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LIMONOIC ACID A-RING LACTONE, A NEW LIMONIN DERIVATIVE IN *CITRUS*

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Abstract—A nonbitter derivative of limonin (the intensely bitter tetracyclic dilactone of *Citrus* seeds) has been found to occur in the fruit tissues of *Citrus sinensis* (L.) Osbeck and *C. paradisi* Macf. The new compound, for which the name limonoic acid A-ring lactone is proposed, is one of the two possible monolactones which can be derived from limonin by partial hydrolysis. It was identified as the A-ring monolactone by TLC and paper electrophoretic comparison with the authentic compound and by its facile conversion into limonin. Indications were found of an enzyme in tissue extracts which converts limonoic acid A-ring lactone into limonin.

INTRODUCTION

WE RECENTLY reported the natural occurrence in the tissues of certain citrus fruits of a limonoid monolactone structurally related to limonin.[1] In addition, it was shown that this nonbitter limonoid was readily converted into the intensely bitter dilactone limonin when juice was prepared from these fruit.

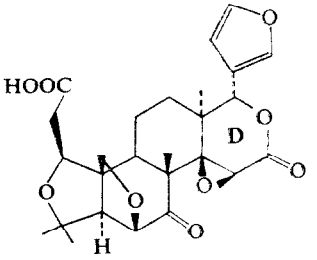
Limonin is a tetracyclic triterpenoid dilactone,[2,3] Fig. 1. The A-, and D-rings are δ-lactones which can be reversibly opened with dilute alkali. The dihydroxy diacid derived from limonin by complete hydrolysis of the lactones, for which we propose the name limonoic acid, can lead to two monolactone forms: limonoic acid A-ring lactone and limonoic acid D-ring lactone (Fig. 1). Methods have now been developed for separating and identifying these two compounds and we report here evidence that limonoic acid A-ring lactone is the naturally occurring monolactone in tissues of citrus fruits.

RESULTS AND DISCUSSION

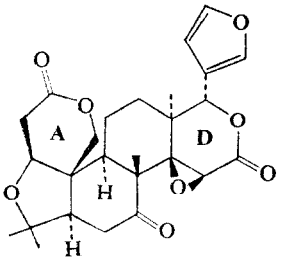
A mixture of limonoate A-ring lactone and limonoate D-ring lactone was prepared from disodium limonoate by acid-catalyzed lactonization at pH 5·0. Limonoic acid D-ring lactone was also prepared as a crystalline solid (contaminated with small amounts of limonin). Efforts to prepare crystalline limonoic acid A-ring lactone have not been successful because of the greater facility with which the D-ring lactonizes as compared with the A-ring. The structure of limonoic acid D-ring lactone (which is unstable in acidic aqueous or non-aqueous solutions) was proven by electrophoretic comparison with limonilic acid (Table 1), a related D-ring monolactone limonoid of known structure[3] and acidity,[4] Fig. 1, and by NMR spectroscopy of methyl limonoate D-ring lactone.

* A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

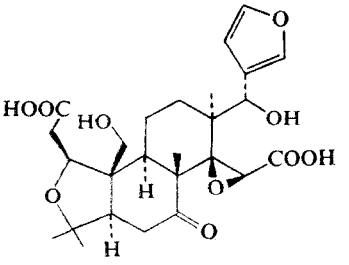
[1] V. P. MAIER and G. D. BEVERLY, *J. Food Sci.*, in press.
[2] D. ARIGONI, D. H. R. BARTON, E. J. COREY, O. JEGER, *et al. Experientia* **16**, 41 (1960).
[3] D. H. R. BARTON, S. K. PRADHAN, S. STERNHELL and J. F. TEMPLETON, *J. Chem. Soc.* 255 (1961).
[4] O. H. EMERSON, *J. Am. Chem. Soc.* **74**, 688 (1952).



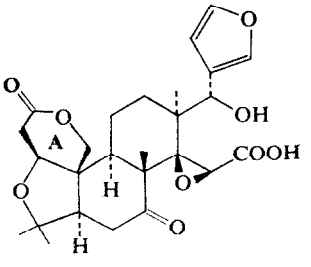
Limonilic acid



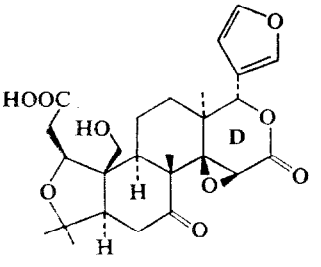
Limonin



Limonoic acid



Limonoic acid A-ring lactone



Limonoic acid D-ring lactone

FIG. 1. STRUCTURES OF LIMONOIDS.

