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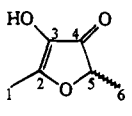
SHORT COMMUNICATIONS

Isolation and Identification of 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone Glucoside from Tomatoes

Keywords: *Tomato; Lycopersicon esculentum; 2,5-dimethyl-4-hydroxy-3(2H)-furanone β-D-glucopyranoside; Furaneol; countercurrent chromatography*

INTRODUCTION

2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone (DMHF, Furaneol) (**1**) is an important flavor compound that can be found in various fruits such as pineapples (Rodin et al., 1965), strawberries (Re et al., 1973; Pickenhagen et al., 1981), arctic brambles (Kallio, 1976), grapes (Rapp et al., 1980), and raspberries (Honkanen, 1980; Pabst et al., 1991). Glycosidically bound DMHF has been found in strawberries (Mayerl et al., 1989), pineapples (Wu et al., 1990, 1991), and raspberries (Pabst et al., 1991). Mayerl and co-workers showed that 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone β-D-glucopyranoside (**2**) exists



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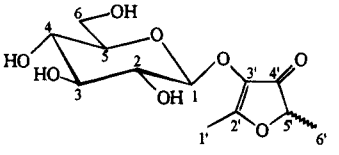

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as a mixture of diastereomers in strawberries. The present study describes the application of adsorption chromatography on XAD-2 followed by countercurrent chromatography (CCC) and preparative high-performance liquid chromatography for the isolation of 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone β-D-glucopyranoside from fresh tomatoes as well as the structure elucidation using NMR spectroscopy and electrospray ionization together with tandem mass spectrometry (ES-MS/MS).

EXPERIMENTAL PROCEDURES

Reagents. The solvents (methanol, 1-butanol, acetonitrile, dichloromethane, diethyl ether) were of HPLC grade from Fisher Scientific Co. (Springfield, NJ). Diethyl ether was distilled before use. 2-Pentanol was obtained from Aldrich Chemical Co. (Milwaukee, WI). Purified water was obtained from a Milli-Q Plus water system (Millipore, Bedford, MA). DMHF was obtained from Aldrich. Amberlite XAD-2 (20–60 mesh) nonionic polymeric adsorbent was purchased from Sigma Chemical Co. (St. Louis, MO). The resin was cleaned by continuous extraction in a Soxhlet apparatus with methanol refluxing for 8 h.

Enzyme-Mediated Hydrolysis. The freeze-dried and preseparated glycosidic extracts (between 0.5 and 5 mg) were dissolved in 2 mL of 0.2 M phosphate buffer (pH 5.0). After almond β-glucosidase (1–3 mg; Sigma; EC 3.2.1.2; 33 units/mg of solid) or Cytolase PCL5 (50 μL; Genencor Inc., South San Francisco, CA) was added, the mixture was incubated at 37 °C overnight. The liberated aglycons were extracted with diethyl ether, dried (anhydrous Na_2SO_4), and concentrated. The extracts were subjected to GC and GC–MS analysis. Control runs without enzyme addition were also performed for each sample.

Isolation of a Glycosidic Extract from Tomatoes. Fresh, vine-ripened tomatoes (*Lycopersicon esculentum* Mill. var. 6203) were obtained in September 1993 from an experimental field in Davis, CA. Tomatoes were stored in ice for 30 min and blended in small portions (approximately 500–600 g) with methanol (110 mL) in a Waring blender for 30 s. The mixture was centrifuged (3000 rpm, 15 min) and the liquid filtered through glass wool. The collected liquid was cooled in an ice bath and maintained under a continuous flow of nitrogen. A total of 15.0 kg of tomatoes was prepared in this manner, yielding approximately 18 L of yellowish-orange solution. The solution was stored at −20 °C covered with argon. In several batches methanol was removed under oil pump vacuum (rotavapor; maximum bath temperature of 30 °C). The aqueous residue was diluted with distilled water to a total volume of 2 L and extracted five times with 200-mL aliquots of dichloromethane (1 L total). The remaining aqueous phase was subsequently extracted with five 200-mL portions of 1-butanol (1 L total). The extracted aqueous solution was concentrated to 300 mL *in vacuo*. Separation of the bound fraction was achieved using two pre-washed (Gunata et al., 1985) Amberlite XAD-2 columns (column 1, 3.8 cm i.d. × 43 cm; column 2, 3.2 cm × 102 cm; flow, 5 mL/min). Column 1 was loaded with 100 mL of solution and washed

