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METABOLISM OF LIMONOIDS IN THE *CITRUS* HYBRID CALAMONDIN

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Abstract—Radioactive tracer work showed that in young seedlings of calamondin deacetylnomilinate was converted to nomilin, obacunone and 6-keto-7β-nomilol, a new limonoid. The latter was further converted to 6-keto-7β-deacetylnomilol and isocyclocalamin, another new limonoid.

INTRODUCTION

Bitterness due to limonoids in a variety of citrus juices is a major problem of the worldwide citrus industry and has significant negative enonomic impact. Substantial progresss has been made in recent years in elucidating the biosynthetic pathways of limonoids in *Citrus* and its hybrids [1–5]. The citrus hybrid calamondin (*Citrus reticulata* var *austera* × *Fortunella* sp.) contains, in addition to the usual citrus limonoids, a group of limonoids oxygenated at the 6-position [6, 7].

We report here that in calamondin seedlings deacetylnomilinate (**1**) is converted to nomilin (**2**), obacunone and a new limonoid, which was identified as 6-keto-7β-nomilol (**3**). Compound **3** was further metabolized to 6-keto-7β-deacetylnomilol (**4**) and another new limonoid. The latter was found to be the 6-hydroxy-7-keto analogue of cyclocalamin (**5**), and we have accordingly named it isocyclocalamin (**6**).

RESULTS AND DISCUSSION

When 25000 cpm [^{14}C]deacetylnomilinate (**1**) was fed to a detached stem of a calamondin seedling, four major radioactive peaks, A–D in the order of A (substrate) as the most polar and D as the least, were observed by TLC using solvent system a. Peak B was not identified, but peak C (35% radioactivity) had an R_f identical to two known limonoids, nomilin (**2**) and 6-keto-7β-deacetylnomilol (**4**). When this peak was scraped from the plate and extracted, it resolved into three separate peaks by TLC in solvent system b. One of them, representing only 4% of the peak, had identical R_fs to **4**. This compound is most likely **4**, but there was insufficient material for positive identification. One of the other peaks, designated peak C-3(33% of peak C) was positively identified as **2** in four solvent systems (Table 1). This shows that **1** is a precursor of **2** in calamondin seedlings, as has already been demonstrated in *Citrus limon* [5].

Peak D (22% of radioactivity) was scraped and analysed by TLC in solvent system b. It resolved into two peaks. One peak, designated D-1 (23% of peak D) was identified by TLC as obacunone (Table 1). Obacunone has been shown to be a metabolite of **2** in *C. limon* [2] and therefore is not a direct metabolite of **1**. The other peak, designated D-2 (77%), did not match any known limonoid. However, the R_fs of this compound were identical to those of an unidentified limonoid that we had previously observed in calamondin seedlings.

To identify this compound, we needed a quantity sufficient for structure determination. The compound was isolated from the roots and stems of 50 calamondin seedlings and crystallized from MeOH. The ^{13}C spectrum of the isolated compound (**3**) was quite similar to that of **4**, except for the presence of two extra carbon signals, a carbonyl at 169 ppm and a methyl at 21 ppm. These signals are characteristic of an acetate ester group. Of the two hydroxylated carbons in **4** which are the possible sites of the acetate group in **3**, the C-7 resonance, at 81 ppm, was unchanged, while that of C-1 moved downfield from 68 to 70 ppm. A shift of the C-2 resonance was also observed from 39 ppm in **4** to 35 ppm in **3**. The C-1 and C-2 resonances in **2**, which contains an acetate ester group at C-1, are found at 71 and 35 ppm, respectively. Thus, the ^{13}C NMR data suggest that **3** is the 1-acetyl derivative of **4**. The ^{1}H NMR spectra also supported this assignment. The H-7 resonance, a singlet, was found at 4.38 ppm in **3** and 4.33 ppm in **4**, while that of H-1 was found at 4.88 ppm (triplet) in **3** and 3.80 ppm (doublet) in **4**. All of

Table 1. Identification of limonoid metabolites by TLC

Compound	R_fs*			
	a	*b*	*c*	*d*
Peak C-3	0.25	0.38	0.60	0.27
Nomilin	0.25	0.38	0.60	0.27
Peak D-1	0.63	0.54	0.89	0.35
Obacunone	0.63	0.54	0.89	0.35
Peak D-2	0.63	0.45	0.80	0.30
6-Keto-7β-nomilol	0.63	0.45	0.80	0.30
Peak E	0.21	0.21	0.48	0.24
6-Keto-7β-deacetylnomilol	0.21	0.21	0.48	0.24
Peak F	0.74	0.50	0.88	0.39
Isocyclocalamin	0.74	0.50	0.88	0.39

*Solvent key: see Experimental.

the other resonances in both the ^{13}C and ^{1}H NMR spectra of **3** were consistent with the structure of **3** for this compound.

