C-Glycosylflavonoids

THE CHEMISTRY OF ORIENTIN AND ISO-ORIENTIN

By B. H. KOEPPEN

Department of Food Science, University of Stellenbosch, Stellenbosch

AND D. G. ROUX

Leather Industries Research Institute, Rhodes University, Grahamstown, South Africa

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1. The structures of orientin and iso-orientin have been investigated by periodic acid and ferric chloride oxidation of the tetra-*O*-methyl derivatives and by identification of the products of oxidation. 2. The preparation and properties of the 3',4',7-trimethyl ethers of orientin and iso-orientin are described, and the nuclear-magnetic-resonance spectra of orientin, iso-orientin and their tri- and tetra-*O*-methyl derivatives are discussed. 3. Orientin and iso-orientin are formulated as 8- and 6-*C*- β -D-glucopyranosyl-luteolin respectively.

In a previous communication (Koeppen, 1962) evidence was presented that orientin and homo-orientin are isomeric *C*-glycosyl-luteolin compounds. In view of this Professor L. J. Haynes (personal communication) has suggested that homo-orientin be renamed iso-orientin, and it is intended to follow this suggestion in the present and in all future references to this compound. Subsequently Koeppen (1964) claimed that both orientin and iso-orientin (homo-orientin) possess *C*-glucopyranosyl residues, their interconvertibility in hot dilute aqueous acid media being due to a Wessely-Moser rearrangement of the flavone portion of the molecule.

The present paper provides details of the experimental methods that led to the above conclusions, and a description is given of the preparation and properties of derivatives that have provided valuable information on the structural interrelationship of the parent compounds. Nuclear-magnetic-resonance (n.m.r.) evidence is also presented that favours the identification of orientin as 8-*C*- β -D-glucopyranosyl-luteolin (I) and iso-orientin as the corresponding 6-*C*- β -D-glucopyranosylflavone (II).

EXPERIMENTAL AND RESULTS

Infrared-absorption spectra were recorded on a Perkin-Elmer model 21 spectrometer by the KBr-disk method (0.3–0.6 mg. of compound/250 mg. of KBr). C, H, methoxyl and acetyl determinations were by K. Jones, Micro-analytical Laboratory, C.S.I.R., Pretoria, South Africa. All melting points are uncorrected and were determined by the Kofler method. Mixed melting points were determined by the method of Roux & Maihs (1960).

Oxidations of 3',4',5,7-tetra-O-methylorientin and 3'-4',5,7-tetra-O-methyliso-orientin

The tetramethyl ethers were prepared as described by Koeppen, Smit & Roux (1962), recrystallized to constant m.p. and dried for 16 hr. over P_2O_5 at 110° under reduced pressure.

Periodic acid oxidation. The method employed was essentially the Fleury-Lange procedure described by Dyer (1956). The compound (14.0 mg.) in aq. 60% (v/v) ethanol (20 ml.) was treated with aq. 0.04N-HIO₄ (20 ml.) in the dark at 15°. A blank was similarly prepared. The rate of oxidation was followed by withdrawing samples (4 ml.) of the reaction mixture and the blank at various intervals and adding to each saturated aq. NaHCO₃ soln. (10 ml.), standard 0.02N-NaAsO₂ soln. (5 ml.) and 20% (w/v) KI in saturated aq. NaHCO₃ soln. (1 ml.). After standing (15 min.) the mixtures were titrated with standard 0.02N-I₂ soln. and starch indicator.

Although the initial uptake of periodate was faster for 3',4',5,7-tetra-*O*-methyliso-orientin than for 3',4',5,7-tetra-*O*-methylorientin, each compound consumed only 2.0 mol./mol. after 23 hr. (Koeppen, 1962). No additional uptake of periodate was observed during a further 24 hr. Control experiments with methyl α -D-glucopyranoside and methyl α -D-galactopyranoside under the above conditions also resulted in the uptake of only 2.0 mol. of periodate/mol. of compound. This was attributed to the expected cleavage of the α -glycol groupings only, as no hydrolysis of the methyl glycosides could be detected after 48 hr. in aq. HCl at the same temperature and pH (2.2) as the HIO₄ solutions.

