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LIMONOID GLUCOSIDES IN CITRUS

SHIN HASEGAWA, RAYMOND D. BENNETT, ZAREB HERMAN, CHI H. FONG and PETER OU

USDA, ARS, Fruit and Vegetable Chemistry Laboratory, 263 South Chester Avenue, Pasadena, CA 91106, U.S.A.

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Key Word Index—*Citrus*, Rutaceae; limonoid glucosides, limonoids, limonin 17-*O*-β-D-glucopyranoside.

Abstract—Citrus contained high concentrations of limonoid glucosides. Four new glucosides, isolated from grapefruit seeds, were identified as limonin 17-*O*-β-D-glucopyranoside, nomilin 17-*O*-β-D-glucopyranoside, deacetylnomilin 17-*O*-β-D-glucopyranoside and obacunone 17-*O*-β-D-glucopyranoside. Grapefruit seeds contained about 0.8% of limonoid glucoside derivatives.

INTRODUCTION

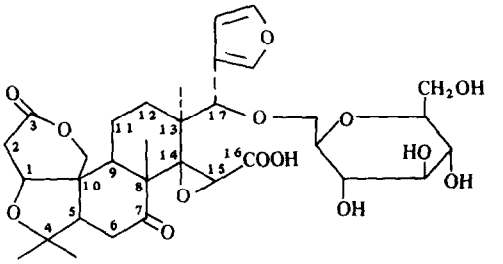
Limonoids are a group of chemically related triterpene derivatives found in Rutaceae and Meliaceae families. Limonin and nomilin, bitter members of the group, are present in citrus juices, and their bitterness reduces juice quality. In general, citrus juices contain limonoids at levels below the bitterness threshold of 6 ppm. During biochemical studies on limonoids in conjuction with our efforts to develop preharvest methods for debittering of citrus juices, we found that citrus tissues and juices contain very high concentrations of limonoid glucosides. We report here the isolation of four major glucoside derivatives from grapefruit seeds and their chemical structures.

RESULTS AND DISCUSSION

Limonoids in citrus tissues and juices have been customarily detected or quantitated by solvent extraction followed by TLC, HPLC or immunoassay methods [1–3]. The aqueous residue from the solvent extraction has been completely ignored. Recently, TLC analyses of such aqueous portions showed that citrus contains very high concentrations of compounds giving an Ehrlich-positive reaction on TLC, which is a typical characteristic of limonoids [4]. For instance, commercial orange juices contained over 300 ppm of Ehrlich-positive compounds. Subsequently, these compounds were isolated from grapefruit seeds and characterized.

TLC analyses showed that grapefruit seeds contain about 1% of limonoid glucosides on a dry weight basis. TLC, HPLC and column chromatographic analyses indicated that grapefruit seed extracts contain at least 15 water-soluble compounds which show an Ehrlich-positive reaction. Four major compounds were isolated in pure form by reverse phase column chromatography. When these compounds were hydrolysed with 0.5 M HCl at 70° for five hours, the limonoid portion of the glycosides was not stable under the acidic conditions used, but the sugar portion was recovered. The sugar was enzymatically identified as D-glucose by a coupled reaction of hexokinase and glucose-6-phosphate dehydrogenase. All of these four isolates were found to possess a D-glucose moiety.

The ^{1}H NMR spectrum of one of these compounds (**1**) in DMSO-d_6 at 25° showed very broad peaks; only at 90° was a typical high resolution spectrum obtained. The spectrum showed four *C*-methyl peaks, thus demonstrating that **1** was a limonoid oxygenated at C-19, like limonin (**2**), as opposed to those such as nomilin (**3**) in which C-19 is methyl. All of the characteristic downfield peaks of **2** could be identified in the spectrum of **1**. However, the chemical shifts of the H-15, H-17, and furan resonances were significantly different from those of **2**, suggesting anomalous structural features in the vicinity of the D-ring. In addition, a highly coupled system of five nonlimonoid proton signals was present in the δ3.0–3.6 region of the spectrum, as well as a doublet at 4.13. These resonances are characteristic of a 6-carbon sugar, with the downfield one being due to the anomeric proton. Thus,



