Metabolism of 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone in Detached Ripening Strawberry Fruits

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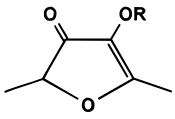
The transformation of *S*-[*methyl*-^{14}C]adenosyl-L-methionine (^{14}C-SAM) and [2-(or 5-)*methyl*-^{14}C]-2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (^{14}C-**1**) was investigated in detached ripening strawberry fruits over a three-day period. Radiochemical analysis of the different fruit parts revealed that 46% and 51% of the applied ^{14}C remained in the stems for ^{14}C-SAM and ^{14}C-**1**, respectively. In samples obtained by solid phase extraction, **1**, 2,5-dimethyl-4-methoxy-3(2*H*)-furanone (**2**), and glycosidically bound **1** were analyzed by reversed-phase high-performance liquid chromatography. Incorporation of ^{14}C-SAM and ^{14}C-**1** into **2** was observed with transformation rates of 0.4% and 0.3% of the applied ^{14}C, respectively. In addition, 0.7% of the applied ^{14}C-**1** was recovered as glycosidically bound **1**. The data support the conclusions that ^{14}C-SAM is the source of the methyl group in the 4-methoxy compound **2** and that **1** is the precursor of **2** and the glycosides studied.

Keywords: *2,5-Dimethyl-4-hydroxy-3(2H)-furanone; 2,5-dimethyl-4-methoxy-3(2H)-furanone; Furaneol; methoxyfuraneol; S-adenosyl-L-methionine; metabolism; strawberry*

INTRODUCTION

2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone (Furaneol) **1**,



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

an important aroma component of a number of fruits including pineapple (Rodin et al., 1965), strawberry (Re et al., 1973), raspberry (Honkanen, 1980), and tomato (Buttery et al., 1995), has been intensively studied during the last decade (Bruche et al., 1991; Guedes de Pinho and Bertrand, 1995; Pérez et al., 1996; Chen et al., 1996). Some researchers consider **1** and its methoxy derivative (mesifurane) **2** as two of the most important aroma contributors to strawberry flavor (Pyysalo et al., 1979; Fischer and Hammerschmidt, 1992; Larsen and Poll, 1992). Recently, the β-D-glucopyranoside **3** (Mayerl et al., 1989; Wu et al., 1990; Krammer et al., 1994) and its malonylated derivative **4** (Roscher et al., 1996) have been identified in fruits whereas the β-D-glucuronide **5** has been detected as the major metabolite of **1**, **3**, and **4** in urine of man (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 formation pathway is still unknown.

On the basis of the determination of the [^{13}C]/[^{12}C] ratio of **1** and **2** in strawberry, revealing a constant difference of about 9‰ (Bruche et al., 1995), two independent biosynthetic pathways for **1** and **2** have been assumed. However, the quantification of **1**, **2**, and **3** during fruit ripening indicated a rapid conversion of **1** into **2** and **3** (Pérez et al., 1996). This report now offers the first conclusive evidence that in strawberries mesifurane **2**, Furaneol glucoside **3**, and the malonylated derivative **4** derive from Furaneol **1**.

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MATERIALS AND METHODS

Chemicals. All chemical reagents were purchased from Sigma and Aldrich. The organic solvents were obtained from Merck. XAD-2 was supplied by Aldrich. L-[1-^{14}C]Fucose (55 mCi/mmol) and *S*-[*methyl*[^{14}C]adenosyl-L-methionine (55 mCi/mmol) (^{14}C-SAM) were purchased from Biotrend.

Fruits. Whole strawberry plants 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 where they received lighting from 8:00 a.m. to 6:00 p.m. daily at 45% humidity and 25−27 °C.

Application of ^{14}C-Labeled Compounds. Maturing strawberries weighing between 5 and 15 g in the orange ripening stage were removed from the plants by severing the stems under distilled water so that about 2.5 cm of the stem remained attached to each berry. Individual berries were placed into 2-mL vials containing the respective radioactively labeled compound (3.8 μCi of ^{14}C-SAM or 1.9 μCi of ^{14}C-**1**) dissolved in 1 mL of tap water. The stems of the berries just reached the bottom of the vessels. Approximately 1 mL of solution was absorbed by a 10 g berry in 24 h under the experimental conditions (10 h of light daily, 45% humidity, and 25−27 °C). The berries were allowed to respire for 72 h while kept in a desiccator fitted with a glass tube containing soda lime granules. Air was pulled through the jar by means of a slight vacuum, and the respired CO_2 was collected totally by the soda lime granules.

