Limonoids in *Citrus ichangensis*

Zareb Herman,* Shin Hasegawa, Chi H. Fong, and Peter Ou

HPLC analyses of limonoids revealed that *Citrus ichangensis* possesses an unusual distribution of limonoids. Fruit tissues and seeds were found to contain very high concentrations of a nonbitter limonoid, ichangensin, which is unique to this species and its hybrids. Unlike most citrus, it contains very low concentrations of bitter limonin and relatively high concentrations of nonbitter deacetylnomilin. Radioactive tracer work is reported, and a possible biosynthetic pathway is proposed.

Bitterness due to limonoids such as limonin (**5**) and nomilin (**1**) in a variety of citrus juices is a major problem of the citrus industry worldwide and has significant negative economic impact. A total of 36 limonoids have been isolated from citrus and its hybrids. Four of these are known to be bitter: limonin, nomilin, ichangin, and nomilinic acid (Maier et al., 1977).

More than 20 years ago, Dreyer (1966) reported that *Citrus ichangensis* contains relatively large amounts of unidentified limonoids. Little work was done, however, to identify these compounds until recently when the major unknown limonoid was structurally identified and named ichangensin (**4**) (Bennett et al., 1988). In this study we analyzed limonoids in various tissues of *C. ichangensis* and found that this citrus possesses an unusual distribution of limonoids.

EXPERIMENTAL SECTION

Materials. Mature stems, leaves, fruit, and seeds were obtained from *C. ichangensis* trees grown at the University of California at Riverside. Seedlings were grown in our greenhouse. They were 4 months old and 8–12 cm tall when used.

Extraction of Limonoids. Limonoids were extracted from weighed portions (1–5 g) of each tissue by the method of Hasegawa et al. (1980). Portions of each sample were treated with diazomethane to methylate acidic limonoids and used for analyses of acidic limonoids.

Identification and Analyses. Identification of limonoids was made using TLC on silica gel plates by the procedure described previously (Hasegawa et al., 1984a; Bennett et al., 1988). Limonoids were analyzed by HPLC using a C_{18} reversed-phase column (4.6 × 250 mm) and eluted isocratically with 49% H_2O, 41% MeOH, and 10% CH_3CN at a flow rate of 1 mL/min. Standards and samples were dissolved in 50% MeOH prior to injection. Limonoids were detected by UV absorption at 210 nm.

Radioactive Tracer Work. [^{14}C]-Labeled nomilin (**1**) (200 000 cpm) in 10 μL of CH_3CN was injected by syringe into the center of a detached *C. ichangensis* fruit. This was incubated for 3 days at room temperature. Limonoids were extracted by the method cited above. The extract was analyzed by TLC on silica gel plates (5 × 20 cm, 250-μm thickness, HLF type) with the following solvent systems: (a) EtOAc–cyclohexane (3:2), (b) CH_2Cl_2–MeOH (97:3), (c) CH_2Cl_2–EtOAc (3:2), (d) toluene–EtOH–H_2O–HOAc (200:47:15:1, upper layer), (e) EtOAc–MeOH (98:2). TLC radiochromatograms were obtained on a Berthold automatic TLC–linear analyzer, Model LB 2832, Wildbad, West Germany.

Radioactive peaks obtained using TLC solvent system a were scraped from the plate. The scrapings were then extracted with EtOAc to obtain radioactively pure compounds. These were then analyzed by TLC in three additional solvent systems. Limonoid standards were spotted on each plate.

RESULTS AND DISCUSSION

Table I shows the results of HPLC analyses of neutral limonoids in various tissues of *C. ichangensis*. In fruit tissues and seeds, ichangensin (**4**) is the predominant limonoid. Compound **4** is, however, only present in trace amounts in leaves and stems of mature trees. The presence of **4** is unique to this species and its hybrids such as Yuzu (*C. ichangensis* × *Citrus reticulata* var. austera) and Sudachi (*C. ichangensis* × *C. reticulata*) (Hashinaga and Hasegawa, 1989).

