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Monitoring Carbonyl-Amine Reaction between Pyruvic Acid and α-Amino Alcohols by FTIR Spectroscopy—A Possible Route To Amadori Products

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The carbonyl-amine reaction between pyruvic acid and α-amino alcohols was monitored by Fourier transform infrared spectroscopy at a temperature range between 20 and 100 °C and under acidic and basic conditions. To avoid interference, the reactions were conducted in the absence of solvent using liquid reactants such as methyl pyruvate, pyruvic acid, ethanolamine, and 1-amino-2,3-propanediol. Analysis of the time- and temperature-dependent spectra indicated that under basic conditions and at room temperature, the initial imine formation and its subsequent isomerization through a 1,3-prototropic shift occur very rapidly and the reaction goes to completion within 12 min. Interestingly, the isomerization product of the initial imine is the so-called Schiff base intermediate formed when the corresponding amino acid and the reducing sugar react during a typical Maillard reaction. Furthermore, the detailed studies also indicated that during the first 30 s, the rate of formation of the initial imine was faster than the rate of its isomerization; however, after 60 s, its rate of isomerization becomes faster than the rate of its formation. The data also indicated that under acidic conditions, this isomerization was prevented from occurring and the reaction was terminated at the initial imine formation stage. In addition, temperature-dependent spectra indicated that the isomerization of the Schiff's base into eneaminol can be achieved at or above 60 °C and its subsequent rearrangement into Amadori product can be attained at temperatures above 80 °C even under basic conditions, thus providing a novel route to Maillard reaction products starting from a keto acid and an amino alcohol. This observation was also confirmed through identification of the common Amadori product in both keto acid/amino alcohol and sugar/amino acid mixtures, by the application of tandem mass spectrometry and chemical ionization techniques.

KEYWORDS: Transamination; Amadori product; pyruvic acid; aminoethanol; imine isomerization; MS/MS

INTRODUCTION

The widely accepted pathway for the formation of Amadori product involves the well-established carbonyl-amine reaction between reducing sugars and amino acids. The first step of this interaction leads to the formation of an imine known as the Schiff's base. The possibility of this imine to be converted into its isomeric imine during the Maillard reaction has been proposed by Høltermand (*1*) and has been referred to as a transamination reaction, since its hydrolysis can generate the corresponding α-keto acid and an amino-sugar. Høltermand (*1*) isolated, for example, trace amounts of pyruvic acid (PA) from the alanine/glucose model system without being able to detect the presence of the corresponding amino-sugar. Similar isomerization of the imine, formed this time between α-keto acids and amino acids, has been observed by Herbst and Engel (*2*), and its mechanism was characterized by Cram and Guthrie (*3*) as base-catalyzed methylene-azomethine rearrangement. Since its first proposition by Høltermand (*1*), no evidence for the occurrence of this type of transamination reaction during Maillard reaction has been provided in the literature. However, the conversion of amino acids into 2-ketoacids in the presence of glyoxal has also been suggested by Davidek et al. (*4*) to occur during Maillard reaction. To provide evidence for the occurrence of isomerization of imines under Maillard reaction conditions, model systems of PA/aminoethanol and alanine/glycolaldehyde were studied by pyrolysis-gas chromatography/mass spectrometry (Py-GC/MS) using variously [^{13}C]-labeled PAs (*5*). Comparison of the pyrolysis products generated at 250 °C and separated on a PLOT column indicated that ∼30% of the peaks between the two model systems were common to both systems. Furthermore, the results of these labeling studies have also confirmed the formation of PA in the reaction mixture of alanine/glycolaldehyde and the presence of alanine and glyco-

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Table 1. MS-MS Optimization Conditions

segment set points	ionization mode	ion preparation
scanned mass range, 33–250 *m/z*	target TIC, 25 000 c	parent ion masses: 175, 161, 160, 144, 143, 129, 101, and 100 *m/z*
scan time, 0.3 s/scan	max ion time, 25 000 μs	isolation window, 3.0 *m/z*
emission current, 80 μA	prescan ion time, 1500 μs	resonant CID waveform
filament delay, 17.2 min	background mass, 44 *m/z*	excitation storage level calculated as parent ion divided by 1.4
	RF dump value, 250 *m/z*	excitation amplitude range, 0.0–2.0 V, increase 0.2

