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FLAVONOIDS OF CITRUS—VII

LIMOCITROL AND ISOLIMOCITROL

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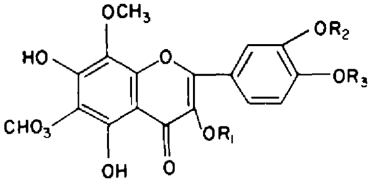
Abstract—The flavonols limocitrol and isolimocitrol, isolated from *Citrus limon*, are shown to be 3,5,7,4′-tetrahydroxy-6,8,3′-trimethoxyflavone and 3,5,7,3′-tetrahydroxy-6,8,4′-trimethoxyflavone, respectively. Each occurs as a 3-β-D-glucoside, as does the previously described flavonol, limocitrin (3,5,7,4′-tetrahydroxy-8,3′-dimethoxyflavone).

Among the flavonoid constituents of the lemon (*Citrus limon*) are three new flavonols that we have named limocitrin, limocitrol[1] and isolimocitrol. The structure of limocitrin (3,5,7,4′-tetrahydroxy-8,3′-dimethoxyflavone) has already been discussed.[2] Here we will be concerned with limocitrol and isolimocitrol, and with the D-glucosyl derivatives of each of the three flavonols.

Limocitrol, isolated from an enzymic hydrolysate of crude lemon flavonoids, is a crystalline yellow solid that contains three methoxy and four hydroxy groups. It yields vanillic acid on treatment with hot alkali. Application of the spectral procedures[3] developed earlier in this Laboratory showed that the compound contains free hydroxy groups at positions 3, 4′ and 7 and no *o*-dihydroxy group. This spectral information, coupled only with the chemical results mentioned, enables one to deduce the structure of limocitrol as 3,5,7,4′-tetrahydroxy-6,8,3′-trimethoxyflavone (I).



I $R_1 = R_3 = H$; $R_2 - CH_3$
II $R_1 = \beta$-D-glucosyl; $R_2 = CH_3$; $R_3 = H$
III $R_1 = R_2 = H$; $R_3 = CH_3$
IV $R_1 = \beta$-D-glucosyl; $R_2 = H$; $R_3 = CH_3$

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[1] R. M. Horowitz and B. Gentili, *J. Org. Chem.* **25**, 2183 (1960).

[2] R. M. Horowitz, *J. Amer. Chem. Soc.* **79**, 6561 (1957); R. M. Horowitz and B. Gentili, *J. Org. Chem.* **26**, 2899 (1961).

[3] L. Jurd and R. M. Horowitz, *J. Org. Chem.* **22**, 1618 (1957). In summary, a free 7-hydroxy group is indicated by a bathochromic shift of the short wavelength band in the presence of fused sodium acetate in absolute ethanol; the simultaneous presence of free hydroxy groups at positions 3 and 4′ is indicated by the rapid disappearance of the long wavelength band in the presence of sodium ethylate or sodium hydroxide; and an *o*-dihydroxy group is indicated by a bathochromic shift in the presence of boric acid–sodium acetate.

Further evidence bearing on structure I was obtained (a) by methylation with excess methyl sulfate, which gives 3,5,6,7,8,3′,4′-heptamethoxyflavone[4] and (b) by methylation with three moles of methyl sulfate, which gives 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone[4]. In addition, the UV spectrum of the tetra-O-acetyl derivative of limocitrol bears a close resemblance to that of tetra-O-acetyllimocitrin. This is to be expected if the compounds differ only by the addition of a 6-methoxy group to one of them. Finally, the structure of limocitrol was proved by synthesis, as described in the following paper.