When 150 000 cpm[^{14}C]**3** was fed to a detached stem of a calamondin seedling, several metabolites were observed by TLC using solvent system a. One of the metabolites (peak E), representing 26% of recovered radioactivity, was isolated by TLC as a radioactively pure compound. The isolate had R_fs identical to those of authentic **4** in four solvent systems (Table 1). From this we conclude that **3** is converted to **4** in calamondin seedlings.

Another metabolite (peak F) represented about 3% of the total radioactivity. This was isolated by TLC as a radioactively pure compound and was found to be a new limonoid. To obtain sufficient material for structure determination, this compound was isolated from an extract of 15 seedlings by column chromatography. The isolated compound (14 mg) (**6**) showed similarities in both its chromatographic mobility and its ^{1}H NMR spectrum to **5** [7]. When it was subjected to CrO_3 oxidation, the product was found to be 7-dehydrocyclocalamin, which is also the product formed by oxidation of **5** [7].

The ^{13}C spectrum of **6** showed a carbonyl resonance, attributable to either a 6- or 7-keto group. This reduces the possible structures for **6** to three: the 7α-isomer of **5** and the two epimers of the 6-hydroxy-7-keto analogue. In the first case the 7-resonance in the ^{1}H NMR spectrum would be a singlet, as it is for **5**. However, no such singlet was observed in the ^{1}H NMR spectrum of **6**. Instead, a coupled pair of doublets (J = 12 Hz) was present, at 1.94 and 4.37 ppm, which must represent H-5 and H-6. Previously we showed that in **5** and 7-dehydrocyclocalamin H-5 has the β-configuration [7]. Since **6** was converted to 7-dehydrocyclocalamin by a reaction which should not affect the configuration at H-5, it follows that **6** is also a 5β-H compound. The large coupling constant between H-5 and H-6 shows a diaxial configuration of these protons. However, in **5** the B-ring is in the chair form [7] and H-5 is equatorial. To accommodate a 5,6-diaxial configuration in **6**, a boat B-ring is required and the 6-hydroxyl group must be β-oriented. Thus, **6** is the 6β-hydroxy-7-keto analogue of **5**, and we have named it isocyclocalamin. The boat form of the B-ring is probably favoured because it allows the 6β-hydroxyl to be equatorial, and thus avoids 1,3-diaxial interactions with the 8- and 10-methyls. In an attempt to interconvert **5** and **6** by enolization, each was treated with 1 M KOH for 1 hr at 25°. In both cases, however, the sole product obtained was methyl isoobacunoate diosphenol (**7**) (after methylation with CH_2N_2 of the acid produced by hydrolysis). The same base-catalysed reaction was observed previously with rutaevin, which, like **5**, is a 6-keto-7-hydroxy-limonoid [8]. Peaks were also observed with R_fs corresponding to **5** and calamin (**8**), but the amounts were insufficient to allow definitive proof of incorporation into these compounds.

We have demonstrated here that **1** is a precursor of both **2** and **3** in calamondin and is most likely an initial precursor of all the limonoids known to be present in *Citrus* and its hybrids. Compound **2** is a precursor of the major citrus limonoids, but it does not seem to be involved in the biosynthesis of the calamin-type limonoids. The latter pathway also originates from **1**, and the first identified intermediate in it is **3**. Conversion of **1** to **3** involves acetylation at C-1, closing of the A-ring, and introduction of the 6-keto-7-hydroxy moiety in the B-ring. The sequence in which these changes occur remains to be determined. The present work shows that **3** is converted to **4** by hydrolysis of the acetate ester group. It seems likely that the incorporation of **3** into **6** demonstrated in this work proceeds with **4**, **8**, and **5** as intermediates. Opening of the A-ring lactone of **4**, followed by methylation of the carboxyl group, would give **8**. Compounds **5** and **6**, unlike the other calamondin limonoids, have the β-configuration at H-5. The chemical conversion of **8** to **5** has been demonstrated, and evidence was presented indicating that the change to 5β-H occurred via a 4,5-unsaturated intermediate [7]. This seems a plausible mechanism for the *in vivo* reaction, although an alternative sequence involving a 1,2-unsaturated intermediate and inversion of configuration by enolization is also possible. In Fig. 1 we have shown a direct conversion of **5** to **6**, probably by an enolization mechanism, but several other possibilities exist. In particular, the role of methyl isoobacunoate diosphenol (**7**) is unclear; this compound, which has been isolated from calamondin seeds [7], could be formed from either **5** or **6**. On the other hand, it could also be a precursor of either of these compounds or perhaps an intermediate between them. These questions will eventually have to be answered by feeding of the appropriate labelled compounds.

