Application of Stable Isotope Ratio Analysis Explaining the Bioformation of 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone in Plants by a Biological Maillard Reaction

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[1-^{13}C]-D-Fructose and [U-$^{13}C_6$]-D-fructose were applied to detached ripening strawberry fruits, and the incorporation into 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone **1**, 2,5-dimethyl-4-methoxy-3(2*H*)-furanone **2**, 2,5-dimethyl-4-acetoxy-3(2*H*)-furanone **3**, 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone *β*-D-glucopyranoside **4**, and 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone 6′-*O*-malonyl *β*-D-glucopyranoside **5** was determined by HRGC−MS and HPLC−ESI MS−MS. The data clearly showed the direct conversion of D-fructose to the furanones without cleavage of the carbohydrate prior to the formation of **1**−**5**, as expected for a biological Maillard reaction. Both, the furanone and the D-glucose moiety of **4** and **5** contained the labels. However, the label was primarily incorporated into the furanone moiety, indicating that D-fructose is a more efficient precursor of the furanones than D-glucose.

Keywords: *2,5-Dimethyl-4-hydroxy-3(2H)-furanone; biosynthesis; stable isotope ratio analysis; biological Maillard reaction; Fragaria ananassa*

INTRODUCTION

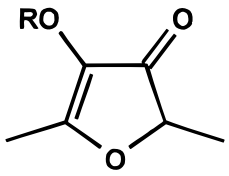
While investigating the browning reaction of reducing sugars with amine salts, also called the Maillard reaction (Ledl and Schleicher, 1990), Hodge et al. (1963) reported for the first time 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone **1**. Today, **1** is considered to be a very important sugar degradation product formed by the Maillard reaction during cooking as well as roasting of foodstuffs. It is found in roasted coffee (Tressl et al., 1978), bread crust (Schieberle and Grosch, 1994), popcorn (Schieberle, 1991), and roasted beef (Cerny and Grosch, 1992).

In addition, **1** has also been described as one of the most important native flavor compounds in various fruits, such as strawberry (Re et al., 1973), pineapple (Rodin et al., 1965), raspberry (Honkanen et al., 1980), and tomato (Buttery et al., 1995). The methoxy derivative **2**, acetoxy derivative **3**, and *β*-D-glucopyranoside of **1** (Mayerl et al., 1989; Wu et al., 1990; Krammer et al., 1994) as well as the malonylated derivative thereof (Roscher et al., 1996) have been identified in fruits, whereas the *β*-D-glucuronide of **1** has been detected as the major metabolite of **1**, **2**, **4**, and **5** in urine of humans (Roscher et al., 1997a).

Although the biosynthesis of **1** has been studied in strawberry callus culture (Zabetakis and Holden, 1996) and the yeast *Zygosaccharomyces rouxii* (Hecquet et al., 1996), the detailed formation pathway is still unknown. On the basis of metabolic studies, in which unlabeled 6-deoxy-D-fructose was fed to strawberry callus culture, Zabetakis and Holden (1996) concluded that methylpentoses are precursor compounds for the biosynthesis of **1**. However, the studies of Hecquet et al. (1996) showed that D-fructose 1,6-biphosphate was effectively transformed into **1** by yeast. Recently, the biosynthesis and metabolism of **1** have been studied in detached ripening strawberry fruits, demonstrating the incorporation of the methyl group of *S*-[*methyl*-^{14}C]adenosyl-L-methionine and [^{14}C] **1** into **2** (Roscher et al., 1997b). Furthermore, radioactively labeled D-fructose 1,6-biphosphate was identified as the most efficient precursor for **1** (unpublished data). However, the localization of the label in **1** could not be unambiguously determined due to the keto−enol tautomerism and known instability of **1**. Therefore, it remained unclear whether **1** was directly generated from the carbohydrate biphosphate or D-fructose 1,6-biphosphate was cleaved by aldolase to two C_3 units serving as building blocks for the formation of the furanone **1**.

