Application of Site Specific ^{13}C Enrichment and ^{13}C NMR Spectroscopy for the Elucidation of the Formation Pathway Leading to a Red 1*H*-Pyrrol-3(2*H*)-one during the Maillard Reaction of Furan-2-carboxaldehyde and L-Alanine

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The intensely red compounds (*S*)-4[(*E*)-1-formyl-2-(2-furyl)ethenyl)]-5-(2-furyl)-2-[(*E*)-(2-furyl)methylidene]-2,3-dihydro-α-methyl-3-oxo-1*H*-pyrrole-1-acetic acid (**1a**) and its 2-[(*Z*)-(2-furyl)methylene] isomer (**2b**) have recently been identified as the main colored compounds formed upon thermal treatment of an aqueous solution of furan-2-carboxaldehyde and L-alanine. For clarification of their formation pathways, a labeling experiment with synthetic site specific ^{13}C-enriched furan-2-carboxaldehyde was performed. The labeled carbon positions in **1a** were unequivocally assigned by ^{1}H broad band decoupled ^{13}C NMR spectroscopy. In contrast to conventional labeling experiments, prior to the reaction with L-alanine, the ^{13}C-labeled furan-2-carboxaldehyde was diluted with the natural ^{13}C abundant analogue to suppress the spectrum complexity due to homonuclear $^{13}C-^{13}C$ spin couplings. This powerful technique has been successfully used for the first time to clarify the formation route of a colored Maillard reaction product.

Keywords: *Maillard reaction; furan-2-carboxaldehyde; alanine; 1H-pyrrol-3(2H)-one; furan-2-[^{13}C]-carboxaldehyde; ^{13}C-labeling experiment*

INTRODUCTION

The formation of color is one of the most important attributes of the Maillard reaction between reducing carbohydrates and amino acids occurring, e.g., during thermal processing of foods, such as roasting of meat, baking of bread, or roasting of coffee. However, due to the complexity of this reaction and the multiplicity of products formed, surprisingly little is known about the chemical nature of these nonenzymatic browning products. It is, therefore, a helpful approach to study suitable model systems for obtaining detailed information on the nature of the chromophoric compounds involved in color formation (Severin and Krönig, 1972; Ledl and Severin, 1978, 1982).

In recent studies (Hofmann, 1997, 1998a), two intensely red 1*H*-pyrrol-3(2*H*)-ones were identified as the main colored compounds formed upon thermal treatment of an aqueous solution of L-alanine and furan-2-carboxaldehyde, a well-known degradation product of pentoses. In addition, it was found that, in the reaction of furan-2-carboxaldehyde with casein, analoguous protein-bound 1*H*-pyrrol-3(2*H*)-one chromophores were generated cross-linked with the lysine side chains of a protein (Hofmann, 1998b). These results indicate that 1*H*-pyrrol-3(2*H*)-one formation is a general pathway in the Maillard reaction producing chromophores. The formation routes leading to such chromophores are, however, as yet not clear. To understand the complex network of reactions leading to nonenzymatic browning, it is a necessary further step to clarify the formation of certain substructures present in the colored Maillard reaction products.

A promising approach for the clarification of these reaction mechanisms is the use of labeled color precursors. Because, due to the possibility of $^{2}H/^{1}H$ exchange in aqueous Maillard systems, deuterated precursors cannot be used, the labeling experiments have to be performed with ^{13}C isotopes. To detect the labeled positions in the colorant generated, mass spectrometry often does not definitely assign the labeled carbon atoms. ^{13}C NMR spectroscopy, however, allows an unequivocal assignment of each labeled carbon site. Due to the low natural abundance (1.1%) of the ^{13}C nucleus, the site specific ^{13}C enrichment of a carbon site has the advantage of increasing the ^{13}C NMR signal of the corresponding resonance up to 90-fold. A signal increase in the ^{13}C NMR spectrum has, therefore, to be interpreted as an incorporation of the label into the carbon position under investigation.

