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C-2 Stereochemistry of Naringin and Its Relation to Taste and Biosynthesis in Maturing Grapefruit

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Circular dichroism and proton magnetic resonance spectroscopy can be used to determine the C-2 chirality of naringin, the main bitter principle of grapefruit. Thus, the aglycone chirality of naringin has been studied as a function of grapefruit maturity. The amount of (2S) isomer is 85–92% in immature grapefruit but only 55–60% in mature grapefruit. These results are discussed in relation to the current postulates relating to flavanone biosynthesis. A naringin sample predominant in the (2R) isomer has been prepared and found by some tasters to be more bitter than the (2S) naringin. It is concluded that the naringin isomerization in ripening grapefruit is not responsible for debittering.

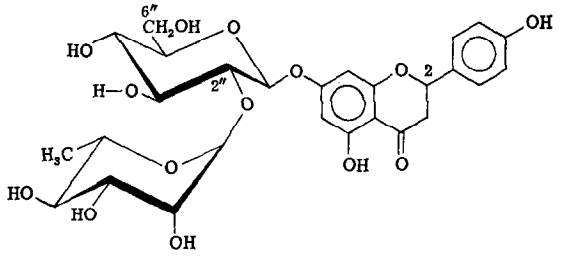
Naringin (**1**) is the chief bitter constituent of grapefruit (*Citrus paridisi*). It is a glycoside composed of the flavanone naringenin and the disaccharide β-neohesperidose (2-*O*-α-L-rhamnosyl-β-D-glucose) (*1*). The C-2 position of the naringenin moiety is a chiral center that can occur in either the (S) or (**R**) configuration. (2S) and (2**R**) naringin [(**2**) and (**3**), respectively] are diastereomeric by virtue of the carbohydrate present.[1] In the course of experiments on the circular dichroism (CD) of optically active flavanones we made the unexpected observation that naringin from different sources contains varying proportions of the (2S) and (2**R**) isomers. Further study has now revealed that the C-2 stereochemistry of naringin undergoes a marked change with increasing maturity of the grapefruit. This change can be accurately followed by either CD or proton magnetic resonance (pmr) measurements. We discuss some possible biosynthetic implications of these findings and report preliminary results on the relation of C-2 stereochemistry to bitterness in naringin.

EXPERIMENTAL AND RESULTS

Extraction of Naringin

All grapefruit were provided by the U.S. Date and Citrus Station, Indio, CA and were taken from the same trees (Marsh or Duncan variety) during the entire season.

[1] Abbreviations used are: Neo, β-neohesperidosyl(2-*O*-α-L-rhamnosyl-β-D-glucosyl); de, diastereomeric excess = D_1–D_2 where D represents mole fractions of diastereomers; see J. D. Morrison and H. S. Mosher, "Asymmetric Organic Reactions," p. 10, Prentice-Hall, Englewood Cliffs, NJ, 1971.



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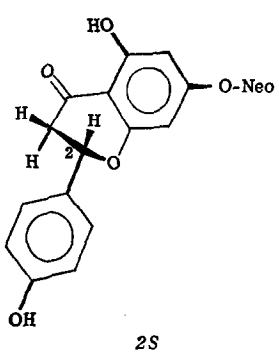

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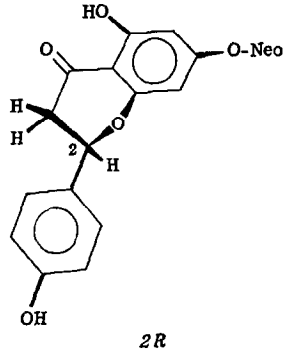

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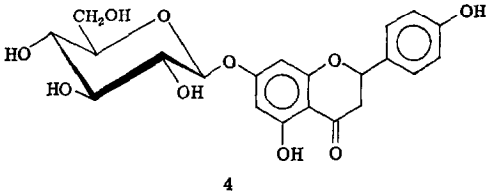

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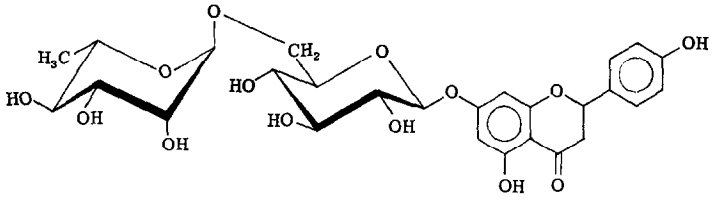

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A total of 40 samples was studied between 1969 and 1971. In a typical experiment, the flavedo of three Duncan grapefruit (diameter 8.26–9.53 cm) collected 9/22/70 was removed and the remaining albedo (197 g) blended for 10 sec in a Waring blendor with 300 ml of methanol–water (1:1) at room temperature. The blended mixture was

filtered immediately under vacuum through a Buchner funnel containing a layer of Celite to give 255 ml of filtrate which was treated as follows.