Recovery and Extraction of Radioactivity. After 72 h, the stem and calyx of the strawberries were removed with a sharp knife and all fruit parts were stored at −20 °C until workup. Frozen strawberries were submerged in 40 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 (150 mL) and subjected to solid phase extraction on XAD-2 (20 cm, 3 cm i.d). After being washed with 250 mL of water, volatiles were eluted by 200 mL of diethyl ether and glycosides were obtained by elution with 200 mL of methanol. The diethyl ether extract was dried with sodium sulfate, carefully concentrated by means of a

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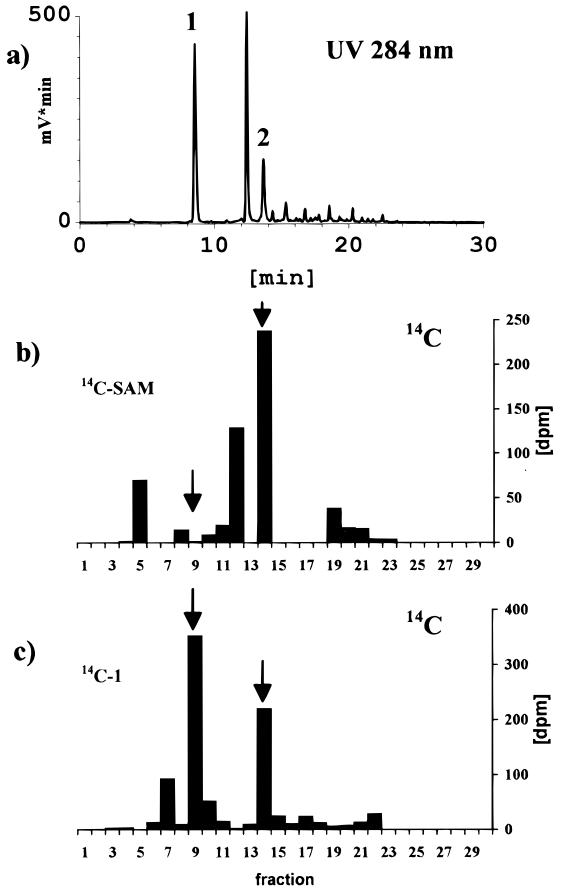

Figure 1. HPLC-RP18 separation (UV trace at 284 nm) of the compounds obtained by solid phase extraction followed by diethyl ether elution of strawberry fruits after the administration of ^{14}C-SAM (a) and LSC chromatograms after the administration of ^{14}C-SAM (b) and ^{14}C-**1** (c).

