Metabolic Fate of Isotopes during the Biological Transformation of Carbohydrates to 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone in Strawberry Fruits

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Isotopically labeled D-glucose, D-fructose, 1-deoxy-D-fructose, and 6-deoxyhexoses were applied to detached ripening strawberry (*Fragaria* × *ananassa*) fruits, and the incorporation of the isotopes into the key strawberry aroma compounds 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF, **1**) and 2,5-dimethyl-4-methoxy-3(2*H*)-furanone (DMMF, **2**) was determined by gas chromatography−mass spectrometry. In contrast to previous reports the data clearly showed that 6-deoxy-D-fructose/6-deoxy-D-glucose and 1-deoxy-D-fructose are not natural precursors of the furanones. However, isotopically labeled **1** and **2** were observed after the application of [1-^{2}H]-, [2-^{2}H]-, and [6,6-$^2H_2$]-D-glucose as well as [U-$^{13}C_6$]-, [1-^{13}C]-, [1-^{2}H]-, [6,6-$^2H_2$]-D-fructose. The isotope label of [4-^{2}H]-D-glucose was not recovered in the furanones. In contrast, [2-^{2}H]-D-glucose was converted to [1- or 6-^{2}H]-**1** and [1- or 6-^{2}H]-**2** by the strawberry fruits. The observed isotope shift can be explained by the catalysis of phosphohexose isomerase in the course of the biogenesis of the hydroxyfuranone (**1**) and the methoxyfuranone (**2**) from D-glucose. Thus, the applied D-glucose is metabolized to D-fructose-6-phosphate prior to the transformation into the furanones.

Keywords: *Strawberry; flavor; biosynthesis; Furaneol; Fragaria* × *ananassa*

INTRODUCTION

Among the 350 compounds already identified in strawberry aroma extracts 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF, **1**) represents a key flavor constituent (*1*). The flavor of the hydroxyfuranone (**1**) has been described as caramel-like, sweet, fruity, and burnt pineapple-like. In diluted solutions **1** exhibits a flavor typical of fresh strawberry. The hydroxyfuranone (**1**) was first discovered in pineapple (*2*) and has also been isolated from strawberry (*3*), raspberry (*4*), tomato (*5*), and other fruits (*1*). In plants **1** is metabolized to 2,5-dimethyl-4-methoxy-3(2*H*)-furanone (DMMF, **2**), DMHF *β*-D-glucopyranoside (*6*, *7*), and the malonylated derivative (*8*, *9*).

The hydroxyfuranone (**1**) is intensively used as a food flavoring, but the detailed formation pathway in fruits is still unknown. On the basis of metabolic studies, precursors of **1** have been postulated. Studies of Pisarnitskii et al. (*10*) and Zabetakis and Holden (*11*) support the hypothesis that 6-deoxysugars such as 6-deoxy-D-fructose, L-rhamnose, and L-fucose are transformed to the hydroxyfuranone (**1**) in strawberry fruits and strawberry callus tissue, respectively. However, in studies of Hecquet et al. (*12*) and Roscher et al. (*13*) D-fructose-1,6-diphosphate is metabolized to **1** by the yeast *Zygosaccharomyces rouxii* and detached strawberry fruits, respectively.

The aim of the present research was to clarify the role of deoxy-D-hexoses as precursors of the hydroxyfuranone (**1**) and the methoxyfuranone (**2**) in detached ripening strawberry fruits, to determine the incorporation of ^{2}H- and ^{13}C-labeled D-glucose and D-fructose into **1** and **2** under different conditions, and to investigate the metabolic fate of the isotopes during the formation of the hydroxyfuranone (**1**) and the methoxyfuranone (**2**).

MATERIALS AND METHODS

Plant Material. Strawberry fruits (*Fragaria* × *ananassa* cv. Elsanta or White D) were picked from garden-grown plants and were kept in a bucket of water until used.

Chemicals. All chemical reagents were purchased from Roth (D-arabinose), Fluka (trifluoroacetic acid, D-fructose-1,6-diphosphate disodium salt), Sigma (D-fructose-2,6-diphosphate tetrasodium salt), and Aldrich (butyl mercaptal, sodium borodeuteride, sodium cyanide). The organic solvents were obtained from Merck (methanol, diethyl ether) and Fisons (acetonitrile). XAD-2 was supplied by Aldrich. [1,2,2,2-$^2H_4$]Acetaldehyde, [^{13}C-*methyl*]-*N*-nitroso-*N*-methyl-4-toluenesulfonamide, [1-^{13}C]-D-fructose, and [U-$^{13}C_6$]-D-fructose were purchased from Cambridge Isotope Laboratory. [1-^{2}H]-D-Fructose, [6,6-$^2H_2$]-D-fructose, [U-$^{13}C_6$]-D-fructose, [1-^{2}H]-D-glucose, [2-^{2}H]-D-glucose, [4-^{2}H]-D-glucose, [6,6-$^2H_2$]-D-glucose, [6-^{13}C]-6-deoxy-D-fructose/-6-deoxy-D-glucose, and [1-^{13}C]-L-ascorbic acid were obtained from Omicron Biochemicals, USA.

