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2,5-DIMETHYL-4-HYDROXY-3[2H]-FURANONE 6′*O*-MALONYL-β-D-GLUCOPYRANOSIDE IN STRAWBERRY FRUITS

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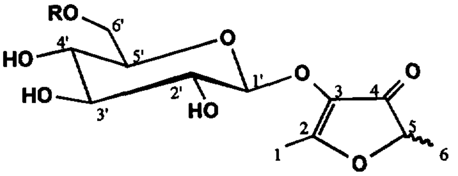
Key Word Index—*Fragaria* × *ananassa*; Rosaceae; strawberry fruits; 2,5-dimethyl-4-hydroxy-3[2H]furanone 6′-*O*-malonyl-β-D-glucopyranoside; furaneol®; HPLC-MS/MS; countercurrent chromatography.

Abstract—2,5-Dimethyl-4-hydroxy-3[2H]-furanone 6′-*O*-malonyl-β-D-glucopyranoside was isolated from a glycosidic extract of strawberry fruit (*Fragaria* × *ananassa*, cv. Senga Sengana) by means of countercurrent chromatography and reverse-phase HPLC. Identification was achieved by comparison of chromatographic and ^{1}H, ^{13}C and 2D-NMR, as well as mass spectral data with those of the synthesized reference compound. In ripe strawberry fruit, a 1 : 1 ratio of the malonylated glucoside to its deacylated glucoconjugate was determined by on-line liquid chromatography-atmospheric pressure chemical ionization-tandem mass spectrometry. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

2,5-Dimethyl-4-hydroxy-3[2H]-furanone (DMHF, Furaneol®) is a well-known flavour compound in various fruits [1] and has also been found as the corresponding β-D-glucopyranoside in strawberry [2], pineapple [3] and tomato [4]. Owing to the importance of DMHF in fruit flavours [5, 6], a complete knowledge of glycosidically bound DMHF is desirable in order to rationalize changes and improvements in flavour during processing and storage of concentrates or juices [7, 8].

In the past, reports on malonylated glyconjugates as flavour precursors were rather scarce [9, 10]. Recently, Moon *et al.* [10] identified linalyl 6′-*O*-malonyl-β-D-glucopyranoside in *Jasminum sambac* and elucidated the complete structure of an underivatized malonylated glycosidic flavour precursor for the first time. These new results stimulated our investigations regarding the composition of glycosidically bound DMHF in strawberry, with special interest on malonylated derivatives. In particular, the natural occurrence of acylated anthocyanins [11] and the recent identification of pelargonidin 3-*O*-(6′-*O*-malonyl-β-D-glucoside) in strawberry fruit [12] supported our efforts.



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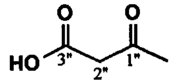
2	

The present paper reports the isolation of 2,5-dimethyl-4-hydroxy-3[2H]-furanone 6′-*O*-malonyl-β-D-glucopyranoside (**2**) from strawberry fruit, its chemical synthesis and structural elucidation by spectroscopic means, as well as the simultaneous detection of **1** and **2** in a glycosidic extract from strawberry by on-line HPLC-atmospheric pressure chemical ionization-tandem mass spectrometry (LC-APCI-MS/MS).

RESULTS AND DISCUSSION

A glycosidic extract from strawberry fruit was obtained by adsorption chromatography on Amberlite XAD-2 and subsequent methanol elution [13]. After concentration and lyophilization, the extract was fractionated by preparative and, subsequently, analytical multilayer coil countercurrent chromatography (MLCCC) guided by TLC and enzymic hydrolysis of the collected fractions. HRGC analysis of liberated aglycones and HPLC (gradient I) of two polar fractions from analytical MLCCC revealed the presence of two DMHF precursors with identical UV spectra (λ_{max} 275 nm). From the less polar MLCCC fraction, gluco-

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side **1** was purified by preparative HPLC. Hydrolysis studies of the most polar MLCCC fraction showed the presence of DMHF, glucose and malonic acid. Out of this fraction compound **2** (1 mg) was purified by HPLC on a RP-18 column.