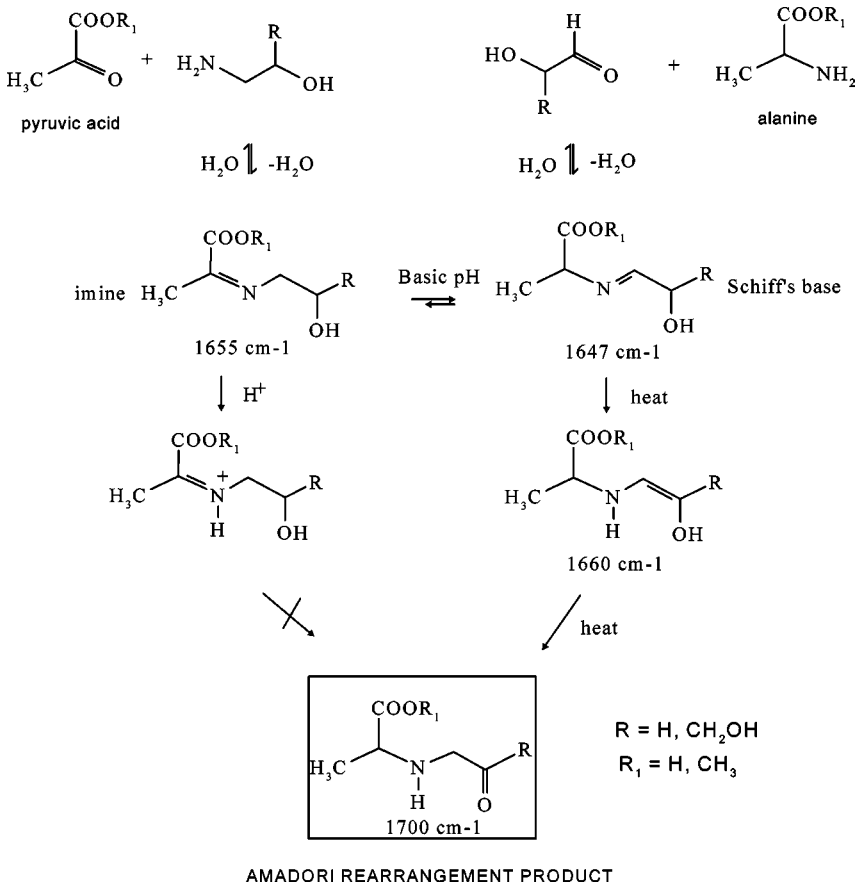

Figure 1. Proposed mechanism of formation of Amadori product from both pyruvate/amino alcohol and amino acid/sugar systems.

laldehyde in PA/ethanolamine mixture, consistent with the occurrence of imine isomerization under the experimental conditions. In this study, detailed investigation of the carbonyl/amine reaction between PA and selected amino alcohols was carried out at different temperatures using Fourier transform infrared spectroscopy (FTIR) and GC-MS/MS to monitor the formation and isomerization of the initial imine and its subsequent rearrangement into Amadori product.

MATERIALS AND METHODS

All chemicals were purchased from Aldrich Chemical Co. (Milwaukee, WI) and used without further purification.

Model Systems. Components of the model systems were mixed at 20 °C at the molar ratio 1.5:1 of amine source to carbonyl, vortexed for 30 s, and placed on either an IR window for scanning or was introduced inside the quartz tube for Py-GC/MS analysis.

FTIR Analysis. Infrared spectra were recorded on a Bomem MB-Series spectrometer (Bomem, Quebec, Canada), equipped with a DTGS detector and purged with dry air. The spectra were acquired on CaF_2 IR windows with no spacer at various temperatures at a rate of one scan per second at 4 cm^{-1} resolution for fast initial reaction monitoring using kinetics scanning and 10 scans per second at 4 cm^{-1} resolution for monitoring the completion of the reaction. Processing of the FTIR data was performed using GRAMS/32 AI version 6.01 (Galactic Industries Corporation, Salem, NH).

Temperature Studies. Sample solutions were placed in a CaF_2 IR temperature-controlled cell with no spacer. The spectra were acquired at a rate of 20 scans per min. A total of 64 scans at 4 cm^{-1} resolution were coadded. The temperature was raised from 20 °C to desired with a rate of 30°C/min controlled with an OMEGA CN8500 Controller. The sample was scanned the moment the desired temperature was reached and then once again after a 30 min incubation. For confirmation of the reversibility of the reaction, after the incubation at a high temperature, the IR cell was cooled to 20 °C with dry air, scanned, and after it was held for 30 min, scanned again, before heating to the next desired temperature. Each sample was scanned at the following temperatures: 20, 40, 60, 80, 100, 120, 140, and 150 °C.