Synthesis of [6,6,6,5-$^2H_4$]-6-Deoxyhexulose-1-phosphate. [6,6,6,5-$^2H_4$]-6-Deoxyhexulose-1-phosphate consisting of a mixture of [6,6,6,5-$^2H_4$]-6-deoxy-D-fructose-1-phosphate and [6,6,6,5-$^2H_4$]-6-deoxy-L-sorbose-1-phosphate was obtained chemoenzymatically from [2,3,3,3-$^2H_4$]-D,L-lactaldehyde and D-fructose-1,6-diphosphate in the presence of aldolase and triose phosphate isomerase according to the method of Durrwachter et al. (*14*). [2,3,3,3-$^2H_4$]-D,L-Lactaldehyde was synthesized from [1,2,2,2-$^2H_4$]acetaldehyde and sodium cyanide according to the methods of Serianni et al. (*15*, *16*). NMR data were in accordance with published data (*14*, *17*). The isotope label was confirmed by ESI MS/MS.

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Table 1. Incorporation of Labeled Carbohydrates into the Hydroxyfuranone (1); Comparison of the Intensities of the Molecular Ions of Unlabeled 1 and Labeled 1

amount applied per strawberry (mg)	substrate	molecular ions (%)				
		m/z 128	*m/z* 129	*m/z* 130	*m/z* 132	*m/z* 134
19.7	D-fructose	100	7.7 ± 1.6	1.0 ± 0.6	<0.1	<0.1
14.7	[1-^{13}C]-D-fructose	100	39.7 ± 1.4	3.9 ± 0.3	<0.1	<0.1
6.0	[1-^{13}C]-L-ascorbic acid	100	6.1 ± 0.8	0.7 ± 0.2	<0.1	<0.1
10.2	[U-$^{13}C_6$]-D-fructose	100	18.9 ± 2.7	4.0 ± 0.4	0.8 ± 0.2	20.1 ± 2.9
15.6	[1-^{2}H]-D-fructose	100	34.4 ± 5.5	3.2 ± 0.2	<0.1	<0.1
15.8	[1-^{2}H]-D-glucose	100	24.7 ± 2.0	2.7 ± 0.2	<0.1	<0.1
14.6	[2-^{2}H]-D-glucose	100	14.1 ± 3.3	1.6 ± 0.3	<0.1	<0.1
15.0	[4-^{2}H]-D-glucose	100	7.2 ± 2.2	1.0 ± 0.3	<0.1	<0.1
6.0	[6,6-$^{2}H_2$]-D-glucose	100	6.6 ± 1.2	3.9 ± 0.7	0.1	<0.1
15.4	[6,6-$^{2}H_2$]-D-fructose	100	11.3 ± 1.0	25.4 ± 2.9	0.4 ± 0.1	<0.1
6.0	[1-^{13}C]-1-deoxy-D-fructose	100	6.8 ± 1.2	0.9 ± 0.3	<0.1	<0.1
6.0	[6-^{13}C]-6-deoxy-D-fructose/[6-^{13}C]-6-deoxy-D-glucose	100	7.0 ± 1.5	1.0 ± 0.7	<0.1	<0.1
6.0	[6-^{2}H]-6-deoxy-D-glucose	100	6.7 ± 1.3	0.9 ± 0.5	<0.1	<0.1
33.0	[6,6,6,5-$^{2}H_4$]-6-deoxyhexulose-1-phosphate	100	7.3 ± 1.2	0.9 ± 0.2	0.1	<0.1

Synthesis of [6-^{2}H]-6-Deoxy-D-glucose. [6-^{2}H]Methyl-6-deoxy-D-glucose was prepared from methyl α-D-glucoside according to the procedure of Cramer et al. (*18*). Hydrolysis of the glucoside was performed in 5% trifluoroacetic acid by refluxing for 1 h. NMR data were in accordance with those of the commercially available 6-deoxy-D-glucose. The isotope label was confirmed by ESI MS/MS.

Synthesis of [1-^{13}C]-1-Deoxy-D-fructose. Tetra-*O*-acetyl dibutyl mercaptal D-arabinose was synthesized from D-arabinose according to the method of Zinner et al. (*19*). [1-^{13}C]-Tetra-*O*-acetyl-1-deoxy-D-fructose was prepared from the mercaptal according to the procedure of Wolfrom et al. (*20*) using [^{13}C-*methyl*]-*N*-nitroso-*N*-methyl-4-toluensulfonamide as the diazomethane precursor. Hydrolysis was performed in 2% ammonia at room temperature for 24 h. NMR spectra were in accordance with published data (*21*). The isotope label was confirmed by ESI MS/MS.

Application of Substrates. Maturing strawberries weighing ∼5 g, in the pink ripening stage, were cut off the plants. Additionally, strawberries of three developmental stages (white, pink, and red) were used. The stem and calyx were carefully removed under distilled water so that the berry remained uninjured. Solutions (50 μL) containing the isotopically labeled compounds (6−33 mg) were injected into the berries from the top, where the stem and calyx had been removed. In a parallel experiment (control) 50 μL of an aqueous solution containing 19.7 mg of unlabeled D-fructose was injected into a berry. The experiments were repeated at least twice. Strawberries were kept in the sunlight behind a window at room temperature for 1−3 days.

