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The Characterization and Properties of Castaprenol-11, -12 and -13 from the Leaves of *Aesculus hippocastanum* (Horse Chestnut)

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The isolation and purification of a mixture of *cis–trans*-polyprenols from the leaves of *Aesculus hippocastanum* (horse chestnut) are described. Results of studies involving mass spectrometry, nuclear magnetic resonance, infrared spectroscopy, micro-hydrogenation and ozonolytic degradation show the mixture to be made up of undecaprenol, dodecaprenol and tridecaprenol with dodecaprenol predominating. Each of the prenols contains three *trans* internal isoprene residues and a *cis* 'OH-terminal' isoprene residue. They differ from each other only in the number of *cis* internal isoprene residues. The trivial names castaprenol-11, castaprenol-12 and castaprenol-13 are proposed to describe these compounds. Gas–liquid-chromatographic and reversed-phase partition thin-layer chromatographic evidence suggest the presence in the mixture of small quantities of castaprenol-10 also.

The presence in Nature of isoprenoid alcohols up to C_{20} in chain length has been a source of diverse interests in both chemistry and biochemistry for many years. However, only recently has the widespread occurrence of polyisoprenoid alcohols (polyprenols) of considerably greater chain length been recognized. Solanesol (all-*trans*-nonaprenol) was isolated from leaves of *Nicotiana tabacum* (tobacco plant) by Rowland, Latimer and Giles (1956). A C_{100} prenol (dolichol-20) present in many mammalian tissues was characterized by Burgos, Hemming, Pennock & Morton (1963). In dolichol(s) the 'OH-terminal' isoprene residue is saturated. Prenols closely related to dolichol have also been described in *Saccharomyces cerevisiae* (baker's yeast) and in *Aspergillus fumigatus* Fresenius (Burgos, Butterworth, Hemming & Morton, 1964). Polyprenols in non-photosynthetic tissue of higher plants have also been reported. For instance the spadix of *Arum maculatum* (lords and ladies) yields a number of different alcohols (Hemming, Morton & Pennock, 1963) and the elegant work of Lindgren (1965) has brought to light the presence of betula-prenols-6, -7, -8 and -9 in the wood of *Betula verrucosa* (silver birch). Apart from solanesol and possibly one of the *Arum* spadix polyprenols, all of these prenols have more isoprene residues in the *cis* than in the *trans* configuration. Thorne & Kodicek (1966) have provided evidence of the presence of bactoprenol (dolichol-11) in extracts of *Lactobacillus casei*. Although this alcohol is the major metabolite of mevalonic acid in this organism, it was present at very low concentrations and the configuration of its isoprene residues has not been determined.

The presence of polyprenols other than solanesol in leaves of higher plants has been known for a few years. The trivial name castaneol was given to a polyprenol preparation isolated from leaves of the *Aesculus hippocastanum* (horse chestnut) by Stevenson (1963). The presence of similar polyprenols in leaves of all higher plants now seems most likely (Wellburn & Hemming, 1966*a*). The concentrations of these compounds vary but in horse-chestnut leaves are up to 110mg./kg. of leaves. Work on the nature of the horse-chestnut polyprenol preparation has coincided with improvements in chromatographic and spectroscopic techniques. As a result of this it is now clear that this preparation contains at least four polyprenols. The three major components have been characterized and have been given the trivial names castaprenol-11, -12 and -13, replacing the earlier term castaneol. The isolation, characterization and properties of these compounds are described in this paper.

METHODS

Isolation and purification of castaprenols. Leaves were taken from horse-chestnut trees (*Aesculus hippocastanum*) in the Liverpool district during September and were stripped free of their main veins. The washed leaf tissue (9·7kg.) was then cut into small pieces and digested by refluxing (250g. at a time) for 1hr. with a mixture of aq. KOH (60%, w/v; 750ml./250g. of tissue) and a solution of pyrogallol in methanol (5%, w/v; 1500ml./250g. of tissue). The hot saponification mixture was then filtered, diluted with water and extracted with ether (3 × 1500ml./250g. of tissue). The ethereal extract was washed free of KOH with water, dried

over anhydrous Na_2SO_4 and evaporated to dryness, finally under N_2. The yield of unsaponifiable lipid was 156g.

A solution of this extract in 25% (v/v) diethyl ether in light petroleum (2l.) was shaken vigorously with a suspension of alumina (1·5kg., Brockmann grade 3) in light petroleum (1l.) and then filtered. The alumina was washed with (25% v/v) diethyl ether in light petroleum (2×2l.). Polar impurities were retained by the alumina. The filtrate was evaporated to dryness and the material redissolved in warm light petroleum. Most of the sterol was then removed by crystallization (twice) from this solution at 0°. The material (17·4g.) in the bulked mother liquors was further purified by chromatography on seven 250g. columns of alumina (acid-washed, Brockmann grade 3; column 15cm.×4·8cm. with a centre rod). A mixture of 3% E/P* eluted hydrocarbons, plastoquinone-9 and other relatively non-polar lipids. The next eluent (10% E/P) contained long-chain prenols, tocopherols and ubiquinone as well as unidentified material. Sterols accounted for most of the weight in the next fraction, eluted by 25% E/P.

The 10% E/P fraction contained 3·45g. of material that was chromatographed preparatively on 70 layers (20cm.× 20cm., 700μ thick) of silica gel G with 2% (v/v) methanol in benzene as developing solvent. A broad band of silica gel corresponding in position to that of long-chain prenols (R_F 0·3–0·5) was scraped off, and the lipid material (1·87g.) was eluted from this with diethyl ether.

A solution of this material in toluene (200ml.) was then mixed with acetic anhydride (A.R., 100ml.) and pyridine (2ml.) and maintained at room temperature for 16hr. The mixture was then poured into ice-cold 2N-HCl (400ml.) and extracted with 50% E/P (3×200ml.). The non-aqueous layer was washed free of acid with water and, after drying over anhydrous Na_2SO_4, it was evaporated to dryness, finally under N_2. This acetylated preparation was dissolved in light petroleum and separated into its components by careful chromatography on a column of alumina (see Table 1). Fraction 4 of this chromatography contained the castaprenyl acetates and this was hydrolysed by dissolving in diethyl ether (5ml.) and refluxing this solution for 30min. with 60% (w/v) KOH in water (25ml.) and 5% (w/v) pyrogallol in ethanol (50ml.). An ether extract of the cooled mixture was washed free of alkali.

A small quantity of solanesol was removed from the unsaponifiable material by preparative chromatography on 14 layers (20cm.×20cm., 700μ thick) of silica gel G with 2% (v/v) methanol in benzene as developing solvent (solanesol, R_F 0·38; castaprenol, R_F 0·45).

