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17-DEHYDROLIMONOATE A-RING LACTONE: A POSSIBLE METABOLITE OF LIMONOATE A-RING LACTONE IN *CITRUS* FRUITS*

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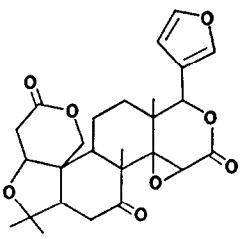
Key Word Index—*Citrus sinensis*; *Citrus limonia*; Rutaceae; 17-dehydrolimonoate A-ring lactone; limonin metabolite; debittering pathway.

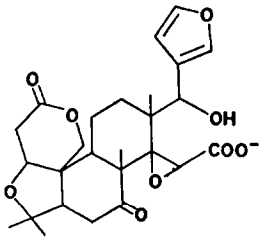
Abstract—A new limonoid, 17-dehydrolimonoate A-ring lactone (III), was isolated from orange peel, juice and lemon seeds and seedlings. This compound which is non-bitter appears to be an initial product of limonoate A-ring lactone (II) metabolism. As such it may be the initial intermediate in at least one debittering pathway in citrus fruits.

INTRODUCTION

BITTERNESS due to limonin (I) in citrus juices has long been regarded as an important economic problem.[1,2] Higby[2,3] first recognized that the limonin content of naval orange juice from mature oranges was substantially lower than that from immature fruit. Later it was shown that the naturally occurring form of limonin in the nonseed tissues of the fruit is limonoate A-ring lactone (II), a non-bitter hydroxy acid form of limonin which converts to limonin after juice extraction.[4] Recently, agents that trigger accelerated metabolism of limonoate A-ring lactone in detached citrus fruits have been reported.[5] However, the pathway by which limonoate A-ring lactone is metabolized has largely remained unknown. Elaboration of this pathway should help lead to a solution of the bitterness problem.



(I)



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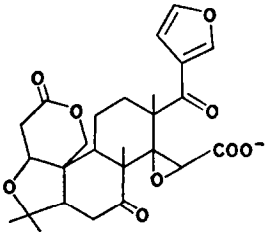

(III)

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[4] V. P. MAIER and D. A. MARGILETH, *Phytochem.* **8**, 243 (1969).
[5] V. P. MAIER, L. C. BREWSTER and A. C. HSU, *Citrograph* **56**, 351 (1971).

In related studies Hasegawa *et al.*[6,7] have shown that limonoate (or limonoate A-ring lactone) is degraded by bacteria via at least two pathways: one through 17-dehydrolimonoate and the other through deoxylimonin. In the former case the enzyme limonoate dehydrogenase has been isolated and characterized.[7] In this paper we report the isolation and identification of 17-dehydrolimonoate A-ring lactone (III) from the peel and juice of navel oranges and from lemon seeds and seedlings. The compound which is nonbitter appears to be an initial product of limonoate A-ring lactone metabolism in citrus.

RESULTS AND DISCUSSION

An acidic isolate was obtained from navel orange peel and juice and from lemon seeds and seedlings by chromatographic procedures. The isolate from each source consisted mainly of one component which had the same mobility as authentic 17-dehydrolimonoate A-ring lactone in TLC solvents 1, 2, 6 and 7 and, after methylation the same mobility as methyl 17-dehydrolimonoate A-ring lactone in solvents 1, 3 and 5 (Table 1). Responses to Ehrlich's reagent[8] and the modified Ehrlich's procedure were identical to those of the authentic compounds.

Further identification of the isolate was made by the action of limonoate dehydrogenase on the navel orange peel isolate.[7] This enzyme is highly specific and attacks reversibly only the 17-keto(hydroxyl) group of limonoids in the presence of NADH (NAD) and requires the furan ring, epoxide group and an open D-ring of the limonoid molecule for its catalytic action. The results showed that approx. 50% of the isolate was converted to a compound whose R_f was identical to that of limonin in solvents 1, 4, 5 and 6. The control (authentic 17-dehydrolimonoate A-ring lactone) also reached the equilibrium condition of 50% conversion. The enzymically converted compound was Ehrlich's reagent positive. In addition, the structure of the isolate was confirmed as 17-dehydrolimonoate A-ring lactone by NMR spectroscopy.

