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APIGENIN 7β-RUTINOSIDE, A NEW FLAVONOID FROM THE LEAVES OF *CITRUS PARADISI*

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Abstract—Apigenin 7β-rutinoside, an isomer of apigenin 7β-neohesperidoside (rhoifolin) has been isolated for the first time from mature leaves of *Citrus paradisi*. Its structure has been verified by spectral, chromatographic and hydrolytic analyses and by comparison with material synthesized from naringenin 7β-rutinoside. Both apigenin glycosidyl isomers have been biosynthesized in the leaves from $^{14}CO_2$.

INTRODUCTION

IN PREVIOUS studies on the biosynthesis of naringin, various parts of the grapefruit plant were screened by thin-layer chromatography (TLC) for flavonoids. Using non-aqueous solvent systems on polyamide plates,[1,2] an unknown compound was observed in mature leaves which seemed to be absent in young leaves, peel, juice, flower and stem of the grapefruit plant. A preliminary analysis of the compound isolated by preparatory TLC indicated the compound to be an apigenin glycoside.

The flavone glycoside, rhoifolin, which is the 7β-neohesperidoside of apigenin (4′,5,7-trihydroxyflavone) has long been known to occur in the fruit and peel of *Citrus*[3–6] and more recently in the leaves of *Poncirus*[7] and *Chorisia* species.[8] The 7β-rutinoside isomer of apigenin until the present time, however, has never been isolated, although the closely related neohesperidosides and rutinosides of the flavanones naringenin, isosakuranetin and hesperetin have all six been reported as constituents of the grapefruit.[9] Recently the 7β-neohesperidoside and 7β-rutinoside of naringenin have been found and biosynthesized in young grapefruit leaves.[10]

The current study is concerned with the isolation and proof of structure of the apigenin glycoside mentioned above and now known to be apigenin 7β-rutinoside. This flavone occurs

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[1] R. E. HAGEN, W. J. DUNLAP, J. W. MIZELLE, S. H. WENDER, B. J. LIME, R. F. ALBACH and F. P. GRIFFITHS, *Anal. Biochem.* **12**, 472 (1965).

[2] H. E. NORDBY, T. J. KEW and J. F. FISHER, *J. Chromatog.* **24**, 257 (1966).

[3] S. HATTORI, M. SHIMOKORIYAMA and M. KANAO, *J. Am. Chem. Soc.* **74**, 3614 (1952).

[4] W. J. DUNLAP and S. H. WENDER, *Anal Biochem.* **4**, 110 (1962).

[5] K. M. ROWELL and C. G. BEISEL, *J. Food Sci.* **28**, 195 (1963).

[6] V. P. MAIER and D. M. METZLER, *Phytochem.* **6**, 1127 (1967).

[7] S. HATTORI and M. SHIMOKORIYAMA, *Sci. Proc. Roy. Dublin Soc.* **27**, 139 (1956).

[8] J. D. COUSSIO, *Experientia* **20** (10), 562 (1964).

[9] J. W. MIZELLE, W. J. DUNLAP, R. E. HAGEN, S. H. WENDER, B. J. LIME, R. F. ALBACH and F. P. GRIFFITHS, *Anal. Biochem.* **12**, 316 (1965).

[10] J. F. FISHER, *Phytochem.* **7**, 769 (1968).

along with its neohesperidoside isomer and the two naringenin isomers in mature grapefruit leaves. The possible role of apigenin rhamnosylglucosides in the biosynthesis of naringin is discussed.

RESULTS AND DISCUSSION

Two samples of the new flavone (I) were obtained chromatographically pure by column chromatography. One of these samples was obtained directly from an extract of "Duncan" grapefruit leaves. The other sample was separated from a mixture of the two apigenin-7-rhamnosylglucosides prepared by dehydrogenating the mixture of the two respective naringenin rhamnosylglucosides in a "Duncan" grapefruit peel extract.[11] As a reference, a third sample rhoifolin (II) was synthesized[11] from purified naringin.

