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GLUCOSYLATION OF EXOGENOUS FLAVANONES BY GRAPEFRUIT (*CITRUS PARADISI*) CELL CULTURES

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Key Word Index—*Citrus paradisi*; Rutaceae; grapefruit; biotransformation; glucosylation; plant cell cultures; flavanones; flavanone glycosides; prunin; hesperitin 7-*O*-glucoside; ^{1}H NMR.

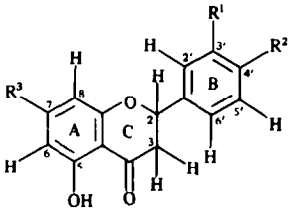
Abstract—Grapefruit (*Citrus paradisi*) cells in suspension cultures did not accumulate flavanone glycosides but were able to specifically *O*-glucosylate exogenous naringenin and hesperitin at position 7. The products were extracted from the cultures using a new technique: absorption on an Amberlite XAD-2 resin and further purification by BioGel-P4 column chromatography. The flavanone glycosides obtained were analyzed by high-resolution ^{1}H NMR and the spectra compared to authentic flavanone aglycones, mono- and diglycosides.

INTRODUCTION

The genus *Citrus* produces coumarins, flavanones, flavones and flavonols which occur in a free and/or as glycosides [1, 2]. Flavanone glycosides have been found in almost every *Citrus* tissue analyzed, including flowers, leaves and fruits [3]. The period of flavanone glycosides accumulation in fruits is correlated with periods of intense cell division [4]. The predominant flavonoid in grapefruits is naringin (naringenin 7-neohesperidoside) (**3**). Narirutin (naringenin 7-rutinoside); neohesperidin (hesperitin 7-neohesperidoside) (**6**); hesperidin (hesperitin 7-rutinoside) (**7**); isosakuranetin 7-neohesperidoside and 7-rutinoside have also been found, but at much lower levels [5].

Little is known about the biosynthetic pathways of the flavanone glycosides in *Citrus*. [^{14}C]Phenylalanine was biotransformed to naringin (**3**) by young grapefruit leaves [6]. Some of the enzymes of the flavonoid pathway have been detected in *Citrus*. They include phenylalanine-ammonia lyase, cinnamate 4-hydroxylase and chalcone-flavanone isomerase (see ref. [7] for review). Chalcone synthase and the enzymes which catalyze the glucosylation and rhamnosylation steps have not been observed. The substrate specificity of a chalcone-flavanone isomerase isolated from grapefruit indicated that both glucosylation and rhamnosylation occur before the naringenin chalcone is closed to the flavanone [8]. Even less is known about the biosynthetic pathway to neohesperidin (**6**). Naringin (**3**) and neohesperidin (**6**) biosyntheses are of interest as they can be chemically converted into artificial sweeteners [9].

Cell suspension cultures are ideal systems to study secondary metabolite biosyntheses, but such undifferentiated cells often do not produce the whole range of desired metabolites [10]. Methoxylated flavones were detected in differentiated calli from orange and lemon flavedo [11]. 'Flavonoid like substances' were detected but not unequivocally identified in proliferating lemon fruit tissue [12]. Flavonoids were not detected in undifferentiated grapefruit calli nor in cell suspensions even though phenylalanine ammonialyase was detected [13].



		R^1	R^2	R^3
1	naringenin	H	OH	OH
2	prunin	H	OH	OGlc
3	naringin	H	OH	OGlc (1→2) Rha
4	hesperitin	OH	OMe	OH
5	hesperitin 7-glucoside	OH	OMe	OGlc
6	neohesperidin	OH	OMe	OGlc (1→2) Rha
7	hesperidin	OH	OMe	OGlc (1→6) Rha

This paper describes the study of grapefruit cell suspensions for their ability to produce and biotransform flavanones. We have investigated the conversion of exogenous naringenin (**1**) to prunin (**2**) and further to naringin (**3**) and narirutin by the action of yet unidentified glucosyl- and rhamnosyl-transferases. We have examined the possibility of converting exogenous hesperitin (**4**) to hesperitin 7-*O*-glucoside (**5**) and the latter to neohesperidin (**6**) and hesperidin (**7**) by the same cell cultures. To perform these studies we had to develop methodologies to efficiently extract and purify the metabolites obtained. We describe here the techniques we have adapted and the results of some biotransformations.