The available evidence shows that 17-dehydrolimonoate A-ring lactone is a natural constituent of citrus and that it occurs in a variety of tissues. Attempts to produce it from limonoate A-ring lactone by chemical means have led to different oxidation products, indicating that other portions of the molecule are more labile than the 17-hydroxyl group.[7] No trace of 17-dehydrolimonoate A-ring lactone was found when large amounts of limonoate were carried through the extraction and isolation procedure or when limonoate or limonin solutions were allowed to stand for long periods.

It was recently pointed out that no authentic degradation products of limonin have been isolated from citrus fruits.[1] Based on the present work and the microbiological studies of Hasegawa *et al.*[6,7] it can now be stated that 17-dehydrolimonoate A-ring lactone and deoxylimonin (previously reported in citrus seeds[8]) are the first authenticated biological degradation products originating from limonin or its hydroxy-acid form, limonoate A-ring lactone, isolated from citrus. Also, no limonoids other than limonoate A-ring lactone, limonin and 'isolimonin'[2] (apparently identical to deacetylnomilin[8]) have been identified in extracts of the nonseed tissues of citrus fruits or in juice. This seems to be due to the very low levels of occurrence of other limonoids as compared to limonoate A-ring lactone (which lactonizes to limonin during extraction and isolation) in these materials. In addition to the identification of 17-dehydrolimonoate A-ring lactone, Bennett[9] has now shown the presence

[6] SHIN HASEGAWA, R. D. BENNETT and V. P. MAIER, *J. Agric. Food Chem.* **20**, 435 (1972).
[7] SHIN HASEGAWA, R. D. BENNETT, V. P. MAIER and A. D. KING, JR., *J. Agric. Food Chem*, **20**, 1031 (1972).
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of minor amounts of the following limonoids in the peel of navel oranges: deacetylnomilin, nomilin, obacunone, deacetylnomilinic acid and nomilinic acid. All had previously been identified as constituents of citrus seeds.[8,10–12] Earlier, another investigator[13] reported the presence in orange juice extracts of four TLC components (in addition to limonin) three of which, from their reported mobilities, could possibly be deacetylnomilin, nomilin, and/or obacunone.

Based on studies of seed limonoids[8,10,14] these other fruit limonoids reported by Bennett appear to be members of the limonoid biosynthetic pathway. On the other hand, 17-dehydrolimonoate A-ring lactone (III) appears to be an initial product of limonoate A-ring lactone (II) metabolism in citrus fruits. The evidence for this is: III is the initial product of II metabolism in *Arthrobacter globiformis*;[7] the organism uses the limonoid as its sole carbon source indicating catabolic metabolism; the conversion is catalyzed by a single enzyme, limonoate dehydrogenase, which has been purified and characterized;[7] the enzyme attacks only those forms of limonin in which the D-ring lactone is open (the 17-hydroxyl group is free); and citrus tissues contain an enzyme which specifically hydrolyzes the D-ring lactone of limonoids.[15] Since II has been shown to be the naturally occurring form of limonin[16] in the non-seed tissues of citrus fruits, III rather than 17-dehydrolimonoate would be the expected product of its metabolism. Since III is a non-bitter compound, metabolism via this route would lead to reduced limonin levels in the juice and consequently reduced bitterness.

Citrus seeds contain two forms of limonin, namely limonin and limonoate A-ring lactone.[16,17] Since the 17-hydroxyl group of limonin is tied up in the lactone ring it would not be a substrate for limonoate dehydrogenase. However, the established presence of limonin D-ring lactone hydrolase,[15] which converts limonin to II, would permit the metabolism of limonin to III in seeds.