The purified material (543mg.) was then crystallized twice from ethanol at −20° to yield 202mg. of material which ran as a single spot on a thin-layer adsorption chromatogram (silica gel G; 1%, v/v, methanol in benzene). A further 254mg. of material was recovered by crystallization from the mother liquors and appeared to be identical in every way with the first batch of crystals. The two samples were combined, giving a yield of 47mg./kg. of leaf. At room temperature the preparation was a colourless, odourless, viscous oil.

Subsequent investigations showed this castaprenol preparation to be a mixture of isoprenologues, principally castaprenol-10, -11, -12 and -13. The last three (only traces of castaprenol-10 were present) were separated from each other by preparative reversed-phase partition thin-layer chromatography as described by Dunphy, Kerr, Pennock & Whittle (1966). Thin layers of kieselguhr (20cm.×20cm., 200μ thick) were saturated with a solution of liquid paraffin in light petroleum (5%, v/v, previously passed through a column of alumina, 250g., Brockmann grade 3) and the solvent was then allowed to evaporate. An acetone solution (0·1ml.) of the castaprenols (1–2mg.) was applied as a line to each paraffin-impregnated plate and the preparation was chromatographed with acetone–water (23:2, v/v) as developing solvent. The developed chromatogram was dried in a stream of air and was then sprayed with an ethanolic solution of fluorescein (0·01%, w/v). After evaporation of the ethanol, accelerated by a stream of air, the plate was dampened with steam from a boiling-water bath and viewed immediately under ultraviolet light. The castaprenols showed up as white bands on a blue–purple background (see also Dunphy *et al.* 1966). The corresponding bands of 40 chromtagrams were removed separately and extracted with ether. Care was taken also to recover material dissolved in the paraffin adhering to the glass plate by wiping with small pieces of cotton wool saturated with ether. Each filtered extract was evaporated to dryness, dissolved in light petroleum and was passed through a column of alumina (acid-washed, Brockmann grade 3, 20g.). A total volume of 200ml. of light petroleum removed all of the paraffin from each column. Castaprenols were eluted by passing 10% E/P (200ml.) through each column. When recovery of castaprenols from reversed-phase partition thin-layer chromatograms was not required, the castaprenols were best visualized by staining with a mixture of anisaldehyde in ethanol and sulphuric acid (see Dunphy *et al.* 1966). The prenols appeared as mauve or grey–green areas on a white background, depending upon the concentration.

Spectroscopy. Infrared spectra were recorded with models

Table 1. *Chromatography of the castaprenol-containing fraction after acetylation*

The fraction (1·87g.) was acetylated and then chromatographed on a column of alumina (100g., acid-washed, Brockmann grade 3). The fractions were eluted as below and the major components identified by infrared and ultraviolet (where appropriate) spectroscopy as well as by chromatographic polarity. P, Light petroleum; %E/P, % diethyl ether in light petroleum.

Fraction	Eluent (l.)	Lipid eluted	
		Weight (mg.)	Major component
1	P	42	Not examined
2	0·25% E/P	22	Phytyl acetate
3	0·5% E/P	53	Phytyl acetate
4	0·5% E/P	670	Castaprenyl acetates
5	1·0% E/P	53	Solanesyl acetate
6	2·0% E/P	655	Tocopheryl acetates
7	3·0% E/P	43	Tocopheryl acetates
8	10·0% E/P	22	Not examined

* Abbreviation: E/P, Diethyl ether in light petroleum at the percentage (v/v) concentration indicated before the abbreviation.

137 and 237 of the Perkin–Elmer Infrared range of instruments. Samples were usually studied as solvent-free films between rock salt plates. High-melting (> 50°) compounds were studied as KBr disks.

Ultraviolet spectra were determined with a Unicam model SP. 800 recording spectrophotometer.

The mass spectra of the castaprenol mixture (at 220°) and of solanesol (at 190°) were obtained by Mr A. E. Williams of Imperial Chemical Industries, Blakely, Manchester. That of castaprenol-12 (at 220°) was determined by Mr J. V. Barkley of the Department of Organic Chemistry, University of Liverpool. All spectra were determined on MS.9 spectrometers (A.E.I., Manchester) with a direct inlet.

Most of the nuclear-magnetic-resonance spectra were recorded in benzene with a Varian HA100 Mcyc./sec. instrument by Dr J. Feeney of Varian Associates Ltd., Walton-on-Thames, Surrey. Dr Feeney also determined some spectra in $CDCl_3$ with a Varian A 60A instrument in the same Laboratory. One spectrum with a Varian HA100 instrument was determined by Dr R. J. Abraham and Miss C. Hale of the Department of Organic Chemistry, University of Liverpool.

Formation of [1-^{14}C]*acetate and the assay of* ^{14}C. A sample of [1-^{14}C]acetic anhydride (0·1 mc) from The Radiochemical Centre, Amersham, Bucks., was diluted to 25 ml. with unlabelled acetic anhydride. Solanesol, β-naphthol and the castaprenol preparation (50 mg. each) were each separately dissolved in toluene (5 ml.) containing [1-^{14}C]acetic anhydride (1 ml.) and pyridine (3 drops). After 16 hr. the acetates were extracted into diethyl ether (as described previously) and each was purified by preparative chromatography on two layers of silica gel G (20 cm. × 20 cm., 700 μ thick) with benzene–cyclohexane (1:1, v/v) as the developing solvent. This was followed by repeated crystallization from ethanol at −20°. About 10 mg. of each preparation was then weighed on a microbalance and dissolved in benzene (10 ml.). Samples (2 ml.) were then evaporated to dryness in counting vials and dissolved in toluene (10 ml.) containing 0·5% (w/v) 2,5-diphenyloxazole and 0·03% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene. The vials were assayed in a Packard Tri-Carb scintillation counter at a counting efficiency of 56·1%.

Micro-hydrogenation. The prenol mixture dissolved in cyclohexane–ethanol–acetic acid (1:1:1, by vol.) was hydrogenated at room temperature and atmospheric pressure in a Towers micro-hydrogenation apparatus. Platinum oxide was used as a catalyst. The apparatus has been shown to give figures 5·2% (± 1·4 S.D.) higher (mean for eight different compounds) than the theoretical figures. The castaprenol figures were corrected to take this error into account.

Ozonolysis. A portion of the castaprenol mixture (42 mg.) was acetylated and dissolved in ethyl acetate (5 ml., redistilled from 2,4-dinitrophenylhydrazone). The solution was ozonized for 30 min. at 0°. Oxygen was then passed through the solution until ozone could not be detected (by starch–iodide paper) in the escaping stream of gas. The ozonides were cleaved reductively by adding zinc dust (50 mg.) and acetic acid (1 drop) and leaving for 16 hr. The resulting solution was filtered into a solution of 2,4-dinitrophenylhydrazine (1 g., freshly extracted with light petroleum) in 2 N-HCl and the 2,4-dinitrophenylhydrazones were precipitated on standing. These were filtered off. When the procedure was repeated with ethyl acetate alone no precipitate was formed at this stage, indicating that all the 2,4-dinitrophenylhydrazones formed were derived from the mixture of castaprenols.