Thin-layer chromatography revealed the three samples to have the same R_f values in five solvent systems. When the three samples were analyzed by TLC on polyamide plates,[2] rhoifolin had a lower R_f characteristic of neohesperidosides[1] (Table 1). Under u.v. light they absorbed light; with aluminum chloride under u.v. light they gave a gold fluorescence, and with the flavanone reagent ($NaBH_4$—HCl)[12] they were negative. When TLC plates were developed in 2-butanone–EtOAc–$HCONMe_2$–H_2O (10:6:3:2), the flavones did not give the greenish white fluorescence under u.v. light characteristic of the common flavanones isolated from citrus. These four TLC reactions show that the aglycone moiety of (I) is a flavone.

TABLE 1. TLC R_f VALUES OF ISOLATED AND SYNTHETIC APIGENIN 7β-RUTINOSIDES (I) AND RHOIFOLIN (II)

Solvent system	Adsorbents	R_f (×100) (I)	R_f (×100) (II)
MeOH–H_2O (1:1)	Polyamide	22	22
2-butanone–EtOAc–$HCONMe_2$–H_2O (10:6:3:2)	Silica gel G	54	54
2-butanone–EtOAc–HOAc–H_2O (100:60:27:20)	Silica gel G	19	19
PrOH–EtOAc–H_2O (3:2:1)	Silica gel G	56	56
MeOH–HOAc–H_2O (18:1:1)	Silica gel G	51	51
Nitromethane–MeOH (5:2)	Polyamide	20	16
Benzene–MeOH (3:1)	Polyamide	25	19
$CHCl_3$–MeOH–2-butanone (5:3:1)	Polyamide	52	46

When examined on TLC plates along with authentic materials, the hydrolysates of synthetic and isolated apigenin 7β-rutinoside (I) and rhoifolin (II) were shown to contain the sugars glucose and rhamnose as well as apigenin. The sugar moieties were further characterized by their colors with the anisaldehyde spray reagent.

Further proof of the structure of (I) was obtained by u.v. analyses. Authentic apigenin, rhoifolin and the two samples of the new flavone gave the same spectrum as rhoifolin in ethanol, (λ_{max} 269, 334 nm[13]). When aluminum chloride was added to alcoholic solutions of the above four compounds the spectra were the same (λ_{max} 278, 301, 347, 381 nm) indicat-

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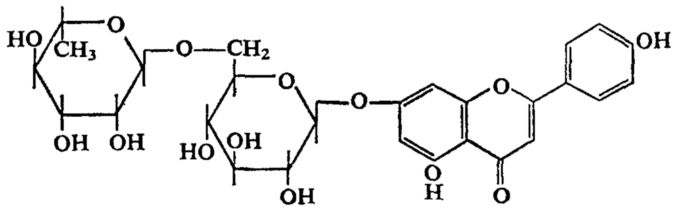
[11] T. R. SESHADRI and N. NARASIMHACHARI, *Proc. Ind. Acad. Sci.* **30**A, 151 (1949).

[12] R. M. HOROWITZ, *J. Org. Chem.* **23**, 1733 (1957).

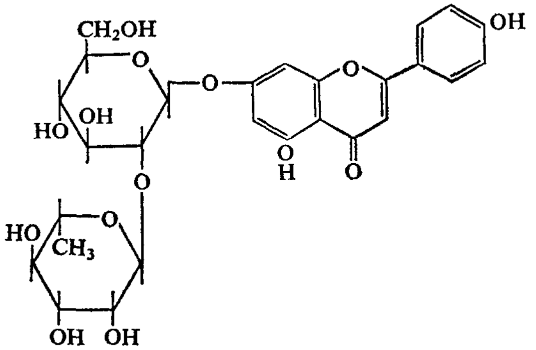
[13] L. JURD, in *The Chemistry of Flavonoid Compounds* (edited by T. A. GEISSMAN), p. 107, Pergamon Press, Oxford (1962).

ing the presence of a free 5-hydroxy group.[13] A bathochromic shift (λ_{max} 271, 304, 390 nm) was obtained in each case when sodium ethylate was added thus characterizing the flavones as having a free 4′-hydroxy group.[13] Addition of anhydrous sodium acetate to alcoholic solutions of the three glycosides failed to change the spectra. The spectrum of apigenin, however, gave the expected bathochromic shift, λ_{max} 278, 383 nm, of a flavone with a free 7-hydroxy group.[13] Thus, the new flavone must have its sugar moieties linked to apigenin at the 7-hydroxy position.

