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THE OCCURRENCE AND SEASONAL DISTRIBUTION OF HIGHER ISOPRENOID ALCOHOLS IN THE PLANT KINGDOM*

A. R. WELLBURN† and F. W. HEMMING

Department of Biochemistry, University of Liverpool

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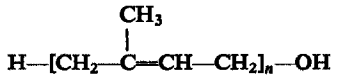
Abstract—Mixtures of higher isoprenoid alcohols, consisting of alcohols having 50, 55, 60 and 65 carbon atoms have been detected in the leaves of the eleven angiosperms examined. In four of these plants, the total amount of these isoprenoid alcohols was found to rise as the leaves aged. Higher isoprenoid alcohols were also found in the alga, *Chlorella pyrenoidosa*, but not detected in the eleven cryptogams examined.

INTRODUCTION

SINCE the first isolation in 1956 of the long-chain isoprenoid alcohol solanesol, from tobacco leaves,[1] a whole series of similar compounds all having the general formula (I) have been



(I)

found and characterized. Solanesol, (I, $n=9$) is not accompanied in tobacco by its congeners whereas other natural long-chain alcohols have been shown to occur as mixtures. The wood from the silver birch, *Betula verrucosa*, contains alcohols termed the betulaprenols (I, $n=6$, 7, 8 and 9).[2] Higher isoprenoid alcohols, where $n=10$, 11, 12 and 13, have been found and characterized in the leaves of the horse-chestnut, *Aesculus hippocastanum*, and called the castaprenols.[3] Castaprenol-12 is the major component. Alcohols similar to the castaprenols have been found in the spadices of *Arum maculatum* and in the leaves of *Ficus elasticus* and *Beta vulgaris*.[4]

In solanesol, the protons on the double bonds are all in the *trans*-position relative to the methyl groups,[5] but in the betulaprenols and the castaprenols more than half of the isoprene residues are in the *cis*-configuration. Whether these *cis* units are distributed at random in each of the molecules or according to a special pattern has not yet been ascertained.

It has been shown earlier that most of the solanesol in tobacco is associated with the chloroplasts.[6] Similar work on horse-chestnut leaves has shown that the castaprenols too are

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[2] B. O. LINDGREN, *Acta Chem. Scand.* **19**, 1317 (1965).
[3] A. R. WELLBURN, J. STEVENSON, F. W. HEMMING and R. A. MORTON, In preparation.
[4] P. J. DUNPHY, F. W. HEMMING and J. F. PENNOCK, Unpublished results.
[5] R. B. BATES, R. H. CARNIGHAN, R. O. RAKUTIS and J. H. SCHAUBLE, *Chem. & Ind.* (*London*) 1020 (1962).
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partly associated with these organelles.[7] In the family of even larger polyisoprenols (dolichols) found in pig liver,[8] over half the molecules are esterified,[9] whereas almost all of the solanesol in tobacco leaves is unesterified.[6] The castaprenols resemble solanesol in that they are almost wholly unesterified[10] whereas most of the betulaprenols occur naturally as esters.[2]

This paper describes how the level of the castaprenols varies with increasing age of horse-chestnut leaf tissue and also reports the occurrence of similar isoprenoid alcohol mixtures in the photosynthetic tissue from a wide range of members of the plant kingdom.

RESULTS AND DISCUSSION

Seasonal Distribution

The results of the seasonal variation of long-chain isoprenoid compounds in horse-chestnut leaves are given in Table 1. It can be seen that throughout the year and with increasing age of leaf there is a corresponding rise in long-chain isoprenoids. The most noticeable increase however was in the castaprenols, the level in October (28 weeks) was almost 100 times that in April (4 weeks). Solanesol and squalene showed about a 14- and 16-fold increase

TABLE 1. VARIATION OF CONCENTRATION OF LONG-CHAIN ISOPRENOID CONSTITUENTS OF HORSE-CHESTNUT LEAVES WITH AGE OF THE LEAVES