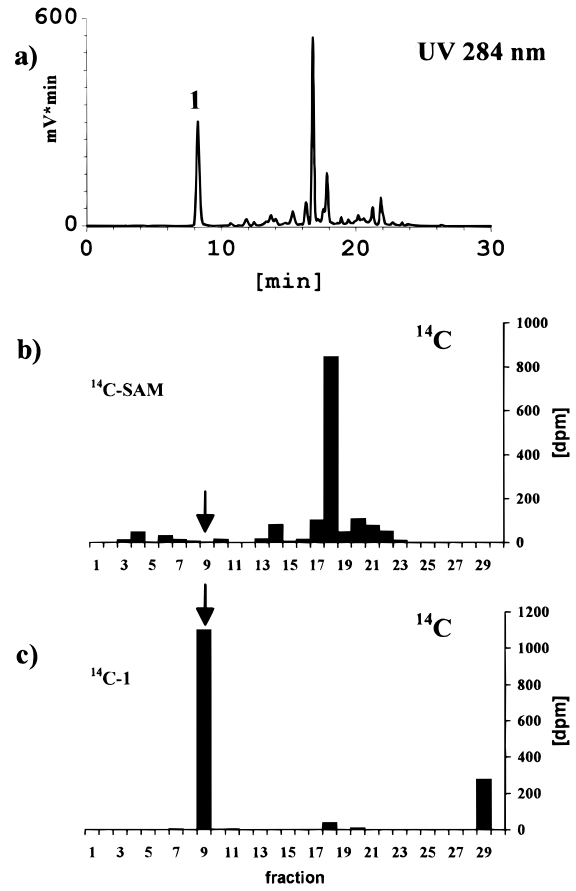

Figure 2. HPLC-RP18 separation (UV trace at 284 nm) of the compounds liberated by glycosidase treatment of the methanol extract obtained by solid phase extraction of strawberry fruits after the administration of ^{14}C-SAM (a) and LSC chromatograms after the administration of ^{14}C-SAM (b) and ^{14}C-**1** (c).

Vigreux column to approximately 1 mL, and transferred into 500 μL of water. The remaining organic phase was removed by a gentle stream of nitrogen and the aqueous phase was finally analyzed by HPLC. Recovery of **1** was 78% ± 2% under these conditions. The methanol extract was concentrated *in vacuo*. Stems and attached calyx as well as the solid residues were dried in an oven at 80 °C. Stems and calyx were homogenised in a mortar with sodium sulfate. $^{14}CO_2$ adsorbed by the soda lime was liberated after addition of 50 mL of HCl (18 %), trapped in 10 mL of Oxysolve 400, and analyzed by liquid scintillation counting (LSC).

Enzymatic Hydrolysis. Aliquots of the methanol extracts were dissolved in 800 μL of 0.2 M phosphate buffer (pH 5.5) and 200 μL of Rohapect D5L (Röhm) 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, 250 μL of water was added, and diethyl ether was removed in a stream of nitrogen.

HPLC Analysis of Metabolites. The transformation of ^{14}C-SAM and ^{14}C-**1** was monitored using high-performance liquid chromatography (HPLC). The HPLC equipment consisted of a Knauer Maxistar pump equipped with a Knauer variable wavelength monitor set at 284 nm and controlled by Knauer Eurochrom 2000 software. The HPLC was fitted with either a Eurospher 100-C18 column (25 cm × 4.0 mm i.d., particle size 5 μm) or a Eurospher 100-DIOL column (25 cm × 4.0 mm i.d., particle size 7 μm). The profiling HPLC gradient for the C18 column was conducted in two linear steps at a flow rate of 1 mL/min, utilizing acetonitrile and acidic water, adjusted to pH 2.5 with 1.0 M sulfuric acid. The gradient proceeded from 5% to 20% acetonitrile in 10 min followed by 20% to 80% acetonitrile in 20 min. The profiling HPLC gradient for the DIOL column was conducted in an isocratic step followed by a linear step at a flow rate of 1 mL/min, utilizing isohexane and 2-propanol. After 5 min at 100% isohexane the gradient proceeded from 0% to 50% 2-propanol in 30 min. Fractions of 1 mL each were collected, scintillation cocktail was added, and the fractions were analyzed by LSC. Total radioactivity was recovered after the HPLC fractionation as confirmed by LSC. Identity of radiochemically labeled **1** and **2** was confirmed as follows: fraction 9, containing **1** (Figures 1a and 2a), or fraction 14, containing **2**, was isolated by HPLC-RP18 separation (Figure 1a), extracted three times with 1 mL of diethyl ether, and re-analyzed by HPLC-DIOL separation. Quantification of **1** and **2** was achieved by means of a calibration curves. Specific radioactivity of **1** and **2** was calculated as quotient of the radioactivity measured for the individual compound by LSC and the amount of the individual compound obtained by HPLC separation with UV detection. The values for the specific radioactivity of **1** and **2** were confirmed by HPLC-DIOL separation followed by LSC.

Liquid Scintillation Counting (LSC). Aliquots of liquid samples were added to 10 mL of scintillation cocktail Emulsifier-safe (Packard). At least four replicates were measured. Solid samples, at least five replicates, were combusted in a biological oxidizer (model Ox 500; Zinsser Analytic). The formed $^{14}CO_2$ was absorbed in 12 mL of Oxysolve 400 (Zinsser Analytic). Recoveries of ^{14}C as [^{14}C]carbon dioxide from test combustions fortified with ^{14}C standards, immediately before combustion, were greater than 90%. All measurements were carried out by means of the liquid scintillation counter LKB Rackbeta 1214 after decaying of the chemiluminescence counts.