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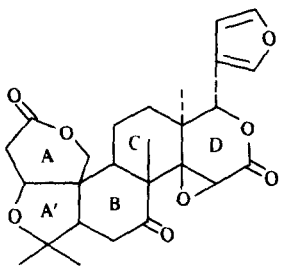

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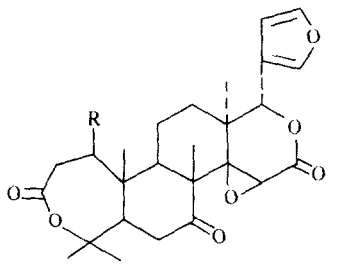

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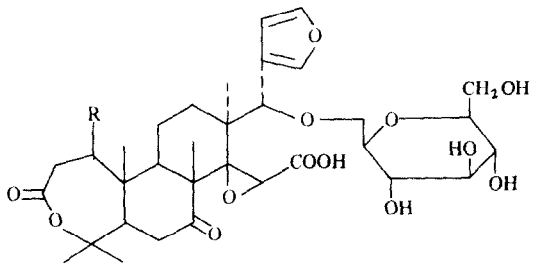
7 R = OH



4 R = OAc
5 R = OH

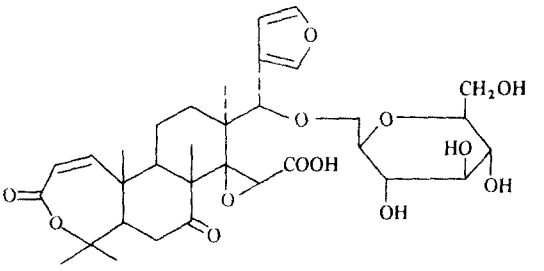
the ^{1}H NMR data suggested that **1** was a glycoside of a limonoid, possibly **2**.

In comparing the ^{13}C NMR spectrum of **1** with that of **2**, the resonances for carbons 1–11 and 19 differed by less than δ1.5, except for that of C-5 which was δ2.5 upfield in the spectrum of **1**. However, large differences were observed for carbons 12–16 and two of the furan carbons, again indicating changes in the D-ring area of the molecule In addition, the following signals ascribable to sugar carbons were observed: a methylene at δ61, four methines between δ70 and 77, and an anomeric methine at δ104. There are no sites at which a sugar can be attached in **2** unless either the A- or D-ring lactone is opened The NMR evidence shows that the latter is the case There are then two possible points of attachment: the carboxyl group at C-16 or the hydroxyl group at C-17. In the former case an ester linkage would be formed, which would be susceptible to base-catalysed hydrolysis. However, when **1** was treated with base it was recovered intact, which establishes the point of attachment as C-17 The configuration of the glycoside linkage was shown to be β by the coupling constant of the sugar H-1 resonance (J=7.1 Hz)

The NMR spectral data leave little doubt that the limonoid portion of **1** was in fact **2**. All of the oxygen functional groups of **2**, and no others, were accounted for in the ^{13}C spectra. The carbon resonances not adjacent to the D-ring were very similar to those of **2**, except for C-5. The latter difference can be rationalized by its presence at the junction of the A'- and B- rings. A Dreiding model of **2** showed that significant strain is involved in closing the D-ring Relief of this strain upon opening the ring to form the glycoside would be transmitted to the other rings and would be especially significant at the ring junctions.

Commercial β-glucosidases including naringinases did not hydrolyse the limonoid glucosides. However, a species of bacterium, capable of metabolizing the glucosides, was isolated from soil on a mineral salt medium containing **1** as a single carbon source. The compound recovered from the **1** growth medium was shown to cochromatograph with **2** on TLC. This confirms that the limonoid portion of **1** is **2**. Thus, we have assigned the structure limonin 17-*O*-β-D-glucopyranoside to **1**

Three more polar limonoids (**4**–**6**) isolated by column chromatography showed ^{1}H and ^{13}C MNR spectra analogous to those of **2**, in respect to the presence of sugar resonances and the unusual chemical shifts of the protons



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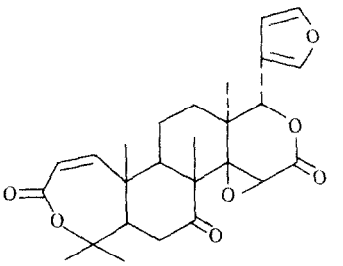

