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FLAVONOIDS OF CITRUS—VI

THE STRUCTURE OF NEOHESPERIDOSE[1]

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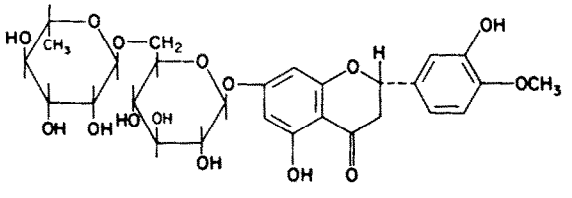
Abstract—The flavanone glycosides naringin, poncirin and neohesperidin all contain the disaccharide neohesperidose, since, on treatment with alkali, they yield the same degradation product, phloracetophenone 4′-neohesperidoside. The structure of neohesperidose is 2-O-α-L-rhamnopyranosyl-D-glucopyranose, as shown by methylation studies and optical rotations. Citrus flavanones that contain neohesperidose are bitter, while the corresponding flavanones that contain the isomeric disaccharide, rutinose (6-O-α-L-rhamnopyranosyl-D-glucopyranose), are tasteless.

A REMARKABLE characteristic of citrus fruits is their high content of flavanone glycosides, which occur principally in the peel. Among the important and easily accessible compounds in this numerous group are naringin, poncirin, neohesperidin and hesperidin. Naringin and poncirin are the exceedingly bitter compounds in grapefruit; neohesperidin occurs in the bitter, or Seville, orange (*Citrus aurantium*); while hesperidin, a non-bitter compound, is the predominant flavonoid in lemons and ordinary sweet oranges (*C. sinensis*). Each of these substances contains a disaccharide unit composed of a molecule of rhamnose and glucose. The disaccharide may assume one of two isomeric forms: rutinose, the structure of which is known, or neohesperidose, the structure of which will be discussed. In addition, it will be shown that there is a definite relation between the structure of the disaccharide and the presence or absence of bitterness.

At the outset of this work hesperidin, which was described as early as 1828,[2] was the only member of the group whose structure had been completely determined. The combined results of many investigations[3] had shown that it must be the 7-β-rutinoside of 2*S*-hesperetin (3′,5,7-trihydroxy-4′-methoxyflavanone; I). Though Zemplén and Gerecs[4] regarded rutinose as being 6-O-β-L-rhamnosyl-D-glucose, recent studies of Gorin and Perlin[5] have shown the need for revising this to 6-O-α-L-rhamnosyl-D-glucose.



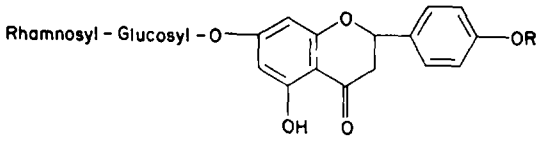
I, Hesperidin

* A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

[1] Preliminary report: R. M. Horowitz and B. Gentili, *Arch. Biochem. Biophys.* **92**, 191 (1961).
[2] P. Lebreton, *J. Pharm. Chim. Paris* **14**, 377 (1828).
[3a] Y. Asahina and M. Inubuse, *J. Pharm. Soc. Japan* **49**, 128 (1929); [b]F. E. King and A. Robertson, *J. Chem. Soc.* **1931**, 1704; [c]G. Zemplén and R. Bognár, *Ber. Dtsch. Chem. Ges* **76B**, 773 (1943); [d] H. R. Arthur, W. H. Wui and C. N. Ma, *J. Chem. Soc.* 632 (1956); [e] H. Arakawa and M. Nakazaki *Chem. Ind.* 73 (1960); and other references cited in these papers.
[4] G. Zemplén and A. Gerecs, *Ber. Dtsch. Chem. Ges.* **67**, 2049 (1934); **68**, 1318 (1935).
[5] P. A. J. Gorin and A. S. Perlin, *Canad. J. Chem.* **37**, 1930 (1959).

Closely allied to hesperidin is the isomeric glycoside neohesperidin, which was isolated in 1936 by Kolle and Gloppe.[6] Hydrolysis of neohesperidin yields rhamnose, glucose and hesperetin, the latter compound occurring again in the 2*S* configuration.[7] Zemplén and Tettamanti[8] found that the probable difference between hesperidin and neohesperidin lies in the point of attachment of rhamnose to glucose and suggested, though they made no attempt to prove rigorously, that in neohesperidin rhamnose is bonded to the C4 hydroxyl group of glucose. This new disaccharide was named *neohesperidose*.