The 2,4-dinitrophenylhydrazones were then extracted with light petroleum in a Soxhlet apparatus for 3 hr. The ultraviolet-absorption spectra of both the petrol-soluble and petrol-insoluble 2,4-dinitrophenylhydrazones, in cyclohexane and chloroform respectively, were compared with those of authentic compounds (see Burgos *et al.* 1963).

Portions of the petrol-soluble and petrol-insoluble materials were analysed by gas–liquid chromatography according to the method of Soukup, Scarpellino & Danielczik (1964). The gas chromatograph and carrier gas were as described in the next section. The stainless-steel tube (6 ft. long, internal diam. 0·096 in.) was packed with silane-treated Chromosorb W coated with SF–96 (10%, w/w). A temperature of 250° was maintained for 4 min., after which it was programmed to rise at 10°/min. for a further 7 min. The temperature was then held at 320°. Authentic samples of the 2,4-dinitrophenylhydrazones of acetone and laevulaldehyde (bis derivative) had a retention time of 1·93 and 19 min. respectively. For some unexplained reason an authentic sample of the 2,4-dinitrophenylhydrazone of glycollic aldehyde acetate failed to give a peak on the gas chromatogram. The petrol-soluble 2,4-dinitrophenylhydrazones were injected as a solution in benzene, whereas the relatively insoluble 2,4-dinitrophenylhydrazones were injected as a suspension in dimethylformamide.

The petrol-soluble and -insoluble preparations were also studied by thin-layer chromatography according to the method of Byrne (1965). With silica gel G as the adsorbent and benzene–tetrahydrofuran (9:1, v/v) as developing solvent, the R_F values of the 2,4-dinitrophenylhydrazones of authentic laevulaldehyde (bis), acetone, acetaldehyde and glycollic aldehyde acetate were 0·78, 0·70, 0·66 and 0·51 respectively. Of a number of chromatographic systems tried, this gave the most successful separation.

Gas–liquid chromatography of castaprenol derivatives. Gas–liquid chromatography was carried out in a manner similar (Wellburn & Hemming, 1966*b*) to that used by Lindgren (1965) for studying derivatives of betulaprenol-6, -7, -8 and -9.

Prenols (10–20 mg.) were acetylated by mixing and leaving for 16 hr. solutions in benzene (5 ml.) with acetic anhydride (5 ml.). The acetates were extracted as described previously. Small portions of these acetates were hydrogenated, also as described previously. The resulting mixtures contained perhydroprenyl acetates and the corresponding saturated hydrocarbons resulting from elimination of the acetyl residue during hydrogenation. The hydrocarbons and perhydroprenyl acetates were separated by preparative chromatography on layers (20 cm. × 20 cm., 200 μ thick) of silica gel G, light petroleum being used as developing solvent. When samples of the perhydroprenyl acetates were required with minimal losses to hydrocarbon it was found best to first hydrogenate the free prenols. The prehydroprenols were then acetylated.

Gas-chromatographic data of the castaprenyl acetates (mixture), perhydrocastaprenyl acetates (mixture) and of the derived saturated hydrocarbons (mixture) were compared with those for similar derivatives of solanesol and a mixture of betulaprenol-6, -7, -8 and -9. The authors are grateful to Dr B. O. Lindgren of Svenska Träforsknings-

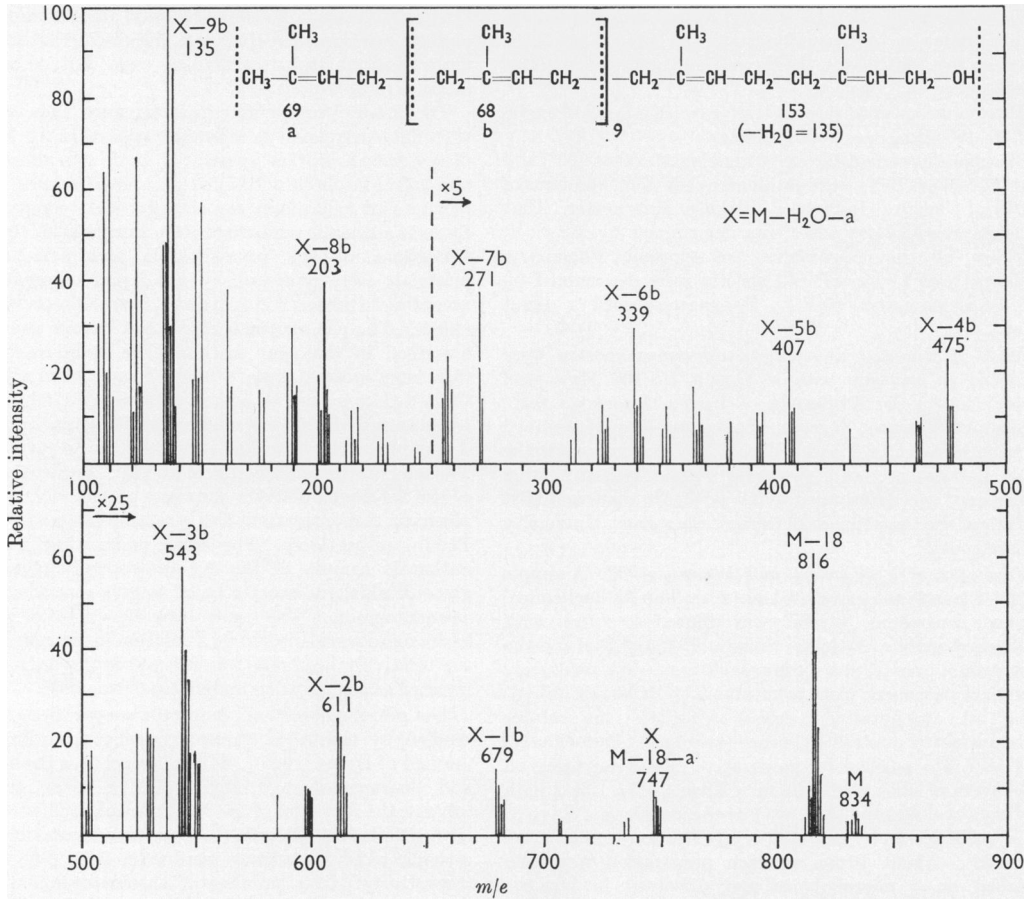


Fig. 1. Histogram of the more prominent peaks in the mass spectrum of castaprenol-12. Only peaks with intensities greater than the following limits (relative to 100 for *m/e* 135) are shown: 10 in range 100–200, 3 in range 200–300, 1 in range 300–550, 0·3 in range 550–700, 0·15 in range 700–900. In the histogram, intensities of the peaks in the range 250–500 and 500–900 have been increased by factors of 5 and 25 respectively. The broken lines in the structure indicate the units that would be produced if the molecule fractured at these points (see text). The unit (ion) X is produced by loss of water and of the ω-terminal isoprene residue from the molecular ion.

institutet, Stockholm, Sweden, for the gift of a sample of his mixture of betulaprenol-6, -7, -8 and -9 and to Dr O. Isler of Hoffmann–La Roche, Basle, Switzerland, for the gift of solanesol.

