Phytochemistry, 1968, Vol. 7, pp. 769 to 771. Pergamon Press. Printed in England

SHORT COMMUNICATION

A PROCEDURE FOR OBTAINING RADIOACTIVE NARINGIN FROM GRAPEFRUIT LEAVES FED L-PHENYLALANINE-^{14}C

JAMES F. FISHER*

U.S. Fruit and Vegetable Products Laboratory,† Winter Haven, Florida, U.S.A.

(*Received* 22 *November* 1967)

Abstract—Radioactive naringin, naringenin-7β-neohesperidoside, the principal bitter constituent of grapefruit (*Citrus paradisi*) and its radioactive tasteless isomer, naringenin-7β-rutinoside, were isolated from young grapefruit leaves fed L-phenylalanine-^{14}C. Radiolabeled L-phenylalanine is taken up by young grapefruit leaves through the petioles and the leaves are then allowed to metabolize the phenylalanine during periods of 12 hr of light and 12 hr of dark. Extraction of the leaves with hot methyl alcohol, followed by concentration and column chromatography afforded both the bitter naringin and its tasteless isomer. Final separation and purification of both compounds were accomplished by thin-layer chromatography. These results demonstrate that L-phenylalanine can serve as a precursor to naringin in grapefruit leaves.

INTRODUCTION

THE principal flavanone glycoside in grapefruit (*Citrus paradisi*) is naringin (naringenin-7β-neohesperidoside)[1,2] the compound primarily responsible for the bitterness of grapefruit. Its isomer, naringenin-7β-rutinoside, also present in grapefruit, is tasteless.[1,3,4] A logical pathway for the biosynthesis of naringin in this plant can be deduced from what is known for similar flavonoids in other plants,[5–7] but confirmation is lacking. An accurate understanding of the biogenesis of naringin will be helpful in the search for an enzymic inhibitor of its biosynthesis.

In order to augment present knowledge concerning the site of synthesis and translocation of naringin, as well as its biosynthesis and metabolism in the grapefruit plant, it became necessary to acquire radiolabeled naringin. Uniformly labeled L-phenylalanine has been incorporated into quercetin by buckwheat (*Fagopyrum tartaricum*).[8] This paper reports a similar procedure for the incorporation of uniformly labeled L-phenylalanine into naringin by grapefruit leaves.

* Present address: Syntex Corporation, Freeport, Grand Bahama Island.

† One of the laboratories of the Southern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture. Mention of firm or brand names is for identification and does not imply endorsement.

[1] R. M. HOROWITZ, in *Biochemistry of Phenolic Compounds* (edited by J. B. HARBORNE), p. 545. Academic Press, New York (1964).
[2] R. M. HOROWITZ and B. GENTILI, *Tetrahedron* **19**, 773 (1963).
[3] W. J. DUNLAP and S. H. WENDER, *Anal. Biochem.* **4**, 110 (1962).
[4] J. W. MIZELL, W. J. DUNLAP, R. E. HAGEN, S. H. WENDER, B. J. LIME, R. F. ALBACH and F. P. GRIFFITHS, *Anal. Biochem.* **12**, 316 (1965).
[5] F. M. DEAN, *Naturally Occurring Oxygen Ring Compounds*, p. 586. Butterworth, London (1963).
[6] J. E. WATKIN and A. C. NEISH, *Phytochem.* **1**, 52 (1961).
[7] G. A. BARBER, *Biochem.* **1**, 463 (1962).
[8] E. W. UNDERHILL, J. E. WATKIN and A. C. NEISH, *Can. J. Biochem. Physiol.* **35**, 219 (1957).

RESULTS AND DISCUSSION

L-Phenylalanine was shown to be a substrate for the synthesis of naringin in 2-week-old grapefruit leaves. When phenylalanine-^{14}C (U.L.) was administered to 3-month-old leaves, the amino acid was taken up through the petioles but subsequent work-up of the leaves afforded very little radioactive naringin; a large portion of the labeled phenylalanine was recovered unchanged. It therefore appears that the biosynthesis of naringin occurs mainly in the young, rapidly-metabolizing grapefruit leaves. Petiole feeding was the most successful of several methods tried for introducing radiolabeled phenylalanine into the grapefruit leaves.

Purification of naringin by TLC followed by autoradiography showed it was radiochemically homogeneous. The naringin was obtained with a specific activity of about 1·0 μc/μmole and labeled in the naringenin moiety. To our knowledge this is the first reporting of the synthesis of radio-labeled naringin by either *vitro* or *vivo* systems.