This report describes for the first time the incorporation of ^{13}C-labeled fructose into the target molecule and its natural derivatives and provides the first evidence that **1** contains the complete carbon chain of the applied fructose molecules.

1 R = H
2 R = CH_3
3 R = CO-CH_3
4 R = ß-D-glucopyranose
5 R = 6′-O-malonyl ß-D-glucopyranose



MATERIALS AND METHODS

Plant Material. Whole strawberry plants (*Fragaria* × *ananassa* cv. Elsanta), carrying fruits of all ripening stages, were kindly provided by CPRO-DLO (Wageningen, The Netherlands). The plants were watered, as required, and were kept in an incubation chamber. They received lighting from 8:00 a.m. to 6:00 p.m. daily at 45% humidity.

Chemicals. All chemical reagents were purchased from Sigma and Aldrich. The organic solvents were obtained from Merck and Fisons. XAD-2 was supplied by Aldrich. [1-^{13}C]-D-Fructose (99% purity) and [U-$^{13}C_6$]-D-fructose (98% purity) were purchased from Cambridge Isotope Laboratory.

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Application of Compounds. Maturing strawberries weighing approximately 5 g in the orange ripening stage were cut off the plants. The stems and calyx were carefully removed under distilled water so that the berry remained uninjured. A solution containing [1-^{13}C]-D-fructose (6.5 mg) or [U-$^{13}C_6$]-D-fructose (6.0 mg) dissolved in 50 μL of tap water was injected into a berry from the top, where the stem and calyx had been removed. In a parallel experiment (control), 50 μL of an aqueous solution containing 6.0 mg of unlabeled D-fructose was injected into a berry. The experiment was repeated twice.

Extraction of Volatiles and Glycosides. After incubation for 72 h at room temperature, the berries were stored at −20 °C until workup. Frozen strawberries were submerged in 10 mL of water, homogenized by means of an Ultra-Turrax apparatus, and centrifuged (2000*g* for 10 min). The residues were washed twice, and the supernatants were combined (40 mL) and subjected to solid phase extraction on XAD-2 (20 cm, 1 cm inside diameter). After washing with 40 mL of water, volatiles were eluted by 50 mL of diethyl ether and the glycosides were obtained by elution with 80 mL of methanol. The diethyl ether extract was dried and concentrated to approximately 100 μL. The methanol extract was concentrated in vacuo to approximately 1 mL.

Enzymatic Hydrolysis. An aliquot of the methanol extracts was dissolved in 800 μL of 0.2 M phosphate buffer (pH 5.5), and 200 μL of Rohapect D5L (Röhm, Darmstadt, Germany), a pectinolytic enzyme preparation exhibiting glycosidase and esterase side activities, was added. After an incubation period of 3 h at 37 °C, the liberated aglycons were extracted three times by 1 mL of diethyl ether each. The combined organic layers were dried over Na_2SO_4 and concentrated to approximately 100 μL.

Synthesis of Reference Compounds [1-^{13}C]- or [6-^{13}C]1 and [U-$^{13}C_6$]1. A solution containing 5 mg of [1-^{13}C]-D-fructose or [U-$^{13}C_6$]-D-fructose and 1 mg of L-alanine, 10 μL of acetic acid and 100 μL of water was prepared and subjected to liquid sampling solid phase microextraction (SPME), according to the procedure of Verhoeven et al. (1997). During thermal desorption in the GC injector, [U-$^{13}C_6$]**1** and [1-^{13}C]- or [6-^{13}C]**1** are formed from [U-$^{13}C_6$]-D-fructose and [1-^{13}C]-D-fructose, via Maillard reaction, respectively.