Extraction of 1 and 2. After incubation at room temperature, the berries were stored at −20 °C until workup. Frozen strawberries (∼3−7 g) were submerged in 10 mL of water, homogenized by means of an Ultra-Turrax, and centrifuged (2000 *g*; 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 i.d.). After washing with 40 mL of water, volatiles were eluted by 50 mL of diethyl ether. The diethyl ether extract was dried and concentrated by a Vigreux column to ∼100 μL.

Capillary Gas Chromatography—Mass Spectrometry (GC-MS). A Fisons MD 800 quadrupole mass spectrometer coupled to a Fisons GC 8060 (Egelsbach, Germany) equipped with Fisons MassLab software (version 1.3) was used. A J&W (Folsom, CA) DB-Wax 20 M fused silica capillary column (25 m × 0.25 mm i.d.; film thickness = 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 2 mL/min of helium gas. Significant MS operating parameters were as follows: ionization energy, 70 eV (electron impact ionization); ion source and interface temperature, 230 and 240 °C, respectively; scan range, 41−250 u; 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 molecular ion ratios was performed as follows: The integrated peak area of the hydroxyfuranone (**1**) in the single-ion trace *m/z* 128 was set to 100%. On the basis of the integrated peak area of **1** the percentages of the intensities of the molecular ions *m/z* 129, 130, 132, and 134 were calculated. The same procedure was applied for the calculation of the molecular ions ratios of the methoxyfuranone (**2**).

ESI MS/MS Analysis. Analysis was performed on a triple-stage quadrupole TSQ 7000 LC-MS/MS system with electrospray ionization (ESI) interface (Finnigan MAT, Bremen, Germany). The temperature of the heated capillary was 220 °C. ESI capillary voltage was set to 3.5 kV, resulting in a 3.4 μA current. Nitrogen served both as sheath (70 psi) and auxiliary gas (10 L/min). Data acquisition and evaluation were carried out on a Personal DEC station 5000/33 (Digital Equipment, Unterföhring, Germany) and ICIS 8.1 software (Finnigan MAT, Bremen, Germany). Loop injection was performed. Mass spectra were acquired in the positive mode.

On-Line Gas Chromatography/Combustion/Isotope Ratio Mass Spectrometry (GC-IRMS). GC-IRMS analyses were performed on an HP 5890 gas chromatograph connected to a Finnigan MAT delta S isotope mass spectrometer via a combustion interface. Isotope ratios were expressed as δ values (‰) versus the PDB standard ([^{13}C]/[^{12}C] isotope ratio = 0.0112372 for CO_2), yielded by the reaction of fossil $CaCO_3$ (Pee Dee Belemnite). The GC was equipped with a poly(ethylene glycol) fused silica capillary column (DB-Wax 20M, 25 m × 0.32 mm i.d.; film thickness = 0.3 μm). Helium was used as carrier gas (14 psi head pressure). Samples were injected in the cool on-column mode. The following temperature program was applied: starting isothermal at 50 °C for 3 min, programmed to 240 °C at 4 °C/min, and finally held for 20 min.

NMR. ^{1}H and ^{13}C NMR spectra were recorded on a 400 MHz/100 MHz spectrometer (Bruker, Karlsruhe, Germany). All NMR data are reported in parts per million (δ) downfield from the internal standard TMS.

RESULTS

Incorporation of Isotopically Labeled Substrates. Aqueous solutions containing the isotopically labeled substrates listed in Tables 1 and 2 were administered to detached ripening strawberry fruits, and the incorporation of the label into the hydroxyfuranone (**1**) and the methoxyfuranone (**2**) was determined by GC-MS after 3 days of incubation. Incorporation of isotope labels into **1** and **2** was shown by comparison of the intensity of the molecular ion of unlabeled **1** (*m/z* 128) and **2** (*m/z* 142), originating from the natural pool with the intensities of the molecular ions of the expected labeled hydroxyfuranone (**1**) (*m/z* 129, 130, 132, and 134) and methoxyfuranone (**2**) (*m/z* 143, 144, 146, and 148). The intensities of the molecular ions of labeled **1** and **2** was

Table 2. Incorporation of Labeled Carbohydrates into the Methoxyfuranone (2); Comparison of the Intensities of the Molecular Ions of Unlabeled 2 and Labeled 2

amout applied per strawberry (mg)	substrate	molecular ions (%) *m/z* 142	*m/z* 143	*m/z* 144	*m/z* 146	*m/z* 148
19.7	D-fructose	100	6.8 ± 0.4	0.8 ± 0.2	<0.1	<0.1
14.7	[1-^{13}C]-D-fructose	100	36.5 ± 0.5	3.8 ± 0.4	<0.1	<0.1
6.0	[1-^{13}C]-L-ascorbic acid	100	7.6 ± 1.0	1.4 +/−0.5	<0.1	<0.1
10.2	[U-$^{13}C_6$]-D-fructose	100	9.7 ± 0.4	1.9 ± 0.2	0.4 ± 0.2	9.1 ± 0.3
15.6	[1-^{2}H]-D-fructose	100	34.1 ± 1.0	4.4 ± 0.5	>0.1	<0.1
15.8	[1-^{2}H]-D-glucose	100	27.0 ± 1.5	3.2 ± 0.4	<0.1	<0.1
14.6	[2-^{2}H]-D-glucose	100	15.6 ± 2.7	1.7 ± 0.3	<0.1	<0.1
15.0	[4-^{2}H]-D-glucose	100	7.9 ± 2.1	1.1 ± 0.3	<0.1	<0.1
6.0	[6,6-$^{2}H_2$]-D-glucose	100	9.2 ± 1.0	6.5 ± 1.1	0.2 ± 0.1	<0.1
15.4	[6,6-$^{2}H_2$]-D-fructose	100	15.3 ± 0.2	33.4 ± 2.4	0.7 ± 0.2	<0.1
6.0	[1-^{13}C]-1-deoxy-D-fructose	100	8.4 ± 0.5	1.0 ± 0.2	<0.1	<0.1
6.0	[6-^{13}C]-6-deoxy-D-fructose/[6-^{13}C]-6-deoxy-D-glucose	100	7.2 ± 1.5	0.8 ± 1.0	<0.1	<0.1
6.0	[6-^{2}H]-6-deoxy-D-glucose	100	7.1 ± 1.3	0.9 ± 0.5	<0.1	<0.1
33.0	[6,6,6,5-$^{2}H_4$]-6-deoxyhexulose-1-phosphate	100	7.2 ± 0.5	0.8 ± 0.2	<0.1	<0.1