Constituent	Age of leaves in weeks, after terminal bud unfolding						
	4 (April)	8	12	16 (July)	20	24	28 (October)
Chlorophyll (μmoles g tissue)	4·7	4 6	4·5	4 5	4·3	4·1	3 4
Castaprenols (μg g tissue)	1 2	2·0	32·0	40 0	88·0	94·0	106 4
Solanesol (mμmoles g tissue)	6	8	44	54	59	73	84
Squalene (mμmoles g tissue)	2	6	15	20	24	32	32

respectively. The results for *Laurus vulgare*, *Arum maculatum*, and *Ficus elasticus* (Table 2) confirm this general feature of increasing amounts of castaprenol-like materials with increasing age of leaf. It has been shown[3, 7, 11] that in horse-chestnut castaprenols exists as a mixture comprising less than 0·5 per cent castaprenol-10, approximately 16 per cent castaprenol-11, 82 per cent castaprenol-12 and 2 per cent castaprenol-13. Using the gas liquid chromatographic method described[11] each alcohol fraction in Table 1 was re-examined after preliminary preparative TLC purification. The results indicated that the pattern of the components of this mixture was essentially the same throughout the year.

Distribution Throughout the Plant Kingdom

The results are summarized in Table 2. Of the comprehensive range of cryptogams and gymnosperms examined, castaprenol-like alcohols were detected only in the alga, *Chlorella pyrenoidosa*. However such castaprenol-like alcohols were detected in each of the diverse angiosperms which were examined. In this survey only photosynthetic tissue was studied.

[7] A. R. WELLBURN and F. W. HEMMING, *NATO Symp. Chloroplasts*, Aberystwyth (1965).
[8] J. BURGOS, F. W. HEMMING, J. F. PENNOCK and R. A. MORTON, *Biochem. J.* **88**, 470 (1963).
[9] P. H. W. BUTTERWORTH, Ph.D. Thesis, University of Liverpool (1964).
[10] A. R. WELLBURN and F. W. HEMMING, Unpublished results.
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However, isoprenoid alcohols have been isolated from the non-photosynthetic spadix of *Arum maculatum* and from yeast[4] and fungi.[12] Lindgren isolated the betulaprenols from the wood of silver birch,[2] but a similar analysis of horse-chestnut bark and hardwood failed to reveal any polyisoprenoid alcohols.[10]

TABLE 2. OCCURRENCE OF CASTAPRENOL-LIKE MATERIALS IN THE PLANT KINGDOM

Tissue			μg/mg chlorophyll	mg/kg wet wt.
Cryptogams	Algae	*Anabaena variabilis*	N.D.†	—
		Euglena gracilis var. *bacillaris*	N.D.	—
		Chlorella pyrenoidosa	0·37*	—
		Fucus serratus	N.D.	—
		Fucus vesiculosus	N.D.	—
		Laminaria digitata	N.D.	—
		Monostroma grevillei	N.D.	—
	Hepaticae	*Conocephalum conicum*	N.D.	—
	Equisetales	*Equisetum arvense*	N.D.	—
	Filicales	*Aspidium felix-mas*	N.D.	—
		Pteridium aquilinum	N.D.	—
Phanerogams	Gymnosperms	*Cupressus lonciae*	N.D.	—
	Angiosperms	*Aesculus hippocastanum*		
		(young, 4 weeks)	0·5	1·2
		(old, 28 weeks)	34·3	106·4
		Ilex equifolium	0·1	0·1
		Laurus vulgare		
		(young)	0·6	0·5
		(old)	1·6	1·0
		Vicia faba	1·4	1·5
		Umbilicus rupestris	0·6	0·2
		Hedera helix	1·2	1·0
		Sambucus nigra		2·0
		Nicotiana virginiana	0·2	0·1
		Polygonum cuspidatum	5·8	3·0
		Ficus elasticus		
		(very young)		2·5
		(young)		27·1
		(old)		150·0
		(very old)		1500·0
		Arum maculatum		
		(young)	3·1	2·0
		(3 weeks later)	8·3	4·0
		(6 weeks later)	90·6	17·2

* Differed from the castaprenols on reverse-phase partition chromatography (see text).
† N.D. not detected. In the cryptogams and gymnosperms the limit of detection varied but was generally of the order of 0·1 μg castaprenol-like alcohol/mg chlorophyll.