Py-GC/MS and GC-MS/MS Conditions. A Varian CP-3800 gas chromatograph equipped with a sample preconcentration trap (SPT) filled with Tenax GR (29.2 cm active bed length) and coupled to a Saturn 2000 mass selective detector and interfaced to a CDS Pyroprobe 2000 unit (CDS Analytical Inc., Oxford, PA) through a valved interface (CDS 1500) was used for Py-GC/MS analysis. The column used was a fused silica DB-5MS (50 m length × 0.2 mm i.d. × 33 μm film thickness; J&W Scientific, ON). Semiliquid samples of the size of 0.2 mg were mixed with silica gel (Merck, grade 60) and were inserted inside the quartz tubes (0.3 mm thickness) and desorbed at 150 °C for 120 s and then concentrated on SPT at 50 °C and subsequently directed toward the GC column for separation and MS/MS analysis. The GC column flow rate was regulated by an electronic pressure controller and set at a pressure pulse of 60 psi for 4 min and then maintained at

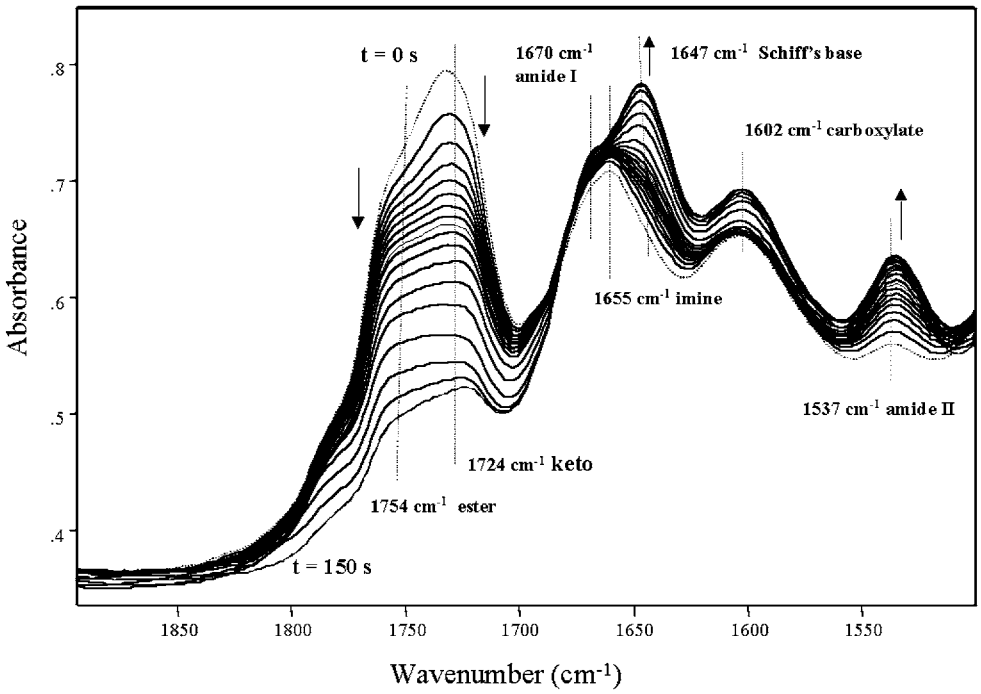

Figure 2. Time-dependent FTIR spectra of a neat mixture of MP and amino propanediol (APD) at room temperature between 1850 and 1500 cm^{-1}. Arrows indicate increase (↑) and decrease (↓) in intensity.

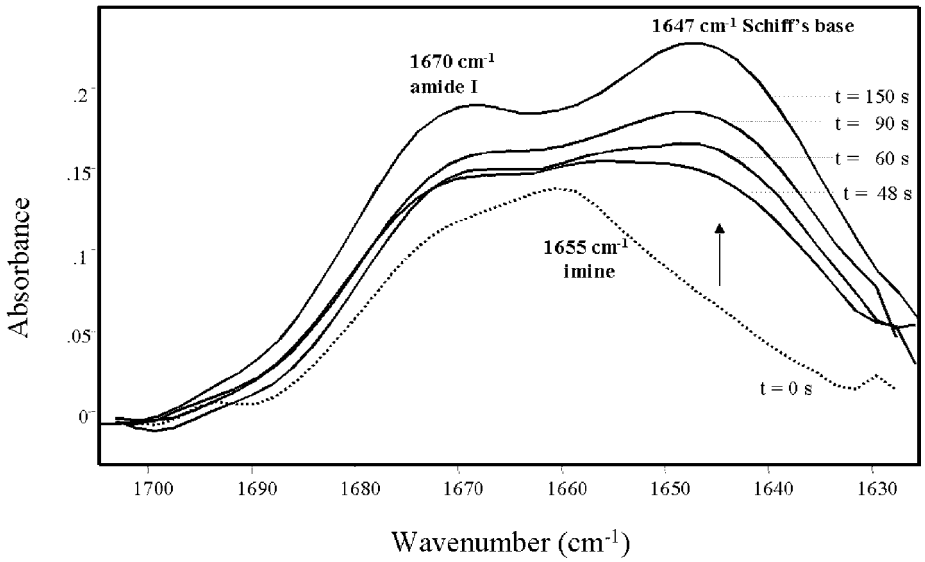

Figure 3. Selected time-dependent FTIR spectra of a neat mixture of MP and APD at room temperature between 1700 and 1630 cm^{-1}. Arrows indicate increase (↑) in intensity.

