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STUDIES ON GLUCOSIDE INTERMEDIATES IN UMBELLIFERONE BIOSYNTHESIS

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Abstract—Two umbelliferone-producing glucosides have been found in *Hydrangea macrophylla* leaf extracts. By chromatographic characterization and comparison with the synthetic compounds, they have been identified as skimmin (7-β-D-glucosyloxycoumarin) and *cis*-2,4-di-β-D-glucosyloxycinnamic acid. Skimmin is by far the major component. Both of these compounds are intermediates in a proposed scheme for umbelliferone biosynthesis consistent with the results of tracer studies. A new technique was developed involving thin-layer chromatography on cellulose powder which has made possible the separation of diglucosides, monoglucosides and phenolic aglycones. A rationale for the observed chemical properties of *o*-glucosyloxycinnamic acids is discussed.

INTRODUCTION

RECENT work has shown that certain coumarins occur in plants predominantly or totally as glucosides (Fig. 1).[1-4] The involvement of glucosides appears to be unique to the biosynthesis of coumarins (see[5]) but very little evidence has been produced to define their role in the biosynthetic scheme. In the biosynthetic route proposed for coumarin, it has been

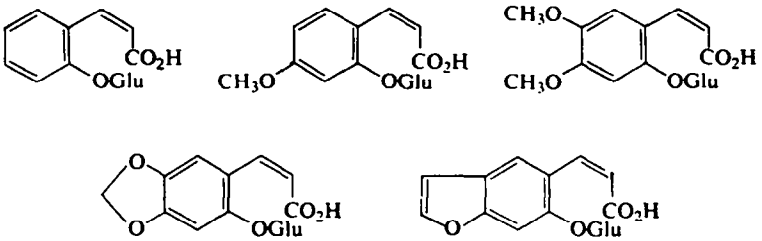

FIG. 1. NATURALLY-OCCURRING COUMARINIC ACID GLUCOSIDES.

suggested that, after *ortho*-hydroxylation of *trans*-cinnamic acid, glucoside formation is necessary to effect the *trans-cis* inversion which must precede the formation of the coumarin lactone ring.[6] We now wish to present the results of our investigations on the nature of the umbelliferone-producing glucosides in *Hydrangea macrophylla* and their function in the biosynthetic sequence.

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[1] F. A. HASKINS and H. J. GORZ, *Crop Sci.* **1**, 320 (1961); G. KAHNT and W. J. SCHÖN, *Angew. Botan.* **36**, 33 (1962).
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[4] A. STOLL, A. PEREIRA and J. RENZ, *Helv. Chim. Acta* **33**, 1637 (1950).
[5] E. E. CONN in *Biochemistry of Phenolic Compounds*, pp. 422–424. (Edited by J. B. HARBORNE) Academic Press, London (1964).
[6] S. A. BROWN, *Can. J. Biochem. Physiol.* **40**, 607 (1962).

RESULTS AND DISCUSSION

In a preliminary communication, we stated that paper chromatograms of hydrangea leaf extracts had not revealed the presence of skimmin (7-β-D-glucosyloxycoumarin).[7] However, the presence of interfering fluorescent substances led to doubts about the validity of this result. An improved separation of hydrangea extracts could be obtained by thin-layer chromatography on unbound cellulose powder with 5% aqueous acetic acid which gave one spot (R_f 0·82) yielding umbelliferone on hydrolysis. However, chromatography of a series of standard cinnamic glucosides in this system (Table 1) showed that all of these compounds have similar R_f values, thus prohibiting the unequivocal identification of the natural umbelliferone glucoside. The ideal solvent for the required separation was found to be the upper phase of *n*-amyl alcohol:acetic acid:water (4:1:5) referred to as AAW. Using this solvent with cellulose powder results in excellent separation of diglucosides, monoglucosides and phenolic aglycones.

TABLE 1 R_f VALUES OF OXYGENATED CINNAMIC ACID DERIVATIVES

	2% HAc on paper	5% HAc on cellulose powder plates	AAW on cellulose powder plates	Fluorescence (350 mμ)*
Umbellic acid	0·22	0·29	0·81	purple
Umbelliferone	0·45	0·59	0·90	blue-purple
o-glucosyloxy-cinnamic acid	0·65	0·76	0·47	— (*a*)
p-glucosyloxy-cinnamic acid	0·64	0·80	0·35	— (*b*)
7-glucosyloxy-coumarin	0·74	0·82	0·28	purple
7-cellobiosyloxy-coumarin	0·68	0·79	0·05	weak purple
trans-DGC†	0·70	0·81	0·035	— (*c*)
cis-DGC†	0·70	0·78	0·035	— (*c*)

* (*a*) Yellow fluorescence in light at 350 mμ after hydrolysis.
(*b*) Purple fluorescence in light at 350 mμ after hydrolysis and exposure to ammonia vapour.
(*c*) Blue-purple fluorescence in light at 350 mμ after hydrolysis.
The compounds which do not fluoresce in light at 350 mμ all exhibit quenching at 254 mμ.

