Biosynthesis of Nomilin from ^{14}C -Acetate in the Stems of Yuzu and Sudachi Seedlings

Fumio HASHINAGA

Faculty of Agriculture, Kagoshima University, Kagoshima 890

Summary

To reduce limonoid bitterness in citrus fruit, the biosynthesis and metabolism of nomilin in stems of Yuzu (*Citrus junos* Sieb. ex Tanaka) and Sudachi (*Citrus sudachi* Hort. ex Shirai) were studied by using ^{14}C -acetate.

Biosynthesis of nomilin from acetate was more active in the stems of seedlings than in shoots of citrus tree. The rate of nomilin biosynthesis in shoots was faster when the leaves were expanding to full size than at budbreak. It was relatively fast in the stem, reaching a maximum in 5.5 hr, whereas after a 10-day incubation period, its activity decreased rapidly.

Biosynthesis of nomilin in stems of Sudachi was slightly more active than that of Yuzu stems. Nomilin was converted mainly to deacetylnomilin; the rate of nomilin metabolism being more active in the shoots than stems of seedlings.

It may be concluded that stems of Yuzu and Sudachi synthesize nomilin from acetate, and then convert it to deacetylnomilin, obacunone, and nomilinic acid.

Introduction

Limonoids, one of the causes of bitterness in citrus, are contained in all citrus fruits, their taste being represented by limonin and nomilin (6). The utilization of citrus fruits for juices is restricted because of limonoid bitterness, which is much stronger than that of naringin (10).

Ichangensin (3), non-bitter limonoid, was found in Ichang Chie (*Citrus ichangensis* Swingle). Its level was higher than that of limonin. A large amount of ichangensin exists in seeds of Yuzu and Sudachi fruits (7,8). This finding indicates that these two species are related to Ichang Chie. A particular pathway from nomilin to ichangensin has been shown to be present in Ichang Chie (9).

Biosynthesis of limonoids was found to be most active in stems of *Citrus limon* seedlings (3), especially in the phloem (11).

For the purpose of clarifying the possible biosynthetic pathways of limonoids in Yuzu and Sudachi, the conditions for limonoid biosynthesis were investigated by feeding ^{14}C -acetate into the stems of citrus seedlings.

Materials and Methods

Materials : Stems of Yuzu and Sudachi seedlings, shoots of Yuzu trees grown in the Citrus Research Center, University of California at Riverside, and a new shoot of Yuzu grafted on to sour orange stock were used for the present studies.

Incorporation of ^{14}C -acetate into the stems : Two μl of ^{14}C -sodium acetate (59 mCi/mmol, 4.4×10^6 cpm) were taken in a small V-type vial and fed into a 3-cm piece of a detached stem. Water was added to the vial to prevent drying. The incubation period at 22°C was 48 hr in most experiments.

Extraction of limonoids : Stems were cut into small pieces and ground in 5 ml of 0.1M Tris buffer (pH 8) with a Politron homogenizer. The supernatant after centrifugation (8,000 rpm, 8 min) was adjusted to pH 2 with 1N HCl, followed by extracting limonoids with ethyl acetate (2 times). After adding a small amount of butylated hydroxytoluene, the ethyl acetate fractions were dried under nitrogen gas. The residue was dissolved in 1 ml of acetonitrile.

Determination of limonoids : The total radioactivity of the extracts was counted with a Beckman Liquid Scintillation System. Another portion of the

extracts was spotted on silica gel plates, which were developed by the following v/v solvent systems: (a) ethyl acetate-cyclohexane (3:2), (b) methylene chloride-methanol (97:3), (c) ethyl acetate-methylene chloride (2:3), and (d) toluene-ethanol-water-acetic acid (200:47:15:1).

Radiochromatograms were scanned with a Berthold Automatic TLC-Linear Analyzer LB 2832. The ratio of the count of each peak to that of the total was measured. Polar compounds extracted with ethyl acetate were rechromatographed after methylation with diazomethane. To identify the separated peaks, authentic limonoids were spotted on both sides of radioactive samples at the origin of the TLC plates. After measuring the radioactivity of the plate, the chromatogram was sprayed with Ehrlich's reagent (0.5% 4-dimethylaminobenzaldehyde in ethanol). The limonoids were then made visible by exposure to HCl fume.

