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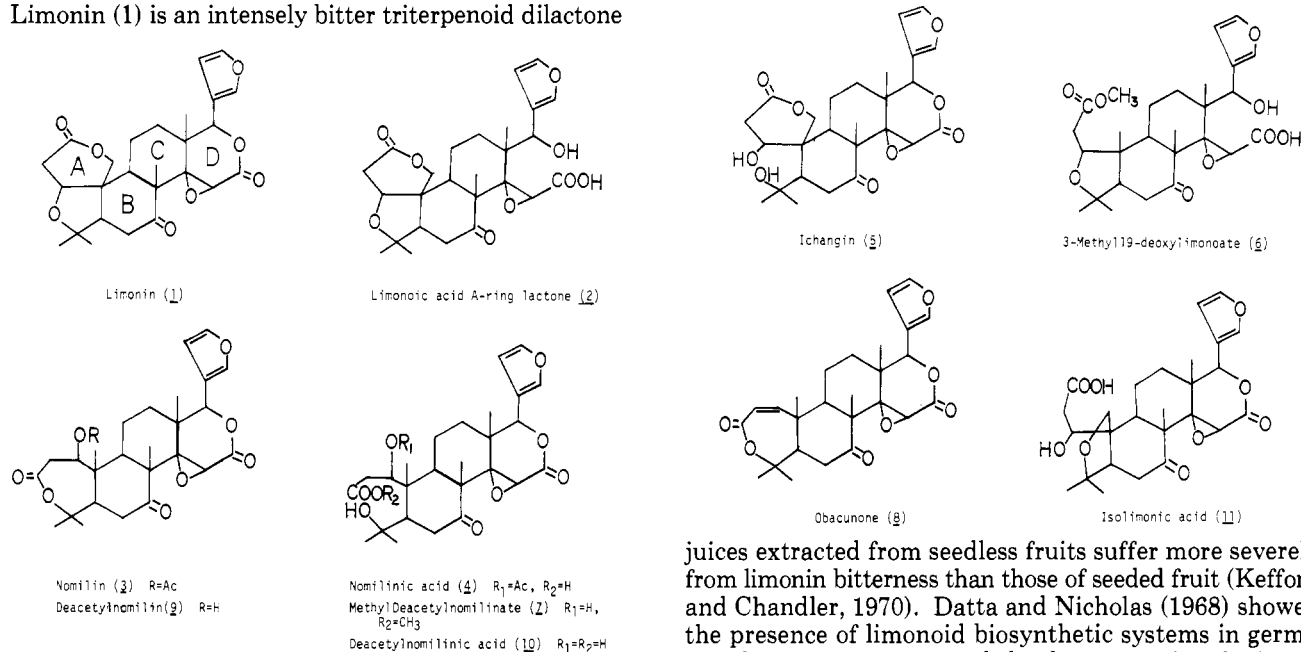
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Limonoids in Citrus Seeds: Origin and Relative Concentration

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Radioactive tracer experiments showed that citrus seeds accumulate limonoids as they are translocated from fruit tissue during growth of the fruit. De novo synthesis of limonoids from ^{14}C -labeled acetate in the seeds could not be demonstrated. The major neutral and acidic limonoids in seeds of various citrus species and hybrids were quantitatively analyzed.

Limonin (1) is an intensely bitter triterpenoid dilactone



present in citrus seeds, and it is largely responsible for the delayed development of bitterness in citrus juices. The intact fruits do not normally contain 1 but rather a non-bitter precursor, limonoic acid A-ring lactone (2), which is converted gradually to 1 in the juice after extraction from the fruit (Maier and Beverly, 1968). This delayed bitterness is a very serious economic problem in the citrus industry.

A group of triterpene derivatives chemically related to 1 found in Rutaceae and Meliaceae families have been named limonoids. Other limonoids such as nomilin (3), nomilinic acid (4), and ichangin (5) are also bitter, but they are insignificant in juice technology because of their low levels in juice.

Citrus seeds contain large amounts of limonoids. It has been suggested that limonoids present in the seeds are translocated from the fruit tissue and that consequently

juices extracted from seedless fruits suffer more severely from limonin bitterness than those of seeded fruit (Kefford and Chandler, 1970). Datta and Nicholas (1968) showed the presence of limonoid biosynthetic systems in germinated Valencia orange seeds by demonstrating the incorporation of ^{14}C -labeled mevalonate into 1. However, it is uncertain whether dormant seeds are capable of synthesizing limonoids, and, if they are, how significant those synthesized are in relation to the total limonoids accumulated in the seeds.

