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Isolation and characterization of limonoate and nomilinoate A-ring lactones

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Abstract

A method combining solid-phase extraction and reversed-phase high-performance liquid chromatography is described for the isolation of two key metabolites in the limonoid biosynthetic pathway critical to citrus quality. Potassium salts of limonoate A-ring lactone and nomilinoate A-ring lactone were isolated from young Chandler pummelo seedlings and characterized on the basis of proton and carbon NMR data.

Keywords: *Citrus glandis*; Rutaceae; Limonoids; Limonoate A-ring lactone; Nomilinoate A-ring lactone

1. Introduction

Limonoids are prominent citrus secondary metabolites that occur in high concentration as aglycones and glucosides in citrus seed and fruit tissues. These complex triterpenoids display significant biological activity and have been speculated to function in plants as protective agents against plant predators (Randall and Murray, 2000). In citrus, considerable research effort has been directed to detecting and modulating limonoids in relationship to the development of "delayed bitterness" (Hasegawa et al., 1996). Delayed bitterness is initiated in citrus subjected to mechanical injury or freeze conditions enzymatically under acid conditions. Limonin (**1**) and nomilin (**2**) (Fig. 1) are the most abundant bitter limonoids generated through the biochemical reactions leading to delayed bitterness. During normal citrus fruit maturation limonoate A-ring lactone (**3**) and nomilinoate A-ring lactone (**4**) are biosynthetically transformed to the tasteless limonoid glucosides limonin glucoside (**5**) and nomilin glucoside (**6**) (Hasegawa et al., 1997). When **3** and **4** pools in immature citrus are exposed to freeze conditions or mechanical damage their metabolism is shunted from the formation of tasteless **5** and **6** to the formation of bitter **1** and **2** in the presence of acid and with catalysis by limonin D-ring lactone hydrolase (Maier et al., 1969).

Studies by Hasegawa and others have suggested that **3** and **4** are key metabolites in the limonoid biosynthetic pathway. Radioactive tracer experiments have demonstrated that **4** is biosynthesized from acetate, mevalonate and farnesyl pyrphosphate and is the starting material for other limonoids in their synthesis (Hasegawa and Herman, 1986; Hasegawa et al., 1984, 1986a,b; Ou et al., 1988).

The existence of **3** and **4** has been known for decades, however their inherent instability has precluded their isolation and characterization as pure compounds (Maier et al., 1969; Maier and Margileth, 1969; Merino et al., 1996, 1997). We now report the first isolation and characterization of **3** and **4**.

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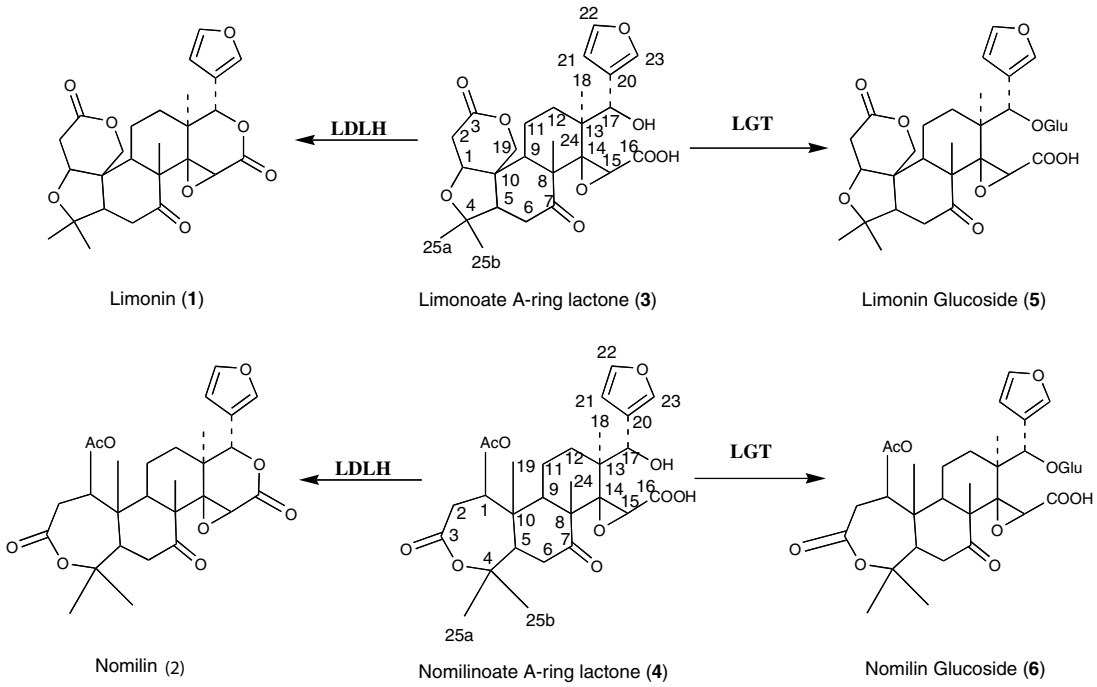