Formic acid determination (cf. Dyer, 1956). The compound (50.0 mg.) in aq. 60% (v/v) ethanol (75 ml.) was treated in the dark with aq. 0.04N-HIO₄ (75 ml.) for 43 hr. at 15°. Aq. 5.6% (w/v) Ba(OH)₂ soln. (8 ml.) was added and the precipitate was removed by centrifugation after 1 hr. at 0°. The precipitate was washed with several portions of water and the combined supernatant and washings (about

220 ml.) were evaporated to dryness under reduced pressure at 60°. The residue was dissolved in water (10 ml.), acidified to litmus by the dropwise addition of 10% (w/v) H_3PO_4 and distilled to dryness under reduced pressure at 70°, the acid distillate being collected in an ice-cooled receiver. The non-volatile residue was dissolved in water (10 ml.) and distilled to dryness three times as above. The four combined acid distillates were titrated to pH 7.0 with standard NaOH. A blank determination was made similarly. The acidity, expressed as formic acid, revealed that 0.8 and 0.9 mol. of acid were produced/mol. of 3',4',5,7-tetra-*O*-methylorientin and 3',4',5,7-tetra-*O*-methyliso-orientin respectively. The neutralized distillate was evaporated to a small volume (about 10 ml.) under reduced pressure at 70° and treated with aq. 10% (w/v) HgCl_2 (2 ml.), saturated aq. sodium acetate soln. (1 ml.) and aq. 4*N*-HCl (0.2 ml.). The mixture was immersed in boiling water in the dark for 1 hr. The calomel formed was equivalent to 0.8 mol. of formic acid/mol. of 3',4',5,7-tetra-*O*-methylorientin. An identical result was obtained for 3',4',5,7-tetra-*O*-methyliso-orientin.

Glycerol detection. Glycerol was detected as a product of HIO_4 oxidation, NaBH_4 reduction and acid hydrolysis of 3',4',5,7-tetra-*O*-methylorientin and 3',4',5,7-tetra-*O*-methyliso-orientin by the procedure described by Koeppen (1965a).

Ferric chloride oxidation. The method of Hay & Haynes (1956) was considerably modified for use on a semi-micro scale. The tetramethyl ether (about 25 mg.) and FeCl_3 (0.2 g.) in water (0.8 ml.) were refluxed gently for 6 hr. The mixture was cooled, the pH adjusted to 8.0 with aq. NaOH soln. and the precipitate was removed by centrifugation. The pH of the supernatant was adjusted to 7.0 with aq. HCl and the solution was desalted in a Shandon electrolytic desalting apparatus (Mark II). The salt-free solution was concentrated to a small volume (about 0.5 ml.) and examined by paper chromatography. Both 3',4',5,7-tetra-*O*-methylorientin and 3',4',5,7-tetra-*O*-methyliso-orientin yielded a mixture of glucose and arabinose in about equal proportions, indistinguishable from authentic *D*-glucose and *D*-arabinose respectively by co-chromatography in benzene–butan-1-ol–pyridine–water (1:5:3:3, by vol.), in butan-1-ol–acetic acid–water (20:5:11, by vol.) and in aq. 75% (w/w) phenol. Oxidation of the parent compounds, orientin and iso-orientin, under the above conditions yielded similar results, but with barbaloin (isolated from commercial aloin) arabinose was the major product and only a trace of glucose could be detected (cf. Hay & Haynes, 1956).

Orientin and iso-orientin trimethyl ethers

3',4',7-Tri-*O*-methylorientin. The most satisfactory method of preparation was found to be by methylation of orientin with dimethyl sulphate as follows. Orientin (130 mg.) was refluxed with dimethyl sulphate (0.3 g.) and anhydrous K_2CO_3 (0.8 g.) in acetone (15 ml.) for 3 hr. The solution was filtered and the residue was washed with three small portions of cold acetone. The filtrate and washings were combined, concentrated under reduced pressure and applied as a band along the short side of two Whatman no. 3 paper sheets (46 cm. $\times$ 57 cm.). The acetone-insoluble residue was dissolved in water (40 ml.) and acidified with aq. HCl. The precipitated orientin (about 60 mg.) was recovered by filtration after 24 hr. at about 5°, washed until free from acid and dried. The methylation procedure

