The Polyphenols in Leaves of *Eucalyptus sideroxylon*

BY W. E. HILLIS AND MASAO HASEGAWA*

Division of Forest Products, C.S.I.R.O., Melbourne, Australia

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The hydroxystilbenes are most commonly found in the woody tissues of certain species. Attempts to study the biosynthesis of hydroxystilbenes in these tissues would be hindered by the partial loss of the labelled precursors through translocation to other tissues. The discovery of an apparent variant of *Eucalyptus sideroxylon* A. Cunn. (Benth.), containing relatively large amounts of hydroxystilbenes in the leaves, provided a more suitable tissue for a study of their biosynthesis. This paper describes the hydroxystilbenes in the leaves of the variant and compares the polyphenols in them with those in the leaves of the more common form.

EXPERIMENTAL

Plant material. The leaves were obtained from five specimens of 5-year-old trees which had been grown from reputedly authentic *E. sideroxylon* A. Cunn. (Benth.) seed. The seedlings were obtained from the Natural Resources Conservation League, Melbourne, Australia. A comparison of the buds, fruits and leaves with authentic herbarium material indicated that the identity was correct. However, leaves collected from other *E. sideroxylon* trees (10 specimens) growing in different parts of Victoria, Australia (in or near Lakes Entrance, Bruthen, Ballarat, Heathcote, Bendigo, Stawell and Melbourne) contained very small amounts, if any, of stilbenes. An examination of the leaves of other species which may have formed a hybrid failed to reveal the presence of hydroxystilbenes.

The different samples of leaves (including 15 samples collected at intervals during 12 months from three specimens of the variant form) were extracted with methanol, and the extracts examined chromatographically.

Paper chromatography. The methods used have been reported by Hillis & Carle (1962). These included two-dimensional co-chromatography with 6% (v/v) acetic acid followed by butan-1-ol-acetic acid-water (6:1:2, by vol.) and one-dimensional co-chromatography with *m*-cresol-acetic acid-water (50:1:49, by vol.) or acetic acid-concentrated hydrochloric acid-water (30:3:10, by vol.). The compounds were revealed by examination under ultra-violet (u.v.) light, before and after exposure to the vapour of ammonia solution (sp.gr. 0.88), and by spraying with at least two different reagents. The compounds identified in this way were catechin, gallic acid, epicatechin, *p*-coumarilquinic acid, chlorogenic acid and quercetin.

On a paper chromatogram, the hydroxystilbenes (spots 1, 2 and 3, Fig. 1) showed under u.v. light very intense blue

fluorescent colours, the shades of which were slightly but significantly different when viewed side-by-side. After exposure of the spots to ammonia the fluorescence of spot 1 was duck-egg blue, of spot 2 light magenta, and of spot 3 greenish blue. The colours with diazotized *p*-nitroaniline were yellow, orange and purple respectively.

The amounts of leaf extracts used were so chosen that each gave the same size of composite spot for components 7 and 8 (Fig. 1) on the two-dimensional chromatogram. The relative concentrations of the different components were estimated by the size of the spots and intensity of the colour reaction.

Preparation of the extracts. Fresh leaves (500 g.) from one of the specimens of the variant form were refluxed

Fig. 1. Two-dimensional chromatogram of the major polyphenols in the leaves of two physiological forms of *E. sideroxylon* run in 6% (v/v) acetic acid, followed by butan-1-ol-acetic acid-water (6:1:2, by vol.). The amounts (++, strong; +, present; tr, trace; -, absent) of the components, relative to catechin, in *a*, the normal, and *b*, the variant forms were: 1: 3,5,4'-trihydroxystilbene glucoside (*a*, -; *b*, ++); 2: 3,5,3'-trihydroxy-4'-methoxystilbene glucoside (*a*, tr; *b*, ++); 3: 3,5,3',4',5'-pentahydroxystilbene glucoside (*a*, tr; *b*, ++); 4: quercetin glycoside (*a*, ++; *b*, tr); 5: flavonol glycoside (*a*, +; *b*, -); 6: quercetin glycoside (*a*, +; *b*, -); 7: catechin (*a*, ++; *b*, ++); 8: gallic acid (*a*, tr; *b*, tr); 9: flavonol glycoside (?) (*a*, tr; *b*, tr); 10: epicatechin (*a*, +; *b*, +); 11: flavonol glycoside (?) (*a*, tr; *b*, tr); 12, 13 and 14: flavans (*a*, tr; *b*, tr); 15: *p*-coumarilquinic acid (*a*, -; *b*, +); 16: unknown (*a*, -; *b*, +); 17: isomers of chlorogenic acid (*a*, tr; *b*, +); 18: unknown (*a*, +; *b*, +).