The radioactive spots on the chromatogram were scraped and the silica gel eluted with ethyl acetate. By respotting the eluates on fresh TLC plates and developing them with the other solvent systems, the identity of each limonoid was reconfirmed.

Preparation of ^{14}C -nomilin : ^{14}C -nomilin was prepared by feeding ^{14}C -acetate to the detached stem of Sudachi seedling. Its metabolites were extracted with ethyl acetate, spotted on TLC plates and developed with the above four solvent systems. The ^{14}C -nomilin was purified repeatedly until only one radioactive spot appeared on the thin-layer plate.

Metabolism of ^{14}C -nomilin : ^{14}C -nomilin was fed into the detached stem of citrus using the same procedure as the incorporation of ^{14}C -acetate. The metabolites extracted with ethyl acetate were identified as described above.

Results and Discussion

Biosynthesis of limonoids in the stem of citrus

Ethyl acetate extracts from ^{14}C -acetate-fed stem of Yuzu which were spotted on TLC plates and developed with solvent system C, yielded a typical radiochromatographic tracing (Fig. 1). Major peak D was identified as nomilin, and peaks C and E, as deacetylnomilin and obacunone, respectively.

Rf value of peak B on the radiochromatogram was almost the same as that of ichangin in most trials. By eluting and developing the radioactive

Fig. 1. Radiochromatographic tracing of an extract obtained from a detached stem of Yuzu seedling fed with sodium ^{14}C -acetate. A: Polar compounds containing nomilinic acid, B: Unknown, C: Deacetylnomilin, D: Nomilin, E: Obacunone, F: Unknown.

constituents several times with solvent system b, the compounds were found to have different Rf values from ichangin. Because the Rf values of the compound differed from those of known limonoids, its true identity is yet known. Since the Rf value of the predominant peak A on the tracing corresponds to that of methyl nomilinate, a major compound within peak A was identified to be nomilinic acid.

The detached stems of Sudachi seedlings were used to determine an optimum incubation time for nomilin biosynthesis. It was found to be relatively short (5.5 hr) for nomilin biosynthesis (Table 1). With a 2- to 10-day incubation period, the level of labelled nomilin decreased; a 2-day incubation period was, therefore, adopted in most experiments.

The influence of stem age on the rate of nomilin biosynthesis was found to be greater in shoots in spring than in autumn, and also slightly greater in more mature shoots than in young shoots, respectively (Table 2). The biosynthesis of nomilin in the seedling and mature stem of Sudachi was found to be slightly more active than that of Yuzu.

The rate of nomilin biosynthesis in young stems of citrus seedlings was much faster than that of shoots. In an extract of very young shoots, one relatively large peak of radioactivity appeared between peaks of nomilin and obacunone and another one near that of ichangin when solvent system b was used. But this latter peak did not coincide

Table 1. Effect of incubation time on nomilin biosynthesis in stems of Sudachi seedlings.

Incubation time	Nomilin % of radiochromatogram	Nomilin incorporation (%)
1 hr	27.3	0.27
5.5 hr	58.1	3.34
2 days	47.5	2.52
10 days	29.8	0.68

Table 2. Biosynthesis of nomilin from sodium [$1\text{-}^{14}\text{C}$] acetate in stems and shoots of Yuzu and Sudachi at various age.

Stems			Percent of total radioactivity		Nomilin incorporation (%)
Age (month)	Height (cm)	No. of leaves	Nomilin	Deacetyl-nomilin	
(Yuzu seedlings)					
2	3	2	8.2	1.9	0.22
2	3	2	9.4	9.1	0.19
2	3	4	34.2	5.6	1.00
2	5	10	53.9	10.8	1.85
6	13	17	45.9	18.9	1.95
5*	15	15	9.8	1.0	0.11
(Yuzu shoots)					
Young**			2.1	—	0.02
Mature**			0.7	—	0.01
Mature*			0.7	—	0.01
(Sudachi seedling)					
2	3	4	27.6	6.1	1.04
2	5	6	40.1	10.4	2.31
4	12	15	33.3	12.1	0.53
(Sudachi shoots)					
Young**			2.3	—	0.02
Mature**			2.3	—	0.05
Mature*			2.5	—	0.03