Synthesis of [2-(or 5-)*methyl*-^{14}C]-2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone (^{14}C-1). In a 1-mL vial a solution consisting of 60 μg of L-[1-^{14}C]fucose (20 μCi, 55 mCi/mmol) dissolved in 30 μL of absolute ethanol was added to a solution of 8.4 μg of piperidine and 14 μg of acetic acid in 70 μL of absolute ethanol. The solution was covered with argon. The

Table 1. Distribution of Recovered Radioactivity in Different Fractions after Application of ^{14}C-SAM and ^{14}C-1 to Detached Ripening Strawberry Fruits

	^{14}C-SAM	^{14}C-**1**
extract	31.9[a]	16.6
flow-through[b]	22.4	13.1
diethyl ether	1.2	0.6
methanol	7.0	3.0
solid residue	24.6	2.8
stem and calyx	45.8	51.1
CO_2	0.0	8.4
recovery	102.3	78.8

[a] Percent of applied radioactivity. [b] Fractions obtained by solid phase extraction (see Experimental Section).

vial was closed and heated to 80 °C in the dark. After 2 h, the vial was cooled to room temperature and the reaction mixture was fractionated by three independent HPLC separations. Solid phase extraction (RP18-cartridge, 100 mg, Supelco) of the pooled fractions containing **1** followed by diethyl ether elution yielded ^{14}C-labeled **1**, exhibiting a radiochemical purity of ≥90%.

RESULTS AND DISCUSSION

Formation of 2,5-Dimethyl-4-methoxy-3(2*H*)-furanone 2. Adenosyl-L-methionine, the universal methyl group donor in plant systems, was applied as ^{14}C-labeled compound to detached ripening strawberry fruits in order to elucidate the biogenetic origin of the methoxy group in **2**. The experimental design corresponded to that described by Loewus et al. (1956) for the biosynthesis of ascorbic acid in detached ripening strawberry fruits. After 3 days the strawberry was frozen and subsequently worked-up. The recovery data for the different fractions are shown as percentages of applied radioactivity in Table 1. Total applied radioactivity of ^{14}C-SAM was recovered; however, $^{14}CO_2$ was not formed. High amounts of ^{14}C remained in the stem and the calyx. Total radioactivity, subjected to solid phase extraction was recovered in the different fractions as confirmed by LSC. The major portion of ^{14}C-SAM was metabolized into highly polar compounds (flow through) or non-extractable residues (Table 1). After fractionation of the aqueous extract by solid phase extraction on XAD-2, **2** was detected in the diethyl ether extract by HPLC-RP18 analysis (Figure 1a), accounting for 42% of the radioactivity in this extract (Figure 1b). The identity and purity of radiochemically labeled **2** was confirmed by a second chromatographic separation using HPLC-DIOL analysis. The total incorporation rate of ^{14}C-SAM into **2** corresponded to 0.4% of the applied radioactivity. As **1**, representing the basic structure of **2** was not ^{14}C-labeled after the application of ^{14}C-SAM (Figure 1b), compound **2**, a derivative of **1** must carry the radiolabel in the methoxy group. Hence, the methyl group in **2** is derived, as in all alkaloids and phenolics, from adenosyl-L-methionine. This result excludes two independent biogenetic pathways leading to **1** and **2** as proposed by Bruche et al. (1995) on the basis of the differences of their $\delta^{13}C_{PDB}$ values. A depletion in the methyl groups originating from the SAM pool ($\delta^{13}C \leq -39‰$) has already been described for natural purine alkaloids (Weilacher et al., 1996).