NMR data (D_2O, see Experimental) of isolated **1** were identical to those of the synthesized reference compound and previously published data [2, 4]. In the ^{1}H NMR spectrum of isolated **2** (D_2O, see Experimental), a characteristic downfield shift of H-6′a (4.44 ppm) and H-6′b (4.31 ppm) compared to the corresponding signals in **1** was observed, indicating acylation at the C-6′ hydroxyl group of the sugar moiety. In combination with the detection of malonic acid and glucose, we assumed the 6′-*O*-malonyl derivative of **1**. For exact structural elucidation of **2**, its synthesis was performed [14]. After the preparation and purification of synthesized **1** and **2** their NMR spectra were recorded in DMSO-d_6 to avoid HD exchange with solvents containing exchangeable deuterium [15] (Tables 1 and 2). In addition to ^{1}H NMR spectral data with the distinct downfield shift of H-6′a and H-6′b in synthesized glucoside **2**, the site of esterification was confirmed by the ^{13}C NMR spectrum of **2** as the chemical shift of C-6′ appeared 2.8 ppm downfield and that of C-5′, 3.6 ppm upfield from the corresponding signals of **1** (Table 2) [14]. Furthermore, the data recorded for **2** revealed that, besides the diasteromeric structure, two conformational isomers occurred in DMSO-d_6 solution, owing to a protonated and deprotonated carboxylic moiety of the malonyl group. This phenomenon has been observed earlier by Matern *et al.* [16] who had proposed that intra-molecular hydrogen bondings are stabilizing the conformers. Feeny *et al.* [17] have postulated a 10-membered boat-chair-boat-ring, formed by hydrogen-bonding of the carbonyl group of the malonyl residue to the 4′-OH group of the glucose. As shown by our experiments, the conformational differences influenced the ^{1}H NMR signals of proton H-6′a1/H-6′a2 and H-6′b1/H-6′b2 in particular, with a shift difference of 12 and 18 Hz (600 MHz), respectively. This observed shift was dependent on the frequency used (200, 400 and 600 MHz NMR spectrometer), while the coupling constants of the specific *dd* signals remained stable. Our 2D-NMR spectroscopic investigations strongly supported the exclusive malonylation at the C-6′ position of glucose and the presence of two conformational isomers of compound **2**. ^{1}H–^{1}H-COSY and ^{13}C-^{1}H-COSY experiments allowed the assignment of the AB part of the ABX

Table 2. ^{13}C NMR spectral data of compounds **1** and **2** (DMSO-d_6)*

C	**1** (400 MHz)	**2** (600 MHz)†
Aglycone		
1	13.8/13.8	13.5/13.6
2	180.1	180.6/180.8
3	132.8	132.3/132.4
4	197.0	196.7/196.9
5	80.1	80.0
6	16.2/16.3	16.1/16.2
Glucose		
1′	102.2/102.5	102.0/102.1
2′	73.4/73.5	73.0/73.1
3′	76.4	75.8
4′	70.0	69.7
5′	77.2	73.6/73.8
6′	61.1	63.9/64.1
Malonyl		
1″		166.7
2‴		41.3
3″		167.7/167.8

*Chemical shifts in ppm.

†Assignments based on results of DEPT and ^{13}C–^{1}H-COSY experiments.