Naringin, which was obtained by De Vry in 1857,[9] is a 7-rhamnoglucoside of naringenin (4′,5,7-trihydroxyflavanone) (II).[3a,10] The detailed structure of the



II, R=H
III, R=CH_3

disaccharide component of naringin has not been examined, though some authors have assumed that it is a rutinoside. This assumption is scarcely in accord with the fact that naringin is bitter while hesperidin is tasteless, for it would appear to be unlikely that the striking difference in taste between the compounds could be due to the relatively small changes in the B-ring substituents.

Some time ago we made the observation that both neohesperidin and naringin yield the same phloracetophenone rhamnoglucoside when heated in 20–25% aqueous potassium hydroxide. Thus, the disaccharide of naringin must be identical with that of neohesperidin and be in the form of neohesperidose rather than rutinose. Furthermore, poncirin,[11] which is a 7-rhamnoglucoside of naringenin 4′-methyl ether (III), also yields this phloracetophenone rhamnoglucoside and must be a derivative of neohesperidose. Following these observations we discovered that neohesperidin is an intensely bitter substance, its bitterness being comparable to that of naringin and poncirin.

The phloracetophenone rhamnoglucoside obtained in these degradations has the structure IV (phloracetophenone 4′-neohesperidoside), as shown by the following experiments. Hydrolysis of IV by acids or enzymes yields rhamnose, glucose and phloracetophenone, while methylation with methyl iodide and potassium carbonate yields a mixture of a monomethyl ether (V) and a dimethyl ether (VI). The latter compound gives 2′,6′-di-O-methylphloracetophenone on hydrolysis and this provides

[6] F. Kolle and K. E. Gloppe, *Pharm. Zentralh.* **77**, 421 (1936).

[7] E. Hardegger and H. Braunschweiger, *Helv. Chim. Acta* **44**, 1413 (1961).

[8] G. Zemplén and A. K. Tettamanti, *Ber. Dtsch. Chem. Ges.* **71B**, 2511 (1938).

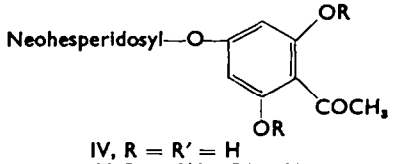
[9] *Cf.* W. Will, *Ber. Dtsch. Chem. Ges* **18**, 1311 (1885).

[10a] J. Shinoda and S. Sato, *J. Pharm. Soc. Japan* **48**, 933 (1928); [b] K. Rosenmund and M. Rosenmund, *Ber. Dtsch. Chem. Ges.* **61B**, 2608 (1928); [c] S. Rangaswami, T. R. Seshadri and J. Veeraraghaviah, *Proc. Ind. Acad. Sci.* **9A**, 328 (1939).

[11a] S. Hattori, *Acta Phytochim. Tokyo* **4**, 219 (1929); [b] S. Hattori, M. Hasegawa and M. Shimokoriyama, *Acta Phytochim. Tokyo* **14**, 1 (1944); [c] A. Sosa and C. Sannié, *C.R. Acad. Sci. Paris* **223**, 45 (1946).

further confirmation that rhamnose and glucose occur as a disaccharide attached to the 7-hydroxy group of the respective flavanones.

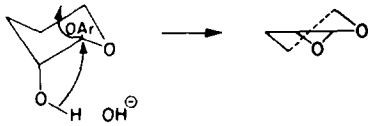
The literature dealing with the alkaline degradation of the flavanone rhamnosides contains a number of statements that we have been unable to verify. Asahina and Inubuse[3a] reported that naringin gives a phloracetophenone rhamnoglucoside



IV, R = R′ = H
V, R = CH_3; R′ = H
VI, R, R′ = CH_3

melting at 149–150° from water. Using various concentrations of alkali and times of contact we have found repeatedly that the product melts at 164–166° when crystallized as the hydrate from water, or at 256–257° when crystallized in an anhydrous form from acetone. A more serious discrepancy arises in the reports that neohesperidin, naringin and poncirin yield phloracetophenone glucoside, in which rhamnose is unaccountably absent.[11b,12] The reported m.p.'s of this product vary from 142–144° to 161–162°. Our own experiments, however, show definitely that the compound contains both rhamnose and glucose and it seems clear that the loss of rhamnose under these conditions would be exceptional.[13]