Limonoid research has been centered mainly on the isolation, structure determination, and, more recently, biochemistry of individual limonoids. Practically no data are available on the relative amounts of individual limonoids in citrus seeds. Dreyer (1966a) reported the occurrence of limonoids in citrus and their hybrids in respect to taxonomy, but only the major neutral limonoids known at that time were reported and they were not reported on a quantitative basis.

Therefore, we studied the mechanism of limonoid accumulation in citrus seeds and also made quantitative analyses of neutral and acidic limonoids in various citrus seeds.

EXPERIMENTAL SECTION

Materials. Calamin, retrocalamin, cyclocalamin, methyl isoobacunoate diosphenol, calaminic acid, retrocalaminic

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acid, and isoobacunoic acid diosphenol were isolated from calamondin seeds and identified as described elsewhere (Bennett and Hasegawa, 1980). Other limonoids were isolated from grapefruit seeds and identified by their NMR spectra. Most of the citrus species used in this study were grown at the USDA Date and Citrus Experiment Station, Indio, CA, the Fruit and Vegetable Chemistry Laboratory, USDA, Pasadena, CA, and the University of California at Riverside. Seeds were removed from the fruits and air-dried before analyses. 3-Methyl- ^{14}C 19-deoxylimonoate (6) was prepared by the procedure of Hasegawa et al. (1974), and methyl- ^{14}C deacetylnomilate (7) was prepared by the procedure of Hasegawa et al. (1980).

Extraction and Analysis of Limonoids. About 5 g of seeds was weighed and thoroughly macerated in 100–150 mL of 0.1 M Tris buffer at pH 8.0 with a Polytron (Brinkman Instruments, Westbury, NY). The mixture was incubated for 18 h at room temperature. During the incubation neutral limonoids, which are insoluble in aqueous solution, were hydrolyzed at the D ring by the limonin D-ring lactone hydrolase present abundantly in the seeds. Subsequently, the acids formed water-soluble solids. The mixture was filtered through Celite to yield a clear filtrate, which was acidified to pH 2.0. Acidification caused the D ring to re-form, and the acidic mixture was extracted with CH_2Cl_2 3 times. The combined CH_2Cl_2 extracts thus obtained contained little nonlimonoid material, whereas if the seeds had been extracted directly with an organic solvent, large amounts of contaminating substances would have been extracted with the limonoids.

Neutral and acidic limonoids were separated on a silica gel column (1.5 × 20 cm). The neutral limonoids were eluted from the column with EtOAc–MeOH (95:5) and the acidic limonoids with EtOAc–MeOH–HOAc (80:20:1). Limonoids were then analyzed by thin-layer chromatography with silica gel plates. Acidic limonoids were first methylated with CH_2N_2 . The chromatograms were developed with cyclohexane–EtOAc (4:6) or CH_2Cl_2 –MeOH (96:4) and sprayed with Ehrlich's reagent (Dreyer, 1965), a highly selective detection reagent for limonoids; exposure to HCl gas revealed the limonoids as orange spots. The relative amounts of limonoids present were estimated visually by comparison with reference compounds (Maier and Grant, 1970).

Feeding Experiments. For experiments on the biosynthesis of limonoids in seeds, aqueous solutions of sodium acetate-2- ^{14}C (2×10^8 cpm) were distributed evenly over 5 g of seeds and the seeds were kept moist for 6 days at room temperature. They were then washed thoroughly with H_2O and analyzed.

For studies on translocation of 3-methyl- ^{14}C 19-deoxylimonoate (6) from lemon fruit tissues to the seeds, an amount of 6 equivalent to about 40 000 cpm was dissolved in 0.2 mL of H_2O and absorbed into the stem of a detached fruit. The stem was then kept immersed in H_2O for 16 h at room temperature. Similar experiments were carried out with calamondin. A fully grown fruit was fed an amount of methyl- ^{14}C deacetylnomilate (7) equivalent to about 1×10^6 cpm.

After incubation, the seeds were removed and the limonoids were extracted by a procedure described previously (Hasegawa et al., 1980). Total radioactivity was counted with a Beckman liquid scintillation system, LS-3133P, and TLC radiochromatograms were scanned with a Packard radiochromatogram scanner, Model 7201.

RESULTS AND DISCUSSION

For the purposes of this paper, it is not necessary to make a distinction between 1 and its open D-ring form 2.