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Table 1 ^{1}H NMR spectra of limonoid glucosides

H	1	4	5	6
α-Furans	7.52	7 50	7 50	7 52
	7 42	7 41	7 41	7 42
β-Furan	6 54	6 53	6 53	6 54
17	5 21	5 23	5 17	5 16
19	4 41	—	—	—
1	4 23 *br s*	4 68 *d*	3 80 *m*	6 39 *d*
		(7 3)		(12.7)
2	—	—	—	5.88 *d*
				(12 7)
15	3 19	3 02	3 09	3 05
C-Methyls	1 37	1 43	1 38	1 38
	1 23	1 36	1 34	1 35
	0.99	1 33	1 28	1 29
	0 71	0 97	0 85	1 05
		0 77	0 78	0 80
Acetate methyl	—	2.03	—	—
Sugar H-1	4 13 *d*	4 12 *d*	4 12 *d*	4 11 *d*
	(7 1)	(7 3)	(7 6)	(7 3)

Table 2. ^{13}C NMR spectra of limonoid glucosides

C	1	4	5	6
1	77 9	70 7	67.4	150 3
2	35 6	35 6	40.1	119 5
3	169.9	169 5	171 0	165.4
4	79 8	84.1	83.5	83 2
5	55 7	47 0	45 3	51 3
6	36.6	38.9	40 1	40.0
7	207 2	208 8	209.9	208.5
8	50 5	50 8	50 8	51.4
9	45.3	42.0	41.5	46 7
10	45 4*	43 1*	44 4*	44.4*
11	17.0	15 1	15.1	17.3
12	26 6	26.4	26.6	26.7
13	44 9*	44.3*	43.9*	44 1*
14	70 8	71 1	71 0	70 8
15	57 1	57 7	57 5	57.4
16	169 6*	169.4	169 3	169.2
17	77 8	77 7	77 7	77.8
19	63 7			
20	125 5	125.7	125 7	125 5
21	141.5	141 5	141.5	141 7
22	112.5	112 6	112.6	112.6
23	140 8	140.6	140.6	140.7
C-Methyls	30.4	32.3	32.1	29.9
	25.1	24 9	25 0	25 4
	21.8	22 0	22 4	23 1
	19 0	19 0	18.7	19 4
		13 3	13 9	15 1
Acetate carbonyl	169.2*			
Acetate methyl	20 5			
Glucose C-1	104.4	104.4	104 5	104.4
Glucose C-6	61 6	61 7	61 7	61.6
Glucose C2–C4	76 9	77 0	77 1	77.0
	76.2	76.1	76.1	76.2
	74 1	74.2	74 2	74.1
	70 6	70 6	70.8	70 6

*Assignments in the same vertical column may be reversed

and carbons in the vicinity of the D-ring. As Tables 1 and 2 show, these signals were so similar that there is no doubt that the sugar attachment was the same in all four compounds. Compounds **4–6** each showed five C-methyl signals in their proton spectra. Based upon the NMR data, the aglycones were found to be as follows: nomilin (**3**) for **4**, deacetylnomilin (**7**) for **5**, and obacunone (**8**) for **6**. Again, the resonances of those carbons not adjacent to the D-ring in the glucosides were very similar to those of the aglycones, with the exception of C-5, and, in the case of **6**, C-1 and C-2. Possibly the conformation of the A-ring in **6** is significantly changed upon opening of the D-ring.

Compounds **2**, **3**, **7** and **8** are the major neutral limonoids present in a variety of citrus seeds [5]. This work showed that their 17-*O*-*β*-D-glucosides are also present in grapefruit seeds as the major derivatives. TLC analyses showed that five commercial orange juices contained an average 320 ppm of glucoside derivatives. Orange juices could provide an excellent source of limonoid aglycones which have been reported to possess anticarcinogenesis activity in mice [6].

TLC analyses showed that there were no detectable amounts of limonoid glucosides in leaves and stems of young *Citrus limon* seedlings, and also in immature 11 and 60 g *Citrus limon* fruits. However, a mature 236 g fruit possessed 330 ppm. This suggests that glucose derivatives are formed during late stages of fruit growth and maturation. The biochemistry of limonoid glucosides in citrus will be studied.