The NMR spectrum* of the methyl limonoate monolactone was similar to that of limonin[5] in many respects. However, an important difference was the resonance for the C-19 methylene group which occurred at 4·59 ppm for limonin and 4·15 ppm for the methyl limonoate monolactone. The upfield shift of the H-19 resonance with the monolactone is consistent with that expected for the carbinol of an alcohol as compared with its corresponding ester.[6,7] Thus, the A-ring of the monolactone is open. In like manner, the identical resonances of the furfurylic H-17 for limonin and the monolactone (5·45 ppm) show the D-ring of the monolactone to be closed (see: Powell[8] for an example of the H-17 resonances of opened and closed D-rings).

* The NMR spectrum determination and interpretation were done by Dr. D. L. Dreyer.

[5] D. L. DREYER, *Tetrahedron* **21**, 75 (1965).

[6] Cf. L. M. JACKMAN, *Applications of Nuclear Magnetic Resonance Spectroscopy in Organic Chemistry*, p. 55, Macmillan, New York (1959).

[7] R. M. HOROWITZ and B. GENTILI, *Chem. & Ind.* 498 (1964).

[8] J. W. POWELL, *J. Chem. Soc.* (C) 1794 (1966).

We have previously shown that limonin, limonoate monolactones and disodium limonoate could be separated from each other by paper electrophoresis at pH 5·7. Thus, disodium limonoate migrated as a doubly-charged compound, limonoate monolactones as single-charged compounds and limonin with zero charge was unmoved. Since the carboxyl groups of limonoic acid have widely different pK_a values, 2·7 and 4·7,[4] electrophoresis at pH 3·1 has now proved to be an effective method of separating the two limonoate monolactones. The D-ring lactone did not migrate at pH 3·1 and therefore is the weaker acid of the two, Table 1. TLC also proved to be an effect method of separating and identifying these compounds, Table 1.

The presence of limonoate A-ring lactone in extracts of citrus tissues is shown by the data in Table 2. The identity of the compound as a limonin type limonoid is shown by its facile conversion into limonin at pH 3·0 or on longer standing at pH 5·6. Paper electrophoresis shows the natural compound to have a charge of −1 at both pH 5·7 and pH 3·1, therefore, it is the strong acid limonoate monolactone, namely, limonoate A-ring lactone. This was

TABLE 1. PAPER ELECTROPHORESIS AND TLC SEPARATION OF LIMONOIDS

Compound	Paper electrophoresis migration distance, cm		TLC, R_f*
	pH 5·7	pH 3·1	
Limonin	0·0	0·0	0·88
Limonoic acid A-ring lactone	6·5	8·0	0·51
Limonoic acid D-ring lactone	6·5	0·0	0·75
Limonoic acid	14·5	7·8	0·21
Limonilic acid	6·5	0·0	0·71
Hydrolyzed limonilic acid	14·5	7·8	—

* Microcrystalline cellulose with isopropanol, ammonia, water; 9:1:1.

confirmed by mixed chromatography and electrophoresis. Attempts to isolate limonoic acid A-ring lactone from acidified aqueous extracts of citrus tissues by partitioning with ethyl ether, ethyl acetate, or chloroform were unsuccessful. In all cases the instability of the A-ring lactone led to the isolation of limonin.

When the tissue extracts of fruit harvested after September were allowed to stand, limonoate A-ring lactone was converted into limonin at a faster rate than that observed with acid catalysis. Evidence suggesting the presence of a lactonizing enzyme in the tissues was obtained by showing the formation of limonin from synthetic limonoate A-ring lactone when this substrate was mixed with an unheated tissue extract. No limonin was formed when the extract was boiled before adding the substrate. The presence of this enzyme requires rapid work-up of the extracts in order to detect the natural occurrence of limonoate A-ring lactone. Work is in progress on the purification and properties of the enzyme.

