The Use of Hydroxystilbene Compounds as Taxonomic Tracers in the Genus *Eucalyptus*

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Hathway & Seakins (1959*a*) reported the isolation of 3,5,4'-trihydroxystilbene and its 3 β -D-glucoside from *Eucalyptus wandoo* heartwood. Enzymic studies show that both these substances behave as active cellulase inhibitors at a concentration of 10⁻⁴ M, and a preliminary communication (Hathway & Seakins, 1959*b*) suggests that these hydroxystilbene compounds may be useful as taxonomic tracers in the genus *Eucalyptus*. In this connexion, Erdtman (1958) has investigated the significance of the incidence of 3,5-dihydroxy- and 3-hydroxy-5-methoxy-stilbenes in pine heartwoods in the taxonomy of the genus *Pinus*, and a similar investigation of eucalypt heartwoods has now been made in the subsection *Longiores* of the genus *Eucalyptus* to which *E. wandoo* belongs.

METHODS

Separation of a fraction containing 3,5,4'-trihydroxystilbene and its 3 β -D-glucoside from eucalypt heartwoods

Heartwood chips (10 g.) were extracted in a Soxhlet apparatus with benzene to remove yellow wax, and the residual wood was extracted with four changes of acetic acid-free ethylacetate for 30 hr. Evaporation of the solvent in nitrogen under reduced pressure below 30° left a solid residue which was dried over P₂O₅ at 20° *in vacuo* for 16 hr.

Paper chromatography

Paper chromatography was carried out in all-glass apparatus in a constant-temperature enclosure at 25°, and the chromatograms were dried at room temperature. Methanolic solutions (5.0%, w/v; 5 μ l.) of the phenols were applied to Whatman no. 2 filter papers, 25.5 cm. square, and chromatographed by the ascending method with N-acetic acid as first-way solvent and butan-1-ol-acetic acid-water (either 6:1:2, by vol., or 4:1:5, by vol.) as the second. Phenols were detected by their fluorescence in ultraviolet light, by the FeCl₃-K₃Fe(CN)₆ and vanillin reagents (Hathway, 1958), and by the tetrazotized benzidine reagent (Koch & Krieg, 1938; Linstedt, 1950).

A portion of the solid residue (50 mg.) was dissolved in methanol (5 ml.) and shaken in hydrogen with 10% palladinized charcoal (15 mg.); 1 mol.prop. was taken up

during 30 min. Methanolic solutions (0.5%; 5 μ l.) of the residues obtained after evaporation were also applied to Whatman no. 2 filter papers, 25.5 cm. square, and chromatographed as described above.

RESULTS AND DISCUSSION

The present work represents a study of the chromatographic properties of hydroxystilbene compounds, and of their provisional identification amongst the heartwood extractives of trees which belong to the subsection *Longiores* of the genus *Eucalyptus*.

The hydroxystilbene compounds were initially detected in extracts by their locations on two-dimensional chromatograms run in alternative pairs of solvent systems (Hathway, 1960; Hathway & Seakins, 1959*a*), by their intense blue fluorescence in ultraviolet light which became more intense after fuming with ammonia, by the red colour with tetrazotized benzidine, and by the abnormal blue colour with vanillin.

On hydrogenation the fluorescence is quenched; the dihydro derivatives formed give the normal red colour with vanillin and their *R_F* values show a characteristic shift in the N-acetic acid solvent. In this solvent, the *R_F* values of 3,5,4'-trihydroxystilbene and of the corresponding dihydro derivative are 0 and 0.50 respectively, and those of 3,5,4'-trihydroxystilbene-3 β -D-glucoside and the corresponding dihydro derivative are 0.27 and 0.70 respectively. On hydrogenation, this *R_F* shift in the N-acetic acid solvent has been found to be characteristic for hydroxystilbene compounds. The locations of a number of hydroxystilbene compounds and of the corresponding dihydro derivatives on a standard two-dimensional chromatogram are shown (Fig. 1).

