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BIOSYNTHESIS OF LIMONOIDS IN *CITRUS*

SHIN HASEGAWA and JOHN E. HOAGLAND

United States Dept. of Agriculture, Western Region, California-Hawaii-Nevada Area, Fruit & Vegetable Chemistry Laboratory, 263 South Chester Avenue, Pasadena, CA 91106, U.S.A.

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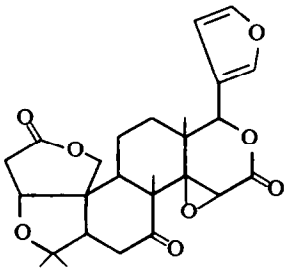
Key Word Index—*Citrus*; limonoids; biosynthesis.

Abstract—Analyses of citrus leaves and fruits, and radioisotope tracer work showed that the leaves could synthesize limonoids and that the trees could translocate limonoids from leaves to the fruit. These results suggest that limonoids in citrus fruit tissues are synthesized in leaves and translocated to the fruit. No evidence was found to support the presence of limonoid biosynthetic systems in the fruit tissues.

INTRODUCTION

Limonin (**1**) is an intensely bitter tetracyclic triterpenoid dilactone which causes bitterness in some processed citrus products. Although studies on the metabolic pathways of limonoids in bacteria and citrus have progressed considerably in recent years [1–7], practically no direct evidence relating to the biosynthesis of limonoids has been reported.



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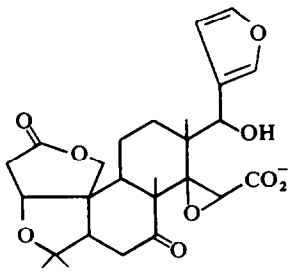
Radioisotope tracer work of Datta and Nicholas [8] showed the presence of biosynthetic systems in germinated seeds of Valencia oranges. However, the very low activity suggested that the seed is not the principal site of limonoid biosynthesis in citrus. In fact juices extracted from seedless fruits, such as navel oranges, appear to suffer more severely from limonin bitterness than those of fruit with seeds [9].

It has been generally accepted that limonoids are synthesized during the growth of the citrus fruits most likely in their albedo tissues [9]. There has been, however, no direct evidence to support this assumption. Datta and Nicholas [8] were unable to demonstrate limonoid biosynthetic systems in fruit tissues.

In the present work we show that citrus leaves are the active site of limonoid biosynthesis, and that the limonoids then appear to be translocated to the fruit.

RESULTS AND DISCUSSION

Limonoate A-ring lactone (**2**), precursor of limonin (**1**), was found to be actively synthesized in young citrus leaves. For instance, 10mg-size lemon leaves contained over 2000 ppm of (**2**). The total content increased as the leaf grew (Fig. 1), but the concentration decreased when expressed as ppm. This indicated that net synthesis of **2** was greater in young leaves than in larger, older ones.



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Second year lemon leaves contained, however, only trace amounts of **2**. Similar results were observed with grapefruit leaves. Pale green, 70mg-size leaves contained 480 ppm, whereas deep green, 480mg-size ones contained 52 ppm.

Navel orange and lemon fruits also contained **2** right after the blossoms dropped. The content then increased as the orange grew (Fig. 2). Similar results were obtained with lemon.

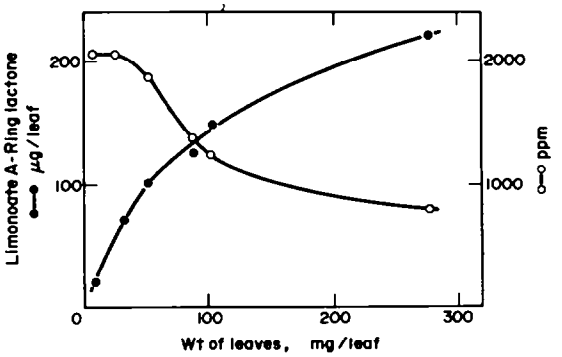


Fig. 1. Changes in limonoate A-ring lactone content during the growth of lemon leaves.