* Sampling in late autumn, ** Shoots after grafting.

with that of ichangensin (2) nor of limonyl acetate (1). When these two unknown radioactive compounds were eluted and administered into another stem of Sudachi seedling, two radioactive peaks reappeared again on subsequent plates. Their R_f values did not correspond with any known limonoids in citrus. Therefore, these two radioactive compounds hardly seem to be intermediates or precursors of limonoids.

Nomilin metabolism in citrus stems

Radiochromatographic tracings of the metabolites of ^{14}C -nomilin in the stem of Yuzu seedlings (Fig. 2) resembled closely a typical pattern of the metabolites in which ^{14}C -acetate was fed into the

stem under good conditions. The polar compound (A) was proven to be nomilinic acid. The unknown compound with a R_f near that of ichangin was also present among the metabolites of ^{14}C -nomilin. Ichangensin was not detected in the stem, although it was present in seeds of Sudachi and Yuzu (8, 9).

Metabolites of nomilin in the stems of Yuzu and Sudachi are shown in Table 3. Percentages of deacetylnomilin and obacunone to the total metabolites was significantly higher in young shoot than in older shoots and stems of seedlings. Therefore, it seems that the activity of enzyme metabolizing nomilin to deacetylnomilin and obacunone was higher in the shoots of mature trees than in the seedlings.

Table 3. Incorporation and metabolites of ^{14}C -nomilin in stems of Yuzu and Sudachi.

Stems	Nomilin fed (cpm)	Total incorpo- ration (%)	Percent of limonoids		
			Nomilin	Deacetylnomilin	Obacunone
(Yuzu)					
Seedling	3.0×10^5	40.0	67.4	2.5	0.5
Seedling	1.4×10^5	36.1	51.6	4.2	3.5
Shoot	3.0×10^5	19.9	54.4	5.6	7.3
(Sudachi)					
Seedling	1.0×10^5	17.7	72.7	3.0	0.2
Seedling	1.0×10^5	50.1	72.7	3.8	0.3
Seedling	1.4×10^5	35.9	71.0	3.6	0.9
Shoot	2.5×10^5	37.6	58.4	9.2	8.1

Fig. 2. Radiochromatographic tracing of metabolites derived from ^{14}C -nomilin fed to a stem of Yuzu seedling. A: Polar compounds containing nomilinic acid, B: Unknown, C: Deacetylnomilin, D: Nomilin, E: Obacunone.

The present study demonstrates that the biosynthetic pathway by which nomilin was synthesized from acetic acid and then converted to deacetylnomilin, obacunone (4) and nomilinic acid (5) present in stems of Yuzu and Sudachi trees.

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ユズとスダチの実生の茎における ^{14}C -酢酸からのノミリンの生合成

橋 永 文 男

鹿児島大学農学部 890 鹿児島市郡元1丁目

摘 要

カンキツ果実におけるリモノイド系苦味物質を制御する目的で、 ^{14}C -酢酸を用いてリモノイドの生合成と代謝をユズ(*Citrus junos* Sieb. ex Tanaka)とスダチ(*Citrus sudachi* Hort. ex Shirai)の茎で研究した。

酢酸からのノミリンの生合成はカンキツの新梢よりも実生の茎が明らかに活発であった。また新梢のノミリンの生合成は展葉期よりも葉が十分に展開したときの方が少し活性があった。茎におけるノミリンの生合成速度は比較的早く、5.5時間で最大近くになり、10日間処理では明らかにそれよりも減少することが認め

られた。

一方、スダチの実生および成熟した茎におけるノミリンの合成速度はユズよりわずかに活発であった。ノミリンは主にデアセチルノミリンに変換され、その代謝速度は実生よりも新梢の方が活発であった。すなわち、ユズとスダチの茎において酢酸からノミリンが合成され、さらにデアセチルノミリン、オバクノンおよびノミリン酸に代謝される経路があることが明らかになった。