Radiotracer Studies on the Formation of 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone in Detached Ripening Strawberry Fruits

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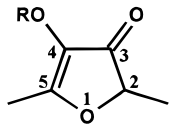
The transformation of 12 radioactively labeled compounds into 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF), glycosidically bound DMHF, and 2,5-dimethyl-4-methoxy-3(2*H*)-furanone (DMMF) was investigated in detached ripening strawberry fruits (*Fragaria* × *ananassa*) over a 3-day period. Radiochemical analysis of the different fruit parts revealed that major portions of the applied radioactivity (up to 66%) remained in the stems and calyx. Incorporation levels of [2-^{14}C]-dihydroxyacetone, D-[1-^{3}H]glucose, D-[U-^{14}C]-glucose, D-[U-^{14}C]glucose 6-phosphate, D-[U-^{14}C]fructose, and D-[U-^{14}C]fructose 1,6-bisphosphate into the total amount of furanone derivatives were 0.022, 0.032, 0.035, 0.147, 0.202, and 0.289% of the radioactivity entering the fruits, respectively. Minor amounts of radioactivity (<0.001%) were detected in the furanone structures after the administration of [1-^{14}C]acetate and [3-^{14}C]pyruvate. L-[1-^{14}C]Fucose, L-[6-^{3}H]fucose, L-[1-^{3}H]rhamnose, L-[U-^{14}C]-threonine, L-[U-^{14}C]lactaldehyde, and [2-^{14}C]malonic acid were not transformed into DMHF or a derivative thereof.

Keywords: *Biosynthesis; carbohydrate metabolism; 2,5-dimethyl-4-hydroxy-3(2H)-furanone; Fragaria* × *ananassa; Rosaceae*

INTRODUCTION

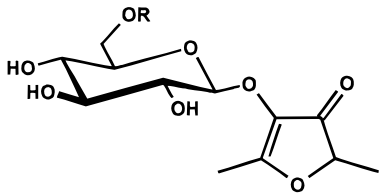
The identification of the volatile flavor constituents in cultivated strawberry fruits (*Fragaria* × *ananassa*) has been the subject of numerous investigations (McFadden et al., 1965; Schreier, 1980; Perez et al., 1992). Approximately 350 compounds have been detected (Latrasse, 1991), including several key components. Two of these important strawberry flavor compounds are 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF) and its methyl ether, 2,5-dimethyl-4-methoxy-3(2*H*)-furanone (DMMF). DMHF, which has been described as possessing a caramel-like, sweet, fruity, and burnt pineapple-like flavor (Pittet et al., 1970; Rodin et al., 1965), exhibits a flavor typical of fresh strawberry in diluted solutions. In addition to strawberry (Re et al., 1973), DMHF has also been found in pineapple (Rodin et al., 1965), raspberry (Honkanen et al., 1980), and tomato (Buttery et al., 1995). Recently, the β-D-glucopyranoside of DMHF (Mayerl et al., 1989; Wu et al., 1990; Krammer et al., 1994) and the malonylated derivative thereof (Roscher et al., 1996) have been identified in fruits, whereas DMHF β-D-glucuronide has been detected as the major metabolite of DMHF, DMMF, and DMHF β-D-glucopyranoside in urine of man (Roscher et al., 1997a).

Although the biosynthesis of DMHF has been studied in strawberry callus culture (Zabetakis and Holden, 1996) and the yeast *Zygosaccharomyces rouxii* (Hecquet et al., 1996), the detailed formation pathway of it in strawberry fruits is still unknown. The biogenetic pathways of natural fruit volatiles can generally be derived from the enzymatically controlled lipid, terpene, amino acid, carbohydrate, and phenylpropane metabolism of the plant. On the basis of metabolic studies in which unlabeled 6-deoxy-D-fructose was fed to straw-



