Minor Furanocoumarins and Coumarins in Grapefruit Peel Oil as Inhibitors of Human Cytochrome P450 3A4

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A new cyclic acetal (**1**) of marmin (6′,7′-dihydroxy-7-geranyloxycoumarin), two new cyclic acetals (**5**, **6**) of 6′,7′-dihydroxybergamottin, and the known compounds marmin (**2**), 7-geranyloxycoumarin (**3**), bergamottin (**4**), and 6′,7′-dihydroxybergamottin (**7**) were isolated from grapefruit peel oil. All compounds were tested for inhibitory activity against intestinal cytochrome P450 3A4, an enzyme involved in the "grapefruit/drug" interactions in humans. Coumarins (**1**−**3**) exhibited negligible inhibitory activity, while the furanocoumarins (**4**−**7**) showed potent in vitro inhibitory activity with IC_{50} values of 2.42, 0.13, 0.27, and 1.58 μM, respectively.

Grapefruit (*Citrus paradisi* MacFad.) (Rutaceae) contains many diverse bergamottin (5-geranyloxypsoralen) derivatives including trace-occurring mixed-species dimers.[1−4] Many of these compounds have been shown to influence the oral bioavailability of prescription drugs whose primary metabolic pathways involve the intestinal cytochrome P450 3A4 (CYP3A4).[2,3,5,6] Although most of the main coumarins and furanocoumarins in grapefruit have been reported,[7−9] many minor constituents remain uncharacterized. This is particularly evident in chromatographic analyses of the nonvolatile constituents of grapefruit peel oil.[10,11] This paper reports our investigation of minor-occurring furanocoumarins in nonvolatile residues of high-vacuum distilled grapefruit peel oil and provides information on three new acetals, including 6′,7′-marmin decanal acetal (**1**), 6′,7′-dihydroxybergamottin octanal acetal (**5**), and 6′,7′-dihydroxybergamottin decanal acetal (**6**), along with the isolation of the known marmin (**2**), 7-geranyloxycoumarin (**3**), bergamottin (**4**), and 6′,7′-dihydroxybergamottin (**7**).[7−10] Neral and geranial acetals of oxypeucedanin, 5-(2′,3′-dihydroxyisopentyloxy)psoralen, represent similar structures reported in lime oil.[11]

The isolation of (furano)coumarin octanal and decanal acetals **1**, **5**, and **6** and the known compounds **2**−**4** and **7** was achieved by sequential normal- and reversed-phase column chromatography and semipreparative HPLC of acetone extracts of grapefruit oil nonvolatile residues. ^{1}H and ^{13}C NMR spectra were obtained for compounds **1**−**7** in DMSO-d_6. NMR data of the known compounds **2**−**4** and **7** were in agreement with literature values.[7−13] Assignments of NMR data for **3**, **5**, and **6** were based on published assignments for related compounds.[11,13−17]

The UV spectrum of **1** closely matched the spectrum of 7-geranyloxycoumarin (**3**), a major coumarin in grapefruit peel oil.[9] The ESIMS spectrum of **1** exhibited an $[M + H]^+$ ion at *m*/*z* 471, major fragment ions at *m*/*z* 315, 297, and 163, and neutral losses of 156, 174, and 308 amu. The major fragment ions at 297 and 163 of **1** are similarly observed in the ESIMS spectrum of **3** (data not shown). HRMS and the ^{13}C and ^{1}H NMR data of **1** (Tables 1 and 2, respectively) are consistent with a molecular formula of $C_{29}H_{42}O_5$. The intense IR absorption band at 1733 cm^{-1} and the ^{13}C NMR resonance at 160.22 ppm are attributed to the presence of a carbonyl functional group. The presence of a linear alkane chain is suggested by the intense methylene IR vibration at 2924 cm^{-1} and the near absence of a methyl stretch at ∼2955 cm^{-1}. This is in agreement with the broad ^{1}H NMR methylene proton resonance at 1.23 ppm with a large integration value and the absence of other terminal methyl group resonances other than those of C-10″ of the alkane substituent and of the geranyl C-3′ and C-7′ methyl groups. The assignment of an acetal group to C-1″ of **1** is consistent with the observation of a ^{1}H NMR resonance at δ 4.77 (H-1″, t, J = 4.72 Hz) and the ^{13}C NMR resonance at δ 101.2.[11] The ^{1}H NMR spectrum revealed three signals associated with protons (H-1′ δ 4.67; H-6′ δ 3.44; H-1″ δ 4.77) covalently bonded to three alkoxy carbons (C-1′, C-6′, and C-1″), whereas the ^{13}C NMR data provided evidence of four alkoxy carbons (C-1′ δ 65.1, C-6′ δ 83.6, C-7′ δ 78.7, and C-1″ δ 101.2). These data supported a structure lacking

