Note

Biosynthesis of Limonoids in
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Bitterness due to limonoids in a variety of citrus lowers the commercial value of their juices and processed products. Among the 37 limonoids isolated from citrus and its hybrids, limonin, nomilin, ichangin, and nomilinic acid are known to be bitter.¹⁾ Meanwhile, ichangensin, a non-bitter limonoid, has been shown to be present in *Citrus ichangensis*²⁾ and its hybrids such as *C. yuzu* (*Citrus junos* sieb. ex Tanaka) and *C. sudachi* (*Citrus sudachi* Hort. ex Tanaka).³⁾ In *C. ichangensis*, nomilin is converted to ichangensin via deacetylnomilin and possibly deacetylnomilate in addition to the common pathway to limonin. The purpose of this study was to investigate the biosynthesis of limonoids in *C. sudachi*, which is closely related to *C. ichangensis*.

C. Sudachi seedlings were grown in the Pasadena laboratory and the fruits were harvested from a 4-year-old *C. sudachi* tree grown in the same laboratory.

A 3-cm long detached stem of a 2–4 months old *C. sudachi* seedling was fed with 2 μ l of [¹⁴C]nomilin (1.0–1.4 $\times 10^5$ cpm) and incubated for 2 days at 22°C. For the feeding experiment with *C. sudachi* fruit, a fruit with a small piece of stem was detached, fed with 5 μ l of [¹⁴C]nomilin (1.0 $\times 10^5$ cpm) through injection into the center core at the stem end of fruit, and incubated for 5 days at 22°C.

Limonoids were extracted from the stem and fruit tissues by the procedure of Hasegawa *et al.*⁴⁾ with some modifications. The tissues were cut into small pieces and macerated in 0.1 M Tris buffer (pH 8) with a Polytron. The homogenate was acidified to pH 2 with 1 N HCl, then extracted twice with ethyl acetate. The extracts were dried, redissolved in acetonitrile, and analyzed by TLC on HLF silica gel plates with four solvent systems: (A) ethyl acetate–cyclohexane (3:2), (B) methylene chloride–methanol (97:3), (C) ethyl acetate–methylene chloride (2:3), (D) toluene–ethanol–water–acetic acid (200:47:15:1, upper layer). TLC radiochromatograms were scanned with

a Berthold Automatic TLC-Linear Analyzer LB 2832. Limonoids were stained with Ehrlich's reagent (0.5% 4-dimethylaminobenzaldehyde in ethanol) and HCl gas.⁵⁾

When the stems of *C. sudachi* seedlings were fed with [¹⁴C]nomilin, five radioactive peaks (peaks A, B, C, D, and E) were observed by TLC with solvent system "C" (Fig. 1). A major peak (peak D), in which 12.9–36.4% of the fed label was recovered, was identified as nomilin by TLC of the compound extracted from the peak. Peaks C (recovery; 0.5–1.9%) and E (recovery; 0.1–0.3%) were identified as deacetylnomilin and obacunone, respectively. Peak A was isolated and methylated, and a major radioactive product was identified as methyl nomilate, which shows that peak A contains nomilinic acid (recovery; 0.4%). Peak B has not been identified yet, but it appeared not to be a limonoid. These results show that the stem tissue of *C. sudachi* seedlings metabolizes nomilin to deacetylnomilin, obacunone, and nomilinic acid.

Similar radioactive tracer work showed that the *C. sudachi* fruit tissue converted nomilin to deacetylnomilin, ichangensin, obacunone, and limonin. When the tissue was fed with [¹⁴C]nomilin, six radioactive peaks were observed

Fig. 1. Radiochromatogram of Metabolites of [¹⁴C]-Nomilin Fed to Sudachi Seedling.

Fig. 2. Radiochromatogram of Metabolites of [¹⁴C]-Nomilin Injected into Sudachi Fruit.

Fig. 3. Possible Biosynthetic Pathways of the Major Limonoids in *Citrus sudachi*.

with solvent system (B) for the extracts of both peel and flesh (Fig. 2). The largest peak, P-5, was identified as nomilin and the polar peak, P-1, was found to contain nomilinic acid (0.1–0.3% incorporation). Peaks P-2, P-3, P-4, and P-6 were identified as deacetylnomilin (0.8–3.7%), ichangensin (0.02–0.1%), limonin (0.03–0.3), and obacunone (0.4–1.6%), respectively.

The conversion of nomilin to deacetylnomilin has been shown to be unique to *C. ichangensis* and no other citrus has been shown to have this pathway. This work shows, however, that the fruit tissue of *C. sudachi* has this pathway

(Fig. 3), which also suggests that *C. sudachi* is a descendant of *C. ichangensis*. On the basis of limonoid composition,³⁾ this pathway is the minor one in *C. sudachi*.

The absence of labeled ichangensin and limonin in the extract of stems fed with labeled nomilin suggests that the stem is not the major site of ichangensin and limonin biosynthesis. Nomilin is biosynthesized in the stem and translocated to other locations such as fruit tissues, seeds, and leaves, where it is further converted to other limonoids.

References

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