† 2,4-di-β-D-glucosyloxycinnamic acid.

If the biosynthetic scheme for umbelliferone is analogous to those proposed for coumarin[6,8] and herniarin,[2] any one or all of the four glucosides shown in Fig. 2 may be present as the "bound" forms of umbelliferone. Two reports have been made of the isolation of a crystalline compound from *Hydrangea paniculata*[9] and *Hydrangea macrophylla* (var. Hortensia)[10] which yields umbelliferone and two molecules of glucose on acid hydrolysis. This material which was named *neohydrangin* appears to be the principal source of umbelliferone in these species of hydrangea, and it is important to determine its structure. It may be either a disaccharide analogue of skimmin or a diglucoside of *cis*-2,4-dihydroxycinnamic acid (*cis*-DGC). The latter structure is an analogue of *cis*-*o*-glucosyloxycinnamic acid and *cis*-2-glucosyloxy-4-methoxycinnamic acid which are known to be the "bound" forms of coumarin[8] and herniarin[2] respectively. Unfortunately we were unable to isolate a crystalline

[7] D. J. AUSTIN and M. B. MEYERS, *Tetrahedron Letters* 765 (1964).
[8] (*a*) T. KOSUGE and E. E. CONN, *J. Biol. Chem.* **236**, 1617 (1961); (*b*) J. R. STOKER and D. M. BELLIS, *J. Biol. Chem.* **237**, 2303 (1962).
[9] H. NAKAHARA, *Chem. Abstr.* **50**, 4926 (1956).
[10] V. PLOUVIER, *Compt. Rend.* **252**, 312 (1961).

glucoside from our hydrangea extracts and were unable to obtain a sample of neohydrangin for comparison purposes.

It was decided, therefore, to undertake the synthesis of *7-cellobiosyloxycoumarin* and *cis*-2,4-di-β-D-*glucosyloxycinnamic acid* in order to compare their chromatographic and chemical properties with the umbelliferone glucosides present in hydrangea.

7-Cellobiosyloxycoumarin was prepared in a straightforward manner by treatment of umbelliferone with acetobromocellobiose in the presence of quinoline and silver oxide. The hepta-acetate so obtained was de-esterified with sodium methoxide. The crystalline compound had the correct analysis for the desired structure and both its i.r. and u.v. spectra were closely similar to those of skimmin (7-β-D-glucosyloxycoumarin). The cellobioside, when chromatographed on paper or cellulose powder thin-layer plates, showed only one spot with a purple fluorescence similar to that of skimmin in u.v. light (350 mμ). It is quickly transformed to umbelliferone when treated with almond emulsing, but more slowly than skimmin which undergoes almost instantaneous hydrolysis on emulsin treatment.

The synthesis of *cis*-2,4-di-β-D-glucosyloxycinnamic acid (*cis*-DGC) presented a more difficult problem. Reaction of umbellic acid with diazomethane gave the methyl ester in good yield. The ester was treated with acetobromoglucose, silver oxide and quinoline to give

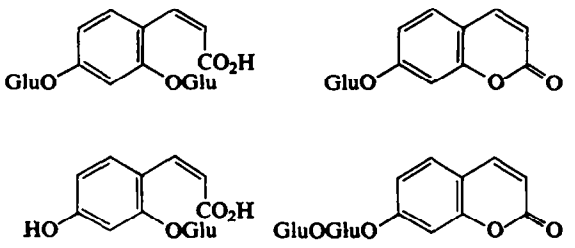

FIG. 2. POSSIBLE "BOUND" FORMS OF UMBELLIFERONE.

an amorphous substance which had the correct analysis for the octa-acetate. Removal of the blocking groups was accomplished by shaking the compound with barium hydroxide solution to produce a methanol and water soluble glass, analysing well for a trihydrate of the anticipated structure.