1) A 5-ml aliquot was removed and analyzed directly by CD within 24 hr. The concentration of naringin in the aliquot was measured spectrophotometrically using the experimentally determined extinction coefficient of 1.76×10^4 at 283 nm (methanol). The data thus obtained are designated as pertaining to the *unfractionated* naringin samples.

2) The remaining filtrate was kept in a refrigerator for 4 weeks or until crystallization was complete. The crystals of naringin were collected and dried under vacuum at room temperature (yield, 1.51 g). Samples prepared in this way are designated as *fractionated* naringin.

Tlc analysis of the fractionated and unfractionated naringins was done with Eastman type K-541-V polyamide plates (CH_3NO_2–methanol, 3:2) using diazotized benzidine as spray reagent to visualize the spots. A trace of poncirin (4′-*O*-methylnaringin) was the only detectable impurity.

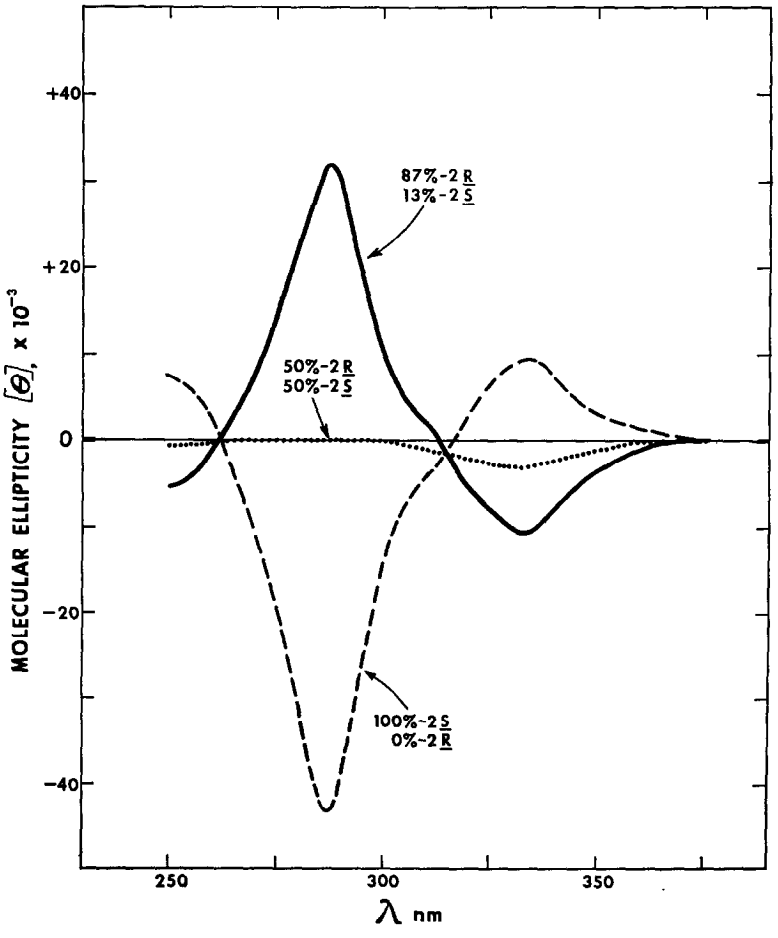


FIG. 1. CD curves, in methanol, of naringin samples diastereomeric at C-2.

