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BIOSYNTHESIS OF LIMONOIDS IN *CITRUS* SEEDLINGS

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Key Word Index—*Citrus* sp.; Rutaceae; limonoids; nomilinoate A-ring lactone; biosynthesis.

Abstract—Radioactive tracer work showed that nomilinoate A-ring lactone was the predominant, if not the only, limonoid biosynthesized and accumulated in seedlings of lemon, Valencia orange, grapefruit and tangerine. Lemon seedlings were excellent tools for biosynthetic preparation of [^{14}C]nomilin.

INTRODUCTION

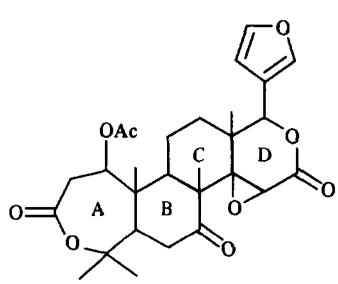
Bitterness due to limonoids such as limonin (**1**) and nomilin (**2**) in certain citrus juices is one of the major problems of the citrus industry worldwide and has significant economic impact. It has been generally accepted that citrus trees biosynthesize and acccumulate limonin (**1**) in seeds and limonoate A-ring lactone (**3**) in leaf and fruit tissues as the major limonoids [1–5]. During the course of our recent studies on limonoid biochemistry, we found that nomilinoate A-ring lactone (**4**) is the predominant limonoid accumulated in the tissues of citrus seedlings. We report here the results of radioactive tracer work showing that nomilinoate A-ring lactone (**4**) is one of the major components of citrus seedlings.

RESULTS AND DISCUSSION

The procedure for limonoid extraction from citrus was designed to extract the hydroxyacid limonoids as their water soluble salts. The predominant limonoids present in leaf and fruit tissues, the monolactones, such as limonoate A-ring lactone (**3**) and nomilinoate A-ring lactone (**4**) were subsequently isolated as their dilactones, limonin (**1**) and nomilin (**2**), respectively, by acidification and extraction with dichloromethane [3].

Nomilinoate A-ring lactone (**4**) was found to be the predominant limonoid present in lemon, and tangerine, and grapefruit seedlings (Table 1). This finding was significant since matured, fruit-bearing citrus trees syn-

*Reference to a product name or company does not imply endorsement of that product or company by the U.S. Department of Agriculture to the exclusion of others that may be suitable.



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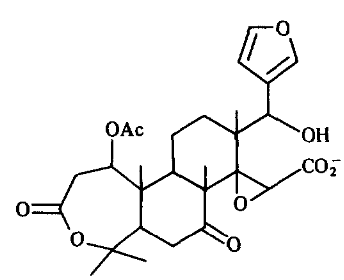

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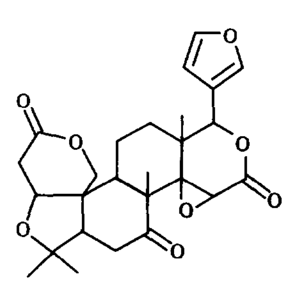

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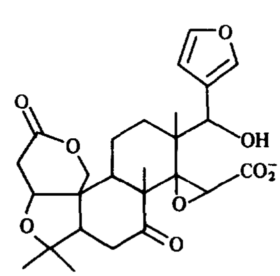

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Table 1. Limonoid content in citrus leaves

Citrus	Limonoate A-ring lactone (3) (ppm)	Nomilinoate A-ring lactone (4) (ppm)
Lemon seedling	31	1031
Lemon seedling	76	951
Lemon seedling	150	876
Tangerine seedling	85	210
Tangerine seedling	65	155
Tangerine seedling	91	514
Grapefruit seedling	86	272
Lemon tree, fruit-bearing	533	207
Lemon tree, fruit-bearing	650	213