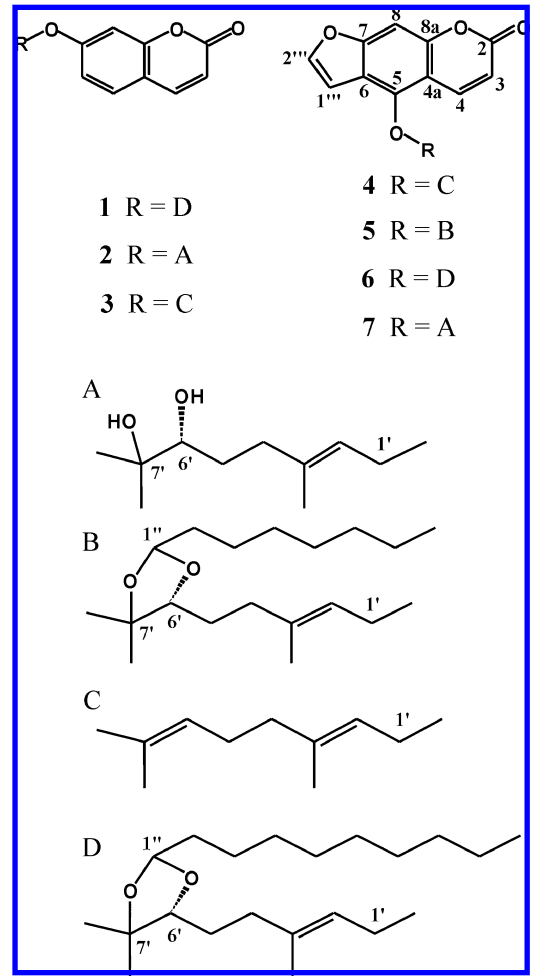

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Table 1. ^{13}C NMR Data (δ) for **1**, **5**, and **6**[a]

position	1	5	6
1			
2	160.2	160.0	160.0
3	112.9	114.0	114.1
4	144.3	139.5	139.6
4a	112.3	106.9	107.0
5	129.4	148.4	148.2
6	112.2	112.3	112.4
7	161.6	157.4	157.4
8	101.4	93.6	93.7
8a	155.3	152.0	152.0
1‴		105.4	105.4
2‴		146.0	146.1
1′	65.1	69.1	69.1
2′	119.1	119.3	119.4
3′	140.9	141.9	142.0
4′	36.0	36.1	36.0
5′	27.2	27.2	27.2
6′	83.6	83.4	83.4
7′	78.7	78.6	78.7
3′-CH_3	16.4	16.3	16.3
7′-$(CH_3)_2$	23.5/23.3	22.0/23.2	22.1/23.3
1″	101.2	101.1	101.1
2″	34.3	34.4	34.4
3″	25.2	25.1	25.2
4″	28.9/28.6	28.9	28.9
5″	28.9/28.6	28.6	28.9
6″	28.9/28.6	31.2	28.9
7″	28.9/28.6	23.5	28.6
8″	31.2	13.8	31.2
9″	22.1		23.5
10″	13.9		13.9

[a] Spectra were recorded in DMSO-d_6.

protons bonded to C-7′ and the involvement of C-6′ and C-7′ in the acetal portion of **1**. The neutral loss of 156 amu in the ESIMS spectrum is consistent with the loss of 1-decanal ($C_{10}H_{20}O$), and the neutral loss of 174 is consistent with the loss of 1-decandiol ($C_{10}H_{22}O_2$). Mild acid hydrolysis of **1** produced 7-geranyloxycoumarin and subsequently 7-hydroxycoumarin (umbelliferone) (data not shown). Facile hydrolysis under mild acidic conditions supports the acetal structure.

To further verify this structure, **1** was synthesized from **2** and decanal using reaction conditions for the synthesis of acetals, similar to that reported by Feger et al.[11] The MS and the ^1H and ^{13}C NMR data of the synthesized product and the originally isolated **1** were identical. Marmin (**2**) was previously shown to possess an *R*-configuration,[18] and thus, **1** was identified as (*R*)-6′,7′-marmin decanal acetal.