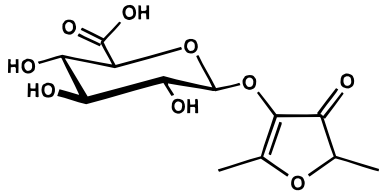
R = H **DMHF**

R = CH_3 **DMMF**



R = H **DMHF ß-D-glucopyranoside**

R = $COCH_2CO_2H$ **DMHF 6-O-malonyl ß-D-glucopyranoside**



DMHF ß-D-glucuronide

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S0021-8561(97)00659-6 CCC: $15.00
Published on Web 03/12/1998

Table 1. Distribution of Recovered Radioactivity (Expressed as Percentage of Total Uptake) in Different Fractions after the Application of Radioactively Labeled Compounds to Detached Ripening Strawberry Fruits

	total uptake (kBq)	extract	flow through	diethyl ether	methanol	solid residue	stem and calyx	CO_2	recovery
[1-^{14}C]acetate	456	19.7	16.62	0.40	1.16	5.5	30.1	17.8	73.1
[3-^{14}C]pyruvate	368	44.5	38.41	0.88	2.34	8.4	26.3	9.0	88.2
[2-^{14}C]dihydroxyacetone	410	12.9	10.93	0.29	1.31	6.6	14.3	48.6	82.4
[2-^{14}C]dihydroxyacetone[a]	308	4.8	4.11	0.07	0.59	2.7	42.5	36.7	86.7
[2-^{14}C]dihydroxyacetone[b]	412	7.7	6.63	0.07	0.67	1.5	44.4	34.0	87.6
L-[1-^{14}C]fucose	340	43.2	40.30	nd[c]	4.70	30.0	33.9	nd	107.1
L-[6-^{3}H]fucose	1432	33.3	31.40	nd	0.60	16.0	43.0	nd	92.3
L-[1-^{3}H]rhamnose	1375	71.1	66.61	0.14	0.15	0.5	4.6	nd	76.2
L-[U-^{14}C]threonine	163	18.2	16.30	0.30	0.89	17.0	54.9	nd	90.1
D-[U-^{14}C]lactaldehyde	110	60.0	56.30	0.71	2.54	3.7	11.5	ndt[d]	75.2
[2-^{14}C]malonic acid	418	58.7	43.04	1.19	8.30	14.1	18.6	10.2	101.6

[a] Incubation after addition of inhibitors. [b] Incubation after addition of citric acid. [c] Not detected. [d] Not determined.

Table 2. Distribution of Recovered Radioactivity (Expressed as Percentage of Total Uptake) in Different Fractions after the Application of Radioactively Labeled Carbohydrate Derivatives to Detached Ripening Strawberry Fruits

	total uptake (kBq)	extract	flow through	diethyl ether	methanol	solid residue	stem and calyx	CO_2	recovery
D-[1-^{3}H]glucose	1415	39.3	37.39	0.13	0.33	0.8	9.0	nd[a]	49.1
D-[U-^{14}C]glucose	331	12.8	11.49	0.08	0.92	2.2	25.0	44.0	84.0
D-[U-^{14}C]glucose[b]	140	14.5	12.8	0.14	1.31	1.5	23.0	44.8	83.8
D-[U-^{14}C]glucose 6-phosphate	258	16.5	14.91	0.14	1.07	3.7	64.4	11.3	95.9
D-[U-^{14}C]glucose 6-phosphate[b]	133	39.1	33.47	0.59	4.13	3.3	66.0	nd	108.4
D-[U-^{14}C]fructose	291	15.1	120.3	0.49	1.79	2.8	50.6	23.1	91.6
D-[U-^{14}C]fructose[b]	135	22.1	18.00	0.67	2.52	5.2	78.3	nd	105.6
D-[U-^{14}C]fructose 1,6-bisphosphate	330	20.2	16.05	1.38	2.26	3.8	15.2	55.0	94.2
D-[U-^{14}C]fructose 1,6-bisphosphate[c]	62	73.3	66.00	0.48	3.54	8.5	nd	16.5	98.3