a constant flow of 1.5 mL/min. The capillary direct MS interface temperature was set at 250 °C, the 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 1750 V. The GC oven initial temperature was set at −5 °C for 5 min and then increased to 50 °C at a rate of 50 °C/min; after that, the temperature was increased to 250 °C at a rate of 8 °C/min and kept at 250 °C for 5 min. The MS fragmentation and peaks identification were estimated with the use of Varian software SatView (ver. 5.52) and NIST Library (ver.1.7). MS/MS dissociation of selected parent ions was optimized to the conditions listed in **Table 1**. Chemical ionization was performed using methanol.

RESULTS AND DISCUSSION

Preliminary analysis using selected model systems containing PA and amino alcohols has indicated the presence of strong spectral interference when solvents or inorganic acids and bases were used to conduct the FTIR experiments and to control the pH of the samples. To overcome these limitations, only neat liquid reactants were selected for further studies, such as methyl pyruvate (MP), PA, amino ethanol, and 1-amino-2,3-propanediol (APD). In addition, excess amino alcohol or PA was used to control the pH of the reaction mixtures. Furthermore, the preliminary studies also indicated that the initial reaction rate at room temperature between the PA and the amino alcohols (see **Figure 1**) was very fast, reaching completion within 10 min of mixing of the reactants. As a result, two types of experiments were carried out, one in which the reactants were mixed directly on the CaF_2 window at 20 °C (room temperature) and immediately scanned using a rate of either one scan per second such that each spectrum represented a passage of 3 s to monitor in detail imine formation and isomerization into the

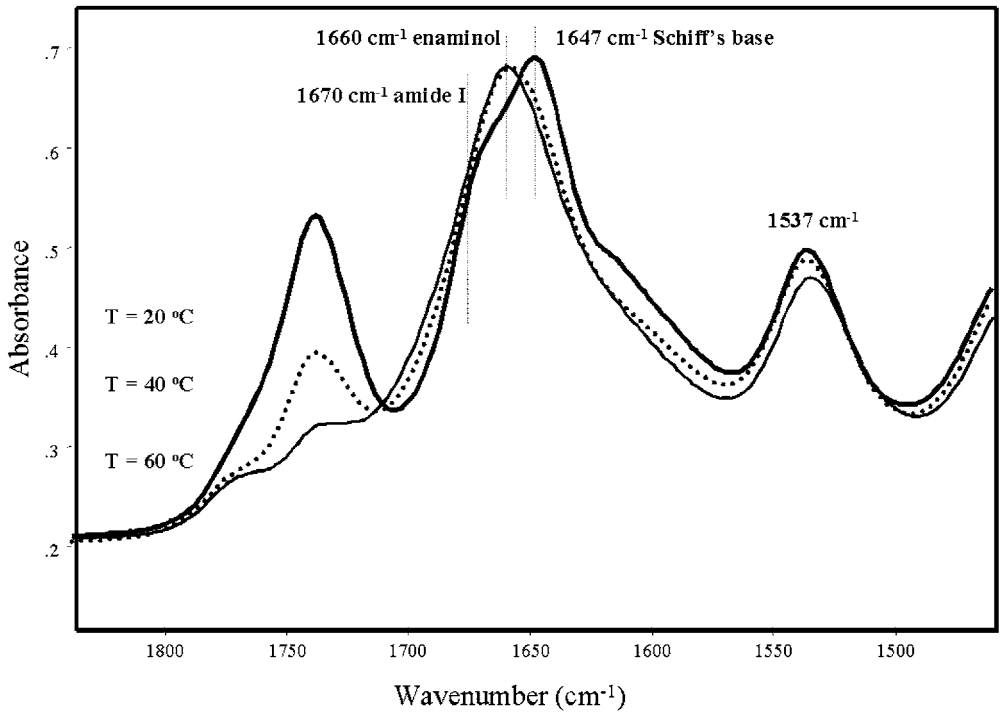

Figure 4. Temperature-dependent FTIR spectra of a neat mixture of MP and APD between 1800 and 1500 cm^{-1}. Solid top line = 20 °C, dotted line = 40 °C, and solid bottom line = 60 °C.