Fig. 1. Simplified biosynthetic pathway for limonoids. Limonoid D-ring lactone hydrolase (LDLH), limonoid UDP-D-glucose transferase (LGT).

2. Results and discussion

Seedlings of *Citrus* sp. were chosen as the raw material for the isolation of **3** and **4** since they were reported to be free of limonoid glucosides that would complicate isolation methods (Ronneberg et al., 1995). LC-MS analysis (data not shown) of extracts from seedlings of several citrus varieties confirmed the predominance of **3** and **4** in seedling tissues. Chandler pummelo (*Citrus grandis* Chandler) (Cameron and Soost, 1961) seedlings displayed the highest relative concentration of **3** and **4** among the seedlings tested and were chosen for extraction.

While **3** and **4** are readily soluble in water (Bennett and Hasegawa, 1981), it was necessary to extract these compounds from macerated Chandler pummelo seedlings with an aqueous buffered potassium phosphate solution (pH 8) in order to avoid the facile conversion of these compounds to their respective A- and D-ring dilactones observed at a pH 7 or less. Compounds **3** and **4** were isolated from the seedling extract by a combination of divinyl benzene solid-phase extraction (SPE) and C-18 chromatography. Initial application of SPE facilitated the concentration of the A-ring lactones by a factor of 400. The resulting extract was subjected to reversed-phase C-18 HPLC chromatography utilizing an aqueous buffer (pH 8): MeOH mobile phase to minimize conversion of the A-ring lactones to their dilactones during chromatographic runs. The procedure yielded potassium salts of **3** and **4**.

Deprotonated molecular ions $[M - H]^-$ observed at *m/z* 487.2 and 531.2 in the electrospray ionization mass spectrometer were consistent with the proposed $C_{26}H_{32}O_9$ and $C_{28}H_{36}O_{10}$ molecular formulas for **3** and **4**, respectively. The open D-ring chemical structures of **3** and **4** (Table 1) were established by comparison of their NMR chemical shifts and proton–proton correlations with reference data of their corresponding aglycones **1**, **2** (Bennett and Hasegawa, 1981; Dreyer, 1965; Manners et al., 2000) and glucosides **5**, **6** (Hasegawa et al., 1989; Manners et al., 2000). The ^1H and ^{13}C NMR spectra of the limonoid portion of **5** and **6** were very similar to those of **3** and **4**, respectively, including the chemical shifts in the vicinity of the D-ring. The known chemical structures of **1** and **2** showed very similar ^1H and ^{13}C NMR spectra to those of **3** and **4**, respectively, except for differences in or near the D-rings. In the case of **3**, the H-17, H-15, and C-17 resonances were observed to shift upfield (δ 5.53 to δ 5.13, δ 4.03 to δ 2.96, and δ 80.52 to δ 72.55 ppm, respectively), in comparison to **1**. In the case of **4**, the proton NMR spectrum displayed chemical shift of resonances associated with H-17, H-15, and C-17 to higher field (δ 5.51 to δ 5.09, δ 3.81 to δ 2.85, and δ 79.72 to δ 72.54 ppm, respectively), in comparison to **2**. Upon acidification, **3** and **4** underwent facile conversion to **1** and **2**, respectively, as determined by LC-MS comparison to standards maintained in our laboratory.