It is significant that the cleavage of the neohesperidose derivatives by alkali occurs smoothly and without apparent deglycosylation to give a high yield of phloracetophenone 4′-neohesperidoside (IV). When this reaction is carried out using hesperidin, however, it is virtually impossible to isolate any discrete fragment containing the sugars. Instead, the reaction yields a tarry mixture that contains phloroglucinol, a glycoside of phloroglucinol, isoferulic and isovanillic acids, and various unidentified compounds, as shown by paper chromatography. It is apparent that deglycosylation followed by other reactions occurs to a large extent. An explanation for this behavior comes from the fact that hesperidin is an aromatic glycoside in which the hydroxyl group on carbon-2 of glucose is *trans* to the adjacent aryloxy group. Glycosides of this type are unstable in alkali and, as shown by McCloskey and Coleman,[14] and others,[15] probably decompose by forming a 1,2-anhydro compound:



[12a] L. K. Wan, *J. Pharm. Soc. Japan* **62**, 466 (1942);[b] S. Hattori, M. Hasegawa and M. Kanao, *J. Chem. Soc. Japan* **65**, 744 (1944).

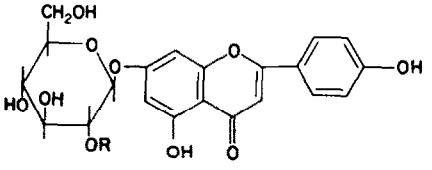
[13] The evidence for the loss of rhamnose is unconvincing. Most of it is based on carbon and hydrogen analyses of hydrated samples. Close scrutiny of the analytical data shows that in one case[12b] an incorrect calculation was made, while in others the data would be equally well accommodated by structure IV. The other evidence[12b] is that (a) authentic phloracetophenone glucoside failed to depress the m.p. of the product (161–162°) and (b) glucosazone was the only osazone isolated on treatment of the hydrolytic products with phenylhydrazine. A sample of phloracetophenone glucoside prepared in this laboratory melted at 140°, then resolidified and remelted at 214–215°.

[14] C. M. McCloskey and G. H. Coleman, *J. Org. Chem.* **10**, 184 (1945).

[15] Reviewed by C. E. Ballou in *Advances in Carbohydrate Chemistry* (Edited by M. L. Wolfrom) **9**, pp 59–95, Academic Press, New York (1954).

The essential feature here is the ionization of the *trans* C2 hydroxyl group, which displaces the aryloxy group by nucleophilic substitution. The resulting anhydro compound cannot be isolated as it rapidly undergoes further changes in the alkaline medium. If the C2 hydroxyl group is blocked by a methyl group as, for example, in phenyl 2,3-di-O-methyl-β-D-glucoside,[14] the displacement of the aryloxy group fails to take place and the glycosidic linkage remains intact.

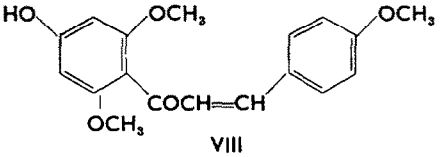
This interpretation provides a clue to the structure of neohesperidose, for it would seem logical that the alkali stability of the glycosidic bond involving neohesperidose could be accounted for by assuming that rhamnose is joined to glucose through the C2 hydroxyl group of glucose. Since the C2 hydroxyl would then be unable to participate in the displacement of the flavanone anion, the only anticipated effect of the alkali would be the cleavage of the flavanone part of the molecule to yield IV. An apparently analogous situation obtains for apiin, a glycoside composed of the disaccharide 2-O-apiosyl-D-glucose ("apiinibiose"), attached to the 7-hydroxyl group of the flavone apigenin (VII).[16] Vongerichten has stated that when apiin is treated with boiling 25%



VII, R = Aplosyl

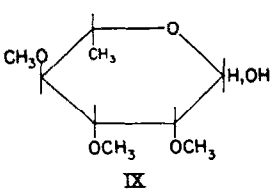
sodium hydroxide the flavone splits to give *p*-hydroxyacetophenone and phloroglucinol glycoside "without any change of the carbohydrate portion."[17]

To obtain definite proof for the structure of neohesperidose, we methylated naringin in one step using methyl iodide and silver oxide in N, N-dimethylformamide.[18] Hydrolysis of the product yielded the known compound, 2',6',4-trimethoxy-4'-hydroxychalcone (VIII),[10c] and a mixture of methylated sugars. The mixture was

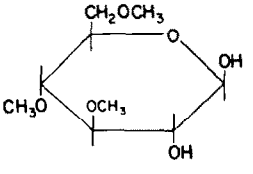


VIII

separated into its components convienently and on a large scale by chromatography on silicic acid, but it could also be partially resolved by partitioning it between water



IX



X

[16] R. Hemming and W. D. Ollis, *Chem. Ind.* 85 (1953).