Thus, ^{13}C enrichment and ^{13}C NMR analysis of each site of labeling is a convenient technique to perform labeling experiments similar to ^{14}C radiotracer studies, however, without the need of a time-consuming carbon-by-carbon degradation.

The ^{13}C-labeling technique has often been reported in the literature as a very useful tool to clarify how certain carbon atoms are shifted during biochemical transformations. For example, the biosynthesis of cholesterol has been successfully clarified by using [^{13}C]-mevalonate (Popják et al., 1977). In addition, the mechanisms of several unusual biochemical reactions have been elucidated, e.g., an NIH shift during the biosynthesis of epoxypseudoisoeugenol 2-methylbutyrate (Martin and Reichling, 1992) or a methyl transfer in the biosynthesis of aporphine and protoberberine alkaloids (Schneider and Zenk, 1993).

This powerful technique, exemplified with L-alanine,

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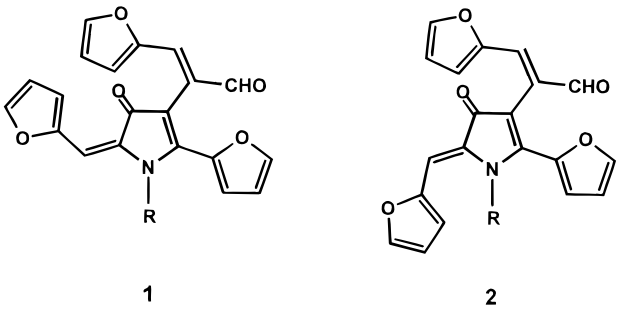

compound	R
1a / 2a	—CH(COOH)—CH$_3$
1b / 2b	—CH$_2$—CH$_2$—CH$_3$
1c / 2c	—CH$_2$—CH$_2$—CH$_2$—CH$_2$—CH(NH$_2$)—COOH

Figure 1. Structures of the red 1*H*-pyrrol-3(2*H*)-ones **1a**−**c** and **2a**−**c**.

was, therefore, applied to gain a more detailed insight into reaction pathways governing the formation of colored 1*H*-pyrrol-3(2*H*)-ones from the Maillard reaction of furan-2-carboxaldehyde and primary amino compounds.

EXPERIMENTAL PROCEDURES

Chemicals. The following compounds were obtained commercially: furan, dimethyl[^{13}C]formamide, phosphoroxy trichloride, and trifluoracidic acid (TFA) were from Aldrich (Steinheim, Germany); L-alanine was from Lancaster (Mühlheim, Germany); and DMSO-d_6 was from Isocom (Landshut, Germany). Solvents were of HPLC grade (Aldrich, Steinheim, Germany).

(*S*)-4-[(*E*)-1-Formyl-2-(2-furyl)ethenyl)]-5-(2-furyl)-2-[(*E*)-(2-furyl)methylidene]-2,3-dihydro-α-methyl-3-oxo-1*H*-pyrrole-1-acetic acid (**1a** in Figure 1) and its 2-[(*Z*)-(2-furyl)methylene] isomer (**2a** in Figure 1) were prepared as recently described by Hofmann (1997, 1998a).

Synthesis of Furan-2-[^{13}C]carboxaldehyde. Phosphoroxy trichloride (3 mmol) was dropped slowly into ice-cooled *N*,*N*-dimethyl[^{13}C]formamide (3 mmol; 99% ^{13}C atomic excess). After 1 h of stirring at room temperature, furan (3 mmol) was added and the mixture was heated for 4 h at 70 °C. The mixture was then poured onto ice−water (20 mL), the pH was adjusted to pH 9.0 with aqueous K_2CO_3 solution (5%), and the mixture was stirred for another 3 h at room temperature. The target compound was extracted with diethyl ether (5 × 20 mL) and then purified by column chromatography (10 × 2 cm) using silica gel (silica gel 60; Merck, Darmstadt, Germany). After application of the crude material onto the silica gel conditioned with pentane, the target compound was eluted using pentane/diethyl ether (70:30, v/v, 100 mL). Pure furan-2-[^{13}C]carboxaldehyde (yield, 53%, 1.6 mmol) was obtained after evaporation of the solvent in vacuo. Comparison of its MS(CI) spectrum (data not shown) with the MS(CI) data of furan-2-carboxaldehyde showing natural ^{13}C abundance demonstrated the incorporation of one ^{13}C atom (99% excess) in the labeled compound.