When a chromatogram which is reasonably heavily loaded with the three hydroxystilbene compounds (Hathway & Seakins, 1959*a*) from *E. wandoo* is run two-dimensionally in the preferred solvents, each of these substances runs as a double spot (Fig. 2), the main spot corresponding to that previously reported. The second spot, of higher *R_F* in the aqueous solvent, is much weaker, and can easily be missed in the presence of other wood

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extractives or with low concentration of the hydroxystilbene compounds. The behaviour of the pairs of spots in ultraviolet light and with the $\text{FeCl}_3\text{-K}_3\text{Fe}(\text{CN})_6$, tetrazotized benzidine and vanillin colour reagents is the same; as judged by these tests, the main spot is 10–20 times as strong as the one with the higher R_F . These facts suggest that the two spots are due to the presence of *cis*- and *trans*-isomers. Since the *trans*-isomers of stilbenes are thermodynamically more stable than the *cis*-isomers, they preponderate in an equilibrium mixture of the geometrical isomers; further, since the *trans*-isomers are more coplanar, they have lower R_F values in aqueous solvents. The spots due to *trans*-hydroxystilbene compounds would accordingly be expected to be stronger and less mobile than those of the *cis*-isomers.

Many stilbene derivatives have been obtained in two forms by irradiation with ultraviolet light; when this treatment was given to solutions of 3,5,4'-

trihydroxystilbene, 3,5,4'-trihydroxystilbene- β -D-glucoside or one of the other naturally occurring hydroxystilbene derivatives, an increase in the relative strength of the higher- R_F spot was observed. As with the *cis*- and *trans*-isomers of cinnamic acid derivatives (Williams, 1955), predominantly aqueous solvents separate the pairs of structural isomers. An attempt to separate the two isomers of 3,5,4'-trihydroxystilbene by chromatography of the irradiated sample on thick paper and elution of the appropriate bands was partially successful; on further chromatography the two extracts were each found to give strong spots in the appropriate position together with a weaker spot of the other isomer. This characteristic separation of the *cis*- and *trans*-isomers of 3,5,4'-trihydroxystilbene is particularly useful in screening chromatograms of eucalypt-heartwood extractives for the presence of these substances. Some of the heartwood extracts examined have a relatively high proportion of the *cis*-isomers, but in no case did the spots due to the *cis*-isomers approach in intensity those of the *trans*-isomers. This is the first reference to the natural occurrence of *cis*-hydroxystilbene compounds, and to the separation of the *cis*- and *trans*-isomers by paper chromatography.

The provisional identification by paper chromatography of 3,5,4'-trihydroxystilbene and of its

Fig. 1. Two-dimensional chromatogram of some naturally occurring hydroxystilbene compounds and the corresponding dihydro derivatives. The chromatogram was run first in *N*-acetic acid, followed by butan-1-ol-acetic acid-water (6:1:2, by vol.). Spots located in ultraviolet light and by $\text{FeCl}_3\text{-K}_3\text{Fe}(\text{CN})_6$ were: 1, *trans*-3,5,4'-trihydroxystilbene; and 2, the corresponding dihydro compound; 3, *trans*-3,5,2',4'-tetrahydroxystilbene; and 4, the corresponding dihydro compound; 5, *trans*-3,5,3',4'-tetrahydroxystilbene; and 6, the corresponding dihydro compound; 7, *trans*-3,5,3',4',5'-penta-hydroxystilbene; and 8, the corresponding dihydro compound; 9, *trans*-3,5,4'-trihydroxystilbene- β -D-glucoside; and 10, the corresponding dihydro compound.

Fig. 2. Two-dimensional chromatogram of the *cis*- and *trans*-hydroxystilbene compounds from the eucalypt heartwoods run in the same solvents as used in Fig. 1. Spots 1 and 2, *cis*- and *trans*-3,5,4'-trihydroxystilbene respectively; 3 and 4, *cis*- and *trans*-isomers of the unknown hydroxystilbene compound respectively; 5 and 6, *cis*- and *trans*-isomers of 3,5,4'-trihydroxystilbene- β -D-glucoside respectively.

Table 1. Occurrence of 3,5,4'-trihydroxystilbene and its 3 β -D-glucoside in the heartwoods of eucalypts belonging to the subsection Longiores of the genus *Eucalyptus*

The Blakely no. is taken from Blakely (1955). +, Present; —, absent.