[17] Quoted by C. S. Hudson in *Advances in Carbohydrate Chemistry* (Edited by W. W. Pigman and M. L. Wolfrom) **4**, pp. 57–74, Academic Press, New York (1949).

[18] R. Kuhn, I. Löw and H. Trischmann, *Chem. Ber.* **88**, 1492 (1955).

and chloroform. Of the two principal components, the first was identified as 2,3,4-tri-O-methyl-L-rhamnose (IX) by its I.R. spectrum, optical rotation and preparation of the aniline derivative. The second was obtained initially as a syrup that crystallized only when seeded with 3,4,6-tri-O-methyl-β-D-glucose (X).[19] In every respect this compound agreed closely in m.p., IR spectrum, mutarotation, R_f value and electrophoretic migration when compared with authentic 3,4,6-tri-O-methyl-β-D-glucose. It took up to one mole of periodate and gave a phenylosazone identical with that obtained from 3,4,6-tri-O-methyl- or 2,3,4,6-tetra-O-methyl-D-glucose. The compound yielded, as a new derivative, a bis-phenylurethane. Thus, the identity of the methylated sugars is clear and it remains only to determine the configuration of rhamnose in the disaccharide. It is known that the glucose in naringin is linked *beta* to naringenin, since partial hydrolysis of naringin by acid gives naringenin 7-glucoside (prunin), which is hydrolyzed by β-glucosidase.[20] The optical rotations shown in Table 1 provide evidence that the rhamnose in neohesperidose has the α-configuration. This agrees with the fact that all the naturally occurring rhamnosides of known structure have, as far as we are aware, the α-configuration. Similarly in steroid compounds it is recognized that glycosides of L-sugars are generally *alpha*.[21] We conclude, therefore, that neohesperidose is 2-O-α-L-rhamnopyranosyl-D-glucopyranose and naringin, poncirin and neohesperidin are the 7-β-neohesperidosides shown in XI, XII, and XIII, respectively.[22,23]

Naturally occurring disaccharides linked through the C2 hydroxyl group of the reducing sugar are not numerous. The best known examples are sophorose (2-O-β-D-glucopyranosyl-D-glucopyranose), kojibiose (2-O-α-D-glucopyranosyl-D-glucopyranose), and the previously mentioned apiinibiose. In addition, a small group of oligosaccharides and polysaccharides are known to contain $1 \rightarrow 2$ linkages.

In flavanone 7-rhamnoglucosides a $1 \rightarrow 2$ linkage between rhamnose and glucose gives rise to unusual taste phenomena. Naringin, poncirin and neohesperidin are all

[19] R. L. Sundberg, C. M. McCloskey, D. E. Rees and G. H. Coleman, *J. Amer. Chem. Soc.* **67**, 1080 (1945).

[20a] D. W. Fox, W. L. Savage and S. H. Wender, *J. Amer. Chem. Soc.* **75**, 2504 (1953); [b] C. W. Nystrom, B. L. Williams and S. H. Wender, *Ibid.* **76**, 1950 (1954).

[21] W. Klyne, *Biochem. J.* **47**, xli (1950).

[22] In a recent paper dealing with the disaccharide of neohesperidin, naringin and poncirin, Nakabayashi[24] has recognized that each of these glycosides contains neohesperidose. However, in accord with Zemplén's suggestion, he considers neohesperidose to be 4-O-β-L-rhamnopyranosyl-D-glucose. This structure is based on the isolation of 2,3,4-tri-O-methyl-L-rhamnose and 2,3,6-tri-O-methyl-D-glucose from the products of methylation of neohesperidin. The 2,3,6-tri-O-methyl-D-glucose was not obtained crystalline, but was said to have the same R_f value as an authentic specimen and to yield a crystalline 1,4-diacetate (m.p. 67°) that did not depress the m.p. of the authentic diacetate.

We have no explanation for the sharp discrepancy in the results obtained by Nakabayashi and ourselves. However, our sample of 2,3,6-tri-O-methyl-D-glucose (m.p. 110–113°) was distinctly different from the trimethylglucose we obtained by methylating naringin (or neohesperidin), as shown by the infrared spectrum, the marked depression of the mixed m.p. and the electrophoretic migration.

[23] Several flavone 7-rhamnoglucosides are thought to be neohesperidosides, including rhoifolin,[25] fortunellin[26] and lonicerin[27]. These contain the aglycones apigenin, 4′-O-methylapigenin and luteolin, respectively.

[24] T. Nakabayashi, *J. Agric. Chem. Soc. Japan* **35**, 942 (1961).