The acetates or hydrocarbons, dissolved in cyclohexane, were injected on to silane-treated Chromosorb W which had been coated with SE–30 (1%, w/w) and had been packed in a 4ft.-long silane-treated stainless-steel tube with internal diameter 0·125in. A dual-column F & M model 810 gas chromatograph fitted with a flame ionization detector was used isothermally at a temperature of either 300° or 340°. The carrier gas was argon at a flow rate of 60ml./min.

RESULTS

Mass spectrometry. A histogram of the more prominent peaks of the mass spectrum of castaprenol-12 is reproduced in Fig. 1. The probable assignments of the most prominent peaks in this spectrum and in those of solanesol (all-*trans*-nonaprenol) and of the mixture of castaprenols are listed in Table 2. In compounds of this type one would expect the molecular ion to lose water and also to fracture at the bond between adjacent methylene groups (dotted lines in structure in Fig. 1). This is in fact what happens. There is a small molecular ion (e.g. at *m/e* 834 in the spectrum of castaprenol-12). The ion corresponding to loss of water (M − 18) is much more prominent. There is then loss of the ω-terminal residue followed by loss of isoprene units, one at a time from the ω-end until a very abundant fragment with *m/e* 135 is left. This fragment is derived from the 'hydroxyl-terminal'

Table 2. *Probable assignments and relative intensities of some of the more prominent peaks in the mass spectra of solanesol, castaprenol-12 and the castaprenols preparation*

N.R., Not recorded

Peak	Intensities (relative to 100 for *m/e* 135)			Assignment*
m/e	Solanesol (190°)	Castaprenol-12 (220°)	Castaprenols preparation (220°)	
41	N.R.	84·2	61·0	$H_2C{=}C^+{-}CH_3$ or $H_2C{-}C^+H{-}CH_2$
55	51·0	84·3	78·0	$(CH_3)_2C{=}C^+H$
69	150	150	200	$(CH_3)_2C{=}CH{-}{}^+CH_2$
81	150	150	200	$^+CH_2{-}C(CH_2){=}CH{-}CH_2{-}C^+H_2$ or $^+CH_2{-}CH{=}C(CH_3){-}CH{=}CH_2$
95	87	106	171	$(CH_3)_2C{=}CH{-}CH_2{-}CH{=}C^+H$
121	57	67·1	144	$^+C(CH_3){=}CH{-}CH_2{-}CH_2{-}C(CH_3){=}CH{-}CH_2OH$ less H_2O
135	100	100	100	$^+b_2$—OH less H_2O
203	24·7	40·0	43·5	$^+b_3$—OH less H_2O
271	10·6	7·6	22·4	$^+b_4$—OH less H_2O
339	6·6	6·0	13·0	$^+b_5$—OH less H_2O
407	5·1	4·6	10·8	$^+b_6$—OH less H_2O
475	3·8	4·6	7·7	$^+b_7$—OH less H_2O
543	2·5	2·3	5·7	$^+b_8$—OH less H_2O
611	—	1·0	3·9	$^+b_9$—OH less H_2O
612	12·3	—	—	$^+H{-}[{-}CH_2{-}C(CH_3){=}CH{-}CH_2{-}]_9{-}OH$ less H_2O
630	1·7	—	—	$^+H{-}[{-}CH_2{-}C(CH_3){=}CH{-}CH_2{-}]_9{-}OH$
679	—	0·58	3·0	$^+b_{10}$—OH less H_2O
747	—	0·40	1·3	$^+b_{11}$—OH less H_2O
748	—	—	4·7	$^+H{-}[{-}CH_2{-}C(CH_3){=}CH{-}CH_2{-}]_{11}{-}OH$ less H_2O
766	—	—	1·8	$^+H{-}[{-}CH_2{-}C(CH_3){=}CH{-}CH_2]_{11}{-}OH$
815	—	—	0·97	$^+b_{12}$—OH less H_2O
816	—	2·2	11·9	$^+H{-}[{-}CH_2{-}C(CH_3){=}CH{-}CH_2]_{12}{-}OH$ less H_2O
834	—	0·20	4·2	$^+H{-}[{-}CH_2{-}C(CH_3){=}CH{-}CH_2{-}]_{12}{-}OH$

Table 2—*continued*

Peak *m/e*	Intensities (relative to 100 for *m/e* 135) Solanesol	Castaprenol-12	Castaprenols preparation	Assignment*
884	—	—	0·44	$^{+}H—[CH_2—\underset{\displaystyle CH_3}{\underset{\vert}{C}}=CH—CH_2—]_{13}—OH$ less H_2O
902	—	—	0·16	$^{+}H—[CH_2—\underset{\displaystyle CH_3}{\underset{\vert}{C}}=CH—CH_2]_{13}—OH$

* $b=[CH_2—\underset{\displaystyle CH_3}{\underset{\vert}{C}}=CH—CH_2]$.
† Or an isomer thereof.

isoprene residue and the isoprene residue adjacent to this. It is possibly stabilized by the loss of water resulting in an extra double bond in this region. Rearrangement of the double bonds, possibly to give

$$^{+}CH_2—\underset{\displaystyle CH_3}{\underset{\vert}{C}}=CH—CH_2—CH=\underset{\displaystyle CH_3}{\underset{\vert}{C}}—CH=CH_2 \qquad (m/e\,135)$$

would result in there no longer being two adjacent methylene groups, thus stabilizing the fragment.

Most of the major peaks below *m/e* 100 (not shown in Fig. 1) probably originate from the ω-end of the molecule, especially as they are also prominent in the spectra of rhodoquinone-9 (Powls & Hemming, 1966) and ubiquinone-4 (unpublished work). Some of the assignments in this region are rather tentative and require migration of double bonds and possibly, in some cases, cyclization.

The mass spectrum of castaprenol-12 is consistent with the compound being dodecaprenol. The spectrum of solanesol is that expected for a nonaprenol and shows a cracking pattern essentially similar to that of the castaprenols. There is no clear difference between the spectra of the two types of prenols that could be used for information regarding the *cis* or *trans* configuration of each isoprene residue. The mass spectrum of the castaprenols preparation is in favour of the presence of castaprenol-11, -12 and -13. The proportions of the intensities of the corresponding molecular ions are 11·5:26·1:1. However, this cannot be used as an accurate guide to the percentage composition of the mixture for these precise proportions vary with changes of the conditions under which the mass spectra are run. For instance, when the spectrum was determined at 230°, these proportions became 1·9:10·9:1.