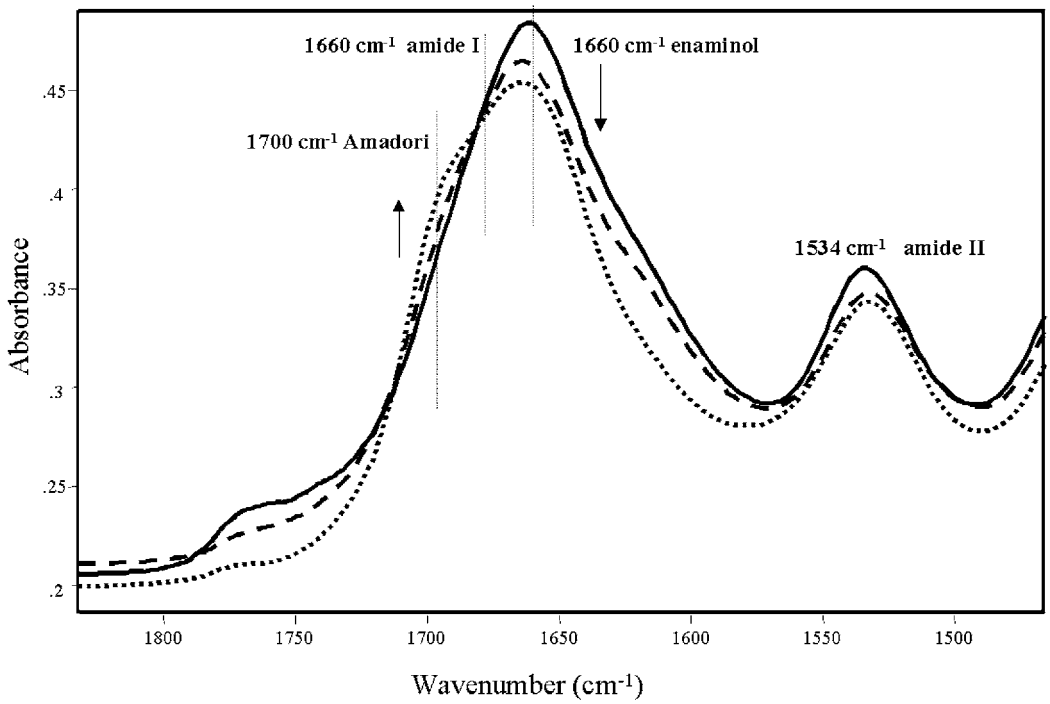

Figure 5. Temperature-dependent FTIR spectra (dotted line = 100 °C, dashed line = 80 °C, and solid line = room temperature) of a neat mixture of MP and APD between 1800 and 1500 cm^{-1}. Arrows indicate increase (↑) and decrease (↓) in intensity.

Schiff's base (see **Figure 1**) or using a rate of 10 scans per second such that each spectrum represented a passage of 45 s to monitor the completion of the reaction. The second type of FTIR experiments involved high temperatures (up to 100 °C) and long time incubations (up to 24 h) to monitor the conversion of the Schiff's base into Amadori rearrangement product as illustrated in **Figure 1**.

Monitoring Imine Formation and Its Isomerization into the Schiff's Base at Room Temperature under Basic Conditions. To prevent salt formation, MP instead of PA was used to monitor the reaction with excess APD. Excess reagent was used to ensure basic conditions. Each reactant was applied separately on each of the CaF_2 windows without spacers and immediately assembled into the cell and scanned at room temperature using a rate of one scan per second such that each spectrum represented the passage of 3 s. **Figure 2** shows time-dependent spectral changes between the nominal time zero and 150 s in the 1850−1500 cm^{-1} range. Inspection of **Figure 2** indicates a dramatic and simultaneous decrease in the intensities of the carbonyl and of the ester bands of MP centered at 1724

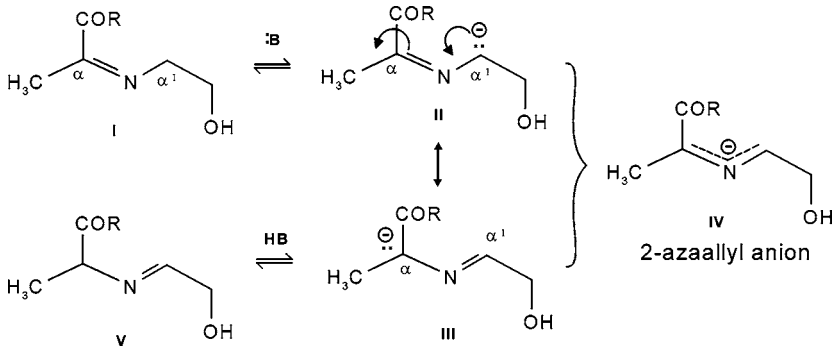

Figure 6. Proposed mechanism of tautomerization of imine and formation of 2-azaallyl anion; R = OR_1, NHR_1.

