Phytochemistry, 1968, Vol. 7, pp. 51 to 56. Pergamon Press. Printed in England

BIOSYNTHESIS OF FLAVONOIDS—XV.*

OCCURRENCE AND BIOSYNTHESIS OF FLAVONOIDS IN *DATISCA CANNABINA*

H. GRISEBACH and H. J. GRAMBOW

Department of Plant Biochemistry, University of Freiburg i.Br., Germany†

(*Received* 21 *June* 1967)

Abstract—The major flavonoids in *Datisca cannabina* L. were characterized as datiscetin 3-rutinoside and galangin 3-rutinoside. The incorporation of 2,2′,4′,6′-tetrahydroxychalcone-4′-glucoside-[β-^{14}C] (I), cinnamic acid-[β-^{14}C] (II) and *o*-hydroxycinnamic acid-[β-^{14}C] (III) into both flavonoids was compared. The dilution of radioactivity upon incorporation of (I), (II) and (III) into datiscetin was in the ratio of 1:3:116. This result proves that the 2′-hydroxyl group in the flavonoid is not introduced until after formation of the chalcone. (II) is a good precursor for galangin whereas the incorporation of (I) and (III) into galangin is negligible. In both cases the glycosides had a higher specific activity than the aglycones.

INTRODUCTION

IN AN earlier investigation we compared the incorporation of 4,2′,4′,6′-tetrahydroxychalcone-2′-glucoside-[β-^{14}C] and 3,4,2′,4′,6′-pentahydroxychalcone-2′-glucoside-[β-^{14}C] into quercetin and cyanidin in buckwheat seedlings.[1] The incorporation rates of both chalcones into the flavonoids and the dilution values were about equal. We interpreted these findings to mean that the 3′-hydroxyl group is not introduced until after formation of the chalcone intermediate. We later found in the same plant that dihydrokaempferol but not kaempferol is a good substrate for the introduction of the 3′-hydroxyl group.[2] On the other hand, Hess has shown that the substitution pattern in ring-B of the anthocyanins in *Petunia hybrida* is probably already determined at the cinnamic acid stage.[3] In support of this assumption is the result that, in *Campanula medium*, 3,4,5-trihydroxycinnamic acid-[α-^{14}C] is a better precursor for delphinidin than *p*-coumaric or caffeic acid.[4]

To investigate the question of the origin of the oxidation pattern in ring B of the flavonoids further it seemed advantageous to study the biosynthesis of a flavonoid with an unusual substitution pattern in this ring. We have therefore compared the incorporation of cinnamic-, *o*-hydroxycinnamic and 2,2′,4′,6′-tetrahydroxychalcone-4′-glucoside-[β-^{14}C] into datiscetin (3,5,7,2′-tetrahydroxyflavone) in *Datisca cannabina* L.; and the incorporation of the same precursors into galangin (3,5,7-trihydroxyflavone), which we have found to be present in the same plant.

* Part XIV: H. GRISEBACH and W. BILHUBER, *Z. Naturforsch.* **22b**, 746 (1967).

† Address: Chemisches Laboratorium d. Universität, 78 Freiburg i.Br., Albertstraße 21.

[1] L. PATSCHKE and H. GRISEBACH, *Z. Naturforsch.* **20b**, 1039 (1965).

[2] L. PATSCHKE, W. BARZ and H. GRISEBACH, *Z. Naturforsch.* **21b**, 45 (1966).

[3] D. HESS, *Planta* **60**, 568 (1964); *Z. Pflanzenphysiol.* **55**, 374 (1966).

[4] H. MEIER and M. H. ZENK, *Z. Pflanzenphysiol.* **53**, 415 (1965).