This study is the first description of **3** and **4** as pure compounds. The SPE/chromatographic isolation

Table 1
^{1}H NMR and ^{13}C NMR chemical shifts for potassium salts of **3** and **4**. *J* values are in Hz

Position	Limonoate A-ring lactone (3)		Nomilinoate A-ring lactone (4)	
	^{1}H	^{13}C	^{1}H	^{13}C
1	4.30 (1H, *s*)	79.6	4.76 (1H, *d*, $J_{1,2b}$ = 8.0)	72.5
2	2.82 (2H, *s*)	36.9		36.4
2a			3.37 (1H, *d*,$J_{2a,b}$ = 16.0)	
2b			3.13 (1H, *dd*, $J_{2a,b}$ = 16.0, $J_{1,2b}$ = 8.0)	
3		175.7		175.4
4		82.1		86.7
5	2.57 (1H, *dd*, $J_{5,6a}$ = 19.2, $J_{5,6b}$ = 5.6)	62.3	3.11 (1H, *dd*, $J_{5,6a}$ = 16.0, $J_{5,6b}$ = 8.4)	48.0
6		38.0		41.8
6a	2.93 (1H, *dd*, $J_{5,6a}$ = 5.6, $J_{6a,b}$ = 14.8)		2.14 (1H, *dd*, $J_{5,6a}$ = 8.4, $J_{6a,b}$ = 18.8)	
6b	2.67 (1H, *d*, $J_{6a,b}$ = 14.8)		3.04 (1H, *dd*, $J_{5,6b}$ = 8.4, $J_{6a,b}$ = 18.8)	
7		211.1		213.7
8		53.2		53.5
9	2.71–2.77 (1H, *m*)	47.2	2.74 (1H, *dd*, $J_{9,11a}$ = 12.4, $J_{9,11b}$ = 7.6)	44.2
10		47.2		45.1
11		19.0		17.2
11a	1.75–1.85 (1H, *m*)		1.58 (1H, *m*, $J_{9,11a}$ = 12.4)	
11b	1.87–1. 99 (1H, *m*)		1.35 (1H, *m*)	
12		31.8		31.7
12a	1.87–1.99 (1H, *m*)		1.83–1.92 (1H, *m*)	
12b	1.25–1.40 (1H, *m*)		1.17–1.24 (1H, *m*)	
13		45.6		44.5
14		72.9		73.0
15	2.96 (1H, *s*)	56.1	2.85 (1H, *s*)	62.5
16		173.7		171.5
17	5.13 (1H,*s*)	72.5	5.09 (1H, *s*)	72.5
18	1.09 (3H, *s*)	20.3	1.23 (3H, *s*)	20.7
19		65.6	1.35 (3H, *s*)	14.1
19a	4.48 (1H, *d*, $J_{19a,b}$ = 13.2)			
19b	4.54 (1H, *d*, $J_{19a,b}$ = 13.2)			
20		127.9		127.9
21	6.50 (1H, *s*)	142.9	6.49 (1H, *s*)	142.9
22	7.37 (1H, *s*)	112.2	7.36 (1H, *s*)	112.1
23	7.55 (1H, *s*)	142.8	7.54 (1H, *s*)	142.8
24	1.07 (3H, *s*)	22.3	1.02 (3H, *s*)	20.9
25a	1.32 (3H, *s*)	30.6	1.41 (3H, *s*)	32.7
25b	1.33 (3H, *s*)	23.2	1.52 (3H, *s*)	23.1
OAc(Me)			2.01 (3H, *s*)	22.2
OAc(CO)				173.2

method described for **3** and **4** provides access to two pivotal compounds in a limonoid biosynthetic pathway. The methodology should provide access to other limonoid A-ring lactones that can yield important information about the biosynthesis of limonoids in citrus.