MS(EI) of furan-2-[^{13}C] carboxaldehyde: 96 (100, $[M - 1]^+$), 97 (98, M^+), 39 (89), 38 (48), 37 (25), 67 (24), 51 (10).

^{13}C Stable Isotope Labeling Experiment. A mixture of furan-2-carboxaldehyde (6.0 mmol), furan-2-[^{13}C]carboxaldehyde (1.5 mmol; 99% ^{13}C atomic excess), and L-alanine (7.5 mmol) in phosphate buffer (0.2 mol/L; pH 7.0; 10 mL) was heated for 1 h at 70 °C. After cooling to room temperature, the ^{13}C-enriched colorant was isolated as described below.

Isolation of ^{13}C-Enriched (*S*)-4[(*E*)-1-Formyl-2-(2-furyl)ethenyl)]-5-(2-furyl)-2-[(*E*)-(2-furyl)methylidene]-2,3-dihydro-α-methyl-3-oxo-1*H*-pyrrole-1-acetic Acid (e-1a). The reacted mixture was extracted with ethyl acetate (4 × 15 mL), and the organic layer was dried over Na_2SO_4 and finally concentrated under vacuum to ∼1 mL. The solution was then subfractionated by column chromatography (35 × 2 cm) on silica gel (silica gel 60, Merck) conditioned in diethyl ether. After application of the crude material, the column was flushed with ethyl acetate (150 mL). Elution with ethyl acetate/methanol (80:20, v/v; 150 mL) then affords a deep red fraction, which was further separated by anion exchange chromatography. The solution was placed onto a column (10 × 1 cm) filled with a slurry of (diethylaminoethyl)cellulose (DE-52, Whatman Ltd., Maidstone, U.K.) in methanol/water (80:20, v/v). First, neutral compounds were eluted with methanol/water (80:20, v/v; 50 mL). The acidic components were then isolated by elution with a mixture (80:20, v/v; 50 mL) of methanol and an aqueous NaCl solution (0.01 mol/L). Water (100 mL) was added to the eluate, the solution was extracted with ethyl acetate (3 × 30 mL), and, after the solvent was distilled off in vacuo, the residue was taken up in methanol (1 mL). For spectral measurements the colorant was finally purified by preparative RP-HPLC. The effluent between 14 and 16 min was collected, diluted with water, and extracted with ethyl acetate. After evaporation of the solvent, the deep red residue was dissolved in DMSO-d_6 and then analyzed by ^{1}H broad band decoupled ^{13}C NMR spectroscopy.

High-Performance Liquid Chromatography (HPLC). HPLC was performed with a gradient system consisting of two pumps type 422 (Kontron, Eching, Germany), a gradient mixer M800 (Kontron), a Rheodyne injector (100 μL loop), and a diode array detector DAD type 440 (Kontron). For preparative RP-18-HPLC (250 × 10 mm; ODS-Hypersil, 5 μm, 10 nm; Shandon, Frankfurt, Germany), a flow rate of 1.8 mL/min was used. Starting with a mixture of methanol (20%) and water (80%), the methanol content was increased to 100% within 45 min.

^{1}H Broad Band Decoupled ^{13}C Nuclear Magnetic Resonance Spectroscopy (^{13}C NMR). NMR spectroscopy was performed on a Bruker AM-360 spectrometer using the following acquisition parameters: transmitter frequency, 90.56 MHz; spectral width, 25000 Hz; pulse length, 10.4 μs; recorded with 64K data points; repetition time, 2.5 s; 1 Hz line broadening; 2000 scans. Processing was done by multiplication with a Lorentz−Gaussian function prior to transformation. The sample was dissolved in DMSO-d_6, and chemical shifts expressed in parts per million (δ) were measured from residual DMSO-d_6 (39.5 ppm). Evaluation of the experiments was done with 1D-WIN-NMR software.