was repeated four times as described. The ten Whatman no. 3 paper sheets containing the acetone-soluble methylation products were developed by downward migration in butan-1-ol–acetic acid–water (20:5:11, by vol.). Examination of the dried chromatograms under u.v. light (3650 Å) revealed a dark band (R_F about 0.45) as the major component. The band was cut out and eluted with aq. 70% (v/v) ethanol. Complete elution of the material took several days. The eluate was evaporated to dryness under reduced pressure and the residue was recrystallized to constant m.p. from water. After drying to constant weight under reduced pressure at 110°, the yield was 138 mg. of pale-yellow prisms, m.p. 224°, solidifying on further heating and remelting at 271–273°; $[\alpha]_D^{24} - 37.7 \pm 0.4^\circ$ (*c* 1.85 in pyridine); λ_{max} 252, 272 and 348 m μ in ethanol ($\log \epsilon$ 4.11, 4.11 and 4.20 respectively), no change in the position of the maxima being observed on the addition of fused sodium acetate (Jurd & Horowitz, 1957); λ_{max} 285, 308 and 360 m μ (inflection) in 2 mm-sodium ethoxide ($\log \epsilon$ 4.12, 4.06 and 3.79 respectively); infrared spectrum 3340 (OH), 2900 (CH aromatic); 1652 (conjugated CO), 1603, 1575 (inflection), 1512, 1452 (aromatic C–C) and 1435 cm^{-1} (unknown) (Found: C, 58.6; H, 5.3; O, CH₃, 19.2. $\text{C}_{24}\text{H}_{26}\text{O}_{11}$ requires C, 58.8; H, 5.3; O, CH₃, 19.0%). The compound had R_F values 0.04 (water), 0.15 [aq. 15% (v/v) acetic acid], 0.59 [aq. 60% (v/v) acetic acid], 0.51 [butan-1-ol–acetic acid–water (20:5:11, by vol.)] and 0.98 [aq. 75% (w/w) phenol].

Acetylation with acetic anhydride–pyridine under a wide variety of conditions failed to yield a crystalline product.

3',4',7-Tri-*O*-methyliso-orientin. The derivative was most satisfactorily prepared by methylation of iso-orientin with diazomethane as follows. A dry ethereal solution of diazomethane (about 100 ml.) generated from nitroso-methylurea (3.5 g.) was added to a solution of iso-orientin (130 mg.) in a mixture of methanol (10 ml.) and acetone (40 ml.) at 5°. After 1 hr. the mixture was evaporated under vacuum to about 25 ml. and left overnight in a desiccator under slightly reduced pressure. The solid that separated was filtered, recrystallized to constant m.p. from ethanol and dried under reduced pressure to constant weight over P_2O_5 at 110°. The final yield was 38 mg. of cream-coloured needles, m.p. 233°, solidifying on further heating and remelting at 272°; $[\alpha]_D^{25} - 10.3 \pm 0.2^\circ$ (*c* 0.97 in pyridine); λ_{max} 245, 274 and 344 m μ in ethanol ($\log \epsilon$ 4.16, 4.16 and 4.28 respectively), no change in the position of the maxima being observed on the addition of fused sodium acetate (Jurd & Horowitz, 1957); λ_{max} 293 and 313 m μ in 2 mm-sodium ethoxide ($\log \epsilon$ 4.01 and 4.00 respectively); infrared spectrum 3340 (OH), 2900 (CH aromatic), 1647 (conjugated CO); 1610, 1570 (inflection), 1512, 1452 (aromatic C–C) and 1493 cm^{-1} (unknown) (Found: C, 58.8; H, 5.5; O, CH₃, 19.0. $\text{C}_{24}\text{H}_{26}\text{O}_{11}$ requires C, 58.8; H, 5.3; O, CH₃, 19.0%). The compound had R_F values 0.28 (water), 0.52 [aq. 15% (v/v) acetic acid], 0.82 [aq. 60% (v/v) acetic acid], 0.72 [butan-1-ol–acetic acid–water (20:5:11, by vol.)] and 0.97 [aq. 75% (w/w) phenol].