Capillary Gas Chromatography−Mass Spectrometry (HRGC−EI-MS). A Fisons MD 800 quadrupol mass spectrometer coupled to a Fisons GC 8060 instrument equipped with Fisons MassLab software (version 1.3) was used. A J&W DB-Wax 20 M fused silica capillary column (25 m × 0.25 mm i.d.; df = 0.25 μm), which was maintained at 50 °C for 3 min and then programmed to 240 °C at 4 °C/min, was used with helium gas at a flow rate of 3 mL/min. Significant MS operating parameters were as follows: ionization voltage, 70 eV (electron impact ionization); ion source and interface temperature, 230 and 240 °C, respectively; scan range, 41−250 mass units; and scan duration, 0.69 s. Constituents were identified by comparison of their mass spectra and retention indices with those of authentic reference compounds. The quantification of the ratio of the fragment ions was performed as follows. The integrated area of **1** in the single ion trace *m*/*z* 128 was set to 100%. On the basis of the integrated area of **1** in the single ion traces of *m*/*z* 129 and 134, the percentages of the intensity of the fragment ions *m*/*z* 129 and 134 were calculated. An analogous procedure was applied for the calculation of the ratios of the fragment ions of **2** and **3**.

HPLC−ESI MS−MS Analysis. Analysis of methanol extracts was performed on a triple stage quadrupole TSQ 7000 LC−MS−MS system with an electrospray ionization (ESI) interface (Finnigan MAT, Bremen, Germany). The temperature of the heated capillary was 220 °C. The ESI capillary voltage was set to 3.5 kV, resulting in a 3.4 μA current. Nitrogen served as both the sheath (70 psi) and auxiliary gas (10 L/min). Data acquisition and evaluation were carried out on a Personal DECstation 5000/33 (Digital Equipment, Unterföhring, Germany) and ICIS 8.1 software (Finnigan MAT). HPLC separations were carried out on an Eurospher 100 C-18 column (100 × 2 mm, 5 μm, Knauer, Berlin, Germany) using a linear gradient with a flow rate of 200 μL/min. Solvent A was 0.05% formic acid in H_2O, and solvent B was 0.05% formic acid in CH_3CN. The gradient program was as follows: 0−10 min, 0 to 40% B; 10−12 min, 40 to 70% B; and 12−15 min, 70 to 100% B. Mass spectra were acquired in the positive mode. Product ion spectra of **3** and **4** were available by collision-induced dissociation (CID) (1.5 mTorr of Argon; − 20 eV). The calculation of the fragment ratios was performed in an analogous fashion as reported for HRGC−MS. However, in the case of HPLC−ESI MS−MS, $[M + 1]^+$ ions were recorded.

Table 1. Incorporation of [1-^{13}C]-D-Fructose into 1−5[a]

	[1-^{13}C]-D-fructose	control
1, *m*/*z* 129/128[b] $[M]^+$	23.2 ± 3.2	7.7 ± 1.8
2, *m*/*z* 143/142[b] $[M]^+$	25.7 ± 4.5	11.5 ± 3.0
3, *m*/*z* 129/128[b] $[M - \text{acetate}]^+$	19.6 ± 0.8	5.4 ± 1.0
m/*z* 171/170[b] $[M]^+$	24.6 ± 1.4	10.8 ± 0.8
4, *m*/*z* 292/291[c] $[M + 1]^+$	38.2 ± 2.7	13.4 ± 0.6
5, *m*/*z* 378/377[c] $[M + 1]^+$	48.8 ± 5.2	17.0 ± 0.4

[a] The intensity of the heavier isotopomer is expressed as a percentage of the lighter isotopomer. [b] Determined by HRGC−MS. [c] Determined by HPLC−ESI MS−MS.

Table 2. Incorporation of [U-$^{13}C_6$]-D-Fructose into 1−5[a]

	[$^{13}C_6$]-D-fructose	control
1, *m*/*z* 134/128[b] $[M]^+$	8.2 ± 3.2	<0.1
2, *m*/*z* 148/142[b] $[M]^+$	7.8 ± 2.1	<0.1
3, *m*/*z* 134/128[b,c] $[M]^+$	9.3 ± 2.5	<0.1
4, *m*/*z* 297/291[d] $[M + 1]^+$	10.9 ± 4.2	<0.1
5, *m*/*z* 383/377[d] $[M + 1]^+$	10.6 ± 2.9	<0.1
1 after hydrolysis of **4** and **5**, *m*/*z* 134/128[b] $[M]^+$	8.3 ± 0.3	<0.1

[a] The intensity of the heavier isotopomer is expressed as a percentage of the lighter isotopomer. [b] Determined by HRGC−MS. [c] Quantification of *m*/*z* 176/170 not feasible due to the coelution of a compound exhibiting the fragment ion *m*/*z* 176. [d] Determined by HPLC−ESI MS−MS.

