Characterization of the Products Formed during Microwave Irradiation of the Nonenzymatic Browning Lysine/(*E*)-4,5-Epoxy-(*E*)-2-heptenal Model System

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Previous studies have shown that a lysine/(*E*)-4,5-epoxy-(*E*)-2-heptenal model system may help to elucidate the nonenzymatic browning produced in foods by lipid peroxidation products during microwave heating. As a continuation of those studies, this research was undertaken to isolate and characterize the different compounds that are produced in the above system in order to elucidate the mechanisms that take place at the molecular level upon microwave irradiation. Six pyrrole derivatives were isolated and characterized by ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy and mass spectrometry. They were 1-[5′-amino-(1′ and 5′)-carboxypentyl]pyrrole (**3** and **4**, respectively), 1-[5′-amino-(1′ and 5′)-carboxypentyl]-2-(1″-hydroxypropyl)pyrrole (**5** and **6**, respectively), and 1-(5′-amino-1′-carboxypentyl)-2-[(*Z* and *E*)-1″-propenyl]pyrrole (**7** and **8**, respectively). Two of these compounds (**7** and **8**) were not previously found during thermic heating at low or moderate temperatures. A possible mechanism that explains the formation of all these compounds is suggested and discussed in relation to the formation of color and fluorescence in this system.

Keywords: *Microwave irradiation; pyrrole products; carbonyl–amine reactions; nonenzymatic browning; fluorescence development*

INTRODUCTION

Nonenzymatic browning is one of the most prevalent chemical reactions that occur in foods (Labuza and Baisier, 1992). It is common for foods containing both reducing sugars and proteins and enough moisture to act as reaction phase (Yeo and Shibamoto, 1991). Nonenzymatic browning causes brown discoloration in foods and also leads to off-flavors, off-colors, and textural changes. It may also cause loss of nutritional value of foods (Hodge, 1953; Labuza and Schmidl, 1986; Sapers, 1993).

In addition to reducing sugars, other carbonyl compounds, particularly lipid peroxidation products, are also able to react with amino groups, producing brown macromolecular pigments in foods (Eriksson, 1987; Karel, 1984). In this respect, previous research from this laboratory has shown that volatile short-chain aldehydes having a 4,5-epoxy-1-oxo-2-pentene system, and particularly (*E*)-4,5-epoxy-(*E*)-2-heptenal, are very reactive with amines, amino acids, and proteins, producing brown color and fluorescence (Hidalgo and Zamora, 1993a). These epoxyenealdehydes, which have been detected in several other systems (Swoboda and Peers, 1978; Grosch, 1993), react with lysine at room temperature, producing different 1-alkyl-2-(1′-hydroxyalkyl)pyrroles and 1-alkylpyrroles, all of which were isolated and identified (Zamora and Hidalgo, 1994). These 1-alkyl-2-(1′-hydroxyalkyl)pyrroles were responsible for the color and fluorescence production via a polymerization reaction. This reaction has been characterized and proposed as an alternative mechanism for production of brown macromolecular pigments, with fluorescent characteristics similar to those of lipofuscins, both in foods and in living organisms (Hidalgo and Zamora, 1993b).

When the lysine/(*E*)-4,5-epoxy-(*E*)-2-heptenal model system was irradiated in a microwave oven, the development of browning and fluorescence was observed (Zamora and Hidalgo, 1992). The browning was faster in this model system than in an analogous glucose/lysine system; however, the color and fluorescence, reached after 100 s of irradiation, were comparable for both systems. These results suggested that oxidized lipid/amino acid reactions might play a role in the nonenzymatic browning reactions of foods during microwave heating. As a continuation of previous studies on the subject, this research aims to isolate and characterize the different compounds that are produced in the lysine/(*E*)-4,5-epoxy-(*E*)-2-heptenal model system during microwave irradiation. The elucidation of the structures of these compounds should contribute to better understanding of the reaction mechanisms at the molecular level. The results reported recently by Zamora and Hidalgo (1995), in their study of the influence of irradiation time, pH, and aldehyde/amino acid ratio on the formation of the various reaction products, will be compared to the production of color and fluorescence in this model system. We believe that an understanding of the nonenzymatic browning reactions produced in this model system by microwave irradiation may provide the basis for future studies to assess the role of certain oxidized lipid/protein reactions in the overall nonenzymatic browning produced in microwave ovens. In addition, these reactions might be used as browning precursors in prepared foods for microwave ovens.