RESULTS

Isolation of Flavonoids from and Identification of Galangin in Datisca cannabina

The presence of a glycoside of datiscetin (datiscin) in *Datisca cannabina* was reported.[5] Charaux[6] identified the sugar components as rhamnose and glucose, and since datiscin and rutin were both hydrolyzed by an enzyme from seeds of *Rhamnus utilis* to the aglycone and an identical amorphous product he concluded that datiscin was a datiscetin rutinoside. The position of attachment of the sugar was not determined. Because of some differences in the chemical properties of datiscetin isolated by different workers, Kalff and Robinson[7] suspected that the compound isolated by Schunck and Marchlewski[5] was a mixture of datiscetin and galangin.

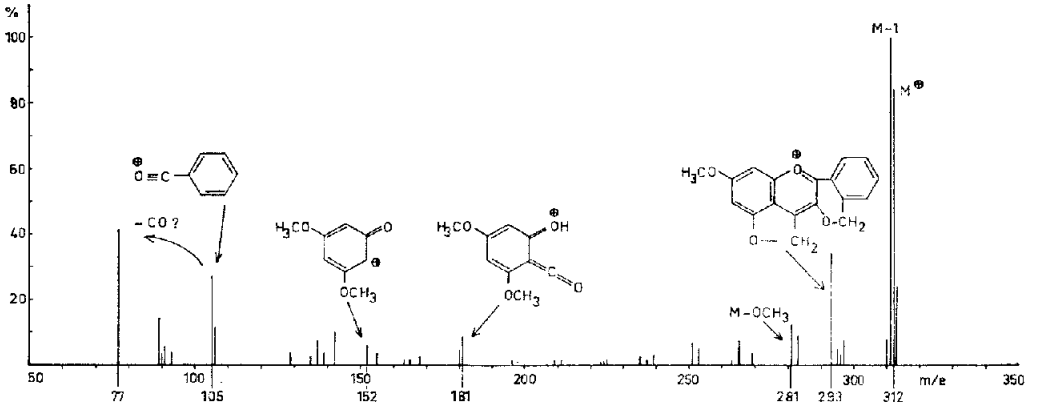

FIG. 1. MASS SPECTRUM OF GALANGIN TRIMETHYLETHER. ATLAS CH 4.

TABLE 1. SPECTRAL PROPERTIES OF FLAVONOIDS FROM *Datisca cannabina*

	λ_{max} nm (log ϵ) in MeOH	λ_{max} nm band I after addition of NaOAc
Galangin	267·5 (4·23); 360 (4·07)	—
Galangin 3-rutinoside	265 (4·23); 315[i] (3·91); 340[i] (3·88)	270
Datiscetin	262·5 (4·14); 360 (3·99)	—
Datiscin	259·5 (4·30); 300 (3·90); 330[i] (3·86)	264·5

i = inflexion.

Fresh roots of 3-yr-old blooming *Datisca* plants were frozen with liquid nitrogen and pulverized in a mortar, and then the powder was immediately put into boiling methanol. The paper chromatogram of the methanolic extract with 10 per cent acetic acid showed five

[5] J. STENHOUSE, *J. Ann. Ch. Pharm.* **98**, 166 (1855); E. SCHUNCK and L. MARCHLEWSKI, *Liebigs Ann. Chem.* **277**, 261 (1893); L. LESKIEWICZ and L. MARCHLEWSKI, *Ber. Dtsch. Chem. Ges.* **47**, 1599 (1914).

[6] C. CHARAUX, *Compt. Rend.* **180**, 1419 (1925); compare G. ZEMPLÉN and A. GERES, *Ber. Dtsch. Chem. Ges.* **68**, 1318 (1935).