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with 5000 mL of water. The glycosidic extract of column 1 was obtained by eluting with 300 mL of methanol. Column 2 was loaded with 200 mL of solution and washed with 7000 mL of water. This column was rinsed with 600 mL of methanol. The methanol fractions were pooled and concentrated *in vacuo*. The remaining residue was diluted with distilled water to a volume of 20 mL. This solution was then freeze-dried to yield 4.1 g of yellowish-brown powder. The powder was then separated by high-speed countercurrent chromatography in portions of 450–580 mg. Each sample was dissolved in the mobile phase and injected into the instrument with a Rheodyne (Cotati, CA) type 50 Teflon rotary valve with a 2-mL sample loop.

High-Speed Countercurrent Chromatography (HSCCC). A high-speed countercurrent chromatograph (Model CCC-1000) from Pharma-Tech Research Corp. (Baltimore, MD) equipped with three coil columns (Tefzel tubing 1.6 mm i.d., total volume 325 mL) was operated at room temperature (ca. 22 °C). Separations were performed in the "head to tail" mode (Conway, 1990) with a rotational rate of 1000 rpm. A 2-pentanol–water (1:1) solvent system was applied using the lower phase as the mobile phase (flow rate, 1 mL/min; Gilson Model 305 pump). The system was operated at a pressure of 552 kPa. The effluent was monitored at 280 nm using a Hewlett-Packard Series 1050 variable-wavelength detector equipped with preparative flow cell. Effluent was collected with a Foxy 200 fraction collector (ISCO, Lincoln, NE) in intervals of 5 min.

High-Performance Liquid Chromatography (HPLC). Preparative HPLC was carried out on a Gilson system 45NC (Gilson Medical Electronics, Middleton, WI) consisting of Model 305 and 306 pumps (Model 25 SC pump heads), a Model 805 manometric module, a Model 811 C dynamic mixer, and a Rheodyne Model 7125 injection valve. A Rainin (Woburn, MA) Dynamax 60A 8 μm C_{18}, 21.4 mm i.d. × 25 cm, column coupled with a 60A 8 μm C_{18}, 21.4 mm i.d. × 5 cm guard column was employed. For the final purification of the preseparated extracts a gradient from 7 to 17% aqueous CH_3CN at a flow rate of 12 mL/min was applied. The program started isocratic with 7% CH_3CN for 10 min, reaching 17% CH_3CN after 40 min.

Capillary Gas Chromatography–Mass Spectrometry (GC–MS). A HP 5890 Series II gas chromatograph equipped with a split/splitless injector was coupled to a HP5971 mass selective detector (capillary direct interface). A 60 m × 0.25 mm i.d. (d_f = 0.25 m) DB-1 (J&W Scientific, Folsom, CA) fused silica capillary column was used with following temperature program: 35 (4 min isothermal) to 225 °C (final hold 15 min) at 4 °C/min. The injector temperature was 190 °C, and the transfer line temperature was 220 °C. Splitless injections were employed (purge delay time was 1 min).

Electrospray Tandem Mass Spectrometry (ES-MS/MS). Electrospray mass spectra were obtained on a VG Quattro-BQ tandem mass spectrometer (Fisons Instruments, Manchester, U.K.) in positive and negative ion mode. An ISCO LC-500 pump was used to deliver a 50:50 (v/v) methanol–water mixture at a flow rate of 10 μL/min. The source temperature was 80 °C. Mass range was 50 to 500 amu. MS/MS experiments were performed using helium for collisional induced dissociation (CID).