Determination of Isomeric Composition by CD

Previous CD studies had shown that (2S) flavanones having equatorial 2-aryl substituents exhibit a positive Cotton effect due to the n → π* transition (~330 nm) and a negative Cotton effect in the π → π* region (~280–290 nm) (*2*). The latter band is probably due to the formally forbidden ^1L_b transition of the aromatic A-ring in conjunction with the π → π* transition of the α,β-unsaturated carbonyl group of the acetophenone chromophore. The magnitude of the Cotton effect in the π → π* region has been shown (*2*) to be related to the (2S) content in naringin and has been utilized to determine aglycone chirality of flavanone glycosides (*3*, *4*). By separating the optical activity of the aglycone from that of the carbohydrate, CD permitted direct determination of C-2 stereochemistry, since the 287-nm Cotton effect of naringin was found to be directly proportional to isomeric composition.

TABLE 1

DETERMINATION OF C-2 CHIRALITY OF NARINGIN

No.	Compound	Chiroptical properties[a]		Isomeric percentages determined by	
		$[\theta]_{287}^{27}$	$[\alpha]_D^{27}$	CD[b] 2S/2R	Pmr[c] 2S/2R
1	Naringin	−43 100	—	100/0	97/3
1a	Naringenin	−42 100	−22.5	—	—
2	Naringin	−5810	—	56/44	52/48
3	Naringin	0	—	50/50	—
3a	Naringenin	0	0	—	—
4	Naringin	+3750	—	46/54	42/58
5	Naringin	+11 500	—	37/63	—
5a	Naringenin	+11 200	+5.9[d]	—	—
6	Naringin	+22 700	—	24/76	21/79

[a] Measurements performed in methanol unless otherwise noted. [θ] is expressed in units of degree cm²/dmol.

[b] Determined using $[\theta]_{287}$ −43 100 as the value for 100% (2S) naringin.

[c] Determined from Fig. 2 by measurement of peak height of the major line of the axial H-3 quartet near 3.0–3.1 ppm.

[d] Measured in acetone.

Values for molecular ellipticity at 287 nm ranged from −43 100 for 100% (2S) naringin to zero for naringin having C-2 racemic, as shown in Fig. 1. As a check on these results, selected naringin samples were hydrolyzed with crude fungal hemicellulase (0.1 *M* acetate buffer, pH 4.6) to naringenin (5,7,4′-trihydroxyflavanone) and the optical rotation of the naringenin determined at the D line. Optical purities determined by CD for naringin agreed well with values calculated from the $[\alpha]_D$ of naringenin (Table 1). For example, the naringenin (entry No. 3a) obtained from 50% (2S), 50% (2R) naringin (entry No. 3) was optically inactive. Assuming the naringenin (entry No. 1a) from naringin (entry No. 1) to be 100% (2S), the naringenin (entry No. 5a)

from naringin (entry No. 5) gave identical isomeric compositions from both CD and $[\alpha]_D$ measurements.

Determination of Isomeric Composition by pmr

All spectral data were obtained at 60°C in deuteriopyridine on an internally locked 100-MHz nuclear magnetic resonance spectrometer. Tetramethyl-silane (TMS) concentration was kept below 1%. The data for H-2, 3_{ax} and 3_{eq} (Table 2) were analyzed with the aid of the iterative, least-squares program LAOCN-3. The parameters listed in Table 2 produced a pmr spectrum having a maximum deviation of 0.1 Hz. Although only the absolute value of geminal coupling can be determined by this procedure, a negative sign has been assigned to $J_{3_{ax}3_{eq}}$ by analogy with similar systems where the absolute sign has been unambiguously determined.