RESULTS AND DISCUSSION

In an aqueous solution of furan-2-carboxaldehyde heated for 1 h at 70 °C in the presence of L-alanine or *n*-propylamine, red Maillard reaction products were recently identified as the previously unknown 2-[(*E*)-(2-furyl)methylene]- and 2-[(*Z*)-(2-furyl)methylene]-4-[(*E*)-1-formyl-2-(2-furyl)ethenyl]-5-(2-furyl)-1*H*-pyrrol-3(2*H*)-ones of L-alanine (**1a** and **2a** in Figure 1) and *n*-propylamine (**1b** and **2b** in Figure 1), respectively (Hofmann, 1997, 1998a). The structure assignment was unequivocally deduced from one- (^{1}H, ^{13}C, DEPT-135) and two-dimensional NMR techniques (DQF−COSY, TOCSY, HMQC, HMBC, NOESY, ROESY), by MS, IR, and UV spectroscopy, and, in addition, by synthetic experiments (Hofmann, 1997).

In studies on the reaction of furan-2-carboxaldehyde with proteins, analoguous 1*H*-pyrrol-3(2*H*)-one chromophores cross-linked with a lysine side chain of a protein (**1c** and **2c** in Figure 1) have also recently been established (Hofmann, 1998b). It was shown that these

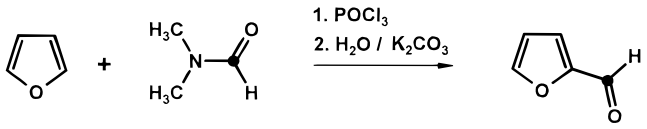

Figure 2. Synthesis of furan-2-[^{13}C]carboxaldehyde.

colorants represent part of the chromophoric substructures in melanoidins formed from heating casein and furan-2-carboxaldehyde in neutral aqueous solution.

Because the 1*H*-pyrrol-3(2*H*)-one chromophore was formed from primary amino components and furan-2-carboxaldehyde independent from the amino moiety, the following studies on its formation route were performed exemplarily with L-alanine.

The structure of chromophore **1a** makes it very likely that besides L-alanine four molecules of furan-2-carboxaldehyde are involved in its formation. It is obvious that three molecules of furan-2-carboxaldehyde were incorporated in **1a** with intact furan ring. The 1*H*-pyrrol-3(2*H*)-one core, however, requires a ring opening of one molecule of furan-2-carboxaldehyde.

To gain a more detailed insight into the formation pathways, the carbon atom of the aldehyde function in the precursor furan-2-carboxaldehyde was tagged with a ^{13}C nucleus by formylation of furan via a Vilsmeyer reaction using dimethyl[^{13}C]formamide (Figure 2). Because four molecules of furan-2-carboxaldehyde are most likely involved in generating the 1*H*-pyrrol-3(2*H*)-one chromophore, the reaction of L-alanine with furan-2-[^{13}C]carboxaldehyde would consequently lead to a 4-fold ^{13}C-labeled isotopomer of **1a**.

This 4-fold labeling would, however, not only increase the intensity of four signals in the ^{13}C NMR spectrum of **1a** but might, moreover, lead to the simultaneous ^{13}C enrichment of consecutive carbon positions in **1a**, resulting in $^{13}C-^{13}C$ spin couplings. These homonuclear couplings detected in the form of line splitting of the resonances into multiplet patterns would increase the complexity of the ^{13}C NMR spectrum of **1a** and, due to the multiplicity of carbon atoms in the range between 100 and 150 ppm of its spectrum, might increase the probability of overlapping resonances. Because the existence of atropisomeric conformers of **1a** leads to diastereomeric splitting of the NMR spectrum (Hofmann, 1997), a double set of signals showing increased