* On leave from the Government Forest Experiment Station, Tokyo, Japan

with methanol (1.5 l.) for 3 hr., and the hot extract was filtered. The extraction was repeated twice. After 1–2 days the extract was again filtered to remove the precipitate and concentrated *in vacuo* to 250 ml. This was extracted continuously with ether for 15–20 hr., then with ethyl acetate for 15–20 hr.

Fractionation of the extracts. The ether extract was evaporated to dryness, triturated several times with petroleum (b.p. 40–60°), followed by hot benzene, water and methanol, the last dissolving all the material left by the previous solvents.

In all cases, the concentrated extracts were examined by two-dimensional chromatography. The chromatograms showed that the precipitate from the original methanol extract contained small amounts of the three hydroxystilbene glucosides. The final aqueous mother liquor after extraction with ether and ethyl acetate contained mainly polymerized material. These fractions were not examined further.

Examination of the hydroxystilbenes

The ethyl acetate extract contained most of the hydroxystilbenes present in the leaves. The extract was evaporated *in vacuo* and dissolved in water (200 ml.). After 1–2 days the colourless needles (1.2 g.) which formed were removed, and after a further 7–14 days a second crop of crystals (4.0 g.) formed. The first crop contained mainly component 2 whereas the second crop contained a much larger proportion of component 1. The crystals were extracted with hot ethyl acetate which dissolved most of component 1. The aqueous mother liquor contained chlorogenic acid, *p*-coumarylquinic acid, and components 3 and 4.

Identification of component 1. Repeated crystallization from water yielded colourless needles (2.5 g.), m.p. 232°, $[\alpha]_D^{19} - 66.0^\circ$ (c 0.5 in methanol). Light-absorption max. in ethanol were 307 m μ (and 322 m μ , unstable) and min. 258 and 314 m μ ; light-absorption max. in ethanol saturated with anhydrous sodium acetate were unchanged (Found: C, 61.7; H, 5.9. Calc. for $C_{20}H_{22}O_8$: C, 61.5; H, 5.6%). Kariyone, Takahashi, Ito & Matsutani (1958, 1959) reported for piceid (3,5,4'-trihydroxystilbene 3-glucoside) m.p. 225–226° and $[\alpha]_D^{27} - 75.7^\circ$ (c 5.18 in ethanol). The m.p. of a sample of piceid provided by Dr M. Takahashi was 225–226°, and the mixed m.p. with the material from *E. sideroxylon* was 227–230°. Hathway & Seakins (1959) reported for 3,5,4'-trihydroxystilbene 3- β -D-glucoside, freshly isolated from *E. wandoo*, m.p. 210°, $[\alpha]_D^{22} - 68.5^\circ$ (c 2.6 in methanol), light-absorption max. 220 and 305 m μ . The m.p. of a sample of this glucoside obtained from *E. wandoo* and provided by Dr D. E. Hathway was 227°, and the mixed m.p. with the glucoside from *E. sideroxylon* was 227–230°. The mixed m.p. of piceid and the aged glucoside from *E. wandoo* was 226–227°. The m.p. of the 3,5,4'-trihydroxystilbene glucoside isolated from fresh *E. wandoo* heartwood in this Laboratory with the methods of Hathway & Seakins (1959) was 232°, and the mixed m.p. with glucoside from *E. sideroxylon* was 232°. Acetylation by the acetic anhydride-pyridine method gave the hexa-acetate, which crystallized from aq. 50% (v/v) methanol as needles, m.p. 156–157°, $[\alpha]_D^{19} - 21.2^\circ$ (c 0.4 in acetone) (Found: C, 59.8; H, 5.5. Calc. for $C_{38}H_{38}O_{14}$: C, 59.8; H, 5.3%). Hathway & Seakins (1959) reported m.p. 107° and $[\alpha]_D^{22} - 21^\circ$ (c 4.4 in acetone).