Paper and thin-layer chromatography of this material with aqueous acetic acid and AAW produced essentially one spot which did not show fluorescence in light at 350 mμ, and exhibited quenching at 254 mμ. The analogous *o*-coumarinylglucoside has similar fluorescence properties.[11] After separating the product with 5% aqueous acetic acid on cellulose powder, it was subjected on the plate to emulsin hydrolysis for 2 hr. Elution in the second dimension with the same solvent showed two spots (R_f values 0·26 and 0·58) with blue-purple fluorescence in light at 350 mμ. The material of R_f 0·26 was by far the major component of the mixture and corresponded in all respects to umbellic acid. That with R_f 0·58 was identified as umbelliferone. This showed that the synthetic diglucoside was predominantly *trans*-DGC with a small amount of the *cis* isomer present. In an attempt to obtain pure *cis*-DGC, a methanolic solution of the synthetic diglucoside was irradiated with u.v. light from a mercury vapour lamp for 1 hr. Thin-layer chromatography of the product with the AAW solvent gave rise to two spots (R_f values 0·04 and 0·27). The material with R_f 0·27 exhibited a purple

[11] T. KOSUGE, *Arch. Biochem. Biophys.* **95**, 211 (1961).

fluorescence in light at 350 mμ and was conclusively identified as skimmin. An analogous lactonization of *cis*-*o*-glucosyloxycinnamic acid to coumarin as a result of u.v. irradiation has been reported.[12] Emulsin hydrolysis of the two irradiation products and development in the second dimension with 5% aqueous acetic acid showed that both produced umbelliferone. Only a very slight trace of umbellic acid could be detected from the spot R_f 0·04.

Chromatography of the *trans*-compound before irradiation shows that no skimmin is present and it would appear that lactonization is indeed induced by the u.v. radiation. The detailed mechanism of this transformation is uncertain but may possibly involve either direct displacement[13] or methanolysis of the glucose residue activated by proton exchange from the carboxyl group, which we postulate is held near the glucose moiety of the *cis*-glucoside.

The emulsin hydrolysis of *cis*-diglucoside proceeds slowly compared to that of skimmin or umbelliferone cellobioside. This is in agreement with previous findings that *cis*-*o*-hydroxycinnamic acid glucosides are slowly hydrolysed by this enzyme.[8] Conversely, several workers have prepared enzyme fractions which rapidly hydrolyse *cis*-*o*-glucosyloxycinnamic acid and are essentially inert towards the *trans* isomer.[8a, 14]

When a hydrangea leaf extract was chromatographed on cellulose powder using the AAW solvent, *two* umbelliferone-producing spots were observed (R_f values 0·24 and 0·02). The material with R_f 0·24 was the major component, had purple fluorescence in light at 350 mμ and was positively identified as skimmin by its rapid hydrolysis with emulsin, yellow fluorescence on treatment with aqueous sodium hydroxide and by comparison of its u.v. spectrum with that of authentic skimmin. The material with R_f 0·02 was present in a much smaller quantity and was only very slowly hydrolysed by emulsin. Because of its position on the chromatogram, it was considered to be a diglucoside (or other disaccharide). This unidentified glucoside was removed as a band from a preparative cellulose powder chromatogram (AAW solvent) and irradiated with u.v. light for 1 hr in methanol solution. On thin-layer chromatographic analysis of the product (AAW solvent) *two* umbelliferone-producing spots were observed (R_f values 0·01 and 0·29). The predominant, faster-moving spot was identified as skimmin by the usual tests. From this evidence, the minor "bound" form of umbelliferone appears to be *cis*-DGC since irradiation of 7-cellobiosyloxycoumarin with u.v. light produces no skimmin. This latter observation should apply equally to other umbelliferone disaccharides. Skimmin itself does not break down under u.v. radiation.

At no time during this work has a glucoside which produces umbellic acid on hydrolysis been detected in hydrangea extracts. In view of the small amount of *cis*-DGC present this is not surprising, because Brown has observed only a trace of a *trans*-*o*-glucoside in lavender where all the herniarin is present as the isomeric *cis*-2-glucosyloxy-4-methoxycinnamic acid (Fig. 1).[15]

Because the *cis*-isomer is a labile compound, the fact that it is the minor umbelliferone-producing constituent in hydrangea does not necessarily prove that skimmin is the main "bound" form. During the extraction of the leaves, spontaneous lactonization of the cinnamic acid may well occur to produce skimmin.