RESULTS AND DISCUSSION

Extraction of flavanone glycosides from *Citrus* tissues and juices [14] is tedious and time-consuming due to their low solubility in water-immiscible solvents. XAD resins

have been used for debittering grapefruit juices [15] as they readily absorbed naringin (3). We found that flavanone glycosides could be quantitatively recovered from XAD columns by elution with MeOH (data not shown). XAD resins did not separate the various flavanone glycosides, thus further purification steps were required. Column chromatography on Sephadex G-25 [16, 17] and LH-20 [18] have been used to purify flavonoids with limited success.

We used BioGel-P4 columns in these studies with very satisfactory results. A typical separation of some authentic flavanones on such a column is presented in Fig. 1. We have established from experiments using different standards the following elution sequence: the aglycones (1) and (4) were eluted first, followed by rhamnoglucosides (3), (6) and (7) and then the monoglucosides (2) and (5). Aglycones, mono- and diglycosides were well resolved from each other but compounds belonging to the same group did not separate that well. ^{1}H NMR was then used to establish the chemical structure of the products. The long retention times of the glycosides enabled very efficient separations from each other and from the many polar contaminants present in the extracts. Hydrophobic interactions between the column support and the eluants are probably responsible for the reverse order of elution for the diglycosides and the monoglycosides.

Identification of products

^{1}H NMR data for many flavonoids are available [19], but are mainly for the TMSi-ether derivatives and not for intact flavonoids. Most ^{1}H NMR data have been obtained at rather low sensitivity and at low spectral frequencies of less than 100 MHz. Previous spectral resolutions have not allowed detailed chemical shift assignments for most of these flavonoids. With the availability of higher magnetic-field NMR spectrometers, it is possible to obtain detailed spectra of intact flavonoids at even lower concentrations (nmol range). We have analyzed naringenin (1) and hesperitin (4) as well as their respective mono- (2), (5) and diglycosides (3), (6), (7) to establish spectral reference standards for the identification of the biotransformed products. The ^{1}H NMR spectra were obtained using a CD_3OD–CD_3CN (1:1) mixture which could dissolve most flavanones, with minimal signal overlap. A ^{1}H NMR spectrum of naringin (3) obtained under these conditions is shown in Fig. 2. The spectral data obtained for authentic and biotransformed flavonoids are shown in Tables 1 and 2.

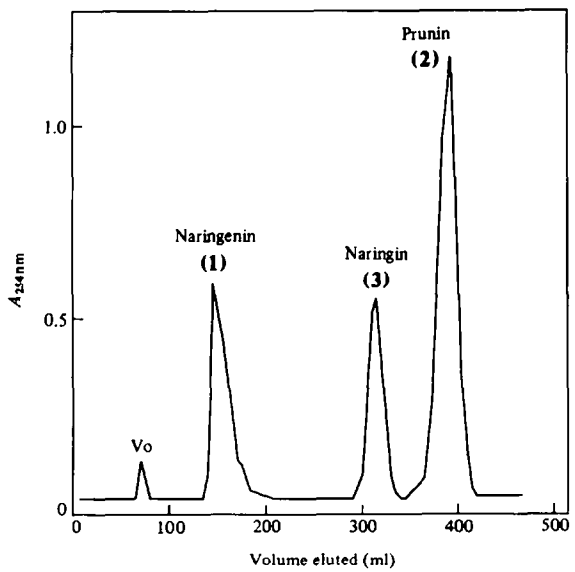


Fig. 1. A typical separation of authentic flavonoids using a 2.5 × 100 cm BioGel-P4 column. Flow rate was 20 ml/hr. A 5 mM solution of $(NH_4)HCO_3$ pH = 7.8 was used as eluant. Note the high resolution of the separation.