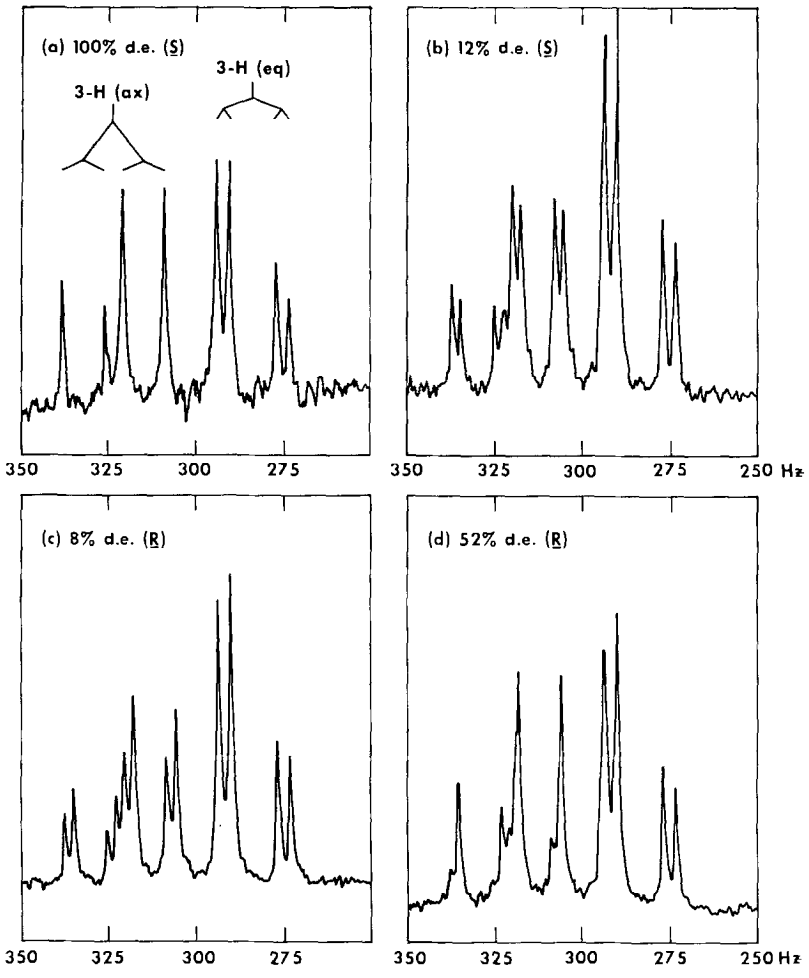


FIG. 2. Partial pmr spectra of diastereomeric naringins in deuteriopyridine at 60°C. The diastereomeric excess (de) listed in each diagram was obtained from CD measurements as described in Table 1, footnote *b*. Thus, the naringin in diagram (b) is 56% (2S):44% (2R) from CD results.

The spectra of diastereomeric samples of naringin contained chemically shifted axial C-3 proton signals from which isomeric compositions could be estimated (Table 1). As shown in Fig. 2, the equatorial C-3 proton signal had the same chemical shift for both (2**R**) and (2**S**) naringin while the axial C-3 proton signal for the (**R**) isomer was 2.9 Hz upfield from the corresponding resonance for the (**S**) isomer (Table 2). The diastereotopic C-3 axial proton signals were positioned too close to permit integration, but the measurement of peak heights allowed estimation of the isomeric composition. The major line of each quartet near 3.0–3.1 ppm was used for peak height estimation, and values obtained from the four examples shown in Fig. 2 are listed in Table 1 (entries No. 1, 2, 4 and 6). The estimate of isomeric composition for a given sample of naringin is 3–4% higher in (**R**) isomer by the pmr method than by CD analysis.

TABLE 2

CHEMICAL SHIFT (δ ppm) AND COUPLING CONSTANTS (Hz) OF H-3 NARINGIN IN DEUTERIOPYRIDINE AT 60°C AS DETERMINED BY COMPUTER SIMULATION

Compound	H-3_{eq}	H-3_{ax}	$J_{3_{ax}3_{eq}}$	$J_{3_{ax}2_{ax}}$	$J_{3_{eq}2_{ax}}$
(2**R**) Naringin	2.861[a]	3.185[b]	−17.2	12.8	3.0
(2**S**) Naringin	2.859[a]	3.214[c]	−17.2	12.3	3.1

[a] Experimentally determined lines of the quartet at 2.942, 2.906, 2.771 and 2.736 ppm.
[b] Experimentally determined lines of the quartet at 3.352, 3.231, 3.182 and 3.059 ppm.
[c] Experimentally determined lines of the quartet at 3.378, 3.259, 3.205 and 3.087 ppm.

It is noteworthy that CD and pmr agree on which diastereomer is present in greater amount in entries No. 2 and 4 (Table 1) since the major isomer is only slightly predominant in these samples. Due to the resolution and sensitivity of the measurements, CD is more suitable for determining isomeric composition when one diastereomer is highly predominant, while pmr is preferable for determining ratios of naringin samples nearly racemic at C-2. For convenience of measurement, the 287-nm CD band was used to monitor the C-2 chirality of naringin present in the unfractionated methanolic extracts to an estimated accuracy of ±3%.