NMR Spectroscopy. NMR spectra were taken on a Bruker ARX 400 spectrometer. ^{1}H NMR spectra were run using a presaturation experiment, presaturating the water signal. ^{13}C NMR spectra were run using a simple one-pulse sequence with broadband WALTZ decoupling.

RESULTS AND DISCUSSION

A clear yellowish-orange liquid was obtained by blending fresh tomato fruits with methanol followed by filtration and centrifugation. A glycosidic extract was isolated from the liquid by use of Amberlite XAD-2 columns (Gunata et al., 1985). After elution with methanol followed by a concentration step and freeze-drying, the glycosidic fraction was subjected to fractionation by high speed countercurrent chromatography (HSCCC) in the head to tail mode (Conway, 1990). The resulting fractions H1–28 were monitored by enzymatic hydrolysis to yield aglycons, which were checked by GC–MS. Enzymatic hydrolysis of fractions H9–11 (retention time = approximately 1 h 50 min) revealed the presence of DMHF (**1**) as confirmed by comparison of its retention index and mass spectrum with that of an authentic reference standard. These fractions obtained by several runs were pooled and purified by preparative HPLC on a reversed-phase C_{18} column. The HPLC chromatogram monitored at wavelength 280 nm as well as parallel enzymatic hydrolysis led to fraction R21 (retention time = 15 min), containing compound **2**.

Table 1. ^{1}H NMR Spectral Data of the Isolated 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone β-D-Glucopyranoside (2) (δ Referenced to 1.5 ppm as the Middle of the C-5′ Methyl Doublet, 400 MHz, D_2O)

δ	signal[a]	assignment	δ[b]
	Aglycon Moiety		
1.49, 1.51	3H, d (7.2)	CH_3-C-5′	1.46
2.39	3H, br t (1)	CH_3-C-2′	2.35
4.75–4.85	obscured	H-5′	4.7–4.8
	Glucose Moiety		
3.32–3.75	4H, m	H-2, H-3, H-4, H-5	
3.79	1H, dd (12; 5)	H-6_b	
3.92	1H, dd (12; 2)	H-6_a	
4.51	1H, d (8.1)	H-1	

[a] Coupling constants (J in Hz) in parentheses. [b] Assignments were made with the aid of published reference data: 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone β-D-glucopyranoside (**2**), the ^{1}H NMR values, particularly for the protons H-$6_{a/b}$ and H-1 are described to be close to those of phenolic β-glucosides (Mayerl et al., 1989).

The ^{1}H NMR spectrum of **2** was measured in D_2O (Table 1). Instead of using an internal shift reference, the center of the doublet pattern for the C-5′ methyl group was referenced to δ 1.5. The scale expansion of this part of the spectrum revealed the presence of two doublets at δ 1.49 and 1.51 having an identical coupling constant of J = 7.2 Hz. According to the work of Mayerl et al. (1989), this was interpreted as two doublets corresponding to the C-5′ methyl signals of two diastereomers of 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone β-D-glucopyranoside. After storing compound **2** dissolved in D_2O for 5 days in the refrigerator, we observed the doublet of the C-5′ methyl group appearing unsymmetrical. This effect has also been described for 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone β-D-glucopyranoside by Mayerl et al. (1989) and is caused by the incorporation of deuterium at C-5′. The high-field branch of the C-5′ methyl doublet and the singlet of the DC-5′ methyl group are overlapping, causing the shape of an unsymmetrical doublet.

For the C-2′ methyl group a triplet at δ 2.39 was found. The reference ^{1}H NMR data (400 MHz; D_2O) of DMHF (**1**) showed for the C-2 methyl group a doublet (J = 1 Hz) due to a long-range coupling with the proton at C-5. Therefore, the observed triplet was interpreted as an overlapping double doublet corresponding to the C-2′ methyl group of the two diastereomers.