Acetylation of the compound (28 mg.) with acetic anhydride (0.2 ml.) and pyridine (0.1 ml.) at about 20° for 12 hr. yielded a white crystalline acetate (19 mg.), which after two recrystallizations from ethanol had m.p. 179° (Found: C, 58.1; H, 5.0; O, CH₃, 13.7; CO, CH₃, 29.2. $\text{C}_{34}\text{H}_{36}\text{O}_{16}$ requires C, 58.3; H, 5.1; O, CH₃, 13.3; CO, CH₃, 30.7%).

Permanganate oxidation of orientin and iso-orientin trimethyl ethers. The compound (50 mg.) was suspended in water (3.0 ml.) and aq. 5% (w/v) NaOH soln. (0.5 ml.) was added. The mixture was heated to boiling point, removed from the flame and a solution of KMnO_4 (0.2 g.) in water (2.0 ml.) was slowly added. The mixture was set aside for 10 min., ethanol (about 3 drops) was added and the MnO_2 was removed by filtration. The filtrate was acidified with aq. HCl and extracted twice with ether (5 ml.). Evaporation of the ether left a crystalline solid (about 6 mg.), which was purified by micro-sublimation under reduced pressure. The purified compound had m.p. 182°, unchanged by admixture with an authentic sample of veratric acid. In addition, the infrared spectra of the compound and of veratric acid were identical over the range 2–15 μ .

Interconversion of orientin and iso-orientin trimethyl ethers. The compound (5 mg.) in ethanol (1 ml.) was refluxed with aq. 2N-HCl (2 ml.) for 1 hr. The solution was cooled and extracted with pentan-1-ol, and the extract was examined by paper chromatography. In each case partial isomerization of the trimethyl ether was observed. The compound formed from 3',4',7-tri-*O*-methylorientin was identical with 3',4',7-tri-*O*-methyliso-orientin by chromatography in water, aq. 15% (v/v) acetic acid, aq. 60% (v/v) acetic acid, butan-1-ol-acetic acid-water (20:5:11, by vol.) and aq. 75% (w/w) phenol. Similarly, the compound formed from 3',4',7-tri-*O*-methyliso-orientin was identical with 3',4',7-tri-*O*-methylorientin in all these solvent systems. Under the above conditions interconversion of orientin and iso-orientin also took place but no interconversion of the 3',4',5,7-tetra-*O*-methyl derivatives could be detected, even after more prolonged acid treatment.

Nuclear-magnetic-resonance spectra of orientin, iso-orientin and various derivatives

The n.m.r. spectra were recorded by Dr K. G. R. Pachler, Chemical Physics Group, C.S.I.R., Pretoria, on a Varian A-60 spectrometer with dimethyl sulphoxide as solvent and tetramethylsilane as internal standard ($\delta = 0.00$ p.p.m.).

The sample concentration was 30–100 mg. to 0.5 ml. of solution. Band positions are expressed as p.p.m. downfield with the standard as origin. The results are summarized in Table 1 and are compared with published data for related compounds.

DISCUSSION

The finding that the tetramethyl ethers of orientin and iso-orientin each consumed 2 mol. of periodic acid/mol. was incompatible with the structures originally proposed for these compounds (Hörhammer, Wagner, Nieschlag & Wildi, 1959). The results suggested further that orientin and iso-orientin were isomeric and analyses of the parent compounds and various derivatives supported this view (Koeppen, 1962). The formation in each case of 1 mol. of formic acid/mol. of tetramethyl ether on periodic acid oxidation of these compounds and the liberation in each case of glycerol on hydrolysis of the periodate-oxidized borohydride-reduced tetramethyl ethers thereupon established that both compounds possess hexopyranosyl residues (cf. Smith & Van Cleve, 1955). The compatibility of these findings with the *C*-glycosyl nature of orientin and iso-orientin has been discussed (Koeppen, 1965a). The final identity of the hexopyranosyl residues of orientin and iso-orientin was established by the detection of D-glucose as a product of ferric chloride oxidation of the tetramethyl ethers, the co-occurrence of D-arabinose being due to oxidative cleavage of the $\text{C}_{(1)}-\text{C}_{(2)}$ bond of the glycosyl residue. As D-arabinose has been isolated as the major water-soluble product of the ferric chloride oxidation of barbaloin (Hay & Haynes, 1956) and as only traces of glucose could be detected on oxidation of this compound under the conditions