EXPERIMENTAL

Materials. Calamondin seedlings (15–18 cm high with 10–12 leaves) were grown from seed in our greenhouse. [^{14}C] Labelled deacetylnomilinate (**1**) was prepared by chemical treatment of [^{14}C] deacetylnomilin by the method of [9]. [^{14}C] Deacetylnomilin was biosynthesized by the procedure described previously [5]. [^{14}C] Labelled 6-keto-7β-nomilol was biosynthesized from [1 – ^{14}C] acetate in calamondin seedlings using previously described procedures [1].

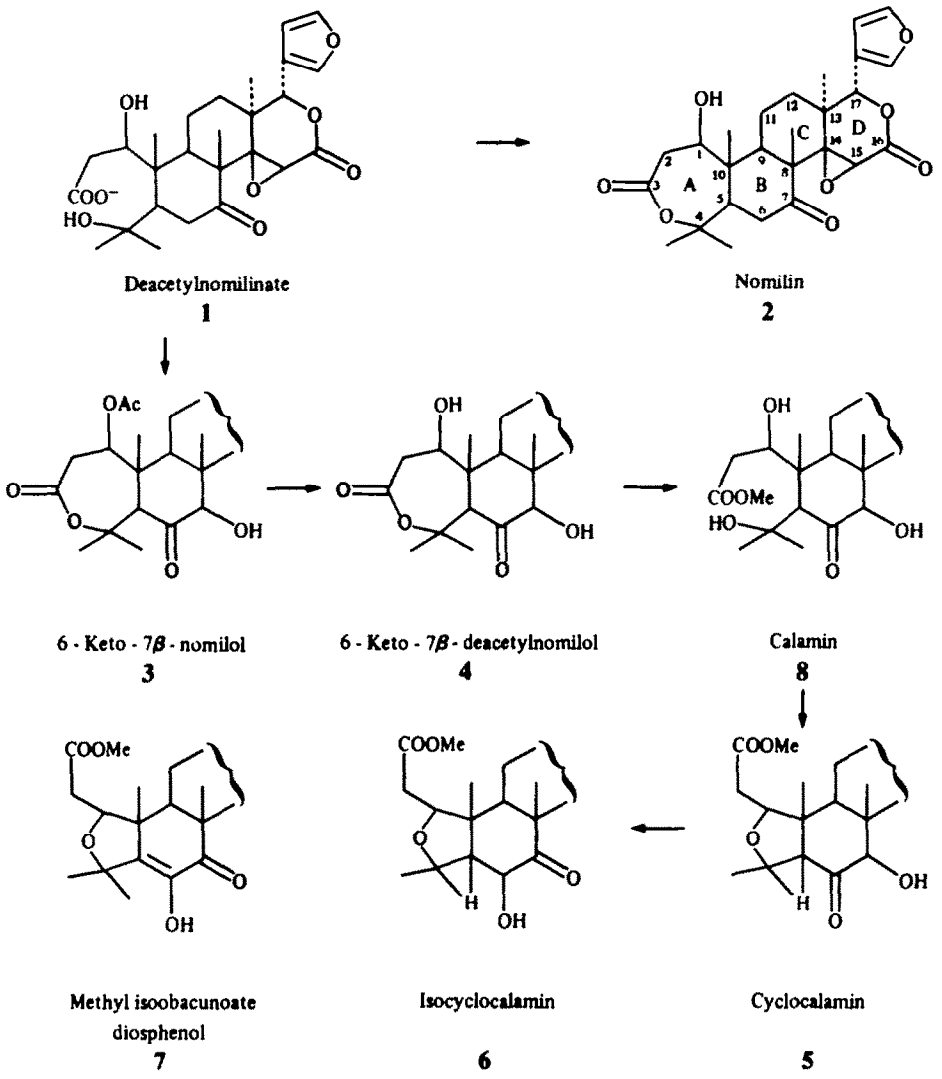
Feeding experiment. The detached stem (3 cm long) of a young calamondin seedling was placed in a small V-shaped vial and fed with labelled compound through the cut area. This was incubated for 2 days at 22°.

Extraction and analysis of labelled compounds. Labelled metabolities were extracted from the stem by the procedure of ref. [1] and analysed by TLC with a Berthold Automatic TLC-Linear Analyser LB2832. Silica gel TLC plates were developed in solvent systems: (a) EtOAc-cyclohexane (3:2), (b) CH_2Cl_2–MeOH (97:3), (c) EtOAc–CH_2Cl_2 (2:3), (d) toluene–EtOH–H_2O–HOAc (200:47:15:1, upper layer).