In addition, ^{14}C-**1** was synthesized from commercially available L-[1-^{14}C]fucose and applied to a detached ripening strawberry in order to reveal the second carbon pool for **2**. Only 80% of the applied ^{14}C was recovered (Table 1) which can at least partly be explained by the volatility of **1** and its degradation products (Chen et al., 1996) which were not recorded in this experimental design. Surprisingly, small amounts of $^{14}CO_2$, accounting for 8% of the applied radioactivity were detected. Several explanations may account for that observation. (i) Compound **1** could be metabolized in some way through the respiration cycle yielding CO_2. (ii) Compound **1** is generally recognized as an unstable molecule (Hirvi et al., 1980; Roscher et al., 1997c), and we observed that radioactively labeled **1** showed an even more pronounced instability (Roscher et al., 1997b). Hence, chemically formed degradation products could be detoxified and finally transformed to CO_2 by the strawberry. (iii) Compound **1** has never been detected before in green plant parts. Therefore, **1** performs as a xenobiotic to the stems and is metabolized as such by the plant defense system. The enzymatically formed degradation products of **1** could serve as a source for CO_2. A high portion of ^{14}C remained in the stem and calyx. Fractionation by XAD-2 followed by HPLC-RP18 analysis revealed that the major portion of ^{14}C-**1** was metabolized into highly polar compounds or non-extractable residues (Table 1) but 0.3% of the applied radioactivity was recovered in **2** (Figure 1c, Table 2). On the basis of these results—incorporation of ^{14}C-SAM and ^{14}C-**1** into the structure of **2** (Figure 1b and 1c, respectively)—the presence of a methyltransferase in strawberry is supposed, transferring the methyl group from SAM to **1** analogously to the formation of methoxy derivatives in the metabolism of phenolics (Kurosaki, 1996).

Table 2. Content, Incorporation Rate, and Specific Radioactivity of 1, 2, and Glycosidically Bound 1 after Application of ^{14}C-SAM and ^{14}C-1 to Detached Ripening Strawberry Fruits

	^{14}C-SAM			^{14}C-**1**		
	μmol/kg	%[a]	dpm/μmol	μmol/kg	%	dpm/μmol
1	41.4	0	0	369.8	0.43	1669
glycosidically bound **1**	26.7	0	0	95.3	0.73	10910
2	16.2	0.41	34343	87.3	0.26	5678
sum	84.3	0.41		552.4	1.41	

[a] Percent of applied radioactivity.

Formation of Glycosidically Bound 1. 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone glucoside **3** and its malonylated derivative **4** have recently been described as natural constituents in strawberry fruits (Mayerl et al., 1989; Roscher et al., 1996). Complete hydrolysis of **3** and **4**, leading to the liberation of **1** has been achieved by treatment with the pectinolytic enzyme preparation Rohapect D5L, exhibiting glycosidase side activity (Roscher et al., 1997c). Hydrolysis of the constituents of the methanol extracts obtained after the application of ^{14}C-**1** provided **1** (Figure 2a), accounting for 0.7% of the applied radioactivity (Figure 2c, Table 2). No radiochemically labeled **1** was detected after the application of ^{14}C-SAM to strawberry fruits followed by the cleavage of **3** and **4** (Figure 2b). Compound **2** was never released from the glycosidic extracts of the cultivar Elsanta, as we confirmed by a two-dimensional HPLC separation technique. Thus, the radioactivity in fraction 14 (Figure 2b) cannot be attributed to **2**. This result confirmed the finding that only the methoxy group in **2** became radiolabeled by ^{14}C-SAM. Recently, Zabetakis and Holden (1996) proposed the exclusive formation of **1** from its glucoside **3** in strawberry callus culture. However, our data clearly show that **1** represents the precursor for **3** and not *vice versa*. On the base of the specific radioactivities (Table 2) one can

generally conclude that, at the beginning, ^{14}C-**1** was effectively metabolized to **2** and glycosidically bound **1**. By this way, the applied radiolabel pulse moved to the next metabolic level. In the course of the experiment, however, the intensive *de novo* synthesis of **1** led to a decrease of the specific radioactivity of **1** compared with **2** and glycosidically bound **1** which are synthesized *de novo*—with a small time lag—from **1**.

ABBREVIATIONS USED

CPRO-DLO, Centrum voor Plantenveredelings en Reproduktieonderzoek; HPLC, high-performance liquid chromatography; HPLC-DIOL, diol-phase high-performance liquid chromatography; HPLC-RP18, reversed-phase high-performance liquid chromatography; LSC, liquid scintillation counting; SAM, *S*-adenosyl-L-methionine; UV, ultraviolet/visible.

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