EXPERIMENTAL

The uniformly labeled L-phenylalanine was purchased from New England Nuclear Corporation. The leaves were monitored with a Nuclear Chicago ratemeter Model 1619A (Labitron). Chromatographic fractions were counted in low potassium 22-ml glass vials using a Packard Tri-Carb Liquid Scintillation Spectrometer Model 4322.

Eighty young grapefruit leaves, about 1–2 weeks old, were removed from outdoor-grown plants. The labeled phenylalanine was administered as follows: the leaves were immersed in water and cut just below their petioles. The fresh-cut ends were placed in eighty microtubes each containing 50 μl of an aqueous solution of $2{\cdot}7 \times 10^{-3}$ μmole phenylalanine-^{14}C (1·0 μc, specific activity 366 μc/μmole). Care was taken to avoid an air block. The solution was absorbed in 30–40 min. During this period the leaves were under constant illumination from both a 15 W fluorescent white and a 100 W incandescent white light placed at a distance of 40 cm from the leaves. The temperature at the surface of the leaves was 27°. The leaves were then placed in glass vials containing water and allowed to metabolize for 12 hr in light as described above and 12 hr in the dark at room temperature. Activity at the surface of each leaf was at least 1000 c.p.m. The leaves were then cut into small pieces and boiled for 40 min in MeOH. After crushing the leaves, the hot mixture was filtered and the filtrate concentrated under N_2 to a small volume. This solution was then chromatographed on 11·0 g of Polyclar AT powder (General Aniline and Film Corp., Dyestuff and Chemical Division) packed into an 800 × 19 mm column as a slurry in 100 ml of distilled water. The column was eluted with water, 10 ml fractions being collected. A 100 μl aliquot from every fifth fraction was placed in a counting vial and evaporated to dryness under N_2. 19 ml of a solution consisting of 10·0 g 2,5-diphenyloxazole and 0·2 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene in 1 l. of toluene was added. The radioactivity of the resulting solution was measured with the scintillation spectrometer. Fractions 35–80, showing the largest activity and appearing to be predominantly naringin by TLC, were combined, rechromatographed on polyamide and the resulting fractions retested. Fractions 40–55 from this second column contained the greatest amount of radioactivity and were further examined by TLC along with authentic naringin.

Thin-layer Chromatography

Polyamide-rich starch[9] and silica gel G were spread separately as 250 μ thick layers on 20 × 20 cm glass plates. Fractions 40–55 were spotted on the above chromatoplates along with both authentic naringin and naringenin-7β-rutinoside and developed at 25° (1) nitromethane–MeOH, 5:2; (2) MeOH–H_2O, 1:1; (3) *n*-propyl alcohol–EtOAc–H_2O, 3:2:1; and (4) dimethylformamide–H_2O–MeCOEt–EtOAc, 1:1:3:5.

Autoradiography and Fluorescence of Naringin

Sheets of Kodak medical X-ray no-screen film were exposed to the above TLC plates for 3 weeks and then developed using General Electric MED, Supermix, X-ray developer and fixer. The TLC plates were then sprayed with 1% $AlCl_3$ in EtOH and exposed to u.v. light (3660 Å). Both naringin and its tasteless isomer appeared as bright yellow fluorescent spots[10] which coincided with the darkened areas on the developed X-ray films. These spots had R_f values identical to those of authentic naringin and naringenin-7β-rutinoside. Fractions 40–55 were then combined.

[9] H. E. NORDBY, T. J. KEW and J. F. FISHER, *J. Chromatog.* **24**, 257 (1966).
[10] J. F. FISHER, H. E. NORDBY and T. J. KEW, *J. Food Sci.* **31**, 947 (1966).

Hydrolysis of Naringin

A portion of the combined fractions 40–55 from column two, previously shown by TLC and autoradiography to be a mixture of naringin and its isomer naringenin-7β-rutinoside, was subjected to acid hydrolysis (2 N HCl) at 100° for 60 min. The reaction mixture was evaporated under N_2 and the residue was dissolved in MeOH and examined by TLC. This methanolic solution was shown to contain radioactive naringenin by autoradiography and comparison of R_f values with authentic naringenin in four solvent systems on silica gel G. The solvents were (3); (4); (5) *n*-propyl alcohol–NH_4OH–H_2O, 6:2:1; and (6) toluene–EtOAc–HOAc, 5:4:1. R_f values were 0·76, 0·56, 0·64 and 0·48, respectively. Naringenin was detected by its yellow fluorescence in u.v. light in the presence of $AlCl_3$.

Acknowledgement—The author wishes to thank Mr. C. Matthew Tynes and Mr. Joe Mathis for technical assistance.