[a] Not detected. [b] Incubation after addition of inhibitors. [c] Application after removing stem and calyx.

berry callus culture, Zabetakis and Holden (1996) concluded that methylpentoses are precursor compounds for the biosynthesis of DMHF. The studies of Hecquet et al. (1996), however, showed that D-fructose 1,6-bisphosphate was effectively transformed into DMHF by yeast. D-Fructose 1,6-bisphosphate was also established as the most important precursor of DMHF formation during thermal treatment of yeast (Schieberle, 1992). Recently, the metabolism of DMHF has 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]DMHF into DMMF (Roscher et al., 1997b). This paper describes for the first time an investigation of DMHF biosynthesis in detached ripening strawberry fruits in which metabolites originating from different radioactively labeled precursors were analyzed by two-dimensional HPLC radiocounting. Radiolabeled precursors were chosen as most of them are commercially available and the calculation of incorporation is readily achievable. The aim of the present paper was to elucidate the carbon pool (carbohydrate, amino acid, fatty acid) used for the generation of furanone derivatives in strawberry fruits and to probe the recently published contradictory theories of DMHF formation. The data obtained enabled us to propose a preliminary biosynthetic scheme for the DMHF formation.

MATERIALS AND METHODS

Plant Material. Whole strawberry plants (*Fragaria* × *ananassa* cv. Elsanta) were kindly provided by CPRO-DLO, Wageningen, The Netherlands (Roscher et al., 1997b).

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-^{14}C]-Acetate sodium salt (2.22 MBq/μmol) was purchased from Amersham International; [2-^{14}C]dihydroxyacetone (2.03 MBq/μmol), D-[U-^{14}C]fructose (11.10 MBq/μmol), L-[1-^{14}C]fucose (2.03 MBq/μmol), L-[6-^{3}H]fucose (2.22 MBq/μmol), D-[U-^{14}C]glucose (11.47 MBq/μmol), D-[1-^{3}H(N)]glucose (740.00 MBq/μmol), D-[U-^{14}C]glucose 6-phosphate (11.10 MBq/μmol), [3-^{14}C]pyruvate sodium salt (0.42 MBq/μmol), and L-[1-^{3}H(G)]rhamnose (185.00 MBq/μmol) were obtained from Biotrend; D-[U-^{14}C]fructose 1,6-bisphosphate (5.68 MBq/μmol), [2-^{14}C]malonic acid (0.09 MBq/μmol), and L-[U-^{14}C]threonine (0.67 MBq/μmol) were purchased from Sigma.

Application of Radioactively Labeled Compounds. Application of the respective radioactively labeled compound (Tables 1 and 2) was conducted as described previously (Roscher et al., 1997b). In parallel experiments, either 8.4 mg of citric acid or 8.4 mg of citric acid, 5.5 mg of ATP disodium salt, and 7.6 mg of NADH disodium salt trihydrate were added to the solution containing the labeled precursor to inhibit glycolysis and the tricarboxylic acid cycle. Approximately 1 mL of solution was imbibed into 10 g of berries in 24 h under these conditions. Although the vials were refilled after 24 h, further uptake of water by the berries was not observed during the following respiration period.

Recovery and Extraction of Radioactivity. Recovery and extraction of radioactively labeled material was performed as reported (Roscher et al., 1997b).

Enzymatic Hydrolysis. Liberation of glycosidically bound compounds was conducted as described previously (Roscher et al., 1997b).

HPLC Analysis of Metabolites. The transformation of the applied compounds was monitored using high-performance liquid chromatography (HPLC) as reported (Roscher et al. 1997b).

Liquid Scintillation Counting (LSC). All measurements were carried out by means of the liquid scintillation counter LKB Rackbeta 1214 after decay of the chemiluminescence counts (Roscher et al., 1997b).

