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METABOLISM OF LIMONOIDS VIA A DEOXYLIMONOID PATHWAY IN *CITRUS*

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Key Word Index—*Citrus microcarpa*; Rutaceae; grapefruit seed; limonoids; metabolism; deoxylimonoids.

Abstract—Methyl deacetylnomilinate was metabolized in leaves of calamondin (*Citrus microcarpa*) to form a deoxy derivative. This reaction indicated the presence in citrus of an epoxidase, which is required for the first step of the deoxylimonoid pathway. The product of the second step, deoxylimonic acid, was isolated from grapefruit seeds. Deoxylimonate A-ring lactone hydrolase, which is involved in the third step of the deoxylimonoid pathway, was also found in grapefruit seeds. These results and the previously reported presence of the first metabolite of this pathway, deoxylimonin, show that limonoids are metabolized in citrus, not only via the 17-dehydrolimonoid pathway as previously established, but also via the deoxylimonoid pathway.

INTRODUCTION

Limonoids constitute one of two classes of bitter constituents in citrus. In bacteria they are metabolized through at least two pathways: one via 17-dehydrolimonoids [1, 2] and the other via deoxylimonoids [3, 4].

In citrus, limonoids have been shown to be metabolized via the 17-dehydrolimonoid pathway. The first metabolite of this pathway, 17-dehydrolimonoate A-ring lactone, was isolated from citrus peel, juice, seeds and seedlings [5], and the enzyme responsible for its formation was shown to be present in navel orange [6].

In bacteria the first three steps in the deoxylimonoid pathway have been identified. First the 14,15-epoxide of limonin is reduced by an epoxidase to a double bond, giving deoxylimonin (**1**) [3]. This compound is then converted by deoxylimonin hydrolase to deoxylimonic acid (**2**) [4], in which the B-ring has been opened. In the third step the A-ring lactone of **2** is opened by deoxylimonate A-ring lactone hydrolase (Hasegawa, S. and Bennett, R. D., unpublished data). Since the product of the first step, compound **1**, has been isolated from grapefruit seeds [7], it seemed possible that the deoxylimonoid metabolic pathway is also present in citrus. We have investigated this possibility by looking for both the metabolites and enzyme activity involved in this pathway in citrus.

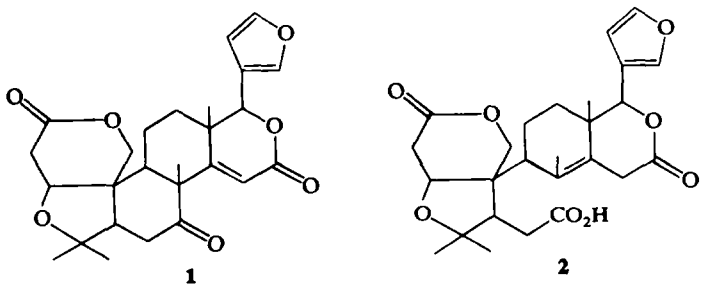
We have now isolated the second metabolite, deoxylimonic acid (**2**), and have demonstrated the presence of enzymes catalysing the first and third steps.

RESULTS AND DISCUSSION

The acidic material of grapefruit seed extract was isolated by extraction with bicarbonate and methylated with CH_2N_2 to facilitate separation of its components by chromatography. TLC showed the presence of a compound corresponding in mobility to deoxylimonic acid (**2**) methyl ester. This component was isolated by Si gel column chromatography and proved to be **2** methyl ester. Since **1** had previously been isolated from grapfruit seeds [7], the products of the first two steps of the bacterial deoxylimonoid degradative pathway (**1** and **2**) have been shown to be natural constituents of citrus.

Citrus leaves are active centres for biosynthesis and biodegradation of limonoids [8]. Thus, leaves of calamondin (*Citrus microcarpa*) were chosen for an experiment with deacetylnomilinate-[methyl-^{14}C] (**3**) to determine whether the deoxylimonoid pathway is active in citrus. Labeled **3** was chosen for the experiment since it is a natural constituent of calamondin [9] and is also readily prepared.

Labeled **3** was metabolized in young leaves of calamondin (Fig. 1) although the proportions of metabolites changed to some extent from one experiment to another. However, the three major peaks, A, B, and C remained relatively unchanged. Compound A was identified as the substrate (**3**). Compound B was isolated by preparative TLC, dissolved in 0.1 M Tris buffer at pH 8.0 and treated with deoxylimonin hydrolase. After 16 hr of incubation, the reaction mixture was acidified and extracted with EtOAc. Radiochromatograms showed that the EtOAc extract of the enzyme-treated sample contained two compounds, whereas the control, which was incubated with boiled enzyme, contained only unchanged metabolite B. About 60–80% of metabolite B was converted to a more polar compound by deoxylimonin hydrolase. This enzyme is highly specific for deoxylimonoids and catalyses cleavage of a C–C bond of B-ring and the double bond of D-ring is shifted to the C-ring [4]. Because of the