Table I. Neutral Limonoids in *C. ichangensis*

tissue	limonoid, ppm				
	4	5	2	1	6
fruit	570	25	150		tr
seeds	2900	250	400	16	34
leaves	tr	47	79	tr	tr
stems	tr	43	230	71	
seedlings					
stems	tr	120	180	350	tr
leaves		170	300	400	

Table II. Acidic Limonoids in *C. ichangensis*

tissue	limonoid, ppm		
	deacetylnomilinic acid	isolimonic acid	nomilinic acid
fruit	tr	tr	tr
seeds	140	90	tr
stems	8.0	14.0	tr
leaves	6.2	15.0	
seedlings			
stems	31	4.5	6.6
leaves	4.8	3.8	

Unlike most other citrus in which limonin (**5**) is predominant (over 50% of total limonoids) (Hasegawa et al., 1980), **5** is not the predominant limonoid in any tissues of *C. ichangensis*. In particular, fruit tissues and seeds were found to contain 22.8 and 11.6 times as much **4** as **5**, respectively.

Table I shows that *C. ichangensis* also contains relatively high concentrations of deacetylnomilin (**2**). In mature stems and leaves, **2** is the most predominant limonoid. In fruit tissues and seeds, **2** is the second most predominant. Also, 180 ppm in seedling stems and 300 ppm in seedling leaves are very high compared to the low or undetectable levels observed in other citrus seedlings.

As in most other citrus (Hasegawa et al., 1984a), **1** was found to be the predominant limonoid in the seedlings. However, *ichangensis* differs from other citrus in that **1** is almost absent in the fruit tissues, and its content in seeds is also relatively low.

Concentrations of acidic limonoids are shown in Table II. Common acidic limonoids such as nomilinic acid, isolimonic acid, and deacetylnomilinic acid were found, but their concentrations were very low compared to those of other citrus (Hasegawa et al., 1980).

In this study, [^{14}C]nomilin (**1**) was used as a radioactive tracer for two reasons: (1) It is an initial precursor in limonoid biosynthesis. (2) It is actively transported throughout citrus tissues (Hasegawa et al., 1986). When

Fruit and Vegetable Chemistry Laboratory, USDA—ARS, 263 South Chester Avenue, Pasadena, California 91106.

Table III. Identification of Radioactive Metabolites by TLC

compound	R_f in solvent system[a]				
	a	b	c	d	e
peak 2	0.15	0.23	0.33		0.73
deacetylnomilin	0.15	0.23	0.33		0.73
peak 3	0.26	0.40	0.53	0.25	
nomilin	0.26	0.40	0.53	0.25	
peak 4	0.59	0.37	0.73	0.27	
ichangensin	0.59	0.37	0.73	0.27	

[a] Solvent key: see the Experimental Section.

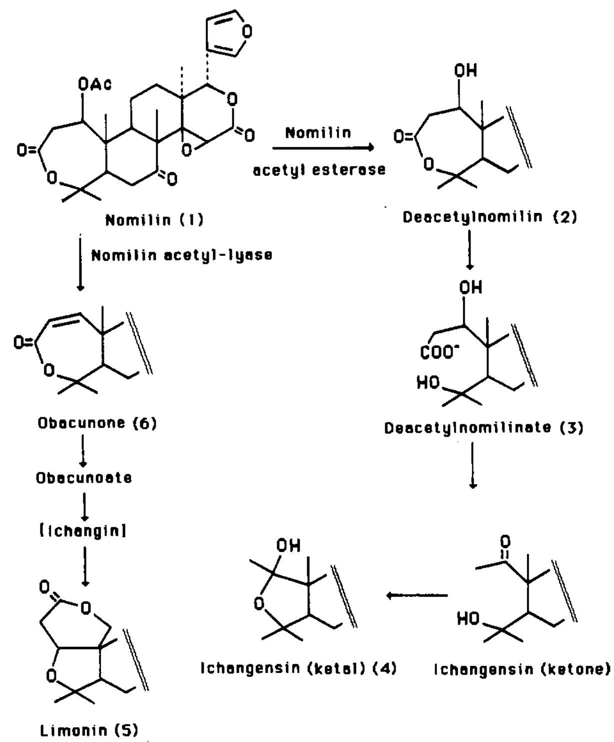

Figure 1. Possible biosynthetic pathways of the major limonoids in *C. ichangensis*.

labeled 1 was injected into an ichangensis fruit, four radioactive peaks were observed by TLC. Peak 1 (12% of radioactivity) was quite polar and did not migrate on the TLC plates. Its identity is unknown. The R_f values for the other three peaks are given in Table III. Peak 2 (55%) matched that of deacetylnomilin (**2**); peak 3 (28%) matched that of the substrate (**1**); and peak 4 (5%) matched that of ichangensin (**4**).