EXPERIMENTAL PROCEDURES

Materials. (*E*)-4,5-Epoxy-(*E*)-2-heptenal was prepared from (*E*)-2-(*E*)-4-heptadienal as described by Swoboda and Peers (1978). (*E*)-2-(*E*)-4-Heptadienal and L-lysine were purchased from Aldrich Chemical Co. (Milwaukee, WI), and 3-chloroperoxybenzoic acid was from Fluka Chemie AG (Buchs, Switzerland). All other chemicals used were of analytical grade and were purchased from reliable commercial sources.

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Sample Preparation. Sample preparation for analytical high-performance liquid chromatography (HPLC) was analogous to the sample preparation for color and fluorescence measurements that was previously described by Zamora and Hidalgo (1992). However, the cooled samples from the irradiation (100 μL) were diluted with 400 μL of 0.1 M potassium phosphate, pH 6.0, and then injected in the chromatograph.

Sample preparation for semipreparative HPLC was carried out by suspending (*E*)-4,5-epoxy-(*E*)-2-heptenal (0.38 mmol) in 3 mL of 0.3 M sodium phosphate, pH 7.0, or sodium borate, pH 10.0, and sonicating it until permanent emulsion was reached. This step was realized by using a Braun Labsonic U sonicator. Lysine (0.78 mmol) was then added, and the resulting solutions were irradiated for 25 or 40 s at the highest setting of a 800-W Panasonic microwave oven (Model NN-8559). At the end of the irradiation period, the samples were cooled and injected in the liquid chromatograph.

High-Performance Liquid Chromatography (HPLC). The HPLC system consisted of a 126 programmable delivery module and a 168 diode array detector module (Beckman, Fullerton, CA). The Software System used in the data acquisition and processing was Gold 7.1 version (Beckman). Analytical separations were carried out on a Spherisorb ODS2 3-μm 25 × 0.46-cm column. A loop of 20 μL and a flow rate of 0.8 mL/min were used in the analytical experiments. Semipreparative separations were carried out on a Spherisorb ODS2 5-μm 25 × 1-cm column. A loop of 500 μL and a flow rate of 5 mL/min were used in these experiments. A temperature of 40 °C and UV detection wavelengths of 220 and 250 nm were used in all experiments. Chromatograms at other wavelengths were obtained using the software ArrayView 1.0 version (Beckman). In the preparative separations, products from successive injections were collected and combined. Solutions were dried under nitrogen flux to remove acetonitrile and then freeze-dried.

HPLC Coupled with Mass Spectrometry Analyses (HPLC–MS). An HPLC system analogous to the one used for analytical and semipreparative separations was used for HPLC–MS analyses. The HPLC column was a C_{18}, 5-μm particle size, 25 × 0.46-cm i.d. reversed-phase column. A 100-μL loop was used in the injector. The UV detector was in-line with the Universal Interface (Vestec, Houston, TX). Thus, the total ion current trace obtained from the MS data system can be directly compared with the UV chromatogram obtained from the UV detector. The Universal Interface was connected to the momentum separator via Teflon tubing, and this separator was connected to a standard VG ion source of the AEI-MS/70VG mass spectrometer via a quartz tube. The temperature of the momentum separator was 135 °C, and the ion source temperature was maintained at 220 °C. In a typical experiment the parameters of the Universal Interface were set analogously to those described by Baczynskyj (1991).