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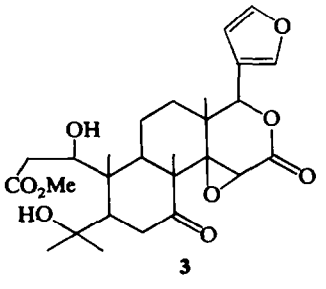

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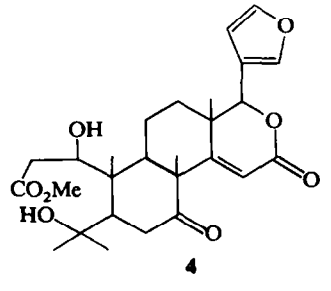

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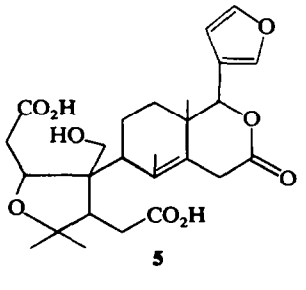

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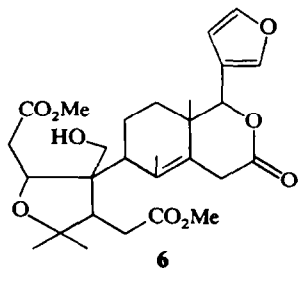

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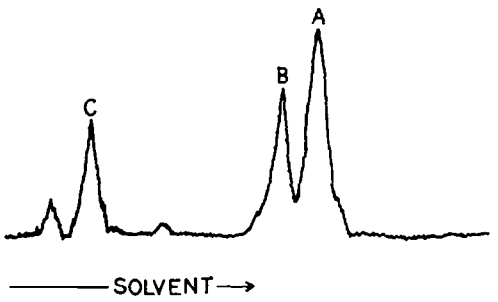

Fig. 1. Radiochromatogram of metabolites of deacetylnomilinate-[methyl-^{14}C] fed to detached leaves of calamondin (*Citrus microcarpa*). TLC was developed with cyclohexane–EtOAc (2:3).

specificity of the enzyme, the metabolite B must have been a deoxylimonoid, most likely compound **4**. This result shows that calamondin possesses epoxidase activity which converts limonoids to deoxylimonoids.

During previous work we observed deoxylimonic acid A-ring lactone hydrolase activity in bacteria (Hasegawa, S. and Bennett, R. D., unpublished data). This enzyme specifically catalyses the opening of the A-ring lactone of **2** to produce deoxylimononic acid D-ring lactone (**5**). (We have named the tricarboxylic acid produced when both lactone rings of **2** are opened 'deoxylimononic acid'.) Since this enzyme is one of those involved in the deoxylimonoid degradation pathway in bacteria, demonstration of its presence in citrus would provide evidence that the same metabolic pathway functions in citrus.

When **2** was incubated with a crude enzyme preparation obtained from grapefruit seeds, compound **5**, isolated as its dimethyl ester, was formed in 65–80% yield. Under the same conditions in the absence of enzyme, **2** was recovered unchanged. Furthermore, the A-ring of limonin was not opened by this enzyme, thus demonstrating its specificity for **2**. Significantly, we have been unable to detect A-ring hydrolase activity for any limonoid except **2** in either citrus or limonin-metabolizing bacteria. The identity of the methylated enzyme product was confirmed by comparison with **6** which we synthesized by the same method used to prepare methyl limonoate D-ring lactone [10]. The D-ring of limonin and similar limonoids closes much more readily than the A-ring, and therefore the carboxyl group of the latter can be selectively methylated. For the synthesis of **6**, compound **2** was dissolved in base and warmed to open both lactone rings. The basic solution was then cooled and acidified to pH 4, giving the monolactone **5**. This was quickly extracted and methylated with CH_2N_2. The product was shown by TLC and ^{1}H NMR to be >90% pure, but attempts to purify it further by crystallization or chromatography resulted in partial conversion to the methyl ester of **2**. Nevertheless, the ^{1}H NMR spectrum provided convincing evidence that the major product was indeed **6**. Two methyl ester signals were observed, demonstrating that only one of the three carboxyl groups had formed a lactone. The H-17

resonances for methylated **2** and **6**, had almost the same chemical shift, indicating that the D-ring of **6** was closed. However, the H-19 signals, observed as an AB quartet at δ 4.30 and 4.08 in methylated **2**, were shifted upfield to 3.68 and occurred as a singlet in **6**. The same phenomenon of an upfield shift of H-19 and a change from a quartet to singlet was observed when the A-ring of limonin was opened with formation of methyl limonoate D-ring lactone [10]. Thus, **6** must have contained a closed D-ring and an open A-ring. TLC with three solvent systems showed that the synthetic product was identical to the methylated enzyme reaction product; and the identity confirms the presence of deoxylimonic acid A-ring lactone hydrolase in grapefruit seeds.

Compound **1** was originally thought to be a side product of limonin biosynthesis rather than a metabolite [7, 11]. However, the present work shows that it is more likely a metabolite of limonin. Thus, both pathways of limonoid metabolism previously demonstrated in bacteria have now been shown to occur in citrus.