Limonoate A-ring lactone was found to be present in the carpellary membrane tissues of immature Washington Navel oranges (*Citrus sinensis* (L.) Osbeck) and the capellary membrane tissues and albedo tissues of Marsh seedless grapefruit and Ruby Red grapefruit (*Citrus paradisi* Macf.). The presence of limonoate A-ring lactone in the carpellary membrane tissues of the fruit of navel oranges grown in the desert region of California (blossom in

TABLE 2. PROPERTIES USED TO IDENTIFY THE NATURALLY OCCURRING LIMONOIC ACID MONOLACTONE

Fruit tissue	Harvest date	Treatment	Paper electrophoresis migration distance,* cm		TLC*, R_f			Extract taste	Identity of compound
			pH 5·7	pH 3·1	MCC-IAW	SG-BEWA	SG-EAW		
Orange membranes	8/29	None†	6·5	8·0	0·51	—	—	Nonbitter	Limonoic acid A-ring lactone
		pH 3+heat‡	0·0	0·0	0·88	0·48	0·73	Bitter	Limonin
Orange membranes	9/20	None†	6·5	8·0	0·51	—	—	Nonbitter	Limonoic acid A-ring lactone
		pH 3+heat‡	0·0	0·0	0·88	0·48	0·73	Bitter	Limonin
Grapefruit membranes	11/5	None†	6·5	8·0	0·51	—	—	NA§	Limonoic acid A-ring lactone
		pH 3+heat‡	0·0	0·0	0·88	0·48	0·73	NA§	Limonin
Grapefruit albedo	11/5	None†	6·5	8·0	0·51	—	—	NA§	Limonoic acid A-ring lactone
		pH 3+heat‡	0·0	0·0	0·88	0·48	0·73	NA§	Limonin

* Positive limonoid test with Ehrlich's reagent, color identical to superimposed authentic compounds. See text for conditions used in electrophoresis and TLC.

† Extract analyzed within 15 min after blending.

‡ Extract adjusted to pH 3 and heated 20 min before analysis.

§ Taste test is not applicable because grapefruit extracts contain naringin, a bitter flavonoid.

March, mature in November) as a function of maturity of the fruit was as follows: 11 July, trace amounts; 29 August, 20 Sept., 11 Nov., significant amounts; 11 January, trace amounts. Juice or extracts of the tissues became bitter after heating at pH 3 for those fruit whose tissues initially showed significant amounts of limonoate A-ring lactone. Where only traces of limonoate A-ring lactone were found no significant bitterness developed. These observations suggested that enzymes are present which lead to the further metabolism of limonoate A-ring lactone as the fruit ripens beyond early maturity. The products of this metabolism are apparently not bitter themselves and are not converted into bitter compounds when juice is prepared from the fruit.

The biosynthetic significance of limonoate A-ring lactone is currently under study. It is tempting to speculate at this time that limonoate A-ring may be the immediate biosynthetic precursor of limonin in those tissues which accumulate limonin, such as citrus seeds.

EXPERIMENTAL*

Preparation of Monolactones

Disodium limonoate (final concentration 400 ppm) was adjusted to pH 5·0 with dilute HCl, made 0·05 M in citrate buffer pH 5·0 and refluxed 2 hr. The solution was cooled at 5° and the precipitated limonin was removed by filtration. TLC analysis showed the filtrate to contain a mixture of limonoate A-ring lactone and limonoate D-ring lactone and a trace of limonin.

Preparation of Limonoic Acid D-Ring Lactone

7·2 g limonin was refluxed with a slight excess of 0·5 N NaOH for 2 hr. The disodium limonoate was cooled to almost 0° and rapidly adjusted to pH 1·5 with 1 N HCl added dropwise and with constant stirring. The copious white precipitate was immediately collected by filtration, washed with water, and air dried to yield 5·32 g product which contained about 5 per cent limonin impurity. The limonin content was reduced by treating the product with MeOH. The D-ring lactone remained in solution while limonin crystallized and was removed. After precipitation by the addition of water, the limonoic acid D-ring lactone still contained small amounts of limonin. Attempts to further purify the product were not successful because of gradual conversion of limonoic acid D-ring lactone into limonin.

Preparation of Methyl Limonoate D-Ring Lactone

0·5 g limonoic acid D-ring lactone in 5 ml MeOH was treated at *ca.* 0° with excess CH_2N_2 and precipitated limonin removed by filtration. The filtrate yielded a viscous oil which was purified several times by solution in ethyl ether and precipitation with light petroleum, giving a white solid. TLC showed only one Ehrlich's reagent positive spot, R_f 0·52, on silica gel N-HR sheets (Brinkmann Instruments, Inc.) developed with ethyl ether–light petroleum (30–60°), 9:1, containing 1·9% HOAc. Under these same conditions limonin gave R_f 0·1 and limonoic acid D-ring lactone gave R_f 0·38. The NMR spectrum was determined in $CDCl_3$ at 60 Mc/s and is given in ppm relative to internal tetramethylsilane: 7·42(d) $J=1$ α-furan, 6·37(d) $J=1$ β-furan, 5·45(s) furfurylic H-17, 4·15(s) H-19, 4·07(s) H-1, 3·79(s) epoxy H-15, 3·69(s) methoxyl, 1·32, 1·22, 1·14, 1·01(s) C-methyls.