Table 1. Assignment of ^{13}C NMR Signals (360 MHz, DMSO-d_6) of Natural ^{13}C-Abundant ($1a_1$/$1a_2$) and ^{13}C-Enriched (e-$1a_1$/e-$1a_2$) 1*H*-Pyrrol-3(2*H*)-one 1a

H at C atom[a]	δ (ppm)[b]				heteronuclear $^1H,^{13}C$ multiple quantum coherence of **1a**[c]	
	$1a_1$	**$1a_2$**	**e-$1a_1$**	**e-$1a_2$**	via ^1J(C,H)	via $^{2,3}J$(C,H)
C(22)	17.8	17.1			CH_3(22)	H−C(21)
C(21)	57.4	57.2			H−C(21)	CH_3−C(22)
C(2)	105.7	104.9				H−C(15), H−C(16)
C(5)	109.7	110.6	109.7	110.6	H−C(5)	H−C(7)
C(12)	112.5	112.4			H−C(12)	H−C(11), H−C(13)
C(8)	113.2	113.2			H−C(8)	H−C(7), H−C(9)
C(19)	113.5	113.5			H−C(19)	H−C(18), H−C(20)
C(11)	115.7	115.8			H−C(11)	H−C(12), H−C(13)
C(7)	115.9	116.0			H−C(7)	H−C(5), H−C(8), H−C(9)
C(18)	116.4	116.5			H−C(18)	H−C(16), H−C(19), H−C(20)
C(14)	130.3	130.1				H−C(15), H−C(16)
C(4)	133.8	132.7	133.8	132.7		H−C(5), H−C(21)
C(16)	137.7	136.7	137.7	136.7	H−C(16)	H−C(15), H−C(18)
C(13)	144.1	144.1			H−C(13)	H−C(11), H−C(12)
C(9)	145.5	145.6			H−C(9)	H−C(7), H−C(8)
C(20)	146.2	146.1			H−C(20)	H−C(18), H−C(19)
C(6)	150.7	150.7				H−C(5), H−C(7), H−C(8)
C(17)	151.1	151.1				H−C(16), H−C(18), H−C(19)
C(10)	151.2	151.2				H−C(11), H−C(12)
C(1)	153.1	153.1	153.1	153.1		H−C(11), H−C(21)
C(23)	171.8	172.3				H−C(21), CH_3(22)
C(3)	180.3	180.5				H−C(5)
C(15)	193.1	193.1			H−C(15)	H−C(16)

[a] Arbitrary numbering of carbon atoms refers to **1a** in Figure 1. [b] The ^{13}C chemical shifts are given in relation to DMSO-d_6. **$1a_1$/$1a_2$** and **e-$1a_1$/e-$1a_2$** are atropisomeric conformers of **1a** and **e-1a**, respectively. [c] Assignments based on HMQC (^1J) and HMBC ($^{2,3}J$) experiments (Hofmann, 1997).