Synthesis of D-[U-^{14}C]Lactaldehyde. L-[U-^{14}C]Threonine (908 kBq, 0.161 mg) and ninhydrin (0.45 mg) were dissolved in 1 mL of P_i buffer (pH 5.3) and were refluxed for 1 h. After cooling, the solution was subjected to solid-phase extraction on an RP-18 cartridge (Supelco, 200 mg). The flow through and water rinse were combined, carefully concentrated in vacuo, and purified by TLC silica gel chromatography using

diethyl ether/methanol (20:1, v/v). Unlabeled D-lactaldehyde was used as reference compound and became visible by spraying with 2,4-dinitrophenylhydrazine. The labeled lactaldehyde was scratched off and eluted by diethyl ether. After addition of 1 mL of water, the organic solvent was evaporated in a weak stream of nitrogen. The aqueous solution containing D-[U-^{14}C]lactaldehyde (110 kBq) was used for the incubation experiment.

Calculation of Incorporation. Incorporation for DMHF, glycosidically bound DMHF, and DMMF were calculated as follows: radioactivity of the target molecule divided by the radioactivity entering the strawberry fruit (corresponding to the sum of the extract, solid residue, and CO_2) multiplied by 100. The incorporation of glycosidically bound DMHF was determined after enzymatic hydrolysis of aliquots of the methanol extract.

RESULTS

Deoxysugars. L-[1-^{3}H]Rhamnose, L-[6-^{3}H]fucose, and L-[1-^{14}C]fucose were applied to detached ripening strawberries as the methylpentoses L-rhamnose and L-fucose have been reported as precursors of DMHF in strawberry fruits (Pisarnitskii et al., 1992). This early work, however, was restricted to a chemical approach to DMHF. They observed a correlation of the amouts of L-rhamnose and L-fucose with the amount of DMHF. Our experimental design corresponded to that described by Loewus et al. (1956) for the biosynthesis of ascorbic acid in detached ripening strawberry fruits. Table 1 shows the distribution of the recovered radioactivity after the application of the deoxysugars. Except for L-[1-^{3}H]rhamnose, recovery of the radioactivity was >92%. Tritium exchange at C1 with water due to an aldose−ketose equilibrium in the course of metabolism might account for the loss of radioactivity (evaporation of [H^3]OH) in the case of L-[1-^{3}H]rhamnose. Analysis of the diethyl ether fraction obtained by solid-phase extraction revealed for all applied deoxysugars the presence of unlabeled DMHF and DMMF. Labeled DMHF and DMMF were not detected. Only unlabeled DMHF was liberated by treatment of the methanol extracts with Rohapect D5L, a pectinolytic enzyme preparation exhibiting β-glycosidase and esterase side activity.

Miscellaneous Compounds. D-Lactaldehyde has been suggested as a precursor of 6-deoxyfructose, which was finally transformed to DMHF in strawberry callus cultures (Zabetakis and Holden, 1996). We synthesized D-[U-^{14}C]lactaldehyde from L-[U-^{14}C]threonine and used both labeled compounds in precursor studies. Malonic acid, a universal biosynthetic building block, was also applied to a detached ripening strawberry. The total recovery of ^{14}C was >90% except for D-[U-^{14}C]lactaldehyde as the amount of $^{14}CO_2$ was not determined (Table 1). Analysis of the diethyl ether and methanol extracts showed that the strawberry fruits contained exclusively unlabeled furanone derivatives.