PE and TLC Separation of Limonoids

High-voltage paper electrophoresis (PE) was run on Whatman No. 1 filter paper at 113 V/cm for 40 min.[1] The buffers used were: 0·05 M citrate, pH 5·7, and 5% acetic acid adjusted to pH 3·1 with NH_4OH. Migration distances are given in Table 1. Thin-layer chromatography (TLC) was run on 0·2 mm layers of microcrystalline cellulose (MCC) (Brinkmann Instruments, Inc.) developed with isopropanol-NH_4OH-HOH, 9:1:1 (IAW). R_f values are given in Table 1. All of these compounds were stable during PE and TLC analysis. Limonoid compounds gave a characteristic orange–brown color when sprayed with Ehrlich's reagent and fumed with HCl.[9] Limonin was differentiated from other limonoids by TLC on silica gel-G using the upper phase of benzene–ethanol–water–acetic acid, 200:47:15:1 (SG-BEWA) and ethyl ether–acetic acid–water, 15:3:1 (SG-EAW).[1]

* Reference to a company or product name does not imply approval or recommendation by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

[9] D. L. DREYER, *J. Org. Chem.* **30**, 749 (1965).

Extraction and Identification of Natural Limonoate A-Ring Lactone

In a typical extraction 5 g of washed citrus fruit tissue was blended at high speed for 3 min in 45 ml water at 0°. The slurry was rapidly filtered through celite (30–60 sec) immediately after blending and the clear extract held in an ice bath. 15–25 μl of extract was spotted for TLC and 62·5 μl/cm was streaked for PE. All extracts were spotted or streaked within 15 min after blending. With the later season fruit, if the clear extracts were allowed to stand for longer periods before PE or TLC, a small amount of limonin was observed in addition to the relatively large amount of limonoate A-ring lactone. This suggested the presence of a lactonizing enzyme. Portions of the clear extracts (pH 5–6) were adjusted to pH 3·0 with 1 N HCl and heated at 94° for 20 min to chemically convert limonoic acid A-ring lactone to limonin for further identification. The original extracts and heated extracts of the orange fruit tissue were tasted by judges experienced in detecting limonin bitterness. The judges had taste thresholds ranging from 2–5 ppm of limonin in aqueous solutions. Authentic compounds were run adjacent to and superimposed upon the extract spots and streaks for TLC and PE identification, Table 2.

Test for Lactonizing Enzyme

Orange and grapefruit carpellary membrane extracts were prepared as in the preceding paragraph. One portion of the extract was boiled for 5 min to inactive enzymes. 5 ml of substrate (roughly 1000 ppm in limonoate A-ring lactone and limonoate D-ring lactone) was combined with an equal volume of the boiled and unboiled extracts (pH 5·7). After 3 hr at 25° each system was analyzed for limonin by TLC-MCC-IAW. The boiled extract system showed little change in the substrate mixture whereas the unboiled system showed a decrease in limonoate A-ring lactone and an increase in limonin.

Isolation of Limonin from Acid-Treated Extract

Carpellary membrane tissue (21 g) was dissected from several grapefruit and extracted with water as outlined above. TLC-MCC-IAW analysis of the clear extract showed the presence of limonoate A-ring lactone. After the extract was acidified and heated as described above, TLC analysis showed the absence of limonoic acid A-ring lactone and the presence of limonin. The solution was extracted with 5×25 ml $CHCl_3$ and the $CHCl_3$ dried (Na_2SO_4) and evaporated to an oily residue. The residue was agitated with hexane, centrifuged and the precipitate taken up in CH_2Cl_2, dried and concentrated. MeOH was added and, on standing, about 2-mg white crystals were deposited which gave R_f and PE values, Ehrlich's reagent color, and an i.r. spectrum identical to authentic limonin. I.r. spectra were determined in Nujol with a Perkin Elmer Infracord spectrophotometer.

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