In contrast to **1**, compound **5** exhibited a UV spectrum closely similar to the furanocoumarins bergamottin (**4**) and 6′,7′-dihydroxybergamottin (**7**), also isolated from the grapefruit peel oil nonvolatile residues.[7,12,13] The ESIMS spectrum of **5** exhibited an $[M + H]^+$ ion at *m/z* 483, major fragment ions at *m/z* 337 and 203, and neutral losses of 146 and 280 amu. The major fragment ions at 337 and 203 are similarly observed in the ESIMS of **4** and **7**. The HRMS and the ^{13}C and ^1H NMR data (Tables 1 and 2, respectively) are consistent with a molecular formula of $C_{29}H_{38}O_6$. Similar to the data of **1**, the IR absorption band at 1732 cm^{-1} and the ^{13}C NMR resonance at δ160.0 are attributed to a carbonyl functional group in **5**. An alkane chain in **5** is suggested by the intense methylene IR vibration at 2922 cm^{-1} and by the ^1H NMR data (Table 2) in a manner similar to **1**. The proton resonance of H-4 (δ 8.17) confirms the 5-*O* substitution.[19] Also similar to **1**, the ^{13}C and ^1H NMR data are consistent with the absence of H-7′ and with the involvement of C-6′ and C-7′ in the acetal portion of **5**. The neutral loss of 146 amu in the ESIMS spectrum is consistent with the loss of 1-octandiol ($C_8H_{18}O_2$), leaving the fragment ion at *m/z* 337 for which we propose the structure of 6′,7′-dehydrobergamottin. Mild acid hydrolysis of **5** produced 6′,7′-dihydroxybergamottin (**7**) and subsequently bergaptol. Compound **5** was thus identified as 6′,7′-dihydroxybergamottin octanal acetal.

Table 2. ^1H NMR Data for **1**, **5**, and **6**[a]

	δ_H (*J* in Hz)		
position	1	5	6
1			
2			
3	6.27, d (9.2)	6.31, d (9.8)	6.32, d (9.6)
4	7.98, d (9.2)	8.17, d (10.0)	8.19, d (9.7)
5	7.61, d (8.6)		
6	6.93, dd (8.8, 2.3)		
7			
8	6.98, d (2.4)	7.35, s	7.38, s
1‴		7.33, d (2.2)	7.34, d (2.2)
2‴		8.04, d (2.1)	8.05, d (2.3)
1′	4.67, d (6.4)	5.02, dd (1.3, 6.7)	5.02, dd (2.6, 6.8)
2′	5.46, dd (5.7, 6.6)	5.46, dd (6.2, 6.8)	5.53, dd (6.1, 6.8)
3′			
4′	1.50, m	1.50, m	1.48, m
5′	2.13, m	2.13, m	2.12, m
6′	3.44, dd (9.7, 3.4)	3.35, dd (9.1, 3.9)	3.32, m
7′			
3′-CH_3	1.74, s	1.67, s	1.66, s
7′-$(CH_3)_2$	1.15, s	1.00, s; 1.1, s	0.99, s; 1.12, s
1″	4.77, t (4.7)	4.73, t (4.7)	4.73, t (4.7)
2″−7″		1.22, m	
2″−9″	1.23, m		1.22, m
CH_3-7″		0.84, s	
CH_3-9″	0.85, s		0.84, s

[a] Spectra were recorded in DMSO-d_6.

The UV and ESIMS spectra of **6** also suggest a furanocoumarin structure with an $[M + H]^+$ ion at *m/z* 511, with neutral losses of 156, 174, and 308 amu, along with fragment ions at *m/z* 355, 337, and 203. The HRMS and the ^1H and ^{13}C NMR data suggest a molecular formula of $C_{31}H_{42}O_6$. Similar to the data for **5**, a comparison of the data of **6** with compounds with related chemical structures indicates a bergaptol (5-hydroxypsoralen) moiety with a substituted geranyl side chain. The IR spectrum as well as the ^{13}C and ^1H NMR spectra (Tables 1 and 2, respectively) of **6** are nearly identical to those of **5**, with the exception of two additional methylene groups in the linear C_{10} alkane substituent of **6**. The neutral losses of 156 and 174 amu in the ESIMS spectrum of **6** are consistent with the loss of 1-decanal ($C_{10}H_{20}O$) and 1-decandiol ($C_{10}H_{22}O_2$), respectively. These neutral losses were similarly observed for **1**. Mild acid treatment of **6** produced 6′,7′-dihydroxybergamottin (**7**) and subsequently bergaptol. The facile hydrolysis under mild acidic conditions further supports an acetal structure. These data allowed us to propose the structure of 6′,7′-dihydroxybergamottin decanal acetal.