EXPERIMENTAL

Materials and methods. Si gel G plates were used for qualitative and radiochromatographic analyses. The solvent systems used were: (1) cyclohexane–EtOAc (1:1), (2) Et_2O and (3) C_6H_6–EtOH–H_2O–HOAc (200:47:15:1, upper layer). ^1H NMR spectra were obtained by use of $CDCl_3$ at 100 MHz with a JEOL PS-100. Compound **2** was isolated from growth media of *Pseudomonas* 321-18 [4]. **3**-[Methyl-^{14}C] was prepared by methylation of deacetylnomilinic acid with $^{14}CH_2N_2$ as described previously [6]. Deacetylnomilinic acid was prepared from deacetylnomilin [12], which was isolated from grapefruit seeds [7].

Isolation of deoxylimonic acid (**2**) *as its methyl ester.* Grapefruit seeds were extracted and limonin was removed from the extract by crystallization as previously described [7]. The mother liquor was evapd to dryness and the residue (66 g) was dissolved in 500 ml EtOAc. This soln was extracted with 5 × 50 ml 2% $KHCO_3$. The emulsions formed each time were broken by filtering through Celite and the two phases were then separated. Each aq. extract was passed through 100 ml EtOAc and immediately acidified with N HCl. The combined acidified extracts were extracted with 3 × 100 ml CH_2Cl_2. The CH_2Cl_2 extracts were washed separately with 100 ml H_2O, combined, and evapd. This acidic fraction (11 g) was methylated with CH_2N_2. The methylated material was partitioned between 100 ml 90% MeOH and 100 ml hexane. The hexane layer was washed with 100 ml 90% MeOH, and the two 90% MeOH layers were washed separately with 100 ml hexane, combined, and evapd to dryness *in vacuo*. This material (4.3 g) was chromatographed on a column of Si gel packed in CH_2Cl_2–Et_2O (49:1) and eluted with gradually increasing amounts of Et_2O in CH_2Cl_2. The fractions eluted with the solvents in 9:1 and 4:1 ratios were combined (500 mg) and rechromatographed on a Si gel column packed in hexane–Et_2O (7:3). A fraction (95 mg) containing >90% of a single component was eluted with hexane–Et_2O (1:4). This material was identical to an authentic sample of methyl deoxylimonate, as determined by TLC and ^1H NMR.

Radioactive tracer work. About 200 000 cpm of labeled **3** were fed to a detached calamondin branch, which had 4–5 young leaves, through the cut end. The branch, supplied with H_2O, was then illuminated for 10 hr per day by fluorescent light. After 3 days the branch was extracted as described previously [8], and a portion of the extract was used for radiochromatographic analyses.

Deoxylimonic acid A-ring lactone hydrolase. Grapefruit seeds (20 g) were soaked in H_2O overnight. The rest of the procedures were carried out at 2–5°. The pellicles were removed, and the seeds were ground in 30 ml 0.1 M phosphate buffer (pH 7.5) with a Polytron. The slurry was centrifuged and the supernatant removed. The residue was re-extracted and centrifuged. The supernatants were combined and brought to 90% saturation with addition of solid $(NH_4)_2SO_4$. The ppt. was pelleted by centrifugation, collected and dissolved in a minimal portion of 0.01 M phosphate buffer (pH 7.0). The soln was then dialysed against 0.01 M phosphate buffer (pH 7.0) for 2 hr, and used as the crude enzyme preparation. A sample of **2** (10 mg) was dissolved in 0.4 ml 0.5 M phosphate buffer at pH 7.0. To the soln, 1.0 ml of the enzyme preparation (3 mg protein) was added. The mixture was then incubated at 22° for 16 hr, acidified to pH 4.0, and then quickly extracted with EtOAc. The extract was methylated with CH_2N_2, and the methylated product was identical to an authentic sample of **6**, as determined by TLC.

Preparation of **6**. Compound **2** (50 mg) was dissolved in 5 ml 0.2 N NaOH. The soln was heated on a steam bath for 20 min, cooled in an ice bath, acidified to pH 4.0 with 0.5 N HCl, and then immediately extracted with 2 ml EtOAc. The extract was quickly treated with excess CH_2N_2 in CH_2Cl_2 and evapd to dryness. TLC of the product (38 mg) with solvent (2) showed one major component, along with a little methylated **2**. ^1H NMR: δ 7.53 and 7.41 (2H, 2*d*, $J = 1$ Hz, α-furans), 6.43 (1H, *d*, $J = 1$ Hz, β-furan), 5.04 (1H, *s*, H-17), 4.51 (1H, *t*, $J = 6$ Hz, H-1), 3.72 and 3.71 (6H, 2*s*, $-CO_2Me$), 3.68 (2H, *s*, H-19), 3.35 (2H, *s*, H-15), 1.69 (3H, *s*, vinyl methyl), 1.34, 1.19, and 1.01 (9H, 3*s*, quaternary methyls).

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