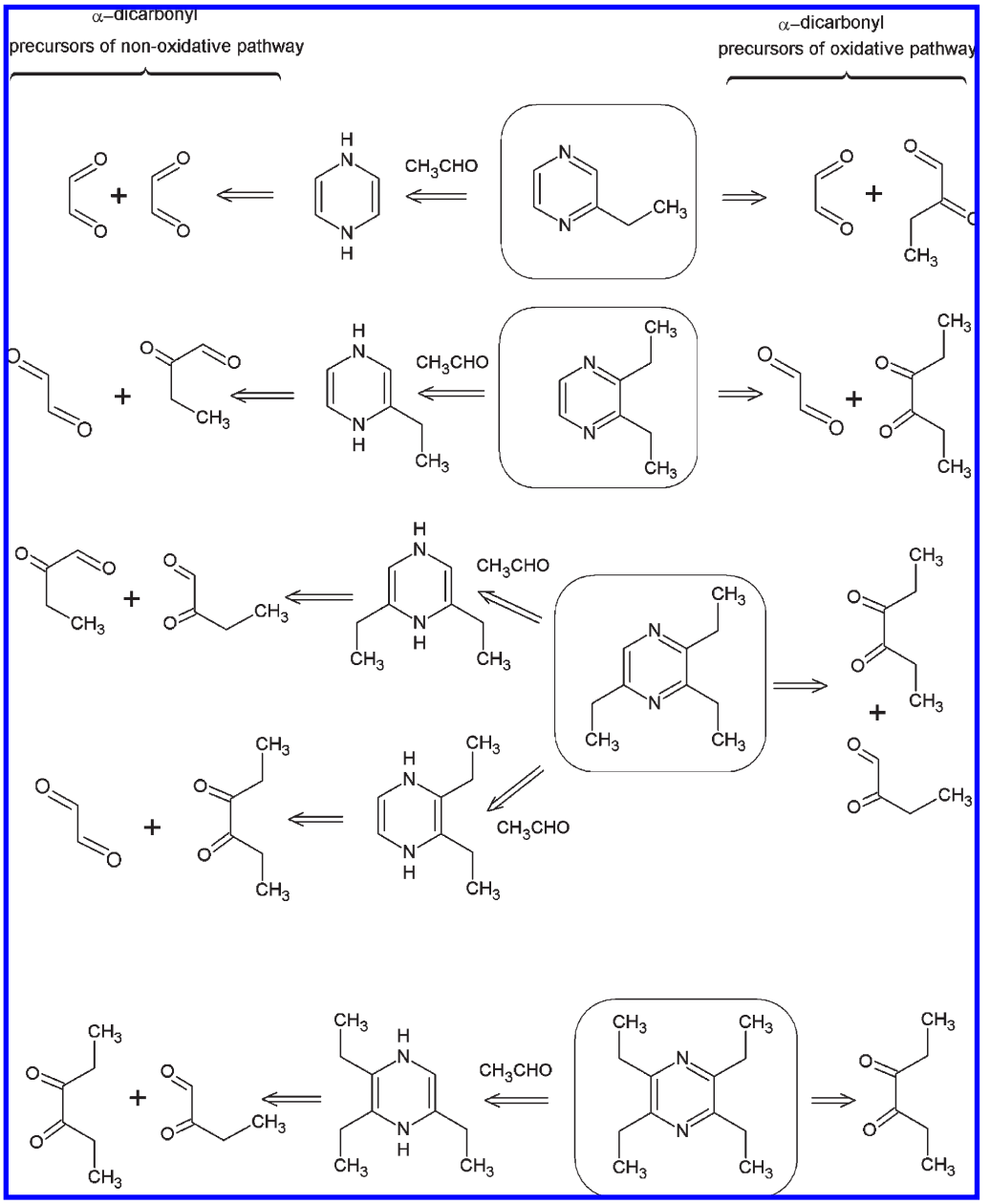



**Figure 6.** α-Dicarbonyl precursors required for oxidative and non-oxidative pyrazine formation through the Strecker reaction in the glyoxal/alanine model system.