[25] S. Hattori and H. Matsuda, *Arch. Biochem. Biophys.* **37**, 85 (1952).

[26] T. Matsuno, *J. Pharm. Soc. Japan* **78**, 11 (1958).

[27] T. Nakabayashi, *J. Agric. Chem. Soc. Japan* **35**, 945 (1961).

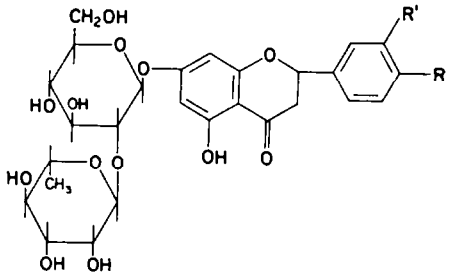
Table 1. Optical rotations of neohesperidose derivatives and related compounds

Compound	Solvent	$[\alpha]_D$	M_D	Calculated M_D for	
				α-Rhamnosyl	β-Rhamnosyl
Methyl α-L-rhamnoside*	Water	−62·5°	−11,140		
Methyl β-L-rhamnoside*	Water	+95·4°	+17,000		
Methyl 2,3,4-triacetyl-α-L-rhamnoside*	s-Tetrachloroethane	−53·7°	−16,340		
Methyl 2,3,4-triacetyl-β-L-rhamnoside*	s-Tetrachloroethane	+45·7°	+13,910		
Naringenin 7-β-D-glucoside (prunin)	Water–methanol (1:1)	−62·0°	−26,910		
Naringin	Water	−84·4°	−48,950	−38,050	−9,910
Phloroglucinol β-D-glucoside**	Water	−76·2°	−22,000		
Phloroglucinol β-neohesperidoside‡	Water	−89·1°	−38,600	−33,140	−5,000
Phloroglucinol β-D-glucoside hexaacetate‡	s-Tetrachloroethane	−24·5°	−13,240		
Phloroglucinol β-neohesperidoside octaacetate‡	s-Tetrachloroethane	−32·9°	−25,350	−29,580	+670

* Data from F. J. Bates, *Polarimetry, Saccharimetry, and the Sugars*, National Bureau of Standards, U.S. Government Printing Office, Washington D.C. (1942).

** Data from M. Cremer and R. Seuffert, *Ber. Dtsch. Chem. Ges.* **45**, 2565 (1912).

‡ The preparation of this compound will be described in a later article.



XI, R = OH; R′ = H: Naringin XII, R = OCH_3; R′ = H: Poncirin
XIII, R = OCH_3; R′ = OH; aglycone configuration as in I: Neohesperidin

remarkably bitter, their bitterness being easily detectable at concentrations as low as 10^{-4} or 10^{-5} M. Also, phloracetophenone 4′-neohesperidoside (IV) and its mono- and dimethyl ethers (V and VI) are bitter, though their bitterness is less than that of the parent flavanones. Data showing the approximate relative bitterness of some of these compounds are given in Table 2.

TABLE 2. MOLAR CONCENTRATIONS HAVING ABOUT THE SAME DEGREE OF BITTERNESS*

	Concentration	Ratio
Phloracetophenone 4′-neohesperidoside	1×10^{-3}	1
Neohesperidin	5×10^{-4}	2
Naringin	5×10^{-5}	20
Poncirin	5×10^{-5}	20

* Hesperidin was not bitter at 2×10^{-3} M.

It will be noted that hesperidin, the rutinose analog of neohesperidin, is not bitter, even at a concentration of 2×10^{-2} M. Similarly, naringenin 7-rutinoside and isosakuranetin 7-rutinoside,[28] the rutinose analogs of naringin and poncirin, respectively, are devoid of bitterness, as is the 7-β-rutinoside of the flavanone eriodictyol (3′,4′,5,7-tetrahydroxyflavanone).[29] No other conclusion is possible but that the point of attachment of rhamnose to glucose is the factor that determines bitterness or nonbitterness in this group of compounds.

The presence of neohesperidose in glycosides is not of itself sufficient to produce bitterness. Some of the transformation products involving the aglycone portions of naringin and neohesperidin are tasteless while others are intensely sweet. On the other hand, all of the derivatives containing rutinose are tasteless. These findings and the possible significance of neohesperidose and rutinose as taxonomic markers in *Citrus* species will be discussed elsewhere.

EXPERIMENTAL

Alkali degradation of naringin, neohesperidin and poncirin

In a typical run, a solution of naringin (17 g) in water (175 ml) containing potassium hydroxide (50 g) was boiled under reflux (nitrogen atm.) for 3·5 hr. The chilled solution, neutralized with cold

[28] These compounds will be described in a later publication.
[29] R. M. Horowitz and B. Gentili, *J. Amer. Chem. Soc.* **82**, 2803 (1960).