RESULTS AND DISCUSSION

Aqueous solutions containing [1-^{13}C]-D-fructose and [U-$^{13}C_6$]-D-fructose were administered to detached ripening strawberry fruits, and the incorporation of the labels into **1**−**5** was determined by HRGC−MS and HPLC−ESI MS−MS. Tables 1 and 2 show the incorporation of the label of [1-^{13}C]-D-fructose and [U-$^{13}C_6$]-D-fructose into **1**−**5**, respectively.

Comparison of the achieved values after the administration of [1-^{13}C]-D-fructose and analysis of the stable isotope ratios with those of the control experiment clearly demonstrated the incorporation of one ^{13}C atom into **1**−**5**. In the case of **3**, we determined the ratios of the intensity of the molecular ions *m*/*z* 171/170 $[M]^+$ and the fragment ions *m*/*z* 129/128 $[M - \text{acetate}]^+$, corresponding to charged **1**, to localize the label in the molecule. The values were an indication of the incorporation of the label into the furanone moiety of **3**.

After the application of [U-$^{13}C_6$]-D-fructose, six ^{13}C atoms were incorporated into **1**−**5** (Table 2). The mass spectra nicely show the transformation of the complete carbon chain of [U-$^{13}C_6$]-D-fructose into **1** (Figure 1). Therefore, D-fructose is not cleaved prior to the formation of **1**. The enzymatic cleavage of the D-fructose metabolite D-fructose 1,6-biphosphate by aldolase yields two C_3 units. Consequently, incorporation of these labeled C_3 units would increase the amount of the isotopomer of **1** exhibiting the molecular ion *m*/*z* 131, which was obviously not the case (Figure 1). The higher incorporation of [U-$^{13}C_6$]-D-fructose into **4** and **5** compared with that into **1**−**3** is consistent with the data obtained for [1-^{13}C]-D-fructose and is caused by the

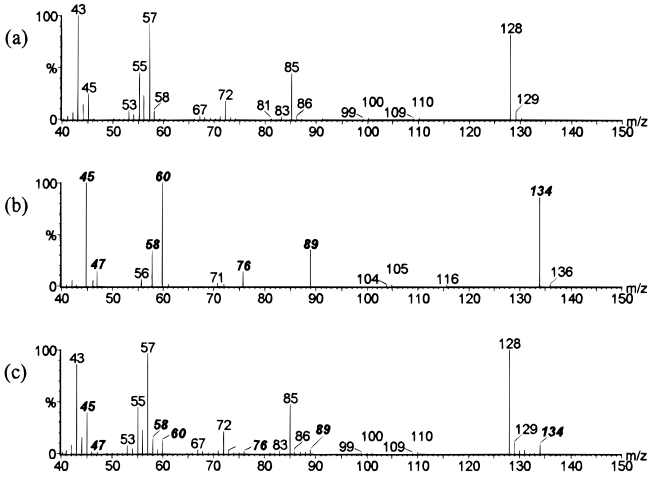

Figure 1. Mass spectrum of unlabeled **1** (a), **[**U-$^{13}C_6$]**1** (b), and **1** obtained after the application of [U-$^{13}C_6$]-D-fructose (c) to detached ripening strawberry fruits.