Carbohydrate Degradation Products. Table 1 presents the distribution of the recovered ^{14}C in the different fractions after the application of [1-^{14}C]acetate, [3-^{14}C]pyruvate, and [2-^{14}C]dihydroxyacetone to detached ripening strawberry fruits. Minor amounts of radioactivity (<0.001%) were detected in the furanone structures after the administration of [1-^{14}C]acetate and [3-^{14}C]pyruvate. [2-^{14}C]Dihydroxyacetone, however, was transformed to DMHF, glycosidically bound DMHF, and DMMF with incorporation of 0.004, 0.010, and 0.006%, respectively (Figure 3). It is generally assumed that DMHF and its derivatives are exclusively synthesized

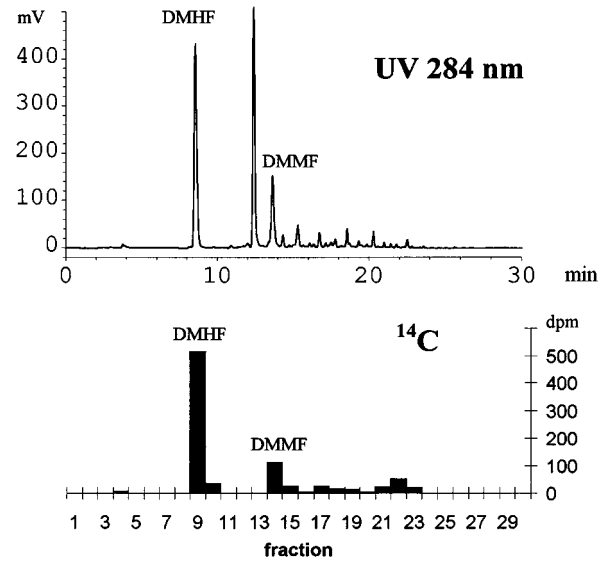

Figure 1. HPLC-RP18 separation of the compounds obtained by solid-phase extraction followed by diethyl ether elution of strawberry fruits after the administration of D-[U-^{14}C]fructose 1,6-bisphosphate.

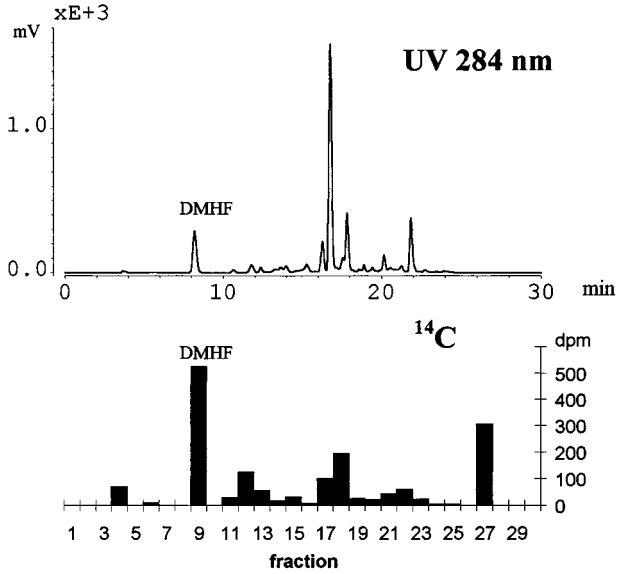

Figure 2. HPLC-RP18 separation of the compounds liberated by glycosidase treatment of the methanol extract obtained by solid-phase extraction of strawberry fruits after the administration of D-[U-^{14}C]fructose 1,6-bisphosphate.

in the strawberry fruit during ripening as the green strawberry plant parts do not contain any DMHF derivative (Perez et al., 1996). Only the radioactively labeled compounds entering the fruit may serve as precursors. Consequently, we calculated the incorporation based on the sum of the radioactivity of the extract, the solid residues, and the CO_2, corresponding to the radioactivity entering the fruit. In a parallel experiment citric acid was added to the solution containing [2-^{14}C]dihydroxyacetone, and in a second parallel experiment citric acid, NADH, and ATP were introduced into the nutrient solution to inhibit glycolysis and the tricarboxylic acid cycle. The incorporation of [2-^{14}C]-dihydroxyacetone into the furanone derivative was doubled by the addition of citric acid (Figure 3).