Compounds **5** and **6** were synthesized from **7** by combining with octanal and decanal, respectively, using reaction conditions for the synthesis of acetals.[11] These syntheses produced single compounds that exhibited identical ESIMS and ^1H and ^{13}C NMR spectra with the originally isolated compounds. These results support the octanal and decanal acetal structures proposed for **5** and **6**, respectively. The absolute configuration of **7** was previously determined as *R*,[20] and thus the absolute configurations of **5** and **6** were similarly assigned.

The IC_{50} values for the inhibition of in vitro CYP3A4 by **1**−**7** (Table 3) show that compounds **5** and **6** are far more potent CYP3A4 inhibitors (IC_{50} values of 0.13 and 0.27 μM, respectively) than the positive controls **4** and **7** (IC_{50} values of 2.42 and 1.58 μM, respectively). This may be due to the significantly higher lipophilicity of **5** and **6** due to the additional octanal and decanal chains. The coumarins, **1**−**3**, are inactive as CYP3A4 inhibitors. The ease of synthesis of **5** and **6** makes these compounds attractive model compounds to further investigate the irreversible CYP3A4 inhibition by grapefruit juice furanocoumarins.

Table 3. IC_{50} (μM) of Cytochrome P450 3A4 Inhibition by Compounds **1**−**7**[a]

compound	IC_{50} values
6′,7′-marmin decanal acetal (**1**)	ND[b]
marmin (**2**)	ND
7-geranyloxycoumarin (**3**)	ND
bergamottin (**4**)	2.42 ± 0.47
6′,7′-dihydroxybergamottin octanal acetal (**5**)	0.13 ± 0.06
6′,7′-dihydroxybergamottin decanal acetal (**6**)	0.27 ± 0.08
6′,7′-dihydroxybergamottin (**7**)	1.58 ± 0.99

[a] Data represent means ± standard deviations of triplicates. [b] ND (not detected) indicates a limited inhibition of CYP3A4 by a compound.

Experimental Section

General Experimental Procedures. Melting points were determined with a Thomas-Hoover Capillary melting point apparatus. UV spectra were recorded on a UV-2401 PC Shimadzu UV−visible spectrophotometer. FTIR spectra were recorded with a Perkin-Elmer Spectrum One with compounds applied to KBr IR cards (International Crystals Lab, Garfield, NJ). Compounds were dissolved in DMSO-d_6, and the ^{1}H and ^{13}C NMR spectra were obtained with a 400 MHz Varian INOVA magnet system with TMS as internal standard. ESIMS were measured with a Waters Micromass ZQ single quadrupole LC mass spectrometer. HRESIMS were measured with a Micromass, Inc. Autospec mass spectrometer (University of Iowa, Iowa City, Iowa). Materials for column chromatography were silica gel (70−230 mesh; Sigma, St. Louis, MO) and Redisep normal- and reversed-phase C18 columns (Teledyne Isco, Inc., Lincoln, NE), attached to either a Horizon Flash Chromatography System (Biotage, Uppsala, Sweden) or an ISCO Combiflash 100 Column Chromatography System with a type 11 UV detector equipped with 340/365 filter. Preparative TLC was run with tapered, glass precoated silica gel plates GF254 (Analtech, Newark, NJ).

Grapefruit Oil Residue. The grapefruit nonvolatile residue from vacuum-distilled oils from mixed grapefruit varieties was obtained from an industrial source.

Assay for Inhibition of Cytochrome P450 3A4. In vitro CYP3A4 inhibition assays of the (furano)coumarins were conducted using a Vivid CYP450 screening kit (Invitrogen, Madison, WI) according to the manufacturer's protocol. Test compounds were added to 100 μL of total reaction volume at different final concentrations, which ranged from 0.24 to 500 ng/100 μL. Two compounds known as CYP3A4 inhibitors, **4** and **7**, were used as controls. All assays were performed using a Synergy HT multidetection microplate reader (BioTek, Winooski, VT) with three replicates. The IC_{50} (50% inhibitory concentration) value of each compound was determined on the basis of fluorescence measurement (emission at 485 nm and excitation at 530 nm) at 30 min after initiation of reaction. In our experiments, fluorescence levels were monitored for 30 min using kinetic assay mode with 3 min intervals. The levels measured at 20 min after initiation of the reaction remained constant (data not shown), indicating a termination of the enzymatic reactions.