[7] J. KALFF and R. ROBINSON, *J. Chem. Soc.* **127**, 1968 (1925).

compounds, which were further purified on paper with the same solvent system. The compound with R_f 0·15–0·2 proved to be datiscetin by comparison with a synthetic sample.[7]

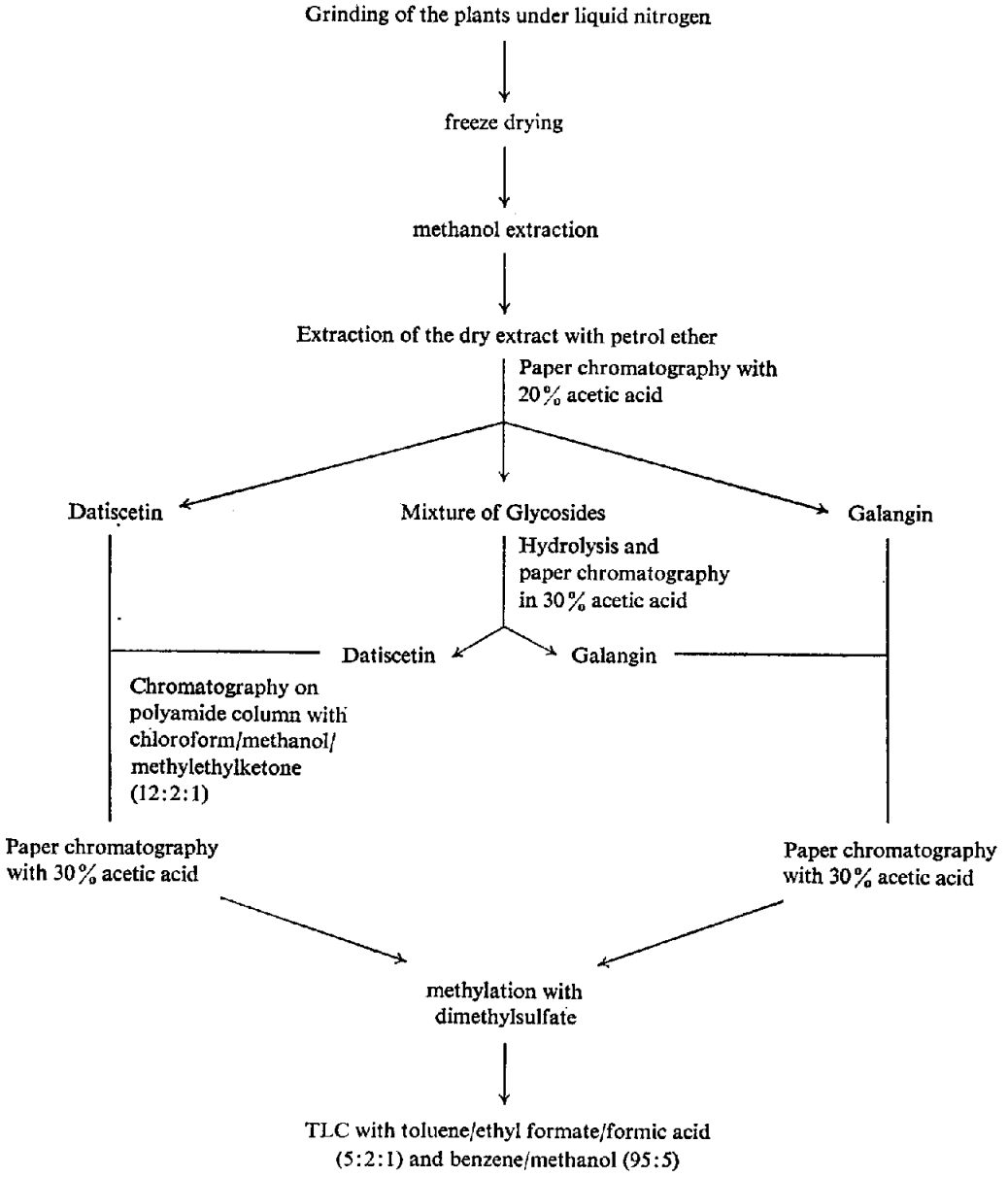

FIG. 2. SCHEME OF PURIFICATION OF THE FLAVONOIDS FROM *Datisca cannabina*.