Table 1. ^{1}H NMR spectral data of compounds **1** and **2** (DMSO-d_6)*

H	**1** (400 MHz)		**2** (600 MHz)†	
Aglycone				
1	2.30/2.29	3H, *s*	2.17/2.16	3H, *d* (1.0/1.0)
5	4.73	1H, *q* (7.0)	4.65	1H, *qq* (7.1; 1.0)
6	1.39/1.38	3H, *d* (7.0/6.9)	1.31/1.30	3H, *d* (7.1/7.1)
Glucose				
1′	4.74	1H, *d* (7.7)	4.66	1H, *d* (7.9)
2′	3.10–3.45	*m*‡	3.05–3.15	1*H*, *m*‡
3′	3.10–3.45	*m*‡	3.20	1H, *t* (8.9)
4′	3.10–3.45	*m*‡	3.05–3.15	1H, *m*‡
5′	3.10–3.45	*m*‡	3.32–3.38	1H, *m*‡
6′	3.71	1H, H-6′a,	4.33	1H, H-6′a1, *dd* (11.9; 2.0)
		d (11.4)	4.31	H-6′a2, *dd* (11.9; 2.0)
	3.51	1H, H-6′b,	4.11	1H, H-6′b2, *dd* (11.8; 6.6)
		d (9.2)	4.08	H-6′b1, *dd* (11.9; 6.7)
Malonyl				
2″			3.35; 3.34	2H, AB system

*Chemical shifts (δ) expressed relative to solvent signal of DMSO-d_6 in ppm; coupling constants in Hz.

†Assignments based on results of a ^{1}H–^{1}H-COSY experiment.

‡Signals overlapped.

system for the methylene protons (4.05–4.35 ppm) at C-6′, clearly indicating that signals at 4.33 ppm (H-6′a1) and 4.08 ppm (H-6′b1) were correlated. On the contrary, the proton signals 4.31 ppm (H-6′a2) and 4.11 ppm (H-6′b2) were related. This correlation pattern has been found by Feeny *et al.* [17] who had assigned H-6′a1/H-6′b1 to the salt structure and H-6′a2/H-6′b2 to the protonated structure of luteolin 7-*O*-(6″-*O*-malonyl-glycoside). Moreover, the $^4C_1$ conformation of the sugar residue in **2** was indicated by the vicinal coupling constant of H-2′ with 8.9 Hz [16]. The signals at 3.35 and 3.34 ppm in the ^{1}H NMR of **2** could be assigned to the malonyl methylene carbon C-2″ by means of a ^{13}C–^{1}H-COSY experiment. Therefore, we concluded an AB system for the malonyl methylene protons [18]. According to Mayerl *et al.* [2] and Krammer *et al.* [4], proton signals of the methyl groups from the aglycone in **1** and **2** were interpreted as two slightly shifted singlets or doublets, due to the diastereomeric structure of **1** and **2**. ^{1}H–^{1}H-COSY data for the observed doublets of the methyl group at C-2 in glucoside **2** (2.17/2.16 ppm, $J = 1.0/1.0$ Hz) revealed the long-range coupling to the proton at C-5. Carbons occurring as two signals in the ^{13}C NMR spectrum were ascribed to the diastereomeric and conformeric structure of **2**.

The new DMHF glycoside **2** isolated from strawberry was identical in retention time and spectral information with that of synthesized **2** using HPLC-MS/MS (positive mode) (Table 3). Mass spectral data obtained with the APCI interface gave distinct structural information, indicating the loss of the malonyl moiety and the presence of DMHF in the molecule. MS/MS experiments focusing on the fragments generated from the pseudomolecular ion $m/z = 377$ $[M + H]^+$ revealed in both cases (APCI or ESI) only two fragments: $[M + H]^+$ (5% relative intensity) and $m/z = 129$ $[DMHF + H]^+$ (100% relative intensity). The same MS/MS fragmentation pattern was observed for glucoside **1** with $[DMHF + H]^+$ as the most abundant product ion generated from the protonated molecular ion $m/z = 291$ $[M + H]^+$.

A glycosidic extract from one strawberry fruit (25 g) obtained by XAD-2 solid phase extraction was used for the simultaneous detection of **1** and **2** using HPLC-ESI-MS/MS. From this crude extract (dissolved in 1 ml distilled water, 5 μl injection) the ratio of **1** to **2** was determined to be *ca* 1 : 1, on the basis of their integrated peak areas obtained in the 'parent scan' mode (detection of all m/z generating the specific product ion $m/z = 129$ by CID). This example demonstrates the high selectivity and sensitivity of HPLC-MS/MS analysis, which has also been used to identify the malonylated glucoside **2** in a number of other fruits, i.e. pineapple, mango and tomato [19].