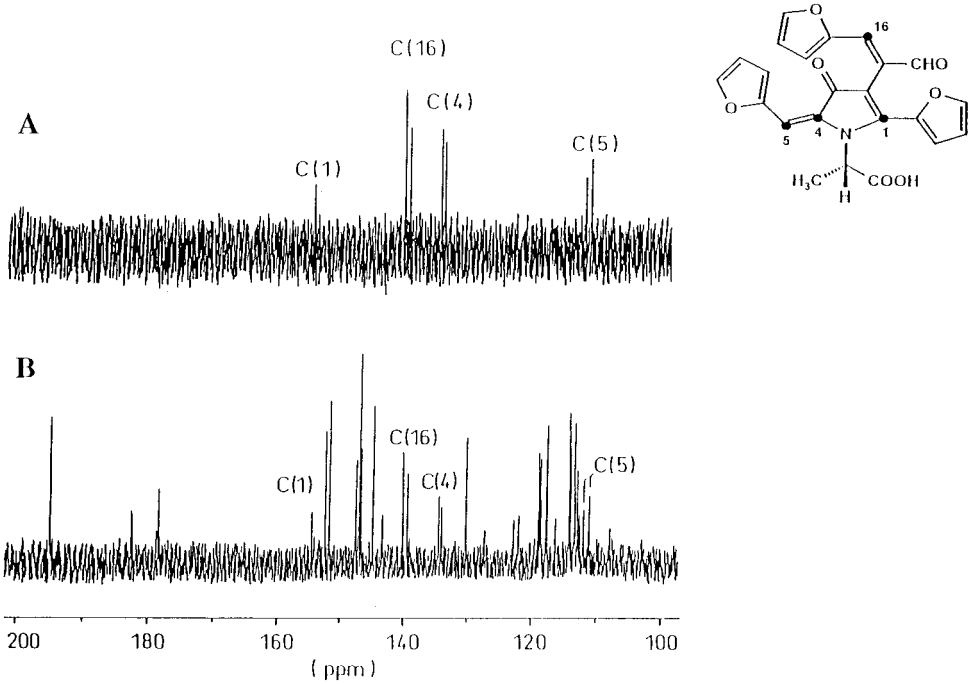

Figure 3. Excerpt of ^{13}C NMR spectrum (360 MHz; DMSO-d_6) of 1*H*-pyrrol-3(2*H*)-one **1a** formed from L-alanine and (A) partially ^{12}C-diluted, site specific ^{13}C-labeled furan-2-carboxaldehyde and (B) furan-2-carboxaldehyde with natural ^{13}C abundance, respectively.