Experiments on the separation of the unhydrolyzed flavonoid glycosides by means of silicic acid chromatography resulted in the isolation of crystalline limocitrol 3-β-D-glucoside (II). This yields a crystalline heptaacetyl derivative and gives D-glucose and limocitrol when hydrolyzed either by acid or β-glucosidase. The sugar is assigned to the 3-position for spectral and phytochemical reasons. The spectrum of the glucoside in alkaline solution contains a high intensity band at 430 mμ. Since this band is stable, either the 3- or 4′-hydroxy group must be substituted by the sugar. In neutral solution the long wavelength band of the aglycone occurs at 378 mμ, while that of the glucoside occurs at 354 mμ. Similarlarge hypsochromic shifts (~10–25 mμ) have been observed frequently in comparing flavonol 3-glycosides with their aglycones.[5] It is significant that in naturally occurring flavonol glycosides the sugar is most commonly situated at the 3-position.

The second new flavonol, isolimocitrol, was isolated in very small quantity (1·5 mg total) as its crystalline D-glucoside. The lack of material precluded the possibility of determining its structure by ordinary procedures of degradation and analysis. Nevertheless, we can be reasonably certain that isolimocitrol is 3,5,7,3′-tetrahydroxy-6,8,4′-trimethoxyflavone (III) and that it occurs as the 3-β-D-glucoside (IV).

Acid hydrolysis of the new glycoside gave D-glucose (identified on paper chromatograms) and crystalline isolimocitrol. The substitution patterns of isolimocitrol and limocitrol must be very closely related to each other, since the UV spectra of the two compounds in neutral ethanol are virtually identical. The spectra obtained by adding sodium acetate or sodium acetate–boric acid show that isolimocitrol further resembles limocitrol in containing a free 7-hydroxyl group and no *o*-dihydroxyl groups. However, an important difference arises in alkaline solution: limocitrol, with its unprotected 3- and 4′-hydroxy groups, decomposes rapidly, but isolimocitrol is relatively stable and its spectrum contains a band of high intensity at 429 mμ.[6] It is reasonable to assume that the difference between the two compounds is simply in the arrangement of substituents in ring B. In limocitrol ring B is derived from vanillin, while in isolimocitrol it is most likely derived from isovanillin. This supposition was verified by a small scale degradation of isolimocitrol in hot alkali; definite chromatographic evidence was obtained for the presence of isovanillic acid. It is, therefore, exceedingly probable that III represents the correct structure of isolimocitrol, although this has not yet been confirmed by synthesis. The relation of limocitrol to isolimocitrol has

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[4] T. R. Seshadri and V. Venkateswarlu, *Proc. Indian Acad. Sci.* **23A**, 192 (1946).

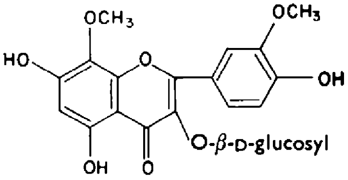
[5] H. W. Siegelman, *J. Biol. Chem.* **213**, 647 (1955).

[6] This band decreases in intensity by about 25%on standing for 30 minutes. In a similarly constituted compound, 3,5,7,3′-tetrahydroxy-4′-methoxyflavone (tamaraxetin), the corresponding band (420 mμ) also decreases in intensity by 25% on standing for 30 minutes. This relatively slow decomposition cannot be confused with the extremely rapid decomposition in alkali of compounds containing both 3- and 4′-hydroxy groups.

already been observed in another pair of lemon flavones, chrysoeriol and diosmetin, which are derived from vanillin and isovanillin, respectively.[1]

The D-glucoside of isolimocitrol is assigned structure IV for reasons similar to those used in assigning structure II to the D-glucoside of limocitrol. Thus, the long wavelength band of isolimocitrol, which in neutral ethanol occurs at 375 mμ, is shifted to 352 mμ in the case of the glucoside. Furthermore, the sugar cannot be located at position 7, as shown by the spectral shift in sodium acetate solution (Table 1).[7]

A third glycoside, isolated from both lemons and oranges (*C. sinensis*), is limocitrin 3-β-D-glucoside (V). It yields a crystalline heptaacetate and, when hydrolyzed with acid or β-glucosidase, D-glucose and limocitrin. The position of the sugar was determined from spectral data, as seen in Table 1.