EXPERIMENTAL

Isolation of glycosidic extract. Ripe strawberry fruits (*Fragaria* × *ananasa*, cv Senga Sengana; 4 kg) from the local market were blended in a mixer with 3 l of 0.2 M Pi buffer (pH 7.0) containing 0.2 M glucono-δ-lactone. After centrifugation (3000 *g*, 5°, 20 min) the supernatant was extracted with pentane–CH_2Cl_2 (2 : 1) in a liquid–liquid extractor overnight and subjected to solid phase extraction on Amberlite XAD-2 using a 4.5 × 1000 mm glass column [13]. After rinsing with dist. H_2O (3.5 l), the absorbed glycosides were eluted with MeOH (3.5 l). The MeOH eluate was concd *in vacuo* and the remaining aq. glycosidic fr. extracted with Et_2O to remove any remaining volatiles, then carefully evapd to give a deep red syrup under red. pres., which was lyophilized for subsequent fractionation by MLCCC (yield, 7.1 g).

Multilayer Coil Countercurrent Chromatography (MLCCC). An Ito multilayer coil separator–extractor (P.C. Inc. Potomac) equipped with a Knauer HPLC pump 64 and a variable-wavelength UV/VIS detector was used. Analytical separations with up to 175 mg of lyophilizate were achieved on a 160 m × 1.6 mm i.d. PTFE column (volume 320 ml), while prep. separations with up to 1.5 g of lyophilizate were carried out with a 75 m × 2.6 mm i.d. PTFE tubing (volume 360 ml). Sepns were performed at 24° using a constant rotational

Table 3. HPLC-MS/MS data of compounds **1** and **2***
(RP-18, gradient: 0.05% TFA in H_2O–0.05% TFA in MeCN)

1 ($R_t = 3.2$ min)		**2** ($R_t = 8.2$)	
+m/z†	Assignment	+m/z†	Assignment
		APCI interface	
291	$[M + H]^+$	333	$[M + H - CO_2]^+$
129	$[DMHF + H]^+$	291	$[M + H - \text{malonyl}]^+$
170	$[DMHF + H + CH_3CN]^+$	377	$[M + H]^+$
		129	$[DMHF + H]^+$
		170	$[DMHF + H + CH_3CN]^+$
		ESI interface	
291‡	$[M + H]^+$	377	$[M + H]^+$
313‡	$[M + Na]^+$		

*Mass spectra obtained from isolated and synthesized compounds **1** and **2**.
†Fragments +m/z are arranged in decreasing order of relative abundance.
‡Identical with ESI mass spectral data previously published [4].

speed of 800 rpm in either the 'head-to-tail' or the 'tail-to-head' elution mode, depending upon which layer of the biphasic system was selected as stationary phase [20]. Flow rates were 1 and 2.5 ml min^{-1} for analytical and prep. applications, respectively. TLC of collected frs was carried out on silica gel 60 F_{254} using the less polar phase of ($CHCl_3$–MeOH–H_2O, 7:13:8) as mobile phase (detection, 254 nm and vanillin–H_2SO_4).

HRGC and HRGC-MS. HRGC analyses were performed using a J&W DB-Wax fused silica capillary column (30 m × 0.25 mm i.d., df = 0.25 μm). Split-injection (1:20) was employed. Temp. programme: 50° for 3 min, then from 50 to 240° at 4°C min^{-1}. Flow rates for the carrier gas were 2.5 ml min^{-1} He, for the make-up gas 30 ml min^{-1} N_2 and for the detector gases 30 ml min^{-1} H_2 and 300 ml min^{-1} air, respectively. Injector and detector (FID) temps were kept at 220° and 250°, respectively. For HRGCMS the same type of column and the same temp. program was used as mentioned above. The temp. of the ion source and all connection parts was 220°; electron energy, 70 eV; cathodic current, 0.7 mA. Volumes of 1 μl were injected.