Unfractionated Naringin Samples from Peel Extract

The percent chirality at C-2 of naringin in the 50% aqueous methanol extracts is shown in Fig. 3. The naringin is practically all (2**S**) at the beginning and during the early part of the season. As the fruit matures more (2**R**) isomer appears until the isomeric ratio at ripeness is about 60% (2**S**):40% (2**R**). The data in Fig. 3 represent samples from both Marsh and Duncan grapefruit and fruit from more than one season. While the naringin tends toward racemization at C-2 as the season progresses, the (2**S**) isomer is present in greater amount at all times.

Fractionated Naringin Samples from Peel Extract

Naringin samples isolated by crystallization from 50% aqueous methanol extracts show somewhat different isomeric compositions, as seen in Fig. 4. As before, the (2**S**)

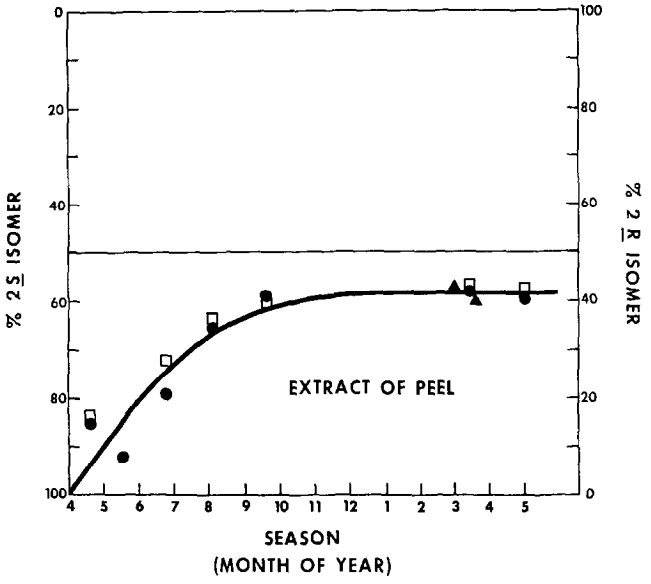


FIG. 3. C-2 chirality of naringin as a function of grapefruit maturity. These unfractionated samples are from the directly extracted (50% aqueous methanol) inner peel. ●, Marsh grapefruit, 1970–71 season; □ Duncan grapefruit, 1970–71 season; ▲ Marsh grapefruit, 1969–70 season.

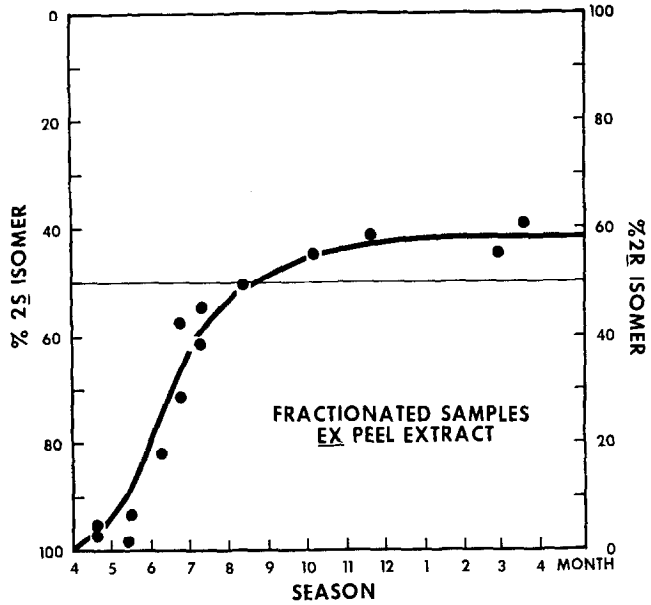


FIG. 4. C-2 chirality of naringin as a function of grapefruit maturity. These samples have been isolated by fractional crystallization of 50% aqueous methanol extracts of inner peel. The samples in this figure are from both Marsh and Duncan grapefruit, 1970–71. CD measurements used to derive data for Fig. 3 and this figure were performed in methanol or 50% aqueous methanol.

isomer is essentially the only isomer detected in the immature fruit and, upon ripening, the amount of (2R) naringin steadily increases. However, unlike the unfractionated peel extracts, the fractionated, isolated naringin samples become slightly predominant in (2R) isomer as maturity proceeds (Fig. 4). The final ratio is approximately 40% (2S):60% (2R). This apparent discrepancy between fractionated and unfractionated naringin extracts is not surprising since diastereomeric flavanone glycosides are known to become preferentially enriched in one isomeric form upon fractional recrystallization (*5*). When second and third fractions of crystallized naringin from late-season peel extracts were taken into account, the overall composition was found to be about 60% (2S):40% (2R), in agreement with the results presented in Fig. 3 for the unfractionated peel extract. Thus, caution must be exercised in arriving at chemical or biosynthetic conclusions from chiral natural products which may have been subjected to fractionation procedures.