V

The flavonoid glycosides described here are the first *glucosides* to be isolated from *Citrus*. In every other case the sugar component is either rutinose or neohesperidose, which are isomeric forms (1 → 6 or 1 → 2) of rhamnosylglucose.[8]

The unusual dimethoxyphloroglucinol type of A-ring found in limocitrol and isolimocitrol has been reported recently in two flavones isolated from *Citrus sudachi* Hort *ex* Shirai.[9] These are considered to be 5,7,4′-trihydroxy-6,8,3′-trimethoxyflavone (sudachitin) and 5,7,4′-trihydroxy-6,8-dimethoxyflavone. It is likely that flavonoids based on dimethoxyphloroglucinol may be of wide occurrence in citrus species. A flavonol, erianthin, obtained from *Blumea eriantha* (Compositae) was reported to be 5,7-dihydroxy-3,6,8,3′,4′-pentamethoxyflavone,[10] but this structure was later questioned.[4]

EXPERIMENTAL

Limocitrol was isolated from an enzymically hydrolysed preparation of "Calcium Flavonate Glycoside, Lemon," as described previously.[1] Crystallized from methanol it was obtained as fine yellow needles, m.p. 210–211°, but was usually obtained from ethanol in a different form, m.p. 221–222°; R_f 0·46 in 50% acetic acid. The compound gave a green color with $FeCl_3$, a magenta color with Mg in ethanolic HCl[2] and reduced Tollens' reagent slightly (as do other flavonols containing

[7] In alkaline solution, the long wavelength band of isolimocitrol 3-β-D-glucoside undergoes a bathochromic shift of only 49 mμ and is much diminished in intensity, while the short wavelength band is greatly increased in intensity. This behaviour is characteristic of flavonols blocked at both the 3- and 4′-hydroxy groups as, for example, in quercetin 3,3′,4′,7-tetramethyl ether.[3] In contrast, the long wavelength band of limocitrol 3-β-D-glucoside is shifted 76 mμ in alkali and both long and short wavelength bands are of high intensity.

[8] R. M. Horowitz in *The Orange: Its Biochemistry and Physiology*, (Edited W. B. Sinclair) p. 334. University of California, Berkeley and Los Angeles (1961).

[9] T. Horie, M. Masumura and F. S. Okumura, *Bull. Chem. Soc. Japan* **34**, 1547 (1961); T. Horie,. H. Shimoo, M. Masumura and F. S. Okumura, *J. Chem. Soc. Japan* **83**, 602 (1962); *Chem. Abstr.* **59**, 6346 (1963).

[10] P. K. Bose and P. Dutt, *J. Indian Chem. Soc.* **17**, 45 (1940).

TABLE 1. UV SPECTRA OF FLAVONOLS AND DERIVATIVES (λ_{max} IN mμ)

Compound[a]	Absolute ethanol	Fused sodium acetate in absolute ethanol[b]	Sodium hydroxide in ethanol[c]	Aluminum chloride in absolute ethanol[b]
Limocitrol	260, 275, ~350, 377	282, 328 (low), 401	Decomposes	268, 378, 441
3,5,6,7,8,3′,4′-Heptamethoxyflavone	254, ~260, 341			
5-Hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone	258, 280, 345	258, 280, 345	296, 312, 410 (low)	268, 291, 370, ~430 (low)
3,5,7,4′-Tetra-O-acetyl-limocitrol	246, 262, 319			
3,5,7,4′-Tetra-O-acetyl-limocitrin	243, ~260, 318			
Limocitrol 3-β-D-glucoside	260, 277, 354	284, 327 (low), 388 (low)	283, 348 (low), 430	272, 287, 373, ~425 (low)
Limocitrol 3-β-D-glucoside heptaacetate	248, ~265, 324			
Isolimocitrol	260, 276, ~350, 375	282, 325 (low), 401	284, 335 (low), 429	271, 381, 441
Isolimocitrol 3-β-D-glucoside	261, 277, 352	283, 322 (low), 387 (low)	282, 401 (low)	265, 290, 365
Limocitrin 3-β-D-glucoside	261, 274, 361	284, 326 (low), 395	~275, 285, ~340, 428	~275, 283, ~305, 362, 418 (low)
Limocitrin	259, ~273, ~340, 378	282, 327 (low), 395	Decomposes	270, ~369, 444