The nature of the linkage between the two sugar moieties of the synthetic and isolated flavone was determined by electrophoresis, nuclear magnetic resonance (NMR) spectroscopy and TLC on polyamide plates (see above). Both Gentili[14] and Mizelle[9] have reported that when mixtures of the neohesperidosides and rutinosides of the same aglycone are subjected to paper electrophoresis, the neohesperidoside have the greater mobility. When compounds I and II were subjected to electrophoresis the ratio between the migration distance of the rutinoside to that of the neohesperidoside was 1 to 2·1. This was nearly the same as the ratio between the two respective naringenin isomers (1 to 2·2). The NMR chemical shifts of the methyl group of the rhamnose moiety of the synthetic and isolated rutinosides (I) in pyridine were the same (94 cps, doublet $J = 5{\cdot}9$ cps). This chemical shift is characteristic of known rutinosides (naringin 7β-rutinoside 92, hesperidin 93, rutin 90 and diosmin 95 cps) and thus supports the evidence that the new flavone is a rutinoside since the neohesperidoside, rhoifolin, gave this doublet at 109 cps. This paramagnetic shift from that of rhamnose (96 cps, $J = 5{\cdot}9$ cps) is characteristic of neohesperidosides such as naringin 104, neohesperidin 105, poncirin 106 and neodiosmin 109 cps. Some of these chemical shifts have also been



Apigenin 7β-rutinoside (I)



Apigenin 7β-neohesperidoside (II)

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[14] B. Gentili and R. M. Horowitz, *Bull. National Inst. Sci. India* 31, 78 (1965).

observed on silylated flavonoids;[15] however, silylation of the hydroxyl groups is not necessary when the samples are run in pyridine and only the rhamnose methyl region is being observed.

When 3-month-old grapefruit leaves were petiole fed phenylalanine-^{14}C,[10] the authors earlier observed that flavones could not be detected by autoradiography. However, in the present work when more mature leaves were subjected to an atmosphere of $^{14}CO_2$, two flavone bands were detected by autoradiography, isolated by TLC and shown by co-chromatography to be the two rhamnosylglucosides of apigenin (I) and (II). There appears, therefore, to be a marked seasonal variation in the biosynthesis of the apigenin rhamnosylglucosides in the grapefruit leaf. It has been postulated[3,6] that in the biosynthesis of naringin rhoifolin is an oxidative or breakdown product of naringin. Further research is needed to elucidate the seasonal variations in the biosynthesis of apigenin 7-glycosides as well as the two naringenin glycosides.

EXPERIMENTAL

Isolation of Apigenin 7β-rutinoside (I)

Six-month-old Duncan grapefruit leaves (0·2 kg) were blended in methanol, heated to inactivate enzymes and extracted with water–methanol (1:3). After filtration, the extract was concentrated in vacuum at 40° and the chlorophylls were removed by extraction with ether. Pectins were precipitated upon diluting the extract with 11 volumes of acetone and removed by decanting. The supernatant after concentration was shown by TLC to contain two naringenin glycosides, rhoifolin and the unknown flavone. This extract (5 ml) was charged onto a Polyclar AT column (General Aniline and Film Corp., Grasselli, N.J.) (3·8 × 30 cm) with the aid of methanol (10 ml). Fractions (100 ml) eluted with distilled water, after evaporation, were assayed by TLC. The order of elution was: pectins, sugars, naringenin 7β-rutinoside, naringin, flavone (I) and rhoifolin. Fractions 65 to 75 containing mainly flavone (I) were further purified by rechromatography on a Polyclar AT column. The product (I) was tested for purity in five solvents. Since the yield was less than 1 mg no attempt was made to crystallize the product.