Carbohydrates. Table 2 shows the results of the incubation experiments with labeled carbohydrates. The recovery of the applied radioactivity was always >83%

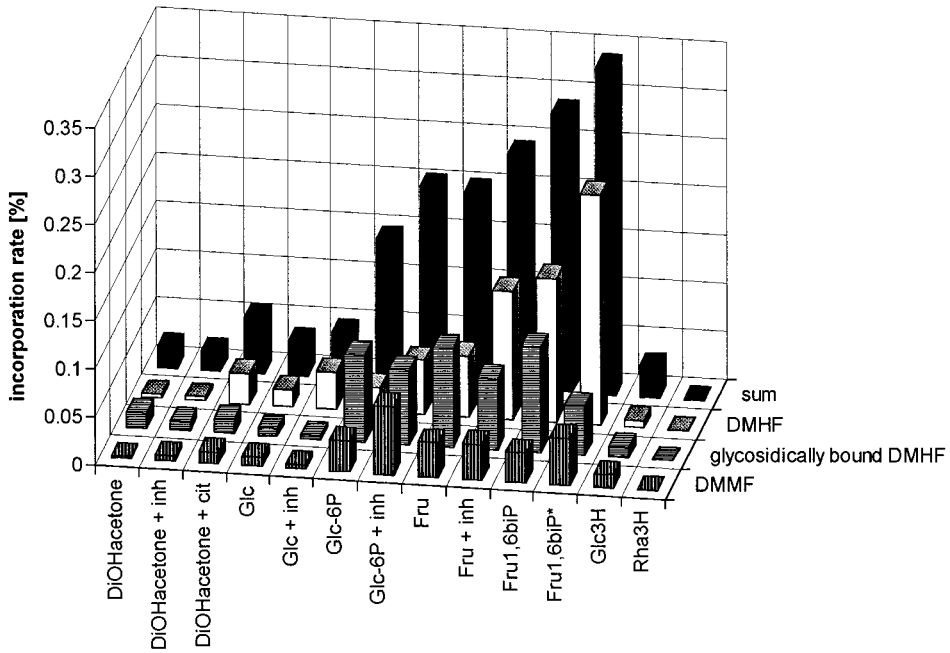

Figure 3. Incorporation of applied substrates into DMHF, glycosidically bound DMHF, DMMF, and the total amount of furanones. Detached ripening strawberries (5−15 g) were placed into 2-mL vials containing the respective radioactively labeled compounds (133−1415 kBq) dissolved in 1 mL of tap water. In parallel experiments either 8.4 mg of citric acid (+ cit) or a combination of 8.4 mg of citric acid, 5.5 mg of ATP disodium salt, and 7.6 mg of NADH disodium salt trihydrate (+ inh) was added to the solution containing the labeled precursor. Incorporation of radioactivity is expressed as a percentage of total radioactivity taken up by the fruits. Glycosidically bound DMHF was quantified after the hydrolysis of the glycosidic extract. DiOHacetone, [2-^{14}C]-dihydroxyacetone; Glc, D-[U-^{14}C]glucose; Glc-6P, D-[U-^{14}C]glucose 6-phosphate; Fru, D-[U-^{14}C]fructose; Fru-1,6biP, D-[U-^{14}C]fructose 1,6-bisphosphate; Fru-1,6biP*, D-[U-^{14}C]fructose 1,6-bisphosphate (application of substrate after removal of the stem and calyx).

except for [1-^{3}H]glucose, indicating a tritium exchange with water (evaporation of [H^{3}]OH) due to an aldose−ketose equilibrium in the course of metabolism. Substantial amounts of the applied ^{14}C-labeled carbohydrates were converted into $^{14}CO_2$. The label of the applied carbohydrates was also recovered in the furanone derivatives (Figure 3). A gradual increase of the incorporation was observed from D-[U-^{14}C]glucose to D-[U-^{14}C]glucose 6-phosphate to D-[U-^{14}C]fructose and to D-[U-^{14}C]fructose 1,6-bisphosphate. The addition of the glycolysis and tricarboxylic acid cycle inhibitors, that is, citric acid, NADH, and ATP, increased all incorporations by 30%. In a parallel experiment, the stem and calyx of a strawberry were removed and a small volume of a solution containing D-[U-^{14}C]fructose 1,6-bisphosphate was put into the formed depression. This experiment yielded the highest incorporation for the furanones (Figure 3).