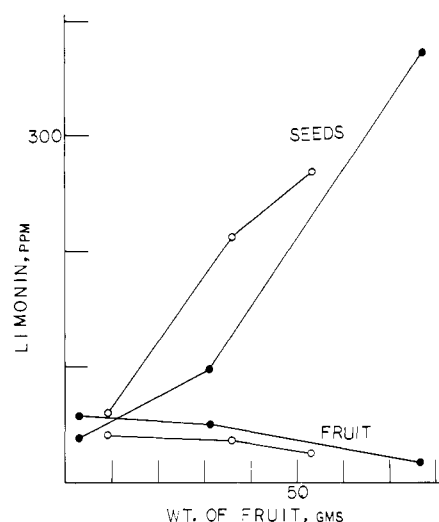


Figure 1. Changes in limonin concentration in fruit tissues and seeds during the growth of lemon fruit. Duplicate experiments were carried out with two different Eureka lemon trees: experiment 1 (○) and experiment 2 (●).

Table I. Ratios of Nomilin to Limonin in Various Tissues of Lemon

tissues	ratios	
	immature	mature
leaves	1:1	1:20
fruit tissues	1:1	1:15
seeds	1:1	1:1.3

Therefore, all analyses for 1 include both 1 and 2. The predominant form in seeds is 1, while in leaf and fruit tissue only 2 is found. It seems likely that this is also the case for the other limonoids, but this has not yet been established.

When lemon seeds, mature or immature, were incubated with ^{14}C -labeled acetate for 6 days, we observed no apparent incorporation of the labeled compound into limonoids. Datta and Nicholas (1968) reported that germinated Valencia orange seeds have limonoid biosynthetic systems. Since limonoids are actively synthesized in citrus leaves, particularly in young, immature leaves (Hasegawa and Hoagland, 1977), we believe that, most likely, the limonoids found by Datta and Nicholas had been synthesized in coleoptiles of the germinated Valencia orange seeds. Because we found no evidence of ^{14}C -labeled limonoids in lemon seeds and because young citrus leaves have been shown to actively synthesize limonoids, we concluded that limonoids found in seeds are not synthesized there.

Analyses showed that the concentration of 1 in lemon seeds increased steadily during fruit growth (Figure 1). On the other hand, the concentration of 1 in the fruit tissues was highest at very early stages of growth and decreased gradually thereafter when expressed as parts per million on a wet weight basis. This trend was similar to that observed for lemon leaves (Hasegawa and Hoagland, 1977). The concentration of 1 was very high in young immature leaves but decreased drastically as they matured.

Nomilin (3) and 1 were the major limonoids found in leaves and fruit tissues of lemon. The ratio of 3 to 1 was 1:1 in the immature leaves, fruit tissues, and seeds (Table I). Since young leaves actively synthesize limonoids and since citrus can translocate limonoids from leaves to fruit tissues (Hasegawa and Hoagland, 1977), we hypothesized that the limonoids found in the seeds had been translocated from the leaves. The ratios of 3 to 1 in mature lemons were different among leaves, fruit tissues, and

Table II. Translocation of 3-Methyl-¹⁴C 19-Deoxylimonate from Fruit Tissues to Seeds in Lemon^a

wt of fruit, g	radioact	
	found in seeds, cpm	translocation, %
10	1010	2.5
28	1120	2.8

^a Labeled limonoid (about 40 000 cpm) was fed through the stem of a detached fruit. The stem was then kept immersed in H₂O for 16 h.

Table III. Concentrations of Limonoids in Citrus Seeds

seeds	limonoids		
	total, % ^a	% neutral	% acidic
grapefruit	1.5	77	23
	1.6	78	22
Valencia orange	1.1	87	13
	1.2		
	0.9	81	19
lemon	1.0	58	42
	1.2	65	35
calamondin	0.73	88	12
	0.77	79	21
kumquat	0.51	55	45
	0.71		

^a Fresh weight percentage.

seeds. The ratio for the mature leaves was 1:20 and indicates that 1 was predominantly synthesized, even though as previously shown (Hasegawa and Hoagland, 1977) 1 is synthesized at a considerably lower rate in mature leaves than in immature leaves (approximately a 20–30 fold reduction). 3 and 1 synthesized in the mature leaves appear to have been continuously translocated to the fruit tissues, for the ratio in the mature fruit tissues changed to 1:15. The difference in ratios between leaves and fruit tissues was most likely due to the fact that limonoids in fruit tissues remain active. Some are metabolized through at least two pathways, one via 17-dehydrolimonoids (Hasegawa et al., 1974) and the other via deoxylimonoids (Hasegawa et al., 1980), and some are translocated to the seeds.