Several features characterize the spectra of the different flavanones. Those containing a *p*-hydroxylated ring B naringenin (1) and its glycosides (2) and (3), show two doublets at $\delta \sim 7.30$ and $\delta \sim 6.8$ with a coupling constant of ~ 8.5 Hz corresponding to protons 2′ and 6′ and to protons 3′ and 5′. Compounds with a 3′-hydroxy-4′-methoxy substitution [hesperitin (4) and its glycosides (5), (6) and (7)] show a multiplet at $\delta \sim 6.9$ corresponding to protons at positions 2′, 5′ and 6′. The doublet of doublets at $\delta \sim 3.1$ represent the axial proton 3, and the doublet of doublets at $\delta \sim 2.7$ represent the equatorial proton 3. The chemical shift of proton 3 (axial) is very sensitive to the configuration of carbon 2 [20]. Glucosylation at position 7 can also be determined by ^{1}H NMR. In naringenin (1) and hesperitin (4) the chemical shift of protons 6 and 8 is $\delta \sim 5.9$, while in the mono- and diglycosides (2), (3), (5), (6) and (7), it is $\delta \sim 6.1$. The chemical shift of the anomeric proton of the glucose is affected by an addition of a rhamnose in the following ways: in the glucosides (2) and (5) (not substituted by a rhamnose) it is $\delta \sim 4.95$, in the neohesperidosides (3) and (6) it is shifted to $\delta 5.07$ while in the rutinoside (7) it is $\delta 4.93$. Similarly, the chemical shift of the anomeric proton of the rhamnose depends on the substitution position at the glucose and it is different in (3) and (6) ($\delta 5.16$) than in (7) ($\delta 4.63$). The position of rhamnose attachment to the glucose affects the chemical shift observed for the C6 methyl protons of the rhamnose. In the neohesperidoside (6) it is $\delta 1.22$ while in the rutinoside (7) it is $\delta 1.12$.

Thus, we have established that the chemical shifts observed in the ^{1}H NMR of underivatized flavonoids are indicative of the structures and substitution patterns of the A, B and C rings. In addition, glucosylation and rhamnosylation can be deduced directly from the chemical shifts of the sugars as explained above.

Biotransformation of precursors

Preliminary small-scale experiments indicated that grapefruit cells neither accumulate nor biotransform 0.1 mM phenylalanine into detectable levels of flavanones when extracts were tested for $AlCl_3$-induced fluorescent spots on TLC.

Exogenous naringenin (1) was biotransformed by the cells. Three major products were detected on TLC which showed $AlCl_3$ positive reaction, indicative of flavonoids with an unsubstituted OH at position 5 [21]. One of the products which had an R_f on TLC identical to that of prunin (2) was purified on XAD-2 and BioGel P4 columns (see Experimental). Its structure was confirmed by its ^{1}H NMR spectrum which was identical to that of authentic prunin (2) (Table 1). The other spots were more polar. Cells which were incubated in basal medium without

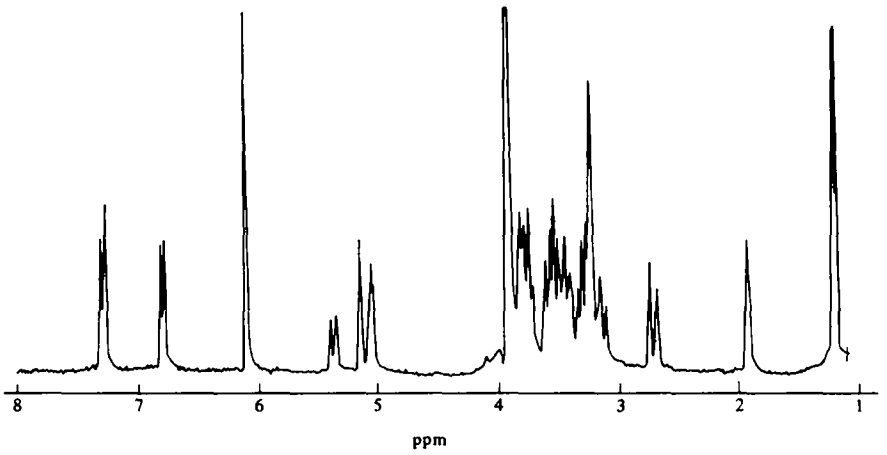


Fig. 2. ^{1}H NMR spectrum of authentic naringin (3) in CD_3CN–CD_3OD (1:1); for details see Experimental.