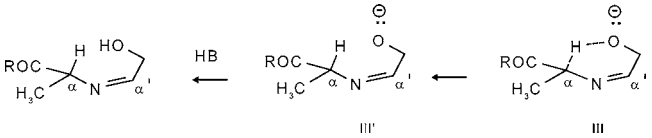

Figure 7. Proposed mechanism of intramolecular proton transfer; R = OR_1, NHR_1.

and 1754 cm^{-1}, respectively. Almost 90% of the intensity of the bands decreased within 2.5 min. The disappearance of the carbonyl band was accompanied by the appearance of an imine band centered at 1655 cm^{-1}, and the disappearance of the ester band was accompanied by the simultaneous appearance of amide (amide I at 1670 cm^{-1}, amide II at 1537 cm^{-1}) and carboxylate bands (1602 cm^{-1}). These observations can be explained by the simultaneous carbonyl-amine reaction and formation of the imine intermediate (see **Figure 1**) and under the presence of excess basic APD, conversion of the ester into an amide. Water released from carbonyl-amine reaction also initiated some hydrolysis of the ester as indicated by the presence of a carboxylate band centered at 1602 cm^{-1}. A closer look at the 1700−1630 cm^{-1} spectral region (**Figure 3**) indicates that the initial imine (spectrum at $t = 0$ s) was formed very fast, even during the preparation and application of the sample on to the CaF_2 windows and the start of spectral acquisition. Thus, at nominal time zero, a substantial amount of imine (1655 cm^{-1}) has already accumulated as shown in **Figure 3**; however, over time, this band disappears with the appearance of another band at 1647 cm^{-1}, which was assigned to the formation of the Schiff base. Furthermore, **Figure 3** also indicates that during the first 60 s, the rate of formation of the imine was faster than the rate of its isomerization; however, after 60 s at room temperature, the rate of isomerization becomes faster than the rate of its formation and continues to be so until the reaction reaches completion after 12 min. Cainelli et al. (*6*) also observed a very fast (20 min) room temperature imine isomerization using

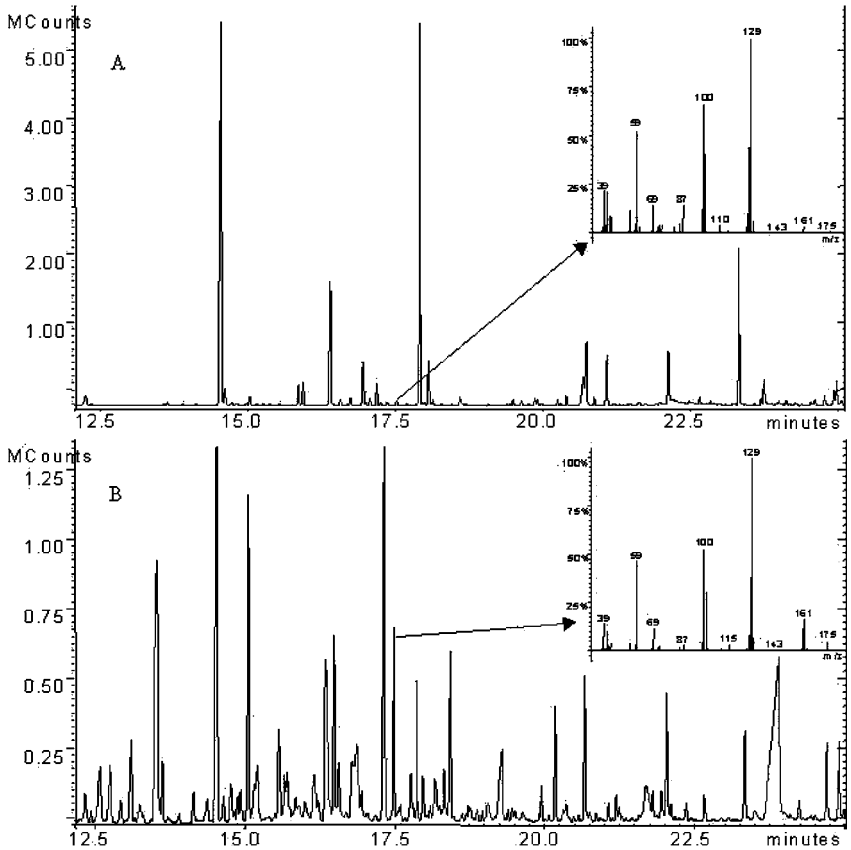

Figure 8. Pyrograms of mixtures of (**a**) MP/APD and (**b**) alanine methyl ester/glyceraldehdye. Inserts are the EI-MS fragmentation patterns of the peak at 17.5 min.