6N hydrochloric acid, yielded a pale yellow precipitate. Two recrystallizations of this material from water afforded small, colorless needles of *phloracetophenone 4'-neohesperidoside* (IV) (6·6 g); m.p. 164–166°; $\lambda_{max}^{C_2H_5OH}$ 282, 325 (low) mμ. (Found: C, 45·3; H, 6·47. $C_{20}H_{28}O_{13}\cdot 3H_2O$ requires: C, 45·3; H, 6·46%).

The compound, when dried in a vacuum at 135°, lost 9·3% in weight; calc. for $3H_2O$: 10·2%. There was no significant change in the m.p. (Found: C, 50·4; H, 5·92. $C_{20}H_{28}O_{13}$ requires: C, 50·4; H, 5·92%).

The compound was obtained from acetone in an anhydrous form that crystallized in small plates, m.p. 256–257°; $[\alpha]_D^{28}$ −111·0° (*c* 0·9 in methanol–water (1:1)). (Found: C, 50·3; H, 5·86%). Recrystallization of the high melting compound from water restored it to the hydrated form, m.p. 164–166°.

Asahina and Inubuse[28] reported a phloracetophenone rhamnoglucoside, m.p. 149–150°; $[\alpha]_D^{21·5}$ −86·51° (alcohol).

The alkali degradation of neohesperidin and poncirin proceeded in the same manner to give phloracetophenone 4'-neohesperidoside (IV) in each case. The products were compared by m.p's. IR and UV spectra, and R_f values.

Hydrolysis of IV in the presence of hemicellulase[29] or acid gave phloracetophenone (m.p. and mixed m.p. 221–222°), glucose and rhamnose. The sugars were identified by paper chromatography, as described earlier.[30]

Alkali degradation of hesperidin

Runs of 1 to 3 hr were made in 10%, 30% and 50% aqueous potassium hydroxide. In general, the products were tarry and very little crystalline material could be isolated except for isoferulic and isovanillic acids. In only one experiment was a trace of solid obtained (m.p. 196–215°) that had spectral characteristics close to those of phloracetophenone 4'-neohesperidoside. Paper chromatograms of the crude reaction mixtures usually showed the presence of phloroglucinol, a possible glycoside of phloroglucinol, and a number of unidentified compounds.

Methylation of phloracetophenone 4'-neohesperidoside

A mixture of phloracetophenone 4'-neohesperidoside (IV; 6·0 g), methyl iodide (34 ml), potassium carbonate (40 g) and acetone (1200 ml) was boiled under reflux for 2 days. The residue obtained after filtering and evaporating the mixture was dissolved in water and allowed to stand. A small quantity (20 mg) of 2'-O-*methylphloracetophenone 4'-neohesperidoside* (V) was obtained, m.p. 240–242° from ethanol; $\lambda_{max}^{C_2H_5OH}$ 287, 335 (low) mμ; $\lambda_{max}^{C_2H_5OH-AlCl_3}$ 309, 376 mμ (complex forms slowly). (Found: C, 51·0; H, 6·42; CH_3O, 6·73. $C_{21}H_{30}O_{13}$ requires: C, 51·4 H, 6·17; $1CH_3O$, 6·33%).

On further standing the aqueous filtrate deposited 1·5 g of *2',6'-di-O-methylphloracetophenone 4'-neohesperidoside* (VI), m.p. 127–128° from acetone; $\lambda_{max}^{C_2H_5OH}$ 270 mμ. (Found: C, 52·0; H, 6·33; CH_3O, 12·1. $C_{22}H_{32}O_{13}$ requires: C, 52·4; H, 6·39; $2CH_3O$, 12·3%).

Hydrolysis of VI by acid or hemicellulase gave glucose, rhamnose and 2',6'-di-O-methylphloracetophenone, m.p. 186–186·5°; reported[31] m.p. 186·5°. (Found: C, 61·2; H, 6·05; CH_3O, 31·7. Calc. for $C_{10}H_{12}O_4$; C, 61·2; H, 6·17; CH_3O, 31·6%).