Table 1. ^{1}H NMR chemical shifts ($\delta \pm 0.02$) of naringenin derivatives*

Proton	Authentic samples Naringenin (1)	Prunin (2)	Naringin (3)	Biotransformed naringenin
2',6'	7.28 (8.4)	7.30 (8.5)	7.30 (8.5)	7.30
3',5'	6.79 (8.4)	6.80 (8.5)	6.80 (8.5)	6.81
6,8	5.87	6.15	6.11	6.15
2	5.32 (13.0, 3.3)	5.38 (12.9, 2.8)	5.38 (13.6, 2.8)	5.38
3(ax.)	3.09 (17.2, 13.0)	3.17 (17.4,12.9)	3.14 (17.2, 13.6)	3.17
3(eq.)	2.66 (17.2, 3.3)	2.68 (17.4, 2.8)	2.73 (17.2, 2.8)	2.72
Glc-H1		4.95 (m)	5.07 (6.9)	4.97
Rha-H1			5.16	
Rha-CH_3			1.21 (6.2)	

*Solvent used: CD_3CN–MeOD (1:1). The acetonitrile signal at 1.93 ppm from TMS was used as an internal standard. Coupling constants in Hz are given in parentheses. m—Unresolved multiplet structure.

The spectral differences between authentic prunin (2) and the biotransformed naringenin were not significant.

Table 2. ^{1}H NMR chemical shifts ($\delta \pm 0.02$) of hesperitin derivatives*

Proton	Authentic samples Hesperitin (4)	Hesperitin-7-*O*-glucoside† (5)	Neohesperidin† (6)	Hesperidin† (7)	Biotransformed hesperitin†
2',5',6'	6.89 (m)	6.91 (m)	6.91 (m)	6.93 (m)	6.92
6,8	5.88 (~1.5)	6.17, 6.15 (~2.2)	6.13, 6.11 (2.2)	6.15	6.12
2	5.31 (12.5, 3.0)	5.36 (12.5, 3.0)	5.37 (12.5, 3.0)	5.39 (12.5, 3.1)	5.39
3(ax.)	3.06 (17.1, 12.5)	3.15 (17.2, 12.5)	3.14 (17.2, 12.5)	3.14 (17.3, 12.5)	3.13
3(eq.)	2.69 (17.1, 3.0)	2.75 (17.2, 3.0)	2.75 (17.2, 3.0)	2.76 (17.3, 3.1)	2.75
OCH_3	3.83	3.83	3.82	3.83	3.84
Glc-H1		4.96 (7.0)	5.07 (7.1)	4.93 (m)	4.94
Rha-H1			5.16	4.63	
Rha-CH_3			1.22 (6.1)	1.12 (6.3)	

*Conditions were identical to those described for Table 1.

†Mixture of 2R and 2S isomers. Chemical shifts given for 2S [20].

The spectral differences between authentic hesperitin-7-*O*-glucoside (5) and the biotransformed hesperitin were not significant.

naringenin (1) did not accumulate any of the above products at detectable levels. Exogenous naringenin (1) supplied at 0.1 mM had no apparent effect on the growth rate of the cells.

One of the products from the biotransformation of exogenous hesperitin (4) was separated by XAD-2 and P4-BioGel column chromatography. It had an R_f on TLC identical to that of hesperitin 7-*O*-glucoside (5) and formed a fluorescent complex with $AlCl_3$. Its structure was confirmed by the ^1H NMR spectrum to be identical with that of an authentic sample (Table 2). Several other unidentified $AlCl_3$ positive spots were present on the TLC plates.

We have shown that *Citrus* cell cultures can glucosylate flavanone aglycones (Tables 1 and 2). Glucosylation in grapefruit cells is specific to the 7 position of the flavanone. Exogenous naringenin (1) was glucosylated to prunin (2) and exogenous hesperitin (4) was glucosylated to hesperitin 7-*O*-glucoside (5). Yields were *ca* 10–20% in the cell line shown.

The products detected have not been previously reported in *Citrus* tissues. Prunin (2) has been detected in *Prunus* calli (Rosaceae) [22] and in other species [23] but not in *Citrus*. We could not find reports of hesperitin 7-*O*-glucoside (5) in any plant. Grapefruit albedo typically accumulates naringin (3), so that prunin (2) and hesperitin 7-*O*-glucoside (5) may serve as precursors for the biosynthesis of the rhamnoglucosides in intact tissue. Some steps in rhamnose transfer and/or biosynthesis are probably inactive in the cell cultures used.