DISCUSSION

The incorporation of a labeled precursor into the target molecule is governed by more factors than proximity of the precursor to the end product in the pathway. The rates are also influenced by duration of feeding, precursor uptake, solubility, lability, and other factors. In the present experiments all precursors were highly water soluble and chemically stable and were taken up by the strawberries within 24 h. As different amounts of radioactivity remained in the stems of the strawberries, the percentages of incorporation into the target molecules were based on the amount of radioactivity taken up by the individual strawberry fruit to eliminate the variability of incorporation in the stem. The comparison of the total incorporation into the furanones in the experiments with and without inhibitors (Figure 3), for example, dihydroxyacetone, shows a quite reasonable reproducibility for the individual precursors. Although the data presented in Tables 1 and 2 were each obtained from a single experiment, they also display reproducible results by comparing the distribution pattern for the experiments with and without inhibitors (for example, [2-^{14}C]dihydroxyacetone, D-[U-^{14}C]glucose, D-[U-^{14}C]glucose 6-phosphate, and D-[U-^{14}C]fructose). Therefore, we conclude that the experimental variability is quite small.

The two most commonly used measures for the quantification of the precursor−product relationship are incorporation and dilution (Floss, 1977). It was impossible to calculate the correct molar specific activity of the precursors for the measurement of the dilution due to the natural pool of the precursors already existing in the strawberry fruits. Therefore, we chose the incorporation for the measurement of the proximity between precursor and product. Additionally, in contrast to the incorporation, the dilution values for DMHF, glycosidically bound DMHF, and DMMF cannot be easily added up to compare the total amount of incorporation.

After the application of [1-^{3}H]rhamnose, no transformation of the label into the DMHF derivatives was observed. In contrast, D-[1-^{3}H]glucose and D-[U-^{14}C]-glucose showed nearly identical incorporations of 0.032 and 0.035%, respectively, indicating the reproducibility of the experiments. Although a minor tritium exchange occurred for D-[1-^{3}H]glucose and L-[1-^{3}H]rhamnose—as indicated by the recovery values—L-rhamnose and the second deoxysugar, L-fucose, do not act as precursors for the biosynthesis of DMHF in strawberries as proposed by Pisarnitskii et al. (1992).

