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METABOLISM OF LIMONOIDS: NOMILIN TO NOMILINATE IN *CITRUS LIMON*

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Key Word Index—*Citrus limon*; Rutaceae; citrus; metabolism; limonoids; nomilin; nomilinate.

Abstract—Nomilinate was found to be the major acidic limonoid present in seedlings of *Citrus limon*. [^{14}C]Nomilin was converted to at least six acidic metabolites in *C. limon*, one of which was identified as nomilinate. The metabolism via nomilinate is the fifth metabolic pathway of nomilin shown to be present in nature.

INTRODUCTION

Bitterness due to limonoids such as limonin and nomilin (**1**) in a variety of citrus juices is a major problem of the worldwide citrus industry. Substantial progress has been made in studies on limonoid biochemistry in citrus during the past two years [1–3]. Recently, biosynthetic pathways of limonoids in citrus have been proposed [3]. Compound **1** is found to be the initial precursor of all the limonoids known to occur in citrus. We report here that **1** is metabolized to nomilinate (**2**) in *Citrus limon* L.

RESULTS AND DISCUSSION

Analyses of acidic limonoids showed that nomilinate (**2**) was the major acidic limonoid present in young seedlings of *C. limon*. Leaf tissues contained 850 ppm of **2** (average of five analyses).

When 1×10^6 cpm of [^{14}C]-**1** was fed to the stem of a young seedling and incubated for 5 days in a green house, it was all converted to acidic metabolites. Methylation of the metabolites with diazomethane showed that there were at least six peaks on a silica gel TLC plate when developed with solvent 2. One, whose R_f was identical to the methyl ester of **2** was isolated by scraping the peak from the preparative plate to obtain 85×10^3 cpm of a radioactively pure compound. The methylated isolate had R_fs identical to those of the methyl ester of **2** with four solvent systems.

The isolate was then suspended in 2 ml of 0.1 M Tris buffer at pH 8.5 and treated with two enzymes, limonin D-ring lactone hydrolase and limonoate dehydrogenase. Limonin D-ring lactone hydrolase converts the methyl ester of **2** to methyl nomilinoate [4], which is the open D-ring form of the ester of **2** and is an excellent substrate for limonoate dehydrogenase [5]. The latter enzyme converts methyl nomilinoate to methyl 17-dehydronomilinoate in the presence of its cofactor NAD. After 10 hr of incubation, the reaction mixture was acidified and extracted with dichloromethane. The extract was methylated with diazomethane and analysed on TLC with four solvent systems. The methylated product had R_fs identical to those of dimethyl 17-dehydronomilinoate (**3**). From these results the isolate was identified as **2**.

Two and a half year old lemon trees also converted **1** to **2**. For instance, when 8.5×10^5 cpm of labeled **1** was fed to the young shoot of the plant, 4500 cpm of activity was incorporated into **2** after 5 days of incubation. Although the rate of conversion from **1** to **2** differed between experiments, it was clear that both young seedlings and 2.5 year old trees of *C. limon* were capable of converting **1** to **2**.

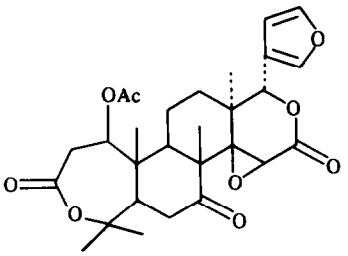
According to the biosynthetic pathways proposed recently [3], **1** is the initial precursor of all the known citrus limonoids. Compound **1** is first converted to obacunone, and then to obacunoate and deacetylnomilinate. Limonin, which is the last major limonoid to be formed, is produced from deacetylnomilinate via ichangin and/or isoobacunoate. Compound **2**, therefore, is not directly involved in the major biosynthetic pathways of limonoids in citrus and is a metabolite of **1**. It has been shown that **1** is metabolized in citrus and bacteria by at least four pathways [5–8]. The metabolism via **2** is the fifth metabolic pathway of **1** found in nature.

EXPERIMENTAL

Citrus limon, both young seedlings (10 cm in height with 8–10 leaves) and 2.5 year old trees, were grown in our greenhouse. [^{14}C]Nomilin (**1**) (1.65 μCi/μmole) was biosynthesized from [1-^{14}C]acetate in young lemon seedlings by the procedure of ref. [1]. Limonin D-ring lactone hydrolase was obtained from grapefruit seeds by the procedure described previously [4]. Limonoate dehydrogenase was obtained from cell-free extracts of *Arthrobacter globiformis* by the procedure of ref. [5].

Feeding experiment. An aq. soln of a radioisotope was fed to the stem of *C. limon* through a wet string by the procedure described previously [1]. The plants were then placed in a greenhouse for 3–5 days.

Extraction and analysis of labeled metabolites. Labeled metabolites were extracted from stems and leaves by the procedure especially designed for extraction of limonoids [1]. Silica gel G plates were used for TLC. The solvents used were: (1) CH_2Cl_2–MeOH (97:3); (2) toluene–EtOH–H_2O–HOAc (200:47:15:1, upper layer); (3) cyclohexane–EtOAc (2:3) and (4)



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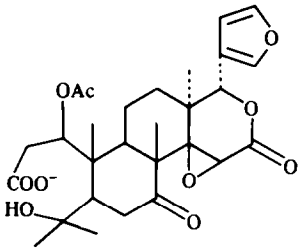

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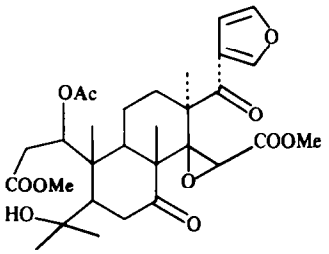

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EtOAc–CH_2Cl_2 (3:2). Radioactivity was located by means of a TLC radiochromatogram scanner. Total radioactivity was measured by liquid scintillation spectrometry.

Isolation of labeled Me nomilinate. The TLC spot, whose R_fs were identical to those of Me nomilinate, was scraped from the preparative plate. The scrapings were then extracted with EtOAc to obtain a radioactively pure compound.

Enzymatic identification of the metabolite. The EtOAc extract obtained above was evaporated and the residue was incubated with limonin D-ring lactone hydrolase and limonoate dehydrogenase in 2 ml of 0.1 M Tris buffer at pH 8.5 in the presence of 10^{-2} M NAD. After 10 hr of incubation at 23°, the reaction mixture was acidified to pH 2 and extracted with CH_2Cl_2. The CH_2Cl_2 extract was methylated with CH_2N_2. The methylated product had the same R_f values as authentic diMe 17-dehydronomilinoate in solvent systems 1 (R_f 0.61), 2 (0.36), 3 (0.56) and 4 (0.63).

Analyses of acidic limonoids. Acidic limonoids in young seedlings of *C. limon* were quantitively analysed by the procedure described previously [9].

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