The compound with R_f 0·08 was shown to be galangin by the mass spectrum of its tri-methyl ether (Fig. 1) and by comparison with a commercial sample. The compounds with R_f 0·75–0·85 and 0·65–0·75 proved to be glycosides of datiscetin and galangin respectively. In both cases glucose and rhamnose were identified by paper chromatography after hydrolysis with

methanol/2 N HCl (1:1). The u.v. spectra of datiscetin, galangin and its glycosides are recorded in Table 1.

The bathochromic shift of 5 nm of the short wave maximum upon addition of sodium acetate proves that the sugars cannot be attached to the 7-hydroxyl group.[8] Both glycosides showed orange-yellow fluorescence in u.v. after treatment with $ZrOCl_2$ which was quenched by citric acid.[9] The glycosides are therefore datiscetin and galangin 3-rutinosides. The compound with R_f 0·6–0·65 was present only in very small concentration; its u.v. spectrum showed no maxima between 230 and 380 nm.

The isolation procedure for the flavonoids of *Datisca* used in the tracer experiments is shown in Fig. 2. Even under the mildest extraction conditions or when the cell sap was directly applied to the paper, datiscetin was always found along with datiscin.

Incorporation of Cinnamic, o-Hydroxycinnamic Acid-[β-^{14}C] and 2,2′,4′,6′-Tetrahydroxychalcone-4′-Glucoside-[β-^{14}C] into Datiscetin and Galangin

Three-month-old *Datisca* plants were used for the tracer experiments. 10·66 μmol of each labeled compound in the form of the soldium salt was dissolved in water and the aqueous solution ($0{\cdot}5 \times 10^{-4}$ M) was taken up by the plants through the roots during 48 hr and under continuous illumination. During this time more than 99 per cent of the compounds were taken up by the plants. Datiscetin and galangin were isolated according to the procedure outlined in Fig. 2. Table 2 shows the incorporation rates into datiscetin and galangin glycosides and the dilution values with the 3 precursors. In Table 3, separate values for glycosides and aglycones are recorded.

TABLE 2. INCORPORATION OF DIFFERENT PRECURSORS INTO DATISCETIN AND GALANGIN IN *Datisca cannabina*

Datiscetin*			
Precursor	Spec. activity (dpm/mmole)	Dilution†	Incorporation Rate (%)
2,2′,4′,6′-Tetrahydroxychalcone-4′-glucoside-[β-^{14}C] (I)	$1{\cdot}77 \times 10^6$	1 728	0·71
Cinnamic acid-[3-^{14}C] (II)	$3{\cdot}33 \times 10^5$	5 123	0·35
o-Coumaric acid-[3-^{14}C] (III)	$2{\cdot}54 \times 10^4$	200 640	0·03
Galangin*			
Precursor			
(I)	0	—	No incorporation
(II)	$3{\cdot}46 \times 10^5$	4 920	0·05
(III)	$0{\cdot}32 \times 10^5$	160 000	0·003

* Isolated as glycoside from the leaves.

† $\frac{\text{Spec. activity precursor}}{\text{Spec. activity product}}$.

[8] L. JURD and R. M. HOROWITZ, *J. Org. Chem.* **22**, 1618 (1957).
[9] L. HÖRHAMMER and R. HÄNSEL, *Arch. Pharm.* **286**, 424 (1953).

TABLE 3. INCORPORATION OF DIFFERENT PRECURSORS INTO DATISCETIN AND ITS GLYCOSIDE

Precursor	Product	Spec. activity (dpm/mmole)	Dilution	Corrected* dilution	Incorporation rate (%)
(I)†	Aglycone, leaves	$9{\cdot}29 \times 10^5$	3 443	27 420	0·02
	Glycoside, leaves	$1{\cdot}77 \times 10^6$	1 728	1 728	0·7
(II)	Aglycone, leaves	$2{\cdot}31 \times 10^5$	7 385	18 465	0·17
	Aglycone, roots	5×10^5	3 386	28 440	0·02
	Glycoside, leaves	$3{\cdot}33 \times 10^5$	5 123	5 123	0·35
	Glycoside, roots	$4{\cdot}2 \times 10^5$	4 069	4 069	0·10
(III)	Aglycone, leaves	$1{\cdot}7 \times 10^4$	294 750	1 625 000	0·003
	Glycoside, leaves	$2{\cdot}4 \times 10^4$	200 640	200 640	0·026

* Corrected for equal concentration of aglycone and glycoside.
† See Table 2.