Subseries	<i>Eucalyptus</i> species	Blakely no.	<i>cis</i> - and <i>trans</i> -3,5,4'-trihydroxystilbene	<i>cis</i> - and <i>trans</i> -3,5,4'-trihydroxystilbene-3 β -D-glucoside
Series <i>Corymbosae</i> (16–55)				
(v) <i>Tesselatae</i>	<i>E. tessellaris</i>	16	—	+
	<i>E. papuana</i>	17	+	+
(vi) <i>Setosae</i>	—			
(vii) <i>Gamophyllae</i>	—			
(viii) <i>Eucorymbosae</i>	<i>E. dichromophloia</i>	26	+	+
	<i>E. erythrophloia</i>	26a	+	+
	<i>E. terminalis</i>	27	—	+
	<i>E. abergiana</i>	28	+	+
(ix) <i>Costatae</i>	—			
(x) <i>Neocorymbosae</i>	<i>E. calophylla</i>	32	+	+
	<i>E. ficifolia</i>	36	+	+
	<i>E. guilfoylei</i>	38	—	—
	<i>E. trachyphloia</i>	40	+	+
	<i>E. polycarpa</i>	41	+	+
	<i>E. haematoxylon</i>	44	+	+
	<i>E. gummifera</i>	45	+	+
	<i>E. intermedia</i>	46	+	+
(xi) <i>Ochrophloiae</i>	<i>E. pellita</i>	48	—	—
	<i>E. torelliana</i>	49	—	—
	<i>E. eximia</i>	50	—	—
	<i>E. bloxsoni</i>	51	—	—
	<i>E. watsoniana</i>	52	—	—
(xii) <i>Maculatae</i>	<i>E. citriodora</i>	53	+	+
	<i>E. maculata</i>	54	+	+
	<i>E. nowraensis</i>	55	+	+
Series <i>Transversae</i> (56–87)				
(xiii) <i>Inclusae</i>	<i>E. jacksoni</i>	56	—	—
	<i>E. diversicolor</i>	57	—	—
(xiv) <i>Leptocarpace</i>	<i>E. grandis</i>	58	—	—
	<i>E. saligna</i>	60	—	—
	<i>E. deanei</i>	62	—	—
	<i>E. botryoides</i>	64	—	—
	<i>E. robusta</i>	67	—	—
(xv) <i>Annulares</i>	<i>E. resinifera</i>	69	—	—
	<i>E. kirtoniana</i>	71	—	—
	<i>E. pellita</i>	73	—	—
(xvi) <i>Pelliculares</i>	<i>E. propinqua</i>	75	—	—
	<i>E. major</i>	76	—	—
	<i>E. shiressii</i>	77	—	—
	<i>E. punctata</i>	78	—	—
	<i>E. canaliculata</i>	80	—	—
(xvii) <i>Pauciflorae</i>	<i>E. longifolia</i>	81	—	—
	<i>E. cosmophylla</i>	85	—	—
Series <i>Obliquae</i> (88–95)				
(xviii) <i>Dealbatae</i>	<i>E. caesia</i>	88	+	+
	<i>E. woodwardi</i>	89	+	+
	<i>E. campaspe</i>	90	+	+
	<i>E. stricklandi</i>	91	+	+
	<i>E. griffithsii</i>	92	+	+
(xix) <i>Cylindratae</i>	<i>E. grossa</i>	94	+	+
Series <i>Cornutae</i> (96–113)				
(xx) <i>Sessiles</i>	<i>E. cornuta</i>	96	—	—
	<i>E. lehmanni</i>	97	—	—
	<i>E. gomphocephala</i>	98	—	—
	<i>E. platypus</i>	99	+	—
	<i>E. nutans</i>	101	+	—
(xxi) <i>Pedicellatae</i>	<i>E. spathulata</i>	103	—	+
	<i>E. eremophila</i>	105	+	+
	<i>E. occidentalis</i>	110	—	+
	<i>E. astringens</i>	112	+	+
	<i>E. sargentii</i>	113	+	+
Series <i>Subcornutae</i> (114–120)				
	<i>E. gardneri</i>	119	+	+
	<i>E. wandoo</i>	120	+	+

3 β -D-glucoside has been checked in the present work by the isolation of pure compounds from *E. gummifera* (Blakely no. 45) and *E. astringens* (Blakely no. 112) heartwoods by the methods (Hathway & Seakins, 1959a) used for the isolation of these substances from *E. wandoo* (Blakely no. 120).