Nuclear Magnetic Resonance (NMR) Experiments. ^1H and ^{13}C NMR at 300 and 75.4 MHz, respectively, were determined by an NMR spectrometer, type Bruker AC-300P (Karlsruhe, Germany), with tetramethylsilane as internal standard. For aqueous solutions, sodium 3-(trimethylsilyl)-1-propanesulfonate was used as internal standard. Two-dimensional NMR was used to assign ^{13}C NMR spectra.

RESULTS

When a mixture of lysine and (*E*)-4,5-epoxy-(*E*)-2-heptenal was irradiated in a microwave oven, brown color and fluorescence were developed as reported by Zamora and Hidalgo (1992). This development, which depended on pH and lipid/amino acid ratio, occurred simultaneously with the appearance of different peaks in the HPLC chromatogram. Figure 1 shows the analytical HPLC elution pattern of a (*E*)-4,5-epoxy-(*E*)-2-heptenal/lysine reaction mixture, at pH 7.0, irradiated in a microwave oven for 100 s. This pattern was very different from that reported by Zamora and Hidalgo (1994), when the (*E*)-4,5-epoxy-(*E*)-2-heptenal/lysine mixture, pH 9.0, was incubated overnight at room temperature. Two main peaks (A and B) were observed at 220 nm. These two peaks corresponded to the two major products of the reaction. When the HPLC elution pattern was monitored at 250 nm (Figure 1B), peaks A and B areas were smaller than at 220 nm. In addition, several other peaks were observed. These results are in accordance with the UV spectra of the different fractions (Figure 2). Fractions A and B, which showed maxima at 204 and 202 nm, respectively, had a relatively high absorbance at 220 nm, but this absorbance was very much lower at 250 nm. On the contrary, the absorbances of fractions C–E were very similar at both wavelengths. The analysis of UV spectra suggested the presence of three groups of compounds with analogous chromophores. These groups were A–B, C–D, and E, and the total identification of these compounds confirmed the existence of these three groups. Fractions containing these peaks were isolated by semipreparative

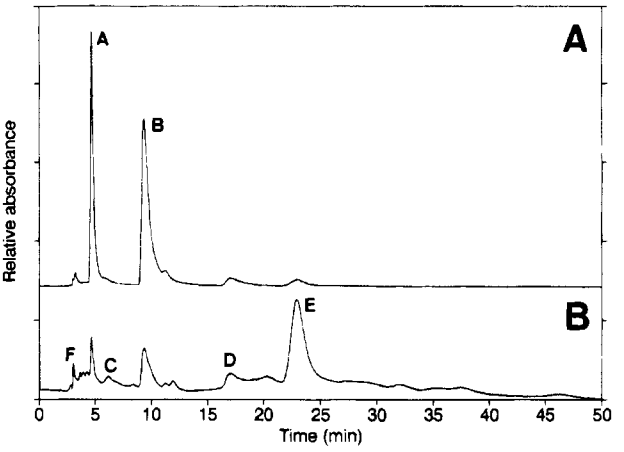


Figure 1. Analytical HPLC elution pattern of the (*E*)-4,5-epoxy-(*E*)-2-heptenal/lysine, pH 7, reaction mixture irradiated at the high setting of a microwave oven for 100 s: (A) detection at 220 nm; (B) detection at 250 nm. Chromatogram at 250 nm was multiplied by 15 to obtain chromatograms comparable at the two wavelengths.