DISCUSSION

The results demonstrate clearly that 2,2′,4′,6′-tetrahydroxychalcone (5,7,2′-trihydroxyflavanone) is the best precursor for datiscetin. The incorporation of cinnamic acid into datiscetin is also quite good whereas *o*-hydroxycinnamic acid is a very poor precursor. Therefore, the introduction of the 2′-hydroxyl group must occur at or after the chalcone/flavanone intermediate and not at the cinnamic acid stage. If this result is valid for all 2′-hydroxyflavonoids and compounds biogenetically derived from them, the pathways to 2′-hydroxyflavonoids and coumarins[10] would diverge at the cinnamic or *p*-coumaric acid stage (Fig. 3).

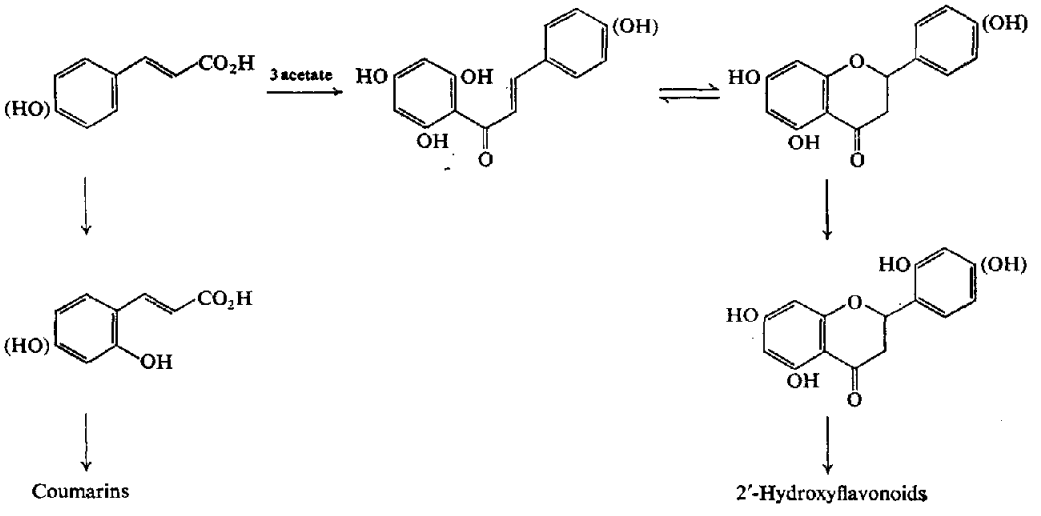

FIG. 3. POSTULATED BIOGENETIC RELATIONSHIP BETWEEN COUMARINS AND 2′-HYDROXYFLAVONOIDS.

[10] S. A. BROWN in *Biosynthesis of Aromatic Compounds*, (edited by G. BILLEK), Vol. 3, p. 15. (Proc. 2nd Mtg. Fed. Eur. Biochem. Soc.), Pergamon Press, Oxford (1966).

The incorporation rates into the glycosides are in all cases higher than into the aglycones and the dilution values are lower. For the dilution values this becomes more significant if one corrects the figures for the different pool sizes of aglycones and glycosides. The similar finding that the flavonoid glycosides of *Prunus mahaleb* had a much lower dilution value than the corresponding aglycones with cinnamic acid-[β-^{14}C] as precursor led Ville and Pachéco[11] to the conclusion that the glycosides were precursors of the aglycones. However, one must be careful in the interpretation of such results because glycosides and aglycones might be present in different compartments in the plant.