Out of 105 species which Blakely (1955) arranges in the subsection *Longiores* of the genus *Eucalyptus*, heartwood specimens of 57 have now been investigated. The remainder are very scarce, and are rarely encountered by botanists and foresters familiar with the facies of *Eucalyptus* species [more than 500 according to Blakely (1955), but possibly reducible to 300–400 according to Carr & Carr (1959)]. A representative selection of heartwoods from the subsection *Longiores* has therefore been examined.

The recognition of the facies has led to the establishment of certain groups ('series'), some of which may be accepted as consisting of closely allied species and therefore 'natural'. The more obviously natural groups, with which this work is concerned, include the series *Corymbosae*, *Transversae*, *Cornutae* and *Subcornutae* (Mueller, 1879). Some series which Blakely (1955) uses are more artificial, and a few species are so imperfectly known that, as pointed out by Carr & Carr (1959), their allocation to series appears to be either intuitive or arbitrary.

The incidence of the occurrence of 3,5,4'-trihydroxystilbene and its 3 β -D-glucoside in the series *Corymbosae* is given in Table 1, which shows that both are usually present in members of this series. (For taxonomic purposes, the presence of the 3 β -D-glucoside may be taken as tantamount to the occurrence of both the glucoside and aglucone in the heartwood.) It follows that *E. guilfoylei* (Blakely no. 38) should be excluded from this group, since it does not contain these compounds, and it might be placed with the series *Transversae*. This conclusion is in agreement with those of a recent taxonomic study (Carr & Carr, 1959) based on comparative morphology. Similarly, the classification of the yellow bloodwoods or series *Ochrophloiae* also appears anomalous on account of the absence of 3,5,4'-trihydroxystilbene from the heartwood extractives of all five species. In this case, Carr & Carr (1959) have concluded on morphological grounds that *E. bloxsomei*, *E. eximia*, *E. peltata*, *E. torelliana* and *E. watsoniana* should be removed from the series *Corymbosae*. They suggest that these yellow bloodwoods should be grouped as a series, for which the name '*Eximieae*' is advanced.

The occurrence of 3,5,4'-trihydroxystilbene and its 3 β -D-glucoside differentiates between the fairly natural series of the subsection *Longiores*. Table 1

shows that none of the species belonging to the series *Transversae* which were examined contain these compounds, and in this respect they differ from the true members of the series *Corymbosae*, and from the series *Obliquae*.

3,5,4'-Trihydroxystilbene and its 3 β -D-glucoside appear to be normally present in the series *Cornutae* and *Subcornutae*, but the subseries *Sessiles* is rather anomalous, since both compounds are completely absent from *E. cornuta*, *E. gomphocephala* and *E. lehmanni*, and *E. nutans* and *E. platypus* contain 3,5,4'-trihydroxystilbene only. In this connexion, Carr & Carr (1959) comment on the precocious loss of a sepaline operculum in *E. lehmanni*, in contrast with other species belonging to the series *Cornutae*.

In nearly every instance where *cis*- and *trans*-isomers of both 3,5,4'-trihydroxystilbene and the 3 β -D-glucoside have been detected, a third hydroxystilbene derivative occurs; this was also detected amongst the heartwood extractives of *E. wandoo*, but was not isolated from this source. The third hydroxystilbene compound was absent from the heartwood extractives of the yellow bloodwoods and of the subseries *Sessiles*.

This work shows that paper-chromatographic methods for the examination of the complicated extracts from plant tissues can afford information which is useful in taxonomy, and in this connexion reference might also be made to the work of Bate-Smith (1956, 1958), and of Roberts, Wight & Wood (1958). This independent method differentiates between the fairly natural series of the genus *Eucalyptus*, with which this work is concerned, and separates odd species and odd subseries which recent work based on comparative morphology (Carr & Carr, 1959) suggests are anomalously classified by Blakely (1955).

SUMMARY

1. Chromatographic properties of hydroxystilbene derivatives have been studied. The corresponding dihydro derivatives formed on hydrogenation of these compounds undergo a characteristic R_f shift in the *N*-acetic acid solvent.

2. The *cis*- and *trans*-isomers of the three hydroxystilbene derivatives of *E. wandoo* heartwood are separated by *N*-acetic acid on a paper chromatogram.