Isolation of metabolites. The TLC spots of interest were scraped from the preparative plate. Scrapings were extracted with EtOAc to obtain radioactively pure compounds.

NMR spectra. ^{1}H and ^{13}C NMR spectra were obtained with a JEOL FT spectrometer, JNM-GX 270 WB. ^{13}C NMR assignments were made on the basis of SFORD spectra, selective heteronuclear decoupling, and comparison with spectra of related limonoids for which assignments had previously been made [9].

Extraction, isolation of 6-keto-7β-nomilol. Limonoids were extracted from the roots and stems of 50 calamondin seedlings by the procedures described previously [1]. The extract was fractionated on a silica gel column (2.5 × 60 cm) using a hexane–EtOAc linear gradient beginning with 9:1 and ending with 1:1. The compound of interest was eluted with 7:3 and was further purified using preparative TLC (silica gel G plate in solvent system b). Crystallization of the isolate from MeOH gave 8 mg, m.p. 249–253° (uncorr.); ^{1}H NMR (270 HMz, $CDCl_3$,



exchanged with D_2O): δ0.92, 1.11, 1.27, 1.44, 1.80 (15H, 5*s*, quaternary Me), 2.10 (3H, *s*, acetate), 2.95 (1H, *m*, H-9), 3.16 (2H, *m*, H-2), 3.41 (1H, *s*, H-5), 4.38 (1H, *s*, H-7α), 4.41 (1H, *s*, H-15), 4.88 (1H, *m*, H-1), 5.62 (1H, *s*, H-17), 6.31 (1H, *d*, J = 1 Hz, β-furan), 7.42 (2H, *d*, J = 1 Hz, α-furans); ^{13}C (67.8 MHz, $CDCl_3$): δ13.5 (*q*, Me), 15.6 (*q*, Me), 16.2 (*t*, C-11), 18.6 (*q*, Me), 20.9 (*q*, acetate Me), 24.7 (*q*, Me), 26.4 (*t*, C-12), 32.5 (*q*, Me), 34.9 (*t*, C-2), 39.4 (*s*, C-13), 40.3 (*d*, C-9), 49.0 (*s*, C-8)*, 49.4 (*s*, C-10)*, 56.1 (*d*, C-15), 61.8 (*d*, C-5), 70.1 (*d*, C-1), 72.2 (*s*, C-14), 78.0 (*d*, C-17), 81.4 (*d*, C-7), 82.0 (*s*, C-4), 109.7 (*d*, β-furan), 120.2 (*s*, β-furan), 141.2 (*d*, α-furan), 143.3 (*d*, α-furan), 167.3 (*s*, C-16), 168.9 (*s*, acetate)*, 169.3 (*s*, C-3)*, 207.6 (*s*, C-6).

Isolation of isocyclocalamin. Limonoids were extracted from 15 seedlings by the procedures described previously [1]. The extract was chromatographed on a 2 × 20 cm column of silica gel. The column was eluted stepwise with increasing concentration of EtOAc in hexane. Fractions containing **6** were combined and further purified by preparative TLC to yield 14 mg of pure material; ^{1}H NMR (100 MHz, $CDCl_3$): δ1.04, 1.04, 1.10, 1.35, 1.44 (15H, 4*s*, quaternary Me), 1.94 (1H, *d*, J = 12 Hz, H-5), 3.57 (3H, *s*, Me ester), 4.04 (1H, *m*, H-1), 4.12 (1H, *s*, H-15), 4.37 (1H, *d*, J = 12 Hz, H-6α), 5.40 (1H, *s*, H-17), 6.33 (1H, *d*, J = 1 Hz, β-furan), 7.39 (2H, *d*, J = 1 Hz, α-furans); ^{13}C NMR (15 MHz, $CDCl_3$): δ17.6 (*s*, Me), 18.4 (*s*, Me), 19.1 (*t*,C-11), 20.0 (*s*, Me), 22.2 (*s*, Me), 31.2 (*t*, C-12), 31.7 (*s*, Me), 35.5 (*t*, C-2), 37.6 (*s*, C-13), 38.6 (*d*, C-9), 47.3 (*s*, C-10)*, 48.1 (*s*, C-8)*, 60.1 (*d*, C-5), 66.5 (*s*, C-14), 72.2 (*d*, C-6), 77.5 (*d*, C-17), 81.0 (*s*, C-4), 82.1 (*d*, C-1), 109.6 (*d*, β-furan), 119.8 (*s*, β-furan), 140.9 (*d*, α-furan), 143.1 (*d*, α-furan), 166.6 (*s*, C-16), 171.6 (*s*, C-3), 212.5 (*s*, C-7).

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*Assignments may be interchanged.

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