EXPERIMENTAL

17-Dehydrolimonoate A-ring lactone. This limonoid and its methyl ester were prepared by the procedure described previously.[7] Aqueous solutions of 17-dehydrolimonoate A-ring lactone were not bitter.

Plant materials. These citrus tissues were used: (a) Washington navel oranges on sweet orange rootstock harvested 27 January 1971. Peel tissues were stored at −20° for 6 months prior to extraction. (b) Washington navel oranges on Cleopatra mandarin rootstock harvested 2 February 1972. The fruit were juiced in the laboratory and the peel and juice were immediately frozen and held at −20° 3 and 5 weeks respectively, before extraction. (c) Lemon seeds from newly harvested fruit. Seeds were washed and air dried immediately after removal from the fruit. (d) Lemon seedlings 3–5 cm in length were harvested 2 weeks after sprouting and immediately extracted.

TLC. Silica gel G (1 mm) and GF (0·25 mm) layers were used with the following solvents: (1) benzene–EtOH–H_2O–HOAc (200:47:15:1); (2) $CHCl_3$–MeOH–HOAc–H_2O (45:15:3:1); (3) CH_2Cl_2–MeOH (24:1); (4) cyclohexane–EtOAc (3:7); (5) Et_2O–HOAc–H_2O (15:3:1); and (6) benzene–hexane–acetone–HOAc (65:22:10:3).[13] Microcrystalline cellulose (0·25 mm) layers were used with (7) isopropanol–NH_4OH–H_2O (9:1:1). Compounds were detected with 50% H_2SO_4-heat, Ehrlich's reagent-HCl[8] or a modified Ehrlich's procedure. The latter method was used to detect 17-dehydrolimonoids which give a negative reaction by the standard Ehrlich's-HCl method. The plate is first sprayed with *p*-dimethylaminobenzaldehyde solution and then with 50% H_2SO_4. Gentle heating produces the characteristic orange color of normal limonoids, while 17-dehydrolimonoids are not revealed. As the temp. is raised the orange color turns first red

[10] R. D. Bennett, *Phytochem.* **10**, 3065 (1971).
[11] O. L. Emerson, *J. Am. Chem. Soc.* **70**, 545 (1948).
[12] O. L. Emerson, *J. Am. Chem. Soc.* **73**, 2621 (1951).
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[15] V. P. Maier, S. Hasegawa and E. Hera, *Phytochem.* **8**, 405 (1969).
[16] V. P. Maier and D. A. Margileth, *Phytochem.* **8**, 243 (1969).
[17] V. P. Maier and G. D. Beverly, *J. Food Sci.* **33**, 488 (1968).

and then green, and at this point the 17-dehydrolimonoids appear as bright yellow spots. Further heating changes this color first to dark orange and then to purple. 17-Dehydrolimonoate A-ring lactone was also detected by its absorption in UV light.

Extraction procedures. (a) 10 kg of lemon seeds were extracted and limonin removed from the extract by crystallization as previously described.[8] The mother liquor was evaporated to dryness and separated into neutral and acidic fractions by column chromatography.[10] (b) Ground tissue (orange peel and lemon seedlings) or juice was macerated in a blendor in acetone several times, the acetone was concentrated *in vacuo* and the aqueous residue was extracted with $CHCl_3$. The combined $CHCl_3$ extracts were taken to dryness *in vacuo*, the residue was dissolved in 50 ml $CHCl_3$ and extracted with 5% $KHCO_3$. This was acidified to pH 2 with HCl and extracted with $CHCl_3$ to obtain an acidic fraction.