(2S) and (2R) Naringin

One hundred percent (2S) naringin was obtained directly by crystallization from the 50% aqueous methanol extract of small (<2 cm diameter) immature grapefruit. To obtain relatively pure (2R) naringin, commercial naringin, containing about 50% each of the (R) and (S) isomers, was recrystallized once from hot acetone. The first crop of crystals usually showed little fractionation but subsequent small crops were somewhat enriched in the (R) isomer. These combined small crops were then fractionally crystallized from ethanol. Eventually samples containing 87 and 83% (2R) naringin were obtained. The isomers cannot be distinguished by melting point. Dried under vacuum at 80°C (2S) naringin melted at 164–166°C, (2R) naringin at 165–167°C and (2R):(2S) naringin at 167–169°C (Fisher–Johns block).

Taste Studies on Diastereomeric Naringins

A group of ten judges made a total of 15 comparisons between the 100% (2S) and 87 or 83% (2R) naringin at concentrations of 10^{-5}–10^{-3} *M*. In seven comparisons it was judged that the (R) isomer was more bitter; in four comparisons that the (S) isomer was more bitter; and in the remaining four comparisons that there was no difference between isomers. The (R) isomer was considered to be distinctly more bitter by four of the judges, while three of the judges considered the (S) isomer to be somewhat more bitter. The other three judges reported no difference between the isomers.

DISCUSSION

Naringin is about one-fifth as bitter as quinine (*6*). Its high level of bitterness is due, in part, to the attachment of an α-L-rhamnosyl residue to the C-2 hydroxy group of β-D-glucose. Removal of the rhamnose to give naringenin 7-β-D-glucoside (prunin) (**4**) results in a threefold decrease in bitterness, while transposition of the rhamnose to the C-6 position to give naringenin 7-β-rutinoside (**5**) results in complete loss of the bitterness. Other structural variations have been studied (*7*, *8*).

Since the isomeric composition of naringin in grapefruit changes during maturation from 92% (2S):8% (2R) to 60% (2S):40% (2R) (Fig. 3) while at the same time bitterness

decreases, it was of interest to determine whether there is a relation between the isomerization and change in taste. Models of (2S) and (2R) naringin show that they have different molecular shapes. As depicted in (**2**) and (**3**), C-2 projects upward in the (2S) isomer and downward in the (2R) compound. Since proton donors and acceptors in surface proteins are the presumed taste receptor sites, slight alterations in substrate structure can markedly affect complex formation or hydrogen bonding of substrate to protein and thereby affect taste.

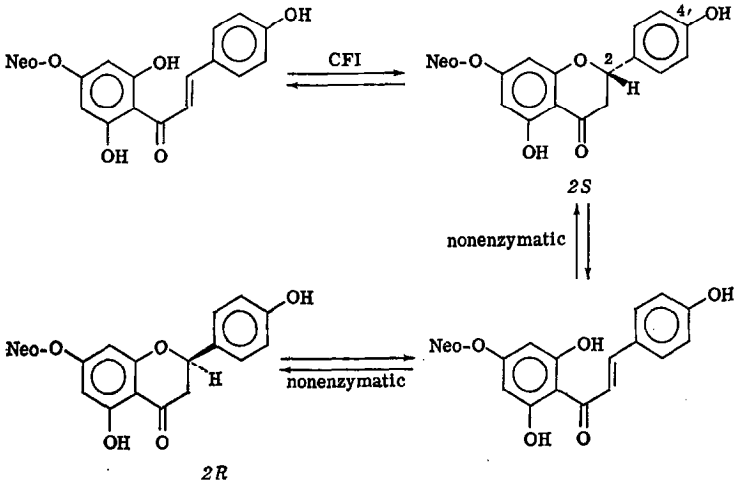
Our results suggest that certain judges do perceive a difference in taste between the (R) and (S) isomers. Although the number of trials was restricted by the limited supply of (2R) naringin, the data indicate that the ten judges in our panel can be tentatively divided into two groups: 1) Those who perceive the (R) isomer as "much" more bitter (four judges) and 2) those who either perceive the (S) isomer as only "somewhat" or "slightly" more bitter (three judges) or who perceive no difference at all between isomers (three judges). The results are similar to those of earlier studies on (2R):(2S) naringin in which (a) it was shown that there is a distribution of individual taste thresholds indicative of a wide range of sensitivity (*9*) and (b) in which a bimodal distribution of sensitivity was observed (G. B. West, personal communication).

