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BIOSYNTHESIS OF LIMONOIDS: CONVERSION OF DEACETYLNOMILINATE TO NOMILIN IN *CITRUS LIMON*

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

Abstract—Radioactive tracer work showed that deacetylnomilinate was converted to nomilin in detached stems of young *Citrus limon* seedlings. This work and the previous findings suggest that deacetylnomilinate is the initial limonoid to be biosynthesized among the limonoids known to be present in *Citrus*. Possible biosynthetic pathways for the formation of limonoids in *Citrus* are proposed.

INTRODUCTION

Bitterness due to limonoids in a variety of citrus juices is a major problem of the worldwide citrus industry. During the past few years, substantial progress has been made in the field of limonoid biochemistry in *Citrus* [1–4]. Deacetylnomilinate has been considered to be a key limonoid directly involved in main limonoid biosynthetic pathways in *Citrus* [4, 5]. In this study we report that deacetylnomilinate is a precursor of nomilin in the detached stems of young *Citrus limon* L. seedlings.

RESULTS AND DISCUSSION

Young *C. limon* seedlings are excellent tools for the preparation of [^{14}C]nomilin from labelled acetate [1]. However, they are not capable of accumulating [^{14}C]deacetylnomilin or deacetylnomilinate from labelled acetate [1]. Therefore, we surveyed various *Citrus* and its hybrids, and found that young seedlings of calamondin (*C. reticulata* cv. 'Austera' × *Fortunella* sp.) biosynthesize deacetylnomilin from acetate as one of the minor limonoids. About 21 000 dpm of labelled deacetylnomilin was obtained from a stem fed with 25 μCi of [1-^{14}C]acetate.

In the radioactive tracer work, we used detached stems of young *Citrus limon* seedlings instead of intact plant tissues. This is because the stem is the major site of limonoid biosynthesis, and the detached stem is capable of biosynthesizing nomilin from acetate as well as the intact stem [6]. Moreover, the major advantage of using the detached stem is that radioactive materials can be recovered very efficiently.

When 20 000 dpm of deacetylnomilinate was fed to the detached stem of a *C. limon* seedling and incubated at room temperature for 3 days, 67% of the substrate was metabolized. There were three major peaks on a TLC plate. The largest peak, 40% of the total activity, was identified as nomilin by the procedure described previously [1]. The second largest, 33%, was identified as deacetylnomilinate, the substrate. The third and most polar peak, 22%, which remained at the origin when developed with three solvent systems (a, b, and c, see Experimental), did not correspond to any of the limonoids known to be present in *Citrus*. The rate of conversion from deacetylnomilinate to nomilin varied among three experiments with results of 25, 40 and 51% under the conditions used.

Based on these findings and data accumulated thus far, the possible biosynthetic pathways of limonoids in *Citrus* are proposed (Fig. 1). The conversion of nomilin to obacunone [2], obacunone to obacunoate [3] and obacunoate to limonin [3] in *Citrus* have been demonstrated. Enzymes responsible for the conversion of nomilin to obacunone, nomilin acetyl-lyase, and for the conversion of obacunone to obacunoate, obacunone A-ring lactone hydrolase, have been isolated from cell-free extracts of *Corynebacterium fasians* [7], but they have not yet been isolated from *Citrus*.

Deacetylnomilinate was previously considered to be one of the key intermediate precursors involved in the proposed pathway between obacunoate and limonin [4, 5]. However, in our previous studies in which labelled nomilin, obacunone or obacunoate was fed to either detached or intact stems of young seedlings or mature *Citrus limon*, we were not able to demonstrate incorporation of radioactivity into deacetylnomilinate [2–4]. Thus, deacetylnomilinate does not seem to be an intermediate between obacunoate and limonin. The results of this study show clearly that deacetylnomilinate is a precursor of nomilin.

The presence of possible intermediates between obacunoate and limonin such as isoobacunoate has not yet been demonstrated. The true pathway between obacunoate and limonin will ultimately have to be determined by incorporation studies with possible precursors in radioactive form.

EXPERIMENTAL

Materials. Seedlings of *Citrus limon* (10 cm in height with eight leaves) and calamondin (12 cm in height with 12 leaves) were grown in our greenhouse. [1-^{14}C]Acetate (54 mCi/mmol) was purchased from Dupont New Products, Massachusetts.