Analyt. HPLC. Reverse-phase HPLC with diode array detection was carried out on an Eurospher 100 C-18 column (250 × 4 mm; 5 μm; Knauer, Berlin, Germany) using a gradient with a flow rate of 1 ml min^{-1}. Gradient I: solvent A (0.1% TFA in H_2O), solvent B (MeCN): 0–7 min, 8–15% B; 7–12 min, 15–20% B; 12–17 min, 20–30% B; 17–20 min, 30–90% B.

Prep. HPLC. Sepns were performed on an Eurospher 100 C-18 column (250 × 16 mm; 5 μm; Knauer, Berlin, Germany) with UV detection at 275 nm. Gradients used were as follows: Gradient II: (flow rate: 5 ml min^{-1}) solvent A (H_2O), solvent B (MeOH): 0–15 min, 0–50% B; 15–20 min, 50–70% B; 20–25 min, 70–100% B. Gradient III: (flow rate: 7.5 ml min^{-1}) solvent A (H_2O), solvent B (MeCN): 0–11 min, 8–15% B; 11–13 min, 15–90% B. Gradient IV: (flow rate 7.5 ml min^{-1}) solvent A (1% HOAc in H_2O), solvent B (MeCN): 0–10 min, 10–15% B; 10–20 min, 15–25% B; 20–22 min, 25–90% B.

Enzymic hydrolyses. Aliquots of monitored frs were dried, dissolved in 5 ml of 0.2 M pi buffer (pH 5.5) and incubated (37°, 24 hr in the dark) with 100 μl of enzyme prep (Rohapet D5L; Röhm, Darmstadt, Germany). Samples were then extracted with Et_2O, dried (Na_2SO_4), carefully concd and analysed by HRGC and HRGCMS.

Isolation of DMHF-glucoside (**1**) *and* (**2**). The glycosidic extract was first separated by prep. MLCCC using $CHCl_3$–MeOH–H_2O (7:13:8) with the less dense layer as mobile phase [21]. Six frs (I–VI) were obtained. Fr. IV (350 mg), yielding a high amount of DMHF after enzymatic hydrolysis, was subjected to analyt. MLCCC using *n*-BuOH–EtOAc–H_2O (2:2:5) with the more dense layer as mobile phase. Thirteen frs (1–13) were collected. Enzymic hydrolyses revealed the presence of DMHF precursors in frs 1 and 2. Compound **1** was isolated (22 mg) from MLCCC fr. 2 by prep. reverse-phase HPLC (gradient II). The most polar MLCCC fr. 1 was further sepd by analyt. HPLC (MeOH–H_2O, 1:3) and subsequently rechromatographed with MeCN–0.1 M HOAc (2:13) yielding compounds **1** and **2** (1 mg) with identical UV spectra. ^1H NMR of isolated **1** (400 MHz, D_2O): δ aglycone moiety: 4.80–4.73 (1H, obscured, H-5), 2.40 (3H, *s*, CH_3-1), 1.45 (3H, *d*, $J = 6.3$ Hz, CH_3-6), glucosyl moiety: 4.80–4.73 (1H, obscured, H-1′), 3.87 (1H, *d*, $J = 12.67$ Hz, H-6′a), 3.73 (1H, *dd*, $J = 5$, 12 Hz, H-6′b), 3.54–3.42 (4H, *m*, H-2′, H-3′, H-4′, H-5′). ^{13}C NMR of isolated **1** (400 MHz, D_2O, referenced to signal of C-1 at 16.6 ppm [4]): δ aglycone moiety: 16.6 (C-1), 18.0, 18.1 (C-6), 84.7 (C-5), 136.0 (C-3), 188.4 (C-2), 203.2 (C-4), glucosyl moiety: 63.3 (C-6′), 72.1 (C-4′), 75.8 (C-2′), 78.3 (C-3′), 79.0 (C-5′), 105.9 (C-1′). ^1H NMR of isolated **2** (400 MHz, D_2O): δ aglycone moiety: 4.90–4.65 (1H, obscured, H-5), 2.30 (3H, *s*, CH_3-1), 1.43 (3H, *d*, $J = 7.2$ Hz, CH_3-6), glucosyl moiety: 4.84–4.70 (1H, obscured, H-1′), 4.44 (1H, *d*, $J = 12.32$ Hz, H-6′a), 4.31 (1H, *dd*, $J = 5$, 12 Hz, H-6′b), 3.85–3.45 (4H, *m*, H-2′, H-3′, H-4′, H-5′), malonyl moiety: 3.32 (2H, *s*, CH_2-2″).