Table 1. δ values for orientin, iso-orientin, related compounds and various derivatives in dimethyl sulphoxide

Compound	δ (p.p.m.)			
	5-OH	3-H	6-H	8-H
Orientin	13.20	6.67	6.30	—
Iso-orientin	13.62	6.70	—	6.54
Vitexin (Horowitz & Gentili, 1964)	—	6.77	6.29	—
Vitexin (Batterham & Highet, 1964)	13.20	6.80	6.28	—
Isovitexin (Horowitz & Gentili, 1964)	—	6.77	—	6.56
Apigenin (Horowitz & Gentili, 1964)	—	6.76	6.24*	6.52*
3',4',7-Tri- <i>O</i> -methylorientin	13.29	6.97	6.49	—
3',4',7-Tri- <i>O</i> -methyliso-orientin	13.47	6.97	—	6.81
4',7-Di- <i>O</i> -methylapigenin† (Lamberton, 1964)	12.92	6.55	6.36*	6.46*
3',4',5,7-Tetra- <i>O</i> -methylorientin	—	6.70	6.57	—
3',4',5,7-Tetra- <i>O</i> -methyliso-orientin	—	6.78	—	7.12
4',5,7-Tri- <i>O</i> -methylapigenin† (Horowitz & Gentili, 1964)	—	6.50	6.30*	6.48*
3',4',5,7-Tetra- <i>O</i> -acetyl-luteolin†	—	6.62	6.88*	7.37*

* Doublet ($J_{6,8} = 2.5$ cyc./sec.).

† Deuteriochloroform used as solvent in these cases.

employed in these Laboratories, it would appear that the glycosyl-aglycone bond is more stable in barbaloin than in orientin or iso-orientin.

As orientin and iso-orientin have both been shown to contain luteolin nuclei (Hörhammer, Wagner & Glogengiesser, 1958; Seikel & Bushnell, 1959; Koeppen *et al.* 1962) and as both contain glucopyranosyl residues, it seemed reasonable to expect that they might differ in the position of attachment of the glucosyl residue to the luteolin nucleus and that their interconvertibility might therefore be due to a Wessely-Moser rearrangement of the flavone nucleus. Such a rearrangement would require the presence of a free 5-hydroxyl group, and the possibility of such a mechanism being operative for the orientin-iso-orientin interconversion is supported by the finding that the 3',4',5,7-tetra-methyl ethers are not interconvertible whereas the 3',4',7-trimethyl ethers are. That the tri-*O*-methyl derivatives prepared are indeed the 3',4',7-trimethyl ethers is confirmed by the following considerations. (1) Permanganate oxidation of the trimethyl ethers yields veratric acid in each case. Thus the 3'- and 4'-hydroxyl groups are methylated. (2) The positions of the ultraviolet-absorption maxima remain unaltered on the addition of fused sodium acetate to ethanolic solutions of the trimethyl ethers. This indicates blockage of the 7-hydroxyl group in each compound (Jurd & Horowitz, 1957). In addition, the spectra of the compounds in sodium ethoxide solution are typical of flavones whose only free hydroxyl group is in the 5-position (Jurd, 1962). (3) The strongly positive brown ferric chloride reactions of the trimethyl ethers indicates the presence of a free 5-hydroxyl group in each case, and the ability of the compounds to form aluminium chelates that fluoresce brightly under ultraviolet light indicates the presence of a *peri*-hydroxycarbonyl grouping in each compound (cf. Koeppen, 1965b). (4) The n.m.r. spectra show a signal at $\delta = 13.2-13.5$ p.p.m. due to a strongly hydrogen-bonded hydroxyl proton in each compound.

The n.m.r. spectra of orientin, iso-orientin and various derivatives (Table 1) support the identification of these compounds as ring-isomeric C-glycosylflavones.

As with vitexin and isovitexin (Horowitz & Gentili, 1964), only one A-ring proton was observed in the spectra of orientin and iso-orientin. In each case this appeared as a simple unsplit signal, indicating the absence of a coupled *meta*-proton. By contrast, the coupled 6- and 8-protons of luteolin tetra-acetate and of apigenin and its methyl ethers appeared as doublets with $J_{6,8} = 2.5$ cyc./sec as expected. These observations support the chemical evidence that the positions *meta* to the A-ring protons in orientin and in iso-orientin are substituted.