Hydrolysis of the glucoside (0.97 g.) with 10% (w/v)

H_2SO_4 on a boiling-water bath for 35 min. followed by extraction with ether yielded 3,5,4'-trihydroxystilbene (resveratrol) (0.44 g.), recrystallized from 6% (v/v) acetic acid as colourless needles, m.p. and mixed m.p. 263–264°; light-absorption max. in ethanol and in ethanol saturated with anhydrous sodium acetate were 307 m μ (and 322 m μ , unstable) and min. 258 and 314 m μ (Found: C, 73.0; H, 5.4. Calc. for $C_{14}H_{12}O_3$: C, 73.7; H, 5.3%). The triacetate crystallized from ethanol as glistening plates, m.p. 109–110° (Found: C, 67.4; H, 5.4. Calc. for $C_{20}H_{18}O_6$: C, 67.8; H, 5.1%). Hathway & Seakins (1959) reported m.p. 118–119°, Kariyone *et al.* (1959) 111–112°. The residual aqueous hydrolysate was percolated through columns of Amberlite 120 and then Duolite A4 ion-exchange resins, and the columns were washed free from sugar. The aqueous solution was concentrated under vacuum on a water bath and finally in a desiccator over NaOH pellets. Chromatographic examination of the material with glucose and galactose as markers in ethyl acetate-pyridine-water (12:5:4, by vol.) and butan-1-ol-acetic acid-water (2:1:1, by vol.), with silver nitrate-ammonia (Partridge & Westall, 1948), revealed that glucose was the only sugar present. The molar ratio of aglucone to glucoside was 1:1 (Found: aglucone, 45.3%. Calc. 58.4%).

An acetone solution (100 ml.) of the glucoside (300 mg.) was treated with dimethyl sulphate (2 ml.) and anhydrous K_2CO_3 (10 g.) for 3 hr. The acetone solution was concentrated and water (100 ml.) added. The oil which settled slowly became a hard mass but could not be crystallized, and an ethanolic solution was hydrolysed with 3% (w/v) H_2SO_4 on a boiling-water bath for 2 hr. and the aglucone then extracted with ether. 5-Hydroxy-3,4'-dimethoxystilbene (30 mg.) was crystallized from light petroleum (b.p. 40–60°) in plates, m.p. 115–116° (Found: C, 73.8; H, 6.6. Calc. for $C_{16}H_{16}O_3$: C, 76.0; H, 6.3%). Hathway & Seakins (1959) reported m.p. 115–116°.

The infrared spectra, the chromatographic behaviour in butan-1-ol-acetic acid-water (6:1:2, by vol.), *m*-cresol-acetic acid-water (4:1:5, by vol.) and 6% (v/v) acetic acid, and the fluorescent colours under u.v. light before and after exposure to ammonia of the 3,5,4'-trihydroxystilbene and its glucoside from *E. sideroxylon* leaves were identical with a sample of 3,5,4'-trihydroxystilbene and an aged sample of its 3- β -D-glucoside isolated from *E. wandoo* heartwood (Hathway & Seakins, 1959).

Identification of component 2. The crude hydroxystilbenes obtained from the ethyl acetate extract were extracted with ethyl acetate. The insoluble portion was repeatedly recrystallized from aq. 50% (v/v) acetone and yielded colourless crystals (1.2 g.) m.p. 245°, $[\alpha]_D^{25} - 65.5^\circ$ [c 1.1 in aq. 50% (v/v) acetone]. Hesse (1899, 1908) reported for raphontin m.p. 230–236° and $[\alpha]_D^{15} - 63^\circ$ [c 2 in aq. 50% (v/v) acetone], Kawamura (1938) m.p. 236–237° and $[\alpha]_D^{32} - 59.5^\circ$ [c 2.1 in aq. 50% (v/v) acetone] (Found: C, 60.0; H, 6.0; O·CH₃, 7.4. Calc. for $C_{21}H_{24}O_6$: C, 60.0; H, 5.7; O·CH₃, 7.4%). Light-absorption max. and min. in methanol were respectively 324 and 260 m μ . The hexa-acetate recrystallized from ethanol as colourless needles, had m.p. 140° [Hesse (1899, 1908) reported 138°, Kawamura (1938) 135–136°], $[\alpha]_D^{20} - 23.4^\circ$ (c 1.8 in acetone) (Found: C, 59.1; H, 5.1; CH₃·CO, 41.9. Calc. for $C_{33}H_{38}O_{15}$: C, 58.9; H, 5.3; CH₃·CO, 38.4%).