To test our hypothesis that limonoids in citrus fruit tissues can be translocated to the seeds, we determined the fate of a radioactively labeled analogue of 2, 3-methyl-¹⁴C-6, fed to detached lemons. We found that 2.5 and 2.8% of the labeled compound were translocated to the seeds of 10- and 28-g size fruits during 16 h of incubation, respectively (Table II). Similar experiments were performed with fully grown calamondin fruits by using radioactive methyl deacetylnomilinate (7) because 7 is a natural constituent of calamondin (Bennett and Hasegawa, unpublished results). In 16 h of incubation, 7500 cpm of the initial 1×10^6 cpm was translocated to the seeds. A radiochromatogram of the seed extract showed that over 90% of its total activity was due to radioactive 7. These radioactive tracer experiments clearly demonstrated that citrus is capable of translocating limonoids from fruit tissues to seeds.

Citrus seeds contain relatively high concentrations of limonoids (Table III). Among the seeds analyzed, *Citrus* species contained larger amounts of limonoids than *Fortunella* (kumquat) and its hybrid (calamondin). Limonoids were highest in grapefruit seeds and low in kumquat and calamondin. Neutral limonoids were higher than acidic limonoids in all species. This is understandable because neutral limonoids such as 1 and obacunone (8) are considered to be the end products of limonoid biogenesis,

Table IV. Relative Composition of Neutral Limonoids in Citrus Seeds

seeds	neutral limonoids			
	limonin (1)	nomilin (3)	oba- cunone (8)	deacetyl- nomilin (9)
grapefruit	76	15	1	8
	83	11	5	1
Valencia orange	50	34	1	15
	75	10	1	5
navel orange	87	10	1	2
sour orange ^a	37	26	1	11
	39	21	1	17
lemon	39	31	29	1
	40	40	20	1
lime	70	28	1	1
tangelo	43	43	13	1
tangerine	63	16	1	20
	60	18	1	21

^a Also ichangin (5) at 25 and 22%.

Table V. Relative Composition of Acidic Limonoids in Citrus Seeds

seeds	acidic limonoids		
	nomilinic acid (4)	deacetyl- nomilinic acid (10)	iso- limonic acid (11)
grapefruit	86	5	8
	85	13	1
Valencia orange	83	17	
sour orange	6	47	47
	3	64	32
lemon	95	5	
lime	76	15	9
tangelo	56	44	
tangerine	33	33	34
navel orange ^a	90	10	

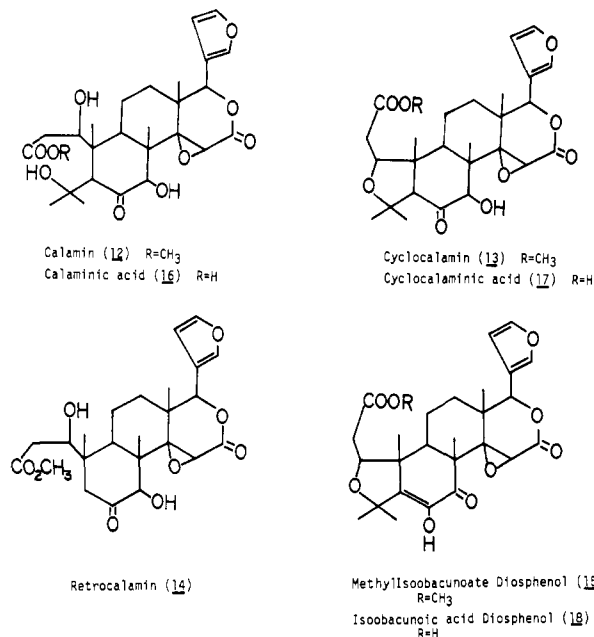
^a Fruit tissues.

whereas acidic limonoids are probably intermediate compounds. About 60% of the total limonoids in grapefruit seeds was 1, which is why grapefruit seeds have been used as the major source of 1 by various investigators.

TLC analyses showed that 1, 3, 8, and deacetylnomilin (9) are the major neutral limonoids in citrus seeds (Table IV). Compound 1 was highest in all species and was followed by 3, except for tangerine seeds, which contained more 9 than 3. Compound 8 was lowest of the four except in lemon and tangelo seeds. Lemon seeds contained a relatively high concentration of 8. A significant amount of 5 was found only in sour orange seeds.

