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# DETECTION OF LIMONOATE DEHYDROGENASE ACTIVITY IN ALBEDO TISSUES OF *CITRUS SINENSIS*

SHIN HASEGAWA, V P MAIER and RAYMOND D BENNETT

Fruit and Vegetable Chemistry Laboratory, Western Region, ARS, USDA, Pasadena, CA 91106, U S A

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**Key Word Index**—*Citrus sinensis*, Rutaceae, navel orange, limonoid metabolism, limonoate dehydrogenase activity

**Abstract**—19-Deoxylimonoic acid 3-methyl-$^{14}$C ester was converted to 17-dehydro-19-deoxylimonoic acid 3-methyl-$^{14}$C ester in albedo tissues of navel oranges This conversion is direct evidence of limonoate dehydrogenase activity in the tissues

## INTRODUCTION

LIMONIN is an intensely bitter tetracyclic triterpenoid dilactone [1,2] The presence of limonin is responsible for bitterness in some processed citrus products, especially orange juices prepared from early- to mid-season California navel oranges and Valencia's grown in Australia Freshly prepared juice from these fruits is nonbitter The bitterness develops only after the juice has been heated or allowed to stand for certain periods of time This delayed bitterness has been explained by Maier *et al* [3,4] limonoate A-ring lactone, a nonbitter precursor of limonin is gradually converted to the latter after juice extraction

It has been shown that limonoid content in citrus fruit decreases with advancing maturity [5–7] For many years attempts to demonstrate limonoid-degrading systems in the fruit have been unsuccessful However, we have recently shown the presence of 17-dehydrolimonoate A-ring lactone in the fruit, suggesting that this compound is the initial metabolite of limonoate A-ring lactone [8]

In this paper, we demonstrate limonoate dehydrogenase activity in albedo tissues of navel oranges This enzyme is undoubtedly responsible for the formation of 17-dehydrolimonoate A-ring lactone in fruits

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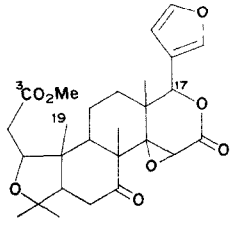
[4] MAIER, V P and MARGILETH, D A (1969) *Phytochemistry* **8**, 243
[5] HIGBY, R H (1938) *J Am Chem Soc* **60**, 3013
[6] SCOTT W C (1970) *Florida State Hort Soc* **83**, 270
[7] MAILR, V P BREWSTER L C and HSU, A C (1973) *J Agric Food Chem* **21**, 490
[8] HSU A C HASEGAWA S MAIER V P and BENNETT R D (1973) *Phytochemistry* **12**, 562

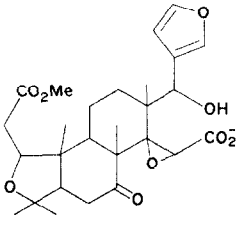
## RESULTS AND DISCUSSION

Attempts to demonstrate limonoate dehydrogenase activity in extracts of acetone powders or extracts directly from fresh tissues of citrus fruits were unsuccessful Consequently we adopted a tissue slice technique in which a radioactive substrate, 19-deoxylimonoic acid 3-methyl-$^{14}C$ ester (**2**) was used This compound was chosen because it is easily prepared, stable and an excellent substrate for limonoate dehydrogenase of bacteria [9]



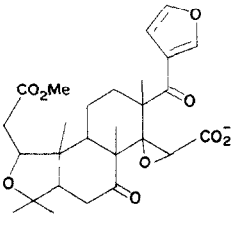
19-Deoxylimonoic acid
D-ring lactone 3-methyl ester

(1)



19-Deoxylimonoic acid
3-methyl ester

(2)



17-Dehydro-19-deoxylimonoic
acid 3-methyl ester

(3)