Component 2 (510 mg.) was hydrolysed on a boiling-water bath with ethanol containing 3% (w/v) of HCl to

yield 3,5,3'-trihydroxy-4'-methoxystilbene (rhapontigenin) (270 mg.) which was crystallized from dilute methanol to give pale-yellow needles (230 mg.), m.p. 187–189° [Hesse (1899, 1908) reported 188°; Kawamura (1938) 186–187°] (Found: C, 69.8; H, 5.7. Calc. for $C_{15}H_{14}O_4$; C, 69.7; H, 5.4%). Light-absorption max. and min. in methanol were 325 and 260 $m\mu$ respectively [Kawamura (1938) reported 320 and 260 $m\mu$ respectively]. The triacetate, prepared with pyridine and acetic anhydride, crystallized from ethanol as colourless needles, m.p. 114–115° [Kawamura (1938) reported 114°]. The only sugar identified in the aqueous solution was glucose. The molar ratio of aglucone to glucoside was 1:1 (Found: aglucone, 52.9%. Calc. 61.4%).

Decomposition of 3,5,3'-trihydroxy-4'-methoxystilbene. The compound (48 mg.) was heated with nitrobenzene (0.1 ml.) and 2N-NaOH (10) in a rotating stainless-steel bomb at 160° for 3 hr. The acidified mixture was then extracted with ether and the extract chromatographed on borate-impregnated paper in (1) di-isopropyl ether-methanol-water (1:1:1, by vol.) and (2) benzene-water (1:1, v/v) (Bland & Stamp, 1955; Bland, 1955). Examination of the chromatograms under u.v. light and after spraying with 2,4-dinitrophenylhydrazine showed that one of the decomposition products behaved identically with authentic isovanillin.

Component 3. The aqueous mother liquor from the separation of components 1 and 2 was concentrated and applied in bands to washed Whatman no. 3 papers (50 sheets, 17 in. $\times$ 20 in.) and the chromatograms were developed in butan-1-ol-acetic acid-water (6:1:2, by vol.) by upward migration. The bands containing component 3 were cut out and extracted with methanol in a Soxhlet apparatus for 5 days. After evaporation under N_2 , 10 mg. of material containing slight traces of impurities was obtained. Fine colourless crystals were obtained from ethyl acetate, methanol and water, but high solubility in these solvents prevented the recovery of sufficient amounts for chemical examination. The substance had R_F values in several solvents identical with those of component 12 in *E. astringens* bark (Hillis & Carle, 1962). Its u.v. spectrum was similar to that of 3,5,3',4',5'-pentahydroxystilbene. After hydrolysis with $N-H_2SO_4$ on the boiling-water bath for 30 min. it yielded glucose and a stilbene with the same chromatographic properties as 3,5,3',4',5'-pentahydroxystilbene in several solvents.

Examination of other fractions

Chromatographic examination showed that the petroleum- and benzene-soluble fractions of the ether extract contained small amounts of 3,5,4'-trihydroxystilbene and traces of the other two hydroxystilbenes. The methanol-soluble portion (0.2 g.) of the ether extract contained small amounts of hydroxystilbenes, chlorogenic acid, *p*-coumarylquinic acid and traces of ellagic acid and the hydroxystilbene glucosides. The water-soluble fraction of the ether extract contained several monomeric polyphenols. Those identified by co-chromatography and colour reactions were chlorogenic acid, *p*-coumarylquinic acid, catechin and gallic acid. One-dimensional chromatography with *m*-cresol-acetic acid-water (50:1:49, by vol.) showed that the last two compounds were present in the approximate ratio of 3:1. Small amounts of the hydroxystilbene glucosides were also present in this fraction.

The yellow fluorescent band corresponding to component 4 (obtained during the purification of component 3) was extracted with methanol. Hydrolysis evidently occurred as only quercetin (identified chromatographically and by its acetate) was present in the extract. The aqueous mother liquor obtained from the ethyl acetate extract of another batch of leaves was passed through a column (1 cm. $\times$ 10 cm.) of aluminium oxide (Savory and Moore Ltd., London) and the column was washed with water (400 ml.). It was eluted with methanol-concentrated HCl (9:1, v/v) (50 ml.), and the eluate heated on a boiling-water bath for 30 min. and finally extracted with ether. Quercetin was identified as above, and traces of kaempferol were identified chromatographically. Component 4 was chromatographically distinct from rutin.