This discovery that hydrangea leaves contain two "bound" forms of umbelliferone, and further that they are skimmin and *cis*-DGC, constitutes the first evidence for the later stages

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[13] J. RUSSELL, R. H. THOMSON and A. G. WYLIE, *Chem. & Ind.* (*London*) **34**, (1964)
[14] F. A. HASKINS and H. J. GORZ, *Science*, **139**, 496 (1963).
[15] S. A. BROWN, *Lloydia* **26**, 211 (1963).

of umbelliferone biosynthesis. Combining this evidence with the results of tracer studies,[16] the overall biosynthetic scheme may now be represented as in Fig. 3. We consider that the proved *in vitro* transformation of *trans*-DGC to *cis*-DGC and thence to skimmin (6→7→8) coupled with our identification of *cis*-isomer and skimmin in hydrangea leaf extracts (the primary site of umbelliferone synthesis),[16] greatly enhances the plausibility of this scheme.

The isomerization of *trans-o*-glucosyloxycinnamic acids to the *cis* form (6→7, Fig. 3) appears to proceed very readily and the equilibrium lies well over to the *cis* form. Haskins and Gorz consider this to be a non-enzymic, light-catalysed reaction.[17] Stoker has presented some evidence for the presence of an isomerase in *Melilotus alba*,[18] but Kahnt has reported an apparent causal connexion between the formation of *cis-o*-glucosyloxycinnamic acid (*cis*-OGC) in *M. alba* and the intensity of illumination of the plants.[19] It has been shown that, in an irradiated aqueous solution of *trans*-OGC, the *cis* form accounts for 80–85 per

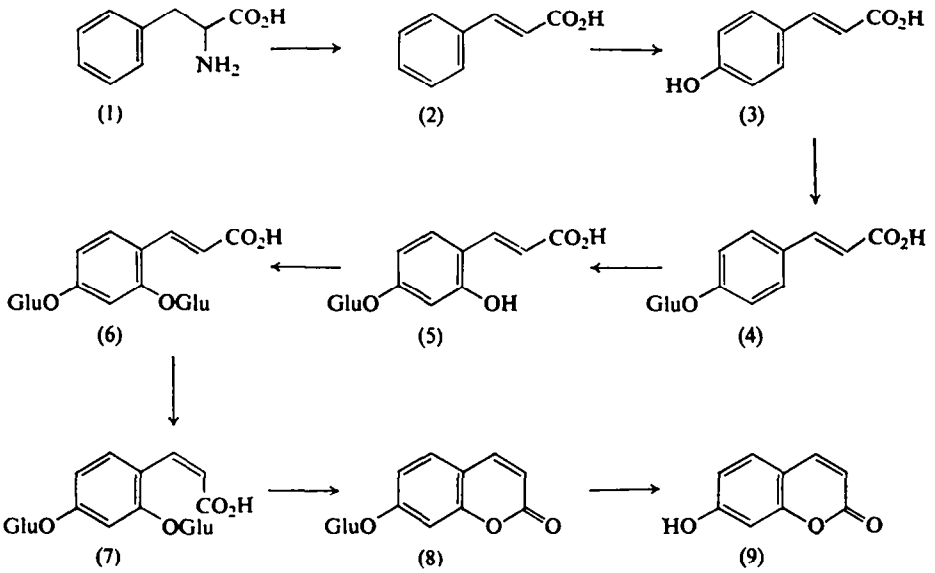

FIG. 3. PROPOSED BIOSYNTHETIC ROUTE TO UMBELLIFERONE.

cent of the glucoside mixture.[12] In contrast, the equilibrium found in *cis-trans* mixtures of cinnamic acids without either *o*-hydroxyl or *o*-glucosyloxy groups lies well towards the *trans* form (65–75 per cent).[20] This striking difference in the stability of the *cis* forms we attribute to the effect of hydrogen bonding in *o*-glucosyloxycinnamic acids. Inspection of Dreiding models of both *cis* and *trans-o*-glucosides shows the possibility of strong intramolecular hydrogen bonding *only* in the *cis* form between the carboxyl group and the 2-hydroxyl of the glucose (Fig. 4). The formation of a "straight" hydrogen bond requires a slight twisting (35–40°) of the acid side-chain out of the plane of the benzene ring and should

[16] D. J. AUSTIN and M. B. MEYERS, *Phytochem.* 4, 245 (1965).
[17] F. A. HASKINS and H. J. GORZ, *Biochem. Biophys. Res. Commun.* 6, 298 (1961).
[18] J. R. STOKER, *Biochem. Biophys. Res. Commun.* 14, 17 (1964).
[19] G. KAHNT, *Naturwiss.* 49, 207 (1962).
[20] W. A. ROTH and R. STOERMER, *Ber.* 46, 260 (1913); D. J. AUSTIN, M. B. MEYERS and J. L. C. WRIGHT, Unpublished results.

have only a small effect on the u.v. spectrum of the cinnamic acid. Because of the asymmetry of the glucose moiety, it can be postulated that the twist of the side-chain can only occur in one direction relative to the plane of the aromatic ring, thus introducing a further element of asymmetry into the molecule.

