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Spectral Characterics of Grapefruit Peel Oil Furanocoumarins and Coumarins

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Grapefruit peel oil (GPO) contains numerous coumarins and furanocoumarins, many of which are uncharacterized. In this study, seven furanocoumarins (FC) and three coumarins were isolated from GPO by silica gel chromatography, preparative TLC, and reversed-phase HPLC. Mass spectral analyses of the FCs showed that these compounds were complex derivatives of bergaptol (5-hydroxypsoralen), the majority of which contained variously substituted geranyl side chains. The three coumarins isolated in this study also contained substituted geranyl side chains. Analyses by Fourier transform infrared (FTIR) spectroscopy provided further information about the functional groups of the compounds isolated in this study.

Grapefruit contains many phenolic compounds, including a number of important classes of flavonoids, coumarins, and related psoralens [furanocoumarins (FC)] (Dugo and McHale, 2002; Horowitz and Gentili, 1977; Kanes et al., 1993; and references therein). While the flavonoids in grapefruit are popularly considered as health-beneficial compounds (Manthey et al., 2001), the FCs have presented challenges to the citrus industry through their interference in the metabolism of certain drugs in humans (reviewed in Paine and Oberlies, 2007). This interference occurs through the irreversible inhibition of intestinal cytochrome P450 3A4, which is responsible for the first pass metabolism of these affected drugs. The result of this interference is an undesirable elevation of the levels of prescription drugs in the consumer (Dresser et al., 2000). This interaction, termed "grapefruit/drug interaction" (Bailey et al., 1998), has contributed to the sharp drop in the domestic grapefruit consumption that has occurred over the past 8 to 10 years (Citrus Administrative Committee, 2006).

There has been a tremendous effort to develop technologies to overcome the grapefruit/drug interactions, and one aspect of this work has been to gain a better understanding of the chemical structures of the active FCs. However, one of the problems associated with this study is the very low concentrations at which many of the active FCs are found in grapefruit, particularly in the juice. These very low concentrations make the isolation of sufficient quantities for structural determination extremely difficult. However, one source of concentrated grapefruit FCs occurs in the peel oil, and chemical characterization of a number of the major FCs in this material has been previously reported (Stanley and Jurd, 1971; Tatum and Berry, 1979). Yet, studies of the FCs in grapefruit juice have shown that these compounds are typically not the same major FCs in grapefruit peel oil (GPO) (Fukuda et al., 2000; Widmer and Haun, 2005). Yet, some FCs in grapefruit juice are also present in the GPO in quantities sufficient for their isolation. Beyond this, there are many additional compounds in GPO that appear to have very similar chemical structures to the juice compounds, and the higher amounts of these compounds in GPO make them attractive targets in the search for active FCs in grapefruit/drug interaction studies as well as for other biological activities. This paper describes the isolation of seven FCs and three coumarins from GPO and their preliminary structural evaluations by UV, MS, and Fourier transform infrared (FTIR) spectroscopy.

Material and Methods

Sample preparation. A nonvolatile residue was obtained from peel oil of mixed grapefruit varieties. The GPO was added to acetone (100 g·L^{-1}) and shaken (250 rpm) for 1 h. The resulting solutions were filtered through 100SH/BX filter papers (Fisher Scientific, Pittsburgh), and the retained acetone-insoluble residues were washed with an additional 300 mL acetone. The filtrates were mixed with 400 g silica gel (70–230 mesh) and dried using a rotary evaporator at 34 °C. Fractions were collected from five different hexane and ethyl acetate solvent mixtures (1 L), consisting of [1:0; 2:1; 1:1; 1:2; and 0:1 (v/v)]. Contents of the fractions were examined by HPLC (described below).

Furanocoumarin isolation. The above fractions were separately mixed with 75 g silica gel 60 (230–400 mesh), and dried under vacuum to obtain a powdered sample. The sample-loaded silica gel (~100 g) was packed into an empty column; the sample loaded column was then attached to 40 g and 120 g Redisep silica gel columns (ISCO, Lincoln, NE) connected in series. The FCs were eluted using a Horizon flash chromatography system (Biotage, Uppsala, Sweden). Elution of the FCs was started with 1 L hexane, followed by sequential linear gradients of hexane/ethyl acetate to (90/10; 1 L), then to (80/20; 1.2 L), then isocratically at (80/20, 1.2 L). Subsequent linear gradients were run to hexane/ethyl acetate (40/60; 1.2 L), to (20/80; 1.2 L), and to 100% ethyl acetate in 1.0 L, and finally acetone (0.5 L). Column fractions (30 mL) were collected and groups of six sequential fractions were pooled and analyzed by HPLC.