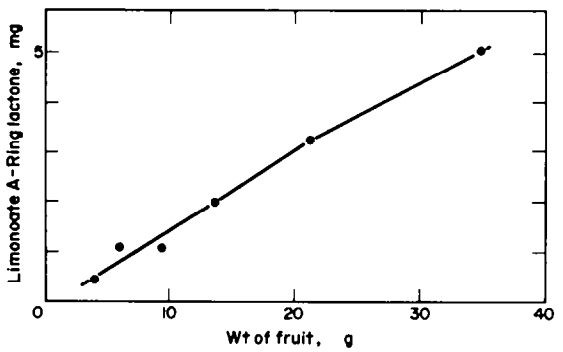
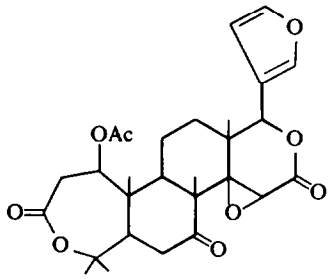


Fig. 2. Changes in limonoate A-ring lactone content during the growth of navel oranges.

Young, immature citrus fruit and leaves also contained large amounts of nomilin **3**, which was easily extractable with 0·1 M Tris buffer at pH 8. Its high solubility in aqueous solutions indicated that **3** was present in the open D-ring form. At early stages of growth, both lemon fruit as well as leaves contained equal amounts of **2** and **3**, but a 70g-size lemon contained only one-tenth as much **3** as **2**.



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Young twigs supporting young leaves and fruit also contained large amounts of limonoids. Lemon twigs which supported leaves containing 1250 ppm of **2** contained 935 ppm of **2**.

The above results clearly show that the citrus leaves tested could synthesize limonoids, but not whether the fruit tissues could. The limonoids present in the fruit could have been synthesized in the fruit or translocated from the leaves. Thus, to solve this question, we fed 5g-size navel oranges with 44×10^6 cpm of sodium acetate-[2-^{14}C], at the rate of 4.4×10^6 cmp per day for 10 consecutive days, either by injection of the fruit or by application on the peels. No labelled **2** was detected in the fruit. Also, no ^{14}C was incorporated into **2** when 5g-size navel oranges were similarly fed 20×10^6 cpm of mevalonate-[2-^{14}C]. Radiochromatography showed that both the acetate and mevalonate were incorporated into many other constituents.

We observed, however, that labelled acetate fed to leaves adjacent to 5g-size navel oranges was incorporated into compound **2** present in the fruit. Upon administration of 88×10^6 cpm of sodium acetate-[2-^{14}C] to the two leaves adjacent to the fruit in doses of 8.8×10^6 cpm per day for 10 consecutive days, compound **2**, isolated as the lactone **1** from fruit extracts by TLC, was labelled to the extent of 5.6×10^4 cpm/950 μg of **1**. This result, and the nonincorporation of labelled acetate or mevalonate into **2** in the navel oranges, suggested that **2** was synthesized in leaves and translocated to the fruit.

Table 1. Translocation of limonoate A-ring lactone-[^{14}C] in lemon

Total amounts fed to a leaf adjacent to 5g-size fruit ... 20000* cpm		
Recovery after 20 hr		
Leaf	2490 cpm	12·5%
Fruit	2670 cpm	13·3%

* About 98% of the labelled compound was absorbed through the surface of the leaf.

To prove the assumption that citrus can translocate **2** from leaves to fruit, we fed labelled **2** in 0.1 M phosphate buffer at pH 7.5 to a leaf adjacent to a 5g-size lemon (Table 1). After 20 hr, the fruit and leaf were extracted separately with 0.1 M Tris buffer at pH 8.0. Radioactivity measurements indicated that about 13.3% of the total radioactivity administered was translocated to the fruit. Radiochromatography showed the presence of labelled **2** in the fruit.

