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LIMONIN BIOSYNTHESIS FROM OBACUNONE VIA OBACUNOATE IN *CITRUS LIMON*

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Key Word Index—*Citrus limon*; Rutaceae; citrus; biosynthesis; limonoids; limonin; obacunone; obacunoate.

Abstract—Radioactive tracer work showed that [^{14}C]obacunone was converted to at least four metabolites in *Citrus limon*. Two were identified as obacunoate and limonin. When [^{14}C]methyl obacunoate was fed, limonin was found to be one of the metabolites. Based on these results and data accumulated thus far, biosynthetic pathways of limonoids in citrus are proposed.

INTRODUCTION

Bitterness due to limonoids in certain citrus juices is one of the major problems of the worldwide citrus industry and has significant negative economic impact. According to biosynthetic pathways [1] proposed previously, which are based upon the limonoids known to occur in *Citrus* and *Citrus hybrids*, deacetylnomilinate (**4**) is the key, initial precursor of all the known citrus limonoids. Nomilin (**1**) and obacunone (**2**) are converted from **4** via nomilinate and/or deacetylnomilin, and limonin (**9**) is converted from **4** via isoobacunoate (**5**) and/or ichangin (**8**). Our recent findings [2, 3], however, showed that among the known citrus limonoids, compound **1** is the first limonoid to be biosynthesized followed by **2** in citrus. We report here that **2** is further converted to obacunoate (**3**) and to compound **9**.

The monolactones such as limonoate A-ring lactone (**10**) are the predominant limonoids present in citrus leaf and fruit tissues, whereas the dilactones such as limonin (**9**) are the predominant limonoids in seeds. For the purpose of this paper, we will not make a distinction between monolactones and dilactones.

RESULTS AND DISCUSSION

In this study we first demonstrated the conversion of obacunone (**2**) to obacunoate (**3**) and further to limonin (**9**) with a radioisotope tracer technique using 2.5 year old lemon trees and young lemon seedlings. When 3.5 $\times 10^5$ cpm of [^{14}C] obacunone (**2**) was fed to a young shoot of a 2.5 year old *Citrus limon* and incubated for 7 days, labeled **2** was converted to at least two major metabolites (Fig. 1). Peak B had R_f values identical to those of authentic **9** in three solvent systems (Table 1). This peak was isolated and treated with sodium hydroxide to hydrolyse the D-ring lactone of **9** to form limonoate A-ring lactone (**10**), which is an excellent substrate of limonoate dehydrogenase of *Arthrobacter globiformis* [4]. This dehydrogenase attacks the open D-ring of limonoids to form 17-dehydrolimonoids such as 17-dehydrolimonoate A-ring lactone (**11**). The base treated isolate was therefore incubated with limonoate dehydrogenase in the presence of NAD. The enzyme treated preparation was then methylated and analysed by TLC. Its R_f values were found to be identical to those of authentic methyl 17-dehydrolimonoate A-ring lactone (Table 1). This result confirmed that peak B was limonin (**9**).

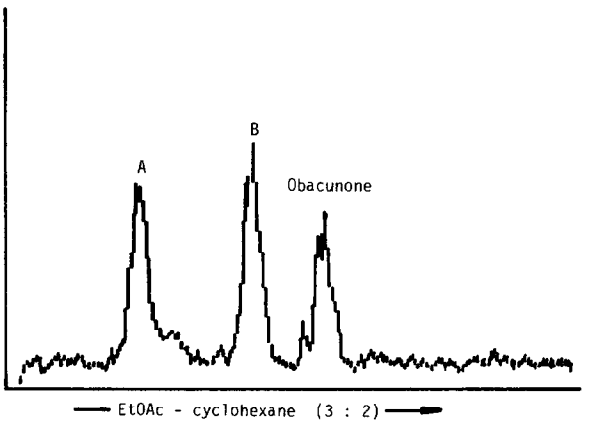


Fig. 1. Radiochromatogram of obacunone metabolites.

Peak A of Fig. 1 did not migrate in the nonacidic solvent systems used. However, when this peak, after isolation, was methylated, three major peaks were observed on TLC using solvent *a*. One peak, after isolation, had the same mobility as an authentic methyl obacunoate (methyl-**3**) sample in four solvent systems (Table 1). From these results we concluded that the acidic isolate was obacunoate (**3**).

When [^{14}C]obacunoate (**3**) was fed to a shoot of a 2.5 year old *C. limon*, very little of the labeled compound was metabolized. One of the metabolites appeared to be limonin (**9**). In an attempt to increase the metabolism of **3**, we fed [^{14}C]methyl obacunoate (methyl-**3**) to a plant. This methylation resulted in a significant increase in **3** metabolism. For instance, when 1.3×10^5 cpm of labeled methyl-**3** was fed and incubated for 5 days, almost 50% of the fed material was metabolized. One of the metabolites