The limit of detection of castaprenols, which was found to be 1 μg when applied as a spot on a chromatogram, was determined by a separate experiment in which decreasing amounts of standard castaprenol mixture were applied to two-dimensional plates. The limiting factor was therefore the weight of lipid that could be applied to each plate to supply the detectable 1 μg of castaprenol-like alcohol. The weight of lipid applied to each plate was exactly 5 mg and so column chromatography and saponification of the 6–8% E/P fraction were employed to

[12] J. BURGOS, P. H. W. BUTTERWORTH, F. W. HEMMING and R. A. MORTON, *Biochem. J.* **19**, 22P (1964).

reduce the general lipid weight in relation to the weight of the castaprenol-like alcohols. It may be possible that the cryptogams and gymnosperms examined contained some castaprenol-

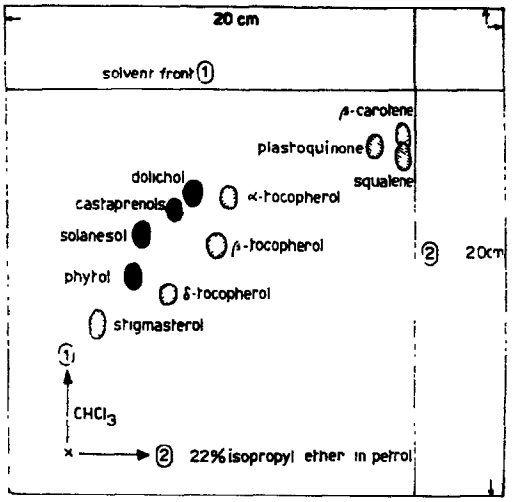

FIG. 1. TRACING OF A TWO-DIMENSION THIN-LAYER CHROMATOGRAM OF AUTHENTIC REFERENCE MATERIALS ON SILICA GEL G.

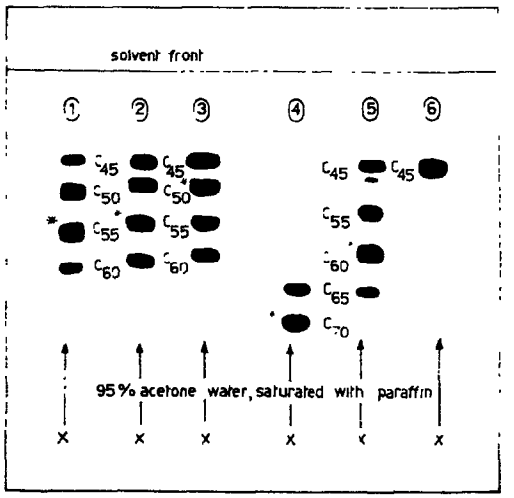

FIG. 2. TRACING OF A REVERSE-PHASE PARTITION CHROMATOGRAM ON A PARAFFIN-IMPREGNATED KIESELGUHR THIN-LAYER PLATE.

Alcohols from (1) *Ficus elasticus*, (2) *Vicia faba*, (3) *Ilex aquifolium*, (4) *Chlorella pyrenoidosa*, (5) castaprenol mixture, (6) authentic solanesol, * major component. The C_{50}-castaprenol (Castaprenol-10) showed up as a very small spot running close behind the spot labelled C_{45}. The "C_{45}-material" has an R_f more in keeping with its being solanesol than a C_{45}-castaprenol. Note that on this particular chromatogram the compounds on the right ran further (higher R_f) than those on the left.

like alcohols; however, if present at all, they must have been at a concentration of less than 0·02 per cent of unsaponifiable lipid of similar polarity. As application of more than 5 mg per plate caused overloading, it was difficult to extend the sensitivity of detection further.

The reversed phase partition chromatography (Fig. 2) shows clearly that the materials termed castaprenol-like isolated from other angiosperms have similar behaviour to the castaprenols of horse-chestnut in this system, indicating that they each exist as a mixture. The pattern of distribution varies considerably. Castaprenol-12 (C_{60}) is the major component of the castaprenols from horse-chestnut while in *Ilex aquifolium* (holly) an alcohol with an R_f corresponding to the C_{50} castaprenol-10 predominates. In *Ficus elasticus* and *Vicia faba*, the major component has an R_f similar to that of castaprenol-11. The quantitative composition of the mixtures of alcohols from the horse-chestnut and *Ficus elasticus* leaves obtained by this method and by gas–liquid chromatography[3, 7, 11] are in good agreement. Leaves of *Beta vulgaris*, another angiosperm, contain a mixture of alcohols closely resembling that found in *Ficus elasticus*.[7, 11]