calculated as a percentage of the intensity of the molecular ion of unlabeled **1** and **2**. Tables 1 and 2 show the incorporation of the labels into the hydroxyfuranone (**1**) and the methoxyfuranone (**2**), respectively.

Comparison of the stable isotope ratios of **1** and **2** obtained after the administration of [1-^{13}C]-D-fructose and [U-$^{13}C_6$]-D-fructose with those of the control experiment (application of unlabeled D-fructose) clearly demonstrated the incorporation of one ^{13}C atom (*m/z* 129 and 143) and six ^{13}C atoms (*m/z* 134 and 148) into the hydroxyfuranone (**1**) and the methoxyfuranone (**2**). After the application of 14.7 mg of [1-^{13}C]-D-fructose, a value of 39.7 ± 1.4% was obtained for *m/z* 129 (molecular ion of [1- or 6-^{13}C]-**1**). This isotope ratio corresponded well with previously published data (*22*) in which 23.2 ± 3.2% was determined after the application of 6.0 mg of [1-^{13}C]-D-fructose. The isotope ratios of **1** and **2** obtained after the application of [U-$^{13}C_6$]-D-fructose also agreed well with the published results (*22*).

After the administration of [1-^{13}C]-L-ascorbic acid, [1-^{13}C]-1-deoxy-D-fructose, a mixture of [6-^{13}C]-6-deoxy-D-fructose and [6-^{13}C]-6-deoxy-D-glucose, and [4-^{2}H]-D-glucose, the values for *m/z* 129 (hydroxyfuranone, **1**) did not significantly change in comparison with the values obtained in the control experiment with unlabeled D-fructose. Therefore, only unlabeled **1** and **2** were detected. Additionally, [6-^{2}H]-6-deoxy-D-glucose and [6,6,6,5-$^{2}H_4$]-6-deoxyhexulose-1-phosphate were synthesized according to known procedures and tested as precursors of the hydroxyfuranone (**1**) and the methoxyfuranone (**2**). However, labeled **1** and **2** were not detected after the administration of [6-^{2}H]-6-deoxy-D-glucose and [6,6,6,5-$^{2}H_4$]-6-deoxyhexulose-1-phosphate into strawberry fruits (Tables 1 and 2). The isotope ratio of the hydroxyfuranone (**1**) and the methoxyfuranone (**2**) obtained after the application of [1-^{13}C]-deoxy-D-fructose and [6-^{13}C]-6-deoxy-D-fructose/-6-deoxy-D-glucose was also determined by GC-IRMS, but the values were not significantly different from those observed for naturally occurring **1** and **2**.

The single-labeled substrates [1-^{2}H]-D-fructose, [1-^{2}H]-D-glucose, and [2-^{2}H]-D-glucose were converted into single-labeled **1** (*m/z* 129) as well as **2** (*m/z* 143), and the double-labeled substrates [6,6-$^{2}H_2$]-D-glucose and [6,6-$^{2}H_2$]-D-fructose were transformed into double-labeled **1** (*m/z* 130) and **2** (*m/z* 144) by the strawberry fruits (Tables 1 and 2).

Variation of Incubation Period. Equal amounts of [U-$^{13}C_6$]-D-fructose were administered to strawberry fruits of the same ripening stage and similar weight.

Table 3. Effect of Incubation Period and Ripening Stage on the Incorporation of [U-$^{13}C_6$]-D-Fructose into the Hydroxyfuranone (1) and the Methoxyfuranone (2); Comparison of the Intensities of the Molecular Ions of Unlabeled 1 and 2 and of [U-$^{13}C_6$]-1 and [U-$^{13}C_6$]-2

amount applied per strawberry (mg)		molecular ions (%) *m/z* 128 (**1**)	*m/z* 134 (**1**)	*m/z* 142 (**2**)	*m/z* 148 (**2**)
	Incubation Period				
10.2	1 day	100	7.9 ± 0.6	100	3.6 ± 0.1
10.2	2 days	100	11.9 ± 1.8	100	6.1 ± 0.1
10.2	3 days	100	20.1 ± 2.9	100	9.1 ± 0.3
	Ripening Stage				
9.1	white	100	16.9 ± 1.0	100	17.7 ± 0.5
9.1	pink	100	17.1 ± 1.2	100	9.4 ± 0.4
9.1	red	100	8.8 ± 0.9	100	4.0 ± 0.3