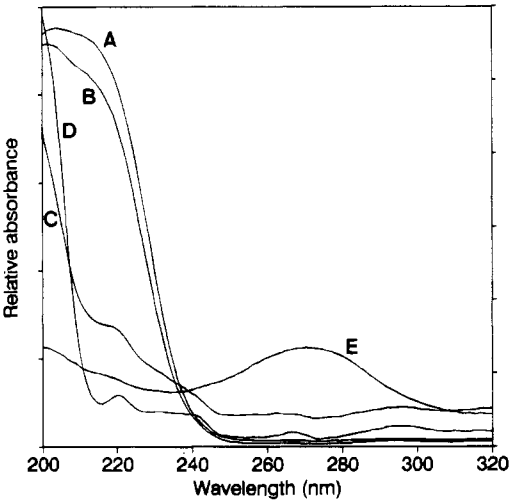
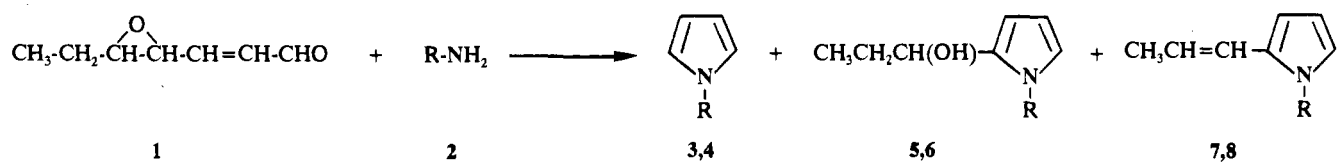


Figure 2. UV spectra of fractions A–E obtained in the HPLC elution pattern shown in Figure 1. They corresponded to the following compounds, which were isolated by semipreparative HPLC and identified by ^1H and ^{13}C NMR and MS: 1-[5′-amino-(1′ and 5′)-carboxypentyl]pyrrole (A and B, respectively); 1-[5′-amino-(1′ and 5′)-carboxypentyl]-2-(1″-hydroxypropyl)pyrrole (C and D, respectively); and 1-(5′-amino-1′-carboxypentyl)-2-[(*Z* and *E*)-1″-propenyl]pyrrole (E).



1 2 3,4 5,6 7,8

Compound	R	Compound	R
2	$(CH_2)_4CH(NH_2)COOH$	6	$(CH_2)_4CH(NH_2)COOH$
3	$CH(COOH)(CH_2)_4NH_2$	7	$CH(COOH)(CH_2)_4NH_2$
4	$(CH_2)_4CH(NH_2)COOH$	8	$CH(COOH)(CH_2)_4NH_2$
5	$CH(COOH)(CH_2)_4NH_2$		

Figure 3. Structure of the compounds identified from the (*E*)-4,5-epoxy-(*E*)-2-heptenal/lysine reaction mixture irradiated at the high setting of a microwave oven. Compounds were isolated by semipreparative HPLC and identified by ^1H and ^{13}C NMR and MS.

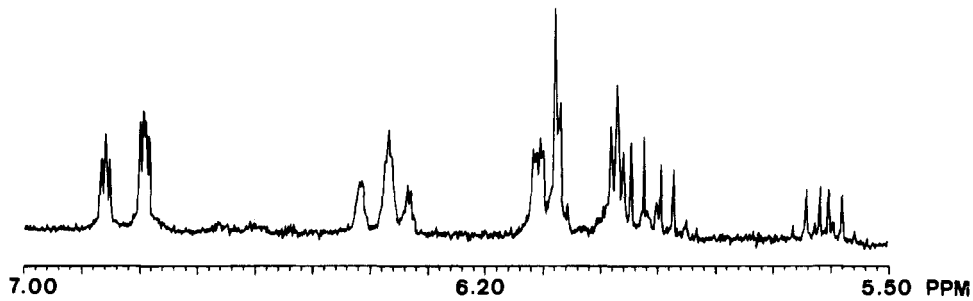


Figure 4. Olefinic and heterocyclic portion of the ^1H NMR spectrum obtained for the mixture of compounds **7** and **8**. The spectrum was taken in CD_3OD.

HPLC of mixtures heated for different periods of time, and the corresponding compounds were identified by ^1H and ^{13}C NMR and MS. The irradiation time depended on the fraction wanted. Thus, shorter irradiation times favored fractions C and D, and higher irradiation times favored fractions A, B, and E as reported by Zamora and Hidalgo (1995). Even though fraction E was a very minor product of the reaction, it was of special interest. In fact, this fraction was the most characteristic product of microwave heating and does not appear in thermic heating at low or moderate temperatures (Zamora and Hidalgo, unpublished results). Most of this work was concentrated on the characterization of this fraction. The structures of compounds identified in this study are given in Figure 3.