Purification procedures. (a) The acidic fraction from seeds was methylated with CH_2N_2 and subjected to column chromatography on silica gel.[10] Fractions were monitored by TLC, using Ehrlich's-HCl and the modified Ehrlich's procedure. After 4 column separations 5 mg of a fraction was obtained which consisted largely of methyl 17-dehydrolimonoate A-ring lactone. (b) The evaporated acidic fraction from (b) above was dissolved in MeCN washed with light petrol. and streaked on preparative silica gel G plates which were developed with solvent 1. The 17-dehydrolimonoate A-ring lactone zone (R_f 0–0·07) was removed, eluted with solvent 2, evaporated, taken up in MeCN, and streaked on silica gel GF plates and developed with solvent 2. The weak UV absorbing band (R_f and UV absorption identical with authentic 17-dehydrolimonoate A-ring lactone) was removed, eluted, taken up in $CHCl_3$, extracted into 5% $KHCO_3$ which was acidified and extracted with $CHCl_3$. The residue obtained after evaporating the final $CHCl_3$ was taken up in MeCN and is referred to as the isolate.

TABLE 1. IDENTIFICATION OF THE ISOLATE AS 17-DEHYDROLIMONOATE A-RING LACTONE BY TLC

Compound	R_f* 1	2	3	4	5	6	MCC 7
Isolate	0·05	0·22				0·00	0·78
17-Dehydrolimonoate A-ring lactone	0·05	0·22				0·00	0·78
Methylated isolate	0·47		0·39		0·43		
Methyl ester of 17-dehydrolimonoate A-ring lactone	0·47		0·39		0·43		
Limonoate dehydrogenase reaction product	0·41			0·34	0·52	0·15	
Limonin	0·41			0·34	0·52	0·15	

* For solvent key, see Experimental.

Identification. (a) The NMR spectrum in $CDCl_3$ of the methyl 17-dehydrolimonoate A-ring lactone isolated from seeds was identical to that of the authentic compound.[7] It also had the same mobility in TLC systems (Table 1) as the standard and the same Ehrlich's-HCl and modified Ehrlich's color reactions. (b) The isolate from (b) above and the CH_2N_2 methylated isolate were run on TLC along with authentic 17-dehydrolimonoate A-ring lactone and its methyl ester (Table 1). Each was negative with Ehrlich's-HCl but positive with the modified Ehrlich's procedure. *Ca.* 300 μg of 17-dehydrolimonoate A-ring lactone was isolated from 5 kg orange peel. This isolate was further identified by use of the specific enzyme limonoate dehydrogenase.[7] The reaction mixture consisted of *ca.* 60 μg of the isolate, 5 μmol NADH, 0·1 M K phosphate buffer, pH 6·5 and 0·05 unit of highly purified limonoate dehydrogenase. After incubating 16 hr at 21° the reaction mixture was brought to pH 1·5 with N HCl, boiled 5 min, extracted with $CHCl_3$, evaporated to dryness under N_2 and taken up in 30 μl MeCN for TLC. Comparative mobilities of the limonoate dehydrogenase reaction product and limonin are listed in Table 1. Each gave a positive Ehrlich's-HCl test

Test for an artifact. Authentic limonin equivalent to the limonin content of 5 kg peel (300 mg) was hydrolyzed to limonoate and carried through the extraction, TLC-purification, and identification procedures to determine whether 17-dehydrolimonoate-A ring lactone arises as an artifact by limonin autoxidation. The $CHCl_3$ soluble acidic fraction on preparative TLC gave several Ehrlich's positive bands, but was negative for 17-dehydrolimonoate A-ring lactone. Nevertheless, the area where 17-dehydrolimonoate A-ring lactone should have been was worked-up and after diazomethane treatment, an amount equivalent to 150 mg of the original limonin was chromatographed two-dimensionally with solvent 1 then 4. No trace of methyl 17-dehydrolimonoate A-ring lactone was detected. The minimum detectable amount of 17-dehydrolimonoate

A-ring lactone per TLC spot by either absorption of UV light on charring with H_2SO_4 is estimated at 0·15 μg. In a similar manner aq. limonoate, mixed limonoate monolactones, and limonin solution which had stood in air for several months were tested and found to contain no trace of 17-dihydrolimonoate A-ring lactone.

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