RESULTS AND DISCUSSION

The mixed melting points of component 1 with piceid (Kariyone *et al.* 1958, 1959) or with an aged specimen of 3,5,4'-trihydroxystilbene 3- β -D-glucoside from *E. wandoo* heartwood (Hathway & Seakins, 1959) were identical. Also, there was no depression in the mixed melting point of the last two substances. These and other properties indicate that these three materials are identical.

The melting points of the 3,5,4'-trihydroxystilbene 3- β -D-glucoside, freshly isolated from *E. wandoo* heartwood by Hathway & Seakins (1959), and of its acetate, are considerably lower than those of the glucoside isolated from *E. sideroxylon* leaves and its acetate. The light-absorption maxima of the glucoside reported by Hathway & Seakins (1959) are lower than those of the glucoside from *E. sideroxylon* leaves. Battersby & Greenock (1961) found that the melting point and the light-absorption maximum of the unstable *trans*-3,4,3',4'-tetramethoxystilbene were higher than those of the *cis*-isomer. The possibility that the glucoside freshly isolated by Hathway & Seakins (1959) and the glucoside isolated from *E. sideroxylon* leaves are the *cis-trans* isomers of 3,5,4'-trihydroxystilbene 3- β -D-glucoside could not be investigated as we were able to isolate only the high-melting-point form from fresh *E. wandoo* heartwood.

The rhapontin (3,5,3'-trihydroxy-4'-methoxystilbene glucoside) isolated by Kawamura (1938) was not available for comparison, but the properties of the sample isolated in this present work are very similar to those he reported. Kawamura showed that the glucose is attached to the 3- or 5-hydroxyl group, as it is in 3,5,4'-trihydroxystilbene glucoside (see Hathway & Seakins, 1959). As the rotations of phenol β -D-glycosides and their acetates [which are *laevo*- whereas the α -D-glucosides and their acetates are *dextro*-rotatory compounds (Jermyn, 1955)], and of 3,5,4'-trihydroxystilbene 3- β -D-glucoside and its acetate are of the same order as the rotations of 3,5,3'-trihydroxy-4'-methoxystilbene glucoside

and its acetate, it is postulated that the latter is also a 3- β -D-glucoside.

Comparable yields of the hydroxystilbene glucosides were obtained from a further six batches of leaves collected from the variants at different times of the year. On chromatographic examination, slight differences in the relative amount of some of the minor components of the leaf polyphenols in the two physiological forms were evident, but these were insignificant when compared with the differences in the major components (Fig. 1).

The existence of different physiological forms of *Eucalyptus* species (and other members of the Myrtaceae) with widely differing content and composition of essential oils is well known (e.g. Penfold, Morrison, McKern & Willis, 1950; McKern, Spies & Willis, 1953; Penfold, McKern & Willis, 1953). The present work shows that wide variations in the composition of the polyphenols in the leaves from an *Eucalyptus* species can occur. In the normal form of *E. sideroxylon* the major monomeric polyphenols are catechin and a quercetin glycoside, whereas in the other physiological form they are catechin and the glucosides of hydroxystilbenes. Therefore in the biosynthetic pathway catechin may be formed before flavonols. This conclusion is supported by other work (M. Hasegawa, unpublished results) which showed that, after *Chamaecyparis obtusa* leaves had been fed with labelled compounds, catechin had a much higher specific activity than the flavonols. Further, the points of divergence from the main pathway leading to quercetin and the hydroxystilbenes appear to be close together and near the end of the pathway, so that when there is a partial blockage in the formation of quercetin, larger amounts of hydroxystilbenes are formed and vice versa.

SUMMARY

1. The leaves of a new physiological form of *Eucalyptus sideroxylon* were found to contain very much greater amounts of three hydroxystilbene glucosides and appreciably lower amounts

of a quercetin glycoside than the leaves of the normal form of this species.

2. The hydroxystilbenes were identified as 3,5,4'-trihydroxy-, 3,5,3'-trihydroxy-4'-methoxy- and 3,5,3',4',5'-pentahydroxy-stilbene.

3. The possible order of biosynthesis of catechin, flavonols and hydroxystilbenes is discussed.

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