The fruits were kept at room temperature in the sunlight. After 1, 2, and 3 days, individual fruits were worked up by homogenization in water and XAD solid phase extraction, and the stable isotope ratio of the hydroxyfuranone (**1**) and the methoxyfuranone (**2**) was determined by GC-MS analysis. The stable isotope ratio of **1** (*m/z* 134) and **2** (*m/z* 148) increased constantly over the 1−3 day incubation period (Table 3). The values obtained for the hydroxyfuranone (**1**) were higher than the ratios determined for the methoxyfuranone (**2**). Longer incubation periods provide furanones with a higher degree of labeling, indicating that the maximum labeling pulse has not yet reached the furanone pool after 3 days.

Application into Strawberries of Different Ripening Stages (White, Pink, and Red). Equal amounts of [U-$^{13}C_6$]-D-fructose were applied to strawberry fruits of three different ripening stages (white, pink, and red). The fruits were kept for 3 days in the sunlight at room temperature. The hydroxyfuranone (**1**) and the methoxyfuranone (**2**) isolated from red strawberry fruits showed the lowest stable isotope ratios (Table 3). Strawberries in the white ripening stage contain lesser amounts of D-glucose and D-fructose when compared with fruits in the red ripening stage. After the application of equal amounts of labeled D-fructose, the isotopic ratio of D-fructose is higher in white strawberries. This difference easily explains the obtained stable isotope ratios of the furanones. It implies that the isotope ratio of the carbohydrate determines the isotope ratio of **1** and **2**.

Application of Increasing Amounts of [1-^{13}C]-D-Fructose. Increasing amounts of [1-^{13}C]-D-fructose (6.1−24.4 mg) were administered to strawberry cv.

Table 4. Effect of the Amount of [1-^{13}C]-D-Fructose Applied on the Incorporation into the Hydroxyfuranone (1) and the Methoxyfuranone (2); Comparison of the Intensities of the Molecular Ions of Unlabeled 1 and 2 and of Labeled 1 and 2

	molecular ions (%)			
amount applied per strawberry (mg)	*m/z* 128 (**1**)	*m/z* 129 (**1**)	*m/z* 142 (**2**)	*m/z* 143 (**2**)
6.1	100	21.0 ± 2.0	100	27.4 ± 2.7
15.4	100	43.0 ± 3.5	100	42.0 ± 4.1
24.4	100	83.3 ± 7.5	100	89.4 ± 8.0

Table 5. Effect of D-Fructose Derivatives (Inhibitors) on the Isotope Ratio of the Hydroxyfuranone (1) and the Methoxyfuranone (2) after the Application of 15 mg of [6,6-$^2H_2$]-D-Fructose into Strawberry Fruits; Comparison of the Intensities of the Molecular Ions of Unlabeled 1 and 2 and of Labeled 1 and 2

	molecular ions (%)			
inhibitor	*m/z* 128 (**1**)	*m/z* 130 (**1**)	*m/z* 142 (**2**)	*m/z* 144 (**2**)
without	100	29.2 ± 5.4	100	20.5 ± 4.0
0.1 mg of D-fructose-2,6-diphosphate	100	19.2 ± 4.3	100	12.6 ± 2.5
11.3 mg of D-fructose-1,6-dichloride	100	5.7 ± 1.1	100	5.4 ± 2.1

Elsanta fruits. The fruits were kept for 3 days in the sunlight at room temperature. The highest isotope ratio for the furanones was obtained after the application of 24.4 mg of [1-^{13}C]-D-fructose (Table 4). Biosynthesis of the furanones is not inhibited by high D-fructose concentrations. The isotope ratio of the carbohydrate pool correlates with the isotope ratio of the furanones. This result was also confirmed by an additional experiment performed with strawberry cv. White D fruits and [U-$^{13}C_6$]-D-fructose (data not shown). High concentrations of D-fructose provided more **1** and **2** (*23*). Thus, carbohydrate-rich strawberry fruits should contain higher levels of the furanones.

Effect of D-Fructose-1,6-dichloride and D-Fructose-2,6-diphosphate. [6,6-$^2H_2$]-D-Fructose and mixtures of [6,6-$^2H_2$]-D-fructose and D-fructose-2,6-diphosphate as well as [6,6-$^2H_2$]-D-fructose and D-fructose-1,6-dichloride were administered to strawberry fruits. The fruits were kept for 3 days in the sunlight at room temperature. Co-injection of D-fructose-2,6-diphosphate (0.1 mg) and D-fructose-1,6-dichloride (11.3 mg) with [6,6-$^2H_2$]-D-fructose resulted in smaller isotope ratios for the hydroxyfuranone (**1**) and the methoxyfuranone (**2**) when compared with the data obtained for [6,6-$^2H_2$]-D-fructose (Table 5).