It is apparent from our taste results that the change in C-2 chirality of naringin upon ripening of grapefruit is unlikely to be a factor in the debittering process. Immature grapefruit contains about 41% naringin per dry weight of fruit and as the fruit matures the naringin turnover approaches a steady state and the weight per unit weight of fruit decreases (*10*). This dilution effect is probably the main factor involved in debittering.

The change in aglycone configuration also has biosynthetic implications. The generally accepted pathway for flavanone formation is enzyme-mediated cyclization of the chalcone (*11*). This proposal has been given credence by the isolation of many flavanones in optically active form (*12*) and by the isolation of chalcone–flavanone isomerases (CFI's) from soybean (*13*), mung bean and garbanzo bean seedlings, from parsley leaves (*14*), tulip and lily anthers (*15*) and *Datisca cannabina* leaves (*16*). Of the plant CFI's examined for substrate specificity, only one has been found which affects cyclization of chalcone glycosides (*17*). Thus, in mung seedlings, garbanzo beans, parsley leaves (*14*) and *Datisca cannabina* leaves (*16*), chalcone aglycones appear to be the natural precursors of flavanones, with an additional glycosidation step necessary to form the flavanone glycosides. The question of whether a chiral or achiral flavanone is obtained upon cyclization of 4,2′,4′-trihydroxychalcone has been answered in studies which showed that the CFI's from soybean (*13*) and mung bean seedlings (*14*) are indeed stereospecific, since (2S)-7,4′-dihydroxyflavanone was obtained (*18*, *19*). The presence of CFI ("flavanone synthease") has been inferred by Shimokoriyama (*17*) in work on citrus enzymes and more recently CFI-type enzymes were isolated from immature grapefruit and partially characterized (W. R. Raymond and V. P. Maier, personal communication, and Ref. (*20*)). It appears from the work cited that flavanone glycosides in grapefruit are formed directly by enzymatic cyclization of chalcone glycosides. Furthermore, since the naringin in immature grapefruit is essentially all (S), it is doubtlessly produced by enzymatic cyclization of its precursor. Our results thus confirm the presence of a CFI in grapefruit.

The formation of increasing amounts of (2R) naringin from the third month of the

season is obviously not consistent with enzyme-catalyzed formation of (2S) flavanone. The Scheme here shows a pathway consistent with our observations. Nonenzymatic chalcone formation is known to occur very readily for flavanones such as naringin



which contain a blocked 7-hydroxyl group and a free 4'-hydroxyl group (*21*). If a (2S) CFI has been inactivated, inhibited, or degraded by the third month of the growing season, then any chalcone present would cyclize nonenzymatically to nearly equal amounts of (2S) and (2R) naringin. The naringin ratio of 60% (2S):40% (2R) at maturity suggests that either the nonenzymatic isomerization of (2S) naringin occurs slowly, so that a preponderance of the (2S) isomer remains, or that a small amount of (2S) CFI activity persists to maturity. It should be noted that, in the nonenzymatic cyclization of the chalcone glycoside, formation of the (2S) isomer is not favored.[2]

Flavanone glycoside accumulation proceeds rapidly until the growing fruit reach a diameter of 5–6 cm (*22*). This fruit size is reached about June 1. At that time accumulation of flavanone glycosides slows and finally ceases. Our *in situ* CD results on the chirality of naringin suggest that CFI may be inactivated, inhibited, or degraded at about the time flavanone glycoside accumulation levels off (see data for June 20 in Fig. 3). A direct relationship between L-phenylalanine ammonia-lyase activity and the rate of naringenin glycoside accumulation in developing grapefruit has been reported (*20*). On the other hand, the activities of other grapefruit enzymes which are involved in the pathway before the chalcone do not correlate with flavanone glycoside accumulation (*23*). As flavonoid biosynthesis slows, the activities of enzymes involved in the pathway should diminish accordingly. It will be interesting to determine whether or not the activity of grapefruit CFI's parallels the isomerization of naringin as the fruit matures.