Table 3. Stable Isotope Ratio Analysis of the Product Ions Formed by the [M + 1]$^+$ Ions of 4 and 5 after the Application of [1-^{13}C]-D-Fructose

	D-fructose 1-^{13}C	control
4, dau *m/z* 291[a] → *m/z* 129/130[b]	100/<0.1	100/<0.1
dau *m/z* 292 → *m/z* 129/130	62.5 ± 3.0/100	100/72.0 ± 2.7
5, dau *m/z* 377 → *m/z* 129/130	100/<0.1	100/<0.1
dau *m/z* 378 → *m/z* 129/130	87 ± 4.1/100	100/69.7 ± 7.2

[a] Precursor ion. [b] Ratio of the intensity of the product ions.

Table 4. Stable Isotope Ratio Analysis of the Product Ions Formed by the [M + 1]$^+$ Ions of 4 and 5 after the Application of [U-$^{13}C_6$]-D-Fructose

	[$^{13}C_6$]-D-fructose	control
4, dau *m/z* 291[a] → *m/z* 129/135[b]	100/<0.1	100/<0.1
dau *m/z* 297 → *m/z* 129/135	34.1 ± 2.5/100	<0.1/<0.1
5, dau *m/z* 377 → *m/z* 129/135	100/<0.1	100/<0.1
dau *m/z* 383 → *m/z* 129/135	27.3 ± 3.1/100	<0.1/<0.1

[a] Precursor ion. [b] Ratio of the intensity of the product ions.

formation of glycosides carrying the additional label in the carbohydrate moiety (Table 2).

The fragment ion ratio *m/z* 129/128 of the aglycon **1** in glycosides **4** and **5** was determined after hydrolysis of the glycosides followed by the extraction of the released aglycon **1**. The value obtained corresponds very well with the data for **1** (Table 2).

In addition, product ion spectra of the molecular ions were recorded to calculate the distribution of the incorporated label in the glucose and aglycon moiety of the glycosides (Tables 3 and 4).

Fragmentation of the pseudo molecular ion of **4** *m/z* 291 yielded exclusively the ion *m/z* 129. The product ion *m/z* 129 (corresponding to **1**) carried the charge. The carbohydrate moiety remained uncharged. As only the ions *m/z* 291 entered the collision cell of the mass spectrometer, the natural isotope distribution of *m/z* 130 could not be observed. However, fragmentation of the pseudo molecular ion *m/z* 292 (correponding to **4** carrying one ^{13}C atom) led to a definite ratio of *m/z* 129/130. In the control experiment, the product ion *m/z* 129 formed by the precursor ion *m/z* 292 was more intense than *m/z* 130. This observation implied that the uncharged glucose moiety of the natural molecule **4** carried the majority of the ^{13}C atoms. Therefore, more ^{13}C atoms are located in the glucose moiety than in the furanone moiety. However, after the application of [1-^{13}C]-D-fructose to detached ripening strawberry fruits, the ratio of *m/z* 129/130 shifted in favor of *m/z* 130. The shifting indicated that the number of labeled **1** in molecule **4** has increased. Now, the incorporated ^{13}C label was primarily located in the furanone moiety of **4**. The data obtained for **5** are consistent with the results of **4**.

The isotope pattern in the product ions of **4** and **5**, after the application of [U-$^{13}C_6$]-D-fructose, showed even more clearly the previous observation. As the natural abundance of the M + 6 isotopomers of **4** and **5** is <0.1%, it is below the detection limit of the mass spectrometer (Table 4). Therefore, the ratio of the intensities of the product ions *m/z* 129 and 135 reflects the incorporation of the six ^{13}C atom into the glucose and aglycon moiety, respectively. It is obvious that the labeled D-fructose isomerized to labeled D-glucose which was finally diluted by the natural pool of D-glucose. This dilution explains the low incorporation of labeled fructose into the glucose moiety of **4** and **5** compared to that of the furanone moiety of **4** and **5**. On the other hand, the high incorporation of labeled D-fructose into the furanone moiety showed that D-fructose rather than D-glucose is the primary precursor for **1** in strawberry fruits which is consistent with our earlier data indicating that D-fructose and especially D-fructose 1,6-biphosphate are the most efficient precursors for **1** (unpublished data). The data provide the first evidence that **1** is formed directly from D-fructose, probably by means of dehydration and reduction reactions rather than by the coupling of two C_3 units as proposed by Zabetakis and Holden (1996).

