Phytochemistry, Vol. 24, No. 9, pp. 1973–1974, 1985.
Printed in Great Britain.

0031-9422/85 $3.00 + 0.00
Pergamon Press Ltd.

BIOSYNTHESIS OF OBACUNONE FROM NOMILIN IN *CITRUS LIMON*

SHIN HASEGAWA and ZAREB HERMAN

U.S. Department of Agriculture, Agricultural Research Service, Western Region, Fruit and Vegetable Chemistry Laboratory, Pasadena, CA 91106, U.S.A.

(*Received* 5 *December* 1984)

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

Abstract—Radioactive tracer work showed that [^{14}C]nomilin was converted to at least four metabolites in *Citrus limon*. One metabolite was identified as obacunone, showing that obacunone is biosynthesized from nomilin in *C. limon*.

INTRODUCTION

Limonoids are a group of triterpenoids found in Rutaceae and Meliaceae. Obacunone (**1**) is one of 29 limonoids found in *Citrus* and *Citrus* hybrids [1] and is one of the major limonoids present in most citrus species [2]. The biosynthetic pathways of limonoids in citrus thus far have been postulated based upon reasonable sequences of known limonoids [1, 3]. No one has actually shown direct evidence to support the proposed pathways. It has been suggested that obacunone (**1**) is synthesized from nomilin (**2**) [4]. We report here that [^{14}C]nomilin was converted to obacunone in *Citrus limon*.

RESULTS AND DISCUSSION

When [^{14}C]nomilin was fed to *Citrus limon* through the stem of a young shoot, it was converted to at least four metabolites (Fig. 1). One, which was less polar than nomilin, was isolated by TLC as a radioactively pure compound. The isolate had R_fs identical to those of

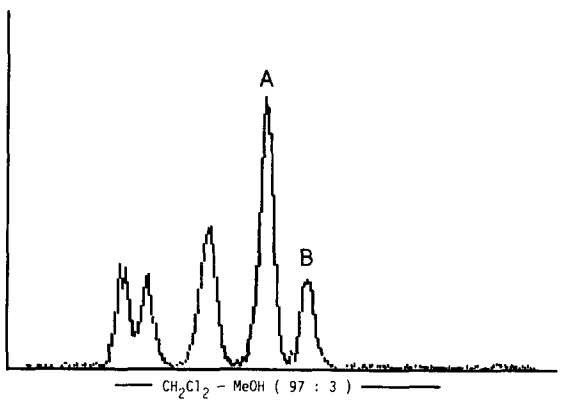
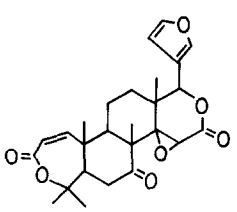
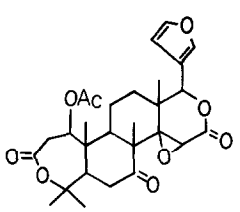


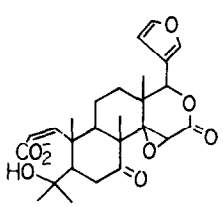
Fig. 1. Radiochromatogram of the extract obtained from *Citrus limon* fed with [^{14}C]nomilin and incubated for 5 days. A, The substrate, nomilin; B, the compound of interest.



1



2



3

authentic obacunone (Table 1). The isolate was then shown to be a substrate of obacunone A-ring lactone hydrolase. This enzyme attacks specifically the A-ring lactone of obacunone and produces obacunoate (**3**). The enzyme treated isolate was methylated and its R_fs were compared with authentic methyl obacunoate. The results showed that in three solvent systems the methylated enzyme product had the same mobility as an authentic

Table 1. Identification of metabolites by TLC

Compound	R_fs* (a)	(b)	(c)
Isolate	0.59	0.51	0.36
Obacunone	0.59	0.51	0.36
Isolate treated with hydrolase and methylated	0.46	0.28	0.25
Methyl obacunoate	0.46	0.28	0.25

*Solvent key: see Experimental.

methyl obacunoate sample (Table 1). This would have been possible only if the metabolite possessed a unique limonoid moiety, the A-ring lactone of obacunone. Therefore, we can assign to it the structure **1**.

The rate of the conversion of nomilin to obacunone in *C. limon* appeared to be slow under the conditions used. Conversion was noticeable after one day of incubation, but was highest, *ca* 5% of the fed substrate, after 5–7 days of incubation. In young seedlings (10 cm height with eight to ten leaves) the conversion was unnoticeable.