Nuclear magnetic resonance. The nuclear-magnetic-resonance spectrum at 100Mcyc./sec. of castaprenol-12 in both carbon tetrachloride and in benzene is reproduced in Fig. 2. The positions of the resonance peaks in the region 7·5–8·5τ of the spectra of castaprenol-11, -12 and -13 and their assignments are listed in Table 3. The relative areas under the peaks (integrated by the instrument) are also given together with those expected by theory. The theoretical figures assume that (1) the structures are as indicated in the Table and (2) that each prenol contains a *cis* 'OH-terminal' isoprene residue (methyl protons resonating at 8·28τ in CCl_4, 8·27τ in $CDCl_3$ and 8·38τ in benzene), three internal *trans*-isoprene residues (methyl protons resonating at 8·42τ in CCl_4, 8·42τ in $CDCl_3$ and 8·38τ in benzene) and, of necessity, methyl groups both *cis* and *trans* to the olefinic proton in the ω-isoprene residues (*cis*: 8·34τ in CCl_4, 8·33τ in $CDCl_3$ and 8·32τ in benzene; *trans*: 8·42τ in CCl_4 and $CDCl_3$ and 8·43τ in benzene). All other isoprene residues are internal *cis* (methyl protons resonating at 8·34τ in CCl_4, 8·33τ in $CDCl_3$ and 8·25τ in benzene). It is clear from the good agreement in Table 1 between the actual and expected relative area that these assumptions are correct. The assignments for the 8·2–8·5τ region of the spectrum in CCl_4 are based on the work of Bates & Gale (1960).

Spectra in $CDCl_3$ are very similar to those in CCl_4 and assignments can be translated directly from the one solvent to the other. The resolution of the methyl protons peaks of the internal *cis* and *trans* residues in these solvents is 0·08–0·09τ. Chen (1962) has reported that in benzene this resolution is increased to 0·13τ and because of this benzene was used in the study of the nuclear magnetic resonance of pig liver dolichol (Burgos *et al.* 1963).

At 60Mcyc. this extra resolution is a significant aid to accurate measurement of the areas of these *cis* and *trans* peaks but it appears that it is not so essential at 100Mcyc. There are complications

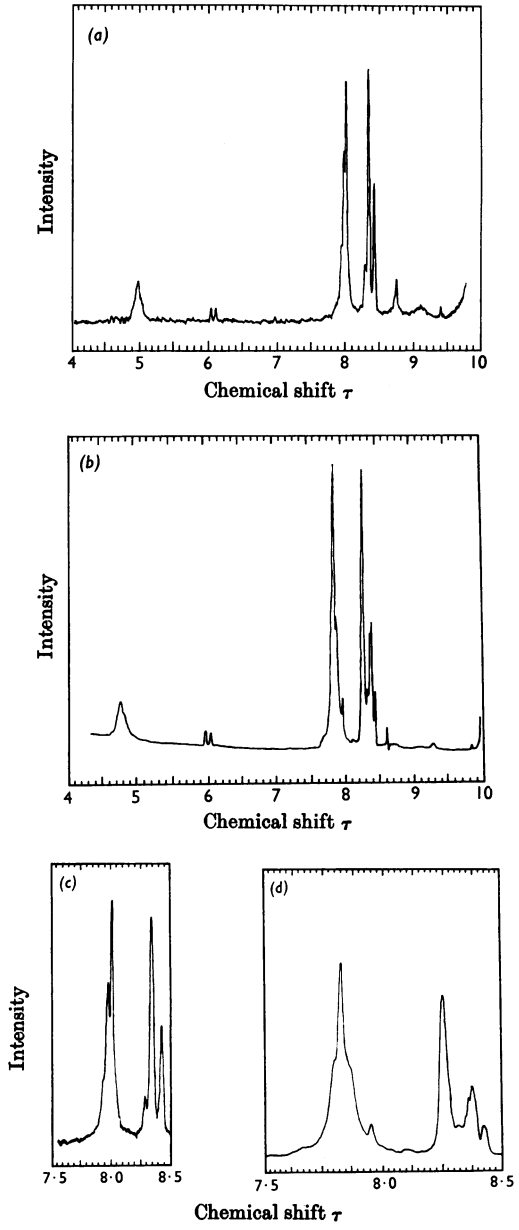


Fig. 2. Tracings of nuclear-magnetic-resonance spectra at 100 Mcyc./sec. of castaprenol-12; (*a*) in carbon tetrachloride; (*b*) in benzene; (*c*) expanded 7·5 → 8·5 τ region in carbon tetrachloride; (*d*) expanded 7·5 → 8·5 τ region in benzene.

when benzene is used. In this solvent the protons of the *cis*-methyl and *trans*-methyl of the ω-isoprene residue of a prenol resonate at 8·32 and 8·43 τ respectively and can be distinguished from those of *cis* and *trans* methyl groups of internal isoprene residues, resonating at 8·25 and 8·38 τ respectively. The methyl group of a *cis* 'OH-terminal' isoprene residue appears to resonate in a position almost indistinguishable from that of the internal *trans* peak at 8·38 τ, while the methyl of a *trans* 'OH-terminal' isoprene gives a peak at 8·52 τ. A peak at 8·355 τ on the side of the 8·38 τ peak in the spectra of the castaprenols may be due to the *cis* 'OH terminal' methyl group. The assignments made for these spectra in benzene are supported by results with phytol, citronellol, geraniol, nerol, solanesol and other naturally occurring *cis–trans*-polyprenols (J. Feeney & F. W. Hemming, unpublished work). Clearly, benzene offers advantages over CCl_4 and $CDCl_3$ when studying internal and ω-isoprene residues but is no more useful than CCl_4 and $CDCl_3$ when studying the configuration of 'OH-terminal' isoprene residues. The differences in pattern between spectra in CCl_4 and benzene are illustrated in Fig. 2.

The assignment of a *cis* configuration for the 'OH-terminal' isoprene residue in the castaprenols is based upon positive evidence from the spectra in CCl_4 and $CDCl_3$. The spectra of castaprenol-11, -12 and -13 show the presence of the peak at 8·27 τ when studied in $CDCl_3$ at 60 Mcyc./sec. but since the spectra of castaprenol-11 and -13 are not good enough for accurate measurement of relative areas they are not mentioned in Table 3. Castaprenol-11 and -13 were not examined in CCl_4. In the CCl_4 spectrum of castaprenol-12 the 8·27 τ peak is too close to the large 8·34 τ peak for its area to be measured separately (see Fig. 2). However, by visual estimation, its relative size appears to be consistent with its being due to the resonance of the protons of one methyl group. The absence of a peak at 8·52 τ in benzene is also evidence in favour of the *cis* configuration of the OH-terminal residue.