The data presented in this paper do not permit a definition of the structure of *neo-hydrangin*. The physical properties reported for this compound, such as the high melting point (204°) and its limited solubility in water and methanol, would appear to correspond with an umbelliferone-7-disaccharide rather than a 2,4-diglucosyloxycinnamic acid. Neo-hydrangin may be a sophoroside, gentiobioside or a maltoside and work is in progress to explore these possibilities.

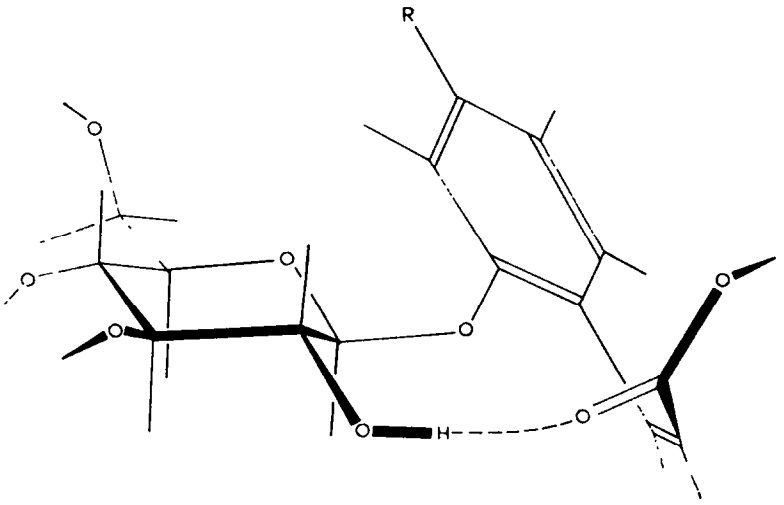

FIG. 4. POSTULATED ELEVEN-MEMBERED HYDROGEN BOND IN COUMARINIC GLUCOSIDES (R = H, OGlu, OCH_3, etc.).

EXPERIMENTAL

Paper chromatography. The ascending technique was used with Whatman No. 1 paper. The solvent was 2% aqueous acetic acid.

Thin-layer chromatography. For analytical purposes and two-dimensional chromatograms 20 × 20 cm plates were coated with 0·25 mm thick layers of Whatman cellulose powder CC41 using a Shandon apparatus. For preparative chromatograms, the layers were 0·5 mm thick. Two solvents were employed; 5% aqueous acetic acid and the upper phase of *n*-amyl alcohol:acetic acid:water (4:1:5), described as the AAW solvent.

Preparation of Synthetic Glucosides

7-β-D-cellobiosyloxycoumarin. To a mixture of acetobromocellobiose (710 mg) and umbelliferone (81 mg) in quinoline (8 ml) there was added silver oxide (500 mg). The reaction mixture was vigorously shaken for 3 hr and then allowed to stand overnight. Chloroform (10 ml) was added and, after stirring, the suspension was filtered through glass fibre paper. The residue was washed with chloroform and the combined filtrate and washings extracted with two portions of dilute hydrochloric acid. The organic layer was washed with brine, dried with sodium sulphate, filtered and evaporated to yield a gum. This gum was placed in

methanol (10 ml) and, on standing, the solution produced 7-heptaacetyl-β-D-cellobiosyloxycoumarin (273 mg) as needles m.p. 194–200°. A second crop (10 mg) was obtained from the mother liquors to give a total yield of 83 per cent. A sample was recrystallized from methanol producing needles, which formed a glass m.p. 196–204° on dissolution in acetone and evaporation of the solvent. The hepta-acetate exhibited ν_{max} (Nujol mull) 1745, 1705 (shoulder), 1230, 1050 and 840 cm^{-1} (Found: C, 53·79; H, 5·38. Calc. for $C_{35}H_{40}O_{20}$ C, 53·84; H, 5·15%).