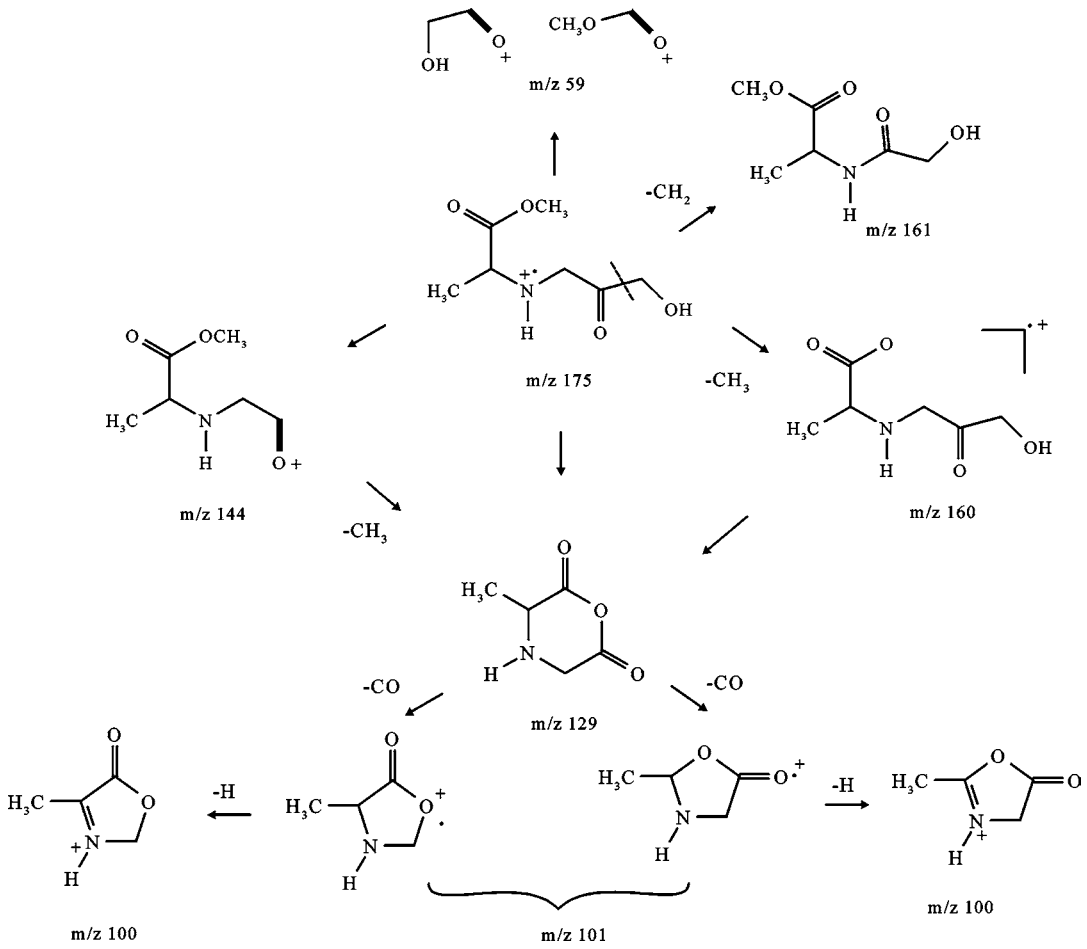

Figure 9. Proposed electron impact fragmentation patterns of Amadori product of methyl alanine with glyceraldehyde.

N-diphenylmethanimines in the presence of potassium *tert*-butoxide and tetrahydrofuran as solvent.

When APD was replaced with excess 1-amino-ethanol (AE), similar spectral changes were observed, except that the conversion of the pyruvate ester into amide occurred very fast, even before imine formation and isomerization. The effect of early amide formation on the absorption frequency of the neighboring carbonyl carbon was observed by a shift from 1724 to 1737 cm^{-1}.

When MP was replaced with excess PA and the reaction was similarly monitored in the presence of either APD or AE, the spectral changes indicated the formation of pyruvate ion (1602 cm^{-1}) and formation of some imminium ion centered at 1632 cm^{-1} confirming requirement for base catalysis for its isomerization.