This work showed that the young citrus leaves were capable of synthesizing limonoids and that the trees were capable of translocating limonoate A-ring lactone to the fruit. These results thus suggest that limonoids in fruit tissues are synthesized in leaves and translocated to the fruit. However, this work does not rule out the possibility that a precursor(s) of limonoids beyond acetate or mevalonate was synthesized in leaves, translocated to fruit and there converted to limonoids. Thus, at present we cannot rule out fruit tissues as sites of limonoid biosynthesis.

Young leaves and fruits would be excellent for use in the study of the biosynthesis of limonoids and in the preparation of labelled limonoids. Sodium acetate-[2-^{14}C] fed to leaves adjacent to immature navel orange fruit was incorporated into compound **2** of the fruit at useful levels. The use of labelled mevalonate in place of acetate should increase the amount of labelled limonoids.

Several biosynthetic pathways of limonoids in citrus have been postulated based upon reasonable sequences of known limonoids [9–13], but confirmation may require the use of radioactive limonoids.

EXPERIMENTAL

Materials. Si gel (0·25 mm) sheets coated on aluminium were used for radiochromatographic analyses. Si gel G plates were used for quantitative analyses of limonoids. The following TLC solvent systems were used: (1) C_6H_6–EtOH–H_2O–HOAc (200:47:15:1, upper layer), (2) cyclohexane–EtOAc (30:70), and (3) CH_2Cl_2–MeOH (97:3). Limonin D-ring lactone hydrolase was isolated from grapefruit seeds according to Maier *et al.* [14]. Na-Acetate-[2-^{14}C] and mevalonate-[2-^{14}C] (DBED salt) were purchased commercially. Robertson navel orange and Meyer lemon trees were used for radioisotope tracer work. Radioactivity was measured by liquid scintillation counting

Quantitative analyses of limonoids. The quantity of **2** extracted from leaves and fruits as the lactone **1** was estimated by TLC. Tissues were ground in 20ml of 0.1 M Tris buffer soln at pH 8 with a Polytron. The mixture was centrifuged and the sediment was ground with 15ml of the same buffer. After filtration, the residue was re-extracted with 10ml of buffer. Combined extracts were acidified to pH 2 with HCl, and extracted with 3 30ml portions $CHCl_3$. Combined $CHCl_3$ extracts were evapd, and residue dissolved quantitavely in a minimum vol. MeCN. The MeCN fraction was spotted on TLC, and the plate was developed with solvent [1]. The plate was then dried, sprayed with Ehrlich's

reagent, and exposed to HCl gas for color development. Intensities of spots were estimated by visual comparison with those of known standards.

Feeding experiments. Aq. solns of labelled acetate and mevalonate were applied to leaves and fruits on trees according to the procedure of Bennett and Heftmann [15]. For studies on translocation of **2** from leaves to fruits, labelled **2** was prepared from labelled **1** with limonin D-ring lactone hydrolase. The D-ring of **1** was hydrolyzed with the hydrolase as follows. Approximately 0.1 mg of labelled **1** was suspended in 0.2ml of 0.1 M Pi buffer at pH 7.5, and incubated with enzyme at room temp. The reaction was completed in 5 hr. The resulting compound **2** was fed to a leaf adjacent to a lemon fruit by the procedure of Bennett and Heftmann [15]. About 98% of the total **2** applied was absorbed through the surface of the leaf.

Isolation of labelled compounds. Labelled **2** was extracted by the procedure described previously, and isolated from MeCN fractions by two preparative TLC runs with solvent (2) and (3). The isolated **1** was homogeneous when analyzed by TLC and a Vanguard automatic chromatogram scanner. The isolated compound **1** was also shown to be homogeneous when it was converted to **2** with limonin D-ring lactone hydrolase.

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