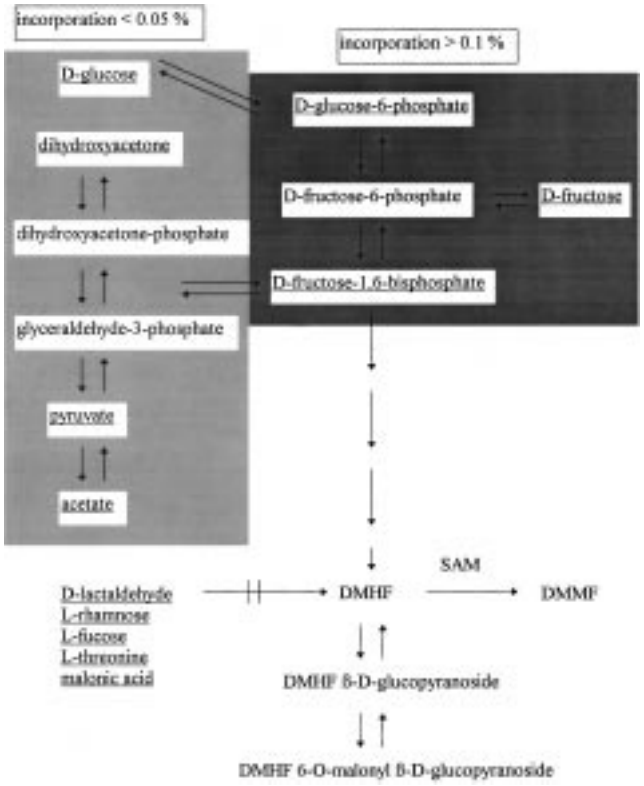

Figure 4. Proposed biosynthesis of DMHF derivatives in strawberry. Underlined compounds were applied as radioactively labeled precursors to detached ripening strawberries. The incorporation into DMHF, glycosidically bound DMHF, and DMMF was analyzed by HPLC radiocounting.

The same conclusion applies for D-lactaldehyde as the label of D-[U-^{14}C]lactaldehyde was not recovered in the furanone derivatives under the experimental conditions of our study. Thus, 6-deoxyfructose, the DMHF precursor suggested by Zabetakis and Holden (1996), is not synthesized by an aldolase reaction from D-lactaldehyde and dihydroxyacetone phosphate.

The C_6 carbohydrates D-glucose, D-glucose 6-phosphate, D-fructose, and especially D-fructose 1,6-bisphosphate showed the highest incorporation into the DMHF derivatives. Additionally, the inhibition of glycolysis and the tricarboxylic acid cycle led generally to an increase of the individual incorporation for the applied radioactively labeled carbohydrates. Carbohydrate and fatty acid degradation products such as acetate, pyruvate, and dihydroxyacetone are also converted to the furanones. As the berries do not photosynthesize, acetate, pyruvate, and dihydroxyacetone are apparently used by the plant, at least to a small extent, for the biosynthesis of carbohydrates serving subsequently as precursors for the DMHF biosynthesis.

It is interesting to note that in all cases phosphorylated carbohydrates such as glucose 6-phosphate and fructose 1,6-bisphosphate show higher incorporation into the furanones than their nonphosphorylated homologues glucose and fructose, respectively. It is generally accepted that phosphate esters do not readily cross the cell membranes but are hydrolyzed due to phosphatase activity. However, Table 2 and Figure 3 exhibit different metabolic profiles for the phosphorylated and nonphosphorylated carbohydrates which cannot simply be explained by experimental and biological variability.

Our data confirm the results of Hecquet et al. (1996), who showed by using unlabeled substrates that the yeast *Z. rouxii* produced DMHF from D-fructose 1,6-bisphosphate. They also obtained higher amounts of DMHF by using phosphorylated carbohydrates instead of their nonphosphorylated homologues. We obtained comparable incorporation in both experiments in which D-fructose 1,6-bisphosphate was added as precursor to strawberry fruit with and without stem. Therefore, metabolism of fructose 1,6-bisphosphate to an unknown precursor in the stem, which is subsequently transformed to DMHF, was excluded. Figure 4 summarizes the results obtained by the present study and our recent investigation of the metabolism of DMHF in strawberries (Roscher et al., 1997b). As only small amounts of labeled acetate and pyruvate were incorporated into the furanone structures, a degradation of the C_6 carbohydrates prior to the formation of the furanone system seems very unlikely.

Attempts were made to locate the radiolabel in the furanones. However, due to the already known instability of DMHF (Rodin et al., 1965; Kunert-Kirchhoff and Baltes, 1990; Chen et al., 1996), a standardized degradation was not achieved without appreciable effort. Furthermore, the keto−enol tautomerization of DMHF leads to a symmetric molecule so that it is impossible to distinguish carbons 2-CH_3/5-CH_3, 2/5, and 3/4. The position of the label and the role of 6-deoxyfructose as proposed precursor of DMHF will be elucidated by the use of ^{13}C-labeled precursors in a future study.

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Received for review July 31, 1997. Revised manuscript received January 20, 1998. Accepted January 23, 1998. Financial support provided by QUEST, Naarden, The Netherlands, is gratefully acknowledged.

JF970659X