Although chemical methods have established unequivocally that the glycosyl residue of vitexin is attached to the 8-position of the flavone nucleus (Evans, McGookin, Jurd, Robertson & Williamson, 1957), all attempts to apply these methods to the determination of the position of the glycosyl residues in orientin and in iso-orientin have so far been without success. However, the solubility and chromatographic properties of orientin strongly suggested that it was more closely related to vitexin than was iso-orientin, and it was accordingly proposed (Koeppen, 1964) that orientin was the 8- and iso-orientin the 6-substituted flavone. The n.m.r. data presented in Table 1 support this view. Thus the spectrum of orientin corresponds closely to that of vitexin, and, similarly, the spectra of isovitexin and iso-orientin are in close agreement, particularly with regard to the resonance position of the A-ring benzenoid proton in each case. In compounds such as apigenin and luteolin tetra-acetate, which possess protons in both the 6- and the 8-positions, the upfield doublet of the characteristic two-doublet pattern obtained has been assigned to the 6-proton (Massicot & Marthe, 1962). This is supported by comparison of the spectra of vitexin and isovitexin with these compounds, the 6-proton of vitexin appearing at higher field than the 8-proton of iso-vitexin. Similarly, the chemical and physical properties of a compound isolated from *Eucalyptus* waxes by Lamberton (1964) indicated that the compound was 5-hydroxy-4',7-dimethoxy-6-methylflavone and not the 8-*C*-methyl analogue. Thus a signal at $\delta = 6.46$ p.p.m. in the n.m.r. spectrum of this compound in deuteriochloroform was ascribed to the 8-proton and this was confirmed by the appearance of an additional signal at higher field ($\delta = 6.37$ p.p.m.) in a Wessely-Moser rearrangement mixture containing the 6- and 8-*C*-methylflavone. In orientin, the benzenoid A-ring proton appears upfield from that in iso-orientin, and the same relative positions of the protons are observed when comparing 3',4',7-tri-*O*-methyl-orientin with 3',4',7-tri-*O*-methyliso-orientin and 3',4',5,7-tetra-*O*-methylorientin with 3',4',5,7-tetra-*O*-methyliso-orientin. Thus comparison of the n.m.r. spectra of the parent compounds and their tri- and tetra-*O*-methyl derivatives all indicate that orientin is the 8- and iso-orientin the 6-substituted flavone. Further evidence to this effect is provided by comparison of the position of the 5-hydroxyl proton resonance in orientin, iso-orientin and their 3',4',7-tri-*O*-methyl derivatives. Thus Batterham & Highet (1964) observed that the low-field resonance of the strongly hydrogen-bonded 5-hydroxyl proton in flavones was shifted to even lower field by substitution of a glycosyl residue in the *para*-position as in vitexin. It might therefore be expected that substitution of such a residue in the

ortho-position might result in even greater unshielding of the 5-hydroxyl proton and this indeed appears to be the case. Thus the 5-hydroxyl proton of orientin is observed at $\delta = 13.20$ p.p.m., the same position as in vitexin, whereas in iso-orientin it appears further downfield ($\delta = 13.62$ p.p.m.). Similarly, in 3',4',7-tri-*O*-methyliso-orientin this signal appears at lower field than in 3',4',7-tri-*O*-methyl-orientin.

The benzylic proton referred to by Horowitz & Gentili (1964) appears in the same position for orientin ($\delta = 4.75$ p.p.m.) and iso-orientin ($\delta = 4.67$ p.p.m.) as for vitexin and iso-vitexin respectively. In each case it is recognizable as a doublet

($J = 10.0$ cyc./sec.), thereby indicating that orientin and iso-orientin, like vitexin, possess β -*C*-glucopyranosyl residues.

By chemical and spectral evidence orientin is therefore formulated as 8-*C*- β -D-glucopyranosyl-3',4',5,7-tetrahydroxyflavone (I) and iso-orientin as the 6-substituted luteolin analogue (II).

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