3. Experimental

3.1. Chemicals

HPLC grade MeOH, $CHCl_3$, EtOAc and MeCN were obtained from Fisher Scientific (Pittsburgh, PA). Water was purified using a Sybron Barnstead water purification system. Potassium phosphate was obtained from J.T. Baker Chemical Co (Phillipsburg, NJ). Limonin and nomilin were available in our laboratory.

3.2. Plant material

Chandler pummelo (*Citrus grandis* Chandler) (Cameron and Soost, 1961) seeds were collected at the University of California at Davis Lincove Research and Extension Center (Exeter, CA). The seeds were planted in soil and grown under greenhouse conditions. Seedlings were harvested as needed, and ranged in age from 1 to 5 months.

3.3. Equipment

Liquid chromatography was conducted on a Waters 2695 chromatography system coupled to a Waters 996 PDA UV detector set at 215 nm. The HPLC utilized a Keystone (Bellefonte, PA) BDS C-18 reversed-phase column (250 mm × 10 mm i.d., 5μm) equipped with a C-18 (50 × 10 mm i.d., 5 μm) guard column.

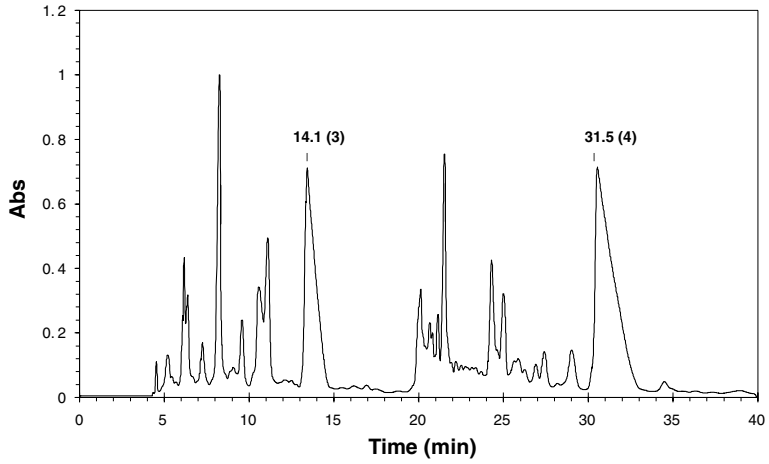

Fig. 2. Separation of limonoate A-ring lactone (**3**) and nomilinoate A-ring lactone (**4**) on a reversed-phase C-18 HPLC column after enrichment on SPE. The other peaks appearing in the chromatogram were not identified.

Mass spectrometric analysis was conducted on a Thermo Finnegan LCQ Advantage (San Jones, CA) ion-trap mass spectrometer equipped with an ESI probe. The mass spectrometer was operated in the negative total ion mode (*m*/*z* 150–1500) with a capillary temperature of 380 °C, capillary voltage of −42 V, and ion spray voltage 4.5 kV. Data were obtained by direct infusion of a sample. High resolution electrospray mass spectrometric analysis (Applied Biosystems Sciex QStar Pulsar i, Foster City, CA) of **3** and **4** were performed by Bay Bioanalytical (Hercules, CA).

^{1}H and ^{13}C NMR spectra were obtained on a Varian-400 (Palo Alto, CA) nuclear magnetic spectrometer operating at 400 MHz. Samples were dissolved in CD_3OD and chemical shifts referenced to tetramethylsilane (TMS) resonance in ppm. Spectroscopic assignments were made on the bases of ^{1}H–^{1}H COSY and DEPT experiments, and comparison with spectra of related limonoids.