Table 1. Identification of metabolites by TLC

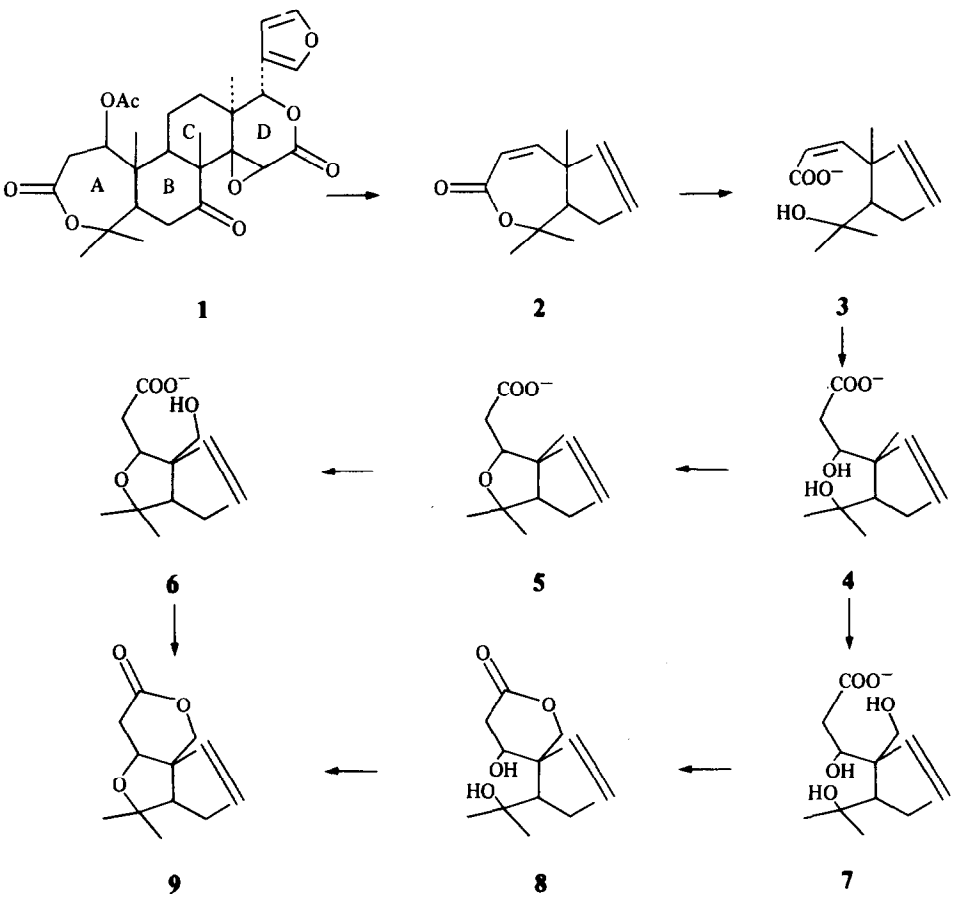
Compound	R_f*			
	Solvent system			
	a	*b*	*c*	*d*
Peak B	0.33	0.32	0.27	
Limonin	0.33	0.32	0.27	
Peak B treated with dehydrogenase and methylated†	0.25	0.28	0.18	
Methyl 17-dehydrolimonoate A-ring lactone†	0.25	0.28	0.18	
Peak A methylated	0.52	0.25	0.28	0.24
Methyl obacunoate	0.52	0.25	0.28	0.24

*Solvent key: see Experimental.
†Negative to Ehrlich's reagent.

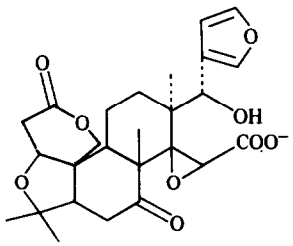
was identified as limonin (**9**) by the procedures similar to those used for identification of **2** metabolites described previously. Apparently, methyl-**3** could reach the site of limonoid biosynthesis more readily than did the free acid. Furthermore, there was TLC evidence that the plant is capable of demethylating some of the fed methyl-**3**. After methyl-**3** diffuses to the location of biosynthesis, the compound is most likely demethylated and then converted to limonin (**9**). The results obtained in this study show that **2** is converted to **3** and further to **9** in *C. limon.*

Based on the results obtained here and data reported thus far [2, 3], biosynthetic pathways of limonoids in citrus are proposed (Scheme 1). Among the known citrus limonoids, **1** is the first limonoid to be biosynthesized [2]. Compound **1** is then converted to **2** [3]. The conversion of **1** to **2** is most likely catalyzed by nomilin acetyl-lyase. This enzyme has been isolated from *Corynebacterium fascians* [5], but it has not been isolated from citrus. In young citrus seedlings such as lemon, grapefruit, Valencia orange and tangerine, **1** is the only limonoid biosynthesized and accumulated [2]. This is most likely due to the lack of nomilin acetyl-lyase activity in young citrus seedlings. In this study we reconfirmed that **1** was not converted to **2** in young lemon seedlings, but we did demonstrate this conversion with more mature trees. However, by the same methods used for the older trees, other limonoid biosynthetic systems, specifically the conversion of **2** to **3** and **3** to **9** (Scheme 1) were present in young seedlings.