Sugar analysis. Isolated glucoside **2** was completely hydrolysed using 4 M TFA in aq. soln. After lyophilization, the liberated glucose was converted into the corresponding methoxyoxime acetate and identified using HRGCMS, as reported previously [22].

Detection of malonic acid. Compound **2** was refluxed for 2 hr in 10 ml of 0.1 M HCl, extracted with Et_2O, methylated with CH_2N_2 and analysed by HRGCMS.

Synthesis of DMHF β-D-glucopyranoside (**1**). Prepn of **1** was achieved using DMHF (9.1 g; 71.4 mmol), α-D-acetobromoglucose (8.2 g; 19.9 mmol), Ag_2O (5.0 g; 21.5 mmol) and Drierite (7.0 g) in CH_2Cl_2 [9]. After desacetylation (24 hr, 2.5% NH_3 in H_2O), the alkaline soln was evapd, the resulting syrup redissolved in H_2O, extracted with Et_2O (24 hr) and subjected to solid phase extraction on XAD-2. The MeOH eluate was concd to dryness (3 g) and purified by prep. MLCCC using *n*-BuOH-EtOAc–H_2O (2:2:5) with the more dense layer as mobile phase, yielding 368 mg of **1**. after prep. HPLC (gradient III), pure **1** was obtained with an overall yield of 2.7% (155 mg; 0.53 mmol; λ_{max} 275 nm).

Synthesis of DMHF 6′-O-malonyl-β-D-glucopyranoside (**2**). The malonylated glucoside **2** was prepared as described recently [14] using synthesized **1** (81.4 mg; 0.28 mmol), malonic acid (87.5 mg; 0.84 mmol) and *tert*-butyl-*iso*cyanide (34.9 mg; 0.45 mmol) in dry MeCN (product control by analytical HPLC, gradient I). Pure **2** was obtained by prep. HPLC (gradient IV) with an overall yield of 54.1% (57 mg; 0.15 mmol; λ_{max} 275 nm).

LC-MS/MS analysis. Analysis of isolated and synthesized glycosides **1** and **2** was performed on a triple stage quadrupole TSQ 7000 LC-MC/MS system with either atmospheric pressure chemical ionisation (APCI) or electrospray (ESI) interfaces (Finnigan MAT, Bremen, Germany). For APCI, the temperatures of the heated vaporizer and inlet capillary in the nebulizer interface were 300 and 150°, respectively. The current of the APCI corona discharge needle was set to 5.0 μA, resulting in 4.59 kV needle voltage. N_2 served both as sheath (10 l min^{-1}) and auxiliary (50 psi) gas. For ESI, the temp. of the heated capillary was 200°. ESI capillary voltage was set to 4.0 kV, resulting in 9.9 μA current. N_2 served both as sheath (50 psi) and auxiliary gas. HPLC separations were carried out on an Eurospher 100 C-18 (100 × 2 mm, 5 μm, Knauer, Berlin, Germany) using a linear gradient with a flow rate of 200 μl min^{-1}. Solvent A (0.05% TFA in H_2O), solvent B (0.05% TFA in MeCN). The gradient program was as follows: 0–7 min, 8–15% B; 7–12 min, 15–20% B; 12–17 min, 20–30% B; 17–20 min, 30–90% B; 20–25 min, 90% B. Runs were performed in positive mode. Product ion spectra of **1** and **2** were available by collision induced dissociation (CID) (1.8 mTorr Ar; −15 eV).

NMR. Spectra were acquired on FT Bruker (200 MHz, 400 MHz, 600 MHz) spectrometer. For the DEPT experiments the Bruker standard impulse sequence was used. NMR spectra were recorded in DMSO-d_6 or D_2O and referenced to the solvent signal, respectively.

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