In the reference ^{1}H NMR spectrum of DMHF (**1**) the signal for the proton at C-5 (1H, qq, J = 7, 1 Hz) was easily determined. In the spectrum of conjugate **2** the signal at 4.75–4.85 was partially obscured by the residual HDO signal. The signal of the anomeric proton was observed at δ 4.52 (J = 8.1 Hz), indicating the presence of β-glycosidic linkage.

Furthermore, from the ^{13}C NMR data obtained for the

Table 2. ^{13}C NMR Spectral Data of the Isolated 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone β-D-Glucopyranoside (2) (δ Referenced to the Signal of C-6 at 63.17 ppm, 100 MHz, Broadband Decoupled, D_2O)

position	δ^a	δ^b
	Aglycon Moiety	
C-1′	16.6	16.57
C-2′	188.8	188.70
C-3′	136.1	136.08
C-4′	203.4	203.30
C-5′	84.8	84.72
C-6′	18.1, 18.2	18.08, 18.17
	Glucose Moiety	
C-1	106.1	106.00
C-2	75.8	75.78[c]
C-3	78.3[d]	78.25
C-4	72.0	71.98[c]
C-5	78.9[d]	78.91
C-6	63.1	63.17

[a] Chemical shifts were assigned on the basis of the data published by Mayerl et al. (1989). [b] 2,5-Dimethyl-4-hydroxy-3(2*H*)-furanone β-D-glucopyranoside (**2**) (Mayerl et al., 1989). [c] Assignment of C-2 and C-4 has been corrected after comparison with the ^{13}C NMR data of methyl β-D-glucopyranoside (Breitmaier and Voelter, 1974). [d] Interchangeable values.

sugar moiety of compound **2**, high coincidence with data published for aryl β-glucosides (Schwab and Schreier, 1988) as well as 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone β-D-glucopyranoside (Mayerl et al., 1989) was evident. For the assignment of C-2 and C-4 the ^{13}C NMR data of methyl β-D-glucopyranoside (Breitmaier and Voelter, 1974) have additionally been considered. The signal for C-6 of the glucose moiety was referenced to δ 63.17 (Table 2). The ^{13}C NMR data for the aglycon were also identical with the data published by Mayerl et al. (1989), clearly showing the presence of two diastereomers.

Further characterization of the isolated glucoside was done by electrospray ionization together with tandem mass spectrometry (ES-MS/MS). The sample was stored in D_2O and diluted with MeOH–water (50:50 v/v) for the analysis. In the positive ion mode prominent protonated as well as pseudomolecular ions at m/z 291 $[M + H]^+$, 292 $[M - H + D + H]^+$, 313 $[M + Na]^+$, 314 $[M - H + D + Na]^+$, and 315 $[M - 2H + 2D + Na]^+$ were observed. Subsequent positive ion electrospray MS/MS experiments on the protonated molecular ion at m/z 291 yielded a daughter ion at m/z 129, corresponding to protonated Furaneol, $C_6H_9O_3^+$. Further MS/MS experiments on the pseudomolecular ion at m/z 314 exhibited daughter ions at m/z 23 $[Na]^+$ and 151 $[Furaneol + Na]^+$. In the negative ion mode the deprotonated molecular ion at m/z 289 $[M - H]^-$, accompanied by an isotope cluster from m/z 290 to 296 due to H/D exchange, was detected.

To our knowledge, this is the first report of 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone β-D-glucopyranoside (**2**) in fresh tomatoes. The content of glucoside **2** in tomatoes seems to be smaller than in strawberries (Mayerl et al., 1989) and pineapples (Wu et al., 1990). To learn about an influence of **2** on the volatile flavor of tomatoes, further quantitative studies are necessary.

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