3. The use of *cis*- and *trans*-isomers of 3,5,4'-trihydroxystilbene and its 3 β -D-glucoside as taxonomic tracers differentiates between the fairly 'natural' series of the subsection *Longiores* of the genus *Eucalyptus*, and separates odd species and odd subseries which are rather artificially classified by Blakely (1955).

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REFERENCES

- Bate-Smith, E. C. (1956). *Sci. Proc. R. Dublin Soc.* **27**, 165.
 Bate-Smith, E. C. (1958). *Proc. Linn. Soc. Lond.* **169**, 198.
 Blakely, W. F. (1955). *A Key to the Eucalypts*, 2nd edition. Canberra: Forestry and Timber Bureau.
 Carr, D. J. & Carr, S. G. M. (1959). *Nature, Lond.*, **184**, 1549.
 Erdtman, H. (1958). In *Proc. IVth int. Congr. Biochemistry, Vienna*, **2**. *Symp. II. Biochemistry of Wood*, p. 1. Ed. by Kratzl, K. & Billek, G. London: Pergamon Press.
 Hathway, D. E. (1958). *Biochem. J.* **70**, 34.
 Hathway, D. E. (1960). In *Chromatographic and Electrophoretic Techniques*, vol. 1, *Chromatography*, p. 308. Ed. by Smith, I. London: William Heinemann Medical Books Ltd.
 Hathway, D. E. & Seakins, J. W. T. (1959a). *Biochem. J.* **72**, 369.
 Hathway, D. E. & Seakins, J. W. T. (1959b). *Biochem. J.* **72**, 37p.
 Koch, J. E. & Krieg, W. (1938). *Chem.-tech. Z.* **62**, 140.
 Linstedt, G. (1950). *Acta chem. scand.* **4**, 448.
 Mueller, F. von (1879). In *Eucalyptographia*. Melbourne: Government Printer.
 Roberts, E. A. H., Wight, W. & Wood, D. J. (1958). *New Phytol.* **57**, 211.
 Williams, A. H. (1955). *Chem. & Ind.* p. 120.

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The Rate of Changes in Inorganic Phosphate Content of Ehrlich Ascites-Tumour Cells

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A good deal is known about the content of inorganic phosphate in the cells of the Ehrlich ascites tumour under different conditions (e.g. Chance & Hess, 1959; Ibsen, Coe & McKee, 1958; Kvamme, 1958; Braun, Erdelyi & Udvardy, 1960), but the changes in inorganic phosphate when the conditions are varied are very rapid, and little is known about the rate at which they occur. This paper gives evidence of the rates, in varying circumstances, at which the inorganic phosphate in these cells is esterified.

Four difficulties had to be dealt with before reliable and reproducible measurements could be made: (1) the inorganic phosphate content of a freshly prepared washed suspension of the cells is variable; (2) at 37° the inorganic phosphate of the cells rapidly leaks out into the medium (Kvamme, 1958); (3) at 37° the rate of the changes is too great to be measured conveniently; (4) phosphate buffer cannot be used for such measurements, and most other buffers inhibit the glycolysis of the cells at 37°.

The first point was dealt with by incubating the cells in nitrogen before beginning the measurements, a procedure which increased the inorganic phosphate content to a fairly constant high value, from

which subsequent reduction under different conditions could be observed. Leakage from the cells was minimized by keeping them at a low temperature throughout, and the rate of the changes in inorganic phosphate was decreased by carrying out the experiments at 12° instead of at 37°. Only one of the common buffers alternative to phosphate did not inhibit the glycolysis of these cells at 37°, namely bicarbonate buffer, but at 12° the inhibitory effect of tris was negligible, and in some experiments at 12° this buffer was used.

EXPERIMENTAL

Abbreviations. P_i, Inorganic phosphate.

Ascites-tumour cells. The Ehrlich ascites tumour [a strain originated by Klein (1951) from a solid Ehrlich carcinoma] was maintained in mice of the BalbC or C3H strain, and the ascitic fluid was removed from mice showing a sufficient accumulation of ascites, usually 7–10 days after inoculation. The combined fluid from anything up to 20 mice was filtered through glass wool, and the cells were washed, without preliminary treatment with water to lyse red blood cells, by centrifuging at 0° for 1–1.5 min. at 500g. Washing was with ice-cold calcium-free Krebs–Ringer solution, and from four to six washes with 5–10 vol. of that solution sufficed to remove ascitic fluid and all but very