These results provide direct evidence to support the hypothesis that obacunone is biosynthesized from nomilin in *Citrus* [4]. In bacteria, obacunone is a metabolite of nomilin [5]. This bacterial conversion is catalysed by nomilin acetyl-lyase, which has been isolated from cell-free extracts of *Corynebacterium fascians* [6]. This suggests that the conversion of nomilin to obacunone in *C. limon* is also catalysed by nomilin acetyl-lyase, but the enzyme has not yet been identified in citrus.

Little is known about the metabolism of limonoids in citrus. We have shown that limonoids are metabolized in citrus through at least two pathways: one is via 17-dehydrolimonoids [7, 8] and the other is via deoxylimonoids [9]. However, practically no evidence related to the biosynthesis of limonoids has been reported. The work presented here is the first direct evidence to show that obacunone is indeed biosynthesized from nomilin.

EXPERIMENTAL

Materials. The *Citrus limon* seeds used for germination were from our laboratory, and seedlings and trees were grown in our greenhouse. [^{14}C]-Labelled nomilin (1.65 μCi/μmol) was biosynthesized from [1-^{14}C]acetate in *C. limon* seedlings by the previously described procedures [10]. Obacunone A-ring lactone hydrolase was purified from a cell-free extract of *Corynebacterium fascians* using a DEAE ion exchange HPLC column [6]. Sodium [1-^{14}C]acetate (56 mCi/mmol) was purchased from New England Nuclear, Boston, Massachusetts.

Feeding experiment. 1×10^5 cpm of nomilin was fed to a young shoot of a 2.5-year old *C. limon* tree through the stem by the procedures described previously [10]. After 7 days of incubation in a greenhouse, the young shoot was harvested and used for analysis.

Extraction and analysis of labelled limonoids. Limonoids were extracted from the stem and leaves by the procedure of Hasegawa *et al.* [10]. The extract was then analysed by TLC on silica gel plates with three solvent systems: (a) EtOAc–cyclohexane (3:2); (b) CH_2Cl_2–MeOH (97:3), and (c) 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 [11].

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

Enzymic identification of the isolate. The EtOAc extract obtained above was evaporated and the residue was incubated with obacunone A-ring lactone hydrolase in 4 ml of 0.1 M Tris buffer at pH 8.5. After 18 hr of incubation at 23°, the reaction mixture was acidified to pH 2 and quickly extracted with EtOAc. The EtOAc extract was methylated with CH_2N_2. The methylated isolate was then identified by TLC.

Acknowledgements—The authors thank Drs. V. P. Maier and R. D. Bennett for their helpful suggestions and Peter Ou and Hung N. Quach for their technical assistance.

REFERENCES

1. Hasegawa, S. and Maier, V. P. (1981) *Proc. Int. Soc. Citriculture* **2**, 914.
2. Hasegawa, S., Bennett, R. D. and Verdon, C. P. (1980) *J. Agric. Food Chem.* **28**, 922.
3. Maier, V. P., Bennett, R. D. and Hasegawa, S. (1977) in *Citrus Science and Technology* (Nagy, S., Shaw, P. E. and Veldhuis, M. K., eds) Vol. 1, p. 355. AVI Publishing Co., Westport, Connecticut.
4. Dreyer, D. L. (1968) *Fortsch. Chem. Org. Naturst.* **26**, 190.
5. Hasegawa, S., Dillberger, A. M. and Choi, G. Y. (1984) *J. Agric. Food. Chem.* **32**, 457.
6. Herman, Z., Hasegawa, S. and Ou, P. (1985) *J. Food Sci.* **50**, 118.
7. Hsu, A. C., Hasegawa, S., Maier, V. P. and Bennett, R. D. (1973) *Phytochemistry* **12**, 563.
8. Hasegawa, S., Maier, V. P. and Bennett, R. D. (1974) *Phytochemistry* **13**, 103.
9. Hasegawa, S., Bennett, R. D. and Verdon, C. P. (1980) *Phytochemistry* **19**, 1445.
10. Hasegawa, S., Bennett, R. D. and Maier, V. P. (1984) *Phytochemistry* **23**, 1601.
11. Dreyer, D. L. (1965) *J. Org. Chem.* **30**, 749.