Extraction and Isolation. The nonvolatile residue obtained from grapefruit peel oil was dissolved in acetone (100 g/L), filtered through 100SH/BX filter papers (Fisher Scientific), combined with 400 g of silica gel, and vacuum evaporated until the furanocoumarin-loaded paste was a free-flowing powder. The furanocoumarin-loaded silica gel was packed in a column and washed with hexanes and EtOAc solvent mixtures [1 L, 1:0; 2:1; 1:1; 1:2; and 0:1 (v/v)]. The resulting fractions were separately mixed with 100 g of silica gel and dried under vacuum to obtain powdered samples. The sample-loaded silica gel was subjected to column chromatography using 40 and 120 g Redisep silica gel columns and eluted successively with hexanes/EtOAc gradient mixtures of increasing polarity. Column fractions (30 mL) were collected and analyzed by HPLC.

Further isolations were performed by preparative TLC using solvent systems previously described.[9] Some compounds were further purified using an Atlantis (19 × 100 mm) 5 μm semipreparative column (Waters, Milford, MA) attached to a Waters 600 HPLC controller and 996 PDA. Fuanocoumarin separations were performed using an 85 min linear gradient of H_2O/MeCN (40:60 to 15:85) at a flow rate of 5 mL/min. The chromatograms were recorded at 310 nm and analyzed using MassLynx software ver. 3.5 (Micromass, Division of Waters Corp., Beverly, MA). ESIMS analyses were performed as described previously.[4]

Synthesis of 6′,7′-Marmin Decanal Acetal (1), 6′,7′-Dihydroxybergamottin Octanal Acetal (5), and 6′,7′-Dihydroxybergamottin Decanal Acetal (6). Marmin (15.0 mg), dissolved in 2 mL of acetone, was combined with 15 μL of decanal and shaken for 15 min at RT. The solution was injected onto a semipreparative Atlantis C18 column equilibrated with H_2O/MeCN (85:15 (v/v)). A linear gradient to 100% MeCN was run over 60 min at 5 mL/min. The reaction resulted in **1** as the sole product, which was collected, analyzed by HPLC-ESIMS, and submitted to ^{1}H and ^{13}C NMR analyses and melting point determination. The same methods were used for the syntheses of **5** and **6**, using 6′,7′-dihydroxybergamottin, and octanal and decanal, respectively.

6′,7′- Marmin Decanal Acetal (1): white, amorphous powder; UV (MeOH) λ_{max} nm (log ε) 324 (4.18); 253 (1.05) nm; IR ν(KBr) cm^{-1} 3073, 2924, 2854, 2733, 1613, 1556, 1506, 1464, 1120, 999, 833; HRESIMS *m/z* 493.2914 $[M + Na]^+$ (calcd for $C_{29}H_{42}O_5Na$, 493.2930); ^{13}C NMR see Table 1, and ^{1}H NMR see Table 2.

6′,7′-Dihydroxybergamottin Octanal Acetal (5): white, amorphous powder; UV (MeOH) λ_{max} nm (log ε) 310 (4.05), 268 (sh), 260 (sh), 251 (4.17) nm; IR ν(KBr) cm^{-1} 3159, 2967, 2922, 2853, 1732, 1625, 1578, 1455, 1127, 1027; HRESIMS *m/z* 505.2577 $[M + Na]^+$ (calcd for $C_{29}H_{38}O_6Na$, 505.2566); ^{13}C NMR see Table 1, and ^{1}H NMR see Table 2.

6′,7′-Dihydroxybergamottin Decanal Acetal (6): white, amorphous powder; UV (MeOH) λ_{max} nm (log ε) 314 (4.15), 267 (sh), 260 (sh), 251 (4.14); IR ν(KBr) cm^{-1} 3160, 2967, 2924, 2854, 1734, 1612, 1579, 1456, 1127, 1074; HRESIMS *m/z* 533.2886 $[M + Na]^+$ (calcd for $C_{31}H_{42}O_6Na$, 533.2879); ^{13}C NMR see Table 1, and ^{1}H NMR see Table 2.

Acknowledgment. Mention of a trademark or proprietary product is for identification only and does not imply a guarantee or warranty of the product by the U.S. Department of Agriculture.

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