From the result that only cinnamic acid is incorporated to a significant extent into galangin it can be concluded that *Datisca* is not able to remove the hydroxyl group in the *o*-position of cinnamic acid or the 2-position of the chalcone (or 2′ of the corresponding flavanone).

EXPERIMENTAL

Labeled Compounds

Cinnamic acid-[β-^{14}C][12] and *o*-coumaric acid-[β-^{14}C][13] had previously been synthesized in our laboratory. *2,2′,4′,6′-Tetrahydroxychalcone-4′-glucoside-[β-^{14}C].* The synthesis described below was first carried out on a preparative scale with unlabeled salicylaldehyde to characterize the compound. The chalcone glycoside crystallized from water with 2 moles of water. (Found: C, 53·92; H, 5·64. $C_{12}H_{22}O_{10}\cdot 2H_2O$ (470·3) required: C, 53·62; H, 5·52%). Ultraviolet spectrum nm (logε) 367 (4·26), 318 (4·08), 253 (3·7). Synthesis of the labeled product was as follows: A solution of *o*-hydroxybenzaldehyde-[carbonyl-^{14}C][13] (prepared from 198 mg (0·93 mmole) *o*-benzyloxybenzaldehyde-[carbonyl-^{14}C]-semicarbazone)[13] in 0·6 ml ethanol was added to a solution of 270 mg (0·54 mmole) 4-tetraacetylglucosidophloracetophenone[14] in 0·12 ml ethanol and 0·8 ml 60% KOH and heated for 10 min to 70–90° under N_2. The solution was cooled in ice/salt and acidified to pH 5 with 15% HCl, and then the solvent removed *in vacuo*. The chalcone glucoside was dissolved in methanol and purified by TLC on prewashed (methanolic HCl) silica gel plates with ethyl acetate/methylethylketone /formic acid/H_2O(5 : 3 : 1 : 1); chalcone glucoside $R_f = 0{\cdot}4$; chalcone $R_f = 0{\cdot}85$. This purification was repeated. The yield (u.v., E_{367}) was 8·13 mg (3% based on the semicarbazone) with a specific activity of $3{\cdot}11 \times 10^9$ dpm/mmole $= 1{\cdot}40$ mc/mmole.

Datiscetin Tetramethylether

100 mg Datiscetin in 40 ml acetone was boiled under reflux with 3 g dry K_2CO_3 and 5 ml dimethyl sulfate for 5 hr and, after addition of another 3 ml dimethyl sulfate, for another 3 hr. After filtration the solution was concentrated *in vacuo* to 5 ml and hot water was added until the solution became turbid. The methyl ether crystallized in colourless needles. Yield 108 mg (85%). The compound was further purified by re-crystallization from acetone–water and TLC (Fig. 2), m.p. 154° (corr.). (Found: C, 6·41; H, 5·49. $C_{19}H_{18}O_6$ (342·3) required: C, 66·66; H, 5·29%.)

Galangin Trimethylether[15]

This compound was obtained in the same way as the datiscetin tetramethylether. Yield 68%, m.p. 200–201° (corr.).

Acknowledgements—This research was supported by Deutsche Forschungsgemeinschaft and by Fonds der Chemischen Industrie.

[11] A. VILLE and H. PACHÉCO, *Compt. Rend.* **261**, 753 (1965); A. VILLE, Doctoral Thesis, University of Lyon (1966).
[12] S. H. BROWN and A. C. NEISH, *Can. J. Biochem. Physiol.* **33**, 948 (1955); H. C. BROWN and B. C. SUBBA RAO, *J. Am. Chem. Soc.* **80**, 5377 (1958).
[13] K. O. VOLLMER and H. GRISEBACH, *Z. Naturforsch.* **21b**, 435 (1966).
[14] G. ZEMPLÉN and R. BOGNAR, *Ber. Dtsch. Chem. Ges.* **75**, 645, 1041 (1942).
[15] G. LINDSTEDT, *Acta Chem. Scand.* **4**, 772 (1950).