Syntheses of Apigenin 7β-rutinoside (I) and Apigenin 7β-neohesperidoside (II) (Rhoifolin)

Duncan grapefruit peel (3 kg) was extracted with hot methanol. The concentrated extract was triturated with acetone to remove pectins and extracted with chloroform to remove pigments. Three crops of naringin precipitated from the aqueous solution upon concentration and storage in a refrigerator, leaving the mother liquor enriched in the more soluble naringenin rutinoside. The mother liquor, containing substantially only the two naringenin isomers (TLC) in a ratio (NMR) of three parts neohesperidoside to one part rutinoside, was reduced to dryness (1·7 g) and oxidized to the flavones with iodine and sodium acetate in ethanol.[11] Upon removal of solvent the reaction products were dissolved in water (50 ml) and the products extracted with butanol (3 × 50 ml). The four flavonoids (TLC) in the butanol extract were individually characterized by analytical and preparatory TLC and u.v. analyses as being the two apigenin glycosides (50 per cent yield) and the unreacted naringenin glycosides. The butanol extract was treated with $NaBH_4$ (0·5 g), which reduced the major portion of the unreacted flavanones to water-soluble flavanes.[5] These flavanes upon acidification with conc. HCl (10 ml) were removed by extraction with water (3 × 50 ml), leaving the butanol solution containing the two apigenin isomers. Final separation and purification of the rutinoside isomer (I) from rhoifolin and other residues was obtained on a Polyclar AT column, the rutinoside preceding the neohesperidoside. No attempts were made to crystallize the final product as the yield was below 1 mg.

Rhoifolin (apigenin 7β-neohesperidoside) (II) was synthesized from purified naringin by the method of Seshadri.[11] The product was crystallized twice from methanol and was shown to be pure by co-chromatography with a rhoifolin sample isolated from grapefruit peel.

Thin-layer Chromatography

Sugars and flavonoids were detected on silica gel plates with an anisaldehyde, sulfuric acid in ethanol (1:1:18 by vol.) spray reagent while flavonoids were detected on polyamide plates by fluorescence-absorbance under u.v. light before and after spraying with aluminium chloride in ethanol (2 per cent w/v).

Hydrolyses

The apigenin glycosides (I and II) were each refluxed 4 hr with methanol–water–conc. HCl (2:1:1), reduced to near dryness under reduced pressure and treated with 20 mg of Dowex 1-XI resin, previously made basic

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[15] H. ROSLER, T. J. MABRY, M. F. CRANMER and J. KAGAN, *J. Org. Chem.* **30**, 4346 (1965).

with 0·1 N NaOH and washed free of excess base to pH 8 with water. The hydrolysate thus treated (neutral to pH paper) was dissolved in four drops of methanol–water (1:1) and used for TLC.

Spectral Analyses and Electrophoreses

NMR spectra (Varian A-60 with a TMS internal standard) were run in pyridine in the methyl chemical shift region (0–150 cps). Samples were prepared in microcells and the signals intensified with the assistance of a Varian C-1024 time-averaging computer. U.v. spectra were obtained on a Cary 14 Spectrophotometer in ethanol alone and with added sodium ethoxide, fused sodium acetate and aluminum chloride. Paper electrophoresis was carried out on Whatman No. 1 filter paper with 0·1 M borate buffer (pH 10) and 26·8 V/cm for 3 hr.

Biosynthesis of ^{14}C-*Apigenin 7β-glycosides*

A portion of a branch still attached to a potted grapefruit tree, approximately 2 years old, was sealed into a sphere containing 1·0 mc of $^{14}CO_2$. The atmosphere in the sphere was circulated with the aid of a finger pump. The leaves and sphere were exposed to light and dark periods of 12 hr duration over 24 hr. The leaves inside the sphere which were approximately 6 months old dropped off after 24 hr. These leaves showed a great amount of activity and thus were extracted for flavonoids. For isolation of the labeled flavonoids, five silica G plates were streaked with the extract and developed in butanol–acetic acid–water (4:5:1). The bands corresponding to co-spotted rhoifolin were eluted with methanol, restreaked on silica gel plates and developed in 2-butanone–EtOAc–$HCONMe_2$–H_2O (10:6:3:2). Final separation of the apigeninrhamnoglucoside mixture into the two isomers was obtained in nitromethane–MeOH (5:2) on polyamide with three developments. The plates were subjected to autoradiography[10] and the hot bands eluted with ethanol for u.v. and chromatographic analysis.

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