[a] Spectra of these compounds in sodium acetate–boric acid[8] were essentially unchanged.

[b] Saturated solution.

[c] 1 drop of 1% aqueous sodium hydroxide in 2·5 ml cuvette.

free hydroxy groups in both the 3- and 4′-positions). (Found: C, 57·5; H, 4·29; CH_3O, 25·0. $C_{18}H_{16}O_9$ requires: C, 57·4; H, 4·29; CH_3O, 24·7%).

A mixture of limocitrol (20 mg) and 60% KOH aq (5 ml) was boiled for 45 min, acidified and extracted with ethyl acetate. Vanillic acid, m.p. and mixed m.p. 206–209°, was recovered from the extract by sublimation.

3,5,7,4′-*Tetra-O-acetyllimocitrol*, prepared in hot acetic anhydride–pyridine, was crystallized from ethyl acetate–ether as colorless needles, m.p. 203–204°. (Found: C, 57·5; H, 4·44; CH_3O, 17·3; CH_3CO, 31·6. $C_{26}H_{24}O_{13}$ requires: C, 57·4; H, 4·44; CH_3O, 17·1; CH_3CO, 31·6%).

3,5,6,7,8,3′,4′-*Heptamethoxyflavone* was obtained by heating limocitrol (36·7 mg) for 20 hrs in a mixture of acetone (85 ml), CH_3I (1 ml) and anhydrous K_2CO_3 (0·5 g). The product was taken up in ethyl acetate and washed with dilute NaOH aq to remove unreacted material. It crystallized from methanol as colorless needles, m.p. and mixed m.p. with a sample[11] isolated from grapefruit oil 128–128·5°; reported[4] m.p. 129–131°. (Found: C, 61·5; H, 5·78. Calc. for $C_{22}H_{24}O_9$: C, 61·1; H, 5·59%).

5-*Hydroxy*-3,6,7,8,3′,4′-*hexamethoxyflavone* was obtained by boiling limocitrol (55·5 mg) for 3 hrs in a mixture containing acetone (100 ml), methyl sulfate (60·8 mg, 3·3 moles) and anhydrous K_2CO_3 (0·5 g). The product crystallized readily from methanol as a bright yellow solid (47·1 mg), m.p. 110–111°; reported[4] m.p. 122–23°. It gave a green ferric test. (Found: C, 60·1; H, 5·20; CH_3O, 43·8. Calc. for $C_{21}H_{22}O_9$: C, 60·2; H, 5·29; CH_3O 44·4%).

Limocitrol 3-*β*-D-*glucoside* was isolated from "Calcium Flavonate Glycoside, Lemon" (CFG) by the procedure described earlier[12] for separating flavonoid glycosides on a column of silicic acid developed with methanol–chloroform. It was eluted from the column when the concentration of methanol reached about 10%. Fractions containing this glucoside (as determined by paper and thin layer chromatography) were taken to dryness and the residue was crystallized from water. Yellow prisms were obtained (160 mg from 6 g of the butanol extract[12] of CFG), m.p. 163°, then resolidifying and remelting at 203–204°; R_f 0·46 in 10% acetic acid (Found: C, 53·0; H, 4·93; CH_3O, 16·6. $C_{24}H_{26}O_{14}$ requires: C, 53·5; H, 4·87; CH_3O, 17·3%).