[2] Several experiments were conducted to test this point. Naringin chalcone was allowed to cyclize chemically in three solvents to naringin which was then assayed for percentage of (2S) and (2R) content without fractionation. The results, which did not indicate significant preferential conversion to either isomer, are as follows: (a) Cyclization in water, 49% (2S):51% (2R) by pmr, 46% (2S):54% (2R) by CD; (b) cyclization in 0.1 *M* acetate buffer, pH 4.45, 51% (2S):49% (2R) by pmr, 50% (2S):50% (2R) by CD; (c) cyclization in 0.1 *M* phosphate buffer, pH 7.0, 47% (2S):53% (2R) by pmr, 46% (2S):54% (2R) by CD.

Further speculation concerning the naringin isomerization must await more definitive chemical and biochemical studies on flavonoid biosynthesis in the grapefruit. The use of CD and pmr to monitor and determine the chirality of substrates *in vivo* should provide useful information on biosynthetic questions.

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REFERENCES

1. R. M. HOROWITZ AND B. GENTILI, *Tetrahedron*, **19**, 773 (1963).
2. W. GAFFIELD, *Tetrahedron*, **26**, 4093 (1970).
3. G. DELLAMONICA AND J. CHOPIN, *Bull. Soc. Chim. Fr.*, 1756 (1971).
4. G. AURNHAMMER, H. WAGNER, L. HORHAMMER, AND L. FARKÂS, *Chem. Ber.*, **103**, 3667 (1970).
5. F. M. DEAN, "Naturally Occurring Oxygen Ring Compounds," p. 351. Butterworths, London, 1963.
6. R. M. HOROWITZ, "Biochemistry of Phenolic Compounds" (J. B. Harborne, Ed.), p. 545. Academic Press, New York, 1964.
7. R. M. HOROWITZ AND B. GENTILI, *J. Agr. Food Chem.*, **17**, 696 (1969).
8. R. M. HOROWITZ AND B. GENTILI, "Sweetness and Sweeteners" (G. G. Birch, L. F. Green and C. B. Coulson, Eds.), p. 69. Applied Science, London, 1971.
9. D. G. GUADAGNI, V. P. MAIER, AND J. G. TURNBAUGH, *J. Sci. Food Agr.*, **24**, 1277 (1973).
10. V. P. MAIER, *Proc. First Int. Citrus Symp.*, **1**, 235 (1969).
11. H. GRISEBACH, *Pure Appl. Chem.*, **34**, 487 (1973).
12. J. W. CLARK-LEWIS, *Rev. Pure Appl. Chem.*, **12**, 96 (1962).
13. E. MOUSTAFA AND E. WONG, *Phytochemistry*, **6**, 625 (1967).
14. K. HAHLBROCK, E. WONG, L. SCHILL, AND H. GRISEBACH, *Phytochemistry*, **9**, 949 (1970).
15. R. WIERMANN, *Planta*, **102**, 55 (1972).
16. H. J. GRAMBOW AND H. GRISEBACH, *Phytochemistry*, **10**, 789 (1970).
17. M. SHIMOKORIYAMA, *J. Amer. Chem. Soc.*, **79**, 4199 (1957).
18. E. WONG AND E. MOUSTAFA, *Tetrahedron Lett.*, 3021 (1966).
19. K. HAHLBROCK, H. ZILG, AND H. GRISEBACH, *Eur. J. Biochem.*, **15**, 13 (1970).
20. V. P. MAIER AND S. HASEGAWA, *Phytochemistry*, **9**, 139 (1970).
21. R. M. HOROWITZ AND L. JURD, *J. Org. Chem.*, **26**, 2446 (1961).
22. J. W. KESTERSON AND R. HENDRICKSON, "Naringin, a Bitter Principle of Grapefruit." Agr. Exp. Station Bull. No. 511A, University of Florida, Gainesville, FL, 1957.
23. S. HASEGAWA AND V. P. MAIER, *Phytochemistry*, **9**, 2483 (1970).