Methylation and hydrolysis of naringin

A solution containing naringin (20 g), methyl iodide (90 ml) and N,N-dimethylformamide (240 ml) was treated with freshly precipitated silver oxide (90 g) added in portions during 15 min. The mixture was stirred overnight at room temp., then was filtered through Celite. The residue was washed with dimethylformamide (50 ml) and chloroform (1000 ml). The combined filtrate was washed with dil. aqueous potassium cyanide (100 ml) and water (4 × 100 ml) and was finally dried (Na_2SO_4), filtered, and evaporated on the steam bath under vacuum. The *permethyl ether* was obtained as a hard amber gum (24 g) that could not be crystallized. An IR spectrum showed only slight absorption in the hydroxyl region. (Found: C, 60·2; H, 7·05; CH_3O, 38·3. $C_{36}H_{50}O_{14}$ requires: C, 61·1; H, 7·12; $9CH_3O$, 39·5%).

[30] R. M. Horowitz, *J. Org. Chem.* **21**, 1184 (1956).

[31] *Dictionary of Organic Compounds* (Edited by I. Heilbron and H. M. Bunbury) Oxford University Press, New York (1953).

The combined permethyl ether from two runs (45·8 g) was boiled overnight in a mixture of methanol (520 ml) and 4 N hydrochloric acid (175 ml). The mixture was steam distilled for 4 hr, while keeping the volume nearly constant by addition of water. The aqueous supernatant, containing the methylated sugars, was filtered from the hard orange gum (19 g), which crystallized as pale yellow needles from ethyl acetate–ligroin or methanol; m.p. 207·5–208°; reported [10c] m.p. of 2′,6′,4-trimethoxy-4′-hydroxychalcone: 206–207°. (Found: C, 68·5; H, 5·86; CH_3O, 29·1. Calc. for $C_{18}H_{18}O_6$: C, 68·8; H, 5·77; CH_3O, 29·6%).

Purification of the methylated sugars

A. *By extraction.* The aqueous filtrate from the hydrolysis was neutralized exactly to pH 7 and was decolorized with Norite. The solution, concentrated to 125 ml, was extracted with many small portions of chloroform (total volume 2000 ml). Evaporation of the separate chloroform extracts gave a total of 21·2 g (85%) of methylated sugars. The later extracts contained almost pure 3,4,6-tri-O-methyl-D-glucose (see below), which is much less soluble in chloroform than is 2,3,4-tri-O-methyl-L-rhamnose. Crystallization of the crude 3,4,6-tri-O-methyl-D-glucose (6·58 g) from isopropyl ether and from benzene–ligroin gave colorless rosettes (4·4 g), m.p. 102–103° (block).

The last chloroform extract yielded a trace of solid, m.p. 118°, tentatively identified as 3,6-di-O-methyl-D-glucose.

B. *By chromatography on silicic acid.* Examination of the mixture of methylated sugars on chromatostrips[33] coated with silicic acid showed that benzene–ethanol would give a good separation. Accordingly, a slurry of 100 mesh silicic acid (925 g, Mallinckrodt's analytical reagent) in benzene (3700 ml) was poured into an 8-cm tube forming a column 36-cm high. The mixture of methylated sugars (11·8 g), dissolved in a minimum of benzene, was added to the column, which was eluted first with 10% ethanol in benzene (5600 ml), then with 15% ethanol in benzene (6300 ml). A total of 400 fractions was collected. Every tenth fraction was examined on a chromatostrip sprayed with anisidine hydrochloride, Tollens' reagent, or aniline phthalate. Fractions 100–180 yielded pure 2,3,4-tri-O-methyl-L-rhamnose (8·6 g); fractions 181–239 yielded a mixture of 2,3,4-tri-O-methyl-L-rhamnose and 3,4,6-tri-O-methyl-D-glucose (0·61 g); while fractions 240–340 yielded pure 3,4,6-tri-O-methyl-D-glucose (1·67 g).

The total recovery of pure trimethyl-D-glucose from A and B was 6·07 g; of pure trimethyl-L-rhamnose 8·6 g.

2,3,4-*Tri*-O-*methyl*-L-*rhamnose*. The product isolated from fractions 100–180 distilled as a colorless oil, b.p. 123–124° at 2·5 mm; $R_{\text{Tetramethylglucose}}$ 1·00 in butanol–ethanol–water (5:1:4); $[\alpha]_D^{24}$ +25·0° (*c* 1 in water). Authentic 2,3,4-tri-O-methyl-L-rhamnose showed a rotation of +24·8° in water and gave an IR spectrum identical with that of the natural compound. (Found: C, 51·7; H, 8·81; CH_3O, 44·6. Calc. for $C_9H_{18}O_5$: C, 52·5; H, 8·80; CH_3O, 45·2%).