After treatment of tissue slices with radioactive **2** and extraction, four peaks were observed on a radiochromatogram The largest was identified as compound **2** (the substrate) Two minor peaks were not identified The compound of the second largest peak was isolated by $KHCO_3$ extraction followed by preparative TLC The isolate had $R_f$ values identical to those of authentic 17-dehydro-19-deoxylimonoic acid 3-methyl ester (**3**) with solvents 1 and 4 (Table 1)

TABLE 1 IDENTIFICATION OF THE ISOLATE BY TLC

| Compound | $R_f$s* | | | |
|---|---|---|---|---|
| | (a) | (b) | (c) | (d) |
| Isolate | 0 07 | 0 | 0 | 0 10 |
| 17-Dehydro-19-deoxylimonoic acid 3-methyl ester | 0 06 | 0 | 0 | 0 10 |
| Isolate treated with limonoate dehydrogenase | 0 56 | 0 52 | 0 73 | 0 84 |
| 19-Deoxylimonoic acid 3-methyl ester | 0 56 | 0 51 | 0 73 | 0 85 |

* Solvent key see Experimental

Further identification of the isolate was made by use of limonoate dehydrogenase of *Arthrobacter globiformis* This enzyme is highly specific and attacks reversibly only the 17-keto (hydroxy) group of limonoids, which possess the furan ring open D-ring and epoxide in the presence of NADH (NAD) [9] The results showed that *ca* 65% of the isolate was converted to a compound whose $R_f$s were identical to those of compound **2** with solvents 1-4 (Table 1) This would have been possible only if the isolate contained a 17-keto group, and therefore we can assign to it the structure **3**

[9] HASEGAWA S BENNETT R D MAIER V P and KING JR A D (1972) *J Agric Food Chem* **20**, 1031

The rate of the conversion of (**2**) to (**3**) in the tissue slices appeared to be directly proportional to the time of incubation, conversion was highest *ca* 5% after 3 days of incubation

These results, coupled with the fact that 17-dehydrolimonoate A-ring lactone is a natural constituent of citrus fruits,[8] prove the previous suggestion[8,10] that the fruit possesses a limonoate dehydrogenase These facts are consistent with the hypothesis that limonoate dehydrogenase is the initial enzyme of at least one pathway responsible for limonoid metabolism during maturation of the fruit Recent evidence[11] that limonoid metabolism in postharvest fruit is interrupted in the absence of oxygen lends further support to the limonoate dehydrogenase pathway In bacteria, limonoate is metabolized via at least two pathways, one through 17-dehydrolimonoate and the other through deoxylimonin [9,10] Whether limonoid metabolism in citrus fruit also proceeds via a deoxylimonin pathway remains to be determined

## EXPERIMENTAL

*Materials* Limonin D-ring lactone hydrolase was isolated from grapefruit seeds as described by Maier *et al*[12] Limonoate dehydrogenase was isolated from cell-free extracts of *Arthrobacter globiformis* by the procedure described previously [9] 19-Deoxylimonoic acid D-ring lactone (commonly referred to as isoobacunoic acid) was prepared as described by Barton *et al* [2] *N*-Methyl-$^{14}$C-*N*-nitroso-*p*-toluenesulfonamide was purchased commercially Silica gel (0 25 mm) sheets coated on aluminum were used Mid-season navel oranges were purchased from a local store

*Preparation of 19-deoxylimonoic acid D-ring lactone 3-methyl-*$^{14}$*C ester* (**1**) A soln of 10 mg (100 $\mu$Ci) *N*-methyl-$^{14}$C-*N*-nitroso-*p*-toluenesulfonamide in 300 $\mu$l $Et_2O$ was treated with 50 $\mu$l 2-(2′-ethoxyethoxy) EtOH and a soln of 2 8 mg KOH in 5 $\mu$l $H_2O$ After 5 min, the mixture was heated at 55° and the $CH_2N_2$ distillate collected in 200 $\mu$l $Et_2O$ and cooled to −10° A soln of 12 mg isoobacunoic acid in 300 $\mu$l $CH_2Cl_2$ then was added to the $CH_2N_2$ soln After 5 min, the soln was evaporated to dryness TLC showed that the product was a mixture of unreacted isoobacunoic acid and the desired methyl ester The latter was isolated by chromatography on a silica gel column with cyclohexane–EtOAc (3 2) as eluent 5 5 mg (30% yield) of chromatographically homogeneous (**1**) was obtained