3.4. Extraction and isolation of compounds

Chandler pummelo seedlings (250 g) were homogenized in a 0.1 M potassium phosphate pH 8.0 buffer (1 L). The resulting extract was filtered through grade 10 cheesecloth and centrifuged (27,000*g*, 1 h). The extract was applied (10 mL/min) in 200 mL batches to Sepra-ZT (Phenomenex, Torrance, CA) sorbent (2.0 g) packed in a glass column (20 × 25 mm), that had been washed with MeOH (20 mL) and preconditioned with deionized water (20 mL). The column was washed in succession with deionized water (20 mL), $CHCl_3$ (20 mL), and EtOAc (20 mL). The analyte containing **3** and **4** was eluted with MeCN (20 mL). The column was washed with MeOH (20 mL) followed by water (20 mL) and the solid-phase extraction procedure repeated with the remaining extract in 200 mL batches. The MeCN elutions were combined and dried on a rotary evaporator. The resulting solid was redissolved in MeOH (0.5 mL) and subjected to a C-18 semi- preparative HPLC/UV. Chromatography of the limonoids was accomplished with a 10 mM potassium phosphate (pH 8.0): MeOH step gradient, flow rate = 3.0 mL/min, column temperature 40 °C. The gradient conditions were: time 0 min, phosphate:MeOH (80:20); time 15 min. phosphate:MeOH (65:35), time 34 min., phosphate:MeOH (80:20). Total chromatographic run time was 40 min. Sample injection volume was 30 μL. Fractions of **3** and **4** were collected at retention time of 14.1 and 31.5 min, respectively (Fig. 2). The fractions containing **3** and **4** were concentrated by solidphase extraction chromatography as soon as they were collected. Two separate Strata-X 200 mg/6 mL columns (Phenomenex) designated for **3** and **4** were washed with MeOH (2 mL) and preconditioned with deionized water (2 mL). Fractions of **3** and **4** (each approximately 9–12 mL) were loaded onto the columns. The columns were washed with deionized water (2 mL), dried at full vacuum for 5 min, and eluted with MeOH (2 mL). MeOH was removed on a rotary evaporator and the resulting amorphous solid collected. The procedure yielded **3** (10 mg) and **4** (8 mg). The obtained compounds were analyzed by ^{1}H, ^{13}C NMR and ESI-MS.

3.4.1. Limonoate A-ring lactone (*3*)

Characterized as potassium salt. White solid m.p. 217–223 °C; $[\alpha]_{589}^{23} - 2.65°$, $[\alpha]_{578}^{23} - 2.91°$, $[\alpha]_{546}^{23} - 3.82°$, $[\alpha]_{436}^{23} - 15.88°$ (MeOH; *c* 0.34); ESI-MS: *m*/*z* 487.3 $[M - H]^-$; HRESI-MS: *m*/*z* 527.1673 $[M + K]^+$ (calc. for $C_{26}H_{32}KO_9^+$, 527.1678); ^{1}H and ^{13}C NMR spectroscopic data (400 MHz, CD3OD) see Table 1.

3.4.2. Nomilinoate A-ring lactone (*4*)

Characterized as potassium salt. White solid m.p. 156–157 °C; $[\alpha]_{589}^{23} + 92.35°$, $[\alpha]_{578}^{23} + 97.06°$, $[\alpha]_{546}^{23} + 111.76°$, $[\alpha]_{436}^{23} + 214.71°$ (MeOH; *c* 0.17); ESI-MS: *m*/*z* 531.2

$[M - H]^-$; HRESI-MS: *m/z* 571.1889 $[M + K]^+$ (calc. for $C_{28}H_{36}KO_{10}^+$, 571.1940); ^1H and ^{13}C NMR spectroscopic data (400 MHz, CD3OD) see Table 1.

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