D-Fructose-2,6-diphosphate is found in the cytosol in minute concentrations, and it exerts a regulatory effect on the cytosolic interconversion of D-fructose-1,6-diphosphate and D-fructose-6-phosphate (*24*). Increased cytosolic D-fructose-2,6-diphosphate is associated with decreased rates of sucrose synthesis because D-fructose-2,6-diphosphate is a powerful inhibitor of cytosolic D-fructose-1,6-diphosphate phosphatase and an activator of the pyrophosphate-dependent phosphofructokinase (*25*). Thus, D-fructose-2,6-diphosphate plays an important role in the cytosolic carbohydrate metabolism by increasing the D-fructose-1,6-diphosphate pool. Higher levels of unlabeled D-fructose-1,6-diphosphate could account for the decrease in the isotope ratio of **1** and **2**. Addition of D-fructose-1,6-dichloride to ripening strawberries seems to inhibit the biosynthesis of **1** and **2** and, thus, provides additional evidence that the hydroxyfuranone (**1**) is the precursor of the methoxyfuranone (**2**) (*9*).

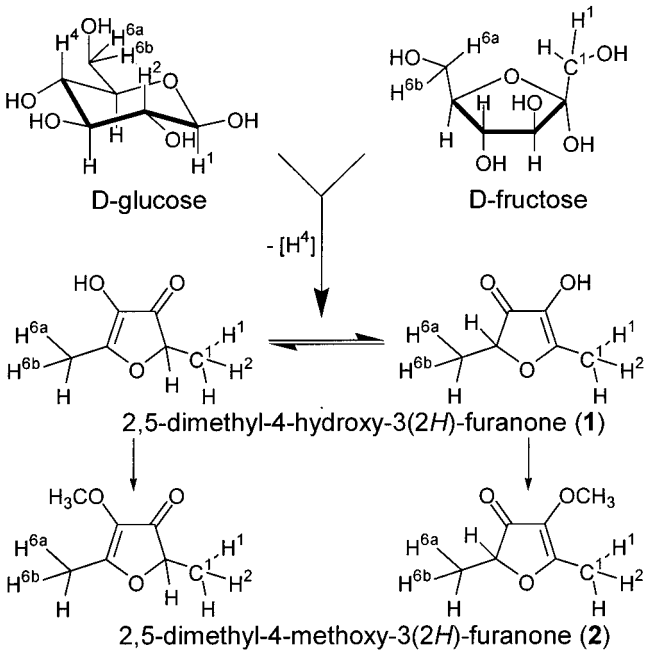

Figure 1. Metabolic fate of isotopes during the biological transformation of carbohydrates into the hydroxyfuranone (**1**) and the methoxyfuranone (**2**).

DISCUSSION

The biogenetic pathways of natural fruit volatiles can be derived from the enzymatically controlled lipid, terpene, amino acid, and phenylpropane metabolism of the plants (*26*). In this respect the hydroxyfuranone (**1**) is an exception as it is enzymatically formed from carbohydrates in strawberry fruits (*13*). We investigated the fate of isotopes during the transformation of carbohydrates into **1** and **2** by using isotopically labeled substrates. On the basis of the obtained mass spectral data and the known fragmentation pattern of the hydroxyfuranone (**1**) and the methoxyfuranone (**2**) (*27*) we were able to follow the fate of the labels into the target molecules (Figure 1). We confirmed that the complete carbon skeleton of D-fructose can be recovered in **1** and **2**. After the application of labeled D-fructose, the hydroxyfuranone (**1**) and the methoxyfuranone (**2**) always showed a higher degree of labeling when compared with the furanones obtained after the administration of labeled D-glucose. As previously shown it appears that D-fructose is a more efficient precursor of DMHF than D-glucose (*13*).

Surprisingly, the label of [2-^{2}H]-D-glucose was recovered in position 1 in the hydroxyfuranone (**1**) and the methoxyfuranone (**2**) (*27*), implying an isotope shift during the formation of the furanones from D-glucose (Figure 1). A hydrogen exchange occurs in the course of the transformation of D-glucose-6-phosphate to D-fructose-6-phosphate mediated by phosphohexose isomerase (Figure 2). The hydrogen at position 2 of D-glucose-6-phosphate is transferred by phosphohexose isomerase to carbon 1 of D-fructose-6-phosphate at the pro-*R* position. Tritium and deuterium labeling experiments confirmed that the transfer from C-2 of D-glucose-6-phosphate to C-1 of D-fructose-6-phosphate is only partly intramolecular in the phosphohexose isomerase reaction