The alga, *Chlorella pyrenoidosa* contains a mixture of alcohols which have a different behaviour on reversed phase chromatography. These alcohols, consisting of a mixture of higher chain length, can be distinguished from the other castaprenol-like alcohols found in the angiosperms. The R_f's of these compounds fall in the same range as those of the dolichols found in *Saccharomyces cerevisiae* (baker's yeast).[13]

The presence of C_{50}–C_{65} isoprenoid alcohols in the angiosperms in amounts sufficient for estimation is interesting as it may indicate a general occurrence of these alcohols in higher flowering plants. The wide range of concentrations found may be, in part, attributable to many factors such as the age of the tissue at time of extraction. Although most angiosperms studied were dicotyledonous, it is interesting to note that *Arum maculatum*, a monocotyledonous plant also contained castaprenol-like alcohols. Analysis of the isoprenoid alcohols by reversed phase and gas–liquid chromatography shows that the pattern of distribution of C_{50}, C_{55}, C_{60} and C_{65} components varies widely between genera and it is possible that a specific pattern may exist for each genus.

Lower isoprenoid alcohols such as all *trans*-geranylgeraniol as its pyrophosphate will probably be present in horse-chestnut leaves as a precursor of the side chain of the plastoquinone-4 found by Eck and Trebst.[14] It will almost certainly also be involved in the biosynthesis of the side chain of vitamin K_1 and the tocopherols. All *trans*-solanesol in the form of the pyrophosphate, probably has as its main function a role as the precursor of the side chain of plastoquinone-9. However, it is difficult to envisage a precursor role for the poly *cis*-compounds. The rise in the level of castaprenols with increasing age would indicate that the lipid composition of the leaf changes as the leaves get older. The function of the castaprenols is not yet known; possibly they have a structural role in cell membranes and it is conceivable that increases in levels of castaprenols could bring about changes in the structure of the leaf which may be an important factor in senescence. Alternatively it is possible that as the leaves age there is a derangement or loss of control of general isoprenoid biosynthesis resulting in high levels of castaprenols.

EXPERIMENTAL

A. Seasonal Variation of Castaprenols in Horse-chestnut Leaves

Leaves were collected from the same tree at four-weekly intervals, after terminal bud unfolding. As the levels of other isoprenoid compounds such as tocopherols are known to change with environmental conditions,[15] the method and time of sampling was kept as

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[14] H. Eck and A. Trebst, *Z. Naturforsch.* **18**, 446 (1963).
[15] V. H. Booth, *Phytochem.* **3**, 273 (1964).

constant as possible. The sample was collected from all round the tree at 4 p.m. and only the first and second pairs of leaves above each annual ring were taken.

The leaves were washed with water prior to removal of the main vascular tissue. Leaf tissue (250 g) was then finely chopped and saponified in 60% (w/v) KOH (750 ml) and 5% (w/v) methanolic pyrogallol (1500 ml) for 1 hr on a water bath. The hot saponification mixture was then filtered, diluted to three times its original volume with water and extracted three times with freshly distilled diethyl ether. The ethereal extract was washed with water, dried (Na_2SO_4), evaporated and the residue was blown to dryness under nitrogen.

The unsaponifiable lipid was taken up in light petroleum (b.p. 40–60°), and chromatographed on a 150 g column of acid-washed alumina (weakened to Brockmann Grade III) using increasing proportions of ether in successive 1 l. portions of eluent. The petrol fraction containing squalene and the 6–8% (w/v) ether in petrol (E/P) fractions containing solanesol and the castaprenols were blown to dryness under nitrogen and dissolved in a suitable volume of cyclohexane prior to chromatography on thin-layer plates of silica gel (275 μ thick). Estimation of each constituent depended on comparing spot size against a range of spots of standard amounts of authentic materials.[16] Petrol was used as a developing solvent for squalene (R_f = 0·50) and 1·5% (v/v) methanol in benzene for solanesol (R_f = 0·37) and the castaprenols (R_f = 0·56). The latter appear as only one spot in this system. On spraying the chromatogram with 20% (w/v) phosphomolybdic acid in ethanol and then heating, each alcohol showed up as a dark blue spot.