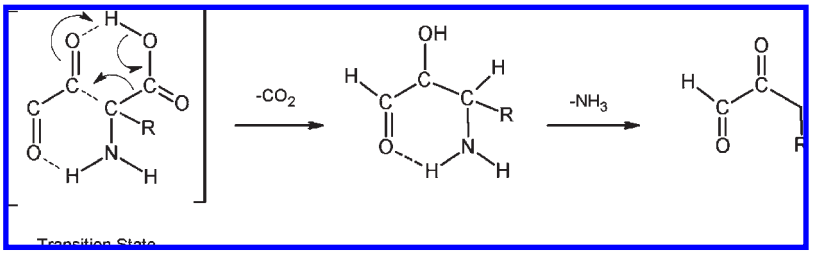



**Figure 7.** Proposed bicyclic transition state for the simultaneous aldol addition and decarboxylation reaction of glyoxal with an amino acid leading to chain elongation of glyoxal.

patterns of the pyrazines; however, much higher concentrations caused 30−50% incorporation of mainly single acetaldehyde units into the pyrazines listed in **Table 1**. These observations indicate that aldol reaction of acetaldehyde does not contribute significantly to the formation of pyrazines but it points mainly to the occurrence of the non-oxidative mechanism of pyrazine formation under unusually high concentrations of the aldehydes, as illustrated in **Figure 2**. When the alanine/glyoxal model system was pyrolyzed under air and in excess acetaldehyde, a 5-fold increase in the total intensities of the pyrazine peaks was observed relative to non-oxidative conditions, indicating the importance of the oxidative pathway. Furthermore, as in the case of pyrazines

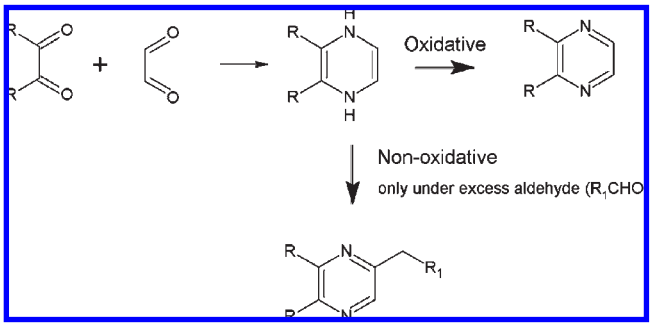



**Figure 8.** Oxidative and non-oxidative pathways of pyrazine formation through the Strecker reaction of the corresponding α-dicarbonyl compounds.

identified in the glucose/alanine system, the pyrazines detected in the glyoxal/alanine model system similarly required 1,2-butanedione and 3,4-hexanedione as preformed α-dicarbonyl compounds irrespective of their mechanism of formation, as illustrated in **Figure 6**.

**How Is Alanine Involved in the Chain Elongation Process of Glyoxal?** Similar to the mechanism proposed earlier (*1*), alanine can interact with the aldehyde end of the α-dicarbonyl as a C-nucleophile through its α-carbon (*15*, *16*) and undergoes aldol-type addition with simultaneous decarboxylation reaction, as shown in **Figure 5**. The resulting adduct produces the 1,2-butanedione after a deamination step. In turn, the 1,2-butanedione undergoes similar alanine-assisted chain elongation to form 3,4-hexanedione, thus adding another set of two carbon atoms from alanine. Perhaps one of the reasons for the efficiency of this type of amino acid interaction with the smaller and terminal end of α-dicarbonyl compounds such as glyoxal and pyruvaldehyde is the possibility and the ease of formation of a six-membered bicyclic transition state (see **Figure 7**) involving two hydrogen bonds to the *trans*-configuration of the α-dicarbonyl moiety: one with the carboxylic acid hydrogen and the other with the hydrogen on the amino group (**Figure 7**). After simultaneous aldol addition and decarboxylation, the resulting 3-amino-2-hydroxycarbonyl compound can undergo deamination and generate the new α-dicarbonyl compound incorporating the carbon atoms of the amino acid. Chain elongation assisted by alanine requires multiple additions to glyoxal to generate longer chain α-dicarbonyls such as the 3,4-hexanedione required for the formation of 2,3-diethyl, 2,3,5-triethyl, and tetraethylpyrazines. As observed, increasing the ratio of alanine relative to glyoxal enhanced the peak areas associated with the above pyrazines. Furthermore, as demonstrated above, it is only under excessive aldehyde concentrations that the dihydropyrazine intermediate deviates from the main oxidative route toward pyrazine formation and reacts with available aldehdyes including Strecker aldehyde to form pyrazine with one more substituent (see **Figure 8**). The same carbon atoms of alanine involved in the formation of Strecker aldehyde are also involved in the formation of α-dicarbonyl compounds through a chain elongation process and/or aldol condensation. However, through the use of appropriately labeled alanine and unlabeled Strecker aldehyde, these two processes were distinguished from each other in this study.

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