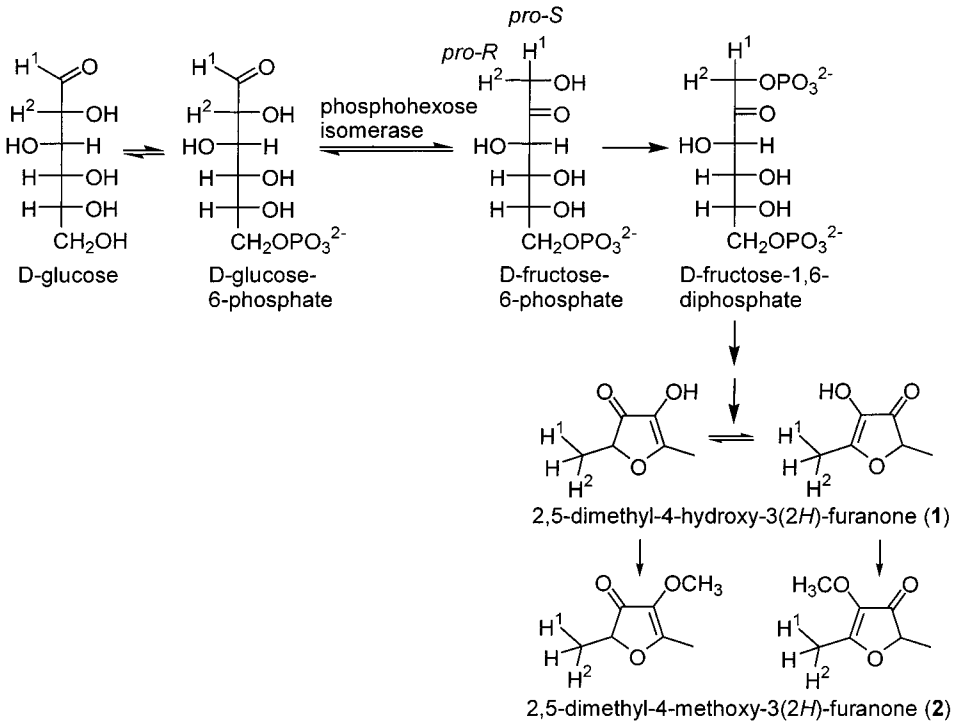

Figure 2. Metabolic fate of hydrogens in the course of phosphohexose isomerase catalysis.

(*28*, *29*). Our results imply that phosphohexose isomerase is involved in the transformation of D-glucose to the furanones, thus confirming previous data in which D-fructose-6-phosphate has been proposed as the natural precursor of **1** and **2** (*13*, *23*). Comparison of the labeling pattern of both furanones showed that in most of the cases the hydroxyfuranone (**1**) exhibited a higher degree of labeling than the methoxyfuranone (**2**) for the same labeled substrate applied. This observation shows that **1** is the direct precursor of **2** as previously supposed (*9*).

Deoxysugars such as L-rhamnose, L-fucose, and 6-deoxy-D-fructose have been proposed as the natural precursor of the hydroxyfuranone (**1**) (*10*, *11*). In microorganisms and plants 6-deoxysugars are synthesized from CDP-D-glucose and UDP-D-glucose, respectively (*30*). Various experiments have shown that the catalysis consists of three discrete steps: oxidation of CDP-D-glucose to the corresponding 4-ketohexose; C5/C6 dehydration to a 4-keto-$\Delta^{5,6}$-glucoseen intermediate; and, reduction at C-6 to give the 4-keto-6-deoxyhexose product. The overall transformation is in fact an intramolecular oxidation−reduction, by which an enzyme-bound NAD^+ receives the 4-H as a hydride in the oxidative half-reaction and passes the reducing equivalents to C-6 of the dehydration product in the reductive half-reaction. An intramolecular hydride shift from C-4 to C-6 can be observed. However, we did not recover any deuterium label in **1** and **2** after the application of [4-^{2}H]-D-glucose. The deuterium was lost during the transformation, implying that L-rhamnose and L-fucose are not natural precursors of the hydroxyfuranone (**1**) as previously shown (*13*). We did not detect any labeled **1** and **2** after the administration of labeled 1-deoxy-D-fructose and 6-deoxy-D-fructose/-6-deoxy-D-glucose. Although one might argue that deoxysugars are not taken up by the strawberry cells, we believe they can be excluded as natural precursors of the furanones.

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LITERATURE CITED

(1) Schwab, W.; Roscher, R. 4-Hydroxy-3(2*H*)-furanones: Natural and Maillard products. *Recent Res. Dev. Phytochem*. **1997**, *1*, 643−673.

(2) Rodin, J. O.; Himel, C. M.; Silverstein, R. M.; Leeper, R. W.; Gortner, W. A. Volatile flavour and aroma components of pineapple. I. Isolation and tentative identification of 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone. *J. Food Sci*. **1965**, *30*, 280−285.

(3) Re, L.; Maurer, G.; Ohloff, G. A simple synthesis of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone (Furaneol) an important aroma component of pineapple and strawberry. *Helv. Chim. Acta* **1973**, *56*, 1882−1894.

(4) Honkanen, E.; Pyysalo, T.; Hirvi, T. The aroma of Finnish wild raspberries. *Z. Lebensm. Unters. Forsch*. **1980**, *171*, 180−182.

(5) Buttery, R. G.; Takeoka, G. R.; Ling, L. C. Furaneol: odor threshold and importance to tomato aroma. *J. Agric. Food Chem*. **1995**, *43*, 1638−1640.

(6) Mayerl, F.; Näf, R.; Thomas, A. F. 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone glucoside: isolation from strawberries and synthesis. *Phytochemistry* **1989**, *28*, 631−633.

(7) Krammer, G. E.; Takeoka, G. R.; Buttery, R. G. Isolation and identification of 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone glucoside from tomatoes. *J. Agric. Food Chem*. **1994**, *42*, 1595−1597.

(8) Roscher, R.; Herderich, M.; Steffen, J. P.; Schreier, P.; Schwab, W. Identification of 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone 6′-*O*-malonyl-*β*-D-glucopyranoside in strawberry fruit (*Fragaria x ananassa*, cv. Senga sengana). *Phytochemistry* **1996**, *43*, 155−160.