The conversion of **2** to **3**, which was demonstrated in this study in young seedlings as well as in 2.5 year old trees of *C. limon* is most likely catalysed by obacunone A-ring lactone hydrolase. This enzyme has been isolated from *C. fascians* [5], but it has not been isolated from citrus.



Scheme 1. Possible biosynthetic pathways of limonoids in citrus.



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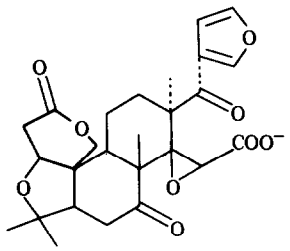

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Compound **9** appears to be the last limonoid to be biosynthesized among the known major limonoids. Between **3** and **9**, there are several possible precursors of **9** present. As suggested previously [1], limonin (**9**) is most likely biosynthesized from deacetylnomilinate (**4**) via isoobacunoate (**5**) and/or ichangin (**8**). Compounds **4**, **5** and **8** have been isolated from citrus seeds [6, 7], but 19-hydroxydeacetynomilinate (**7**) and limonoate (**6**) have not been isolated. The latter two may not accumulate in the tissues, and if accumulated at low concentration levels, they could be difficult to isolate because their open A-ring is unstable.

Previously, **4** was considered to be the initial and key precursor of all the limonoids [1], but this present work coupled with our recent work [2, 3] clearly demonstrates that **1** is the initial precursor of other limonoids.

EXPERIMENTAL

Materials. The *Citrus limon* seeds used for germination were from our laboratory. Seedlings and trees were grown in our green house. [^{14}C]Nomilin (**1**) (1.65 μCi/μmole) was biosynthesized from [1-^{14}C]acetate in young *C. limon* seedlings (10 cm in height with 8–10 leaves) by the procedure of Hasegawa *et al.* [2]. [^{14}C]Obacunone (**2**) was prepared enzymatically from [^{14}C]-**1** with nomilin acetyl-lyase, which was obtained from *Corynebacterium fascians* by the procedure of Herman *et al.* [5]. [^{14}C]Obacunoate (**3**) was prepared enzymatically from [^{14}C]-**2** with obacunone A-ring lactone hydrolase, which was obtained from *C. fascians* [5]. [^{14}C]Methyl obacunoate (methyl-**3**) was prepared by methylating [^{14}C]-**3** with CH_2N_2.

Feeding experiments. An aq. soln of a radioactive precursor was fed to a young shoot of a 2.5 year old tree or a young seedling through the stem by the procedure of Hasegawa *et al.* [2]. After 5–7 days of incubation, the shoot was harvested and used for analysis.

Extraction and analysis of labeled limonoids. Limonoids were extracted from the stem and leaves by the procedure of Hasegawa *et al.* [2]. The extracts were analysed by TLC on silica gel G plates with solvent systems: (*a*) EtOAc–cyclohexane (3:2), (*b*) CH_2Cl_2–MeOH (97:3), (*c*) CH_2Cl_2–EtOAc (7:3) and (*d*) toluene–EtOH–H_2O–HOAc (200:47:15:1, upper layer). TLC radiochromatograms were scanned with a Berthold Automatic TLC-linear Analyzer LB 2832. Limonoids were visualized by spraying plates with Ehrlich's reagent followed by exposure to HCl gas [8]. 17-Dehydrolimonoate A-ring lactone (**11**), which is negative to Ehrlich's reagent, was visualized by spraying H_2SO_4 followed by heating.

Isolation of labeled limonoids. The TLC spots, whose, R_f values were identical with the limonoids of interest, were scraped from the plates. The scrapings were then extracted with EtOAc to obtain radioactively pure compounds.

Enzymatic identification of the limonin isolate. The EtOAc extract obtained above was evaporated and the residue was dissolved in 4 ml of 0.1 N NaOH. This was heated for 10 min at 90° to convert limonin (**9**) to limonoate A-ring lactone (**10**). The pH was then adjusted to 9.5 with Tris buffer. The soln was incubated with limonoate dehydrogenase in the presence of 10^{-3} M NAD. After 2.5 hr of incubation at 23°, the reaction mixture was acidified to pH 2 and extracted with EtOAc and the extract was methylated with CH_2N_2. This methylated isolate was used for identification.

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REFERENCES

1. Hasegawa, S. and Maier, V. P. (1981) *Proc. Int. Soc. Citriculture* **2**, 91.
2. Hasegawa, S., Bennett, R. D. and Maier, V. P. (1984) *Phytochemistry* **23**, 1601.
3. Hasegawa, S. and Herman, Z. (1985) *Phytochemistry* **24**, 1973.
4. Hasegawa, S., Bennett, R. D., Maier, V. P. and King, A. D. Jr. (1972) *J. Agric. Food Chem.* **20**, 1031.
5. Herman, Z., Hasegawa, S. and Ou, P. (1985) *J. Food Sci.* **50**, 118.
6. Bennett, R. D. (1971) *Phytochemistry* **10**, 3065.
7. Dreyer, D. L. (1966) *J. Org. Chem.* **31**, 2279.
8. Dreyer, D. L. (1965) *J. Org. Chem.* **30**, 749.