To a solution of sodium methoxide in methanol (0·5 g of sodium in 30 ml of methanol) there was added a solution of 7-heptaacetylcellobiosyloxycoumarin (225 mg) in methanol (20 ml). The mixture was allowed to stand in the refrigerator for 2 hr and a small excess of glacial acetic acid was added. Evaporation of the solvent gave a residue which was extracted with six 30-ml portions of boiling acetone. Removal of the acetone gave a microcrystalline powder (40 mg). This was 7-β-D-cellobiosyloxycoumarin, m.p. 248–252°. Recrystallization from aqueous-methanol gave colourless rods, m.p. 228–235°, λ_{max} (MeOH) 216 (ε 11,400), 239 (3500) 249·5 (2900), 293 (9900) and 317·5 mμ (12,700), ν_{max} (Nujol mull) 3500, 1705, 1620, 1160, 1080 (broad multiplet), 870, 845 and 715 cm^{-1} (Found: C, 51·86; H, 5·38. Calc. for $C_{21}H_{26}O_{13}$ C, 51·81; H, 5·39%).

Trans-2,4-di*β*-D-*glucosyloxycinnamic acid.* A solution of umbellic acid (1·0 g) in dioxane (70 ml) was added to a solution of 15 mmole of diazomethane in ether. After 10 min at room temperature, excess glacial acetic acid was added and the mixture poured into aqueous sodium bicarbonate solution (500 ml). Ether extraction and removal of the solvent gave an oil which, on standing in benzene solution, gave *methyl umbellate* as tan crystals (0·650 g) in 60 per cent yield, m.p. 171–173°. Recrystallization from aqueous methanol produced fronds of m.p. 168–171°, λ_{max} (MeOH) 242 (ε 8300), 294 (12,100), and 326 mμ (16,700); ν_{max} (Nujol mull) 1675 (shoulder), 1650, 1200 and 1175 cm^{-1} (Found: C, 62·22; H, 5·68. $C_{10}H_{10}O_4$ required C, 61·85; H, 5·19%).

To a solution of methyl umbellate (194 mg) and acetobromoglucose (1·64 g) in quinoline (4 ml) there was added silver oxide (1·2 g). After shaking for 30 min, the reaction mixture was allowed to stand overnight at room temperature. The resulting paste was triturated with 50% aqueous acetic acid (25 ml) and the extract poured into methanol (100 ml). The mixture was adjusted to pH 6 with dilute sulphuric acid. After filtration through celite, the filtrate was warmed and an equal volume of water slowly added. The solution was placed in an ice bath for 2 hr, giving on filtration methyl 2,4-*di*-(tetraacetyl-β-D-glucosyloxy)-cinnamate as an amorphous tan solid (603 mg, 70 per cent). It exhibited ν_{max} (Nujol mull) 1745 (broad), 1705 (shoulder), 1635, 1605, 1580, 1240 (broad), 1060 and 1040 (broad) cm^{-1}. An analytical sample was prepared by dissolving the crude product in methanol, filtering and evaporating the solvent to produce a glass, liquefying at 95° (Found: C, 53·14; H, 5·59. $C_{38}H_{46}O_{22}$ required C, 53·39; H, 5·43%).

A solution of methyl 2,4-di-(tetraacetylglucosyloxy)-cinnamate (50 mg) in methanol (3 ml) was added to a saturated aqueous solution of barium hydroxide (10 ml). After shaking the mixture overnight, the solution was adjusted to pH 3 with dilute sulphuric acid and centrifuged to remove barium sulphate. The centrifugate was evaporated to dryness to yield *trans*-2,4-di-β-D-*glucosyloxycinnamic acid* as a glass, liquefying slowly with gas evolution from 115–155° which was insoluble in ether and acetone, and soluble in methanol and water, λ_{max} (MeOH) 233 (ε 9700), 286 (8250) and 314·5 mμ (6800); λ_{max} (MeOH + NaOH) 277 (ε 9580), 289 (8250) and 310 mμ (4750) (Found: C, 45·46; H, 6·64. $C_{21}H_{28}O_{14}.3H_2O$ required C, 45·16; H, 6·14%).

Preparation of leaf extracts. Hydrangea leaves were blended in boiling 80% ethanol and the suspension heated under reflux for 2 hr. The hot solution was filtered through celite, reduced in volume and refiltered. The filtrate was subjected to constant ether extraction to remove free coumarins and free acids. The extracted aqueous phase was reduced to dryness under vacuum and the residue treated with boiling methanol. The filtered methanol extract was cooled to 0° overnight, then refiltered and reduced to give a brown syrup which was analysed by thin-layer and paper chromatographic techniques.

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