(9) Roscher, R.; Schreier, P.; Schwab, W. Metabolism of 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone in detached ripening strawberry fruits. *J. Agric. Food Chem*. **1997**, *45*, 3202−3205.

(10) Pisarnitskii, A. F.; Demechenko, A. G.; Egorov, I. A.; Gvelesiani, R. A. Methylpentoses are probable precursors of furanones in fruits. *Appl. Biochem. Microbiol.* **1992**, *28*, 97−100.
(11) Zabetakis, I.; Holden, M. A. The effect of 6-deoxy-D-fructose on flavour bioformation from strawberry (*Fragaria* x *ananassa*, cv. Elsanta) callus culture. *Plant Cell Tissue Org. Cult.* **1996**, *45*, 25−29.
(12) Hecquet, L.; Sancelme, M.; Bolte, J.; Demuynck, C. Biosynthesis of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone by *Zygosaccharomyces rouxii*. *J. Agric. Food Chem.* **1996**, *44*, 1357−1360.
(13) Roscher, R.; Bringmann, G.; Schreier, P.; Schwab, W. Radiotracer studies on the formation of 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone in detached ripening strawberry fruits. *J. Agric. Food Chem.* **1998**, *46*, 1488−1493.
(14) Durrwachter, J. R.; Drueckhammer, D. G.; Nozaki, K.; Sweers, H. M.; Wong, C.-H. Enzymatic aldol condensation/isomerization as a route to unusual sugar derivatives. *J. Am. Chem. Soc.* **1986**, *108*, 7812−7818.
(15) Serianni, A. S.; Clark, E. L.; Barker, R. Carbon-13-enriched carbohydrates. Preparation of erythrose, threose, glyceraldehyde, and glycolaldehyde with ^{13}C-enrichment in various carbon atoms. *Carbohydr. Res.* **1979**, *72*, 79−91.
(16) Serianni, A. S.; Nunez, H. A.; Hayes, M. L.; Barker, R. Chemical synthesis of monosaccharides enriched with carbon isotopes. *Methods Enzymol.* **1982**, *89*, 64−73.
(17) Hecquet, L.; Bolte, J.; Demuynck, C. Chemoenzymatic synthesis of 6-deoxy-D-fructose and 6-deoxy-L-sorbose using transketolase. *Tetrahedron* **1994**, *50*, 8677−8684.
(18) Cramer, F.; Otterbach, H.; Springmann, H. A synthesis of 6-desoxy-6-amino-glucose. *Chem. Ber.* **1959**, *92*, 384−391.
(19) Zinner, H.; Brandner, H.; Rembarz, G. Derivate der Zucker-mercaptale, II. Mitteil.: Acyl- und Tritylverbindungen der D-Lyxose- und der D-Arabinose-mercaptale. *Chem. Ber.* **1956**, *89*, 800−813.
(20) Wolfrom, M. L.; Crum, J. D.; Miller, J. B.; Weisblat, D. I. The action of diazomethane on the tetraacetates of aldehydo-D(and L)-arabinose. *J. Am. Chem. Soc.* **1959**, *81*, 243−244.
(21) Angyal, S. J.; Bethell, G. B.; Cowley, D. E.; Pickles, V. A. Equilibria between pyranoses and furanoses. IV. 1-deoxyhexuloses and 3-hexuloses. *Aust. J. Chem.* **1976**, *29*, 1239−1247.
(22) Schwab, W. Application of stable isotope ratio analysis explaining the biotransformation of 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone in plants by a biological Maillard reaction. *J. Agric. Food Chem.* **1998**, *46*, 2266−2269.
(23) Pérez, A. G.; Olías, R.; Olías, J.; Sanz, C. Biosynthesis of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone and derivatives in in vitro grown strawberries. *J. Agric. Food Chem.* **1999**, *47*, 655−658.
(24) Huber, S. C. Fructose-2,6-diphosphate as a regulatory metabolite in plants. *Annu. Rev. Plant Physiol.* **1986**, *37*, 233−246.
(25) Buchanan, B. B. Photosynthesis: Carbon Reactions In *Plant Physiology*; Taiz, L., Zeiger, E., Eds.; Sinauer Associates: Sunderland, MA, 1998; pp 195−225.
(26) Schreier, P. *Chromatographic Studies of Biogenesis of Plant Volatiles*; Hüthig Verlag: Heidelberg, Germany, 1984.
(27) Fay, L. B.; Huynh-Ba, T.; Blank, I. Study of the fragmentation of 3[2*H*]-furanones by mass spectrometry. *J. Agric. Food Chem.* **1997**, *45*, 4057−4064.
(28) Willem, R.; Malaisse-Lagae, F.; Ottinger, R.; Malaisse, W. J. Phosphoglucoisomerase-catalysed interconversion of hexose phosphates. Kinetic study by ^{13}C NMR of the phosphoglucoisomerase reaction in $^{2}H_2O$. *Biochem. J.* **1990**, *265*, 519−524.
(29) Yunianta, B. L. Z.; Martin, M. L. Site-specific isotope fractionation in the characterization of biochemical mechanisms. *J. Biol. Chem.* **1995**, *270*, 16023−16029.
(30) Hallis, T., M.; Liu, H.-W. Learning Nature's strategies for making deoxy sugars: pathways, mechanisms, and combinatorial applications. *Acc. Chem. Res.* **1999**, *32*, 579−588.

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