The results show some parallels with the data obtained by Schieberle (1992) for the formation of **1** in heat-processed foods. His model experiments indicated that fructose 1,6-biphosphate was the predominant precursor of **1**. However, dry heating was necessary to liberate **1** from D-fructose and D-glucose.

Recently, the bioformation of 3-deoxy-D-fructose was reported in diabetic rat lenses (Lal et al., 1995) where D-fructose 3-phosphate was produced by a 3-phosphokinase from D-fructose. Transformation of D-fructose 3-phosphate led to the reactive 3-deoxyglucosone which was finally detoxified to 3-deoxy-D-fructose. In an analogous reaction, 1-deoxyglucosone and subsequently 1-deoxy-D-fructose and their phosphorylated derivatives, possible biogenetic precursors of **1**, would be formed starting from D-fructose 6-phosphate or D-fructose 1,6-biphosphate, respectively. The 1-deoxyglucosone was also discussed as a possible progenitor of **1** in the Maillard reaction (Ledl and Schleicher, 1990), and this has been demonstrated by trapping experiments with *o*-phenylenediamine (Huber and Ledl, 1990), recently.

On the other hand, 6-deoxy-D-fructose was identified as an efficient progenitor of **1** in strawberry callus cultures and yeasts (Zabetakis and Holden, 1996; Hecquet et al., 1996). The aldolase-catalyzed reaction of dihydroxyacetone phosphate and D-lactaldehyde was proposed as the formation pathway of 6-deoxy-D-fructose (Zabetakis and Holden, 1996). Recently, we applied radioactively labeled precursors to detached strawberries (unpublished data). Although we detected incorporation of dihydroxyacetone into **1** and its derivatives, however, with lower incorporation compared with that of D-fructose, we found no radioactivity in the furanones after the administration of D-lactaldehyde. Therefore, this reaction does not operate in detached

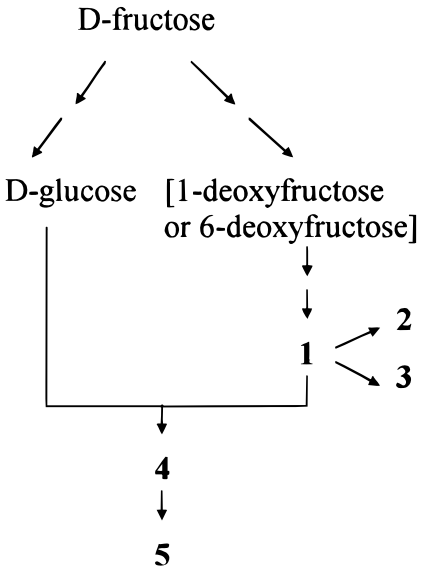

Figure 2. Proposed biosynthetic pathway of **1**−**5**.

ripening strawberry fruits. Of course, an alternative biogenetic pathway, e.g., the biogenesis of 6-deoxy sugars, cannot be excluded. If that is the case, the 1-deoxyglucosone derivative of 6-deoxy-D-fructose could be formed in a reaction analogous to the reaction in diabetic rat lenses.

On the basis of the fact that D-fructose (Shaw et al., 1968), D-fructose 1,6-biphosphate (Schieberle, 1992), and 6-deoxyfructose (Wong et al., 1983) are efficient progenitors of **1** in model Maillard reactions, our data demonstrate for the first time that **1** and its derivatives are formed by a biological Maillard reaction (Figure 2).

ABBREVIATIONS USED

HRGC, high-resolution capillary gas chromatography; HRGC−MS, high-resolution capillary gas chromatography−mass spectrometry; HPLC, high-performance liquid chromatography; HPLC−ESI MS−MS, high-performance liquid chromatography−electrospray ionization tandem mass spectrometry; CPRO-DLO, Centrum voor Plantenveredelings-en Reproduktieonderzoek; XAD-2, nonionic polymeric adsorbent.

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