Preliminary studies indicated that some limonoid glucosides such as **1** and **4** are mildly bitter as compared to the intense bitterness of their limonoid aglycones. The possible impact on citrus juice flavours will be investigated.

EXPERIMENTAL

Materials. Grapefruit seeds were obtained from Bordo Citrus Products, Winter Haven, FL. Seedlings and a mature tree of *Citrus limon* were grown at the Pasadena Laboratory Orange juices were purchased from a local store Adsorbent XAD was purchased from Accurate Chemical & Scientific Corpn, Westbury, NY, and Amberlite XAD-2 was obtained from Sigma Chemical Co, St Louis, MO. An enzyme system for D-glucose determination (Cat No 716251) was purchased from Boehringer Mannheim Biochemica Silica gel TLC plates were used for analyses of limonoids and limonoid glucosides Solvent systems used for glucoside analysis were EtOAc–MeCOEt–HCO_2H–H_2O (5:3:1·1) or BuOH–NH_4OH–H_2O (9 1 1) For limonoids, three solvent systems were used· cyclohexane–EtOAc (2.3), CH_2Cl_2–MeOH (49·1) or EtOAc–CH_2Cl_2 (2·3) Plates were sprayed with Ehrlich's reagent and a typical pink colour was developed in a HCl gas chamber [4].

Extraction of glucosides Grapefruit seeds were washed thoroughly with H_2O and dried at first in open air, then in a 60° oven Seed meals obtained from dried seeds were extracted first with hexane to remove oily materials Then, glucoside-free limonoids were extracted with Me_2CO The residue was finally extracted with MeOH to obtain limonoid glucosides. All extractions were carried out at about 55–60° in a custom made extractor

Quantitation of glucosides. Total limonoid glucosides in grapefruit seeds and commercial orange juices were estimated by TLC analyses. A 5 g portion of the grapefruit seed meal was treated first with hexane to remove oily materials and then refluxed with 125 ml MeOH overnight This was centrifuged at 10000 *g* for 10 min and filtered The residue was washed thoroughly with MeOH. The combined filtrate was used for quantitation of total glucosides by TLC using a standard of **1** For orange juices, a 2 ml portion of centrifuged juices was treated with a Sep-Pak and the MeOH eluant was used for analyses.

Isolation of glucosides. The MeOH extract was evapd to near dryness and the residue was dissolved in a minimal portion of H_2O. The soln was then transferred to the top of a column (5 × 47 cm) packed with Amberlite XAD-2. The column was washed thoroughly with H_2O and the glucosides were eluted with MeOH. After the MeOH fraction was evapd, the residue was dissolved in a minimal portion of H_2O. A portion of the soln was then fractionated on a column (2 × 90 cm) packed with XAD The column was eluted linearly with 500 ml each of 10% and 55% MeOH Four major limonoid glucoside fractions were collected in this order· **1**, **5**, **4** and **6**. Each fraction was further fractionated on XAD columns (3 × 30 cm or 2 × 90 cm) with linearly increasing concentrations of MeCN in H_2O. If necessary, the fractions were again fractionated on the XAD column,

which was eluted with linearly increasing concentrations of MeOH in H_2O. After repeated CC, four limonoid glucosides were obtained in pure form.

Acid hydrolyses. Each isolate (5 mg) was dissolved in 1 ml 0.5 M HCl and heated at 70°. After 5 hr of heating, the soln was brought to pH 7.0 and a portion was used for identification of the sugar moiety.

Enzymic identification of the sugar moiety D-Glucose in the acid hydrolysate was enzymatically determined with a hexokinase, glucose-6-phosphate dehydrogenase system according to the procedure given by Boehringer Mannheim Biochemica.

NMR analyses. All NMR spectra were run in DMSO-d_6 at 90°, at 270 MHz for ^1H and 67.8 MHz for ^{13}C ^{13}C spectral assignments were made on the basis of SFORD and DEPT spectra, selective heteronuclear decoupling, and comparison with spectra of related limonoids for which assignments had previously been made [7, 8] Spectra for compounds **2** and **8** are in ref [7], and those for **3** and **7** in ref [8]

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