Other resonance peaks not included in Table 3 occur at lower field (see Fig. 2). Olefinic protons give broad peaks at 4·95 τ in CCl_4, 4·86 τ in $CDCl_3$ and 4·73 τ in benzene. In most spectra there is evidence of a triplet at 4·58, 4·66 and 4·47 τ in CCl_4, 4·44, 4·55 and 4·66 τ in $CDCl_3$ and 4·52, 4·59 and 4·66 τ in benzene which corresponds to the olefinic proton on the carbon atom β to the hydroxyl group (J. Feeney & F. W. Hemming, unpublished work). The two protons on the carbon atom α to the hydroxyl group give doublets at 6·02 and 6·10 τ in CCl_4, 5·85 and 5·96 τ in $CDCl_3$ and 5·98 and 6·05 τ in benzene. The splitting of these peaks and their relative areas is as expected for the proposed structures.

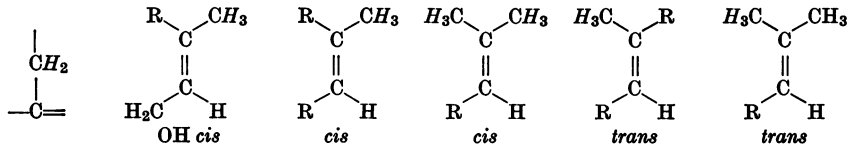
Infrared spectroscopy. The infrared spectra of castaprenol-11, -12 and -13 are reproduced in Fig. 3. The spectra are almost identical, the only noticeable differences being in the relative intensities of the O—H stretching bands at 3575 and 3310 $cm.^{-1}$ and the C—O stretching (O—H deformation) bands

Table 3. *Relative areas of the peaks in the region 7·50–8·50 τ of the nuclear-magnetic-resonance spectra of castaprenol-11, -12 and -13*

Figures in parentheses are the expected values, see text.

100 Mcyc., CCl_4						
Peak positions (τ)	7·79, 8·00	8·28	8·34	8·34	8·42	8·42
Castaprenol-12 (6 mg.)	44·5 (44)		26·7 (27)		11·7 (12)	
60 Mcyc., $CDCl_3$						
Peak positions (τ)	7·93, 7·99	8·27	8·33	8·33	8·42	8·42
Castaprenol-12 (26 mg.)	45·8 (44)		28·2 (27)		10·8 (12)	
100 Mcyc., benzene						
Peak positions (τ)	7·83, 7·95	(8·38)	8·25	8·32	8·38*	8·43
Castaprenol-11 (1 mg.)	40·43 (40)		18–20 (18)		16–18 (18)	
Castaprenol-12 (26 mg.)	43·4 (44)		20·8 (21)	2·8 (3)	12·2 (12)	3·2 (3)
Castaprenol-13 (3 mg.)	46·7 (48)		25·2 (24)	13·9 (15)		2·8 (3)

Assignment†



* In benzene this peak also includes the resonance peak for the protons of the methyl group *cis* to the olefinic hydrogen in a 'OH-terminal' isoprene residue. This methyl group resonates at 8·28 τ in CCl_4 and at 8·27 τ in $CDCl_3$.

† Resonating protons in italics. R, Isoprene unit. The configuration of the methyl groups is relative to the olefinic protons.

at 1000 cm^{-1}. As expected these bands become relatively less intense as the number of isoprene units in the molecule increases. The infrared spectra of other polyisoprenoid alcohols has been discussed in detail by Burgos *et al.* (1963). Comparison of these spectra with those of solanesol and pig liver dolichol is in agreement with the castaprenols being polyisoprenoid primary alcohols containing more *cis* than *trans* isoprene units. The assignments of the main bands in the infrared spectra are given in Table 4. Confirmation of the band at 1000 cm.$^{-1}$ being due to a primary but allylic alcohol was gained from the observation that upon acetylation it was replaced by a peak at 1020 cm.$^{-1}$ and that after hydrogenation (of the alcohol) it was replaced by a peak at 1055 cm.$^{-1}$, the usual position for C—O stretching of a saturated primary alcohol.

Some of the small peaks in the infrared spectra of polyisoprenoid compounds give an indication of the net situation regarding the configuration of the isoprene units. In Table 5 the positions of these peaks (arrowed in the spectra, Fig. 3) are compared with those in solanesol (all-*trans*) and pig liver dolichol (mainly *cis*). It is clear that the castaprenols' spectra are in keeping with a majority of their isoprene units being in the *cis* configuration (see also Burgos *et al.* 1963). Another relevant point is that when all *trans*-polyisoprenoids are studied as oils the prominent broad band at 835 cm.$^{-1}$ always carries a marked inflexion at 795 cm.$^{-1}$, whereas the inflexion is absent from the spectra of poly-*cis*-isoprenoids. The inflexion is not apparent in the spectra of both the castaprenols and dolichol.

Gas–liquid chromatography of derivatives of the castaprenols mixture. The retention times for the acetates of the castaprenols mixture, for solanesyl

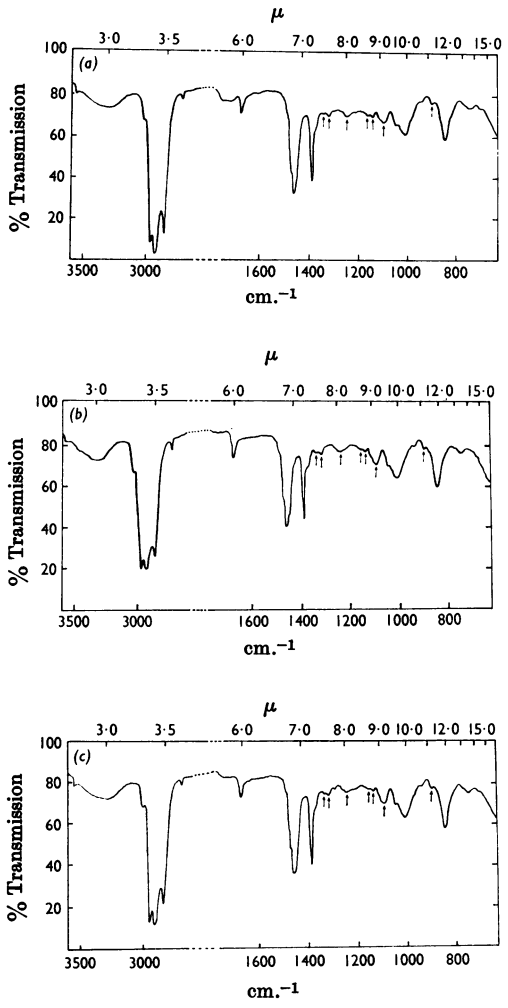


Fig. 3. Infrared spectra of castaprenol-11, -12 and -13 (*a*, *b* and *c* respectively) determined as solvent-free films between rock salt plates.