Fraction A was identified as 1-(5′-amino-1′-carboxypentyl)pyrrole (**3**). Compound **3** had the ^1H and ^{13}C NMR and MS described previously (Zamora and Hidalgo, 1994). The HPLC–MS *m/e* (relative intensity, ion structure) was as follows: 178 (28, $M^+ - H_2O$), 152 (84, $M^+ - CO_2$), 135 (51, 152 − NH_3), 122 (55), 94 (77, ethylpyrrole − H), 81 (96, methylpyrrole), 68 (100, pyrrole + H^+).

Fraction B was identified as 1-(5′-amino-5′-carboxypentyl)pyrrole (**4**). Compound **4** had the ^1H and ^{13}C NMR and MS described previously (Zamora and Hidalgo, 1994). The HPLC–MS *m/e* (relative intensity, ion structure) was as follows: 196 (65, M^+), 178 (11, $M^+ - H_2O$), 134 (61, $M^+ - CO_2 - NH_4$), 122 (47, $M^+ - C_2H_4NO_2$), 109 (23, propylpyrrole), 94 (26, ethylpyrrole − H), 81 (100, methylpyrrole).

Fraction C was identified as 1-(5′-amino-1′-carboxypentyl)-2-(1″-hydroxypropyl)pyrrole (**5**). This compound had the ^1H and ^{13}C NMR and MS described previously (Zamora and Hidalgo, 1994). The HPLC–MS *m/e* (relative intensity, ion structure) was as follows: 236 (10, $M^+ - H_2O$), 218 (11, 236 − H_2O), 179 (25, 236 − 2-propenylamine), 134 [37, 1-ethyl-2-(1-propenyl)pyrrole − H], 84 (100, tetrahydropyridine + H^+).

Fraction D was identified as 1-(5′-amino-5′-carboxypentyl)-2-(1″-hydroxypropyl)pyrrole (**6**). This compound had the ^1H and ^{13}C NMR and MS described previously (Zamora and Hidalgo, 1994). The HPLC–MS *m/e* (relative intensity, ion structure) was as follows: 236 (37, $M^+ - H_2O$), 162 (34, 236 − $C_2H_4NO_2$), 134 [42, 1-ethyl-2-(1-propenyl)pyrrole − H], 120 [49, 1-methyl-2-(1-propenyl)pyrrole − H], 84 (62, tetrahydropyridine + H^+), 44 (100).

Fraction E was identified as 1-(5′-amino-1′-carboxypentyl)-2-[(*Z* and *E*)-1″-propenyl]pyrrole (**7** and **8**, respectively). This fraction was a (2:1) mixture of isomers *E* and *Z*, respectively, as indicated by the integral of the olefinic protons in the ^1H NMR. The olefinic and heterocyclic portion of the ^1H NMR spectrum of this mixture is shown in Figure 4. ^1H NMR (CD_3OD) δ 1.30 m (2H, H3′), 1.7–2 m (4H, H2′ and H4′), 1.81 dd (1.98H, $J_{1'',3''}$ = 1.7 Hz, $J_{2'',3''}$ = 6.7 Hz, H3″ of *E*-isomer), 1.85 dd (1.02H, $J_{1'',3''}$ = 1.8 Hz, $J_{2'',3''}$ = 7.0 Hz, H3″ of *Z*-isomer), 2.80 m (2H, H5′), 4.54 dd (1H, J = 6.5 Hz, J = 8.8 Hz, H1′), 5.61 dq (0.34H, $J_{1'',2''}$ = 11.5 Hz, $J_{2'',3''}$ = 7.0 Hz, H2″ of *Z*-isomer), 5.91 dq (0.66H, $J_{1'',2''}$ = 15.5 Hz, $J_{2'',3''}$ = 6.7 Hz, H2″ of *E*-isomer), 5.97 t (0.66H, J = 3.2 Hz, H4 of *E*-isomer), 6.08 m (0.68H, H3 and H4 of *Z*-isomer), 6.11 *dd* (0.66H, $J_{3,4}$ = 3.5 Hz, $J_{3,5}$ = 1.8 Hz, H3 of *E*-isomer), 6.35 dq (0.34H, $J_{1'',2''}$ = 11.5 Hz, $J_{1'',3''}$ = 1.8 Hz, H1″ of *Z*-isomer), 6.38 dq (0.66H, $J_{1'',2''}$ = 15.5 Hz, $J_{1'',3''}$ = 1.7 Hz, H1″ of *E*-isomer), 6.79 dd (0.66H, $J_{3,5}$ = 1.8 Hz, $J_{4,5}$ = 2.6 Hz, H5 of *E*-isomer), 6.86 t (0.34H, J = 2.2 Hz, H5 of *Z*-isomer). HPLC–MS *m/e* (relative intensity, ion structure) 236 (11, M^+), 218 (77,