*Preparation of 19-deoxylimonoic acid 3-methyl-*$^{14}$*C ester* (**2**) The D-ring of compound **1** was hydrolyzed with limonin D-ring lactone hydrolase 5 5 mg compound **1** was suspended in 2 ml 0 1 M phosphate buffer soln at pH 7 5 and incubated with limonin D-ring lactone hydrolase at room temp After 17 hr of incubation, the reaction was completed

*Preparation of 17-dehydro-19-deoxylimonoic acid 3-methyl ester* (**3**) Compound **3** was prepared enzymically from **2** and used as a reference 0 35 ml soln of 0 1 M phosphate at pH 7 5 containing *ca* 1 mg of **2** was incubated with 0 01 unit of limonoate dehydrogenase in the presence of 5 $\mu$mol NAD at room temp After 17 hr of incubation all of the **2** was converted to **3**

*Incubation of* **2** *with albedo tissues of navel oranges* 15–20 g of albedo tissue slices of navel oranges were placed evenly in 2 Petri dishes and incubated at 25° with 10 ml 0 1 M phosphate buffer soln, pH 7 0, containing *ca* 0 3 $\mu$Ci **2**, 50 000 units of penicillin and 0 1 mmol each of NAD and NADP for 1–3 days Then, the slices were washed thoroughly with $H_2O$ and ground in 50 ml 0 05 M phosphate buffer at pH 7 5 with a Polytron The mixture was filtered The filtrate was acidified to pH 2 with HCl, boiled for 5 min, and extracted with two 50-ml portions of $CHCl_3$ The combined extracts were evaporated and the residue was dissolved in a minimal portion of MeCN and analyzed by TLC TLC sheets were developed with (a) $C_6H_6$–EtOH–$H_2O$–HOAc(200 47 15 1), (b) cyclohexane–EtOAc (1 1), (c) $CH_2Cl_2$–MeOH (49 1) and (d) $CHCl_3$–MeOH–HOAc–$H_2O$ (45 10 1 1) After the sheets were developed and dried, radioactive peaks were located and their relative intensities were estimated by a Vanguard Automatic Chromatogram Scanner

*Isolation of the metabolite* The combined $CHCl_3$ extracts obtained above were extracted with 5% $KHCO_3$ The extract was then acidified to pH 2 with HCl and extracted with $CHCl_3$ to obtain an acidic fraction The evaporated acidic fraction was dissolved in MeCN and streaked on a preparative silica gel plate which was developed with solvent 1 The 17-dehydro-19-deoxylimonoic acid 3-methyl ester zone ($R_f$ 0–0 1) was removed, eluted with EtOAc–HOAc (19 1) evaporated, taken up in MeCN and was referred to as the isolate

*Conversion of the isolate to* **2** *with limonoate dehydrogenase* The reaction mixture consisted of a half of the isolate obtained above, 5 $\mu$mol NADH, 0 1 M phosphate buffer at pH 6 0 and 0 01 unit of limonoate dehydrogenase After incubation at room temp for 17 hr, 65% of the isolate was converted to a compound whose $R_f$s were identical to those of compound **2** with solvents 1–4

[10] Hasewaga, S, Maier, V P and King, Jr, A D (1973) *J Agric Food Chem* in press
[11] Hsu, A C, Brewster, L C and Maier, V P unpublished results
[12] Maier, V P, Hasegawa S and Hera E (1969) *Phytochemistry* **8**, 405