Further isolations were performed by preparative thin layer chromatography (TLC) using precoated silica plates (Uniplate

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#81013, tappered layer, Analtech, Newark, DE), with fluorescence indicator. TLC was performed as previously described (Tatum and Berry, 1979). Samples containing similar sets of compounds were combined, mixed with LRP C18 resin (Whatman, Florham Park, NJ) (13 g), and dried under vacuum. The sample-loaded resin was packed into an empty column and attached to an ISCO RediSep Flash C18 reverse phase (130g) column. Separations were performed with an ISCO Combiflash 100 chromatography system, using linear gradients of methanol/water from (20:80 v/v) to 100% methanol. Final compound purifications were achieved with an Atlantis (19 × 100 mm) 5 µm semi-preparative column (Waters, Milford, MA) attached to a Waters 600 HPLC controller and 996 PDA. FC separations were performed using linear gradients of water/acetonitrile (40/60) to (15/85) at a flow rate of 5 mL·min^{-1}. The chromatograms were recorded at 310 nm, and analyzed using MassLynx software ver. 3.5 (Micromass, Division of Waters Corp., Beverly, MA).

HPLC–PDA analysis. FC fractions were analyzed with a Varian ProStar (HPLC) equipped with a Varian 335 photodiode array (PDA) detector. Chromatography of the FCs was performed on a Discovery RP Amide C16 column (150 × 3 mm) (Supelco, Bellefonte, PA), with multistep linear gradients at a flow rate of 0.75 mL·min^{-1}. The gradients of aqueous 2% acetic acid/acetonitrile started at 90/10 (v/v), then increased to 60/40 (v/v) by 15 min, then to 30/70 (v/v) by 40 min, then to 5/95 (v/v) by 52 min. Data handling was done with Star Chromatography Work Station ver. 6.41

HPLC–PDA–MS analysis. A previously published HPLC–ESI–MS method of analysis of grapefruit FCs was used with modifications (Manthey et al., 2006).

Structural analysis. Preliminary analyses of the structures of the 10 unknown compounds were carried out using ESI-mass spectrometry, UV spectroscopy, and FTIR. MS analyses were performed as described previously (Manthey et al., 2006). FTIR spectra were recorded with a Spectrum One FTIR spectrophotometer (PerkinElmer, Waltham, MA). Compounds were dried on KBr cards (International Crystal, Garfield, NJ) for FTIR analysis.

Results

Isolation and characterization. Ten compounds were isolated from a GPO residue obtained from mixed grapefruit varieties. Preliminary structural analyses of these compounds were performed by UV, MS, and FTIR spectroscopy.

Compound 1. MS: 493 $(M+Na)^{+23}$, 471$(M+H)^{+1}$, 391, 315, 297, 163. UV λ_{max} nm: 323. IR ν_{max} cm^{-1}: 3073, 2924, 2854, 1733 ($\nu_{C=O}$), 1613 ($\nu_{C=C}$), 1556 ($\nu_{C=C}$), 1506, 1464, 1120, 999, 833. The fragment ion at 297 *m/z* is proposed to arise from the collision-induced formation of dehydrogeranyl-7-oxycoumarin (Fig. 1). The coumarin portion of **1** is indicated by the 163 *m/z* fragment ion, i.e., the $(M+H)^{+1}$ of 7-hyroxycoumarin (umbelliferone). The absence of a free hydroxyl group in **1** is demonstrated by the absence of the ν_{O-H} stretch between 3500 and 3200 cm^{-1}. The methylene ν_{CH2} stretches at 2924 and 2854 cm^{-1} are significantly higher in intensity over the corresponding methyl ν_{CH3} vibrations. This indicates that the number of methylene groups in **1** is substantially greater than the number of methyl groups. This suggests the possibility of an alkane chain (>C4) as a portion of **1**.

Compound 2. This compound exhibits an identical HPLC elution time, MS, and FTIR as authentic bergamottin (Tatum and Berry 1979). MS: 339 $(M+H)^{+1}$, 203 (hydroxypsoralen (bergaptol)). UV λ_{max} nm: 309, 263 (sh), 259 (sh). IR ν_{max} cm^{-1}: 3159, 3126, 2967, 2922, 2853, 1732 ($\nu_{C=O}$), 1625 ($\nu_{C=C}$), 1578 ($\nu_{C=C}$), 1455, 1127, 1027.