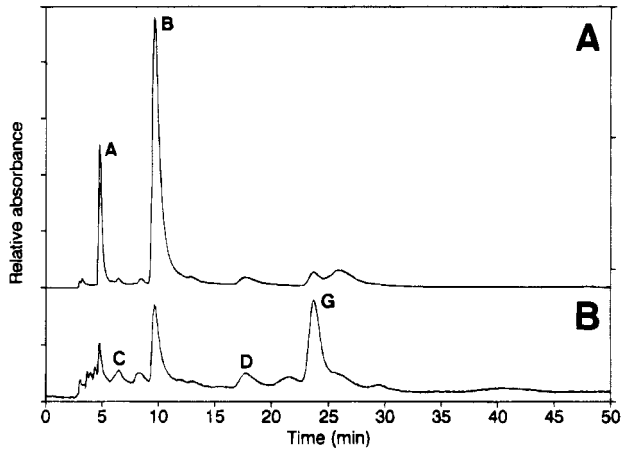


Figure 5. Analytical HPLC elution pattern of the (*E*)-4,5-epoxy-(*E*)-2-heptenal/lysine, pH 10, reaction mixture irradiated at the high setting of a microwave oven for 100 s: (A) detection at 220 nm; (B) detection at 250 nm. Chromatogram at 250 nm was multiplied by 18 to obtain chromatograms comparable at the two wavelengths.

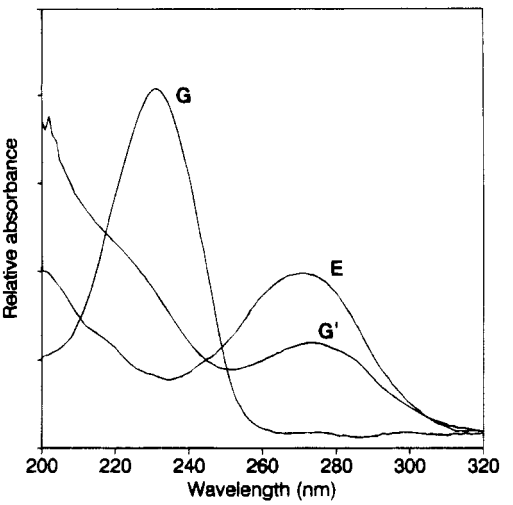


Figure 6. UV spectra of fractions E and G obtained in the HPLC elution patterns shown in Figures 1 and 5, respectively. Fraction G evolved the G′ spectrum during the purification process.

$M^+ - H_2O$), 192 (15, $M^+ - CO_2$), 120 [74, 1-methyl-2-(1-propenyl)pyrrole − H], 106 [81, 2-ethenyl-1-methylpyrrole − H or 2-(1-propenyl)pyrrole − H], 84 (75, tetrahydropyridine + H^+), 58 (100).