Effect of Temperature on the Stability of the Schiff's Base under Basic Conditions—Rearrangement into Amadori Product. Incubation of MP with excess APD or with AE at 20 °C has been demonstrated above to effect the formation of imine (1655 cm^{-1}) and its subsequent base-catalyzed isomerization into the Schiff base (1648 cm^{-1}) within 12 min. Under acidic conditions, Schiff bases are known to undergo facile rearrangement into Amadori product and/or hydrolyze depending on the reaction conditions. To study the temperature stability of the Schiff base, the reaction mixture of MP with excess APD was monitored at different temperatures and times. **Figure 4** indicates the formation of the Schiff base at 20 °C as was demonstrated above; however, as the temperature was raised to 40 °C and subsequently to 60 °C, the absorption peak centered at 1647 cm^{-1} was shifted to 1660 cm^{-1}. This was attributed to the isomerization of the Schiff base and formation of enaminol moiety (*7*) as indicated in **Figure 1**. Formation of enaminol moiety was accompanied to a small extent by an intramolecular esterification as was evidenced by the peak centered at 1776 cm^{-1}. Further heating and incubation at 100 °C indicated a decrease in the intensity of the eneaminol band and emergence of a shoulder centered at 1700 cm^{-1} (see **Figure 5**). The new band was assigned to the formation of Amadori product as shown in **Figure 1**.

Table 2. Daughter Ions Observed in the Mass Spectrum of Peak at m/z 175

parent ion	daughter ions[a]
175	**161**, **160**, 144, 143, **129**
161	**129**, 128, 100
160	144, 143, **129**, 101, 59
144	**129**, 69
129	116, **101**, **100**, **59**
101	**85**, 59
100	85

[a] Bold characters represent major daughter ions.

Mechanism of Isomerization of the Imine. Base-catalyzed tautomerization of imines such as I in **Figure 6** involves the formation of a delocalized 2-azaallyl anion (*6*) (structure IV in **Figure 6**). This anion can be protonated by the conjugate acid (HB) formed in the reaction mixture at both α- (see structure III) and α′- (see structure II) positions with the establishment of an equilibrium between imine I and imine V (**Figure 6**). This type of imine isomerization has been recognized as a prototype of the biochemical transamination between amino acids and

pyridoxal (*6*). Recently, these intermediates have been generated under mild conditions at room temperature through deprotonation of imines using potassium *tert*-butoxide (*6*). A concerted mechanism was proposed (*3*) for this isomerization process in which the base removes a proton from one carbon (α or α′) synchronously with the donation of a proton to the other carbon by its conjugate acid. In the specific case of the imines generated from keto esters and amino ethanols such as I (**Figure 6**), the β-hydroxy group can act as a proton donor to the α-position through the formation of a H-bond in a stable six member ring intermediate as shown in **Figure 7** (structure III). This process will protonate the α-position preferentially due to the entropy factor and thus lead the reaction to completion rather than to equilibrium.

Detection of Amadori Intermediate by Py-GC/MS, Py-GC/CI-MS, and GC/MS-MS. To provide direct evidence for the ability of the PA pathway to generate Amadori product, the alanine methyl ester/glyceraldehyde model system was analyzed under the same experimental conditions as that of the MP/APD model by Py/GC-MS. Both systems are expected to produce a common Amadori product as shown in **Figure 1**. Spectral comparison of both chromatograms has led to identification of a common peak at retention time 17.5 min having an identical electron impact fragmentation pattern and the expected molecular mass of *m*/*z* 175 (see **Figure 8**) as confirmed by chemical ionization. Furthermore, tandem mass spectrometry was used to provide evidence that the electron impact mass spectral fragmentation pattern shown in **Figure 8** is consistent with the proposed structure of the methyl ester of the Amadori product. Data obtained from optimized EI-MS/MS of *m*/*z* 175 are shown in **Table 2**.

According to the EI-MS spectrum shown in **Figure 8**, it seems that ion at *m*/*z* 129 is the most stable and can be envisaged to be formed through three pathways as shown in **Figure 9**. One pathway was through a single step cyclization from the parent ion or through stepwise formation from either demethylated parent ion at *m*/*z* 160 or dehydroxymethylated parent ion at *m*/*z* 144. The ion *m*/*z* 129 will in turn lose CO to give two isomeric ions at *m*/*z* 101, which consequently after losing of a proton can generate isomeric ions at *m*/*z* 100. Finally, ion *m*/*z* 59 can be formed directly from *m*/*z* 175 by cleavage of carbon atom in the α-position to the nitrogen atom.

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