Compound 3. MS: 505 $(M+Na)^{+23}$, 483 $(M+H)^{+1}$, 355, 337, 203 (bergaptol). UV λ_{max} nm: 309, 263 (sh), 260 (sh). IR ν_{max} cm^{-1}: 3155, 3125, 2924, 2854, 1732 ($\nu_{C=O}$), 1624 ($\nu_{C=C}$), 1578 ($\nu_{C=C}$), 1455, 1124, 1071, 823. The high-energy vibrational spectrum (4000–2600 cm^{-1}) of **3** is nearly identical to that of **1**. The fragment ion at 203 *m/z* supports the presence of bergaptol as a portion of **3**. Bergaptol is a product of mild acid hydrolysis of **3** (data not shown). It is proposed that the 337 *m/z* fragment ion is due to the formation of 5-dehydrogeranylbergaptol (Fig. 1B) from the molecular ion, similar to the fragmentation pathway proposed for **1** in Fig. 1A.

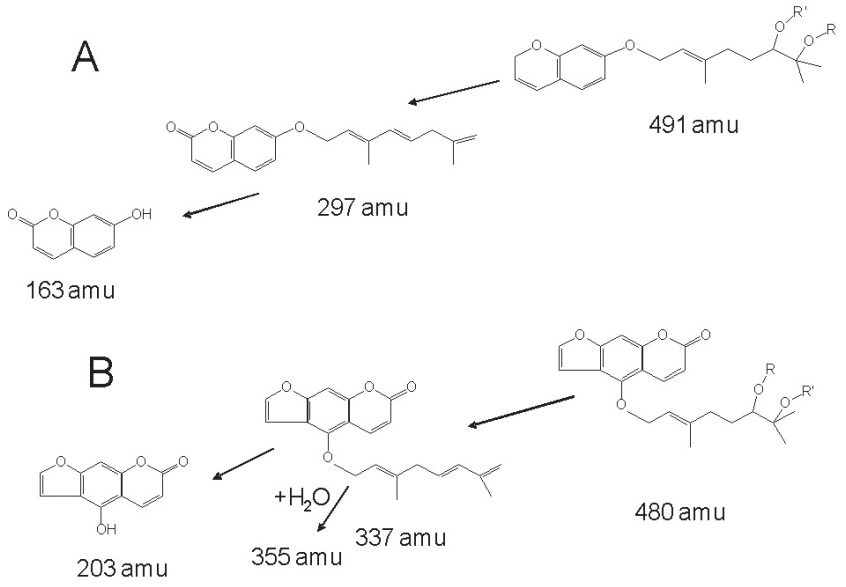

Fig. 1. (**A**) Proposed scheme for the production of the 297 amu fragment ion from **1**. (**B**) Proposed scheme for the production of the 337 amu fragment ion from **3**. Addition of H_2O to the 337 adduct produces the 355 amu species.

Compound 4. MS: 595 $(M+Na)^{+23}$, 573 $(M+H)^{+1}$, 355, 337, 203 (bergaptol). UV λ_{max} nm: 309, 264 (sh), 260 (sh). IR ν_{max} cm^{-1}: 3449, 2924, 2853, 1735 ($\nu_{C=O}$), 1624 ($\nu_{C=C}$), 1578 ($\nu_{C=C}$), 1456, 1126, 1073, 825. The presence of a free hydroxyl in **4** is evident by the intense ν_{O-H} vibration at 3449 cm^{-1}. Similar to **1** and **3**, the intensity of the ν_{CH2} vibration is far greater than for ν_{CH3}, suggesting the presence of a linear alkane (>C4) attached to the geranyl substituent of **4**.

Compound 5. MS: 533 $(M+Na)^{+23}$, 511 $(M+H)^{+1}$, 355, 337, 203 (bergaptol). UV λ_{max} nm: 309, 264 (sh), 260 (sh). IR ν_{max} cm^{-1}: 3160, 3127, 2924, 2854, 1734 ($\nu_{C=O}$), 1612 ($\nu_{C=C}$), 1579 ($\nu_{C=C}$), 1456, 1127, 1074, 826. Similar to **1** , **3**, and **4,** the intenisty of the $\nu_{methylene}$ is far greater than for the ν_{methyl}, suggesting the presence of an (>C4) alkane substituent attached to the geranyl side chain. The mid- to low-energy vibrational spectrum of **5** is nearly identical to **3** and **4.**

Compound 6. MS: 519 $(M+Na)^{+23}$, 497 $(M+H)^{+1}$, 355, 337, 203 (bergaptol). UV λ_{max} nm: 309, 264 (sh), 259 (sh). IR ν_{max} cm^{-1}: 3160, 3125, 2960, 2920, 2851, 1741 ($\nu_{C=O}$), 1626 ($\nu_{C=C}$), 1580 ($\nu_{C=C}$), 1456, 1259, 799. Similar to **1**, **3**, and **5**, there is an absence of ν_{O-H} between 3500 and 3200 cm^{-1}. Unlike these earlier compounds, the symmetric ν_{CH3} stretch at 2960 cm^{-1} is far more prominent, and suggests that there is a closer match in the relative numbers of methyl and methylene groups. This distinguishes **6** from the earlier compounds, and suggests the occurrence of key differences in the side group(s) attached to the geranyloxy substituent.