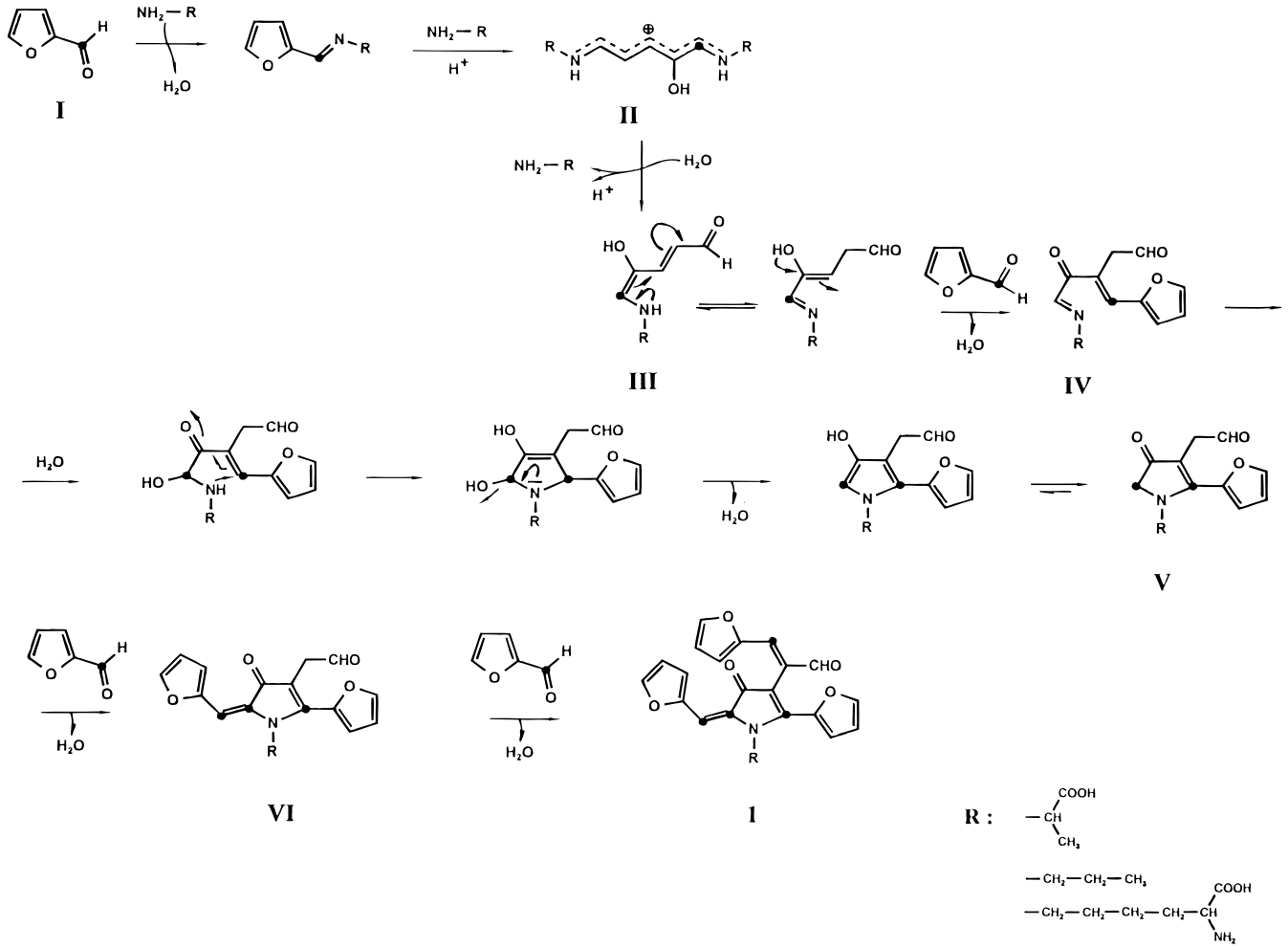

Figure 4. Reaction pathway leading to colored N-substituted 2-[(*E*)-(2-furyl)methylene]-4-[(*E*)-1-formyl-2-(2-furyl)ethenyl]-5-(2-furyl)-1*H*-pyrrol-3(2*H*)-ones from furan-2-carboxaldehyde and primary amino compounds (●, ^{13}C-labeled carbon atom).

intensity would be expected, leading to an additional increase of complexity of the NMR spectrum. An unequivocal assignment of the ^{13}C-enriched positions in **1a** might not be, therefore, guaranteed. These data imply that the 100% ^{13}C enrichment of the aldehyde group of furan-2-carboyaldehyde is not the optimum labeling strategy for studying the 1*H*-pyrrol-3(2*H*)-one formation.

To overcome this problem, furan-2-carboxaldehyde was, therefore, not used in 100% ^{13}C atomic excess, but, prior to reaction with L-alanine, the site specific ^{13}C precursor was 1 + 4 diluted with furan-2-carboxaldehyde showing natural ^{13}C abundance. This dilution results in a high probability that in the mean only one ^{13}C-enriched carbon atom is incorporated in **1a** and in a very low probability of ^{13}C−^{13}C spin couplings between two or more enriched carbon atoms. If the colorant will be generated from the precursors without isotope discrimination, then, in the mean, several singly labeled isotopomeres should be formed preferentially showing the label at four different carbon sites.

The ^{12}C-diluted furan-2-[^{13}C]carboxaldehyde was, therefore, reacted with L-alanine, and the enriched isotopomeres of the colorant (**e-1a**) were then extracted with ethyl acetate. The colorant was then isolated by column chromatography on silica gel followed by anion exchange chromatography and was finally purified by preparative RP-HPLC.

The isolated mixture of the ^{13}C-enriched isotopomers **e-1a** was then analyzed by ^{1}H broad band decoupled ^{13}C NMR spectrocopy (Table 1). In comparison with nonlabeled **1a**, the ^{13}C NMR spectrum of labeled 1*H*-pyrrol-3(2*H*)-one **e-1a** displayed in Figure 3 exhibits seven signals at 109.7, 110.6, 132.7, 133.8, 136.7, 137.7, and 153.1 ppm. Heteronuclear correlation experiments (HMQC, HMBC) given in Table 1 (Hofmann, 1997) led to the assignment of the increased signals as C(1), C(4), C(5), and C(16). Due to the diastereomeric conformers **e-1a$_1$** and **e-1a$_2$**, the resonances of C(4), C(5), and C(16) in **e-1a** were split into two signals each. Because the C(1) of both diastereomers resonated at the same chemical shift, a sole signal at 153.1 ppm was detected.