Preparation of labelled deacetylnomilinate. Acetate (25 μCi) was fed to the stem of a calamondin seedling by the procedure

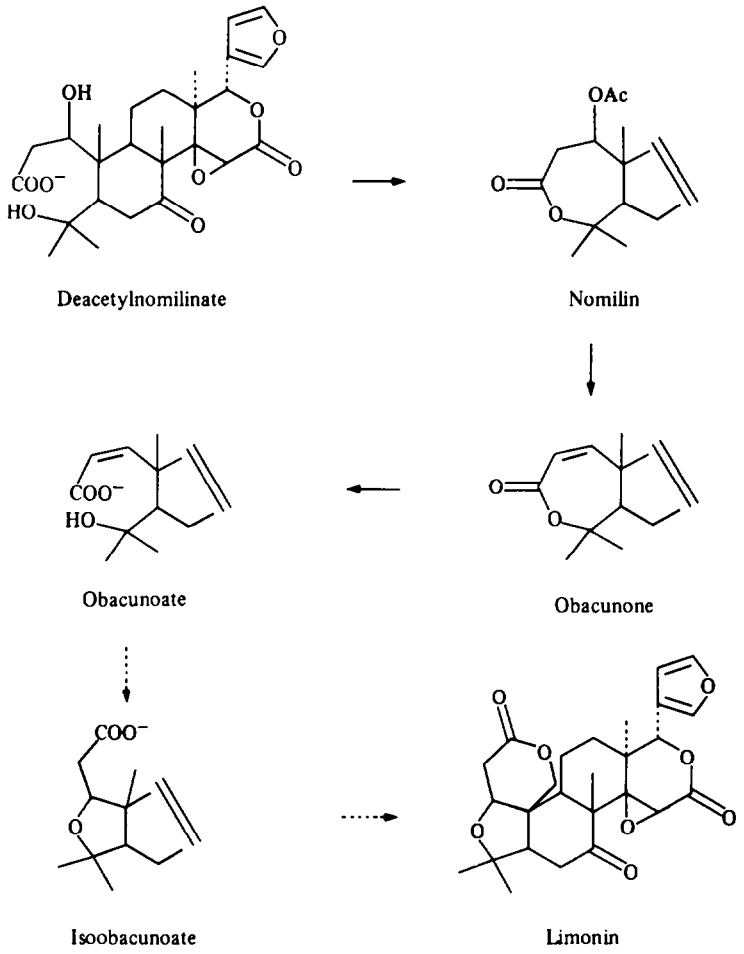

Fig. 1. Proposed biosynthetic pathways for the formation of limonoids in *Citrus*.

described previously [1]. After 2 days of incubation in the greenhouse, radioactive materials were extracted from the stem by the procedure previously described [1]. The extract was fractionated on a silica gel column (0.7 × 10 cm). The column was eluted by a linear gradient system. The mixing chamber consisted of 20 ml of 20% EtOAc in CCl_3CF_3 (v/v) and the reservoir consisted of 20 ml of 90% EtOAc in CCl_3CF_3 (v/v). The flow rate was 0.25 ml/min. The isolate was 95% pure (TLC radiochromatogram). Further purification was performed by prep. TLC, which was developed with solvent system b (see below). The isolate was then chemically converted to deacetylnomilinate by the procedure of Bennett [8]. The final yield was relatively consistent with about 21 000 dpm per experiment.

Feeding of labelled deacetylnomilinate to detached stems. The detached stem (1 cm long) of a young *C. limon* seedling was placed in a small V-shaped vial and fed with labelled compound through the cut area. This was incubated for 3 days at 22°.

Extraction and analysis of labelled limonoids. Labelled metabolites were extracted from the stem by the procedure described previously [1] and analysed by TLC with a Berthold Automatic TLC-linear Analyzer LB 2832. Silica gel TLC plates were developed with solvent systems: (a) EtOAc–cyclohexane (3:2), (b) CH_2Cl_2–MeOH (97:3) and (c) EtOAc–CH_2Cl_2 (2:3). Total radioactivity was counted with a Beckman liquid scintillation system, LS-3133P.

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