2,3,4-*Tri*-O-*methyl*-L-*rhamnose anilide*, obtained by warming the methylated sugar with a mixture of aniline and aniline hydrochloride in methanol, crystallized from pet. ether as colorless needles, m.p. and mixed m.p. 123–123·5°; $[\alpha]_D^{24}$ +136·1° → +10·6° (*c* 1 in ethanol plus a drop of hydrochloric acid). The reported[18] constants are: m.p. 124–125°; $[\alpha]_D^{23}$ +137·0° → +16·0°. The IR spectrum of the authentic anilide was identical with that of the natural compound, as well as with that reported by Polonsky, *et al.*[33] (Found: C, 63·9; H, 8·23; CH_3O, 33·1; N, 5·01. Calc. for $C_{15}H_{23}O_4N$: C, 64·2; H, 8·23; CH_3O, 33·1; N, 4·98%).

3,4,6-*Tri*-O-*methyl*-D-*glucose*. The products of parts A and B (above) crystallized from isopropyl ether or from benzene–ligroin as hard, colorless rosettes, m.p. and mixed m.p. 95–96·5° (capillary); 103–104° (block); $[\alpha]_D^{25}$ + 49·5° (11 minutes) → +77·4° (*c* 1·6 in water); $R_{\text{Tetramethylglucose}}$ 0·84 in butanol–ethanol–water (5:1:4); M_{Glucose} 0·40 in 0·1 M sodium borate at 20 volts per cm on Whatman No. 1 paper. The reported[19,34] constants of 3,4,6-tri-O-methyl-β-D-glucose are: m.p. 97–98°; $[\alpha]_D^{25}$ +46·4° (interpolated) → +78·0°; M_G 0·37. The compound took up exactly 1·00 mole of periodate per mole and had an infrared spectrum identical with that of authentic[35] 3,4,6-tri-O-methyl-β-D-glucose. (Found: C, 48·7; H, 8·02; CH_3O, 42·2. Calc. for $C_9H_{18}O_6$: C, 48·6; H, 8·16; CH_3O, 41·9%).

[32] J. G. Kirchner, J. M. Miller and G. J. Keller, *Analyt. Chem.* **23**, 420 (1951).
[33] J. Polonsky, E. Sach and E. Lederer, *Bull. Soc. Chim.* 880, (1959).
[34] H. B. Wood, Jr., R. Allerton, H. W. Diehl and H. G. Fletcher, Jr., *J. Org. Chem.* **20**, 875 (1955).
[35] Samples provided by Dr. Chester McCloskey and Dr. Hewitt G. Fletcher, Jr.

The mother liquors yielded a second compound, which was obtained as silky needles, m.p. 74–78° after several recrystallizations from isopropyl ether; $[\alpha]_D^{25}$ +79·7° → +76·2° (*c* 0·07 in water). The reported[19] constants of 3,4,6-tri-O-methyl-α-D-glucose are: m.p. 76–77°; $[\alpha]_D^{25}$ +91·9° → +77·4°. The IR spectrum of this compound was similar to that of the β-form, except that it showed a band at 859 cm^{-1} that is presumably due to the α-configuration.

The *phenylosazone* of 3,4,6-tri-O-methyl-D-glucose was obtained from dil. ethanol as a hydrate, m.p. 75–80°, and from pet. ether in an anhydrous form, m.p. 105–106° then resolidifying and remelting at 133–135°. The reported[26] m.p.'s of the osazone are 80–82° (hydrated) and 137–138° (anhydrous). A sample of the phenylosazone prepared from authentic 3,4,6-tri-O-methyl-D-glucose or from 2,3,4,6-tetra-O-methyl-D-glucose (by prolonged heating) agreed closely in m.p. behaviour and in IR spectrum with the natural compound.

The *bis-(phenylurethane)* derivative of 3,4,6-tri-O-methyl-D-glucose was formed on warming the latter compound (206 mg) with phenyl isocyanate (0·5 ml) over a small flame for 2 min. The solution was cooled, treated with ligroin and filtered. The solid, recrystallized from benzene–ligroin and from ethanol, was obtained as needles, m.p. 177·5–178·5°. (Found: C, 60·2; H, 6·13; CH_3O, 20·0; N, 6·04. $C_{33}H_{38}O_8N_2$ requires: C, 60·0; H, 6·13; CH_3O, 20·2; N, 6·09%).

The ethanol liquors yielded a small amount of a second compound, obtained as silky needles, m.p. 202·5–206°. (Found: C, 60·1; H, 6·18; CH_3O, 19·9%). These compounds are presumably the α and β epimers. The IR spectra were similar but characteristic.

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[26] W. N. Haworth and A. Learner, *J. Chem. Soc.* 619 (1928).