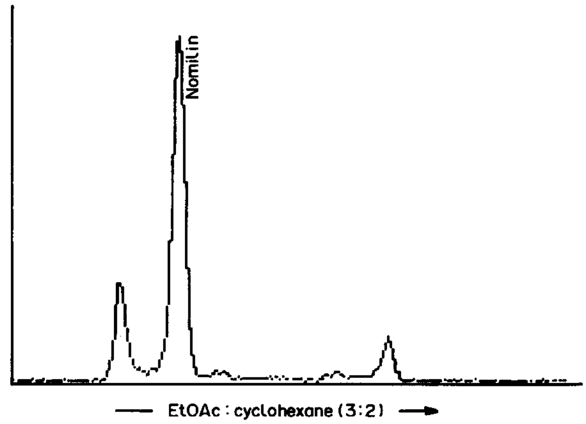

Fig. 1. Radiochromatogram of the stem extract obtained from a lemon seedling fed with sodium [1-^{14}C]acetate and incubated for 2 days.

thesize and accumulate limonoate A-ring lactone (3) as the major limonoids in their leaf and fruit tissues [2, 6]. Therefore, limonoid biosynthesis in the seedlings was investigated further.

When sodium [1-^{14}C]acetate was fed to lemon seedlings, 1.20–1.73% of the activity was incorporated into nomilinoate A-ring lactone (Table 2). A typical radiochromatogram of the stem extract is shown in Fig. 1. The major peak was identified as nomilin. Other peaks have not been identified yet, but none of them appeared to be limonoids. In particular, no peak was found in the limonin zone, showing that no activity was incorporated into limonoate A-ring lactone. [^{14}C]-Labelled nomilin was isolated on a silica gel column from an extract of a lemon seedling, fed with sodium [1-^{14}C]acetate. Analysis by TLC showed that the isolate had the same mobility as an authentic nomilin sample with three solvent systems. Furthermore, the association of the radioactivity with nomilin was confirmed by crystallization to constant specific activity (Table 3). After a small initial drop, the specific activity remained constant.

Similar radioactive tracer work showed that seedlings of grapefruit, Valencia orange and tangerine also biosynthesized and accumulated nomilinoate A-ring lactone in their tissues, but there was no evidence of limonoate A-ring lactone biosynthesis. These results suggested strongly that citrus seedlings, unlike matured, fruit-bearing trees, synthesize and accumulate nomilinoate A-ring lactone as the predominant limonoid. The limonoate A-ring lactone present in the seedlings as the minor limonoid (Table 1) could have been translocated from their seeds during germination and early growth, and may not be the product of biosynthesis in the tissues.

The incorporation of acetate into nomilinoate A-ring lactone was always much higher in the stems than in the leaves (Table 2). The specific activity of the nomilin isolated from the stems was also higher. In one experiment in which both the radioactivity and amounts of nomilin were measured quantitatively, the specific activity of the stem nomilin was 3.7 times greater than leaf nomilin. This means that little or none of the nomilin isolated from the stem could have been synthesized in the leaves. Therefore, the stem tissue must be a major site of limonoid biosynthesis. This is consistent with our finding that attempts to feed acetate directly to the leaves gave much lower yields of radioactive nomilin than did stem feeding.

Lemon seedlings were found to be excellent tools for the biosynthetic preparation of radioactively labelled nomilin. Over 5×10^6 cpm of [^{14}C]nomilin was isolated from the stem fed with 200 μCi of sodium [1-^{14}C]acetate.

Table 2. Biosynthesis of nomilinoate A-ring lactone in citrus seedlings

Citrus seedlings	Radioisotopes	Amounts fed (μCi)	Incubation (days)	Nomilinoate A-ring lactone (4) (cpm)		Total Incorporation (%)
Lemon	[1-^{14}C]acetate	30	6	stem	724 300	
				leaves	170 700	1.49
Lemon	[1-^{14}C]acetate	5	4	stem and leaves	120 000	1.20
Lemon	[1-^{14}C]acetate	200	2	stem	5 770 900	
				leaves	1 140 800	1.73
Lemon	[2-^{14}C]mevalonate	60	2	stem	513 900	
				leaves	58 300	0.48
Lemon	[1-^{14}C]farnesyl pyrophosphate	0.5	2	stem	15 000	0.15
Valencia orange	[1-^{14}C]acetate	30	2	stem and leaves	207 800	0.35
Grapefruit	[1-^{14}C]acetate	30	2	stem and leaves	178 100	0.30
Tangerine	[1-^{14}C]acetate	30	2	stem and leaves	330 000	0.55

Table 3. Recrystallization of [^{14}C]-labelled nomilin*

Solvents used for crystallization	Wt of crystals (mg)	cpm (mg)†
None	50.6	363 ± 8
MeOH	36.3	333 ± 9
MeOH	27.4	323 ± 7
Me_2CO	5.3	343 ± 8

* Purified labeled nomilin (120 000 cpm) obtained from the leaf and stem extract of lemon seedling was added to 50.6 mg of pure nomilin and crystallized.