The positions of the four ^{13}C-enriched carbon atoms in **e-1a** clearly show that the resonance signal at 193.1 ppm assigned as the aldehyde function in **1a** (Table 1) cannot originate from the carbonyl group of furan-2-carboxaldehyde. The aldehyde carbon atom of furan-2-carboxaldehyde was, however, incorporated at C(4) of **1a**.

On the basis of these results the reaction pathway displayed in Figure 4 can be proposed for the formation of **1a**. To follow the fate of the ^{13}C label of furan-2-carboxaldehyde throughout its incorporation into the colorant, the ^{13}C-enriched atoms are marked in the structures displayed in Figure 4. As recently shown for the reaction of furan-2-carboxaldehyde with L-proline (Hofmann, 1998a) also in the presence of primary amino compounds, the furan ring of one molecule of furan-2-carboxaldehyde (**I**) is cleaved, giving rise to 5-(alkylamino)-2-hydroxy-(*E*,*E*)-2,4-pentadienal alkylimine (**II**).

Hydrolysis yields the methylene active 5-(alkylamino)-4-hydroxy-2,4-pentadienal (**III**), which, after enolization, reacts with a further molecule of furan-2-carboxaldehyde, resulting in 5-(alkylimino)-3-[(2-furyl)methylidene]-4-oxopentanal (**IV**). Hydratization and nucleophilic cyclization, followed by water elimination, lead to the formation of an N-substituted (*S*)-4-(formylmethyl)-5-(2-furyl)-2,3-dihydro-α-methyl-3-oxo-1*H*-pyrrole (**V**). Further condensation with furan-2-carboxaldehyde then gives rise to an N-substituted (*S*)-4-(formylmethyl)-5-(2-furyl)-2-[(*E*)-(2-furyl)methylidene]-2,3-dihydro-α-methyl-3-oxo-1*H*-pyrrole-1-acetic acid (**VI**). Such condensation reactions of 5-(2-furyl)-1*H*-pyrrol-3(2*H*)-ones with furan-2-carboxaldehyde were recently shown to run very easily, also under mild conditions (Hofmann, 1997). A further condensation of the methylene active formylmethyl moiety of **VI** with another molecule of furan-2-carboxaldehyde then results in the N-substituted (*S*)-4-[(*E*)-1-formyl-2-(2-furyl)ethenyl)]-5-(2-furyl)-2-[(*E*)-(2-furyl)methylidene]-2,3-dihydro-α-methyl-3-oxo-1*H*-pyrrole (**1**). Because condensation of the intermediate **V** with furan-2-carboxaldehyde can run in the *E* as well as in the *Z* orientation, it can be assumed that its 2-[(*Z*)-(2-furyl)methylene] isomer (**2a**) will be formed via the same reaction pathway.

After ring opening of one molecule of furan-2-carboxaldehyde, the three methylene active functions might also condense with other carbohydrate-derived carbonyl compounds, e.g. acetaldehyde, glycerinaldehyde, 2-oxopropanal, or deoxyosones; a wide range of colored 1*H*-pyrrol-3(2*H*)-ones can be assumed to be formed via this reaction pathway. Also, the amino group might be substituted by a variety of different amino acid moieties and, in addition, can represent a cross-link between the chromophoric system and proteins in melanoidin-type colorants (Hofmann, 1998b). The formation pathway presented might, therefore, indicate a major reaction route leading to 1*H*-pyrrol-3(2*H*)-one chromophores in Maillard systems as well in thermally processed foods.

CONCLUSIONS

The dilution of a site specific ^{13}C-enriched precursor with a natural ^{13}C abundant precursor was for the first time successfully used to clarify the formation pathway of a Maillard reaction product by identifying the enriched carbon atoms using ^{13}C NMR spectroscopy. The application of this powerful technique offers the possibility to disentangle the complex network of reactions producing chromophores and will help to construct a route map of reactions leading to nonenzymatic browning during thermal processing of foods.

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