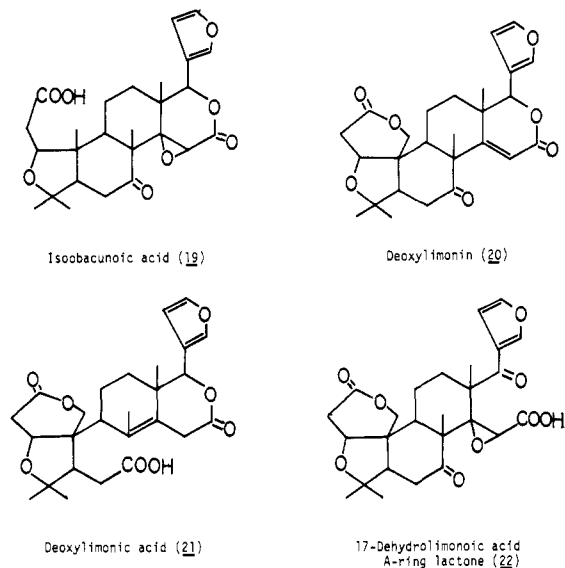
Relative concentrations of acidic limonoids in citrus seeds are summarized in Table V. Compound 4 and deacetylnomilinic acid (10) were the major acidic limonoids in citrus seeds. Compound 4 was highest in all species, followed by 10, except for sour orange seeds, which contained much more 10 than 4. They also contained a large amount of isolimononic acid (11) (Bennett and Hasegawa, 1980). Tangerine also contained 11, in an amount equivalent to 4 and 10.

Kumquat (*Fortunella margarita*) and calamondin (*Citrus reticulata* cv. "Austera" × *Fortunella* sp.) contained, in addition to limonoids found in citrus species, a group of limonoids which were inherited from *Fortunella* (Table VI). They are calamin (12), cyclocalamin (13), retrocalamin (14), methyl isoobacunoate diosphenol (15), and 7 and the corresponding acids of these neutral limonoids, such as calaminic acid (16) and cyclocalaminic acid (17). In kumquat seeds, 12 was the predominant neutral limonoid, 4 times as much as 1. The seeds also contained



a relatively large amount of 3. Compound 16 was the major acidic limonoid in kumquat seeds, which also contained considerable isobacunoic acid diosphenol (18). In calamondin seeds, 1 and 12 and 10 and 16 were the major neutral and acidic limonoids, respectively.

The occurrence of other minor limonoids in various citrus species has been reported. Isoobacunoic acid (19)



and epiisobacunoic acid have been isolated from grapefruit seeds (Bennett, 1971). Deoxylimonin (20) (Dreyer, 1965) and deoxylimonic acid (21) (Hasegawa et al., 1980) have also been isolated from grapefruit seeds. These two compounds are most likely metabolites of limonoid biodegradation systems via a deoxylimonoid pathway (Hasegawa et al., 1980). 17-Dehydrolimononic acid A-ring

Table VI. Relative Composition of Limonoids in Kumquat and Calamondin Seeds

	composition in	
	calamondin	kumquat
Neutral Limonoids		
limonin (1)	56, 51	17, 17
nomilin (3)	7, 10	15, 15
deacetylnomilin (9)	3, <1	1, 2
obacunone (8)	<1, <1	1, <1
calamin (12)	28, 27	67, 60
retrocalamin (14)	1, 2	1, 2
cyclocalamin (13)	2, 1	1, 2
methyl isobacunoate	2, 1	1, 1
diosphenol (15)		
methyl deacetylnomilate (7)	1, 5	1, 1
Acidic Limonoids		
calaminic acid (16)	40, 37	76
cyclocalaminic acid (17)	5, 7	1
retrocalaminic acid	5, 9	1
deacetylnomilinic acid (10)	50, 46	<1
isobacunoic acid	<1, 1	22
diosphenol (18)		

lactone (22), which gives no color reaction with Erlich's reagent, has been isolated from lemon seeds (Hsu et al., 1973). This compound is also considered to be a metabolite of a 17-dehydrolimonoid pathway (Hasegawa et al., 1974). Ichangin (5) has been found as a minor limonoid in Ichangin lemon (Dreyer, 1966b) and grapefruit (Bennett and Hasegawa, 1980) seeds. These minor limonoids have been isolated from one or two species, but they may also be present in others. In most cases their concentrations are quite low.

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