Chlorophyll was determined by a separate extraction of a known weight of leaf with methanol and estimated using the method of Vishniac.[17]

B. Identification of Castaprenol-like Materials in Other Plant Tissues

The tissues were extracted three times by maceration in acetone (approx. 2 ml g tissue) in the liquidizer of a Kenwood Chef and filtered through cloth of coarse mesh or a sintered glass funnel under reduced pressure. The acetone extract, diluted with water (1:1), was extracted with ether three times. The combined ethereal extract was then treated as above and the whole lipid fraction chromatographed on an alumina column as indicated previously using 10 g alumina for each 100 mg lipid. The 6–8% E/P fractions from each tissue were then saponified to reduce the weight of lipid. Prior experiments had shown that isoprenoid alcohols are unaffected by this treatment.

Using the two-dimensional TLC system devised for tocopherols by Pennock *et al.*[18] the identification of castaprenol-like materials was possible. In turn, 5-mg portions of saponified 6–8% E/P fraction from each tissue were applied to three separate plates A, B and C. To this spot were added 5 μg each of α, β and δ-tocopherol and the plate developed in the first dimension by chloroform followed by 22% (v/v) isopropyl ether in petrol in the second dimension. A, B and C were stained as follows: A, 20% (w/v) phosphomolybdic acid in ethanol; B, iodine vapour; C, 5% (w/v) anisaldehyde in a 1:19 (v/v) mixture of conc. H_2SO_4 and 90% (v/v) ethanol. The tocopherols were located easily by their immediate reduction (at room temperature) of phosphomolybdic acid (plate A) to a deep blue colour and by their yellow colour on heating plate C. With reference to the standard plate of known spot locations (Fig. 1) and using the three added tocopherols as an internal guide, the location of the spots of castaprenol-like materials could be predicted. If the spot had the same staining character-

[16] E. V. TRUTER, *Thin Film Chromatography*, p. 112. Cleaver-Hume, London (1963).
[17] W. VISHNIAC, *Methods of Enzymology*, Vol. 4, p. 342. Academic Press, New York (1957).
[18] J. F. PENNOCK, F. W. HEMMING and J. D. KERR, *Biochem. Biophys. Res. Commun* 17, 542 (1964).

istics as the castaprenols, that is intense yellow with iodine vapour, blue–black on heating with phosphomolybdic acid and blue–green on heating with anisaldehyde, it was then termed castaprenol-like. The amount of castaprenol-like materials in the fractions which satisfied these conditions was estimated by chromatography, against standards, on thin layers of silica gel using 1·5% methanol in benzene as the developing solvent.

Chlorophyll was determined in 80% acetone using the method of Arnon.[19]

C. Reversed-phase Partition Chromatography of Castaprenol-like Materials

The saponified fractions from *Ficus elasticus*, *Ilex aquifolium*, *Vicia faba* and *Chlorella pyrenoidosa* were chromatographed in bulk, preparatively on layers of silica gel G (600 μ thick). About 50 mg lipid in cyclohexane was applied to each 20 × 20 cm plate and using 22% (v/v) isopropyl ether in petrol as the developing solvent. The position of the alcohol band, moving with a marker spot of authentic castaprenol, was indicated by spraying with 0·01% (w/v) fluorescein in ethanol[20] and was then eluted with ether and blown to dryness under nitrogen. This alcohol-containing fraction was then taken up in acetone and, using the method of Pennock *et al.*,[21] was chromatographed on a paraffin-impregnated thin layer of kieselguhr with 95% (v/v) acetone–water, saturated with paraffin, as the mobile phase. Applying amounts of 1·5 μg alcohol as a spot and using the anisaldehyde stain, the castaprenol-like alcohols separated into their component spots instead of running together as in the adsorption chromatography (Fig. 2) (see also Wellburn *et al.*[3]).

Acknowledgement—We wish to thank Dr. C. Bucke for the supply of some of the lipid extracts used in this work.

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[20] P. J. Dunphy, K. J. Whittle and J. F. Pennock, *Chem. & Ind. (London)* 1217 (1965).
[21] J. F. Pennock, Personal communication.