Compound 7. MS: 529 $(M+Na)^{+23}$, 507 $(M+H)^{+1}$, 355, 337, 203 (bergaptol). UV λ_{max} nm: 309, 263 (sh), 260 (sh). IR ν_{max} cm^{-1}: 2957, 2854, 2825, 1735 ($\nu_{C=O}$), 1625 ($\nu_{C=C}$), 1579 ($\nu_{C=C}$), 1457, 1377, 1127, 825. The infrared spectrum indicates the absence of a free hydroxyl group, and a closely matching number of methyl and methylene groups. Significant differences occur in the mid- to low-energy vibrations relative to **6**.

Compound 8. MS: 355 $(M+Na)^{+23}$, 333 $(M+H)^{+1}$, 315, 297. UV λ_{max} nm: 322, 229. IR ν_{max} cm^{-1}: 3416, 3082, 3043, 2967, 2851, 1726 ($\nu_{C=O}$), 1705, 1613 ($\nu_{C=C}$), 1507 ($\nu_{C=C}$), 1403, 1350, 1128, 841. This compound exhibits the identical HPLC elution time, MS, and FTIR as authentic marmin (6'7'-dihydroxy-7-geranyloxycoumarin (Chatterjee et al., 1967).

Compound 9. MS: 505 $(M+Na)^{+23}$, 483 $(M+H)^{+1}$, 355, 337, 203 (bergaptol). UV λ_{max} nm: 309, 264, 260. IR ν_{max} cm^{-1}: 2951, 2916, 2848, 1700($\nu_{C=O}$), 1626 ($\nu_{C=C}$), 1463, 1124, 942. The IR spectrum of **9** shows an absence of a free hydroxyl. A key difference between **9** and the other compounds is the lower energy of the carbonyl stretch $\nu_{(C=O)}$ at 1700 cm^{-1}. This lower energy of the carbonyl bond may possibly result from increased resonance stabilization in the adjoining aromatic rings. This may arise from electron delocalization by a hydroxyl phenolic substituent. Yet, no evidence of a free hydroxyl is indicated in the IR spectrum, nor by any significant difference in the UV spectra of **9** and the other FCs isolated in this study. Although **9** and **3** have identical molecular weights and MS fragmentation, significant differences are observed in the mid to low-energy IR spectra of these compounds (Fig. 2).