The tracer results show that **1** is converted to **2** and **4** in fruit tissue. The fact that 55% of the recovered radioactivity appeared as **2** suggests that **2** is a direct metabolite of **1**. We presume that the conversion of **1** to **2** is catalyzed by nomilin acetyl esterase. The likely presence of this enzyme activity in *C. ichangensis* appears to be unique to this species because this activity has not been reported in other species of citrus or in microorganisms. In other citrus or microorganisms, **1** is mainly converted to obacunone (**6**) by nomilin acetyl-lyase (Hasegawa et al., 1984b; Hasegawa and Herman, 1985). The presence of nomilin acetyl esterase activity probably explains why *C. ichangensis* contains a relatively large amount of **2**.

Possible biosynthetic pathways of the major limonoids in *C. ichangensis* are shown in Figure 1. Compound **4** is present in two isomer forms, ketone and ketal, in $CHCl_3$ (Bennett et al., 1988). In aqueous solution, it is present as ketal. The tracer work shows that **1** is converted to **2** and **4**. The probable conversion of **2** to **4** appears to require two biosynthetic steps. Compound **2** is possibly converted to deacetylnomilinic acid (**3**) and then to **4**. On the basis of the findings of this study, the likely pathway of **1** to **4** via **2** appears to be the major one in fruit tissues and seeds of *C. ichangensis*. This pathway, if correct, is unique to this species. In most other citrus, **1** is converted to limonin via obacunone, obacunoic acid, and possibly ichangin (Herman and Hasegawa, 1985).

If the genes for the pathway from **1** to **4** or even from **1** to **2** (nomilin acetyl esterase) could be transferred by genetic engineering or breeding techniques to other citrus species with the limonoid bitterness problem, nonbitter **4** or nonbitter **2** might accumulate as the major limonoid in the fruit tissues rather than bitter **5**. This presents a possible new approach to the limonoid bitterness problem.

ACKNOWLEDGMENT

We thank Dr. V. P. Maier for his helpful suggestions in preparation of this manuscript.

Registry No. **1**, 1063-77-0; **2**, 3264-90-2; **4**, 115777-76-9; **5**, 1180-71-8; **6**, 751-03-1.

LITERATURE CITED

Bennett, R. D.; Herman, Z.; Hasegawa, S. Ichangensin: a new citrus limonoid. *Phytochemistry* **1988**, *27*, 1543–1545.
Dreyer, D. L. Citrus bitter principles. V. Botanical distribution and chemotaxonomy in the Rutaceae. *Phytochemistry* **1966**, *5*, 367–368.
Hasegawa, S.; Herman, Z. Biosynthesis of obacunone from nomilin in *Citrus limon*. *Phytochemistry* **1985**, *24*, 1973–1974.
Hasegawa, S.; Bennett, R. D.; Verdon, C. P. Limonoids in citrus seeds: origin and relative concentration. *J. Agric. Food Chem.* **1980**, *28*, 922–925.
Hasegawa, S.; Bennett, R. D.; Maier, V. P. Biosynthesis of limonoids in citrus seedlings. *Phytochemistry* **1984a**, *23*, 1601–1603.
Hasegawa, S.; Dillberger, A. M.; Choi, G. Y. Metabolism of limonoids: conversion of nomilin to obacunone in *Corynebacterium fascians*. *J. Agric. Food Chem.* **1984b**, *32*, 457–459.
Hasegawa, S.; Herman, Z.; Orme, E.; Ou, P. Biosynthesis of limonoids in citrus: sites and translocation. *Phytochemistry* **1986**, *25*, 2783–2785.
Hashinaga, F.; Hasegawa, S. Limonoids in Sudachi seeds. *J. Jpn. Soc. Hortic. Sci.* **1989**, in press.
Herman, Z.; Hasegawa, S. Limonin biosynthesis from obacunone via obacunoate in *Citrus limon*. *Phytochemistry* **1985**, *24*, 2911–2913.
Maier, V. P.; Bennett, R. D.; Hasegawa, S. In *Citrus Science and Technology*; Nagy, S., Shaw, P. E., Veldhuis, M. K., Eds.; AVI: Westport, CT, 1977; Vol. 1, pp 355–396.

Received for review September 26, 1988. Accepted December 28, 1988.