Table 4. *Assignments of the main peaks in the infrared spectra of the castaprenols*

Position (cm.$^{-1}$)	Assignment*
3575	O—H stretching, free
3310	O—H stretching, polymeric association
3024	C—H stretching of =CH—
2956	C—H stretching of CH_3
2918	C—H stretching of CH_2, CH_3
2845	C—H stretching of CH_2
1660	C=C stretching
1450	C—H deformation of CH_3, CH_2
1365	C—H deformation of CH_3
1000	C—O stretching of allylic primary alcohol
835	C—H deformation of trisubstituted olefin

* See Bellamy (1958).

Table 5. *Infrared-absorption peaks relevant to the configuration of isoprene residues*

~, Shoulder; W, weak.

Solanesol (cm.$^{-1}$)	Castaprenols (cm.$^{-1}$)	Dolichol* (cm.$^{-1}$)
1328	1330~	1326~
	1307	1307
1235~	1238	1242
1221	1221~	1221~
1148	1149W	
	1130	1126
1099	1089	1086
891~	888	888

* Isolated from pig liver, Burgos *et al.* (1963).

acetate and for the acetates of the betulaprenols mixture together with those for the derived perhydro-acetates and saturated hydrocarbons are given in Table 6. In Fig. 4 the logarithms of the retention times for the perhydroacetates are plotted against the number of isoprene units (now saturated) believed to be present in each molecule. The resultant straight line confirms that each mixture chromatographed contains a series of saturated isoprenologues. The saturated hydrocarbons give a similar graph. The unsaturated acetates of the castaprenols fall on one straight line when plotted in this way but this is slightly different from and not parallel to the straight line joining the betulaprenyl acetates. Solanesyl acetate falls close to both but on neither of the lines (see Fig. 5). It appears that *cis–trans* differences have a small effect on the retention times of long-chain prenyl acetates.

The fact that each castaprenyl acetate falls on a straight line when plotted in this way supports the evidence that these compounds are members of a series of isoprenologues.

The areas under the peaks of the castaprenol derivatives suggest the percentage composition of the mixture of castaprenols to be 0·5% castaprenol-10, 16% castaprenol-11, 82% castaprenol-12 and 2% castaprenol-13. These figures can only be taken as an approximate guide as the response of the detector to each component of the derived mixture has not yet been checked. However, the figures agree quite well with estimates of spot size and intensity of stain after reversed-phase partition thin-layer chromatography of a spot of the mixture. They are also confirmed by the weights of each

Table 6. *Retention times for derivatives of the mixture of betulaprenols, of solanesol and of the mixture of castaprenols during gas–liquid chromatography*

N.R., Not recorded.

Source	Perhydroacetates		Saturated hydrocarbons	Acetates	
	300°	340°	340°	300°	340°
Betulaprenol-6	0·48	0 29	N.R.	0·57	N.R.
Betulaprenol-7	1·28	0·47	N.R.	1·10	N.R.
Betulaprenol-8	2·86	0·82	N.R.	2·15	N.R.
Betulaprenol-9	6·47	1·47	N.R.	*	N.R.
Solanesol	6·47	1 47	0·78	5·85	N.R.
Castaprenol-10	*	2·80	1·42	*	1·85
Castaprenol-11	32·2	5·48	2·80	22·0	3·82
Castaprenol-12	69·0	10·00	5·42	47·2	7·40
Castaprenol-13	*	18·20	7·50	*	13·10

* Insufficient sample was applied to the column for this peak to be observed.

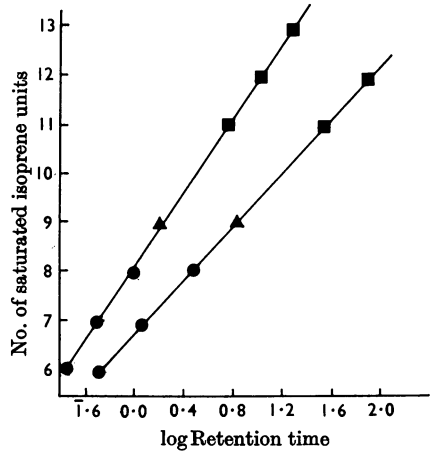


Fig. 4. Relationship between $\log_{10}$ retention time at 300° (lower line) and at 340° (upper line) and the number of saturated isoprene residues per molecule of each component observed during the gas–liquid chromatography of a mixture of the acetates of perhydrobetulaprenol-6, -7 and -8 (●), perhydrosolanesyl acetate (▲) and the perhydrocastaprenyl acetates (■).

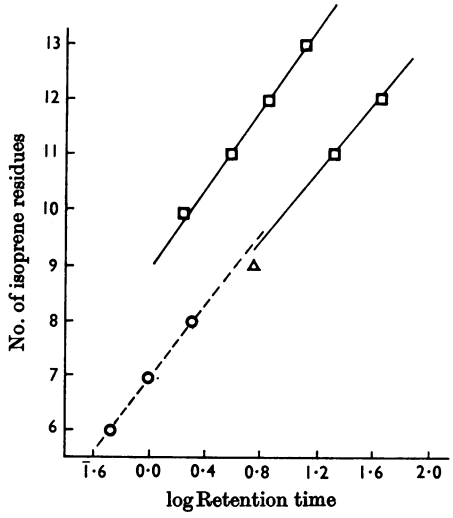


Fig. 5. Relationship between $\log_{10}$ retention time at 300° (lower lines) and at 340° (upper line) and the number of isoprene residues per molecule of each component observed during the gas–liquid chromatography of a mixture of the acetates of betulaprenol-6, -7 and -8 (○--), solanesyl acetate (△) and the castaprenyl acetates (□).

component recovered after preparative reversed-phase partition thin-layer chromatography of the mixture. It is interesting that crystallization of the castaprenols appeared not to alter the gas-chromatographic ratios of areas.

Thin-layer chromatography. The three main castaprenols were isolated by preparative reversed-phase partition thin-layer chromatography. The individual prenols were shown to be essentially free of contaminating material by adsorption and reversed-phase partition thin-layer chromatography. Each of the castaprenols had essentially the same R_F (0·38) on adsorption chromatography. However, on reversed-phase partition thin-layer chromatography, the individual castaprenols were well separated, running with R_F values 0·53 (castaprenol-11), 0·39 (castaprenol-12) and 0·27 (castaprenol-13). When the mixture of castaprenols was run in the latter system an additional spot with R_F 0·67 corresponding to castaprenol-10 was quite clear.

Microhydrogenation of the mixture of castaprenols. Three samples, each approximately 5mg., of the castaprenol mixture were subjected to quantitative microhydrogenation and gave a mean hydrogen uptake of 1·454moles of hydrogen/100g. at S.T.P. The theoretical figure for the mixture is 1·439moles of hydrogen/100g. The results confirm that all of the isoprene units are unsaturated.