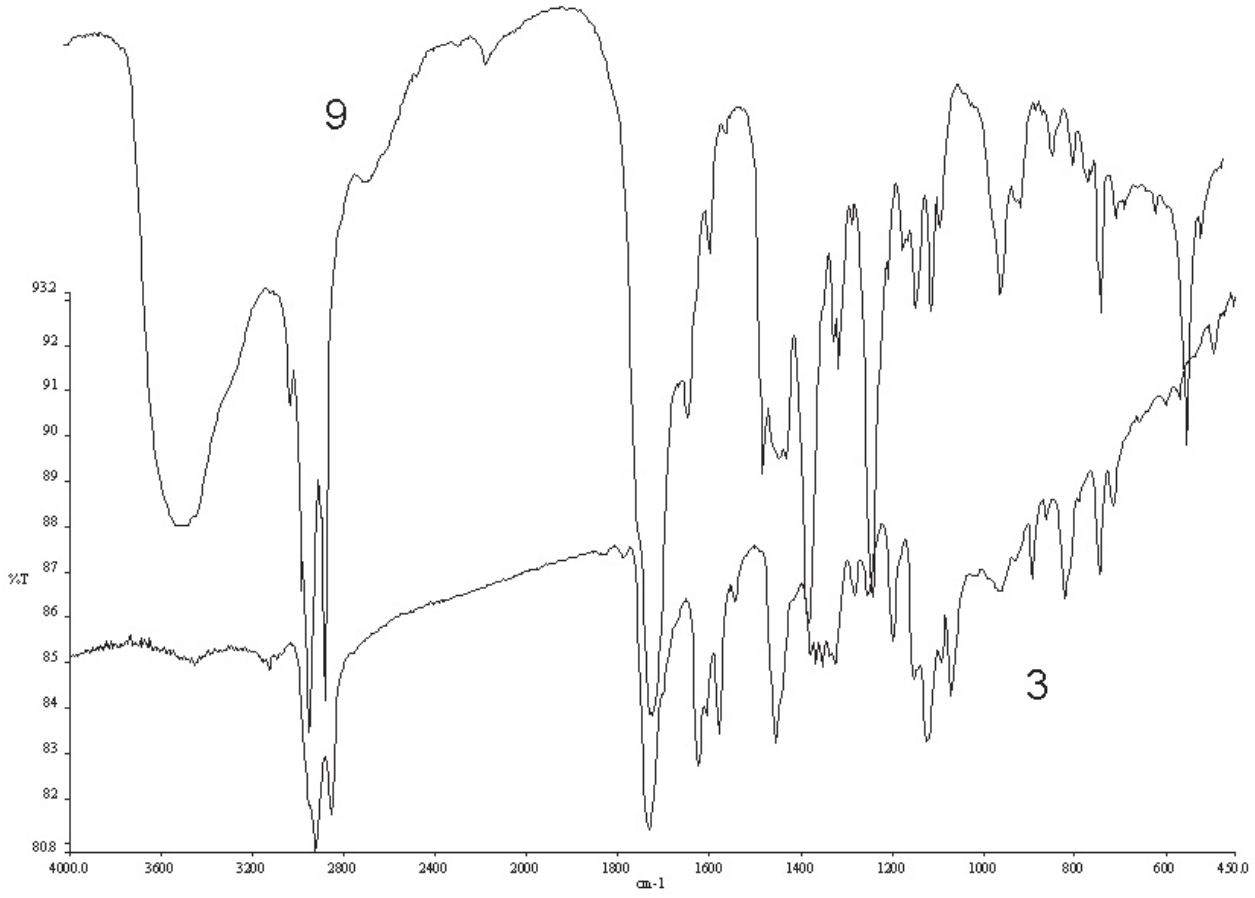

Fig. 2. Differences in the IR spectra of **3** and **9**. The prominent differences in the spectra of these two compounds in the 1700 to 600 cm^{-1} range demonstrate the occurrence of significant differences between these two compounds although they exhibit identical molecular weights, and nearly identical UV and MS fragmentation.

Compound 10. MS: 543(M+Na)$^{+23}$, 521 (M+H)$^{+1}$, 261, 243. UV λ_{max} nm: 321, 257. IR ν_{max} cm^{-1}: 3444 (ν_{O-H}), 2968, 2927, 2852, 1721 ($\nu_{C=O}$), 1607 ($\nu_{C=C}$), 1567 ($\nu_{C=C}$), 1497, 1251, 1098, 832. The IR spectrum demonstrates the presence of a free hydroxyl group. The UV spectrum of **10** is consistent with a coumarin structure, and the 261 *m/z* fragment ion indicates the occurrence of 7-geranyloxycoumarin as a major portion of this molecule.

Discussion

The results of the spectral analyses of **1**–**10** indicate that these compounds are coumarins and FCs. Among the FCs the UV spectra are nearly identical, and this suggests close structural similarities among these compounds, particularly among the aromatic and the geranyl side chain portions of the molecules. This similarity is further supported by the nearly identical MS fragmentation exhibited by each of these compounds. MS fragmentation of **3**–**7**, and **9** produced ions at 337, 355, and 203 *m/z*, and an interpretation of these fragment ions involving the production of a 7-dehydrogeranylbergaptol fragment intermediate is proposed in Figure 1. From the above observations, it is believed that the main structural differences in these FCs occur primarily in the portions of these molecules linked to the geranyl side chain, and we propose that this occurs via linkages at the geranyl 6' and 7' positions. Information about these latter portions is gained by IR analysis of these compounds, particularly in the high-energy portions (4000–2600 cm^{-1}) of the spectra. As discussed for a number of these compounds, the high intensities of the C-H $\nu_{methylene}$ vibrations vs the C-H ν_{methyl} vibrations provide an indication of a high methylene/methyl group ratio. One possible interpretation of this is the occurrence of linear (>C4) alkane chains as portions of these molecules. The occurrence of such alkane-linked FCs would represent the discovery of a new set of compounds in GPO. High resolution MS analysis, and ^{1}H and ^{13}C NMR measurements of the compounds isolated in this study are underway, and will assist in the structural characterizations for these compounds.

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