† 90% confidence level.

The isolate obtained by a single column chromatography was radiochemically pure and can be used for studies of limonoid biosynthesis and biodegradation. Among substrates used, sodium [1-^{14}C]acetate was the best followed by [2-^{14}C]mevalonate and [1-^{14}C]farnesyl pyrophosphate. The latter was fed to a seedling in 10^{-3} M potassium phosphate buffer (pH 7.0). Without phosphate ions the substrate was not incorporated into nomilinoate A-ring lactone. Phosphate ions appeared to inhibit phosphatase activity present in the wounded tissues of the stem where the feeding string penetrated, and thereby prevented hydrolysis of farnesyl pyrophosphate before it could be incorporated. A similar effect of phosphate ions was observed when [1-^{14}C]farnesyl pyrophosphate was fed to a culture of *Cercospora rosicola* for studies of abscisic acid production [R. D. Bennett, unpublished results].

Up to 1.73% of labelled acetate was incorporated into nomilinoate A-ring lactone. This high value of incorporation shows that nomilinoate A-ring lactone is one of the major compounds synthesized and accumulated in the seedlings. At present biological functions of limonoids are unknown. Nomilin, limonin and obacunone, however, have been shown to possess antifeeding activity against lepidopteran pest, fall armyworm and cotton ballworm [7].

EXPERIMENTAL

Materials. The citrus seeds used for germination were obtained from the former U.S. Department of Agriculture, Date and Citrus Experiment Station, Indio, California and the U.S. Department of Agriculture, Fruit and Vegetable Chemistry Laboratory, Pasadena, California. Seedlings (about 10 cm in height with 8–10 leaves) were used for feeding experiments. Sodium[1-^{14}C]acetate (56 mCi/m mol) and [2-^{14}C]mevalonic acid DBED salt (50 mCi/m mol) were purchased from New England Nuclear, Boston, Massachusetts. [1-^{14}C]Farnesyl pyrophosphate was prepared by the procedure of Norman *et al.* [8]. Silica gel (Polygosil 60–2540, Machery-Nagel) was used for column chromatography. Silica gel G plates were used for TLC. Plates were developed with EtOAc–cyclohexane (3:2), CH_2Cl_2–MeOH (97:3) or toluene–EtOH–H_2O–HOAc (200:45:15:1, upperlayer).

Feeding experiments. An aq. soln of a radioisotope was fed to a seedling through a wet string penetrating the stem at about 3 cm above the ground. Both ends of the string were placed in a reservoir bottle where the feeding soln was supplied. Seedlings were then placed in the open field for 2–6 days.

Extraction and analysis of labeled limonoids. Seedlings were cut into small pieces and macerated thoroughly in 0.1 M Tris buffer at pH 8 with a Polytron. The mixture was filtered to yield a clear filtrate, which was then acidified to pH 2. Acidification caused the D-rings of the limonoids to close (for instance, nomilinoate A-ring lactone became nomilin). The acidic mixture was extracted ($\times 3$) with CH_2Cl_2. The combined extracts were brought to dryness and redissolved quantitatively in MeCN and used for analysis. Total radioactivity was counted with a Beckman liquid scintillation system, LS-3133 P and TLC radiochromatograms were scanned with a Berthold Automatic TLC-linear Analyzer, LB 2832.

Isolation of labelled nomilin (**2**). The stem extract (5.77×10^6 cpm) was dissolved in 0.5 ml of EtOAc–CCl_3F (1:4) and transferred to the top of a silica gel column (0.6 × 7 cm). The column was eluted, stepwise, by increasing concentrations of EtOAc in CCl_3F. The nomilin (**2**) fraction (5.12×10^6 cpm) was radiochemically pure as shown by TLC with three solvent systems.

Analysis of limonoids in citrus leaves. Leaves were extracted using the procedures described above and limonoids were quantitatively analysed by the method of Maier and Grant [9].

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