The infrared spectrum of the fully hydrogenated mixture was compared with that of perhydrosolanesol (e.g. see Burgos *et al.* 1963). Quantitatively, the spectra were identical. Only the relative intensities of the O—H and C—O stretching bands were noticeably different. These differences were in accordance with the differences in chain lengths.

Castaprenyl [1-^{14}C]acetates. The specific activities of the [1-^{14}C]acetates of the mixture of castaprenols, solanesol and β-naphthol, prepared from portions of the same sample of [1-^{14}C]acetic anhydride, were compared. The mean figure for β-naphthyl acetate was 2155·1 (±4·1), for solanesyl acetate was 588·8 (±0·4) and for the mixture of castaprenyl acetates was 464·3 (±0·6). With β-naphthyl acetate as a standard, the mean equivalent weight of the mixture of castaprenols was calculated to be 822, whereas on the basis of the solanesyl acetate figure this became 810. The mean molecular weight of the mixture of castaprenols is 824 and it therefore follows that the castaprenols contain only one acetylatable hydroxyl group per molecule. The infrared spectrum of the castaprenyl acetates mixture showed the total absence of a hydroxyl group. It can thus be concluded that the castaprenols contain only one hydroxyl group per molecule.

Ozonolysis. The mixture of castaprenols (42mg.) was acetylated and ozonized. Reductive cleavage of the ozonides was followed by formation of the 2,4-dinitrophenylhydrazones of the products. These were divided into petrol-soluble and petrol-insoluble portions.

The petrol-soluble portion of these derivatives gave an ultraviolet-absorption spectrum in cyclohexane almost identical with that of acetone 2,4-dinitrophenylhydrazone. Thin-layer chromatography gave one major spot with the same R_F (0·70) as the authentic acetone derivative. Only traces of other material, including the 2,4-dinitrophenylhydrazone of acetaldehyde, were present. Gas–liquid chromatography confirmed this, giving one major peak with retention time 1·93min.

The ultraviolet-absorption spectrum of the petrol-insoluble material in chloroform was very similar to that of the authentic laevulaldehyde bis-2,4-dinitrophenylhydrazone (see Burgos *et al.* 1963). Gas–liquid chromatography gave one large peak with retention time (19·0min.) corresponding to that of the authentic laevulaldehyde derivative. Thin-layer chromatography of this fraction confirmed the major component to be the laevulaldehyde derivative and this was accompanied by small amounts of material running with the same R_F (0·51) as the authentic 2,4-dinitrophenylhydrazone of glycollic aldehyde acetate.

Portions of both the petrol-soluble and -insoluble derivatives were crystallized from ethanol (redistilled from 2,4-dinitrophenylhydrazine) and from acetonitrile–toluene (1:1, v/v) respectively. The ultraviolet-absorption spectra, in cyclohexane and chloroform respectively, both qualitatively and quantitatively proved the crystals to be respectively acetone 2,4-dinitrophenylhydrazone and laevulaldehyde bis-2,4-dinitrophenylhydrazone (see Burgos *et al.* 1963). It was calculated from extinction values at 345mμ (acetone derivative in cyclohexane, $E^{1\%}_{1cm.}$ 911) and at 357mμ (laevulaldehyde derivative in chloroform, $E^{1\%}_{1cm.}$ 911) that in the original mixture of 2,4-dinitrophenylhydrazones the molar ratio of these two derivatives was 1:12·9. The expected figure for this mixture is 1:11·9.

It can be concluded that the results of ozonolytic degradation of the castaprenyl acetates were close to those expected for the proposed structures.

DISCUSSION

The evidence presented is strongly in favour of the mixture of castaprenols containing *cis–trans*-undecaprenol, -dodecaprenol and -tridecaprenol. The size of the alcohols is shown clearly by mass spectrometry. The poly-isoprenoid nature of each component is confirmed by mass spectrometry, nuclear magnetic resonance, infrared spectroscopy and ozonolytic degradation. Nuclear magnetic resonance and infrared spectroscopy show that each component is a primary allylic alcohol. This is confirmed by ozonolytic degradation of the mixture. Nuclear magnetic resonance, mass spectrometry, isotope dilution of [1-^{14}C]acetic anhydride and infrared spectroscopy of the acetates all indicate the presence of only one hydroxyl group per molecule. That each isoprene residue is unsaturated is proven by mass spectrometry, nuclear magnetic resonance, hydrogen uptake and ozonolytic degradation. Infrared spectroscopy indicated that all three compounds had more of their isoprene residues in the *cis* configuration than in the *trans* configuration. Nuclear magnetic resonance enabled a quantitative assay of the configuration of each component which showed that common factors were the presence of a *cis* 'OH-terminal' isoprene residue and three *trans* internal isoprene residues. The alcohols differed only in the number of *cis* internal residues.

Chromatographic evidence suggested the presence

of a fourth isoprenologue, castaprenol-10. Insufficient of this component could be isolated to determine the configuration of its isoprene residue. However, it seems likely that it will differ from the other castaprenols only in the number of *cis* internal isoprene residues. This would be a logical situation that is indeed met in the family of ficaprenols isolated from the leaves of *Ficus elasticus* (Stone, Wellburn, Hemming & Pennock, 1967), and in the family of heveaprenols isolated from the leaves of *Hevea brasiliensis* (J. F. Pennock, P. J. Dunphy & K. J. Whittle, personal communication).

The castaprenols are to be found mainly in the chloroplasts of horse-chestnut leaves. Only as the leaves age does the proportion outside the chloroplasts become significant. Also both wood and bark of the tree appear to be lacking in castaprenols (A. R. Wellburn & F. W. Hemming, unpublished work). The site of localization of these prenols makes it tempting to assume that in each molecule all three *trans* internal isoprene residues are adjacent to each other and to the ω-terminal isoprene residue. The carotenoids (Goodwin & Williams, 1965) and phytol (Wellburn, Stone & Hemming, 1966), both characteristic components of chloroplasts, are biogenetically all-*trans* and are almost certainly formed from all-*trans*-geranylgeranyl pyrophosphate. It may well be that the castaprenols are also derived by the action of a poly-*cis* synthetase system adding *cis* isoprene residues to the same precursor. This would result in the three *trans* internal isoprene residues being immediately adjacent to the ω-terminal isoprene residue which itself would also be biogenetically *trans*. X-ray crystallographic techniques or carefully controlled biosynthetic studies with labelled all-*trans*-geranylgeranyl pyrophosphate may settle this point.

Most of the polyprenols in silver birch wood (Lindgren, 1965) and 50% or more of pig liver dolichol are esterified in Nature. The castaprenols, on the other hand, occur in horse-chestnut leaves in the unesterified form. Esterification of an alcohol is often a biological method of inactivation or of converting a compound into a storage form. Whether or not this has any bearing on the biochemical importance of the castaprenols is not known. As yet it is difficult to see a function for these compounds.

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