In addition to these five fractions, other fractions that did not correspond clearly to single structures, as deduced by NMR and MS, were also observed. These fractions, analogously to similar fractions obtained when the reaction between (*E*)-4,5-epoxy-(*E*)-2-heptenal and lysine was incubated overnight at room temperature, are thought to be undefined complexes between lysine molecules and some lipid ligands. Finally, fraction F (Figure 1) contained a polymer that was responsible for the color and fluorescence produced in the reaction.

HPLC elution patterns of mixtures irradiated for different periods of time at other pHs and epoxyenealdehyde/lysine ratios were slightly different. Figure 5 shows the analytical HPLC elution pattern of the reaction mixture at pH 10 irradiated in a microwave oven for 100 s. The comparison between peaks obtained in different chromatograms was carried out by using both retention times and UV spectra. To confirm that identifications obtained using these two criteria were correct, the mixture incubated at pH 10 was fractionated by semipreparative HPLC and the isolated fractions were studied by ^1H and ^{13}C NMR and MS. Although there were significant differences in peak areas and weights between pH 7.0 and 10.0 [see accompanying paper in this issue, Zamora and Hidalgo (1995)], the compounds identified at pH 10 were mostly the same as those found at pH 7.0. The only difference was the peak G that had a retention time analogous to that of peak E (see Figure 1), but its UV spectrum was very different (Figure 6). The attempts carried out for the complete characterization of this compound were not successful because of a transformation during the purification process. Its UV spectrum evolved a spectrum similar to that of compounds **7** and **8** (G′ in Figure 6). In addition, the HPLC-MS obtained when the fraction was being purified was analogous to that obtained for compounds **7** and **8**.

DISCUSSION

The results presented in this study show that, although HPLC elution patterns of (*E*)-4,5-epoxy-(*E*)-2-heptenal/lysine reaction mixtures irradiated in a microwave oven for short periods of time or incubated overnight at room temperature (Zamora and Hidalgo, 1994) were different, the main products of both reactions were similar. The only compounds that were produced exclusively by microwave irradiation were compounds **7** and **8**. These compounds can be considered derivatives of compound **5** by dehydration, and, therefore, the mechanism found for the pyrrole formation in the reaction of (*E*)-4,5-epoxy-(*E*)-2-heptenal and lysine at room temperature might also be valid in the case of microwave irradiation. This mechanism is presented in Figure 7. It implies the formation of an imine at a first stage that would produce the pyrrole ring by intramolecular attack of the nitrogen at one of the epoxy carbons. A rearrangement, which may be accompanied by formation of propanal, would produce the pyrroles (**3**−**6**) isolated. Although studies for detection of propanal have not been carried out specifically for microwave irradiation, it was detected as a product of the reaction of (*E*)-4,5-epoxy-(*E*)-2-heptenal with lysine at room temperature (Zamora and Hidalgo, 1994). Analogously to nonheated reactions, the produced 1-alkyl-2-(1′-hydroxypropyl)pyrroles (**5**, **6**) are supposed to be responsible for the color and fluorescence formation in these reactions, via a polymerization mechanism similar to that previously described (Zamora and Hidalgo, 1993b). However, microwave irradiation produced a second reaction involving 1-alkyl-2-(1′-hydroxypropyl)-pyrrole (**5**) that was not reported in previous studies. Compound **5** was dehydrated to produce compounds **7** and **8**. Surprisingly, this reaction did not take place on compound **6** (the corresponding derivatives were neither isolated nor detected), suggesting that the presence of the α-carboxyl group might be necessary for such reaction. Additional studies are underway to confirm if this dehydration is an effect of microwave irradiation or a consequence of the superheating caused by absorption of microwaves. These studies are also trying to determine whether or not high temperatures in conventional ovens can produce similar products.