A solution of limocitrol 3-β-D-glucoside in 0·1M acetate buffer (pH 4·8) containing β-glucosidase was allowed to stand 24 hrs at room temp. Paper chromatography showed about 60% hydrolysis to limocitrol. No hydrolysis occurred in the absence of β-glucosidase. D-Glucose was identified in acid hydrolysates (a) by paper chromatography[13] and (b) by preparation of the osazone and comparison of its IR spectrum with that of an authentic sample of glucosazone. Limocitrol was identified in the usual way by mixed m.p. and spectra.

Limocitrol 3-*β*-D-*glucoside heptaacetate*, prepared in boiling acetic anhydride–sodium acetate, crystallized from ethanol as needles, m.p. 159·5–160·5°. (Found: C, 54·8; H, 4·84; CH_3O, 11·2; CH_3CO, 36·2. $C_{38}H_{40}O_{21}$ requires: C, 54·9; H, 4·79; CH_3O, 10·8; CH_3CO, 35·6%).

Isolimocitrol 3-*β*-D-*glucoside* was isolated in the same manner as limocitrol 3-β-D-glucoside. It preceded the latter compound from the column, being eluted with 9% methanol in chloroform. Crystallized from a small volume of aq methanol it had m.p. 220–225° (yield 1·5 mg); R_f 0·50 in 10% acetic acid (the spot appeared dark under UV light).

Isolimocitrol was obtained by warming the preceding glucoside on the steam bath for several min in a small volume of methanolic HCl. Upon cooling, yellow needles separated, m.p. 236–238°; R_f 0·50 in 50% acetic acid. The mother liquors were extracted with ethyl acetate, then concentrated and chromatographed on paper irrigated with 1-butanol:methanol:water (5:2:4); on spraying with aniline phthalate a spot was observed that had the same R_f and color as those of glucose.

Isolimocitrol was heated with 60% KOH aq, as in the case of limocitrol. The ethyl acetate extract was chromatographed (together with isovanillic, vanillic and protocatechuic acids) on a paper irrigated with benzene:acetic acid:water (2:2:1). Spraying with $FeCl_3$–$K_3Fe(CN)_6$ reagent revealed a spot at R_f 0·50. This R_f value is characteristic of isovanillic acid in this solvent system and distinguishes it from vanillic (R_f 0·65) and protocatechuic acids (R_f 0·04).

Limocitrin 3-*β*-D-*glucoside*, isolated in the same manner as limocitrol 3-β-D-glucoside, was eluted from the column immediately after the latter compound with 10% methanol in chloroform. Evaporation of the combined fractions containing this glucoside gave a water-soluble gum which crystallized

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[11] We are indebted to Dr. W. L. Stanley and Mrs. S. H. Vannier for this compound.

[12] R. M. Horowitz and B. Gentili, *J. Amer. Chem. Soc.* **82**, 2803 (1960).

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

as a yellow solid (40 mg) when the aqueous solution was boiled briefly. It had m.p. 150°, then resolidified and remelted at 240–245°; R_f 0·38 in 10% acetic acid.

The glucoside was hydrolyzed by acid or β-glucosidase exactly as in the case of limocitrol 3-β-D-glucoside. Glucose was identified on paper chromatograms in the usual way[12] and limocitrin by mixed m.p. and spectra.

Limocitrin 3-β-D-*glucoside heptaacetate* was obtained by heating the glucoside in acetic anhydride–sodium acetate. The product, dissolved in ethyl acetate, was treated with decolorizing carbon and then was crystallized twice from ethanol as colorless needles, m.p. 167–168°. (Found: C, 55·6; H, 4·98; CH_3O, 7·65; CH_3CO, 36·2. $C_{37}H_{38}O_{20}$ requires: C, 55·4; H, 4·78; CH_3O, 7·73; CH_3CO 37·5%).