EXPERIMENTAL

Materials. Naringenin (1), hesperitin (4), hesperidin (7), naringinase and hesperidinase were purchased from Sigma. Neohesperidin (6) and naringin (3) were kindly supplied by Jafora Co., Rehovot, Israel. Hesperidin (7) was recrystallized from DMF–H_2O [24]. Prunin (2) and hesperitin 7-*O*-glucoside (5) were prepared enzymatically as described below.

Enzymatic preparation of prunin (2) *and hesperitin* 7-*O-glucoside* (5). Prunin (2) was prepared from naringin (3) by the following procedure modified from [25]. 1 U/ml naringinase and 1.1 mg/ml naringin (3) were dissolved in 0.1 M citrate buffer (pH = 4.0) and incubated for 1 hr at 37°. The reaction was terminated by boiling the soln for 5 min, then it was extracted with EtOAc and purified on a BioGel-P4 (Bio-Rad) column [2.5 × 90 cm, 5 mM $(NH_4)HCO_3$ (pH = 7.8) 20 ml/hr].

Hesperitin 7-*O*-glucoside (5) was prepared by dissolving hesperidin (7) (0.8 mg/ml final vol.) in a few drops of dilute NaOH, neutralized and added to a 0.2 U/ml hesperidinase soln in 10 mM aspartate buffer (pH = 3.8). The soln was incubated for 30 min at 42°, freeze-dried and purified on BioGel-P4 as described for prunin (2).

Plant material. Callus cultures of *Citrus paradisi* Macf. cv 'Duncan' (*ca* 3-years old) were grown on a modified Murashige and Tucker medium [26] supplemented with 4% sucrose and 1% agar [27], except that the Fe^{2+} level was lowered to 33 μM to prevent its precipitation in the medium [28]. Cultures were irradiated with 10 $\mu E/m^2s$ (PAR) continuous cool-white light at 25°.

Suspension cultures were initiated by transferring pieces of callus to the above medium without agar (basal medium). Cells were grown at 30° in a gyratory shaker (125 rpm) and illuminated with 70–80 $\mu E/m^2s$ (PAR) cool-white light. The cultures were transferred at weekly intervals, with a packed-volume dilution of *ca* 2.5-fold. One week old, mid-exponential cell suspensions were used.

Biotransformations. Grapefruit cell suspensions (*ca* 40–60 ml packed volume) were transferred into ten 250 ml flasks each containing 100 ml of 0.3 mM naringenin (1) or hesperitin (4) in basal medium, and cultured as above. After a 48 hr incubation, the cultures were assayed for flavanones. The cells and the supernatant medium were boiled for 5 min and then centrifuged (5200 *g*, 4 hr). The supernatant was passed through an Amberlite XAD-2 (Rohm & Haas) column (5 × 10 cm) and washed with *ca* 1 l. of H_2O. Flavonoids were eluted with MeOH. The column was regenerated with 250 ml of 1 M NaOH, followed by washing with at least 3 l. of H_2O [15]. The MeOH extract was dried, dissolved in *ca* 5 ml of 5 mM $(NH_4)HCO_3$ pH = 7.8 eluting buffer and applied to a BioGel-P4 column (conditions as for prunin). The fractions absorbing at 254 nm were freeze-dried and analysed by TLC, repurified on the same BioGel-P4 column and analysed by ^1H NMR.

Thin layer chromatography. The following systems were used: silica gel (Riedel de Haen, without fluorescence indicator) Me_2CO–$CHCl_3$–H_2O (8:2:0.48) [29], MeOH–*n*-BuOH–EtOAc–CH_2Cl_2 (1:1:1:1), *iso*-PrOH–CH_2Cl_2 (9:1). Micro-polyamide (Schleicher & Schuell), CH_3NO_2–MeOH–H_2O (5:2:0.25) [17]. Spots were observed in 366 nm light after developing the plates in ammonia vapour and spraying them with a 1% $AlCl_3$ soln in MeOH [6].

^1H NMR. The samples were dissolved in 99.8% D_2O and freeze-dried three times. After high-vacuum drying the samples were dissolved in dry CD_3OD–CD_3CN (1:1) and placed in a 5 mm o.d. NMR tube. The spectra were run on a Bruker instrument operating at 270 MHz at ambient temperature. A spectral window of 2700 Hz, 8192 data points, recycling time of *ca* 1.5 sec and a pulse width of 80° were used. We took from 300 to 900 scans for the standard compounds and from 3000 to 19 000 scans for the biotransformed products.

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