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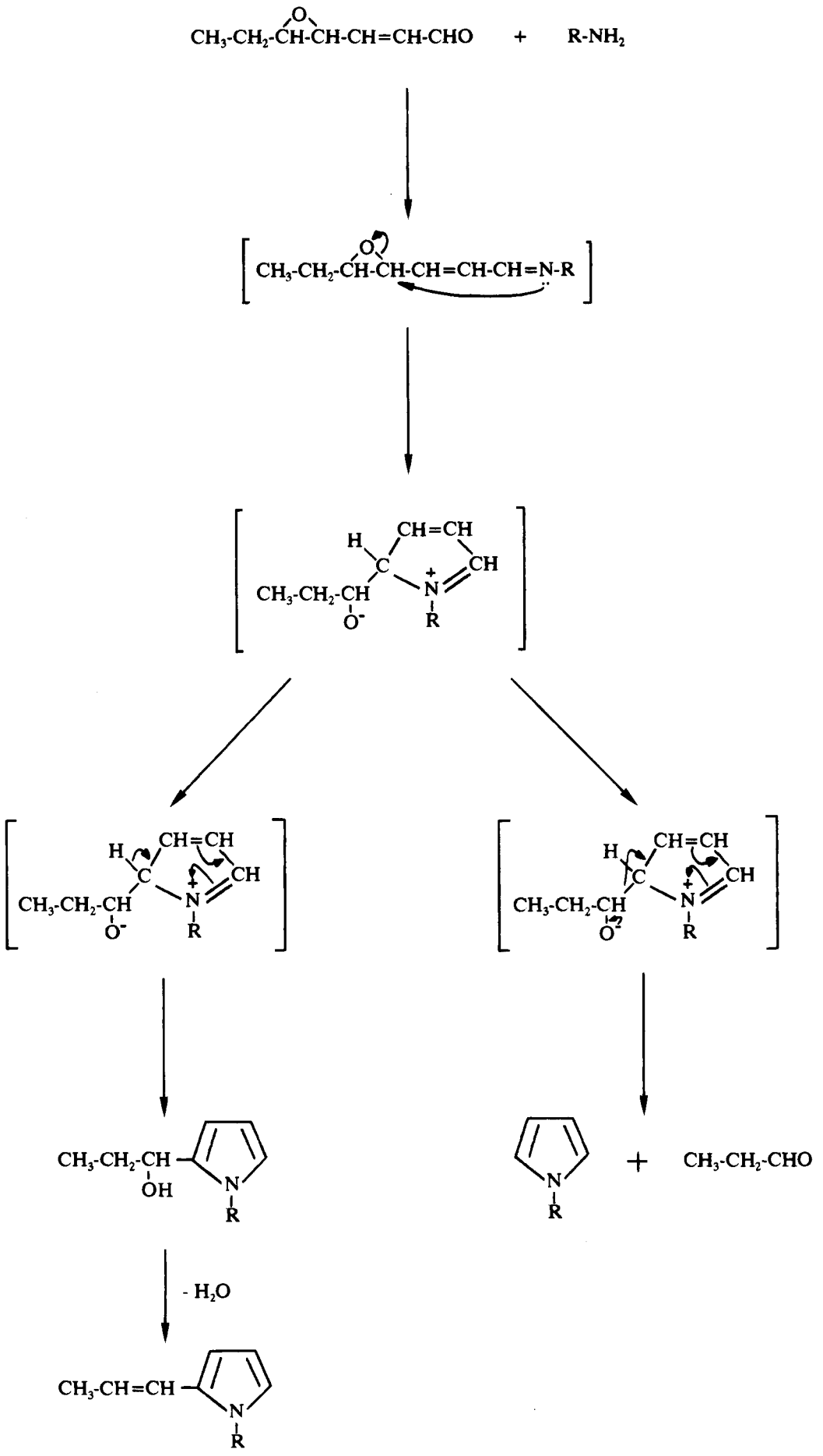

Figure 7. Proposed mechanism of pyrrole formation in the (*E*)-4,5-epoxy-(*E*)-2-heptenal/lysine reaction mixture irradiated at the high setting of a microwave oven.

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